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(**6) Results**.

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Cover Page, Introduction, Contents, Call for Papers, All in one file

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CONTENTS

Abstract: The effects of farmyard manure on the dry matter content of two cucumber varieties (Ashley and Palmetto) was evaluated at the Teaching and Research Farm of the Ambrose Alli University, Ekpoma, Nigeria Lat 6^{o} 45^{1} N and Long 6^{o} 08^{1}E..The farmyard manure was applied at the rates of 0, 5 and 10t/ha. The layout was a 2 x 3 factorial scheme with three replicates. The result of the study showed that increasing the farmyard rates led to an increase in the dry matter weights of the two varieties of cucumber. Farmyard manure at 10t/ha increased the dry matter content of cucumber. Palmetto was however more responsive to FYM application, with the rate of 10t/ha out yielding the control by 60% in contrast to Ashley, in which 10t/ha of FYM out yielded the control by only 30 %. [Nature and Science. 2010;8(5):16-22]. (ISSN: 1545-0740).

Keywords: Farmyard manure, two cucumber varieties, dry matter accumulation and yield

3

Study effect drought stress and different levels potassium fertilizer on K^{+} accumulation in corn

Full Text

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Abstract: the necessity of exertion of irrigation regimes proportional to absorb process of nutritious element of potassium appear necessary duo to famine periods, saltines problem, and the shortage of drainage irrigation net in agricultural lands of Khuzestan province(southwest of Iran). This research is performed in the form of split plot test in accidentally complete bloke plan (main treatment: various levels in irrigation I. = 7, I_1 =12, I_2 = 17, I_3 = 22 day and secondary treatment: potassium fertilizer various levels K_1 =50, K_2= 100, and K_3=150(kg/ h) with 3 repetition. Plant date of first half of 2008 August is done in Shahid Salami irrigation institute farm in Ahvaz County. According to variance breakdown results the effect of irrigation cycles, different potassium and their interaction at level %5 effects on biological performance, seed function, harvest index and potassium assembling process were meaningful. But in case of the row number of maize, the effect of different levels of potassium fertilizer and reciprocal effect of water tension and different levels caused reduction of biological performance, seed function, harvest index, and row number of maize, seed number of row and weight of thousand seed. Treatment with seed yield of 15/5 ton in hectare has dedicated highest function to itself. And I_3 with 10/33 ton in hectare has lowest function which regarding to ware shortage and famine phenomena, it is under consideration. [Nature and Science. 2010;8(5):23-27]. (ISSN: 1545-0740).

Key Words: corn, drought stress, potassium

4

Cytogenetic and Biochemical Studies On the Protective Role of *Rhodotorula glutinis* And its Autoploidy Against the Toxic Effect of Aflatoxin B_1 in Mic

Full Text

Inas S.Ghaly [1], M.M.Hassanane[1], E.S.Ahmed[1], W.M.Haggag[2] S., A. Nada[3] and I. M. Farag[1].

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Abstract: The present study was designed to investigate the effect of *Rhodotorula glutinis* and its autoploidy on cytogenetic and biochemical analyses and to evaluate the protective role of these yeasts against aflatoxin B_1- in mice. Eight groups of male mice were used. Three of them were treated with three strains (wild type G1 and two autoploidy (G2 and G3) of *Rhodotorula glutinis*. In addition, one group was treated the suspension of growth

	medium of yeasts (served as control),three groups were treated with the G1, G2 and G3 after an hour of injection with the aflatoxin. Cytogenetic analyses revealed that the treatment with the wild type of yeast (R.g, G1) and its two mutants (G2 and G3) had improved the genetic materials in normal somatic and germinal animal cells by decreasing chromosome aberrations and increasing the mitotic and meiotic indices compared to control group. On the other hand, the chromosomal aberrations were more frequent of mitotic and meiotic indices were depressed in the animals treated with aflatoxin alone. In contrast, the frequencies of the chromosome aberrations were significantly decreased and mitotic and meiotic indices were increased in animals treated with the wild type (G1) and its two autoploidy (G2 and G3) plus aflatoxin B_1. Biochemical results showed that the treatment with yeast strains especially the treatment with two autoploidy G2 and G3 did not induce changes in liver and kidney functions in normal animals. The treatment with the three strains wild type G1 and two autoploidy G2 and G3 had enhanced the TP compared to control group. The treatment with aflatoxin B1 significantly increased the liver enzymes (GGT, ALT and AST), kidney function markers(uric acid and creatinine) and significantly decreased the TP compared to control group. In contrast, the treatment with yeast strains plus aflatoxin B_1 succeeded in diminishing the elevated value of liver enzymes and kidney functions and normalized TP level. [Nature and Science. 2010;8(5):28-38]. (ISSN: 1545-0740). **Keywords**: Aflatoxin,*Rhodotorula glutinis*,autoploidy,chromosome aberrations,biochemistry.	
5	**Practical Aspects and Immune response of Probiotics Preparations Supplemented to Nile Tilapia (*Oreochromis Niloticus*) Diets** H.M.Ali1; A.A. Ghazalah1; E.A. Gehad2; Y.A. Hammouda1 and H.A. Abo-State1 1- Animal Production Department, National Research Center, Dokki, Giza, Egypt 2- Animal Production Department, Fac. of Agric., Cairo University, Cairo Egypt dr_mona_zaki@yahoo.co.uk **Abstract:** This study was carried out for 7 months at fish Laboratory of Animal Production Department, National Research Center, Dokki, Egypt. The experiment aimed to investigate the effect of two commercial probiotics (Premalac and Biogen) each at 1,2 and 3 g/Kg diet on growth performance and immune response of Nile tilapia fingerlings. Premalac is a dried fermented product of Lactobacillus acidophilus, Aspergillus oryzae extract, Bifedobacterium bifedum, Streptococcus faecium, Torula yeast, Skim milk, Vegetable oil and CaCo3. Biogen is a dried natural product composed of Allicin, high unit hydrolytic enzymes, Bacillus subtilis and Ginseng extract. A total of 420 fingerlings with a uniform size and weight (1 gram) were used of which 60 fingerlings represent the control group. The rest (360 fingerlings) distributed randomly into two blocks (probiotics), each block included three treatments (probiotic levels). Each treatment in addition to the control one were represented in three replicates (aquaria) in which 20 fingerlings were kept in each aquarium. The best results of growth and feed utilization of tilapia were obtained by fish diet supplemented with Biogen followed by those having Premalac, each at 2g/Kg diet. However, fish fed on Biogen-supplemented diets exhibited significantly higher values of nutrients digestibility. On the other hand, fingerlings fed either Premalac or Biogen at 2g/Kg had significantly higher total leucocytes count than the control which indicating highs immune response of tilapia fingerlings. In conclusion, it is suggested that the tested probiotics preparations are suitable for mixing with tilapia diets to improve their performance and immunity. [Nature and Science. 2010;8(5):39-45]. (ISSN: 1545-0740). **Key words**: Oreochromis Niloticus , Immune response, Probiotics Preparations.	Full Text
6	**Effect of Probiotics on performance and nutrients digestibility of Nile tilapia *(Oreochromis niloticus*) Fed Low Protein Diets** A.A. Ghazalah[1]; H.M. Ali[2]; E.A. Gehad[1]; Y.A.Hammouda[2] and H. A. Abo-State[2] 1- Animal Production Department, Fac. of Agric. Cairo University, Cairo, Egypt 2- Animal Production Department, National Research Center, Dokki, Giza, Egypt dr_mona_zaki@yahoo.co.uk **Abstract:** This study was carried out at fish laboratory of Animal Production Department, National Research Center, Dokki ,Egypt using two commercial probiotics (Premalac and Biogen) to study their effects on growth the	Full Text

	performance of Nile Tilapia fed diets with slightly lower levels of crude protein. Premalac is a dried fermented product of *Lactobacillus acidophilus*, *Aspergillus oryzae* extract , *Bifedobacterium bifedum* , *Streptococcus faecium* , Torula yeast , skim milk , vegetable oil and $CaCo_3$. Biogen is a dried natural product composed of Allicin , high unit hydrolytic enzymes, *Bacillus subtilis* and Ginseng extract. The basal diets were formulated to contain 30, 27.5 and 25.0% crude protein (CP), each was either supplemented or not with either Premalac or Biogen at 2g /kg diet. The experiment was conducted in 3x3 factorial design and included nine treatments each in three replicates (aquaria) in which 20 fingerlings mono sex Nile tilapia of the same size and weight (1 gram) were stocked in each aquarium. The actual experimental feeding trials durated for four months. Results indicated that the lowest CP level (25%) in tilapia diets without or with the tested probiotics recorded the worst values of growth performance parameters and nutrients digestibility. The use of either Premalac or Biogen at 2g/kg diet in diets with 27.5% crude protein was more efficiently than those containing 30% CP,. in addition, it gave best values of economic efficiency. However, Biogen was more superior than Premalc. In other words, these probiotics spared nearly 2.5% CP of the recommended level for tilapia. This result would be effective from the economical point of view, since protein is the most expensive feed nutrient in all live stock feeding, particularly fish. [Nature and Science. 2010;8(5):46-53]. (ISSN: 1545-0740). **Keywords**: Probiotics; Oreochromis niloticus; nutrients digestibility.	
7	**Ovarian activity, biochemical changes and histological status of the dromedary she-camel as affected by different seasons of the year** M.A. El-Harairy[1], A.E.B. Zeidan[2], A.A. Afify[2], H.A. Amer[3], and A.M. Amer[1] 1. Department of Animal Production, Faculty of Agriculture, Mansoura University, Egypt. 2. Animal Production Research Institute, Dokki, Giza, Egypt. 3. Department of Theriogenology, Faculty of Veterinary Medicine, Zagazig University, Egypt. dr_mona_zaki@yahoo.co.uk **Abstract**: The present study aimed to investigate the effect of different seasons of the year on body thermoregulation (rectal temperature, respiration rate and pulse rate), blood hematology (hemoglobin, packed-cell volume, red blood cells and white blood cells counts), blood components (total protein, albumin, globulin, aspartate-aminotransferase, alanine-aminotransferase, alkaline phosphatase, acid phosphatase, cholesterol, sodium, potassium, calcium, total phosphorus, testosterone and oestradiol-17β hormone concentrations of the dromedary she–camel. Histological changes of the right and left ovaries were also recorded. The obtained results showed that, rectal temperature and respiration rate in the dromedary she-camels increased significantly ($P<0.05$) during summer as compared to the other seasons. However, pulse rate showed significantly ($P<0.05$) lower during winter than other seasons. The highest ($P<0.05$) values of hemoglobin, packed-cell volume and red blood cells count were recorded during summer, while the lowest ($P<0.05$) value of the white blood cell's was recorded during autumn season. Total protein, albumin and globulin concentrations (mg/dI) were increased insignificantly during summer season as compared to other seasons. Aspartate-aminotransferase, alanine-aminotrasferase enzymes, sodium and calcium concentrations of the dromedary she–camels increased significantly ($P<0.05$) during summer, while potassium and total phosphorus concentrations (mg/dl) increased significantly ($P<0.05$) during spring as compared to other seasons. The lowest ($P<0.05$) value of alkaline phosphatase and acid phosphatase enzymes were recorded during winter season. Testosterone, oesterdiol-17β hormone and cholesterol concentrations were significantly ($P<0.05$) higher during winter than other seasons of the year. The histological examination of the left and right ovaries in different seasons of the year revealed higher activity in spring and winter than summer and autumn seasons. The left ovary showed more growing and mature follicles and higher activity than the right one. *In conclusion,* the female dromedary camels display ovarian activity during the non-breeding season. So, the environmental temperature, relative humidity and daylight length seemed to play the major role in the regulation of seasonal ovarian activity in the female dromedary camels. [Nature and Science. 2010;8(5):54-65]. (ISSN: 1545-0740). **Key words**: Seasons, She-camel-ovaries, testosterone, oesterdiol-17β, cholesterol	Full Text
8	**Ethnomedicinal Plant Diversity in Kumaun Himalaya of Uttarakhand, India**	Full Text

K. K. Gangwar*, Deepali** and R. S. Gangwar***
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Abstract: Kumaun Himalaya of Uttarakhand State is characterized by a rich diversity of ethnomedicinal plants a well as a rich heritage of traditional medicine system. The present study reveals the status of ethno-medicinal flo and their importance preserved by the local population in Kumaun region. During the study it was observed that 1(species of ethno-medicinal plants belonging to 48 families are being used in the folk-medicine system by th indigenous people of this region. For the present study, an intensive and extensive survey was made for four selecte districts of Uttarakhand, viz. Almora, Champawat, Bageshwar and Pithoragarh. The neighboring villages of the stud areas were also visited for identification of plant species and to explore the traditional knowledge about the use t indigenous medicinal plants. Therefore, the ethnobiological knowledge of people and listing of plants of particul region are important tools that may help in understanding human environment interactions. [Nature and Scienc 2010;8(5):66-78]. (ISSN: 1545-0740).

Keywords: *e*thno-botany; folk medicines; Kumaun region; local communities

9

ENVIRONMENTAL STUDIES ON THE MUDSKIPPERS IN THE INTERTIDAL ZONE OF KUWAIT BAY

Full Text

Bahija E. Al-Behbehani* & Hussain M. A. Ebrahim
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Abstract: This work deals with monitoring mudskippers in their natural environment (intertidal zone) along the Kuwait Bay muddy shores in the State of Kuwait. This is to provide information is concerning the environmental factors effecting mudskipper diversity in Kuwait Bay. Kuwait Bay is a large mud-flat with a fascinating associated fauna of mud-skippers and crabs provide rewarding feeding-grounds for many birds. A number of fifty mudskipper samples are collected during the hot summer season (July and August) of the year 2009 and examined for parasites and to evaluate the different environmental factors controlling the biodiversity in this marine environment. The results of the present study indicate the abundance of the mudskippers allover the intertidal mud flat of the Bay and the total absence of either external and/or internal parasites in the mudskipper tissues and organs. Mudskippers are found to be completely amphibious fish that are adapted to live in the intertidal environment. Mudskippers are very active when they are outside the water, feeding and interacting with one another. The mud in the Kuwait Bay environment is very good for burrowing in, since the particles are very sticky, unlike sand. Often, the mudskipper form mixed colonies with digging crabs (Fiddler crabs-Caidae). Specific physiological and behavioural changes in bioindicators are used to detect changes in environmental health, so Mudskippers can be considered as bioindicators of marine pollution in Kuwait Bay, this needs further studies. [Nature and Science. 2010;8(5):79-89]. (ISSN: 1545-0740).

Key words: Mudskippers, Intertidal Zone, Kuwait Bay

10

Genetic Variation Between Horse Breeds Using RAPD Markers

Full Text

Karima F. Mahrous*, Sally S. Alam and Aziza M. Hassan
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Abstract: Genetic diversity is the basis for present day diversified living systems and future genetic improvement needs. Within the framework of breed conservation, genetic characterization is important in guarding breeds and is a prerequisite for managing genetic resources. The objective of the present study is to estimate the genetic diversity and phylogenetic relationships among Egyptian horse breeds (Native and Arabian) and an exotic breed

	(Thoroughbred) using Random Amplified Polymorphic DNA – Polymerase Chain Reaction (RAPD) technique. Initially, 25 primers were screened among all the breeds of which 14 primers amplified the genomic DNA. Four primers generated reproducible and distinct RAPD profiles and were used for further analysis. A total of 40 loci were amplified, of which 37 were found polymorphic (92.5%), useful for genetic variation study between breeds. The genetic diversity had the highest value (0.2048) in Arabian and the lowest value (0.0116) in Native breed. The genetic distance was found highest between Arabian and Thoroughbred (D=0.5442) and lowest between Native and Arabian (D=0.3280). However, genetic identity was highest (I=0.7204) between Native and Arabian and lowest (I=0.5803) between Arabian and Thoroughbred. UPGMA dendrogram based on Nei's genetic distance grouped the investigated horse breeds genotypes into two clusters. The first cluster includes Egyptian breeds (Native and Arabian) where as the second cluster include Thoroughbred which appeared to be most distant from the other breeds. In conclusion, these results indicated the effectiveness of RAPD in detecting polymorphism between horse populations and their applicability in population studies and establishing genetic relationships among the horse populations. [Nature and Science. 2010;8(5):90-99]. (ISSN: 1545-0740). **KeyWords**: Genetic diversity, Horse breeds, RAPD-PCR.	
11	**The Role of Hyperthermia in Potentiation of Chemotherapy and Radiotherapy in Mice Bearing Solid Tumor** Amal I. Hassan Radioisotopes Department, Atomic Energy Authority, Egypt. dr_mona_zaki@yahoo.co.uk **Abstract:** Hyperthermia is procedure in which body tissue is exposed to a high temperature up to 41°C and is an effective tool in cancer treatment. Hyperthermia also is a therapy applied together with other modalities in the treatment of cancer. The aim of this study was to determine if there was a change in immunological and biochemical parameters after using each of hyperthermia, radiotherapy or chemotherapy separately and the combined treatments in mice bearing solid tumor. Seventy females Albino mice weighing (20-25g) were used in the current study. The animals were divided into five groups. Group I: served as a control animals. Group II: animals were cancered by solid tumor and were untreated. Group III: animals exposed to WBH alone. Group IV: animals administered doxorubicin (Dox) 3mg/kg body weight i.p. once a week. Group V: animals were exposed to fractionated whole body gamma rays (WB-γ) at a dose level of 0.5 Gy once a week. Group VI: animals were exposed to WBH and administered doxorubicin (Dox) 3mg/kg body weight (i.p.) once a week. Group VII: animals were exposed to WBH then fractionated whole body gamma rays (WB-γ) at a dose level of 0.5 Gy once a week. After four weeks (the end of treatments), blood samples were collected from orbital venous plexus in heparinized tubes from all animal groups. The results of the present study indicated that WBH with or without radio- and chemotherapy induced significant increase in TNF-α, IL-2 and HSP70 values as compared to cancered group. As well as WBH with or without radio- and chemotherapy induce significant increases of phagocytosis and killing cells percent as compared to untreated cancered group. On the other hand WBH alone or with radiotherapy and (Dox) induced significant decrease of α -FP as compared to cancered group. Also, the results revealed that WBH with or without radio- or chemotherapy induced apoptosis for cancer cells. It could be concluded that, WBH enhances the response of tumor cells to radiation and chemotherapy and it has an important role in potentiation of radio- and chemotherapy in solid tumor treatment. [Nature and Science. 2010;8(5):100-108]. (ISSN: 1545-0740). **Key Words:** Hyperthermia, mice bearing solid tumors, doxorubicin, whole body gamma irradiation, apoptosis, immune responses.	Full Text
12	**Effect of Calcium and Some Antioxidants treatments on Storability of Le Conte Pear Fruits and its Volatile Components** Omaima, M. Hafez[1]; H. A. Hamouda [2]; and Magda A. Abd- El- Mageed [3] [1]Pomology Research Dept., [2] Fertilizer Technology Dept., and [3]Chemistry of Flavour and Aroma Dept., National Research Center- Dokki, Giza, Egypt. dr_mona_zaki@yahoo.co.uk	Full Text

Abstract: The possibility of calcium nitrate and / or some antioxidants i.e. citric acid and ascorbic acid as preharvest treatment alone or in combination to control decay and its role in improvement the quality of Le Conte pear fruits as well as volatile components under cold storage condition and marketing period during to successive seasons 2007 and 2008.Le Conte pear trees were foliar spraying twice with calcium nitrate at concentration of (0.0 and 1700 ppm), citric acid at concentration of (0.0,50 and 100 ppm) and ascorbic acid at concentration of (0.0,50 and 100 ppm), ten treatments were used including control. All treated and untreated pear fruit were stored at 0 ± 1 ºC and 85 – 90% relative humidity (RH) for 75 days and additional one week at room temperature (20-25ºC) as stimulated marketing period. Fruit quality assessments i.e. weight loss and decay percentage, fruit firmness, total soluble solids %, total acidity %, total sugars, fruit calcium content and volatile components were evaluated. Results showed that treated and control fruits withstand free from chilling injury and pathogenic rot up to 45 days of cold storage. While, almost treatments prevented chilling injury symptoms and fruit deterioration up to 60 days of cold storage. Moreover, all treatments alone or in combination decreased the weight loss (%), total acidity % and fruit softening, while increasing fruits content of TSS %, total sugars and calcium (%) as a good keeping fruit conditions for along time. Furthermore, the same trend was observed during marketing period. Therefore, it can be concluded that prolonging storage period of the Le Conte pear fruits by using the considered treatments. However, the combined treatments with calcium nitrate + citric acid, calcium nitrate + ascorbic acid or /and the single treatment of calcium nitrate could be recommended because its gave the best results for keeping fruits and their volatile components under cold storage and marketing period extinction. The headspace volatiles of fresh and stored Le Conte Pear were collected and subjected to GC and GC-MS analysis. 27 volatile components were identified: 15 esters, 8 alcohols, 3 aldehydes and one terpene. Volatile components varied considerably both quantitatively and qualitatively between fresh and stored samples. The best treated samples at fresh were (Ca + CA1) and (Ca + CA2) compared to the control treatments. Although all samples retain a good quality during storage period, Ca(NO3)2, AsA1, (Ca + CA1), (Ca + CA2), and (Ca + AsA1) treated samples were the best compared to the control samples because of the highest content of esters which exhibit it more fruity aroma and cause it more acceptable for consumer. [Nature and Science. 2010;8(5):109-126]. (ISSN: 1545-0740).

Keywords: Le Conte pear, Calcium, Citric Acid, Ascorbic Acid, Volatile Components, Storage, Quality Assessments

13

A comparative Study On Different Carbon Source Concentrations And Gelling A Gent On In Vitro Proliferation Of Pineapple (Ananas colossus)

Abd El Gawad. N.M.A *Zaied. N.S *. and. M.A Saleh.
* Pomology Res. Dept., National Res. Center, Dokki, Egypt.
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Abstract: The shoots regenerated from shoot tip of Pineapple Ananas Comosus Cv. Smooth cayenne) plantlets from the establishment stage were cultured individually on Ms medium supplemented with 200 mg/L 6- benzylamin opurine (BAP). Sucrose, fructose and mannitole with concentrations (20, 30 and 40 gl/L) were tested. Various kinds of gelling agent i.e. Agar and Gerlited were tested. Data indicated that all sucrose treatment (20, 30 and 40 g/L) enhanced the proliferation percentage and shoot number compared with other treatment and control except treatment of mannitol at 40g/L improved the shoot length only. Moreover, agarasa gelling agent was better than Gelrite at proliferation stage. The best shoot length, shoot number and growth percentage were obtained when 2.0 mgL Gelrite was added to the medium. [Nature and Science. 2010;8(5):127-130]. (ISSN: 1545-0740).

Key words: Carbon Source-gelling-pineapple

14

Relation between Glycoprotein and EA4 – Time Mechanism in *Secamia creatica*

N.B. Aref[(1)] and H.A. Ahmed[(2)]
National research Center, (1) Plant protect. Dept. (2) Biochemistry Dept.
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	Abstract: ATPase (EA4) seems to measure time- interval as a diapause – duration timer in the seasonal cycle of the Sesamia cretica. A peptide named peptidyl – inhibitory needle (PIN) seems to regulate the time measurement of EA4. We characterize the EA4 in the first step to analyse its interaction with PIN. Matrix – assisted laser desorption/ ionization – time of flight- mass spectrometry shows EA4 of an equimolar complex with PIN. The binding affinity of EA4 for PIN is about 460nM, measured by surface plasmon resonance. Western blot analysis of EA4 with a variety of biotynylated lectins suggest that EA4 is a glycoprotein containing N- linked oligosaccharide. By enzymatic cleavage of the glycosyl chain the carbohydrate is revealed to be essential for the regulation of EA4-time measurement through the interaction with PIN. PIN holds the timer by binding to EA4, and the dissociation of the complex could constitute the cue for the time measurement. [Nature and Science. 2010;8(5):131-138]. (ISSN: 1545-0740). **Key words**: Timer protein, Glycoprotein, Time – EA4, ATPase	
15	**Flavone-5-O-Glycosides from *Cheilanthes dalhousiae* (Hook)** [1]Rachana Mishra and D. L. Verma[2] [1]Department of Chemistry, Kumaun University, DSB Campus, Naini Tal-263001, (Uttarakhand) India. [2]Department of Chemistry, Kumaun University, SSJ Campus, Almora-263601, (Uttarakhand) India. 09411102476m@gmail.com **Abstract:** Fern fronds (about 500gm) of Cheilanthes dalhousiae Hook. Vouch. Sp. No. 21 was collected from Pindari glacier routes (2200-2800m above sea level) of Almora district of Uttarakhand state. It was extracted with acetone-water (1:1, V/V) and extract was concentrated under reduced pressure until H2O layer (up to 50ml) remained. The H2O layer was partitioned with CH2Cl2, EtOAc and BuOH Successively. The CH2Cl2 fraction gave antibacterial tests against Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus epidermidis and Escherichia coli by the standard method of disc-diffusion using DMSO-d6 solution of CH2Cl2 residue impregnated on Whatman No. 3, paper disc (6 nm) and base plates containing 10ml MH agar. Antibacterial activity was expressed as the ratio of the inhibition zone produced by CH2Cl2 extract and the inhibition zone caused by the reference, neomycin (2µg). No antibacterial activity was observed in ethyl-acetate and n-butanol fractions. EtOAc fraction was evaporated to dryness and residue obtained was dissolved in MeOH. The MeOH soluble of EtOAc fraction was fractionated on Whatman No. 3 chromatographic papers using BAW (n- BuOH-AcOH-H2O, 4:1:5, V/V, upper layer) as an eluent. Two blue UV fluorescent flavone-5-O-glycosides: Quercetin-3-methyl ether-5-O-glycoside and Kaempferol-5-O-(6”-O-malonyl)-glycoside were isolated by RPPC from EtOAc fraction of acetone-H2O (1:1) extract of fern fronds of Cheilanthes dalhousiae. The structural elucidation of the compounds was carried out by UV, 1HNMR and MS spectral studies. [Nature and Science 2010;8(5):139-143]. (ISSN: 1545-0740). **Keywords:** Kumaun Himalaya, *Cheilanthes dalhousiae* (Hook), Medicinal plants	Full Text
16	**Physiological Studies on the Risk Factors Responsible for Atherosclerosis in Rats** *Ahmed M Shehata and **Olfat M Yousef * Physiology Department, National Organization for Drug Control and Research, Giza 12553 – Egypt ** College for Women, Arts, Sciences and Education, Ain Shams University, Cairo, Egypt ahmedmshehata@yahoo.com, olfat_mohamed711@yahoo.com **Abstract:** Despite the well established correlation between hypercholesterolemia and coronary artery disease (CAD), a substantial body of evidence challenge this relationship. The study aimed to examine whether hyperlipidemia per se constitutes the principal risk factor for atherosclerosis or just a coordinator to other critical mediators. Hyperlipidemia was produced by feeding rats with high-fat diet for two months. The occurrence of hyperlipidemia was determined by measuring lipid profile. The hyperlipidemic rats were subdivided into two groups i) hyperlipidemic rats ii) hyperlipidemic rats injected with single dose of *Escherichia coli* (*E. Coli)* (and kept for two weeks to develop bacteremia and its subsequent effects. Result showed that hyperlipidemia significantly increased total cholesterol, triglycerides, low density lipoprotein (LDL) and homocysteine levels, whereas decreased high density lipoprotein cholesterol (HDL) levels. Moreover, hyperlipidemia induced mild oxidative stress in terms of elevated levels of malondialdehyde (MDA) and nitric oxide (NO) and decreased level of	Full Text

	reduced glutathione (GSH) in blood. In addition, hyperlipidemic rats exhibited high plasma viscosity, altered hematological indices and caused histological abnormalities manifested as perivascular hemorrhage, vacuolation of the tunica media and minor thickening in aorta wall. Bacteremia provoked inflammatory reactions and oxidative stress, elevated plasma homocysteine and caused noticeable considerable thickening of media-intema layer suggesting the commencement of atherosclerosis. Hyperlipidemic-bacteremic rats showed an additive effect. The study indicated that although hyperlipidemia is an apparent risk factor, homocysteinmia, the inflammatory component and the oxidative stress emerge to be the underlying mechanisms of atherosclerosis pathogenesis. [Nature and Science 2010;8(5):144-151]. (ISSN: 1545-0740). **Key words:** Hyperlipidemia- Bacteremia- inflammation- Atherosclerosis	
17	**Comparative study of endo-parasitic infestation in *Channa punctatus* (Bloch, 1793) collected from Hatchery and Sewage lagoon** Md. Jobaer Alam[1], Md. Rakibuzzaman[1], Mehedi Mahmudul Hasan[2] [1]Department of Fisheries, University of Dhaka, Dhaka-1000, Bangladesh [2]Department of Fisheries and Marine Science, Noakhali Science and Technology University, Sonapur, Noakhali-3802, Bangladesh Email: jobaer_alamdu@yaho.com, rakib_214@yahoo.com, mehedi_nstu@yahoo.com **Abstract:** The study was conducted to collect and identify endoparasites of *Channa punctatus* (Bloch,1793) from different water bodies of varying water quality in Bangladesh and to determine the prevalence and intensity of infestation brought about by the endoparasites in the hosts. The host fishes were collected from polluted water at sewage treatment lagoon in Narayangong and fresh water at Tongi Hatchery in Gazipur, Bangladesh. The prevalence of endoparasites in the host fish *Channa punctatus* was 91.30% in female and 88.88% in male fishes, among them in polluted water fishes the prevalence was 85.71% in female and 86.66% in male and in fresh water fishes the prevalence was 100% in both the male and female fishes. The intensities of infestation in *Channa punctatus* was 6.78 in female and 6.55 in male fish collected from hatchery; and in sewage water fishes the intensity was 3.50 in females and 1.15 in males respectively. Six parasite species were found from polluted water fishes and seven species of parasites were recorded from fresh water fishes. The parasite groups were trematodes (*Genarchopsis bangladensis, Allogomtiotrema attu, Phyllodistomum* sp., *Neopecoelina saharanpuriensis*), nematodes (*Ascaridia* sp., *Procamallanus* sp.) and Acanthocephalan (*Pallisentis nandai*). Acanthocephalans were found in the fishes collected from sewage lagoon. Liver, stomach, intestine and body cavity of the host fishes were examined for parasites. Females were more infected than the males. The intensity and the prevalence were higher in host fishes collected from hatchery than the sewage water host fishes. The hosts of intermediate length and weight group were found to be more infected than smaller and larger length groups. [Nature and Science 2010;8(5):152-156]. (ISSN: 1545-0740). **Key words:** Endoparasite, *Channa punctatu*, Hatchery, Sewage Lagoon	Full Text
18	**Comparison of digesting capacity of nitric acid and nitric acid-perchloric acid mixture and the effect of lanthanum chloride on potassium measurement** Molla Rahman Shaibur[1], Abul Hasnat Md. Shamim[2, 3], SM Imamul Huq[4] and Shigenao Kawai[5] [1]Department of Environmental Science & Health Management, Jessore Science & Technology University, Bangladesh [2]School of Agriculture & Rural Development, Bangladesh Open University, Gazipur-1705, Bangladesh [3]Graduate School of Environmental Science, Okayama University, 1-1, Tsushima-Naka, 3-Chome, Okayama700-8530, Japan [4]Department of Soil, Water & Environment, University of Dhaka, Dhaka-1000, Bangladesh [5]The United Graduate School of Agricultural Sciences, Iwate University, Morioka 020-8550, Japan **Abstract:** Nitric acid-perchloric acid mixture is the renowned digesting reagent in the scientific world of plant nutrition. Beside this, some other inorganic acids can be used as the digester of plant samples. Therefore, this experiment was conducted to find out if there is any difference between the digesting capacity of nitric acid (HNO_3)	Full Text

	and nitric acid-perchloric acid mixture (HNO_3-$HClO_4$) or not. The hydroponic experiments were conducted with barley (*Hordeum vulgare* L. cv. Minorimugi) and rice (*Oryza sativa* L. cv. Akihikari) seedlings. At suitable stage, the plant samples were collected, washed with deionized water, separated into shoot and root, dried, grinded and then divided into two groups for shoot and root individually for two types of seedlings. One group was for only HNO_3 acid and the other group was for HNO_3-$HClO_4$ acid mixture. Phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu) were measured after digesting the samples. There was no significant difference between the digesting capacity of HNO_3 acid and HNO_3-$HClO_4$ acid mixture. Potassium was measured by diluting the samples (200-600 times) containing lanthanum chloride ($LaCl_3$) or without $LaCl_3$. Lanthanum chloride did not have any significant effect on K measurement in this dilution system. [Nature and Science 2010;8(5):157-162]. (ISSN: 1545-0740). ***Key words**: Concentration, Lanthanum chloride, Nitric acid and nitric acid-perchloric acid mixture*	
19	**Study on Seed Germination and Growth Behavior of Brinjal** *Solanum melongena* var.BR 112 **in Admiration to Effect of C.M.L. (Country Made Liquor)** Sanjeev Sharma[1] and Kapil Sharma[2] School of Biosciences 1. Lecturer, Biotechnology, School of Biosciences, IMS Ghaziabad, Uttar Pradesh, 201009, INDIA. 2. Research Scholar, School of Biosciences, IMS Ghaziabad, Uttar Pradesh, 201009, INDIA. For correspondence: sanjeevsharma@imsgzb.com **Abstract:** In India a leading News paper Times of India published an unconfirmed report citing the use of Country made Liquor by the farmers in the National Capital Region Gurgaon for the cultivation of Brinjal crop. It was reported that use of CML increased the production of Brinjal by 06to 08 times. The brinjal thus produced were reported to be of good quality and appearance. An experiment was conducted to study the seed germination and growth behavior of brinjal (*Solanum melongena* L.) with country made liquor under environmental conditions. Seeds of *Solanum melongena* L. var. BR 112, were sown at the depth of 2.5 cm. with different treatments i.e. S1 (Control- Without Country made liquor 36 %V/V), S2 (Soil + 10% solution Spray of Country made liquor at intervals of 03 days with original Concentration 36% V/V). 1000 replicates of each treatment were used for the study. Total numbers of germinated plants were counted from each set of all treatments, at the interval period of 5 days after sowing, and reported as emergence count. For growth study plant height, number of leaves, length and width of leaves and root length were measured from all the treatments. Result revealed that CML treated batch showed maximum germination% i.e. 700 plantlets from 1000 seeds then control i.e. 500 plantlets. After 20 days of Growth plantlets also showed maximum plant height (7.1 cm.), number of leaves (4.5) length of leaves (2.5 cm.), width of leaves (2.6 cm.) and root length (4.3 cm.) in S2 treatment then control S1 plant height(6.5 cm), number of leaves(3.8), length of leaves(2.0 cm), width of leaves(1.0 cm), root length(4.7 cm). [Nature and Science 2010;8(5):163-166]. (ISSN: 1545-0740). **Key Words**: *Solanum melongena* BR 112, Country Made Liquor, Tharra	Full Text
20	**ON THE REALIZATION OF FLOATING INDUCTORS** **AHMED M SOLIMAN** Electronics and Communications Engineering Department, Cairo University, Egypt E-mail: asoliman@ieee.org **ABSTRACT:** Floating inductor circuits using minimum number of passive elements namely two resistors and one capacitor is reviewed in this paper. All the circuits considered in this paper are floating. Previously reported non-floating circuits are modified to be floating and new floating circuits are introduced as well. The active elements used in this paper are floating conveyor building blocks as well as pairs of non-floating conveyor blocks acting as a floating pair. Simulation results of second order lowpass filters realized using different types of floating inductors are included. [Nature and Science 2010;8(5):167-180]. (ISSN: 1545-0740).	Full Text

	Keywords: Floating inductors, current conveyors, gyrator, DVCC, FDVCC	
21	**Functional-Food Supplementation and Health of Broilers** Hussein A. Kaoud Dept. of Veterinary Hygiene, Environmental Pollution and Management, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt. *Postal Code: 1221, Fax: 202-5725240* Email: ka-oud@link.net **Abstract:** This study was conducted to evaluate the effect of a probiotic mixture and chromium as food supplementation on broiler chicks' performance. The experiment was conducted, to determine the effect of a probiotic mixture (BiovetYC) and chromium chloride supplementation on growth performance, carcass traits and immune response against Avian Influenza virus from 0- to 42-d-old broiler chicks as well as to determine the anti-stress effect of the dietary probiotic mixture and chromium chloride supplementation on broiler chicks (0-42days old) when subjected to high stocking density (15 birds/m^2 in open-system) as a stress factor. Growth performance, carcass traits and Avian Influenza immune response were recorded. At 42-d of age, 50 birds were randomly selected from each group for blood samples collection and slaughtered for carcass traits. Stress indicators in blood (cortisol and L/H ratio) were measured. The current results revealed: (1) The activation effect of the probiotic mixture on growth performance (2) Chromium chloride supplementation improves growth performance, carcass traits, and immune response and had a strong anti-stress effect. [Nature and Science 2010;8(5):181-189]. (ISSN: 1545-0740). **Keywords:** Functional food; Performance; Immune response	Full Text
22	**Epidemiology of Brucellosis Among Farm Animals** H.A. Kaoud[1], Manal.M. Zaki[1], A.R. El-Dahshan[1] , Shaaima[1] .A. Nasr[1] Department of Veterinary Hygiene, Environmental Pollution and Management, Faculty of Veterinary Medicine, Cairo University, Giza 11221, Egypt *:Correspondence to: Dr. H. A. Kaoud , Dept. of Veterinary Hygiene ,Environmental Pollution and Management ,Faculty of Veterinary Medicine ,Cairo University ,Giza ,Egypt. *Postal Code: 1221, Fax: 202-5725240,* Email: ka-oud@link.net **Abstract:** : In this article we studied the epidemiology and the role of risk factors of Brucella infection in ruminants, besides the methods concerning the evaluation of biosecurity measures which are taken against the disease in farms. A cross sectional study was carried out on different Governorates representing allover Egypt to evaluate the potential major risk factors, mal- biosecurity practices and their role in the maintenance of the disease among farm animals. Serum samples (1670) were collected from 126 Herds / Flocks of sheep, goats and cattle and analyzed using Rose Bengal Plate test and iELISA test. A structured questionnaire was designed to identify and evaluate the role of risk factors for Brucellosis. .The results pointed out that, prevalence of brucellosis among herds/flocks of sheep, goats and cattle were; 26.66%, 18.88% and 17.22% respectively. And the seropositive percentages in blood samples were 21.20%, 14.5 % and 2.16% respectively. Major risk factors play a very important role in the prevention and maintenance of the disease among farm animals. The role and magnitude of risk factors varied but the presence of good sanitary measures in farms are considered as a protective factor, where R.R was less than 1 and the attributable risk was -0.01. [Nature and Science 2010;8(5):190-197]. (ISSN: 1545-0740). **Keywords:** Seroprevalence, Questionnaires, role and magnitude of risk factors, Relative and Attributable risk, Brucellosis. Abbreviations: R.R: Relative risk A.R: Attributable risk: Omega	Full Text

23	**Physico-Chemical Evaluations And Trace Metals Distribution in Water-Surficial Sediment of Ismailia Canal, Egypt** M. H. Abdo[1], S. M. El-Nasharty[2] [1]National Institute of Oceanography and Fisheries and water research center. [2]National Water Research Center **Abstract:** Ismailia Canal is considered as one of the most important irrigation and drinking water resources. During drought period the water level decreased and the concentrations of the most physico-chemical parameters were increased. Physical parameters include (air and water temperatures EC,TS,TDS and TSS).Chemical parameters (pH, DO, BOD, COD, CO_3^{--}, HCO_3^-, Cl^-, SO_4^{--}, Ca^{2+}, Mg^{2+}, Na^+, K^+, NO_2^-, NO_3^-, NH_3, PO_4^{3-}, TP and SiO_2^-). In addition to some trace metals (Fe, Mn, Zn. Cu, Pb, Cd, Al, As, Ba, Co, Cr, Ni, Sb, Se, Sn, Sr, V and Mo) in water and surficial sediment of Ismailia Canal during drought period were studied. [Nature and Science 2010;8(5):198-206]. (ISSN: 1545-0740). **Keywords:** Chemical evaluation, trace metals, water, sediment, Ismailia Canal	Full Text

Histopathologic study of the Antiestrogenic Nolvadex Induced Liver Damage in Rats and Vitamins Ameliorative Effect

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ABSTRACT: This study was designed to evaluate the effects of antiestrogenic Nolvadex (used for breast cancer treatment) on rat liver and the possible protective effects of vitamin C and/ or E. Material and methods: A total of 140 adult female albino rats were used; divided into seven groups; each containing 20 rats: First group: as control. Second group: orally daily dosed with Nolvadex 20 mg/kg b. w. for three weeks. Third group: orally given vitamin C (0.02 g/100 g b wt), 15 min before daily Nolvadex administration. Fourth group: given vitamin E (120 mg/Kg b.w), 15 min prior to daily Nolvadex administration. The fifth group was given combination of the two vitamins C & E (0.02 g/100 g b.w.) and (120 mg/kg b.w.) respectively, 15 min before daily Nolvadex administration. Each of the remaining two groups was daily given vitamin C (0.02 g/100 g b.w.) and/or E (120 mg/kg b.w.) for two weeks. Paraffin sections were used for histopathological , quantitative image analysis DNA ploidy and histochemical studies .Electron microscopy was performed. Results: Histopathological degenerative effects in the form of vacuolar degeneration, fatty changes and hydropic degeneration were noticed in Nolvadex treated rat liver. Karyolysis and karyorrhexis were also seen.Dysplasia and chromatin clumping were observed in scattered hepatocytes together with a decrease in DNA content (hypoploidy) and marked diminution of protein and mucopolysaccharides content.Histopathological, histochemical and ultra structural changes were diminished in rats treated with vitamins C and/ or E prior to Nolvadex. Conclusion: The treatment of rats with vitamins C and/or E prior to Nolvadex resulted in amelioration of the histopathological ,histochemical and ultrastructural changes of liver. [Nature and Science. 2010;8(5):1-15]. (ISSN: 1545-0740).

Key words: Histopathology – antiestrogenic - Nolvadex – liver – rat – Vitamins

1. Introduction

Breast cancer remains the most common malignancy in women world wide. Estrogen levels appear to be associated with an increased risk for the development of breast cancer (Lo. and Vogel 2004). In 1998 the National Surgical Adjuvant Breast and Bowel Project (NSABP) demonstrated that Nolvadex treatment reduced the incidence of both invasive and non -invasive breast cancer in population at high risk for disease (Tan- Chiu et al., 2003).

Tamoxifen (TAM), a non steroidal antiestrogen is used as a chemotherapeutic and chemopreventive agent for breast cancer (Goss and Stresses-Weipple, 2004). Tamoxifen is a nonsteroidal triphenylethyl compound that belongs to a class of selective estrogen receptor modulators (SERMs), binds to estrogen receptors (ERs) and elicits estrogen agonist or antagonistic responses, depending on the target tissue. Its estrogen antagonistic properties have made Nolvadex an important treatment modality for patients with breast cancer, especially those whose tumors are positive for ERsDray et al., 2000 reported a case of non-alcoholic steatohepatitis with cirrhosis in a woman receiving tamoxifen as adjuvant treatment for breast cancer. Nolvadex has been used as an agent for the treatment and prevention of breast cancer. However, long-term treatment of Nolvadex in women increases the risk of the developing endometrial cancer. The secondary cancer may be due to the genotoxicity of TAM (Kim et al., 2006). Nolvadex -induced non-alcoholic steatohepatitis (NASH) may increase the demand on oncologists, not only with regard to screening for diabetes, but also for the suggested link of NASH with high incidence of coronary heart disease (Osman et al., 2007). The incidence of toremifene-induced fatty liver was significantly lower than that induced by tamoxifen. Accordingly, in terms of fatty liver and NASH, toremifene is considered to be more appropriate agent than Nolvadex. (Hamada et al., 2000).

Nolvadex is liver carcinogenic in rats and has been associated with an increased risk of endometrial cancer in women (Curtis et al., 2004). Furthermore Nolvadex use has been associated with a 35% decrease in incidence of osteoporotic bone fractures (Decensi et al., 1998). In mice, TAM produced proliferative lesions in the oviduct and uterus (Srinivas et al., 2004) followed by uterine carcinoma (Newboid et al., 1997).

Nolvadex affects some types of visual pathway (Eisner et al., 2004). Woman taking Nolvadex suffer from damage of retina and corneal opacities. These changes may have no immediate effect on visual acuity, but may predispose the eye to latter problems including

cataracts (Epstein et al., 1997). Nolvadex induces menstrual irregularities in premenopasual woman. Amenorrhae (absence of menstrual cycle) often results and can be permanent (Sellman, 1998. Nolvadex can induce multinucleated giant cells and germinal epithelial sloughing, seminiferous tubules distortion and these changes are detrimental to male fertility (Dsouza, 2003).

Nolvadex and its metabolites, 4-hydroxytamoxifen (4OH-TAM), N-desmethyltamoxifen (DMT) and 4-OH-N-desmethyltamoxifen (endoxifen) exhibit antiestrogenic activities by competitively inhibiting the binding of potent agonists to the estrogen receptor (ER), thus antagonizing their proliferative effects (Johnson et al, 2004). Despite the high therapeutic index of TAM for breast cancer, there are concerns regarding the increased occurrence of uterine cancer as early as 2 years after initiating treatment (Fisher, 1994). Nolvadex is classified as a selective estrogen receptor modulator (SERM) as a result of its differential effects in breast and uterine tissues. A number of factors influence the specificity and efficacy of SERM-bound, ER-mediated gene expression, and the subsequent physiological effects (Fong et al., 2007).

Nolvadex has demonstrated genotoxic activity in the rat liver causing DNA adducts (Divi et al., 2001) unscheduled DNA synthesis, hepatic aneuploidy and mitotic spindle disruption (Phillips, 2001). For the formation of DNA adducts, metabolic activation of tamoxifen is indispensable; the metabolites α-hydroxytamoxifen (Beland et al, 1999) and its O-sulfate (Shibutani et al, 1998) are characterized as proximate and ultimate carcinogens, respectively. On the other hand, major metabolites such as N-desmethyltamoxifen, tamoxifen N-oxide and 4-hydroxytamoxifen are generally characterized as detoxification forms, although the further metabolites, α- hydroxyl forms of the N-desmethyltamoxifen and tamoxifen N-oxide, are able to produce the DNA adducts (Umemoto et al, 2000). Long term administration of Nolvadexinduced hepatoproliferative lesions and hepatocellular tumors in rats (Hirsimaki et al., 1993). In the stage before the formation of hyperplastic nodules in the liver, the genes of several hepatic enzymes responsible for not only detoxification but also activation of Nolvadex were activated and that in the later stage (in the nodules), the gene activation of detoxification enzymes was selectively maintained, while that of activation enzymes was suppressed. Thus, the overall change in the gene expression of the Nolvadex-metabolizing enzymes by Nolvadex treatment appears to be reasonable for the formation and growth of the hepatic hyperplastic nodules, because the increase in detoxification enzymes in the later stage would be expected to confer tamoxifen resistance to the induced nodules (Kasahara et al., 2002).

One of the proposed pathways for the metabolic activation of Nolvadex involves oxidation to 4-hydroxy tamoxifen, which may further oxidize to electrophilic Quinone methide (Costa et al., 2001). Nolvadex is well tolerated but causes steatosis in 43% of recipients (Nishino et al., 2003). Steatohepatitis can develop, particularly in overweight women (Bruno et al., 2005), and can lead to cirrhosis (Oien et al., 1999). Nolvadex administration decreases fatty acid synthase (FAS) expression in rat liver (Lelliott et al., 2005), and tamoxifen both uncouples and inhibits mitochondrial respiration (Larosche et al., 2007)

Antioxidants have been reported to play a significant role in protection against lipid peroxidation (Steenvooden and Henegouwen, 1999). Some investigators reported that antioxidants inhibit chemical carcinogenesis when the antioxidants are administered either prior or with carcinogen (Ames, 1983). Vitamin C (ascorbic acid) has a considerable antioxidant activity: it scavenges reactive oxygen species and may, thereby, prevent oxidative damage to the important biological macromolecules, such as DNA, proteins, and lipids (Konopacka, 2004). Ascorbic acid (vitamin C) exerts protective role against acute ultraviolet B-rays (Sunburn cell formation) (Meves et al., 2002), organophosphorous pesticides (Kurata et al., 1993), and could reduce aflatoxin induced liver cancer (Yu et al., 1994). Moreover vitamin C abolishes chromosome damage resulted from the effect of toxic substances (Trommer et al., 2002), and help to protect the body against pollutants (Masaki et al., 2000).

Because vitamin C is a biological reducing agent, it is also linked to preventive of degenerative diseases such as cataracts, certain cancer and cardiovascular disease (Barros et al., 2004 and Wang & sun, 2004). Increased vitamin C intake could possibly reduce and prevent nephrotoxic effect (Nagyova et al., 1994). It assists in the prevention of blood clotting and bruising; it strengthens the walls of the capillaries (Tousoulis et al., 2003) and it is also needed for healthy gum (Ambros et al., 1998). Vitamin C helps to reduce cholesterol levels, high blood pressure and preventing atherosclerosis (Napoli et al., 2004 and Zureik et al., 2004). It protects susceptible cells from genotoxicity associated with antiestrogen metabolite-4- hydroxy tamoxifen (4-OH tom) (Sharma and Slocum, 1999), and inhibit DNA adduct induced by tamoxifen (Sierens et al., 2001 and Sharma et al., 2003).

Vitamin E (alpha tocopherol) is the naturally occurring lipid soluble antioxidant (Butterfield et al., 1999). It is a powerful antioxidant that combats damaging free radical. It is important for reproduction, prevention of various diseases (Biri et al., 1998). It appeared significant for reduction of hot flash (Barton et al., 1998), toxicity of doxorubicin in tissue of rats

(Geetha et al., 1990). Alpha tocopherol prevents oxidation of LDL cholesterol and help to protect against atherosclerosis. Vitamin E exerted protective effects against cyanide induced tissue lesions in rabbits (Okolie and Iroanya, 2003) and protected neurons against oxidative cell death in vitro (Behl, 2000). Vitamin E maintained bone mineral density in ovariectomized rats and caused conflicting effect on bone calcium content (Norazlina et al., 2000), and could enhance the proliferative status of prostate gland (Yao et al., 1996). Moreover the increased level of enzyme in fibrosarcoma in rats was reduced by vitamin E administration (Vinitha et al., 1995). Vitamin E is used in the treatment of Elzheimer's disease through preventing brain cell damage by destroying toxic free radicals (Klatte et al., 2003 and Zandi et al., 2004) and in the treatment of disorders in the central nervous system (Vatassery et al., 1999).

Co administration of vitamin C and vitamin E reduces the Nolvadex induced hypertriglyceridemia (Babu et al., 2000). Vitamin C and alpha tocopherol alone reduce the growth of human melanoma (sk-30) cells in culture (Prasad et al., 1994).

The aim of the present study is to investigate the possible protective effect of vitamin C and vitamin E to ameliorate antiestrogenic Nolvadex induced histopathological, histochemical and ultrastructure changes in liver of rats.

2. Material and Methods

140 female albino rats weighting 130-160g were used in this study. The animals were divided into seven groups. Each group contained 20 rats.

Group (1): was kept as control.

Group (2): was given Nolvadex daily for two weeks at dose level of 20mg/kg b.w.

Group (3): was given vitamin C only at dose level of 0.01g/100g b.w. by stomach tube for two weeks.

Group (4) was given vitamin E only 100 mg/kg b.w. by stomach tube daily fro two weeks.

Group (5) was given vitamin C at dose level of 0.01g/100g b.w. (Padget and Barnes, 1964) by stomach tube, 15 min before Nolvadex administration daily for two weeks.

Group (6) was given vitamin E at dose level of 100 mg/kg b.w., 15 min before Nolvadex administration.

Group (7) was given combination of vitamin C at dose level of 0.01g/100g b.w. and vitamin E at dose level of 100mg/kg b.w., 15 min before Nolvadex administration daily for two weeks.

Histopathological and histochemcial studies:

The liver of different groups were removed and fixed in 10% formal saline. Paraffin sections 5 μm thick were stained with haematoxylin and eosin (Drury and Wallington, 1980) and Masson trichrome stain to demonstrate the collagen fibers (Masson, 1929). All sections were investigated by the light microscope.

DNA Ploidy studies:

Further sections were stained for DNA (Feulgen and Rosenbeck, 1942) and counterstained with Light Green. DNA analysis was performed by lecia Qwin 500 image cytomery in the department of pathology, National Research Center. For each section (100-120 cells) were randomly measured. The threshold values were defined by measuring control cells. The results are presented as histograms and tables which demonstrate the percentage of the diploid cells (2C), the triploid cells (3C), the tetraploid cells (4C) and the aneuploid cells (>5C). The DNA histogram classified according to Danque et al., (1993). Protein stain (Mazia et al., 1953) and mucopolysaccharids stain (Mac-Manus and Cason, 1950) were also performed.

The ultrastructural studies:

Sample processing for electron microscopy together with examining the thin sections and getting the electron micrographs was done in the Electron Microscope Unit, Institute of Ophthalmology. Small pieces of liver, about 1mm3 in size were prepared.

Thin sections 60-90 nm thick were prepared by using ultra cats/ FCS; the thin sections were mounted on copper grids, stained with uranyl acetate and lead citrate (Watson, 1958) and finally examined with transmission JEM- 100x IL electron microscope. Photographs using Kodak films and photographic paper were taken and examined. Further sections were stained for DNA (Feulgen and Rosenbeck, 1942) and counterstained with Light Green. DNA analysis was performed by lecia Qwin 500 image cytomery in the department of pathology, National Research Center. For each section (100-120 cells) were randomly measured. The threshold values were defined by measuring control cells. The results are presented as histograms and tables which demonstrate the percentage of the diploid cells (2C), the triploid cells (3C), the tetraploid cells (4C) and the aneuploid cells (>5C). The DNA histogram classified according to Danque et al., (1993). Protein stain (Mazia et al., 1953) and mucopolysaccharids stain (Mac-Manus and Cason, 1950) were also performed.

3. Results

- Histopathological results:

The liver of control rats revealed the characteristic hepatic architecture (Fig. 1, A).

No pathological changes could be noticed in the liver of rats treated with either vitamin C or vitamin E.

The liver of rats treated with Nolvadex only showed hydropic degeneration, nuclei with variable sizes and dysplastic cells (Fig. 1,B). Fatty changes, vacuolar degeneration, mitotic figure and fibrosis were seen (Fig.1,C). Dilation, congestion of blood sinusoid and peripheral chromatin clumping were also observed (Fig.1, D).

Concerning rats treated with vitamin C and Nolvadex in combination for two weeks, examination of liver sections showed marked diminution of hydropic degeneration, fatty changes and mitotic figure. No fibrosis and no chromatin clumping were noticed. While some hepatocytes still showed hypertrophy, others showed signs of degeneration in the form of karyolysis and karyorrhexis. The kupffer cells showed mild hypertrophy (Fig.1, E).

The rats treated with vitamin E and Nolvadex in combination, showed some protective effects as compared to the group of rats subjected to Nolvadex only. Examination of liver sections showed moderate hypertrophy of kupffer cells. Red blood cells are seen in the blood sinusoids. Focal area of necrosis was also noticed. The liver of rats subjected to combination of vitamin C and vitamin E prior to administration of Nolvadex showed some histological changes, but these changes were somewhat less than those of rats treated with Nolvadex only. Examination of liver sections showed focal necrosis and a number of binucleated cells (Fig. 1, F).

Examination of control liver sections showed normal distribution of collagen, which showed small amount of wavy fibrils (Fig. 2,A). Treated group with Nolvadex showed collagen fibrils that occurred as wavy fibrils either singly or fused together in dense bundles (Fig. 2, B). The liver of rats subjected to vitamin C and / or vitamin E prior to administration of Nolvadex showed improvement in collagen deposition and connective tissue fibers as compared to liver of rats treated with Nolvadex only (Fig. 2, C).

- **DNA Ploidy results:**

DNA content in all the studied groups:

In the present study, the Qwine 500 image analyzer was used to evaluate the DNA content. The image analysis system automatically express the DNA content of each individual cell measured then gave the percentage of each cell out of the total number of cells examined. Also, it classifies the cells into four groups; diploid (2C), proliferating cells (3C), tetraploid (4C) and aneuploid cells (>5C). The proliferating cells were further classified according to Lee et al. (1999) into; (<10%) low proliferation index, (10-20%) medium proliferation index and (>20%) high proliferation index.

Normal distribution of DNA content in the liver of the control group showed that 20.18 % of the examined cells contained DNA (<1.5C), 65.13% of the examined cells contained diploid DNA value (2C), 12.84% of the examined cells contained (3C) DNA value (medium Proliferation Index) and 1.83% of the examined cells at (4C) area (Histogram 1). The group treated with Nolvadex showed that 93.54% of the examined cells contained DNA (<1.5C) this means decrease in DNA content (hypoploidy) compared to the control. (Histogram 2).

In the present work the treatment of rats with Nolvadex along with vitamin C showed that 31.63% of the examined cells contained DNA (<1.5C), 61.22% of the examined cells contained diploid DNA value (2C), 6.12% of the examined cells contained (3C) DNA value (low Proliferation Index) and 1.02% of the examined cells at (4C) area (Histogram 3).

The group treated with Nolvadex along with vitamin E showed that 43% of the examined cells contained DNA (<1.5C), 51% of the examined cells contained diploid DNA value (2C), 6% of the examined cells contained (3C) DNA value (low Proliferation Index) The group treated with Nolvadex along with combination of vitamin C and E showed that 9.90% of the examined cells contained DNA (<1.5C), 86.13% of the examined cells contained diploid DNA value. (2C), 3.96% of the examined cells contained (3C) DNA value (low Proliferation Index). These results indicate that treatment with vitamin C & E showed DNA values comparable to the control values while, the group treated with Nolvadex showed decreased DNA values (hypoploidy).

- **Histochemical Results**

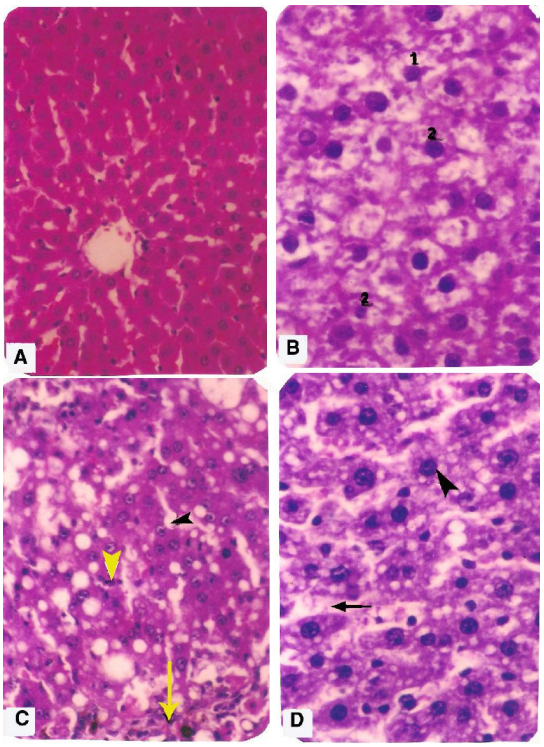
Examination of control liver sections showed moderate protein content in the cytoplasm of hepatocytes. Some nuclei showed deep protein content. After daily treatment of rats with an oral dose of Nolvadex for two weeks, the protein inclusions showed marked diminution in the cytoplasm of hepatocytes and staniability was mostly diffused. Slight increase in protein content was noticed in the case of rats subjected to vitamin C in combination with Nolvadex as compared to liver of rats subjected to Nolvadex only. Moderate increase in protein content in the cytoplasm of hepatocytes was also recorded in the case of rats treated with vitamin E in combination with Nolvadex as compared to rats subjected to Nolvadex only. The pretreatment of rats with combination of vitamins (vitamin C and vitamin E) along with Nolvadex showed marked increase in protein content in the cytoplasm of hepatocytes.

Examination of control liver sections stained with periodic acid schiff's (PAS) showed

mucopolysaccharide granules in the cytoplasm of hepatocytes; the peripheral zonal cells showed higher mucopolysaccharide content than the central zonal cells (Fig.3, A). Daily treatment of rats with tamoxifen only for two weeks induced marked decrease in stainability of PAS +ve materials (Fig.3 B). Daily administration of vitamin E in combination with tamoxifen showed moderate increase in mucopolysaccharides content in the cytoplasm of hepatocytes and mild increase in mucopolysaccharides content could be noticed in the case of rats subjected to vitamin E and tamoxifen as compared to rats subjected to Nolvadex only (Fig. 3 C). Co administration of vitamins (vitamin C and vitamin E) in combinatin with Nolvadex showed marked generalized increase in mucopolysaccharides content ino the cytoplasm of hepatocytes (Fig. 3 D).

Electron microscopic results:

Figure (4 A) showed electron micrograph of control liver cells. Hepatocytes of rats treated with Nolvadex only show areas of cytoplasmic dissolution, partial clumping of nuclear chromatin and corugated nuclear membranes. Mitochondria were swollen with dense matrix (Fig.4 B). Endoplasmic reticulum dilated cisternae with no obvious attached ribosomes (Fig.4 C). The treatment of rats with vitamin C or vitamin E showed improvement in the ultrastructural changes in the form of diminution of cytoplasmic dissolution and restoration of nuclear normal shape (Fig. 4 D).



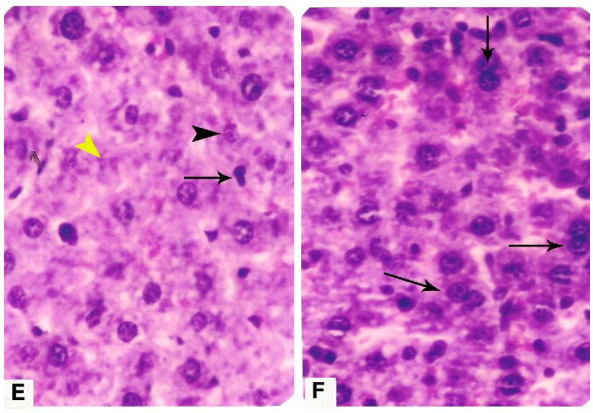


Figure (1): (A) Section in the liver of control rat showing normal histological structure of hepatic lobules and central vein (Hx.&E. X 200). (B) Section of the liver of a rat treated with Nolvadex showing hydropic degeneration (1), variable sized nuclei (2). Also seen, many pyknotic nuclei and some hepatocytes are devoid of nuclei (Hx.&E. X400). (C) Section of the liver of the same group showing lymphocytic infiltration (arrow), fatty changes, vacuolar degeneration (arrow head) and mitotic figures (yellow arrow head) (Hx.&E. X 200). (D) Section of the liver of a rat treated with Nolvadex showing dilation and congestion of blood sinusoids (arrow) and peripheral chromatin clumping (arrow head) (Hx.&E. X400). (E) Section in the liver of a rat treated with vitamin C along with Nolvadex showing no fibrosis, no fatty changes and no vacuolar degeneration. Karyolysis (black arrow head), karyorrhexis (yellow arrow head) & mild hypertrophy of Kuppfer cells (arrow) were noticed. The same results were obtained with Vitamin E along with Nolvadex (Hx.&E. X400). (F) Section of the liver of a rat treated with Nolvadex along with vitamin C and vitamin E showing scattered binucleated cells (arrows). (Hx & E X 400).

4. Discussion

Nolvadex is a triphenyl ethylene derivative commonly used in the treatment of breast cancer (Kennel et al., 2003 and Mati & Chen, 2003). Nolvadex is known to have varied biological effects ranging from complete estrogen antagonism to pure estrogen agonism dependeing upon its concentrations, the sex of animals and target organ (Williams, 1984). In humans and rats Nolvadex is predominantly antiestrogenic with residual estrogenic activities (Furr and Jordan, 1984).

Rat liver is an organ with especial sensitivity of developing tumors after exposure to many chemicals and drugs (Maronpot et al., 1995). The rat at which the liver tumors develop is known to be strongly influenced by Nolvadex's promoting effect on hepatocyte proliferation where sustained proliferation has also been associated with chronic cell death (Carthew et al.,

1996).

The microscopical appearance of liver in rats receiving 20mg/kg b.w. of Nolvadex by an oral route for two weeks was characterized by vacuolar degeneration and hydropic degeneration. Results of this work go in agreement with Hirsimaki et al., (1993) who noticed that the treatment of rats with Nolvadex at dose level of 45 mg/kg b.w. for two weeks caused vacuolar degeneration in the liver of rats. In controversy Kasahara et al., (2002) stated that no pathological changes could be noticed in the liver of rats treated with Nolvadex at dose level of 20 mg/kg b.w. for two weeks. Pathological altered cell foci and placental form of glutathione s-transferase (GST-P) positive foci were observed in the liver after 12 weeks. Treatment for 52 weeks resulted in the formation of liver hyperplastic nodules that strongly expressed GST-P. According to Badawy et al., (2002) the treatment of rabbit with Nolvadex at dose level of 14 mg/kg b.w. daily for 60 days induced histopathological changes in the testis in the form of vacuolar degeneration of spermatogenic cells, atrophied and collapsed seminiferous tubules with asospermia.

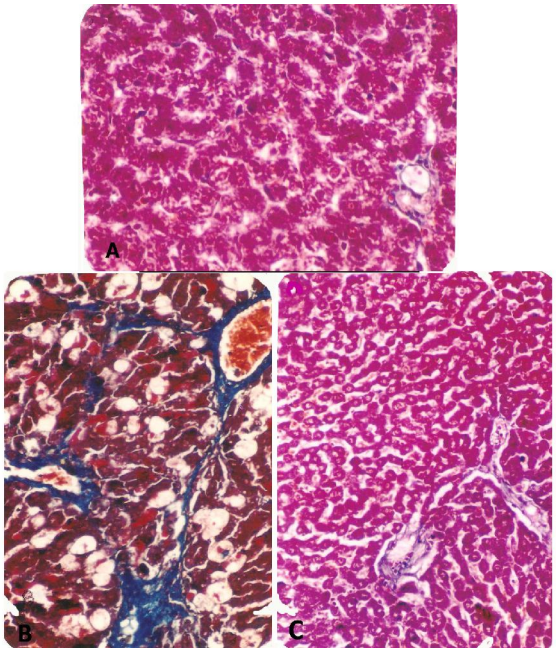


Figure 2: Section of the liver of a rat showing collagen (A): control. (B): treated group with Nolvadex showing collagen fibrils occurred as wavy fibrils either singly or fused together in dense bundles especially in and around the portal area, around the central vein and in-between hepatocytes. (C): treated group with Nolvadex along with vitamin C showing mild amount of fibrous tissue in the portal area. (Masson trichrome stain x 200).

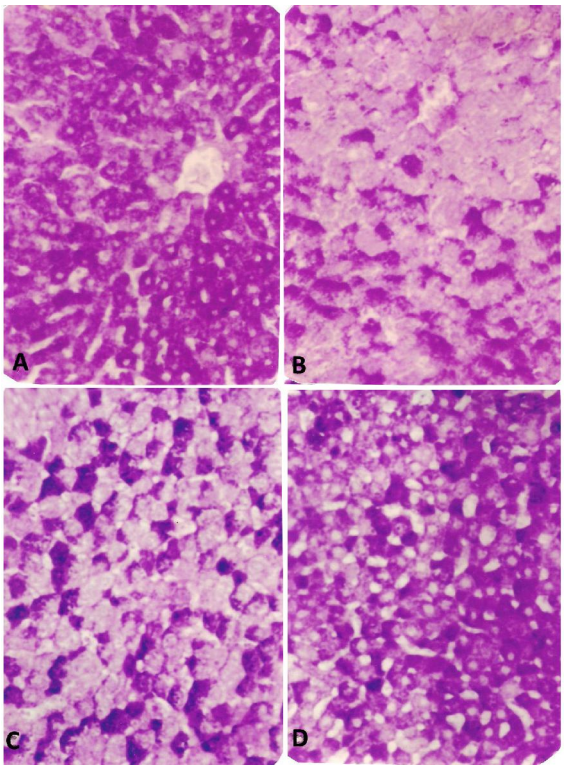
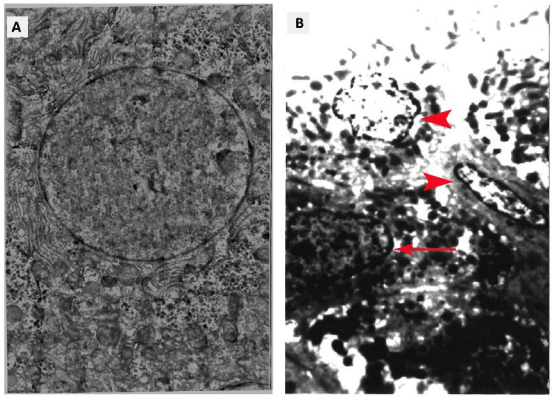


Figure (3): Section of the liver of a rat showing PAS+ ve materials in the cytoplasm of hepatocytes (A) control. (B): Treated with Nolvadex: showing decreased stainability of PAS + ve materials. (C): Treated with vitamin E along with Nolvadex showing mild improvement in PAS +ve materials. The same results were obtained from the group of rats treated with Vitamin C along with Nolvadex. (D): Treated with Nolvadex along with vitamin C and vitamin E showing increased PAS+ ve materials. (PAS reaction x 400).



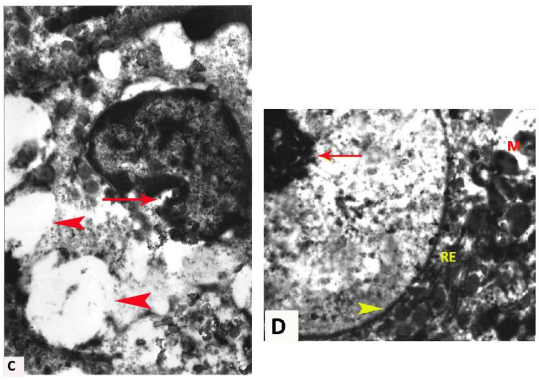
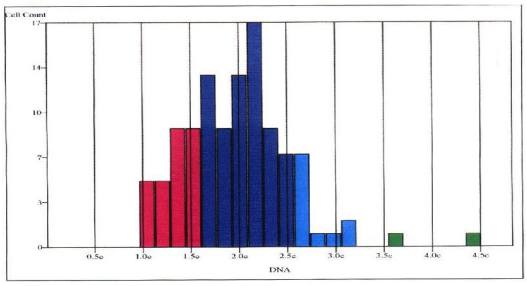


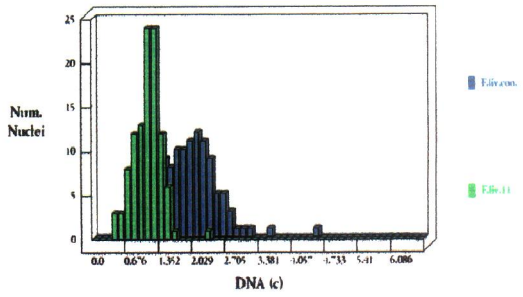
Fig. 4: (A): Photoelectron micrograph of a control adult albino rat (x8000), (B): Electron micrograph of hepatocytes treated with Nolvadex showing swollen mitochondria with dense matrix, partial clumping of nuclear chromatine (arrow) and nuclear shrinkage (arrow head) (x6000). (C): Another field of electron micrograph of hepatocytes treated with Nolvadex showing dilatation of endoplasmic reticulum with no obvious ribosomes (arrow head) the nuclear membrane is corrugated. (arrow) (x 10000). (D): treated group with a combination of vitamin C & E along with Nolvadex showing well-defined nucleolus (arrow) and nuclear envelope (arrow head). Also seen normal-shaped rough endoplasmic reticulum (RE), although mitochondria (M) are still dilated. (x 6000).

Histogram (1): DNA Ploidy of the Control Rat Liver



Liver

Histogram (2): DNA Ploidy of Rat Liver Treated with Nolvadex.



Histogram (3): DNA Ploidy of Rat Liver Treated with Nolvadex along with Vitamin C .

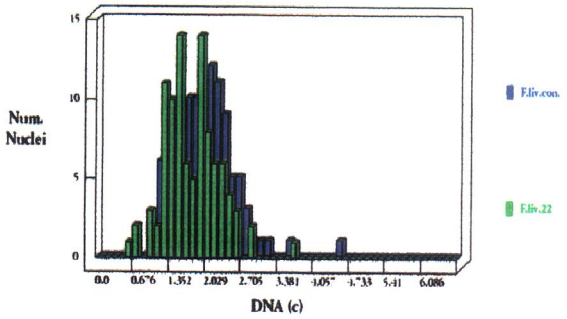


Fig. 5: DNA Ploidy of Rat Liver

Vacuolation observed in the present study may be due expansion of the mitochondrial intermembrane space and extension of the outer mitochondrial membrane (Higgins et al., 2003) consistent with ultrasturctural changes observed in the present study. Vacuolation may be due to disturbance of ionic milieu of the cell with consequent retention of water and sodium leading to cellular swelling (Jaarsma et al., 2001 and Wiedemann et al., 2002).

Nolvadex was associated with higher risk of development of non-alcoholic steatohepatitis only in overweight and obese women (Bruno et al., 2005). Adjuvant Nolvadex increases the incidence of fatty liver in patients with breast cancer (Liu et al., 2006). In the present work fatty changes were observed in the liver after treatment of rats with Nolvadex only. Fatty change observed in the present work may be due to damage in rough endoplasmic reticulum confirmed by electron microscopic changes observed in the present work, impaired protein synthesis and inhibition of lipoprotein manufacture. The latter is involved in the transport to hepatic triglycerides to extrahepatic tissue and its inhibition results in accumulation of fat in the cytoplasm (Deboyser et al., 1989). According to Marzouk (1995) mitochondria are known to contain many of the enzymes necessary for the metabolism of triglycerides (i.e. fatty acid oxidases). This leads to another explanation that the fatty changes observed in the present work may be due to mitochondrial damage.

Nolvadex decreases hepatic triglyceride secretion, and it accumulates electrophoretically in mitochondria, where it impairs β-oxidation and respiration. Nolvadex also inhibits topoisomerases and mitochondrial DNA synthesis and progressively depletes hepatic

mitochondrial DNA in vivo. These combined effects could decrease fat removal from the liver, thus causing hepatic steatosis despite the secondary down-regulation of hepatic fatty acid synthase expression (Larosche et al; 2007).

In the present work the treatment of rats with Nolvadex only induced foci of necrosis, signs of degenereation in the form of, karyolysis, karyorhexis and fibrosis of hepatocytes. Displastic cells could be noticed. These results were in agreement with Hirsimaki et al. (1993) they noticed that the treatment of rats with Nolvadex only induced area of hepatic necrosis and apoptosis. In controversy Coe et al., (1992) reported that subcutaneous injection of Nolvadex alone at dose level of 0.1mg (5mg) did not cause degenerative changes or neoplastic lesions in Armenian hasmster. Also Sauvez et al. (1999) they found that the treatment of rats with Nolvadex induced biliary proliferation and peribiliary fibrosis and degeneration of hepatocytes. Coinciding with Smith et al. (2000), they reported that Nolvadex revealed tissue damage and carcinogenic changes in rats by an oral route.

The hepatic fibrosis observed in the present study may be due to increased level of malondialdehyde (MDA) and decreased production of superoxide dismutase (SOD) and glutathione peroxidase in the liver cells. Oxidative stress plays a role in the development of hepatic fibrosis and degeneration (Duthie et al., 1995). Accordin to Hu et al. (2003) one of the proposed pathways for the metabolic activation of Nolvadex involves oxidation to 4- hydroxytamoxifen which may futher oxidized to an electrophilic quinone methide and may affect cytochrome P-450. However, Badawy et al. (2002) they reported that the administration of Nolvadex caused the production of reactive oxygen species (ROS) which can damage the cellular elements. Oxidative modifications of DNA, protein and lipid by ROS play a role in ageing and disease.

In the present work the pathological changes observed in the liver of rats due to oral route of Nolvadex may be due to lipid peroxidation and free radicals. Free radical may propagate damage in the endoplasmic reticulum and oxidation of membrane component of the liver cells consistent with ultrastructural changes observed in the present work. Oxidation has been shown to be associated with apoptosis (Programmed cell death) (Mohan et al., 2003). The effects of Nolvadex may be neutralized by radical scavenger antioxidants such as vitamin C and /or vitamin E (Babu et al., 2000).

In the present work the oral administration of vitamin C prior to administration of Nolvadex showed some improvement in pathological changes in comparison with group of rats subjected to Nolvadex only. According to Okolie and Iroanya (2003) the supplementation of vitamin C led to marked reduction of histopathological degeneration in tissues by toxic agents. Vitamin c exerted antioxidant action and free radical scavenger (Barros et al., 2004). Coinciding with Sharma and Slocum (1999), they reported that vitamin C adverse some pathological changes induced in liver of rats treated with Nolvadex. According to Sharma et al., (2003) ascorbic acid reduced the level of alpha hydroxytamoxifen substantially (68.9%) when exposure of endometrial explanted culture to 100 micro M Nolvadex and 1mM ascorbic acid.

The treatment of rats with vitamin E conditioned the adverse effect of Nolvadex in liver of rats. According to Custodio et al. (1994) oral administration of antioxidant such as vitamin E has a high protective capacity of vitamin against lipid peroxidation. Also Inal and Kahraman (2000) they reported that vitamin E exerted the antioxidant action and can interfer with the production of reactive oxygen species and other reactive oxygen species scavengers such as glutathione peroxidase, superoxide dismutase, xanthine and increase glutathione in cells.

In the present work the treatment of rats with vitamin E prior to administration of Nolvadex showed marked diminution of vacuolar degeneration and fatty changes. Crewe et al. (2002) found that the vitamin E may be possible therapeutic agent with potential applications against pathological states due to reactive oxygen species. Coinciding with Babu et al. (2000) they demonstrated that vitamin E adverse some pathological changes induced in tissue of rats treated with Nolvadex Vitamin E also decreased dimethyl valeronitrile induced phospholipid peroxidation. Also Mohan et al. (2003) reported that the pretreatment of rats with vitamin E inhibited apoptosis by acting a quite upstream in the apoptosis cascade at the mitochondrial level as well as down stream at the caspase.

In the present work the treatment of rats with combination of the two vitamins (vitamin C and vitamin E) before an oral route of Nolvadex showed more improvement in the pathological changes. Results of this work go in agreement with Prasad et al. (1994) who reported that a mixture of vitamins (vitamin C and vitamin E) were more effective in reducing the effect of Nolvadex on tissue damage and they were more effective in reducing growth of human melanoma cells. In the previous studies of Babu et al. (2000) showed that the combined effect of Nolvadex, vitamin C and vitamin E encumber the abnormalities investigated by Nolvadex.

The measurement of DNA ploidy has the advantages of being precise, rapid and quantitative, (Filipe et al., 1991). Image cytometry for DNA

quantification has become an established technique in the field of analytical cellular pathology, used as an important parameter providing significant information about the biological behavior of tumors (Cohen, 1996). Concerning ploidy results, the treatment of rats with Nolvadex only resulted in decreased nuclear DNA content, 93.54% of the examined cells contained DNA <1.5c i.e hypoploidy and low proliferation index. 6.54% of the examined cells contained diploid DNA value. These results go in agreement with (Phillips, 2001 and Cardoso et al. 2003) who reported that, the mechanism by which Nolvadex causes liver cancer in rats is through accumulation of DNA damage, caused by adduct formation between Nolvadex and hepatocytes DNA. According to Süzme et al. (2001) Nolvadex injections induced DNA aneuploidy, but did not stimulate proliferation in the liver as estimated by S-phase fraction. Friedlander et al. (1984) found that the normal human somatic cell contains 46 chromosomes which is referred diploid (2n), the gametes contain one set of chromosomes (23) referred to as haploid. While a cell with fewer or more than 46 chromosomes is described as hypoploid or hyperploid respectively. Also Umenoto et al. (2001) found that DNA adduct is formed when chemical carcinogen or their metabolites bind covalently with DNA. On the other hand Sierens et al. (2001) and Kasahara et al. (2003) found that Nolvadex has demonstrated genotox activity in rat liver causing unscheduled DNA synthesis and hepatic aneuploidy. They added that tamoxifen causes hepatic tumors through a genotoxic mechanism. Moreover Dragan et al. (1998) found that the treatment of rats with Nolvadex resulted in a shift of DNA from tetraploid to diploid. Marrero et al. (1996) reported that the cellular DNA content is abnormal at an early stage in dysplasia and may even predate it. Increasing value of abnormal DNA content is related to the severity of dysplasia. Also Carthew et al. (1997) explained that the endogenous DNA damage was not generated by estrogen receptor mechanisms but by microsomal cytochrome p-450 mediated redox cycling of catechol estrogen.

In the present work the treatment of rats with vitamin C along with Nolvadex showing improvement in DNA content as compared to group of rats treated with Nolvadex only. According to Nefic (2001) vitamin C (ascorbic acid) is an antioxidant that can scavenge free radicals and protect cellular macromolecules, including DNA, from oxidative damage induced by different agents. Some studies indicated that vitamin C is much more than just an antioxidant; it regulates the expression of some genes participating in apoptosis or DNA repair processes (Konopacka, 2004). Also,vitamin C provides high ability to decrease the number of aneuploid DNA value. Tarin et al., (1998) reported that the DNA aneuploid and diploid were highly increased in mouse treated with some toxic agents and decreased DNA aneuploid after administration of vitamin C. They added that mixture of vitamins C and vitamin E induced more improvement in DNA content.

In the present work the treatment of rats with Nolvadex only showed marked diminution in protein content. These results disagreement with Kulesar and Gergely (1991) stated that Nolvadex caused protein synthesis in healthy and in injured liver. These results were in agreement with Gong et al. (1999) who reported that the Nolvadex or 4-hydroxytamoxifen caused decrease in mRNA and protein levels depending on time and dose. Also Divi et al. (2001) noticed that Nolvadex induces the formation of hepatic enzyme altered foci that have lost the capacity to metabolize the drug to DNA binding species. Nolvadex induced modified mitochondrial DNA or Nolvadex modified protein. On the other hand Matin et al. (1987) showed that subcellular fractionation of mouse liver showed that 82% of the antiestrogen binding protein was associated with the rough endoplasmic reticulum where it was confined to the membranous component. The antiestrogen binding protein was also present in smooth endoplasmic reticulum, nuclei and cytosol. High affinity of protein was recorded in tissue of mouse treated with Nolvadex In the present work the treatment of rats with vitamin C and/or vitamin E prior to administration of Nolvadex produced more improvement in protein content. These results are in agreement with Sierens et al. (2001) who stated that the antioxidant species may act in vivo to decrease damage of protein content in tissues. However Sharma et al. (2003) noticed that the antioxidants vitamin C & vitamin E play an important role in stimulating intercellular signals indirectly for activation of gene responsible for protein synthesis related to DNA repair.

Results of the present work showed that the oral administration of rats with Nolvadex produced marked diminution in mucoploysaccharides content. Results of this work go in agreement with Kulesar and Gergely (1991) who found that Nolvadex caused moderate glycogen loss in liver lesion. According to Hirsimaki et al. (1993) the amount of smooth endoplasmic reticulum appeared to be increased in some cells after administration of Nolvadex at the dose level of 45 mg/kg at total period of 52 weeks.

Depletion of glycogen that was observed in the present study was most probably consequent to hydropic and fatty degeneration manifested in this work, or due damaging effect of Nolvadex on the cytoplasmic organelles and the associated enzymes. However Poop and Cattley (1991) reported that the decrease in mucopolysaccharides content in tissues may be due to disturbed role of Golgi apparatus which is responsible

for synthesis of polysaccharides.

In the present work the treatment of rats with Nolvadex only showed swollen mitochondria with dense matrix. According to Hirota (1997) Nolvadex induced mitochondrial disappearance of cristae. However Hoyta et al. (2000) stated that high concentration of Nolvadex (100micro M) caused mitochondrial depolarization. Also Andreassen et al. (2000) reported that mitochondrial dysfunction can lead to energy deficiency, ionic imbalance, elevated reactive oxygen species (ROS) and oxidative damage. The mitochondrial vacuolation and swelling represent an accelerated form of mitochandrial damage caused by high level of mutant superoxide dismutase accumulation (Wang et al., 2002).

The liver of rats treated with Nolvadex only showed dilated endoplasmic reticulum with no obvious attached ribosomes. According to Hirota (1997) Nolvadex induced damage of granular endoplasmic reliculae. The dilatation of endoplasmic reticulum may be the cause of vacuolation of the cytoplasm observed by light microscope in the liver of rats treated with Nolvadex only. The dilatation of rough endoplasmic reticulum was considered by Robbin et al. (1984) to be reaction to cell injury. Detachment of ribosomes most probably reflected a disturbance in protein synthesis confirmed by histochemical changes observed in the present work. According to Traynor and Hall (1981) the increase of protein catabolism is a major effect of the body's response to stress.

Conclusion:

Nolvadex treatment induces liver damage that was performed by histopathological, histochemical and ultrastructural changes. These changes may be due to the production of reactive oxygen species (ROS) which could damage the cellular elements. The using of vitamin C and/ or vitamin E ameliorate the harmful effects of Nolvadex. This protection may be due antioxidant action which can interfere with production of reactive oxygen species scavengers such as glutathione peroxidase, superoxide dismutase, xanthine and increase glutathione in cells.

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anure on the dry matter components of ucumber varieties

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Abstract: The effects of farmyard manure on the dry matter content of two cucumber varieties (Ashley and Palmetto) was evaluated at the Teaching and Research Farm of the Ambrose Alli University, Ekpoma, Nigeria Lat 6^{o} 45^{1} N and Long 6^{o} 08^{1}E..The farmyard manure was applied at the rates of 0, 5 and 10t/ha. The layout was a 2 x 3 factorial scheme with three replicates. The result of the study showed that increasing the farmyard rates led to an increase in the dry matter weights of the two varieties of cucumber. Farmyard manure at 10t/ha increased the dry matter content of cucumber. Palmetto was however more responsive to FYM application, with the rate of 10t/ha out yielding the control by 60% in contrast to Ashley, in which 10t/ha of FYM out yielded the control by only 30 %. [Nature and Science. 2010;8(5):16-22]. (ISSN: 1545-0740).

Keywords: Farmyard manure, two cucumber varieties, dry matter accumulation and yield

Introduction

Cucumber (*Cucumis sativus* L [2n = 24]) is a high nutrient demanding crop and performs poorly on nutrient deficient soils leading to low yields, bitter and misshapen fruits (Grubben and Denton, 2004). The fruits that result are not marketable hence farmersô income is reduced. Farmyard manure which is a source of plant nutrition has been used as a soil conditioner since ancient times and its benefit has not been fully harnessed due to large quantities required in order to satisfy the nutritional needs of crops (Makinde *et al.*, 2007). The need for renewable forms of energy and reduced cost of fertilizing crops, have revived the use of organic manures worldwide (Ayoola and Adeniran, 2006). Improvement in environmental conditions and public health are important reasons for advocating increased use of organic materials (Ojeniyi, 2000; Maritus and Vleic, 2001). Also because it is bulky, the cost of transportation and handling constitute a constraint to its use by peasant farmers. Farmyard manure acts as an alternative source of fertility enhancement for inorganic fertilizers as they release nutrients slowly and steadily over longer periods of time and also improve the soil fertility status by activating the soil microbial biomass (Ayuso *et al*., 1996; Belay *et al*., 2001).

Dry matter is one of the measures of plant growth (Noggle and Fritz, 1983) and it reflects the relative growth rate as regards to net assimilation rate (Ibeawuchi, 2004). Jones (1976) stated that dry matter can be influenced by farmyard manure application and this is a function of crop species and soil fertility. Silva *et al*. 2004 verified that manure increased green ear yield and grain yield in two corn cultivars. Ramamurthy and Shivashankar (1996) in their experiment found that organic matter improved the plant height, dry matter production at different stages of crop growth and yield attributing characters of corn like number of cobs per plant, shelling percentage, 100 grain weight, protein content of grains and yield per hectare.

The objective of this study was to determine the effects of farmyard manure on the dry matter components and yield of two cucumber varieties.

Materials and Methods

The experiment was conducted at the Teaching and Research farm of the Ambrose Alli University Ekpoma on Lat. 6^{0} 45^{1} N and Long. 6^{0} 08^{1} E in a forest, savanna transition zone of Edo State, Nigeria. The area is characterized by a bimodal rainfall pattern with a long rainy season which starts in late March and the short rainy period extends from September to late October after a dry spell in August .The soil order is a ultisol and the site is classified locally as kulfo series (Moss, 1957).

The site was left fallow for three years after it was cropped to maize, yam and cassava for two years prior to the establishment of the experiment. A composite soil sample was collected from 0-30 cm depth prior to planting before the incorporation of farmyard manure to determine the pH and the nutrient status of the soil. Soil pH was analyzed by 1:2 in H_2O, total N content was determined by Kjeldahl method (Bremner, 1965); available phosphorus was analyzed using the modified method of Walkley and Black (Nelson and Sommers, 1982). The farmyard manure was collected from a deep litter pen of the Poultry Unit of the Teaching and Research Farm of Ambrose Alli University Ekpoma and left to decompose for three months.

ugust, ey and 75cm by 75cm and later thinned after two weeks to one seedling per stand to give a population of 17,777.8 plants per hectare. The organic fertilizer treatments imposed were three levels of farmyard manure, (0, 5 and 10t/ha).

The experiment was laid out in a 2 x 3 factorial scheme with three replicates. The plot size was 3.75 x 3.75 metres with 2 - metre pathways. The farmyard manure was uniformly spread on the plots and a West Indian hoe was used to turn the manure into the soil two weeks before planting. Hoe weeding was carried out at 3 and 5 weeks after planting. Insect pests were controlled with lamdacyahalothrin as Karate at biweekly intervals for effective insect control.

Cucumber vine length was measured by using a flexible tape rule. Number of leaves was assessed by visual count of the green leaves and the leaf area was assessed by the use of dry weight disc method based on the work of Rhoads and Bloodworth (1964).

For dry matter determination, two plants were cut from their bases at each sampling period of 4, 6 and 8 weeks after planting and separated into leaves, stems and reproductive parts (flowers and fruits). These parts were enclosed in labelled envelops and oven dried at 70^0C until a constant weight was attained. These parts were then weighed and recorded (leaf, stem and fruit and total dry matter weights). At every harvest, the fruit girth was assessed by using a vernier calliper, the fruit length was measured by using a flexible tape before the fruits were weighed using a 10kg scale. The cumulative weights of the entire harvests (10 times) were summed up and extrapolation made of total yield /ha.

Results

The soil of the experimental site was a loamy soil with a pH of 5.8. The organic matter content was medium and the Nitrogen content was moderate. The soil available P was moderate and the exchangeable K was low (Table 1).
The farmyard manure had an almost neutral pH.. The organic matter content was adequate and the N and P contents were high and the K content was medium

The mean vine length of two cucumber varieties at 4, 6 and 8 WAP as affected by farmyard manure rates are shown in Table 2a. At 4 WAP, the mean vine length of two cucumber varieties ranged from 20.28 to 41.83cm. The Palmetto variety produced the longest vine when 10t/ha of FYM was applied and Ashley variety, the shortest. The differences between the various rates of FYM were significant ($P<0.05$). The mean vine length for the two cucumber varieties at 6 WAP ranged from 88.26 to 173.66cm. The Palmetto variety produced the longest vines when 10t/ha of FYM was applied and Ashley variety, the shortest and there were significant differences between the various rates of FYM ($P<0.01$). The mean vine length of two cucumber varieties assessed at 8 WAP ranged from 191.51 - 287.50cm. The Palmetto variety produced the longest vine when 10t/ha of farmyard manure was applied which was similar to the Ashley variety at the same rate of application and the shortest vine was recorded in control plots of Ashley variety.

The mean number of leaves for the two cucumber varieties as affected by the application of farmyard manure at 4, 6, and 8 WAP are shown in Table 2a. At 4 WAP, the number of leaves ranged from 5.74 to 13.81. The highest number of leaves was recorded in the Palmetto variety at the 10t/ha FYM rate and the control of both varieties, the lowest and there were significant differences between the treatment means ($P<0.05$). The mean number of leaves at 6 WAP, ranged from 26.69 to 51.77. The Palmetto variety had the highest number of leaves at 10t/ha FYM rate that was significantly different ($P<0.05$) from the same rate for the Ashley variety. Applying FYM rate at 5t/ha resulted in similar number of leaves for both varieties. At 8 WAP, the number of leaves ranged from 31.60 to 55.79. The Palmetto variety had the highest number of leaves at the 10t/ha FYM rate which was similar to the Ashley variety at the same level of farmyard manure application. At 5t/ha rate both varieties were not significantly different from each other.

The mean number of branches as affected by farmyard manure rate (FYM) is shown in Table 2b. At 4 WAP, the mean number of branches of two cucumber varieties ranged from 0.68 to 1.95. The Palmetto variety had the highest number of branches at 10t/ha rate of FYM, which was however similar to the Ashley variety at the same rate of application. At 5t/ha FYM rate, both varieties had similar mean number of branches, which were significantly different from the control ($P<0.05$). At 6 WAP, the mean number of branches ranged from 9.52 to 18.56. The Palmetto variety had the highest number of branches which was significantly different from Ashley variety ($P<0.05$) at 10t/ha of FYM application. At 5t/ha rate of FYM, both varieties had similar mean number of branches which was significantly different from the control ($P<0.05$). The mean number of branches at 8 WAP ranged from 10.32 to 19.04. The highest number of branches was recorded at 10t/ha for both varieties, which was significantly different ($P<0.05$) from the other rates of FYM application. At 5t/ha, both varieties had

for the 5t/ha lmetto control (P<0.05).

The mean leaf area per plant of two cucumber varieties at 4, 6 and 8 WAP as affected by farmyard manure rates are shown in Table 2b. At 4 WAP, the mean leaf area ranged from 531.45 to 1017.56cm^2. Applying FYM at the rate of 5t/ha and 10t/ha for both varieties resulted in values which were similar but were significantly different from the control (P<0.05). The mean leaf area per plant at 6 WAP ranged from 3025.93 ï 4210.30cm$^{2.}$ The Palmetto variety produced the highest leaf area at 10t/ha which was similar to the Ashley variety at the same rate and the lowest value was observed in the control of both varieties. The differences between the various farmyard manure rate were significant (P<0.05). The mean leaf area at 8 WAP ranged from 3458.14 to 5035.68 cm^2. The Palmetto variety had the highest leaf area at 10t/ha and the Palmetto control, were the lowest. There were significant differences between the various treatments (P<0.05).

The mean dry weight of leaves per plant of two cucumber varieties as affected by farmyard manure rate at 4, 6 and 8 WAP are presented in Table 3a. At 4 WAP, the mean dry weight of leaves ranged from 4.04 to 10.74g. . The application of 5t/ha of farmyard manure on the two cucumber varieties significantly (P<0.05) produced more leaf dry weight than the control. The 10t/ha rate of farmyard manure significantly produced the highest mean leaf weight per plant for the two varieties. The application of farmyard manure to the cucumber varieties at 6 WAP produced a mean leaf dry weight of 18.50 - 25.18g. The Palmetto variety produced the highest mean leaf dry weight, which was significantly different (P<0.05) from the Ashley variety when 10t/ha FYM was applied. The 8 WAP sampling period witnessed a range of 34.95 to 48.54g of leaf dry weight. The highest mean dry weight was recorded at the 10t/ha farmyard manure rate for both varieties. The Palmetto variety at 5t/ha produced leaf dry weight that was significantly different (P<0.05) from the Ashley variety and the control.

The mean stem dry weight per plant of two cucumber varieties as affected by farmyard manure rate is presented in Table 3a. At 4 WAP, the mean dry weight ranged from 1.97 to 2.38g. The application of farmyard manures at 10t/ha produced the highest stem dry weight for the two varieties. At lower FYM rate of 5t/ha, the weights of the two varieties were similar but significantly different from the control (P<0.05). At 6 WAP, the stem dry weight of two varieties ranged from 5.17 to 8.10g. The highest mean stem dry weight was recorded at 10t/ha FYM for the two varieties and they were significantly different (P<0.05) from lower rate of 5t/ha and the control. At 8 WAP, the mean stem dry weight ranged from 13.07 to 15.13g. The highest stem dry weight was recorded in the Ashley variety at 10t/ha FYM rate which was similar to the Palmetto variety at the same rate of FYM application. At lower rate of 5t/ha, the two varieties had values, which were similar but was significantly different (P<0.05) from the control.

The mean fruit dry weights per plant as affected by farmyard manure rate are shown in Table 3b . At 6 WAP, the fruit dry weight ranged from 2.42 - 3.368g. Increasing FYM rate increased the cucumber fruit dry weight. The application of farmyard manure at 5t/ha and 10t/ha was similar for both varieties but was significantly different from the control (P<0.05). At 8 WAP, the fruit dry weight of two cucumber varieties ranged from 16.87 to 31.09g. The application of farmyard manure at 10t/ha rate resulted in the highest fruit dry weight that was similar for the two varieties.

The mean total dry weight of two cucumber varieties at 4, 6 and 8 WAP as affected by farmyard manure rate are shown in Table 3b. At 4 WAP, the mean total dry weight ranged from 6.01 to 13.12g. The use of farmyard manure at the rate of 10t/ha resulted in total dry weight which had similar values for both varieties and significantly different (P<0.05) from the rate of 5t/ha and 0t/ha. At 6 WAP, the response of two cucumber varieties to varying rates of farmyard manure ranged from 26.11 - 36.96g. The highest total dry weight was observed in the Palmetto variety when 10t/ha of FYM was applied and the control had the lowest. The differences between the treatment means was significant (P<0.05). At 8WAP, the total dry weight ranged from 64.89 ï 94.73g. As the rates of application increased, the total dry weight also increased. The Palmetto variety at 10t/ha FYM rate produced the highest total dry weight while the control of Palmetto produced the lowest. The differences between the treatment means was significant (P<0.05).

The mean number of fruits per plant as affected by varying rates of farmyard manure is presented in Table 4. The fruit number per plant ranged from 5.05 to 11.51. The Ashley variety produced the highest mean fruit number per plant at the 10t/ha of FYM application rate and the lowest number was from the control plots.

The mean fruit length and mean fruit girth of the two cucumber varieties as affected by farmyard manure rate are presented in Table 4. The fruit length ranged from 15.07 to 15.95cm. The application of 5t/ha and 10t/ha of farmyard manure produced longer fruits which were significantly different (P<0.05) from the control. Data on the fruit girth ranged from

10t/ha
10t/ha
were
significantly different (P<0.05) from the control.

The fruit weight per plant and fruit yield per hectare of the two cucumber varieties as affected by farmyard manure rate are presented in Table 4. The fruit weight per plant ranged from 1.39 to 2.24kg. The application of farmyard manure at 10t/ha for the two varieties produced the highest number of fruits per plant which was significantly different (P<0.05) from the 5t/ha and the control. The fruit yield per hectare of two cucumber varieties ranged from 24,881.46 to 39,853.18kg/ha. The Palmetto variety had the highest fruit yield per hectare at 10t/ha of farmyard manure application and the control of Palmetto produced the lowest. Fruit weight and yield increased significantly (P<0.05) with increase in the rate of FYM applied. There was no significant difference in the fruit weight and the yield of the two varieties. Palmetto was however more responsive to FYM application, with the rate of 10t/ha out yielding the control by 60%, in contrast to Ashley, in which 10t/ha of FYM out yielded the control by only 30 % (Table 4).

Discussion

The two varieties of cucumber responded significantly to the applied farmyard manure in terms of growth parameters, dry matter accumulation and yield and yield components. This is in line with Jones (1976) who stated that dry matter can be influenced by farmyard manure application and this is a function of crop species and soil fertility. The growth characters and dry matter of two cucumber varieties at 4, 6 and 8 weeks after planting and the number of fruits per plant, fruit weight per plant and yield per hectare were influenced by the application of farmyard manure and this is in line with the findings of Raramurthy and Shivashankar (1996) who stated that organic matter improved the plant height, dry matter production at different stages of crop growth and yield attributing characters of corn like number of cobs per plant, shelling percentage, 100 grain weight, protein content of grains and yield per hectare.

The application of farmyard manure increased the dry matter and yield. This increase could be attributed to nutrient availability and its uptake by the plants. This dry matter accumulation is a result of nutrient uptake and one of the measures of plant growth (Noggle and Fritz, 1983).The result of the study has shown that farm yard manure at any rate increased dry matter accumulation of the two varieties of cucumber and response was up to the highest level applied (10t/ha). This dry matter accumulation affected the number of fruits per plant, fruit length and fruit girth and the yield per hectare. This is in agreement with the findings of Badaruddin *et al.* (1999) who posited that the application of farmyard manure resulted in significant increase in final above ground biomass in wheat. Increasing the farmyard manure rates for the two varieties resulted in an increase in the dry matter accumulation and an increase in the number of fruits per plant, fruit weight per plant , fruit length and fruit girth and yield per hectare. This is in line with Jarvan and Edesi (2009) who opined that the use of FYM on cultivated crops have higher dry matter content than those grown conventionally and hence increased yield.

The nutrients absorbed by the cucumber plants were effectively utilized in the formation of fruit, leaf and stem tissues and this is in agreement with Ibeawuchi *et al.* (2007) who posited that dry matter accumulation affected the grain yield and 1000 maize grain and dry matter accumulation is a measure of relative yield.

Conclusion

Farmyard manure had a clear and consistent effect on dry matter accumulation and yield of the two varieties of cucumber. Increasing the farmyard manure resulted in the increase of growth, dry matter accumulation and yield of cucumber.

Table 1. Chemical analysis of soil and farmyard manure

	Soil sample	Farmyard manure
pH (in 2: 1 water)	5.80	6.0
Organic matter content	24.15g/kg	53.73g/kg
Organic carbon	14.0g/kg	31.13g/kg
Nitrogen	1.290g/kg	2.23g/kg
Ca	8.80mg/kg	39.08cmol/kg
Mg	0.96mg/kg	4.32cmol/kg
Available P	10.40cmol/kg	61.29mg/kg
Exchangeable K	0.29cmol/kg	2.53cmol/kg

...e vegetative traits of two cucumber varieties evaluated at 4, 6 and 8

Varieties	FYM Applied (t/ha)	Vine length (cm) WAP			Number of leaves/plant WAP		
		4	6	8	4	6	8
Ashley	0	20.28	88.26	191.51	5.74	26.69	31.61
	5	26.25	132.53	235.57	10.36	39.74	44.52
	10	33.97	143.01	286.14	12.84	46.04	52.78
	Mean	26.84	121.25	237.25	9.65	37.49	42.97
Palmetto	0	20.52	101.92	201.88	5.74	27.80	33.93
	5	40.15	140.91	257.93	10.81	39.91	46.85
	10	44.38	173.66	287.50	13.81	51.77	55.97
	Mean	34.17	138.83	249.10	10.12	39.83	45.52
LSD (P<0.05)FYM means		3.176*	13.151*	20.181*	1.168*	3.352*	3.788*
Variety means		2.593	10.738	16.477	0.954	2.737	3.093
Interaction means		4.491	18.599	28.540	1.652	4.740	5.356

Table 2b. The effects of farmyard manure on the vegetative traits of two cucumber varieties evaluated at 4, 6 and 8 WAP

Varieties	FYM Applied (t/ha)	No of branches/plant WAP			Leaf area (cm^2)/plant WAP		
		4	6	8	4	6	8
Ashley	0	0.71	9.80	12.00	531.45	3076.47	3540.66
	5	1.23	13.24	13.64	944.90	3793.50	4440.65
	10	1.79	16.84	17.70	994.92	4208.23	4640.20
	Mean	1.24	13.28	14.40	823.76	3692.94	4192.08
Palmetto	0	0.68	9.52	10.32	688.48	3025.93	3458.14
	5	1.54	12.88	13.44	914.64	3685.74	4783.41
	10	1.95	18.56	19.04	1017.56	4210.30	5035.68
	Mean	1.39	13.64	14.28	873.56	3640.66	4425.51
LSD (P<0.05)FYM means		0.192*	1.501*	1.728*	100.192*	433.263*	342.174*
Variety means		0.156	1.228	1.412	81.807	353.758	279.384
Interaction means		0.270	2.124	2.444	141.693	612.726	483.906

Table 3a. The effects of farmyard manure on dry matter partition to plant parts of two cucumber varieties assessed at 4, 6 and 8 weeks after planting

Varieties	FYM Applied (t/ha)	Dry weight of leaves g/plant WAP			Dry weight of stem (g)/plant WAP		
		4	6	8	4	6	8
Ashley	0	4.16	18.61	36.59	1.98	5.30	13.22
	5	8.55	21.41	41.47	2.27	7.23	14.33
	10	10.64	23.60	48.04	2.36	8.03	15.13
	Mean	7.78	21.21	42.03	2.20	6.85	14.24
Palmetto	0	4.04	18.50	34.95	1.97	5.17	13.07
	5	8.74	22.13	44.24	2.26	7.15	14.52
	10	10.74	25.18	48.54	2.38	8.10	15.09
	Mean	7.84	21.94	42.58	2.20	6.81	14.23
LSD (P<0.05)FYM means		0.633*	0.993*	1.247*	0.058*	0.490*	0.582*
Variety means		0.517	0.811	1.018	0.048	0.400	0.475
Interaction means		0.895	1.404	1.764	0.080	0.694	0.823

y matter partition to plant parts of two cucumber varieties assessed at

Varieties	FYM Applied (t/ha)	Dry weight of fruits (g)/plant WAP		Total dry matter (g)/plant WAP			
		6	8	6	8	6	8
Ashley	0	2.42	16.96	2.42	16.96	2.42	16.96
	5	3.15	26.50	3.15	26.50	3.15	26.50
	10	3.62	30.54	3.62	30.54	3.62	30.54
	Mean	3.06	24.67	3.06	24.67	3.06	24.67
Palmetto	0	2.44	16.87	2.44	16.87	2.44	16.87
	5	3.25	27.25	3.25	27.25	3.25	27.25
	10	3.68	31.09	3.68	31.09	3.68	31.09
	Mean	3.12	25.07	3.12	25.07	3.12	25.07
LSD (P<0.05)	FYM means	0.633*	0.625*	1.550*	0.625*	1.550*	0.625*
	Variety means	0.517	0.510	1.266	0.510	1.266	0.510
	Interaction means	0.895	0.884	2.192	0.884	2.192	0.884

Table 4. Effects of farmyard manure application on yield and yield components of two varieties of cucumber.

Varieties	FYM applied (t/ha)	Fruit No/plant (kg)	Fruit length (cm)	Fruit girth (cm)	Fruit wt/plant (kg)	Yield kg/ha
Ashley	0	5.16	15.05	4.64	1.53	27278.33
	5	8.79	15.77	5.14	1.85	32823.61
	10	11.55	15.92	5.15	2.03	35353.25
	Mean	8.49	15.59	4.98	1.80	31818.40
Palmetto	0	5.05	15.24	4.87	1.39	24881.46
	5	8.47	15.78	5.42	1.86	32911.37
	10	11.35	15.95	5.44	2.34	39853.18
	Mean	8.29	15.66	5.24	1.83	32548.67
LSD (P<0.05)	FYM means	1.050	0.273	0.245	0.226	4055.481
	Variety means	0.858	0.223	0.200	0.184	3311.286
	Interaction means	1.485	0.386	0.347	0.318	5735.316

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Study effect drought stress and different levels potassium fertilizer on K^+ accumulation in corn

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Abstract: the necessity of exertion of irrigation regimes proportional to absorb process of nutritious element of potassium appear necessary duo to famine periods, saltines problem, and the shortage of drainage irrigation net in agricultural lands of Khuzestan province(southwest of Iran). This research is performed in the form of split plot test in accidentally complete bloke plan (main treatment: various levels in irrigation I. = 7, I_1 =12, I_2 = 17, I_3 = 22 day and secondary treatment: potassium fertilizer various levels K_1 =50, K_2= 100, and K_3=150(kg/ h) with 3 repetition. Plant date of first half of 2008 August is done in Shahid Salami irrigation institute farm in Ahvaz County. According to variance breakdown results the effect of irrigation cycles, different potassium and their interaction at level %5 effects on biological performance, seed function, harvest index and potassium assembling process were meaningful. But in case of the row number of maize, the effect of different levels of potassium fertilizer and reciprocal effect of water tension and different levels caused reduction of biological performance, seed function, harvest index, and row number of maize, seed number of row and weight of thousand seed. Treatment with seed yield of 15/5 ton in hectare has dedicated highest function to itself. And I_3 with 10/33 ton in hectare has lowest function which regarding to ware shortage and famine phenomena, it is under consideration. [Nature and Science. 2010;8(5):23-27]. (ISSN: 1545-0740).

Key Words: corn, drought stress, potassium

1. Introduction

Having flat and productivity lands as well as great solar energy Khuzestan is potentially an appropriate area for cultivating the plant like corn. In many years, due to insufficient water availability or when it grows with such plants as rice and melon ground simultaneously, the corn is affected by water deficits especially during growing phase. Adverse effect of water deficit on growth, development and yield in corn much more depend on occurrence time, severity of the stress, growth phase and genotype (Lack et al 2007).

Saki nejad (2002) reported that seasonal drought is the most important factor limiting corn production in the world, so that in average it decreases 17 percent of global corn yield annually. At some areas, decreased yield induced by drought has been reported by 70 percent. Moisture stress also affects the plant growth through anatomical, morphological, physiological, and biochemical changes.

The resistance of cultivating plant to accumulated elements in roots environment is very different. This resistance depends on such factors as ion accumulation degree in tissue, prevention of entering some ions into plant and capability of generating osmotic regulators (Heidari et al 2007).

In stress condition and presence of high sodium ion, the uptake of potassium ion will be disordered because the sodium removes the entire cell membrane and decreases the selective cell uptake (Izzo 1999).

Jose et al (2000) by studying the effect of drought-induced stress on corn reported that if it is irrigated properly a high yield will be obtained. Hugh et al (2003) by examining the effect of moisture stress on corn stated that intermediate and severe stress in 2000 and 2001 decreased grain corn yield by %63, %85 and %13, %26 respectively.

Besides the water deficits, the nitrogen deficits could also multiply the stress on growth and yield so that mismanaging of irrigation and nitrogen is regarded as the most basic factor decreasing corn yield. (Norwood 2000, Winhold 1995).

Asborne et al (2002) reported that the water – induced stress at the pre-flowering, flowering, post-flowering phases, compared to control plants, decreased the corn yield by 21%, 5%, 25% respectively. Water deficit at flowering pollination phases induces intense yield decrease through abnormal embryonic sac development, grain pollen abortion and ultimately reduces the number of productive grain (Denmead & Shaw 1999).

Rashidi (1999) by studying the effect of different irrigation regimes 0, 40, 70, 90cm evaporation from wash tube Class A on grain corn yield at Jiroft of Iran area has obtained the maximum grain yield in irrigation cycle 40mm from the wash tube.

Almost 90 percent of countries farmlands are located in arid and semi- arid regions, drought–

induced stress are more obvious especially in Khuzestan due to high temperature. The aim of this research is to evaluate the accumulation and transport trend of the nutritional element (potassium) grain yield and dry matter of single cross corn 704 in the different irrigation regimes and levels of potassium fertilizer and understand a part of plant changes in order to prevent of negative effects on metabolic process.

2. Material and methods

This experiment was been considered at the field of Ahvaz's shahid salami Irrigation institute in summer of 2008 as spilt plot within basic plan of random completely blocks with main care (various periods of irrigation I_0=7, I_1=12, I_2=17 I_3=2.2 days and auxiliary care of different levels of potassium fertilizer k_1=50, $k_2$100, k_3=150kg/h) with 3 times repetition (Table 1).

Table 1.Soil Analysis Results

Depth	Potash %	Organic %	PH	EC	Soil Type
0-30	123	0.45	7.51	1.9	**Loam**
30-60	107	0.53	7.52	4.4	**Clay Loam**

The land preparation at late May was conducted by plowing, disc, fertilizing (before planting the phosphate ammonium and during planting the pure nitrogen and phosphorus fertilizers were used 200 kg and 90kg /ha respectively) and again a disc was done. In maintenance stage, the weeds were removed by an extirpator three times. The irrigation was done based on experiment treatment in the field and a half of N was applied as a topdressing fertilizer during establishing the stem into ground.

Estimating of seed performance and its components complete randomly sampling method from existing section of field was done on the basis of requirement and plan of experiment and in each experimental section, seven line were planted that two middle lines were examined in order to final performance studying. To obtain seed performance and its components, the sampling was done as follow. At each plot, its two middle line with its sampling margins as FHA(Final Harvest Area) was considered and seed performance was obtained.

Performance components of corn were studied and calculated. Row number of maize, seed number of row and weight of thousand seed were calculated and then on the basis of product of this components, seed performance was prided

For estimating of potassium element, every 14of day ,3 plant from each plot were sent to Determining and Analyzing food Elements laboratory after putting in plastic bags that potassium estimation was done by photometry way. In every sampling.3 plants were harvested to analyze potassium element and in period of growing, minimum 8 samples were got at on interval of 14 days. Variance Analysis was done by SAS software and the diagrams were drawn by Excel 2007 software program and average comparison was done as Duncan's multi- slope test at5% level.

3. Results

The results of variance analysis of various irrigation cycles, different potassium and their reciprocal effect on biological performance, seed function, harvest index and potassium assembling process were meaningful. But in case of the row number of maize, the effect of different levels of potassium fertilizer and reciprocal effect of water tension and different levels caused reduction of biological performance, seed function, harvest index, and row number of maize, seed number of row and weight of thousand seed (Table 2).

3.1. Biological yield: in Duncan test, the highest assembling of day substance obtained at 5% level of I_0 care with average of 29/71 Tons per Hectare and the lowest assembling was obtained at I_3 care with 21/4 tons per Hectare (Table 3). Increasing of plant biomass in terms of desirable irrigation has been due to more development and better duration that is caused to produce enough strong physiological resource in order to using of much received light and production of day substance.

3.2. Seed yield: I_0 care with 15.5 ton per Hectare allocated the highest seed function to itself and I_3 care wint 10.33 T ph had the lowest seed function, (Table3). The main reason of reed function reduction at different irrigation cycles can be assigned to LAI reduction and decline of photosynthesis in order to filling of seeds that caused decreasing of seed weights.

3.3. Row number of seed: the highest row number at I_0 care with numerical value of 17/3 row and the lowest of it at I_3 care with numerical value 15/4 row was obtained (table3). It is appeared that the cause of reduction of row nub men of maize at different cycle of irrigation relate to compare of physiological purposes for receiving photosynthetic materials. But against this theory, Richie and Henry (1997) reported that with regard to determining of row number of maize, there has not been comparison between other components to use of photosynthetic materials and in result row number of maize have a relative stability.

3.4. Seed Number of row: I_0 care with numerical value of 28/9 assigned the highest seed number of row and I_3 care with numerical value of 22/9 assigned the lowest seed number of row. (table3). The reasons of reducing of growing stages of plant, especially at stage of 12 leafing after that and productive growing stage, with drought tension.

3.5. Weight of thousand seeds: the highest value of weight of thousand seed obtained at I_0 car with 310 g and the lowest of it obtained at I_3 care with 2/92, g (table 3).

3.6. Harvest Index: the highest index percent of harvest obtained at I_0 care with value of 52/1% and the lowest of it obtained at I_3 care with value of 48/2% and by performing of different irrigation cycles, the percent of harvest reduced (table 3). In terms of severe drought tension, high decreasing of seed performance brought meaningful reduction of harvest index.

3.7. Potassium accumulation process: I_3 care with numerical value of 2/47 showed the highest percent of potassium assembling in plant, in the other hand, by increasing of tension intensity, potassium assembling percent in plant showed meaningful increasing. By performing different levels of water tension, potassium element assembling showed significant increasing to example care (without water tension) (table 3). One of reasons of it can be allocated to plant need to this element at various water conditions and drought tension.

4. Discussion

Liker man (2002) stated that under drought tension, the degree of potassium uptake is increased 2-3 times above desired conditions, also with presence of potassium ion, the water stress and its effect on dry matter accumulation trend, leaf area index, plant height is adjusted. The reason of such phenomenon can be attributed to high capability of photosynthesis by increasing the capturing carbon and enzyme robisco and encouraging synthesis and matter transport.

Smith (2003) the trend of potassium accumulation during initial growth stages is very severe compared to accumulation of dry matter. The maximum potassium uptake is taken place when the grain begins to get milky and before grain formation the accumulation is completed.

Smith (1999) showed that the trend of increased maize product during drought, ordinary and wet years is 4800 kg, 900 kg and 5400 kg respectively. Therefore, under drought condition, the trend of potassium absorption in order to improve the plant resistance against water deficit shows move severity and as the potassium uptake increases when emerging maize tuft, the product will increase. When the water stress is applied the potassium uptake be increased during flowering stage or by root or the transport of this element from lower leaf toward upper leaf is quickly conducted, so that the water potential in aerial point is greatly decreased and water moved in to these organs.

Rafiee (2002) stated that relative moisture content of leaf at time of flowering of corn plant have high correlate with seed function, negative correlation between drought tension with leaf surface index and potential of leaf water have provided reduction of leaf surface and reduction of photosynthesis at leaf water unit at level of sinking and result, reduction of supplying processed substances and negative effect of it on seed production in maize was led to the result of seed performance reduction. Sepehry et al (2002) declared that the most effect of drought tension on seed weight was during filling of seeds and tensions would happen after giving silk cause being small of seed by reducing the duration of seed filling.

Khokpour (1994) observed that strict drought tension caused reduction of corn harvest index, but aligned this reduction to more declination of seed function to total dry weight.

Likely (1999) said that at water tension conditions, process of potassium absorption perform with consuming energy and in from of active. Sony et al (2001) have reported the reason of increased potassium uptake under drought stress can be attributed to active uptake mechanism of this ion by which the plant in order to promote its resistance to drought, increases the potassium concentration in root and other organs through energy consumption. The other reason is attributed to extended drought and wet periods in the dried and semi-dried regions which it causes potassium release through day layers. Guiyang (2002) stated that a large amount of the plant energy spent for adjusting osmosis pressure of the leaf tubing & rising the stomas ‘insistence; in other words, the dry tension created conditions with which spent a lot of energy to confront it; for example about adjusting osmosis pressure, it took approximately one month to decreasing its osmosis potential that a lot of energy have been spent for increasing active absorption of potassium& also protein polymers.

Table2: summary of variance analysis results (square averages) and meaningful level of components of corn function in examination.

Potassium accumulation	Harvest index	Seed yield	Weight of thousand	Seed number of row	Row number of seed	Performance of dry material	df	Changes resources
$0.96^{n.s}$	0.19^{*}	$0.014^{n.s}$	0.89^{**}	$0.15^{n.s}$	$0.80^{n.s}$	$0.061^{n.s}$	2	Repetition
8.02^{**}	56.91^{**}	41.02^{**}	48.9^{**}	10.76^{**}	$15./01^{**}$	137.45^{**}	3	I treatment
0.086	0.024	0.015	0.58	0.59	0.46	0.039	6	Error (I)
11.32^{**}	0.50^{**}	1.35^{**}	0.99^{**}	5.36^{**}	$0.79^{n.s}$	8.39^{**}	2	K treatment
14.33^{**}	0.30^{**}	0.30^{**}	24.6^{**}	4.16^{**}	$0.68^{n.s}$	1.94^{**}	6	Interaction I×K
0.053	0.015	0.011	0.32	0.37	0.23	0.021	16	Error (K)
9.91	12.20	10.45	13.65	13.4	13.95	10.55	-	CV%

*, **, ns show meaning fullness at level of 1%, 5% and unmeaning fullness, respectively

Table3: comparison of average with Duncan test wag at 5% level

Potassium accumulation	Harvest index	Seed yield	Weight of thousand seeds	Seed number of row	Row number of seed	Performance of day material	Treatment
							Drought stress(I)
1.91b	52.1A	15.5A	310A	28.9A	17.3A	29.71A	I_0
2/05b	51.4B	14B	309A	26.3B	17.2A	27.21B	I_1
2.33ab	50C	13.6C	305B	26.8B	16.6AB	27.2B	I_2
2.47a	48.2D	10.33D	292C	22.9C	15.4AB	21.4C	I_3
						Various levels of potassium fertilizer(k)	
1.61a	43.2B	12.24B	307Ab	22.1C	18A	28.31B	K_1
2.62b	49.7A	15.12A	308A	27.5B	17.8A	30.11A	K_2
2.61b	51.5A	15.51A	306B	28.4A	17.8A	30.38A	K_3

In each column, being on common article between 2avernage show unmeaning fullness 5% level.

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Cytogenetic and Biochemical Studies On the Protective Role of *Rhodotorula glutinis* And its Autoploidy Against the Toxic Effect of Aflatoxin B_1 in Mic

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Abstract: The present study was designed to investigate the effect of *Rhodotorula glutinis* and its autoploidy on cytogenetic and biochemical analyses and to evaluate the protective role of these yeasts against aflatoxin B_1- in mice. Eight groups of male mice were used. Three of them were treated with three strains (wild type G1 and two autoploidy (G2 and G3) of *Rhodotorula glutinis*. In addition, one group was treated the suspension of growth medium of yeasts (served as control),three groups were treated with the G1, G2 and G3 after an hour of injection with the aflatoxin. Cytogenetic analyses revealed that the treatment with the wild type of yeast (R.g, G1) and its two mutants (G2 and G3) had improved the genetic materials in normal somatic and germinal animal cells by decreasing chromosome aberrations and increasing the mitotic and meiotic indices compared to control group. On the other hand, the chromosomal aberrations were more frequent of mitotic and meiotic indices were depressed in the animals treated with aflatoxin alone. In contrast, the frequencies of the chromosome aberrations were significantly decreased and mitotic and meiotic indices were increased in animals treated with the wild type (G1) and its two autoploidy (G2 and G3) plus aflatoxin B_1. Biochemical results showed that the treatment with yeast strains especially the treatment with two autoploidy G2 and G3 did not induce changes in liver and kidney functions in normal animals. The treatment with the three strains wild type G1 and two autoploidy G2 and G3 had enhanced the TP compared to control group. The treatment with aflatoxin B1 significantly increased the liver enzymes (GGT, ALT and AST), kidney function markers(uric acid and creatinine) and significantly decreased the TP compared to control group. In contrast, the treatment with yeast strains plus aflatoxin B_1 succeeded in diminishing the elevated value of liver enzymes and kidney functions and normalized TP level. [Nature and Science. 2010;8(5):28-38]. (ISSN: 1545-0740).

Keywords: Aflatoxin,*Rhodotorula glutinis*,autoploidy,chromosome aberrations,biochemistry.

1-Introduction

Fungal deterioration of stored seeds and grains is a chronic problem in the Egyptian storage system because of the tropical hot and humid climate. Harvested grains are colonized by various species of *Aspergillus*, under such conditions leading to deterioration and mycotoxin production. *Aspergilli* are the most common fungal species that can produce mycotoxins in food and feedstuffs. Mycotoxins are well known for their health - hazardous effects in human beings and animals (Probst *et al.,* 2007; Reddy and Raghavender, 2007; Reddy *et al.,*2009). Among all the mycotoxins, particularly aflatoxin is the most toxic form for mammals. This aflatoxin (AF) is a group of structurally similar polysubstituted coumarins produced by the common moulds *Aspergillus flavus* and *Aspergillus parasiticus*. The main biological effects of aflatoxins are carcinogenicity, immunosuppression, and teratogenicity (Betina, 1989; Abdel- Wahhab *et al.*, 1998; Santos *et al*., 2001; Reddy *et al*., 2009). Moreover, aflatoxin is a potent mutagenic food component and has been found to be an inhibitor factor in mitosis (Hall *et al*., 1988 ; Abdel- Wahhab *et al*., 1998). It is metabolized by the mixed function oxidase system to a number of hydroxylated metabolites and to aflatoxin 8, 9 epoxide which binds to DNA, forming covalent adducts (Busby and Wogan, 1984) and disturbs DNA replication causing chromosomal aberrations (Sinha and Prasad, 1990). Many trails were conducted for minimizing the effects of mycotoxins on human and animal health as well as increasing the animal productivity and performance. In animals, many additives such as specific types of clay could be added to their food to bind or reduce the harmful effects of mycotoxins (Ramos *et al*., 1996 and Abdel – Wahhab *et al*., 1998). The situation is different

in humans, especially after the discovery of yeasts which could be added to their food in order to maintain their health by contributing in fighting toxicants and illness (Koleva *et al*., 2008). Recently, an antagonistic yeast strain of *Rhodotorula glutinis* have been reported as an effective biocontrol agent (in vivo) against post-harvest decay of fruits and vegetables (Qin *et al*., 2003; Zhang *et al*., 2007; Zhang *et al*., 2008; Malisorn and Suntornsuk, 2008). Moreover, the yeast strain of *Rhodotorula glutinis* and its autoploidy have been reported as an effective biocontrol agent (in vivo) and (in vivo) against grey mould of greenhouse sweet pepper (Haggag, *et al*., 2005). However, there are no informations available (to our knowledge) about the use of *Rhodotorula glutinis* or its autoploids as protective agents on mammalian cells (in vivo) against the toxic effect of mycotoxins. Therefore, the aim of the present study is to investigate the protective role of *Rhodotorula glutinis* and its two autoploidy against aflatoxin B_1- intoxication in mice by using biochemical and cytogenetic analyses.

2. Materials and Methods

2.1. Materials

2.1.1. Mice: Random bred of forty - eight adult male Swiss albino mice weighing about 25 grams were used. These animals were obtained from animal house laboratory, National Research Centre, Cairo, Egypt. Apparently health acceptable animals were randomly assigned into eight groups (6 mice in each).

***Environmental conditions*:**

Chosen mice were kept in stainless steel wire mesh cages on a bedding of wood chips. They were housed in an ambient temperature of 25± 3 ^{0}C, on a light/ dark cycle of 12/12 hours and supplied with mice chew and fresh water ad-libitum.

2.1.2. Mycotoxins:

Aflatoxin B_1 was purchased in a pure crystalline form from Food Toxicology and Contaminants Department, National Research Centre, Egypt. The toxin was dissolved in corn oil.

2.1.3. Types of used yeasts:

The strains of yeast G2 (Col- 1R1) and G3 (Col-1R3) have been genetically improved and isolated from the wild type yeast strains *Rhodotorula glutinis* (G1) after colchicines treatments, (Haggag, *et al*. 2005). Briefly, wild type yeast isolates were cultured in flasks containing Yeast Nitrogen Base (YNB) liquid medium supplemented with 5% glucose and incubated at 30ºC for 18 h. Then, colchicine was added to the yeast cell suspensions to give final concentrations (0.2%). The treated cell suspensions were incubated for further 18 h. The treated cells were inoculated on YMPG medium plates and incubated for 2 weeks at 30ºC. A glance on colonies grown on the agar plates showed that large colonies were always composed of large cells than that of small colonies. Consequently, large cells from about 270 initial large colonies of *R. glutinis* were selected. The types of used yeasts and their properties were shown in Table (1). The growth of the tested yeasts was estimated by measuring the optical density at 610 nm. Hemocytometer was used to count yeast cells. The cell diameter was measured microscopically using an eyepiece micrometer. Protein content in the supernatant was determined at 595 nm by Bradford(1976) method using bovine serum albumin as a standard. Protein content was determined at 595 nm.

Table 1 shows the properties of *Rhodotorula glutinis* strains (wild type and its two mutants).

GROUPS	*R. GLUTINIS* (WILD TYPE) G1	G2	G3
Asperigillus niger inhibition (inhibition zone, mm)	127	22.4	24.6
Aperigilus flavus inhibition (inhibition zone, mm)	13.6	18.9	21.7
Cell diameter (µm)	6.20 (4.12-7.15)	8.51 (6.15-11.2)	8.30 (6.00-10.0)
Cell volume (μm^3)	158.61 (90.05-230.30)	352.44 (287.25-404.3)	333.51 (255.3-307.4)
DNA content %	100 $156.1\ \mu g / 10^8$ cells	225.08	194.85
Growth rate (h^{-1})	0.0771	0.1122	0.1140
Cell concentration (OD 610 nm)	1.490	1.975	1.910
Total Protein $\mu g^{-1} / ml^{-1}$	216.8	300.7	367.8

2.2 Methods:

2.2.1. Preparation of mycotoxin and strains of yeast:

The strains of yeast (G1, G2 and G3) and growth medium were suspended in gum accacia (5% w/v).

2.2.2. Experimental design:

Eight groups of adult mice (6 animals in each) were used in this work. Three groups of them were given orally with three strains (G1, G2 and G3) of yeast (A strain/ A group). Dose of 50.000 cells of each strain/ 1 ml of solution/ 100 gm of body weight were administrated daily to each animal for 15 days. In addition, one group of animals was given orally with the suspension of growth medium (1 ml of solution/ 100 gm body weight) daily to each animal for 15 days. This group was served as control. Also, one group of

animals was given orally with aflatoxin B_1 (1 mg/ 1 kg body weight) daily to each animal for 15 days.

Moreover, three groups of animals were given orally with three strains (G1, G2 and G3) of yeast (A strain/ A group). Then after an hour of injection, the animals in each group were given orally with the toxin (1 mg/ kg b.w). The strains of yeast and toxin were given daily for 15 days.

2.2.3. Cytogenetic analyses: Two hours before sacrifice, the animals were injected with 0.5 mg of colchicine.

2.2.4. Chromosome analysis in somatic cells: Femurs sere were removed and bone marrow cells were aspirated using saline solution. Metaphase spreads were prepared by using Preston *et al*., (1987) method. Fifty metaphase spreads per animal were analyzed for studying the chromosome aberrations.

2.2.5. Mitotic index: The mitotic activity of bone marrow cells was investigated by recording the number of dividing cells/ 1000 cells/ animal" the mitotic index".

2.2.6. Chromosome analysis in germ cells: spermatocyte cells were prepared according to **Brewen and Preston (1978)** for meiotic chromosomal analysis.

2.2.7. Meiotic index: The meiotic activity of spermatocyte cells carried out by recording the number of dividing cell/ 1000 cells/ animal"meiotic index".

2.2.8. Biochemical studies: At the end of experimental period (after 15 days) blood samples were collected from all animals from the retro-orbital venous plexus for biochemical analyses. These analyses included, serum GGT (Rosalki *et al*., 1970), ALT, AST and creatinine **(**Thefeld *et al*., 1974), total proteins (Bradford, 1976) and uric acid (Haisman and Muller, 1997).

2.2.9. Statistical analysis:

The obtained results of cytogenetic examinations or biochemical analysis were statistically analyzed by ANOVA (one or two-way) using Excel 2003 Microsoft Crop (11.5612.5606), Redmond, WA software package.

3. Results

3.1. Cytogenetic analyses:

3.1.1. Chromosome examination in bone marrow cells:

Cytogenetic results showed that the frequencies of total structural chromosome aberrations were low in the three animals groups which treated with the three yeast strains G1, G2 and G3 compared to those found of the control group (5.5, 5.5 and 5.25 vs. 6.0% respectively). However, statistical analysis for the frequencies of chromosome aberrations showed that there were no significant differences between the three yeast groups and control group. When comparing the cytogenetic results between the three yeast groups with each other, it can be seen that the proportion of frequencies of chromosome aberrations were similar and there were no significant differences between the three

3.1.2. Mitotic index: The mitotic activity (Table 2) was significantly increased in G1 and G3 groups compared with those found in the control. While mitotic activity in G2 was approximately similar with those found in the control (9.03 vs 9.95 respectively) and there were no significant differences between the two groups. Also, there were no significant differences for mitotic activities between G1 and G3. However, mitotic activity was significantly decreased in G2 compared to those found in G1 or in G3.

3.1.3. Chromosome examination in spermatocyte cells: .

Chromosome aberrations in spermatocyte cells (Table 3) consisted of autosomal univalent, sex univalent and translocation. Total chromosomal aberrations (especially autosomal univalent) were lowered in the three animal groups which treated with the three yeast strains (G1, G2 and G3) than those observed in the control group. The G3 group followed by G2 group had the lowest proportion of the total structural chromosome aberrations. However, statistical analysis showed that there were no significant differences for the frequencies of chromosome aberrations between control group and the three animal groups that treated with three yeast strains. Also, there were no significant differences for the cytogenetic results between the three groups (G1, G2 and G3) with each other.

3.1.4. Meiotic activity: The meiotic activity was significantly increased in the three animal groups which treated with the three yeast strains (G1, G2 and G3) compared to those found in the control group (11.65; 8.47 and 10.98 vs. 6.93 respectively).

On the other hand, the meiotic activity was approximately similar with those found in G3 group and there was no significant differences between the two groups. However, the meiotic activity was significantly increased in G2 group compared to those found in G1 or G3.

3.2. The protective role of yeast strains against toxic effect of aflatoxin B_1 in animal cells:

3.2.1 Chromosome examination in bone marrow cells:

The cytogenetic results (Table 2) showed that the frequencies of total structural chromosome aberrations in mice treated with AFB1, were more than those observed in the control group (12.25 vs 6.6, respectively). Statistical analysis showed that there were highly (0.001) significant differences between the AFB1 and control groups.Moreover, the frequencies of deletions and centric fusions were highly significant in AFB1 group compared to those found in control group. On the other hand, cytogenetic examination showed that the frequencies of total structural chromosome aberrations were lowered in mice treated with AFB1, plus three yeast strains (G1, G2 and G3) than those found in mice treated with AFB1, alone (9.25; 7.75 and 8.75 vs 12.25 respectively). AFG2 group followed by AFG3 had the lowest proportions of total structural chromosome aberrations. Statistical analysis showed that there were significant differences between AFG1 , AFG2 , AFG3 groups and AF group. When comparing the cytogenetic results between groups of AFG1, AFG2 or AFG3 with each other, it was found that the frequencies of total structural chromosome aberrations were significantly decreased in AFG2 group than those found of AFG1 group. However, there were no significant differences between AFG1 and AFG3 groups. Also, the treatment with G2 plus AF significantly decreased the frequencies of deletions compared to AFG1 or AFG3 groups. Moreover, the treatment with G3 plus AF significantly decreased the frequencies of deletions than the treatment with G1 plus AF. On the other hand, statistical analysis showed that there were no significant differences for the frequencies of each of gaps, Breaks, centric fusion and end to end association between the three groups AFG1, AFG2 and AFG3 with each other.

3.2.2. Mitotic index: cytogenetic examination observed that the mitotic activity has been increased in mice treated with AF plus G1 or G2 or G3 compared to those found in mice treated with AF alone (9.25, 8.96 and 9.0 vs. 8.87 respectively). However, statistical analysis showed that there were no significant differences for mitotic activity between AF group and AFG1 or AFG2 or AFG3 groups. Also, there were no significant differences for mitotic activity between animal treated with AF plus G1 or AF plus G2 or AF plus G3 with each other.

3.2.3. Chromosome examination in spermatocyte cells:

Cytogenetic results showed that the mice which treated with AF has more frequencies of autosomal univalent (AU), six univalent (SU), translocation (Tr) and total structural chromosome (TSA) aberrations than the control group. Statistical analysis showed that there were significant differences between AF and control groups for the frequencies of AU, Tr and TSA. However, there were no significant differences for the frequencies of SU between AF and control groups.

On the other hand cytogenetic examination found that the treatment of with G1 or G2 or G3 plus AF significantly decreased the frequencies of each of total structural chromosome aberrations and autosomal univalent comparing with the treatment of AF alone (4.0, 5.0 and 4.5 vs. 875, respectively or 2.25, 1.5 and 2.0 vs. 5.25, respectively).

Also, the treatment with G1 or G3 plus AF significantly decreased the frequencies of translocation comparing with the treatment of AF alone. However, there were no significant differences for the frequencies of translocation between animals treated with G2 plus AF and those treated with AF alone. Moreover, there were no significant differences for the frequencies of sex univalent between animals treated with G1 or G2 or G3 plus AF and animals treated with AF alone.

3.2.4. Meiotic activity: Cytogenetic examination showed that the meiotic activity decreased in animals treated with AF than those observed in the control group (5.75 vs. 6.93, respectively). However, this decrease was not significant. On the other hand, the meiotic activity in animals treated with G1 or G2 or G3 plus AF significantly increased than those found of animals treated with AF alone (11.05, 8.46 and 10.52 vs. 5.75, respectively).

The animals treated with G1 plus AF followed by G3AF had the highest proportion of meiotic activity and there were no significant differences between the two groups. However, the meiotic activity was significantly decreased in AFG3 compared to those found in AFG1 or AFG3 groups.

3.3. Biochemical analysis:

Biochemical analysis of serum (table 4) showed insignificant changes in liver markers (GGT, ALT, AST) in normal groups treated with G2 and G3 when compared with the control values. While treatment with G1 significantly elevated GGT, ALT and AST activities. AST values did not changes by all treatment with G1, G2 and G3. However, serum TP level was normal in groups of animals treated with G1 and G3 ; whereas, G2- treatment significantly increased TP value than the other treated groups.

Kidney functions revealed that the tested yeast Rh1(G1) and its two mutants (G2 and G3) had no effect on creatininee concentration. As well as, uric acid values did not altered in groups of mice administrated G2 and G3 when compared with the control group. While, G1 showed significant elevation in uric acid level comparing with the control or with the two mutants (G2 and G3).

AFB1- treated group had significant increase in liver enzymes: GGT, ALT, AST and kidney functions:creatinine and uric acid. Moreover, AFB1-administration caused significant decrease in TP value when compared with the normal control animals or with the combined treated with the three yeasts.

Generally, the combined treatment of AFB1 with G1, G2 and G3 significantly inhibited the liver enzymes, as well as, they decreased the elevated values of creatinine and uric acid in kidney functions (table 1).

Mice treated with the wild yeast suspension concomitant with AFB1 (G1+ AFB1) significantly decreased GGT, ALT, AST and creatinine than AFB1 – treatment alone, while it had no effect on TP and uric acid values.

Furthermore, the treatments with G2 and G3 in combination with AFB1 nearly have similar effects on liver and kidney functions; both of them significantly decreased the elevated values induced by AFB1(GGT, ALT, AST, creatinine and uric acid) and significantly increased serum TP towered the normal values when compared with AFB1- treatment alone.

Table (2): Effect of treatment of *Rhodotorula glutinis* (G1) and its two autoploidy (G2 and G3) with or without aflatoxin B_1 on chromosomes and mitotic index in bone marrow cells of mice.

Treatment	Structural abnormalities					Total structural chrom. Aberrations		Mitotic Index
	Gaps	Deletions	Breaks	Centric fusions	End to end associations	including gaps	excluding gaps	
Control	1.25 ± 0.2 ^{A}	4.0 ± 0.4 ^{A}	6.93 ± 0.32 ^{A}	6.93 ± 0.32 ^{A}	6.93 ± 0.32 ^{A}	6.93 ± 0.32 ^{A}	6.93 ± 0.32 ^{A}	9.95 ± 0.52 ^{A}
G1	1.25±0.3 ^{A}	4.0 ± 0.4 ^{A}	11. 65± 0.43 ^{C}	11. 65± 0.43 ^{C}	11. 65± 0.43 ^{C}	11. 65± 0.43 ^{C}	11. 65± 0.43 ^{C}	11. 2± 0.34 ^{B}
G2	1.0±0.4 ^{A}	3.75± 0.3 ^{A}	8.47± 0.51 ^{B}	8.47± 0.51 ^{B}	8.47± 0.51 ^{B}	8.47± 0.51 ^{B}	8.47± 0.51 ^{B}	9.03± 0.88^{A}
G3	0.75±0.2 ^{A}	3.75± 0.3 ^{A}	10.98± 0. 4 ^{C}	10.98± 0. 4 ^{C}	10.98± 0. 4 ^{C}	10.98± 0. 4 ^{C}	10.98± 0. 4 ^{C}	11.32± 0. 53 ^{B}
A F	1.5±0.28 ^{A}	7.75± 0.62 ^{C}	5.75± 0.72 ^{D}	5.75± 0.72 ^{D}	5.75± 0.72 ^{D}	5.75± 0.72 ^{D}	5.75± 0.72 ^{D}	8.87± 0. 38 ^{A}
A F 1+ G1	0.5±0.3 ^{A}	7.5± 0.5 ^{C}	11.05± 0.67 ^{C}	11.05± 0.67 ^{C}	11.05± 0.67 ^{C}	11.05± 0.67 ^{C}	11.05± 0.67 ^{C}	9.25± 0. 25 ^{A}
A F 2+ G2	1.5±0.3 ^{A}	4.25± 0.3 ^{A}	8.47± 0.32 ^{B}	8.47± 0.32 ^{B}	8.47± 0.32 ^{B}	8.47± 0.32 ^{B}	8.47± 0.32 ^{B}	8.96± 0.41 ^{A}
A F 3+ G3	0.5±0.3 ^{A}	5.75± 0.47 ^{B}	10.52± 0.16 ^{C}	10.52± 0.16 ^{C}	10.52± 0.16 ^{C}	10.52± 0.16 ^{C}	10.52± 0.16 ^{C}	9.0± 0.42 ^{A}

ANOVA single way. The different alphabetical superscripts are significantly different at $p \leq 0.05$.

Table (3): Effect of treatment of *Rhodotorula glutinis* (G1) and its two autoploidy (G2 and G3) with or ithout flatoxin B_1 on chromosomes and meiotic index in spermatocyte cells of mice.

Treatment	Structural abnormalities			Total chromosomal aberrations	Meiotic index
	Autosomal univalent	Sex univalent	Translocation		
Control	1. 25± 0.25 ^{AB}	1. 5± 0.28 ^{A}	0.5± 0.28 ^{A}	3.25± 0.25 ^{AB}	6.93 ± 0.32 ^{A}
G1	1. 0± 0.4 ^{AB}	1. 5± 0.28 ^{A}	0. 5± 0.28 ^{A}	3. 0± 0.4 ^{AB}	11. 65± 0.43 ^{C}
G2	1.0± 0.4 ^{AB}	1.25± 0.25 ^{A}	0.5± 0.28 ^{A}	2.8± 0.62 ^{AB}	8.47± 0.51 ^{B}
G3	0.75± 0.25 ^{A}	1.25± 0.47 ^{A}	0.5± 0.28 ^{A}	2.5± 0.64 ^{A}	10.98± 0. 4 ^{C}

A F	5.25± 0.47 ^{C}	2.0± 0.57 ^{A}	1.5± 0.28 ^{B}	8.75± 0.47 ^{E}	5.75± 0.72 ^{A}
A F 1+ G1	2.25± 0.47 ^{B}	1.25± 0.25 ^{A}	0.5± 0.3 ^{A}	4.0± 0.4 ^{BCD}	11.05± 0.67 ^{C}
A F 2+ G2	1.5± 0.28 ^{AB}	2.0± 0.4 ^{A}	1.5± o.3 ^{B}	5.0± 0.4 ^{D}	8.47± 0.32 ^{B}
A F 3+ G3	2.0± 0.4 ^{AB}	2.0± 0.4 ^{A}	0.5± 0.3 ^{A}	4. 5± 0.28 ^{CD}	10.52± 0.16 ^{C}

ANOVA single way. The different alphabetical superscripts are significantly different at $p \leq 0.05$.

Table(4): Effect of treatment of *Rhodotorula* glutinis (G1) and its two autoploidy (G2 and G3) with or without Aflatoxin B_1on serum biochemical analysis in mice.

Group	Control	G1	G2	G3	AF	AF+G1	AF+G2	AF+G3
GGT IU/ ml	1.42 ± 0.15 ^{A}	2.8 ± 0.2 ^{B}	1.46 ± 0.13 ^{A}	1.93 ± 0.07 ^{A}	5.74 ± 0.24 ^{C}	4.1 ± 0.17 ^{D}	3.84 ± 0.18 ^{D}	3.92 ± 0.19 ^{D}
ALT IU/ ml	28.7± 0.76 ^{A}	37.3 ± 1.01 ^{B}	28. 37± 0.77 ^{A}	29. 06± 0.93 ^{A}	54. 25± 1.61 ^{C}	41. 81± 1.89 ^{D}	40. 73± 1.37^{D}	39. 15± 1.47 ^{BD}
AST IU/ ml	49.4± 1.42 ^{A}	51.7± 1.47 ^{A}	49.3± 1.01 ^{A}	48.8± 1.03 ^{A}	99.34± 2.33 ^{B}	83.98± 1.18 ^{C}	80.22± 2.01 ^{C}	64.48± 1.09^{D}
TP IU/ ml	6.99± 0. 08 ^{AE}	7.24± 0.18 ^{A}	8.09± 0. 2 ^{B}	7.17± 0. 16 ^{A}	5.85± 0. 26 ^{C}	6.37± 0. 2 ^{CD}	6.78± 0. 21 ^{AD}	6.5± 0. 25 ^{DE}
Creatininee IU/ ml	0.45± 0.01 ^{A}	0.52± 0.02 ^{AD}	0. 51± 0.02 ^{A}	0.49± 0.02 ^{A}	1.87± 0.11 ^{B}	0.82± 0.02 ^{C}	0.73± 0.02 ^{C}	0.64± 0. 01 ^{CD}
Uric acid IU/ ml	3.0± 0.1 ^{A}	4.1± 0.23 ^{B}	3.07± 0.19 ^{A}	3.22± 0.18 ^{A}	5.87± 0.16 ^{C}	5.17± 0.19 ^{C}	4.42± 0.17 ^{B}	4.33± 0. 25 ^{B}

T.C.A= total chromosome aberrations
M.I.= mitotic activity

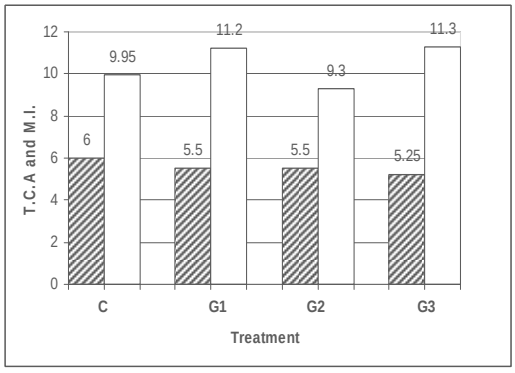

Fig.1: The effect of treatment with the three strains of yeasts on chromosome aberrations and mitotic activity in normal somatic animal cells.

C= control. G1= wild type strain of yeast.

G2= Co1-1R1. G3= CoI-1R3.

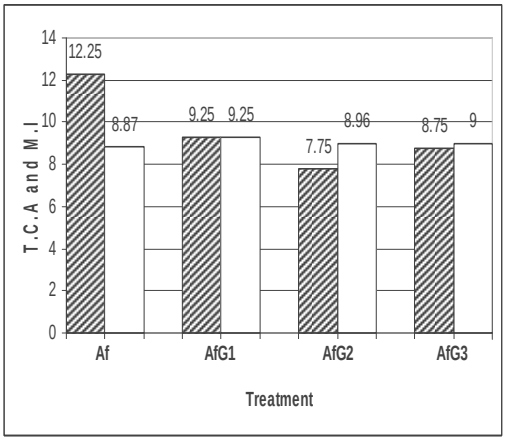

Fig. 2: The effect of treatment with the three strains of yeasts as protective agents against the mutagenecity of Af in somatic animal cells.

C= control. G1= wild type strain of yeast

G2= Co1-1R1. G3= CoI-1R3.

Af = aflatoxin. Af G1= aflatoxin plus G1.

Af G2= aflatoxin plus G2. Af G3= aflatoxin plus G3.

T.C.A= total chromosome aberrations
M.I.= meiotic activity

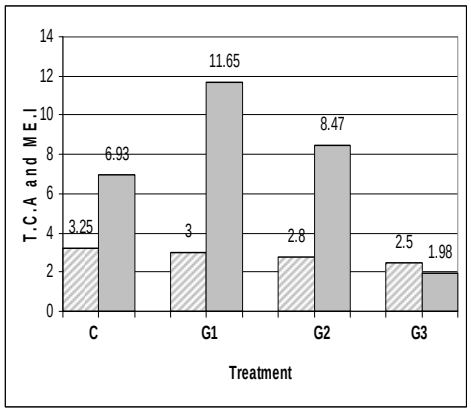

Fig.3: The effect of treatment with the three strains of yeasts on chromosome aberrations and meiotic activity n normal germinal animal cells.

C= control. G1= wild type strain of yeast

G2= Co1-1R1. G3= CoI-1R3. Af = aflatoxin

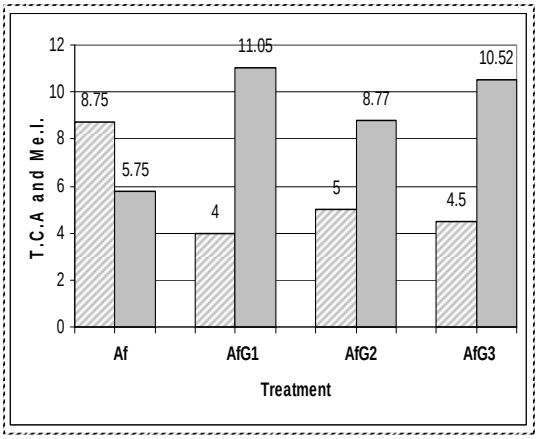

Fig.4: The effect of the three strains of yeasts on chromosome aberrations and meiotic activity in germinal animal cells.

C= control. G1= wild type strain of yeast

G2= Co1-1R1. G3= CoI-1R3.

Af = aflatoxin. Af G1= aflatoxin plus G1.

Af G2= aflatoxin plus G2. Af G3= aflatoxin plus G3.

4. Discussion

In the present study, cytogenetic analyses revealed that the treatment with wild type strain of yeast (R.9, G1) and its mutants (G2 and G3) in normal somatic and germinal animal cells has improved the genetic materials by decreasing chromosome aberrations and increasing the mitotic and meiotic indices. To our knowledge the effect of R.9 (G1) or its mutants on cytogenetic and biochemical analyses in animal cells (in vivo) is not previously studied. Also, the biochemical results found that the treatment with R. yeast strains especially the treatment with G2 and G3 did not induce changes in liver and kidney functions in normal animals. The treatment with the three strains (G1, G2 and G3) has enhanced the TP compared to control group. However, the safe treatment especially with G2 and G3 strains and some enhancement of liver and kidney functions as well as the improvement of genetic materials by treatment with the three strains of yeast which showed in the present study may be due to that the yeast cells contain high amounts of carotenoids, vitamins, minerals and essential amino acids (Hussein *et al*., 1996). These constituents are considered as antioxidant agents (Omara *et al*., 2009). The antioxidant defense system has both enzymatic and nonenzymatic components that prevent free radical formation, remove radicals before damage can occur, repair oxidative damage, eliminate damaged molecules and could be effective means for preventing changes in liver and kidney functions as well as they are protective agents against inducing mutagenic effects in genetic materials (Chandra and Sarchielli, 1993; Kubena and Mc Murray, 1996; Cramer *et al*., 2001; Nicolle *et al*., 2003; Devaraj *et al*., 2008).

In previous cytogenetic studies, the carotenoids were safe on mammalian genetic materials (Agarwal *et al*. 1993 ; Farag *et al*., 2001). Agarwal *et al*. (1993) showed that the treatment with β-carotene did not induce depression of mitotic index in material and foetal cells of animals treated with β-carotene compared with control group. Farag *et al*., (2001) demonstrated that the treatment with β-carotene did not cause inducing chromosome abnormalities or depression in mitotic index in maternal and foetal cells of mice.

Also, in another studies, carotenoids have been found to be protective agents on the liver and kidney functions (Nicolle *et al*., 2003; Devaraj *et al*., 2008) from oxidative stress. Nicolle *et al*., (2003) investigated the effect of 3- week supplementation of the diet with carrot on lipid metabolism and antioxidant status in rats. They observed a significant decrease of cholesterol level together with a reduction of the level of triglycerides in liver. Also, the results revealed that carrot consumption improved the antioxidant status by decreasing the urinary excretion of thiobarbituric acid reactive substances (TBARS), reducing TBARS levels in heart and increasing vitamin E plastimatic level as compared to control. Devaraj *et al*., (2008) tested the effects of different doses of purified lycopene (natural

carotenoid) supplementation on biomarkers of oxidative stress in healthy volunteers. Their results revealed a significant decrease in DNA damage and a significant decrease in urinary 8- hydroxyl deoxaguanosine (8-ohdG) at 8 weeks versus baseline with 30 mg hycopene/ day.

Few literatures demonstrated that some vitamins associated with diets have shown to improve immune status in healthy individuals. Meydani *et al.* (1997) showed that Vitamin E supplementation improved response to delayed- type hypersensitivity tests in healthy individuals > 65 y of age. Moreover, in previous study, Duthie *et al*. (1996) showed a significant decrease in endogenous oxidative base damage in the lymphocyte DNA of both smokers and nonsmokers with a 20- wk daily supplement of vitamin C (100 mg) and B- carotene (25 mg).

Concerning the effect of some amino acids such ascorbic acid on oxidation condition, some studies showed that the addition of ascorbic acid - rich foods to a controlled experimental diets led to inhibition of endogenous formation of N-nitroso compounds (oxidative and mutagenic agent) in humans (Knight and Forman, 1987; Bartsch *et al*., 1988).

In the present study, the treatment with aflatoxin B_1 significantly increased the liver (GGT, ALT and AST) and kidney (uric acid and creation) functions and significantly decreased the TP compared to control group. In contrast, the treatment with the yeast strains (G1, G2 and G3) together with aflatoxin B_1 succeeded in diminishing the elevated values of liver enzymes (GGT, ALT,AST) and kidney function tests (uric acid and creatinine) and normalized TP level. On the other hand, the cytogenetic results showed that the chromosome aberrations were more frequent and the mitotic and meiotic indices were depressed in the animals treated with aflatoxin B_1 alone. In contrast the frequencies of the chromosome aberrations were significantly decreased and mitotic and meiotic indices were increased in animals treated with AFB1 concomitant with yeast strains (G1, G2 and G3).

As known the liver is considered the principal target organ for aflatoxin. The activity of ALT and AST are sensitive indicators for acute hepatic necrosis (Kaplan, 1987) and decrease in concentration of TP as indicators of the alternation in protein synthesis observes in aflatoxicosis (Kubena *et al*., 1993; Abo-Norage *et al*., 1995; Abousadi *et al*., 2007).

Changes in liver and kidney functions induced by aflatoxin have been documented previously by Heathcote and Hibbert (1979) and Abdel- Wahhab *et al*. (1998). Some studies showed that aflatoxins have induced oxidative damage and caused for generation of free radicals which reacted with cellular component and led to pathological changes in liver and kidney functions (Vasankari *et al*., 1997; Abdel- Wahhab *et al*., 1998; Abousadi *et al*., 2007).

Ito *et al*., (1989) and Abdel-Wahhab *et al*., (1998) had previously reported that aflatoxin B_1, has a potent activity to induce chromosome aberrations in bone marrow of rats. The mutagenicity of aflatoxin arising from the toxin molecules might form covalent N7 guanine- adducts which disturb DNA replication (Bonnett and Taylor, 1989), resulting in anomalies in the chromosomes (Sinha and Prasad, 1990).

The protective effect of yeast which observed on animal cells (in vivo), in the present study has not been discussed previously. However, as reported above, the protection role of the yeast may be due to that the yeast cells contain antioxidant components such, carotenoids, vitamins, minerals and essential amino acids (Hussein *et al*., 1996) that interrupts the free radical- initiated chain reaction of oxidation or scavenge and disable free radicals before they react with cellular components (Vasankari *et al*., 1997). β-carotene showed protective activity against AFB1-induced hepatotoxicity (Khorshid *et al*., 2008), hepatic inflammation, fibrosis and attenuating cirrhosis (Seifert *et al*.,1995) in rats. The protective mechanism of β-carotene may also enhance immunity and down regulation of key cytokines (He *et al*., 2004). Kim (1995) reported that carotenoids have protective effect on oxidant-induced liver injury, improved the cell viability of hepatocytes, increased catalase activities and glutathione levels in hepatocytes from chronically ethanol-fed rats.

Moreover, many previous studies provided the presence of B-D- glucans in adequate percentage on the cell wall of yeasts, which had immuno – modulatory effect (Vetvicka 2001; Brown and Gordon 2003). As well as, B-D-glucans has antioxidant activity, scavenging the free radicals, reduced DNA-oxidative damage (Oliveira *et al*., 2009 and Sener *et al*. 2007).

Furthermore, in another studies (in vitro) an antagonistic yeast strain of *Rhodotorula glutinis* has been reported as an effective biocontrol agent against post- harvest decay of apples (Qin *et al*., 2003), pears (Zhang *et al*., 2008; Zhang *et al*., 2009), strawberries (Zhang *et al*., 2007), sweet cherries (Tian *et al*., 2004) and oranges (Zhang *et al*., 2005). Also, an antagonistic yeast strain of *Rhodotorula glutinis* and its autoploidy have been reported as an effective biocontrol agent (in vitro) and (in vitro) against grey mould of greenhouse sweet pepper (Haggag, *et al*., 2005).

The mode of action of the yeast strains (G1, G2 and G3) or their constituents antioxidant compounds against mutagenic or toxic effect of aflatoxin B_1 in animal cells (in vivo) may be due to binding with the

mutagens or inhibition of activation of enzymes of cytochrome systems- meiated N- hydroxylation with consequently enhancement of liver and kidney functions and reduction of abnormalities of genetic materials (Wang *et al*., 2004; Devaraj *et al*., 2008). It is well known that some of economically important yeasts are high ploidy. The increment of ploidy leads to a larger cells than that of the wild strain. Colchicine treatment of cells is an easy and simple method for breeding a microorganism possessing a high ploidy (Jibiki *et al.*,1993). So that, in the present study, when *R. glutinis* strains treated by 0.2% colchicine many ploidy level increments have been generated and showed increased in their cells diameter, size and density as well as DNA contents and growth rate. Convincing experimental evidence in support of the existence of a ploidy-driven mechanism of gene regulation was reported by Galitski *et al*. (1999). The aneuploidy can confer selective advantages by increasing the copy number of beneficial genes and protecting against lethal or deleterious mutations. The increases of yeast number and other unicellular organisms have been induced by a process of cell growth followed by division (Guijo *et al*., 1997). However, the present biochemical and cytogenetic results showed approximately similar effects of *Rhodotorula glutinis* and its autoploidy on normal animal cells (in vivo) or their use as protective agents against toxic effect of aflatoxin B_1 in mice.

5. Conclusion

In conclusion, the present study adds evidence that the treatment with yeast Strains *of Rhodotorula glutinis* is safe on animal cells and could be protective agent against toxic effect of aflatoxin (in vivo).

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Practical Aspects and Immune response of Probiotics Preparations Supplemented to Nile Tilapia (*Oreochromis Niloticus*) Diets

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Abstract: This study was carried out for 7 months at fish Laboratory of Animal Production Department, National Research Center, Dokki, Egypt. The experiment aimed to investigate the effect of two commercial probiotics (Premalac and Biogen) each at 1,2 and 3 g/Kg diet on growth performance and immune response of Nile tilapia fingerlings. Premalac is a dried fermented product of Lactobacillus acidophilus, Aspergillus oryzae extract, Bifedobacterium bifedum, Streptococcus faecium, Torula yeast, Skim milk, Vegetable oil and CaCo3. Biogen is a dried natural product composed of Allicin, high unit hydrolytic enzymes, Bacillus subtilis and Ginseng extract. A total of 420 fingerlings with a uniform size and weight (1 gram) were used of which 60 fingerlings represent the control group. The rest (360 fingerlings) distributed randomly into two blocks (probiotics), each block included three treatments (probiotic levels). Each treatment in addition to the control one were represented in three replicates (aquaria) in which 20 fingerlings were kept in each aquarium. The best results of growth and feed utilization of tilapia were obtained by fish diet supplemented with Biogen followed by those having Premalac, each at 2g/Kg diet. However, fish fed on Biogen-supplemented diets exhibited significantly higher values of nutrients digestibility. On the other hand, fingerlings fed either Premalac or Biogen at 2g/Kg had significantly higher total leucocytes count than the control which indicating highs immune response of tilapia fingerlings. In conclusion, it is suggested that the tested probiotics preparations are suitable for mixing with tilapia diets to improve their performance and immunity. [Nature and Science. 2010;8(5):39-45]. (ISSN: 1545-0740).

Key words: Oreochromis Niloticus , Immune response, Probiotics Preparations.

1. Introduction

The use of probiotic products as feed supplements has attracted considerable attention by feed manufactures as means of improving livestock performance. The diverse range of bacteria has been examined as probiotics for possible use in aquaculture **(Watson *et al,* 2008)**.

Most studies concerned with the effects of probiotics on cultured aquatic animals have emphasized a reduction in mortality or, conversely, increased survival (**Change and Liu, 2002**), improved resistance against disease (**Villamil *et al.*, 2003)**; enhance the ability to adhere and colonize the gut (**Vine *et al.*, 2004; Abo-State 2009)**; improved the ability to antagonize other organisms (**Burgents *et al.*, 2004; Li *et al.*, 2004; Brunt and Austin, 2005; Panigrahi *et al.*, 2005 and Shelby *et al.*, 2006)** also, the ability to reduce the number of bacterial cells in kidneys (**Park *et al.*, 2001)**, the production of polyamines and digestive enzyme activity (**Tovar *et al.*, 2002 and Hidalgo *et al.*, 2006)** and the development of the non specific immune system by means of Cellular systems like increased phagocytic and lysozyme activities (**Irianto and Austin, 2002)**.

The use of probiotics in aquaculture has been accompanied by an increase in nutrient utilization through providing enzymes capable of converting certain components of the diet into more digestible nutrients for the host. In this connection, **Geovany *et al.*, (2007)** showed that feeding probiotics may improve appetite and growth performance of the farmed fish or what?? species. Hwever, the specific function of probiotics may differ depending on the host animal and more on the characteristics of the probiotics. **Guillian *et al.*, (2004) El-Haroun *et al.*, (2006) and Eid and Mohamed (2008)** found that *Bacillus* spp. may modulate the mucosal immune system enhancing their resistance to enteric pathogens and so improved survival and growth of tilapia. Also, **Venkat *et al.*, (2004)**found that *Lactobacillus* spp. have an inhibitory effect on the harmless gram negative bacteria present in gut microflora of *Macrobrachium rosenbergii* post-larvae, and so reduced the mortality significantly.

The objectives of this study are to investigate the effect of two commercial probiotics (Premalac and Biogen) on growth performance and immune response of Nile tilapia fingerlings.

2 - Materials and Methods

This experiment was conducted for seven months, using Nile tilapia (*Oreochromis niloticus)* all male fingerlings, one gram average weight obtained from Kafr El-sheikh fish hatchery, Egypt.

A total number of (420) tilapia fingerlings were distributed at randoum into seven experimental dietary treatments including the control one, each in three aquaria (60x30x40 cm^3) as replicates.

Water temperature ranged between 27.4-27.6ºC with photo period 12h light and 12 h darkness. The disolved oxygen level was 6.6 mg/L, while records for total ammonia, nitrite, nitrate and pH levels were appropriate for tilapia cultivation, being 0.07 mg/L, 0.06mg/L, 5.93mg/L and 7.6, respectively (**APHA, 1992)**.

Experimental design:

A conventional corn-SBM-fish meal basal diet was formulated to meet the minimum requirements of fish (**NRC, 1993)**, and used with supplementation representing the control unsupplemented group (Table 1). The basal diet was supplemented with two commercial probiotic preparations being Premalac and Biogen. Premalac is a dried fermentation product of *Lactobacillus acidophilus*, *Aspergillus oryzae* extract, *Bifedobacterium bifedum*, *Streptococcus faecium*, Torula, yeast, Skim milk, vegetable oil and $CaCO_3$. Biogen is a dried natural product composed of Allicin, high unit hydrolytic enzymes, *Bacillus subtilis* and Ginseng extract.

Each one of the tested probiotics was applied at 1, 2 and3 g/Kg diet as recommended by the producers. The viability of the tested probiotics were tested before using according to the method outlined by **Martin *et al.*, (1981)** . The viable contents were determined by counting the CFU (Colony Forming Unit), which is considered as an indication for the viability of the microorganisms present viable in these commercial probiotics and so represents their growth promoting effect. Such determination showed the presence of $8.5x10^6$ and $2.5x10^7$ CFU, for Premalac and Biogen, respectively. Then, the feeding experiment started and durated for 7 months. Feeding level was 4% of the total biomass of the fish /day. The amount of feed was divided into three equal portions at 9 a.m , 1 p.m and 5 p.m. Every fourteen days, the fish in each aquarium were weighed and the amount of feed was corrected according to the new fish biomass (**Annet, 1985)**.

At the end of the feeding period, digestibility trials were conducted using 8 fish /aquarium and offered the experimental diets at a rate of 2% of the total biomass/day. The collected feces were directly spread with 10% sulfuric acid and 10% formalin and kept in deep freezer at -4ºC. Analysis of CP, EE, CF and Ash in the collected feces were carried out in pooled dried samples. Proximate chemical analysis were made according to **A.O.A.C. (1990)** methods while their energy contents were calculated according to **Jobling (1983)**. Apparent digestion coefficient (ADC %) of the nutrients were calculated using crude fiber as an inert marker as described by **Tacon and Rodringus (1984)** as follows:

$$ADC\% = 100 - 100 X \left[\frac{\%\,Marker\ in\ feed}{\%\,Marker\ in\ Feces} X \frac{\%\,Nutrient\ in\ feces}{\%\,Nutrient\ in\ feed} \right]$$

Data of the study include the growth performance parameters, feed utilization, nutrients digestibility of dietary treatments and the immune response of tilapia as affected by the tested probiotics.

The immune response test was done by subjecting the experimental fish prefed the tested probiotics to injection with one of the most common bacterial infection among cultured fish being *Aeromonas hydrophilla* for determining the differential count of blood film.

A total number of 168 fish (8 fingerlings/aquarium) obtained from the stock were used for immune response test. The fish were injected with *A. hydrophilla* inter-peritoneally (I.P.) at dose of 0.2 ml/fish representing $2x10^8$ bacterial cell /ml for each fish. After two weeks of injection, blood films from duplicate samples were prepared (**Sakai *et al.*, 1995)** and examined at X400 to determine the number of leucocytes, being indicator for immunity.

Statistical analysis:

Growth performance and feed utilization efficiency parameters were statistically compared using the SAS programme (1992) SAS/STAT users guide, Release 6.03 Edition SAS inst. INC. Cary, NC. USA.

Duncan's New Multiple Range Test was conducted to determine the significant differences between means (**Duncan, 1955)**.

3. Result Analysis

This experiment was extended for 7 months to study the effect of two commercial probiotics on performance and feed utilization as well as the immune response of tilapia fingerlings. These commercial probiotics were Premalac which is commonly used for poultry at 1g/Kg diet being the recommended level, however, because it would be applied in a different media being water, therefore, it is proposed to use Premalac in addition to Biogen which is specific for fish, each at 1,2 and 3g/Kg diet.

The average values of initial live weight, final weight, weight gain, specific growth rate and average daily gain of Nile tilapia are given in Table(2).

It appears that all dietary treatments have commenced with a nearly similar initial body weight which ranged between 1.10 to 1.33 g with slight

(P>0.05) differences, so confirmed the appropriate randomization process.

Results indicated that Nile tilapia fingerlings which received diet supplemented with Biogen at 2g/Kg diet have highest final live body weight, weight gain, average daily gain (20.11, 18.88 and 0.12g), respectively compared to other treatments. However, there were no significant differences (P<0.05) between treatments in SGR (Table 2). Feed intake, feed conversion ratio and nutrients utilization are shown in Table (3). Results obtained indicated that feed intake increased gradually as the level of the probiotics incorporation increased. However, results of the values obtained for feed intake among all the experimental groups revealed that diet supplemented with Biogen at 2 or 3 g/Kg diets was superior than other dietary treatments.

The results indicated that there were no significant differences among most groups in FCR and nutrients utilization expressed as PER PPV, FPV and EU. Meanwhile, data obtained showed the superiority of dietary treatments having either Premalac or Biogen at the level of 2g /Kg diet compared to other dietary treatments. Moreover, increasing the supplemental level of any of the tested probiotics up till 3g/Kg diet become worth the feed conversion ratio to become the worst, besides, it lowered also the utilization of other nutrients (protein, fat and energy). Therefore, a dose effect relationship indicates that dosage of probiotics should be defined carefully to avoid over dosing with resultant lower efficiency and consequently unnecessary costs (**Nikoskelainen *et al*., 2001)**.

This means that Nile tilapia fingerlings grew well when either Biogen or Premalac at 2g/Kg diet has been used as growth promoters in fish feeding.

However, by comparison, the probiotic Biogen (Specific for fish) was superior than Premalac (commonly used for poultry) in aquatic environments. Therefore, the intimate relationship between bacteria and their host should be considered. In this respect, **Olafsen (2001)** stated that the use of probiotics which has proven advantageous in domestic animal or poultry production and microbial management may also have a potential in aquaculture. This because the gastrointestinal microbiota of fish are peculiarly dependent on the external environment, due to the water flow passing through the digestive tract. Most bacterial cells are transient in the gut, with continuous intrusion of microbes coming from water and food (**Gatesoupe, 1999)**. In addition**,** by feeding fish with probiotics bacteria, these bacteria will also be present in the surrounding water and also colonise the fish skin and other parts of the body (**Nikoskelainen *et al*., 2001)**. The most likely explanation of the effective role of probiotics is their effect in suppressing pathogenic coli forms in the stomach and intestine and improving the absorption of nutrients by reducing the thickness of intestinal epithelium (**Venkat *et al*., 2004)**.

The improvement in live body weight in probiotic treated groups of fish is mainly due to maintaining the beneficial bacteria such as Lactobacillus in the intestinal tract which can competes with the undesirable organisms for space and nutrients as reported by **Jena *et al*., (1996)**. Such useful bacterial growth facilitates the fermentation process which is of nutritional significant such as producing various types of vitamins (**Fuller, 1997)** and organic acids which provide energy to the host as well as stimulate the growth.

Average apparent digestion coefficient (ADC) of DM, CP, EE, NFE and energy using CF method as an internal marker are presented in Table (4). Statistical analysis of these data showed significant differences ($P \leq 0.05$) among all the treatments.

The highest figures of DM, CP, EE, NFE and energy digestibility were obtained form groups of fish fed diets supplemented with Biogen at level of 2g/Kg being (90.93, 88.42, 92.62, 97.31 and 91.95% respectively, while the corresponding values of the control group were 87.14, 85.89, 89.42, 93.50 and 88.17% at the same order, followed by Premalac .

The data obtained declared that tilapia were able to utilize dietary nutrients when the tested probiotics had been added particularly at 2g/Kg diet. However, the probiotic Biogen was found to be the most successful one followed by Premalac for Nile tilapia fingerlings.

This result go paralleled with the results obtained by **De Schrijver and Ollevier (2000)** who reported a positive effect on apparent protein digestion with supplementing turbot feeds with the bacteria *Vibrio proteolyticus.* They attributed this effect to the proteolytic activity of bacteria. In this connection, **Lara Flores *et al*., (2003)**found that the digestibility results for the control groups were lower than those having the probiotics supplemented diet.

Determining the immune response of experimental fish to tested Probiotics had been done by detecting the differential count of blood film, to asses the changes in blood content of white cells following spontaneous infection of tilapia to evaluate the serosity of the disease on the basis of blood alterations (Table 5). The results were listed in Table (5). Both Premalac and Biogen treated fish groups had significantly greater mean total leucocytes count than the control group, particularly when added to fish diets at 2g/Kg.

When Probiotics and control fish groups were challenged with *Aeromonas hydrophilla*, there probiotics which containing *Lactobacillus and Bacillus* sp. supplied fish with more immunity compared with the control. These findings are in agreement with those reported by **Rengpipat *et al.*, (2000)** who stated that *Bacillus* sp provided disease protection to shrimp by activating both cellular and humoral immune defenses. Also, **Nikoskolainen *et al.*, (2001)** found that Lactobacillus strain increased the immune response of rainbow trout challenged with *Aeromonas salmonicida* sp.

In general, the increase in leukocytes count during infection of tilapia fed either Premalac or Biogen probiotics, containing organism released defense against pathogens as indicated by **Caruso *et al.*, (2002)** and **Benli and Yildiz (2004)** . However, the strength of immunity obtained depend to a large extent on the active microorganisms still viable after mixing with feed. The viability of the tested probitoics were $8.5x10^6$ and $2.5x10^7$ CFU for Premalac and Biogen, respectively.

Table (1): Composition and calculated chemical composition of the basal diet used in the experiment.

Ingredient	%
Yellow corn	48.0
Soybean meal (44%)	18.5
Fish meal (72%)	26.0
Corn oil	5.0
Vit.& Min. Mix.	2.5
Total	**100**
Calculated analysis (on DM basis) %	
DM	93.70
CP	32.13
EE	11.01
CF	1.83
Ash	5.59
NFE	49.44
Nutritive value	
GE kcal/kg **	4906.6
E:P ratio	152.7

* each 1 kg. contains vitamin A, 4.8 mI.U.; vit D_2 0.8 mI.U/ vit E, 4.0g/ vit. K, 0.8 g; vit B_1, 0.49 vit. B_2 , 1.6 g; vit. B_6, 0.6; vit. B_{12}, 4 mg; Pantothenic acid 4g; Nicotinc acid 8g; Folic acid, 4000 mg; Biotin 20 mg; Choline chloride, 200 mg; Copper, 4.0 g; Iodine, 0.4g; Iron, 12 mg; Manganese, 22g; Zinc 22 g and Selenium 0.04g.

** Gross energy value was calculated from their chemical composition, using the fac5os 5.65 , 9.45, 4.00 and 4.00 (k cal/g) for protein, fat, fiber and NFE, respectively (**Jobling, 1983**).

Table (2): Performance of Tilapia (*O.n)* as affected with the tested probiotics.

Treat	Initial weight g	final wt. g	Wt. gain g	SGR	ADG g
Cont	1.15 ^{a} ±0.04	18.93 ^{ab} ±1.14	17.78 ±1.15 ^{ab}	1.80 ^{a} ±0.08	0.84 ^{ab} ±0.01
P_1	1.11 ^{a} ±0.04	15.75 ^{bc} ±21.14	14.64 ^{bc} ±1.15	1.69 ^{a} ±0.01	0.07 ^{bc} ±0.01
P_2	1.10 ^{a} ±0.04	13.51 ^{bc} ±1.14	12.41 ^{c} ±1.15	1.61 ^{a} ±0.08	0.06 ^{c} ±0.01
P_3	1.18 ^{a} ±0.04	16.38 ^{c} ±1.14	15.20 ^{abc} ±1.15	1.69 ±0.08	0.07 ^{c} ±0.01
B_1	1.25 ^{a} ±0.04	16.07 ^{bc} ±1.14	14.82 ^{bc} ±1.15	1.65 ^{a} ±0.08	0.07 ^{abc} ±0.01
B_2	1.23 ^{a} ±0.04	20.11 ^{a} ±1.14	18.78 ^{a} ±1.15	1.80 ^{a} ±0.08	0.89 ^{A} ±0.01
B_3	1.23 ^{a} ±0.04	18.77 ^{ab} ±1.14	17.54 ^{b} ±1.15	1.71 ^{a} ±0.08	0.083 ^{Ab} ±0.01
Proba	0.0226	0.0132	0.0170	0.6912	0.0314

a, b: Means within column with different superscripts are significantly different ($P<0.05$).

P = Premalac, B = Biogen.

SGR = specific growth rate

ADG = average daily gain

Table (3): Feed consumption, feed conversion ratio and nutrients utilization of tilapia (*O.n*) as affected with probiotics supplementation.

Treat	Feed consump. g	FCR	PUE	PPV	FPV	EU
Cont	35.59 ^{abc} ±2.15	2.04 ^{a} ±0.14	1.83 ^{a} ±0.08 ^{a}	24.13 ^{a} ±1.278	55.83 ^{a} ±3.10	15.90 ^{a} ±0.85
P_1	33.79 ^{a} ±2.14	2.37 ^{a} ±0.14	1.51 ^{a} ±0.08	21.94 ^{a} ±1.27	50.28 ^{ab} ±3.10	15.03 ^{ab} ±.851
P_2	33.04 ^{a} ±1.154	2.18 ^{a} ±0.14	1.63 ^{a} ±0.08 ^{a}	20.55 ^{a} ±1.27	57.63 ^{a} ±3.10	15.53 ^{ab} ±0.85
P_3	31.14 ^{c} ±1.15	2.51 ^{a} ±0.14	1.41 ^{a} ±0.08	19.07 ^{a} ±1.27	44.55 ^{b} ±3.10	12.91 ^{a} ±0.85
B_1	33.36 ^{a} ±1.15	2.24 ^{b} ±0.17	1.55 ^{a} ±0.08	20.36 ^{a} ±1.27	44.67 ^{b} ±3.10	13.45 ^{b} ±.85

B_2	38.75 ^{ab} ±1.15	2.06 ^{a} ±0.14	1.70 ^{a} ±0.08	21.47 ^{a} ±1.27	50.90 ^{ab} ±3.10	14.40 ^{ab} ±0.85
B_3	40.80 ^{a} ±1.16	2.33 ^{a} ±0.14	1.50 ^{a} ±0.08	19.28 ^{a} ±1.27	47.11 ^{a} ±3.10	13.06 ^{b} ±0.85
Proba	0.0674	0.2466	0.6912	0.0433	0.0650	0.1295

A, b: Means within column with different superscripts are significantly different (P<0.05).

P = Premalac, B = Biogen.

FCR = Feed conversion ratio

PUE = Protein utilization efficiency.

PPV = Protein productive value.

FPV = Fat productive value.

EU = Energy utilization.

Table (4): Apparent digestiion coefficient (ADC%) of nutrients of the experimental diets.

	ADC %				
	DM	**CP**	**EE**	**NFE**	**Energy**
Cont	87.14 ^{a} ±0.66	85.89 ^{a} ±0.914	89.42 ^{b} ±0.28	93.50 ^{a} ±0.77	88.17 ^{a} ±0.69
P_1	85.07 ^{c} ±0.66	74.08 ^{c} ±0.91	80.41 ^{c} ±0.58	93.14 ^{bc} ±0.77	82.28 ^{c} ±0.69
P_2	88.08 ^{a}b ±0.66	79.86 ^{b} ±0.91	90.52 ^{b} ±0.58	96.15 ±0.77 ^{a}	87.77 ^{b} ±0.69
P_3	84.12 ^{c} ±0.66	59.78 ^{de} ±0.91	72.66 ^{e} ±0.58	90.84 ^{cd} ±0.77	75.40 ^{e} ±0.69
B_1	85.52 ^{c} ±0.66	61.67 ^{d} ±0.91	76.94 ^{d} ±0.58	93.79 ^{b} ±0.77	78.49 ^{d} ±0.69
B_2	90.93 ^{a} ±0.66	88.42 ^{a} ±0.91	92.62 ^{a} ±0.58	97.31 ^{a} ±0.77	91.95 ^{a} ±0.69
B_3	84.19 ^{c} ±0.66	57.58 ^{e} ±0.91	75.22 ^{d} ±0.58	88.56 ^{d} ±0.77	74.34 ^{e} ±0.69
Proba	0.0001				

a, b: Means within column with different superscripts are significantly different (P<0.05).

P = Premalac, B = Biogen.

DM = Dry matter, CP= crude rpotein, EE= Ether extract,

NFE = Nitrogen free extract

Table (5): Mean immunity index values for Nile tilapia fed the experimental diets.

	Differential count of white blood cells ($1x10^3$ cell/ml)				
	Total	**Hetero**	**Lympho**	**Mono**	**Eosino**
Cont	52.00 ^{d} ±0.51	16.40 ^{d} ±0.40	32.50 ^{c} ±0.38	2.60 ^{bc} ±0.13	0.69 ^{d} ±0.03
P_1	52.30 ^{d} ±0.51	23.80 ^{b} ±0.40	24.30 ^{e} ±0.39	3.30 ^{a} ±0.13	0.94 ^{c} ±0.03
P_2	70.50 ^{a} ±0.51	29.90 ^{a} ±0.40	36.70 ^{a} ±0.38	2.10 ^{d} ±0.13	1.73 ^{a} ±0.03
P_3	53.50 ^{b} ±0.516	23.08 ^{b} ±0.40	26.50 ^{d} ±0.38	2.40 ^{cd} ±0.13	1.60 ^{b} ±0.03
B_1	52.70 ^{cd} ±0.51	15.60 ^{d} ±0.40	35.30 ±0.38 ^{b}	1.10 ±0.13 ^{e}	0.52 ±0.03 ^{e}
B_2	67.70 ^{b} ±0.51	29.70 ^{a} ±0.40	34.40 ±0.38 ^{b}	2.90 ±0.13 ^{b}	0.66 ±0.03 ^{d}
B_3	54.00 ^{c} ±0.51	19.13 ^{c} ±0.401	32.90 ±0.38 ^{c}	1.30 ±0.13 ^{e}	0.53 ±0.03 ^{e}
Proba	0.0001				

a, b: Means within column with different superscripts are significantly different (P<0.05).

P= Premalac, B = Biogen.

4. Conclusions

In conclusion, all results obtained indicated that either Biogen or Premalac at 2g/Kg produced a positive effect on growth and feed utilization of tilapia fingerlings. In addition, the immune responses were substantial in both treatment groups following the challenge with bacterial infection. However, the probiotics Biogen when added to fish diet at 2g/Kg, produce a steady improvement of tilapia growth compared to Premalac.

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Effect of Probiotics on performance and nutrients digestibility of Nile tilapia *(Oreochromis niloticus*) Fed Low Protein Diets

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Abstract: This study was carried out at fish laboratory of Animal Production Department, National Research Center, Dokki ,Egypt using two commercial probiotics (Premalac and Biogen) to study their effects on growth the performance of Nile Tilapia fed diets with slightly lower levels of crude protein. Premalac is a dried fermented product of *Lactobacillus acidophilus, Aspergillus oryzae* extract , *Bifedobacterium bifedum , Streptococcus faecium* , Torula yeast , skim milk , vegetable oil and $CaCo_3$. Biogen is a dried natural product composed of Allicin , high unit hydrolytic enzymes, *Bacillus subtilis* and Ginseng extract. The basal diets were formulated to contain 30, 27.5 and 25.0% crude protein (CP), each was either supplemented or not with either Premalac or Biogen at 2g /kg diet. The experiment was conducted in 3x3 factorial design and included nine treatments each in three replicates (aquaria) in which 20 fingerlings mono sex Nile tilapia of the same size and weight (1 gram) were stocked in each aquarium. The actual experimental feeding trials durated for four months. Results indicated that the lowest CP level (25%) in tilapia diets without or with the tested probiotics recorded the worst values of growth performance parameters and nutrients digestibility. The use of either Premalac or Biogen at 2g/kg diet in diets with 27.5% crude protein was more efficiently than those containing 30% CP,. in addition, it gave best values of economic efficiency. However, Biogen was more superior than Premalc. In other words, these probiotics spared nearly 2.5% CP of the recommended level for tilapia. This result would be effective from the economical point of view, since protein is the most expensive feed nutrient in all live stock feeding, particularly fish. [Nature and Science. 2010;8(5):46-53]. (ISSN: 1545-0740).

Key words: Probiotics; Oreochromis niloticus; nutrients digestibility.

1. Introduction

Nile tilapia is an economically important cultured species in several areas of the world **(El-Husseny *et al.*, 2007 and El-Saidy and Gaber, 2005)**. Egypt made an impressive increase in aquaculture tilapia production, from 24 916 mt in 1990 to 487000 mt in 2005m accounting for 55% of Egyptian total fish production (879000, mt $year^{-1}$). **(FAO 2004 and GAFRD, 2006)**.

As the principal and most expensive component in tilapia diets, protein has received the most attention in nutritional requirement studies. Tilapia need continuous supply of protein which is necessary for maintenance, growth and reproduction (**NRC, 1993)**. A study evaluating the least –cost dietary protein level for four species of tilapia (*Oreochromus mossambicus, O. niloticus, O. aureus and Tilapia Zillii)* showed that the dietary protein level from 34% to 36% provided maximum growth of young tilapia (1.5 g) , but the most cost-effective protein level was 25% to 28% (**De Silva *et al.*, 1989)**.

Practical feeds for grow out of tilapia usually contain 25 to 35% CP. However, it has been reported that the dietary CP requirements of fish vary with species, size or age , protein quality, dietary energy level, water quality, feeding and culture management. **Ogunji and Wirth (2000)** found that the diet containing 33.32% dietary protein DM with a protein: energy ratio of 16.26mg/kJ appeared optimal for the protein requirement of tilapia. **Meyer and Pera (2001)** indicated that tilapia efficiently utilize dietary protein at level between 25 and 35%. The same authors added that , the fish were less efficiently at utilizing 45% crude protein in the diet for growth.

Ogunji and Wirth (2002) concluded that a dietary deficiency in protein results not only in a deficiency of essential amino acids in the body but also affects transport and storage of lipids within the fish body

The probiotics of live microbes have shown their effectiveness to mitigate the effects of stress , resulting in a greater production. **Olvera *et al.*, (2001)** concluded that yeast have a positive effect on fish performance when cultured under stress condition of lowering dietary protein, leading to improving growth and feed efficiency. In contrast , **Hidalgo *et al.*, (2006)** found that growth and feed conversion of juvenile dentex were not significantly influenced by probiotics which is in agreement with the findings, **Shelby *et al.* (2006)** who found that the probiotic used with juvenile channel catfish diet had lack effect on specific growth promoting or immune stimulating aspects.

On the other hand, many studies concluded the positive effect of using viable microorganisms in probiotic mixtures into diets of fish (**Li. and Gatlin 2004; Brunt and Austin, 2005; Pangrahi *et al.*, 2005; Barnes *et al.*, 2006**; **Abo-State *et al.*, 2009).**

Regarding the effect of interaction between dietary CP and probiotics, **Lara- Flores *et al.*, (2003)** evaluated the effects of probiotics on growth performance in Nile tilapia under two stress factors. These stressors were dietary protein level and stocking density. They found that the fry fed diets with a probiotic supplement exhibited greater growth than those fed the control diet without probiotic.

The aim of this study is to find out the effect of Premalac and Biogen as commercial probiotics on growth and protein utilization of Nile tilapia fingerlings fed on diets with different levels of CP.

2. Material and Methods

The experiment was conducted for four months, using a total number of 540 Nile tilapia (*Oreochromis niloticus)* all male fingerlings with one gram average weight obtained from Kafr El-Sheikh fish hatchery Egypt.

The fingerlings were distributed at random into nine experimental dietary treatments , each in three aquaria (60x30x40 cm^3) as replicates in which fingerlings were stocked at a rate of 20 fish/ aquarium. Water temperature in the experiment of aquaria ranged between 27.4-27.6ºC with photo period of 12h light and 12h darkness, the dissolved oxygen level was 6.6 mg/L, and the pH value was about 7.5 representing the alkali media needed for tilapia. Water samples were weekly taken for analysis of total ammonia nitrite, nitrate and pH levels using standard methods (**APHA , 1992**) to adjust the appropriate water quality parameters for tilapia cultivation .

Experimental diets and design

Two tested commercial probiotics being Premalac and Biogen were used to study their effects on growth performance of Nile tilapia fingerlings fed diets with slightly decreasing levels of crude protein (30, 27.5 and 25%). Premalac is a dried fermentation product of *Lactobacillus acidophilus* , *Aspergillus oryzae* extract , *Bifedobacterium bifedum*, *Streptococcus faecium* , Torula yeast , skim milk , vegetable oil and $CaCO_3$. Biogen is a dried natural product composed of Allicin , high unit hydrolytic enzymes, *Bacillus subtilis* and Ginseng extract . Three basal diets were formulated to contain the recommended 30% and two lower 27.5 and 25% crude protein levels (Table 1). Each of the tested diets was either supplemented or not with either Premalace or Biogen at 2g /kg diet level. Therefore , The experiment was conducted in 3x3 factorial design and included nine treatments . Feeding level of all experimental diets was 4% of the total biomass of the fish per day. The amount of feed was divided into three equal portions and distributed by hand in one side of the aquaria three times daily at 9 a.m, 1p.m. and 5 p.m. Every fourteen days, the fish in each aquarium were weighed and the amount of feed was readjusted according to the new fish biomass **(El-Banna, 1991).** The performance parameters included weight gain, average daily gain (ADG), feed conversion ratio (FCR) and protein utilization efficiency (PUE). After running the feeding experiment, digestibility trails were conducted using 8 fish /aquarium and fish were offered the same experimental diets at a rate of 2% of the total biomass/day.

During the digestibility trail the daily meal was offered one time at 9.00 a.m. and the siphoning method was undertaken to collect the settled feces from the bottom of the aquarium. The collection of feces was made for three weeks, one time daily in the morning before the meal was provided. Consequently, the collected feces were directly spread with 10% sulfuric acid and 10% formalin and kept in a deep freezer at -4C till analysis to avoid the fermentation.

Analysis of protein, ether extract, crude fiber, and crude ash in the collected feces were carried out in pooled –dried samples. Proximate chemical analysis were made according to **A.O.A.C (1990)** methods.

Apparent Digestion Coefficients (ADC%) of the nutrients were calculated using crude fiber as an inert marker as described by **Tacon and Rodringus (1984)** as follows:

$$\%\ ADC = 100 - 100 \times \left[\frac{\%\ Marker\ in\ feed}{\%\ Marker\ in\ feaces} \times \frac{\%\ Nutrient\ in\ feces}{\%\ Nutrient\ in\ feed} \right]$$

The economical efficiency of dietary treatments were calculated to estimate the cost of feed needed to produce one kg of fish weight gain. The cost of experimental diets has been calculated in L.E according to the local market prices at year 2004.

Growth performance and feed utilization efficiency parameters were statistically compared using the **SAS programme (1992)** SAS/STAT users guide, release 6.03 Edition SAS Inst. INC. Cary, NC. USA.

Considering the interaction effect between dietary CP level and added probiotics , the used model was :

$$Xijk = M + Pi + Lj + Pi\,Lj + Eijk$$ where ,

- M the overall mean
- Pi the effect of probiotic supplementation
- Lj the effect of cp level

- Pi Lj the effect of interaction between p and l
- Eijk the experimental error .

Duncans New Multiple Range Test was conducted to determine the significant differences between means (**Duncan, 1955)**.

3. Result Analysis

Growth and feed utilization:

The effects of both dietary CP, probiotic supplementation and their interaction on growth and feed utilization of Nile tilapia fingerlings are summarized in Tables (2 and 3). The initial live body weight of all fish used was almost similar, which confirmed appropriate randomization process . Meanwhile, it created suitable condition to appraise the effect of dietary treatments on the performance of fish .

Results showed that the diet contained 27.5% CP irrespective of probiotic supplementation gave better ($P<0.05$) values for final body weight, FCR, PUE of fish when compared with those containing 30% and 25% CP. Regarding the tested probiotics, results showed that adding either Premalac or Biogen at 2g/kg recorded significantly ($P<0.05$) higher values compared to the control without supplementation .However , The best values of live body weight, FCR and PUE were obtained with Biogen followed by Premalac.

The combined effect of dietary CP with the tested probiotics showed that the diet contained 27.5% CP and supplemented either Premalac or Biogen (T5 , T6) had recorded best values for growth performance and improved the feed utilization when compared to diets with the same probiotics but contained the recommended level of CP as 30% (T_2, T_3). Moreover, the diets with 27.5% CP (T_5, T_6) were superior than the corresponding diet with the same CP level but without probiotic supplementation (T_4). This may be due to the effect of the tested probiotics which improved absorption of nutrients and depressed harmful bacterial affects that causes growth depression. These results are in agreement with those obtained by **Hoyos and Cruz (1990)** who stated that the positive effect of probiotcs may be due to their beneficial effects since its microbial constituents produce natural lactic acid that helps in maintaining an optimum low pH in the digestive tract which inhibit growth of undesirable bacteria leading to optimum enzyme activity. Similar results were observed by **Fernandes and Shahani (1990)**, who indicated that probiotic preparations contain multiple strains of *Lactobacillus* which are highly active against a wide range of stress conditions in the fish gastrointestinal tract, resulting in higher immunity and higher rate of utilizing nutrients and accordingly higher growth rate.

In this connection, **Noh *et al.* (1994)**; **Bogut *et al.* (1998)** and **Nikoskelainen *et al.* (2001)** obtained better growth response with diets supplemented with probiotics containing bacteria.

Although , diets with the high CP (30 %) and supplemented with any of the tested probiotics had recorded significantly better values than the corresponding diet without supplementation, nevertheless, diets with 27.5 % CP supplemented with probiotics were superior. These results may be explained as these probiotics (Premalac or Biogen) spared nearly 2.5% crude protein so, optimizing protein utilization for growth. These findings are in good agreement with those obtained by **Ringø and Gatesoupe (1998)** , who found that probiotics performed more efficiently in case of stress conditions like that of lowering dietary CP.

Nutrients digestibility:

Results of the effect of either dietary CP, probiotic supplementation or their interaction on nutrients digestibility are listed in table (4). Results indicated that tilapia fingerlings utilized the nutrients content of diets with 30% or 27.5% CP at equal rate. While, that contained 25% CP failed to obtain similar findings. Seamingly, Premalac and Biogen both have the same effect on nutrients digestibility. Regarding the interaction effect of dietary CP and probiotics supplementation on nutrients digestibility (Table 4), results revealed that either Premalace or Biogen when supplemented to diet containing 30% or 27.5% CP recorded significantly ($P<0.05$) higher values of nutrient digestibility than the non supplemented diets, with no significant differences among those treatments. However, the use of the same probiotics with diets containing 25% CP recorded significantly the lowest values.

Similar results were obtained by **De Schrijver and Ollevier (2000)** who found positive effect on apparent protein digestion when turbot (*Scophthalmus maximus*) diets were supplemented with bacteria *Vibrio proteolyticus*. They attributed this effect to the proteolytic activity of bacteria.

Economical evaluation:

Data in Table (5) show that the best values of economical efficiency expressed as feed cost /kg gain in weight and relative economic efficiency were for diets containing 27.5% CP and supplemented with either Premalac or Biogen. Meanwhile, the worst values were recorded with diets containing the lowest CP (25%) level .

By comparison, the results obtained confirmed the higher adequacy of Biogen for tilapia than Premalac,

which is commonly used in poultry feeding. It is obvious that the bacterial strain *Bacillus subtilis* mainly present in Biogen is more convenient to the digestive system of fish than *Lactobacillus* strain in Premalac. Moreover, Biogen contains ginseng extract that needed by fish to keep their internal biological functions at sufficient levels. In addition, Biogen characterized by the presence of Allicin which helps to increase the activity of endocrine glands, therefore, secretion of various hormones especially growth hormone are increased. It contained also high-unit hydrolytic enzymes such as photolytic, biolytic and amylolytic that have the capacity to increase the digestibility and utilization of nutrients and decrease ammonia production by fish, which results in higher growth with lower feed consumption, otherwise healthy environment.

Table (1): Composition and calculated analysis of the experimental diets.

Ingredient , %	**Recommended CP**	**Lower CP**	
	30%	**27.5%**	**25.0%**
Yellow corn	48.0	52.0	56.0
Soy bean meal (44%)	18.5	18.5	18.5
Fish meal (72%)	26.0	22.0	18.0
Corn oil	5.0	5.0	5.0
Vit. Min. Mix*	2.5	2.5	2.5
Total	100	100	100
Calculated analysis , %			
Moisture	6.30	7.00	7.60
DM Composition:			
CP	32.13	29.62	27.11
EE	11.01	10.34	10.34
CF	1.83	1.89	1.94
Ash	5.59	5.07	4.55
NFE	49.44	52.75	56.06
GE kcal / kg **	4906.6	4867.4	4828.8
E : P ratio	152.7	164.3	178.1
Price / ton (L . E)	2932.2	2708.6	2485.0

* Each 1 kg. Contains vitamin A, 4.8 I.U.; vit D_2 , 0.8 m I.U; vit E, 4.0 g; vit. K, 0.8 g; vit B1, 0.49, vit. B2, 1.6 g; vit. B6, 0.6 g; vit. B12, 4 mg; Pantothenic acid 49; Nicotinc acid 8 g; Folic acid, 400 mg; Biotin, 20 mg; Choline chloride, 200 mg; Copper, 4.0 g ; Iodine,0.4g ; Iron, 12 mg ; Manganese, 22 g; Zinc 22 g and Selenium 0.04 g.

** Gross energy value was calculated from their chemical composition, using the factors 5.65, 9.45, 4.00 and 4.00 (k cal/g) for protein, fat, fiber and NFE respectively **(Jobling, 1983).**

Table (2): Effect of dietary protein level, probiotics supplementation and their interaction on growth parameters of Nile tilapia.

Treatment			**Initial wt. g/fish**	**Final wt. g/fish**	**Total gain g/fish**	**ADG g/fish**
Tr No.	**CP %**	**Probiotic**				
(1)	**30**		1.12	17.73^{b}	16.61^{b}	0.138^{b}
	27.5		1.15	19.87^{a}	18.72^{a}	0.156^{a}
	25		1.13	14.98^{c}	13.85^{c}	0.115^{c}
(2)		_____	1.14	16.38^{b}	15.24^{b}	0.127^{b}
		Premalac	1.13	18.03^{a}	16.90^{a}	0.141^{a}
		Biogen	1.14	18.55^{a}	17.41^{a}	0.145^{a}

T1	**30**	**Control**	1.14	17.95^{bc}	16.81^{bc}	0.140^{bc}
T2	**30**	**Premalac**	1.13	17.64^{c}	16.51^{c}	0.138^{bc}
T3	**30**	**Biogen**	1.14	18.65^{bc}	17.51^{bc}	0.146^{bc}
T4	**27.5**	**_____**	1.16	17.05^{bc}	15.89^{bc}	0.132^{bc}
T5	**27.5**	**Premalac**	1.12	21.10^{a}	19.98^{a}	0.167^{a}
T6	**27.5**	**Biogen**	1.18	21.60^{a}	20.42^{a}	0.170^{a}
T7	**25**	**_____**	1.12	14.15^{d}	13.03^{d}	0.109^{d}
T8	**25**	**Premalac**	1.14	15.36^{d}	14.22^{d}	0.118^{d}
T9	**25**	**Biogen**	1.12	15.40^{d}	14.28^{d}	0.119^{d}
SEM			0.028	1.98	1.82	0.020
Prob.			0.3014	0.0008	0.0007	0.0029

a, b .... Means in the same column within each factor with different superscripts are significantly different (P<0.05).

(1) Effect of CP level regardless to probiotics

(2) Effect of probiotic regardless to CP level

Table (3): Effect of dietary protein level, probiotics supplementation and their interaction on feed conversion ratio (FCR) and protein utilization efficiency (PUE) by Nile tilapia during whole period.

Treatment			**FCR**	**PUE**
Tr. No.	**CP %**	**Probiotic**	**feed / gain**	**gain / protein intake**
(1)	**30**		1.746^{a}	1.782^{b}
	27.5		1.695^{a}	1.991^{a}
	25		2.695^{b}	1.368^{c}
(2)		**_____**	2.185^{b}	1.556^{b}
		Premalac	1.926^{a}	1.765^{a}
		Biogen	1.934^{a}	1.756^{a}
T1	**30**	**Control**	1.812^{a}	1.717^{c}
T2	**30**	**Premalac**	1.723^{a}	1.806^{bc}
T3	**30**	**Biogen**	1.717^{a}	1.812^{bc}
T4	**27.5**	**_____**	1.919^{a}	1.759^{c}
T5	**27.5**	**Premalac**	1.634^{a}	2.066^{a}
T6	**27.5**	**Biogen**	1.623^{a}	2.079^{a}
T7	**25**	**_____**	2.986^{c}	1.235^{d}
T8	**25**	**Premalac**	2.570^{b}	1.434^{d}
T9	**25**	**Biogen**	2.648^{b}	1.393^{d}
SEM			0.324	0.203
Prob.			0.2487	0.0001

a, b .... : Means in the same column within each factor with different superscripts are significantly different (P<0.05).

(1) Effect of CP level regardless to probiotic

(2) Effect of probiotic regardless to CP level

Table (4): Effect of dietary protein level, probiotics supplementation and their interaction on apparent digestion coefficient (ADC) of dietary nutrients of Nile tilapia.

Treatment			ADC , %			
Tr. No.	**CP %**	**Probiotic**	**DM**	**CP**	**EE**	**NFE**
(1)	**30**		86.07[a]	85.23[a]	91.45[a]	82.21[a]
	27.5		86.13[a]	85.69[a]	92.00[a]	83.02[a]
	25		76.99[b]	76.28[b]	85.33[b]	77.87[b]
(2)		**_____**	78.20[b]	77.79[b]	86.71[b]	78.09[b]
		Premalac	84.30[a]	83.06[a]	90.85[a]	82.54[a]
		Biogen	86.55[a]	85.92[a]	90.92[a]	82.71[a]
T1	**30**	**Control**	80.85[b]	79.60[c]	89.45[b]	79.70[c]
T2	**30**	**Premalac**	86.95[a]	86.05[b]	92.11[a]	83.50[ab]
T3	**30**	**Biogen**	89.66[a]	89.61[a]	92.62[a]	84.21[a]
T4	**27.5**	**_____**	80.15[b]	79.82[c]	88.73[b]	78.45[c]
T5	**27.5**	**Premalac**	87.53[a]	87.15[ab]	92.50[a]	85.64[a]
T6	**27.5**	**Biogen**	89.74[a]	89.70[a]	93.04[a]	84.87[a]
T7	**25**	**_____**	73.60[c]	73.95[d]	81.95[c]	76.12[c]
T8	**25**	**Premalac**	78.42[bc]	75.98[d]	87.94[b]	78.48[c]
T9	**25**	**Biogen**	80.25[b]	78.45[c]	87.10[b]	79.05[c]
SEM			3.44	3.17	2.97	2.65
Prob .			0.0011	0.0024	0.0130	0.0260

a, b .... : Means in the same column within each factor with different superscripts are significantly different ($P<0.05$).
(1) Effect of CP level regardless to probiotic
(2) Effect of probiotic regardless to CP level

Table (5): Effect of dietary protein level and probiotics supplementation on the economic efficiency of the experimental treatments.

Treatment			**Cost /ton L.E ***	**Feed intake g/ fish**	**Total gain g/fish**	**Feed cost / kg gain L.E**	**Relative feed cost / kg gain % ****
Tr No.	**CP %**	**Probiotic**					
T1	**30**	**Control**	2932.2	30.47	16.81	5.31	100.00
T2	**30**	**Premalac**	3086.2	28.45	16.51	5.31	100.00
T3	**30**	**Biogen**	3042.2	30.07	17.51	5.22	98.30
T4	**27.5**	**_____**	2708.6	30.50	15.89	5.20	97.92
T5	**27.5**	**Premalac**	2862.6	32.66	19.98	4.68	88.13
T6	**27.5**	**Biogen**	2818.6	33.16	20.42	4.58	86.25
T7	**25**	**_____**	2485.0	38.92	13.03	7.42	139.73
T8	**25**	**Premalac**	2639.0	36.55	14.22	6.78	127.68
T9	**25**	**Biogen**	2595.0	37.82	14.28	6.87	129.37

* The price of Premalac = 77 L.E / Kg , of Biogen = 55 L.E / Kg
** Relative to T1 which represents the recommended CP level

4. Conclusions

Ether Premalac or Biogen spared nearly 2.5 % CP of the recommended level for tilapia. This result would be effective from the economical point of view, since protein is the most expensive feed nutrient in all livestock feeding , particularly in fish feeding .

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Ovarian activity, biochemical changes and histological status of the dromedary she-camel as affected by different seasons of the year

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Abstract: The present study aimed to investigate the effect of different seasons of the year on body thermoregulation (rectal temperature, respiration rate and pulse rate), blood hematology (hemoglobin, packed-cell volume, red blood cells and white blood cells counts), blood components (total protein, albumin, globulin, aspartate-aminotransferase, alanine-aminotransferase, alkaline phosphatase, acid phosphatase, cholesterol, sodium, potassium, calcium, total phosphorus, testosterone and oestradiol-17β hormone concentrations of the dromedary she–camel. Histological changes of the right and left ovaries were also recorded. The obtained results showed that, rectal temperature and respiration rate in the dromedary she-camels increased significantly (P<0.05) during summer as compared to the other seasons. However, pulse rate showed significantly (P<0.05) lower during winter than other seasons. The highest (P<0.05) values of hemoglobin, packed-cell volume and red blood cells count were recorded during summer, while the lowest (P<0.05) value of the white blood cell's was recorded during autumn season. Total protein, albumin and globulin concentrations (mg/dI) were increased insignificantly during summer season as compared to other seasons. Aspartate-aminotransferase, alanine-aminotrasferase enzymes, sodium and calcium concentrations of the dromedary she–camels increased significantly (P<0.05) during summer, while potassium and total phosphorus concentrations (mg/dl) increased significantly (P<0.05) during spring as compared to other seasons. The lowest (P<0.05) value of alkaline phosphatase and acid phosphatase enzymes were recorded during winter season. Testosterone, oesterdiol-17β hormone and cholesterol concentrations were significantly (P<0.05) higher during winter than other seasons of the year. The histological examination of the left and right ovaries in different seasons of the year revealed higher activity in spring and winter than summer and autumn seasons. The left ovary showed more growing and mature follicles and higher activity than the right one. *In conclusion,* the female dromedary camels display ovarian activity during the non-breeding season. So, the environmental temperature, relative humidity and daylight length seemed to play the major role in the regulation of seasonal ovarian activity in the female dromedary camels. [Nature and Science. 2010;8(5):54-65]. (ISSN: 1545-0740).

Key words: Seasons, She-camel-ovaries, testosterone, oesterdiol-17β, cholesterol

1. Introduction

Reproduction is an important factor in economics of the animal production. The camel is a domesticated animal whose full agricultural reproductive potential has not yet been achieved. It is fully adapted to the rigours of the extreme diurnal variations of temperature of the arid zones of Africa and Asia and therefore requires little expenditure in terms of housing or shelter. The *dromedarius* and *bactrianus* camels are both regarded as seasonal breeders (Wilson, 1984). The impression gained is that decreasing day length is the stimulus to seasonally, but it is obvious that, in dromedary camels near the equator factors such as rainfall, nutrition and management (Wilson, 1984), may override the effect of photoperiod (Merkt *et al.,* 1990) and allow breeding to occur throughout the year (Arthur *et al.,* 1982). The breeding season of camels varies geographically, since the environmental factors affect temporally the pattern of reproduction in this species (Gombe and Okela, 1977). Camels are induced ovulators and exhibit follicular cycles with follicles developing and regressing successively and ovulation will occur only when mating takes place (Elias *et al.*, 1984 and Ismail, 1987). Daylight ratio and temperature are the two main climatic factors influencing the annual sexual cycles. However, numerous investigations have shown that the most efficient climatic factors are the variation in the daylight ratio (Hafez, 1987), although the length of daylight seems to be the primary stimulus for seasonally in reproduction. On the other hand, the respective activity of the left and right ovary has attracted interest from different scientists because of the fact that, the majority of pregnancies are established in the left horn of the uterus (El-Wishy, 1987and Shalash, 1987). The blood components are

the mirror which reflects the healthy condition of animals. So, the biochemical studies under different fluctuating climatic conditions are very important for clinicians in the field during interpretation of their findings. Minerals and trace elements has long been known to be important in animal nutrition as they may be dietary essential and vital to enzyme processes of living cells or have some metabolic activity, bone formation and reproductive performance. However, very few studies have demonstrated the endocrinological and physiological bases of seasonality in the female camels. The present study aimed to investigate the effect of different seasons of the year on body thermoregulation, blood hematology and blood components of the dromedary she–camel. Histological status of the right and left ovaries, were also studied.

2. Materials and Methods

The present study was conducted in the Laboratory of Physiology in the Department of Animal Production, Faculty of Agriculture, Mansoura University, in co-operation with Animal Production Research Institute, Egypt. The present work was carried out in the Private Camels Farm, Belbies City, Sharkiya Governorate, located in the North Eastern part of the Nile Delta (30ºN).

The present work aimed to investigate the effect of seasons of the year on body thermoregulation (rectal temperature, respiration rate and pulse rate), blood hematology (hemoglobin, packed-cell volume, counts of red blood cells and white blood cells), blood components (total protein, albumin, globulin, aspartate-aminotransferase: AST, alanine-aminotransferase: ALT, Alkaline phosphatease: ALP and acid phosphatase: ACP, sodium, potassium, calcium, total phosphorus, testosterone and oestradiol-17β hormones). A total number of 220 clinically healthy she-camels were used in this study. The age of these camels varied from 5 to 10 years and their weights were approximately 500 kg.

Minimum and maximum values of air temperature (ºC), relative humidity (%), temperature–humidity index (THI) and length of daylight (hours) of different seasons of the year are shown in Table 1. The temperature – humidity index (THI) was estimated according to the following formulae:

THI=Td-(0.55-0.55×RH) (Td -58.00) where Td= dry bulb temperature in Fahrenheit and RH = relative humidity percentage in decimals as the method described by West (2003).

Thermal parameters:

Rectal temperature, respiration rate and pulse rate were measured three times daily at 0800, 1200 and 1500h during different seasons of the year. Rectal temperature (ºC) was obtained gently by inserting the digital liquid thermometer for 15 –20 cm in the rectum for two minutes. Respiration rate (r.p.m.) was determined by counting the frequency of flank movement per one minute. Pulse rate (p.p.m.) was determined by counting the frequency of the jugular vain with hand per minute. All possible precautions were taken in consideration to avoid disturbing the animal, including counting the respiration breaths and pulse rate just before measuring the body temperature.

Blood hematology:

Blood samples were collected from each animal in dry clean screw caped tube and divided into two portions. The first portion was taken to determine hemoglobin concentration (g/dl),packed-cell volume (%), counts of red blood cells($x10^6/mm^3$) and white blood cells ($x10^3/mm^3$).The second portion was centrifuged at 600*g* for 15 minutes for the separation of serum and stored in a deep freezer at –20°C for assaying of total protein , albumin, globulin, AST, ALT, ALP, ACP, sodium, potassium, calcium, total phosphorus, testosterone and estradiol-17 β concentrations.

Hemoglobin concentration was determined in fresh blood samples using haemoglobinometer according to Tietz (1982). Packed–cell volume (%) was estimated by haematocrit capillary tube and centrifuged at 600 *g* for 20 minutes. Haematocrit value was read and recorded according to Wintrobe (1965). Red blood cell's (RBC's)and white blood cell's (WBC's) were counted in fresh blood sample using haemocytometer and counted at x40 objective of phase contrast microscope according to Hawakey and Dunnett (1989).

Blood serum components:

Total protein was determined colourimetrically according to Biuret method as described by Welchselbaum (1946). Albumin concentration was determined colourimetrically according to Weis (1965).Globulin level was calculated by subtraction of albumin content from the total protein value.

Aspartate-aminotransferase (AST), alanine-aminotransferase (ALT) activities were determined colourimetrically using the method described by Reitman and Frankle (1957). Alkaline phosphatease (ALP) and acid phosphatase (ACP) activities were determined colourimetrically using commercial kits purchased from Bio-Merieux (Marcy L'Eltoile, Charbonnieres, Les Bains, France) according to Graham and Pace (1967).

Sodium, calcium, potassium and total phosphorus concentrations were determined colourimetrically according to the method described by Kuttner and Liechtenstein (1930), Trinder (1951), Sunderman Jr. and Sundarman (1958) and Gindler (1972).

Testosterone and oesterdiol-17β concentrations were determined in blood serum by Radiommunoassay Technique (RIA) of Coat-Ab-Count Kits (Diagnostic Products Corporation-Los Angles, USA) according to Abraham (1977) and Pratt (1978).

Histological changes in the ovaries:

For histological studies, the ovaries, were taken and put in formalin solution (10%) to preserved, then it passes in ordinary histological set (by putting small pieces of the fresh tissue in the proper fixative as 10%formalin saline). Then the fixed tissues are washed in running tap water to remove fixative from them, and then the water was removed by treatment with ethyl alcohol (70, 90 and 100%). These ascending grades of alcohol prevent shrinkage of tissues and it removes the water completely from the fixed tissues. Then the tissues are treated with clearing agents as xylol or benzol to remove alcohol and to allow the fixed tissues to be miscible with paraffin which will be used in the next step. Then the tissues are put in melting soft paraffin wax at 50 °C in then oven. The paraffin will penetrate in between the cells of the tissues. This process of paraffin in filtration is a necessary step to harden the tissues before their embedding. The tissues are then embedded in the center of melted and hard paraffin. The paraffin was then allowed to be cooled-down in order to form a block of hard paraffin with tissues in its center. The block of hard paraffin with the tissues in its center was then cut into thin sections by mean of a rotatory micro tome. The thin paraffin sections are then put on clean glass slides smeared with albumen glycerin to flow beneath the sections and the then we warm the slides on hot plate. Thereafter the sections were preserved for several hours in the incubator to dray. The sections are now fixed on the slides and are ready to be stained by haematoxlin and eosin (H&E) according to Carleton and Drurg (1967). After the staining, the slide was examined by binuclear microscope and photographed by magnification x10 and 40.

Statistical analysis:

Data were statistically analyzed using least squares Analysis of Variance according to Snedecor and Cochran (1982). Percentage values were transformed to arc-sin values before being statistically analyzed. Duncan's New Multiple Range test (Duncan, 1955) was used for the multiple comparisons.

3. Result Analysis

Data presented in Table (2) showed that the effect of different seasons of the year on rectal temperature and respiration rate was significant ($P<0.05$), being higher during summer than in winter, spring and autumn seasons. The highest ($P<0.05$) value of the rectal temperature was recorded during summer and the lowest ($P<0.05$) value during winter season. However, the effects of different seasons of the year on pulse rate was also significant ($P<0.05$), being lower during winter than summer, autumn and spring seasons. The highest ($P<0.05$) value of the pulse rate was recorded during summer and the lowest ($P<0.05$) value during winter season. The overall mean of pulse rate was 51.66.

Data presented in Table (3) showed that hemoglobin percentage (Hb %) in the dromedary she-camel during summer and autumn was significantly ($P<0.05$) higher than spring and winter seasons. The highest ($P<0.05$) value of hemoglobin was recorded during summer and the lowest ($P<0.05$) value during winter season.

Table (1): Mean air temperature (°C), daylight length, relative humidity (%) and temperature-humidity index (THI) values, during the different seasons of the year.

Seasons of the year	Air temperature (°C)		Relative humidity (%)		Temperature-humidity index (THI)		Length of daylight (hours)
	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	
Winter	8.86±0.21	19.15±0.35	48.62±0.35	4.33±1.15	45.11	64.81	11.55
Spring	13.6±0.18	24.16±0.18	37.41±0.43	2.64±1.21	56.08	70.93	14.13
Summer	20.84±0.32	34.3±0.46	38.83±0.48	53.66±0.95	65.64	84.63	15.24
Autumn	15.43±0.12	28.62±0.42	42.67±0.62	58.42±1.32	59.21	77.68	13.00

Table (2): Rectal temperature, respiration rate and pulse rate in the dromedary she–camels, during different seasons of the year.

Items	Season of the year			
	Spring	Summer	Autumn	Winter
Rectal temperature (˚C)	37.46 ± 0.16 [b]	38.83 ± 0.67 [a]	36.80 ± 0.37 [b]	36.33 ± 0.33 [b]
Respiration rate(r.p.m.)	14.66 ± 0.63 [b]	23.66 ± 0.47 [a]	14.70 ± 0.43 [b]	12.73± 0.29 [b]
Pulse rate (p.p.m.)	52.26 ± 0.31 [a]	52.41 ± 0.44 [a]	52.30 ± 0.43 [a]	49.66± 0.4 [b]

Means bearing different letters within the same row, differ significantly (P<0.05).

Table (3): Blood hematology in the she-camels, during different seasons of the year.

Items	Season of the year			
	Spring	Summer	Autumn	Winter
Hemoglobin (g/dl)	10.93 ± 0.43 [b]	12.30± 0.29 [a]	12.10 ± 0.45 [a]	10.36 ± 0.35 [b]
Packed-cell volume (%)	31.28 ± 0.33 [b]	34.24±1.46 [a]	30.64 ± 1.02 [b]	30.43 ± 0.84 [b]
White blood cell's ($x10^3/mm^3$)	12.16± 0.41 [a]	11.35 ± 0.61 [a]	9.12 ± 0.36 [b]	10.78 ± 0.34 [a]
Red blood cell's ($x10^6/mm^3$)	10.25 ± 0.24 [bc]	11.84 ± 0.21 [a]	11.23±0.67 [ab]	9.37± 0.34 [c]

Means bearing different letters within the same row, differ significantly (P<0.05)

Table (4): Some blood serum components in the dromedary she-camels, during different seasons of the year.

Item	Seasons of the year			
	Spring	Summer	Autumn	Winter
Total protein (mg/dl)	8.53 ± 0.83 [a]	8.80 ± 0.21 [a]	8.43 ± 0.26 [a]	8.36 ± 0.33 [a]
Albumin (mg/dl)	5.50 ± 0.61 [a]	5.54 ± 0.12 [a]	5.24 ± 0.43 [a]	5.22 ± 0.21 [a]
Globulin (mg/dl)	3.03 ± 0.14 [a]	3.26 ± 0.49 [a]	3.19 ± 0.65 [a]	3.14 ± 0.17 [a]
AST(U/L)	16.53 ± 1.77 [b]	26.51 ± 1.59 [a]	15.54 ± 1.29 [b]	17.35 ± 1.52 [b]
ALT(U/L)	5.16 ± 0.66 [b]	7.33 ± 0.64 [a]	5.79 ± 0.26 [b]	5.04 ± 0.71 [b]
ALP(U/L)	88.48± 1.61 [a]	88.64 ± 1.99 [a]	86.43 ± 1.83 [a]	67.40 ± 1.30 [b]
ACP(U/L)	25.20 ± 0.27 [b]	27.43 ± 0.86 [a]	25.08 ±0.49 [b]	22.76 ± 0.61 [c]
Cholesterol (mg/dl)	72.10 ±2.10 [b]	72.66 ± 1.45 [b]	74.33 ± 2.09 [b]	78.65± 1.04 [a]
Sodium (mg/dl)	128.66 ± 3.37 [c]	137.10 ± 4.05 [a]	125.66 ± 2.13 [c]	133.20± 2.32 [b]
Calcium (mg/dl)	10.21 ± 0.43 [b]	11.30 ± 0.52 [a]	6.86 ± 0.87 [d]	8.63 ± 1.04 [c]
Potassium (mg/dl)	6.60 [a] ± 0.42	4.43 [b] ± 0.12	4.90 [b] ± 0.33	4.93 [b] ± 0.15
Total phosphorus	6.23 [a] ± 0.48	5.10 [b] ± 0.34	5.21 [b] ± 0.89	5.14 [b] ± 0.27
Testosterone (pg/ml)	7.30[c] ± 0.63	5.80 [d] ± 0.43	10.70[b] ± 0.32	31.20[a] ± 1.48
Oestradiol -17ß	56.15[b] ± 1.25	20.13 [d] ± 1.02	28.16 [c] ± 1.31	62.18[a] ± 1.16

a,b,c and d means bearing deferent letters within the same row, differ significantly (P<0.05).
AST: Aspartate-aminotransferase.
ALT: Alanine-aminotransferase.
ALP: Alkaline phosphatase.
ACP: Acid phosphatase.

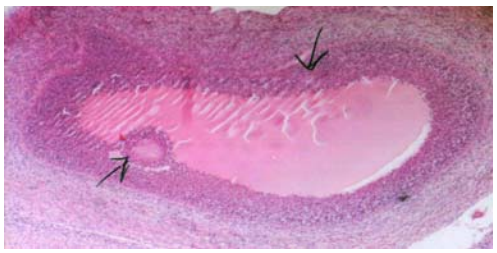

Plate 1. Cross section in the left ovary of she-camel during winter showing mature graffian follicles (Stained by H & E x 40).

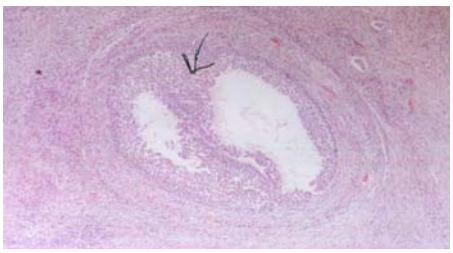

Plate 2. Cross section in the left ovary of she-camel during spring showing growing follicles (Stained by H & E x 40).

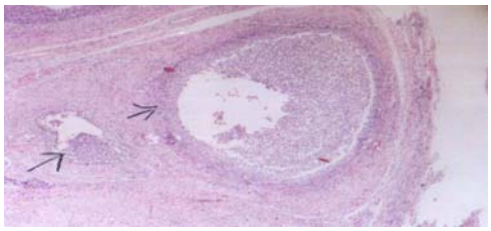

Plate 3. Cross section in the left ovary of she-camel during autumn showing a growing follicle (Stained by H & E x 40).

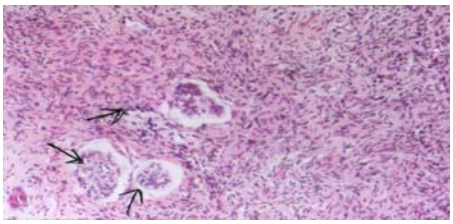

Plate 4. Cross section in the cortex of left ovary of she-camel during summer primordial follicles (Stained by H & E x 40).

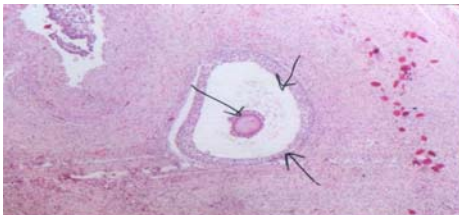

Plate 5. Cross section in right ovary of she-camel during winter showing degenerated follicles (Stained by H & E x 40).

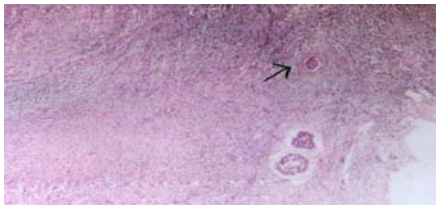

Plate 6. Cross section in right ovary of she-camel during spring showing secondary follicles (Stained by H & E x 40).

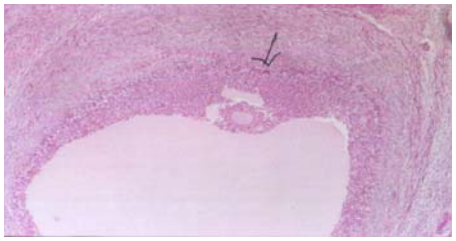

Plate 7. Cross section in right ovary of she-camel during autumn showing mature follicles (Stained by H & E x 40).

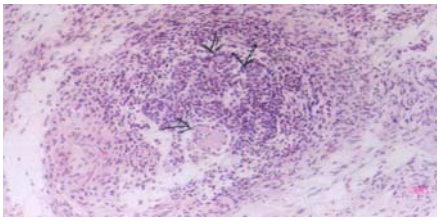

Plate 8. Cross section in right ovary of she-camel during summer showing primordial follicles (Stained by H & E x

The effects of the different seasons of the year on packed-cell volume (PCV) of the dromedary she-camel were significant (P<0.05). Packed-cell volume in the dromedary she-camel was significantly (P<0.05) higher during summer and significantly (P<0.05) lower during winter season. The overall mean of PCV during all seasons was 31.65 %, The white blood cells count ($x10^3/mm^3$) of the dromedary she-camel was significantly (P<0.05) higher during spring, summer and winter than autumn season`. The highest (P<0.05) count of white blood cells (WBC's) was recorded during spring and the lowest (P<0.05) count during autumn season. The values of WBC's tended to be were higher during summer than winter season. The white blood cells count ($x10^3/mm^3$) of the dromedary she-camel was significantly (P<0.05) higher during spring, summer and winter than autumn season. The highest (P<0.05) count of white blood cells (WBC's) was recorded during spring and the lowest (P<0.05) count during autumn season. The values of WBC's tended to be were higher during summer than winter season. The effects of seasons of the year on red blood cells count($x10^6/mm^3$) of the dromedary she-camel was highly significant (P<0.05) being the higher during summer and autumn than winter and spring seasons. The highest (P<0.05) value of red blood cells (RBC's) was recorded during summer and the lowest (P<0.05) value during winter season.

Data presented in Table (4) showed that, the effects of the different seasons of the year on total protein concentration of the dromedary she-camels were insignificant. The highest value of the total protein was recorded during summer and the lowest value during winter season. The effect of seasons of the year on albumin and globulin concentrations of the dromedary she-camels was insignificant. The highest values of the albumin and globulin were recorded during summer, and the lowest values were recorded during winter and spring seasons, respectively. The effects of different seasons of the year on cholesterol concentration of the dromedary she-camels were significantly (P<0.05) higher during winter than summer, spring and autumn seasons. The highest (P<0.05) value of cholesterol was recorded during winter and the lowest (P<0.05) value during summer season.

The effects of different seasons of the year on AST and ALT enzymes activity of the dromedary she-camels were significantly (P<0.05) higher during summer than winter, autumn and spring seasons. The highest (P<0.05) values of AST and ALT enzymes were recorded during summer and the lowest (P<0.05) values during autumn and winter seasons, respectively. The effect of different seasons of the year on alkaline phosphates (ALP) activity of the dromedary she-camels was significantly (P<0.05) higher during summer, spring and autumn than winter season. The highest (P<0.05) value of ALP activity was recorded during summer and the lowest (P<0.05) value during winter season. With regard to acid phosphatase (ACP), the effect of different seasons of the year on acid phosphatase concentration of the dromedary she-camels was significantly (P<0.05) higher during summer than winter, autumn and spring seasons. The highest (P<0.05) value of ACP was recorded during summer and the lowest (P<0.05) value was recorded during winter season.

The effect of different seasons of the year on sodium concentration of the dromedary she-camels was significantly (P<0.05) higher during summer than spring, winter and autumn seasons. The highest (P<0.05) value of sodium was recorded during summer and the lowest (P<0.05) value during autumn season. In respect to calcium, the effect of seasons of the year on calcium concentration of the dromedary she-camels was significantly higher (P<0.05) during summer and spring than winter and autumn seasons. The highest (P <0.05) value of calcium concentration of the dromedary she camel was recorded during summer and the lowest (P<0.05) during autumn season. According to the results of the present work, there was a marked increase in calcium of she-camel during summer and autumn.

The effect of different seasons of the year on potassium concentration of the dromedary she-camels was significantly higher (P<0.05) during spring, winter, and autumn than summer seasons. The highest (P<0.05) value of potassium was recorded during winter and the lowest (P<0.05) value during summer season. With regard to total phosphorus, the effect of different seasons of the year on phosphorus concentration of the dromedary she-camels was significantly (P<0.05) higher during spring than other seasons. The highest (P< 0.05) value of the phosphorus was recorded during spring and the lowest (P< 0.05) value during summer season. The obtained results in showed that the effect of different seasons of the year on testosterone hormone concentration of the dromedary she-camels was significantly (P<0.05) higher during winter than spring, summer and autumn seasons. The highest (P<0.05) value of the testosterone concentration was recorded during winter and the lowest (P<0.05) value daring summer season.

The effect of different seasons of the year on testosterone hormone concentration of the dromedary she-camels was significantly (P<0.05) higher during winter than spring, summer and autumn seasons. The highest (P<0.05) value of the testosterone concentration was recorded during winter and the

lowest (P<0.05) value daring summer season. With regard to ostradiol-17β hormone, the effects of different seasons of the year on oestradiol-17β hormone concentration of the dromedary she-camels were significantly (P<0.05) higher during winter than spring, summer and autumn seasons. The highest (P<0.05) value of the oestradiol-17β hormone was recorded during winter and the lowest (P<0.05) value during summer season.

The histological examination in the left and right ovaries of the dromedary she–camel at different seasons of the year revealed that, camel's ovary showed higher activity in spring and winter than summer and autumn seasons (Plates 1 to 8). The photographs show more ovarian follicles at different stages, primary, secondary, growing, mature and graffian follicles as well as corpora lutea, also corpora hemorrhagic are present. Many follicles were present in the breeding season (winter). The follicles are very clear cell obvious and cell division are also present. The interstitial tissues cells were clear and highly active and many ovulations occur rapidly at the peak of the breeding (spring and winter) compared to the non-breeding season (summer). It was observed that, the ovary in non-breeding season (summer) in comparison with that of other seasons, showed less activity, lower follicle number and higher interstitial tissue, so the ovary in the summer is considered in dormant phase. In respect to ovary side, the left ovary contains growing and mature follicles more than the right one. It can be noticed that, there are no much differences between the left and right ovaries activity in the same season, while the differences became greater among different seasons**.**

3. Discussion

In this study, the effect of different seasons of the year on rectal temperature and respiration rate was significant, being higher during summer than in winter, spring and autumn seasons. The highest value of the rectal temperature was recorded during summer and the lowest value during winter season. Similar trend was observed by Guirgis *et al*. (1992) who found that season had a significant effect on rectal temperature of the dromedary she-camel in Egypt (low in winter and high in summer). The increase in rectal temperature during the hot summer conditions may be minimized temperature gradient between the body and the environment, that resulted in reduce of body heat gain (Abdel-Samee and Marai, 1997), this could be minimized the heat-stress on animals. However, the effects of different seasons of the year on pulse rate were also significant, being lower during winter than summer, autumn and spring seasons. The highest value of the pulse rate was recorded during summer and the lowest (P<0.05) value during winter season. These results are in agreement with those of Zeidan *et al.* (2008). However, Abdel-Samee and Marai (1997) showed that, the pulse rate (counts/minuts) was insignificantly declined as a function of heat stress. The overall mean of pulse rate was 51.66, however, Sarwar *et al.* (1998) reported that, mean pulse rate was 43.46 (counts/minutes) for dromedaries (*Camelus dromedarius*) during the summer season.

Hemoglobin percentage (Hb %) in the dromedary she-camel during summer and autumn was significantly higher than spring and winter seasons. The highest value of hemoglobin was recorded during summer and the lowest value during winter season. These trends are in agreement with those of Zeidan and Abbas (2004). The increase of hemoglobin during non-breeding season may be due to that iron and copper essential for hemoglobin synthesis, since camels during breeding season lose their appetite and body condition with diarrhea (Schalm *et al*., 1975). The effects of the different seasons of the year on packed-cell volume (PCV) of the dromedary she-camel were significant. Packed-cell volume in the dromedary she-camel was significantly higher during summer and significantly lower during winter season. The overall mean of PCV during all seasons was 31.65 %, which was similar to that reported by Nyangao *et al.* (1997) who found that, mean PCV was 27.1, while Rezakhani *et al.* (1997) and Zeidan and Abbas (2004) found that, PCV was 28.94% in dromedary camel. The white blood cells count ($x10^3/mm^3$) of the dromedary she-camel was significantly higher during spring, summer and winter than autumn season`. The highest count of white blood cells (WBC's) was recorded during spring and the lowest count during autumn season. The values of WBC's tended to be were higher during summer than winter season. These results are in agreement with those obtained by Kataria *et al*. (2002) and Zeidan and Abbas (2004). However, disagree with those reported by Rezakhani *et al*. (1997).

The effects of seasons of the year on red blood cells count($x10^6/mm^3$) of the dromedary she-camel was highly significant being the higher during summer and autumn than winter and spring seasons. The highest value of red blood cells (RBC's) was recorded during summer and the lowest value during winter season. The mean value of RBC's was higher during summer than winter, similar to that reported by Zeidan and Abbas (2004). The reduction of blood hematological parameters during winter may be due to reduced oxygen intake caused by increasing ambient temperature, thus reducing metabolic heat

production. In addition, Ashour *et al*. (1995) suggested that, heat-stress decreased the level of adrenocorticotrphic hormone (ACTH) which in turn decreases the value of hemoglobin, RBC's, WBC's and PCV, due to the stimulatory effect of ACTH on erythropoiesis.

The highest value of the total protein was recorded during summer and the lowest value during winter season. These results are in agreement with those of Abdel-Samee and Marai (1997) who recorded that the total protein, in camels did not show significant change during different seasons of the year. However, these results disagreed with those obtained by Amin (1993) and Ahmadi (2001) who found significant increase in total protein concentration during summer as compared to the other seasons in camels. The increase of total protein during summer may be attributed to exposure to heat-stress which represented the potent stimulus for growth releasing hormones (Maxwell and Kleemon, 1980) which lead increase plasma protein that considered important in maintaining plasma water (Horowiz and Adler, 1983) or due to haemoconcentration during summer. Moreover, physiological hypothyroidism during summer was accompanied by protein deposit for retaining plasma water (Ganong, 1979). They stated the effect of seasons of the year on total proteins revealed a significant increase during summer in camels.

Non significant effect of seasons of the year on albumin and globulin concentrations of the dromedary she-camels was observed. The highest values of the albumin and globulin were recorded during summer, and the lowest values were recorded during winter and spring seasons, respectively. These results are in agreement with those of Gupta (1994) and Abdel-Samee and Marai (1997) who found that the albumin and globulin concentrations in camels did not show significant change between the seasons. These results for blood components may be reflect the greater ability of camels to adapt to heat stress.

The effects of different seasons of the year on cholesterol concentration of the dromedary she-camels were significantly higher during winter than summer, spring and autumn seasons. The highest value of cholesterol was recorded during winter and the lowest value during summer season. These results are in agreement with those of Nazifi and Gheisari (1999) and Zeidan *et al.* (2008) who found that, the concentration of serum cholesterol was significantly higher in winter than in summer months.

Significantly higher effects of different seasons of the year on AST and ALT enzymes activity of the dromedary she-camels were observed during summer than winter, autumn and spring seasons. The highest values of AST and ALT enzymes were recorded during summer and the lowest values during autumn and winter seasons, respectively. Mobilization of the liver functions may be partially affected by heat-stress during non-breeding season (Abd El-Samee and Marai, 1997). However, these results disagreed with those of Ahmadi (2001) who found that, the effects of seasons of the year on AST and ALT concentrations of the male dromedary camels were significantly higher during summer than spring, winter and autumn seasons.

The effect of different seasons of the year on alkaline phosphates (ALP) activity of the dromedary she-camels was significantly higher during summer, spring and autumn than winter season. The highest value of ALP activity was recorded during summer and the lowest value during winter season. These results are in agreement with those of Sarhan (2007) and Zeidan *et al*. (2008) who reported that, alkaline phosphatase concentration was higher during non-breeding than breeding season.

With regard to acid phosphatase (ACP), the effect of different seasons of the year on acid phosphatase concentration of the dromedary she-camels was significantly higher during summer than winter, autumn and spring seasons. The highest value of ACP was recorded during summer and the lowest value was recorded during winter season. These results are in agreement with those of Kataria *et al.* (1991) who showed that, activities of ACP was significantly higher during extremely hot conditions (May-June) than in extreme cold (December-January) .

Generally, the blood enzymes are easily and often influenced by the external environment including feeding practices, type of shelter and many other aspects of herd management, since they are intimately related to metabolism. Accordingly, seasonal changes of the enzymes are very important and must be considered. In addition, it is also important to control carefully all experimental conditions, especially environmental ones when measuring the enzyme activity in any animal (Boots *et al.,* 1969).

The effect of different seasons of the year on sodium concentration of the dromedary she-camels was significantly higher during summer than spring, winter and autumn seasons. The highest value of sodium was recorded during summer and the lowest value during autumn season. These results are in agreement with those of Ahmadi (2001) and Zeidan *et al*. (2008) who found that, the highest value of sodium was recorded during summer. Amin (1993) confirmed that the normal sodium values of adult male camel were 158.10, 162.60, 139.25 and 135.20m. Equiv/L during spring, summer, autumn

and winter seasons, respectively. These results may be attributed to the combined effect of both absorption and reabsorption of sodium and chloride from the alimentary tract and kidney, under the effect of aldosterone which had higher level in the summer and this was accompanied by an increase of plasma sodium level (Yagil and Etzion, 1979).

In respect to calcium, the effect of seasons of the year on calcium concentration of the dromedary she-camels was significantly higher during summer and spring than winter and autumn seasons. The highest value of calcium concentration of the dromedary she camel was recorded during summer and the lowest during autumn season. According to the results of the present work, there was a marked increase in calcium of she-camel during summer and autumn. Similarly, Abbas and Musa (1989) reported that there was a marked increase is calcium of the camel during spring and summer, while this increase was highly significant during spring in compared with that during winter season.

The effect of different seasons of the year on potassium concentration of the dromedary she-camels was significantly higher during spring, winter, and autumn than summer seasons. The highest value of potassium was recorded during winter and the lowest value during summer season. These results are in agreement with those of Zeidan and Abbas (2004) who reported that, potassium concentration was higher during breeding than non-breeding season. Amin (1993) confirmed that the average value of potassium in adult male camel were 4.48, 3.95, 4.61 and 5.22 m Equiv./L during spring, summer, autumn and winter seasons, respectively. The decrease of potassium concentration during summer may be attributed to an increase of aldosterone secretion in hot and dry climate which enhanced by remain-angiotensin system in response to changes in effective circulating fluid volume where aldosterone balance largely plasma potassium, through its effect on renal resorption of sodium in exchange for potassium and hydrogen ion (Kaneko,1980).

With regard to total phosphorus, the effect of different seasons of the year on phosphorus concentration of the dromedary she-camels was significantly higher during spring than other seasons. The highest value of the phosphorus was recorded during spring and the lowest value during summer season. Similarly, Abrams (1951) found that phosphorus level was higher in camel during the green season (winter) than the dry one (summer).

The effect of different seasons of the year on testosterone hormone concentration of the dromedary she-camels was significantly higher during winter than spring, summer and autumn seasons. The highest value of the testosterone concentration was recorded during winter and the lowest value daring summer season. These results are in agreement with those of Abd El-Azim (1996) who found that the testosterone levels increased during winter and decreased during summer season. The increase of testosterone may be due to the increase of androgen level is parallel to the increase of sexual activity in winter and spring seasons. In addition, the decrease of androgen production during the non-breeding season could be explained by the effect of environmental cause as phosphoperiodism, rainfall, temperature and humidity. Bedrak *et al.* (1983) recorded a significant low levels in testosterone during the non-breeding season which holds good with the results of the present study attributed that to the low gonadotropins and high prolactin levels in the blood. At the same time, the low gonadotropins level in the non-breeding season could be explained by the inhibitory effect of prolactin secretion (Gold and Ganong, 1967). The seasonal rhythm of prolactin secretion is influenced by photoperiodism in which concentrations being high under long days and low under short days (Almeida and Lincoln, 1984).

With regard to ostradiol-17β hormone, the effects of different seasons of the year on oestradiol-17β hormone concentration of the dromedary she-camels were significantly higher during winter than spring, summer and autumn seasons. The highest value of the oestradiol-17β hormone was recorded during winter and the lowest value during summer season. These results are in agreement with those of Agarwal *et al.* (1987) who found that the oestradiol-17β levels elevated during breeding and low non-breeding seasons, Abd El-Azim (1996) in dromedary camel showed that the highest level of oestradiol-17β hormone was recorded in winter and spring and the lowest level in autumn and summer. These results may be attributed to the involvement of estrogens in modulation of sexual behaviour (McEwen, 1976) and testosterone secretion (Eiler and Graves, 1977). It is hypothesized that decreasing light hours and probably low temperature might be instrumental in triggering the hypothalamic hypophysical axis as it observed in other short day breeders like sheep (Turrek and Campbell, 1979). In addition, Bedrak *et al.* (1983) observed that the relative activity of several enzymes associated with testosterone and its conversion to estrogen in the blood plasma of dromedary camel was significantly lower during the non mating season than that of the mating one.

The histological examination in the left and right ovaries of the dromedary she – camel at different seasons of the year revealed that, camel's ovary showed higher activity in spring and winter than summer and autumn seasons. The photographs show

more ovarian follicles at different stages, primary, secondary, growing, mature and graffian follicles as well as corpora lutea, also corpora hemorrhagic are present. Many follicles were present in the breeding season (winter). The follicles are very clear cell obvious and cell division are also present. The interstitial tissues cells were clear and highly active and many ovulations occur rapidly at the peak of the breeding (spring and winter) compared to the non-breeding season (summer). It was observed that, the ovary in non-breeding season (summer) in comparison with that of other seasons, showed less activity, lower follicle number and higher interstitial tissue, so the ovary in the summer is considered in dormant phase. Similar trends were recorded by Amer (2004), Sarhan (2007) and Zeidan *et al.* (2008). In respect to ovary side, the left ovary contains growing and mature follicles more than the right one. It can be noticed that, there are no much differences between the left and right ovaries activity in the same season, while the differences became greater among different seasons. Similar trend was reported by Amer (2004) and Zeidan *et al.* (2008). The respective activity of the left and right ovary has attracted interest form different scientists because of the fact that the majority of pregnancies are established in the left horn of the uterus. Many authors have tried to explain the predominance of left- horn pregnancies in the camel date by a difference in follicular activity and incidence of ovulation between the left ovary and the right ovary or by an increased incidence of ovulation between the left ovary and the right ovary or by an increased incidence of embryo mortality for the right ovary horn pregnancies (El- Wishy, 1987, Shalash 1987 and Zeidan *et al.,* 2008)

4. Conclusion

The female dromedary camels (*Camelus dromedarius*) display ovarian activity during the non-breeding season. Body temperature, blood components, hormonal patterns and histological status in the ovarian she-camel showed better, during the breeding season in winter (short daylight) than the non- breeding season in summer (long daylight). In addition, the left ovary appears more active than the right one. So, the environmental temperature, relative humidity and daylight length seemed to play the major role in the regulation of seasonal ovarian activity in the female dromedary camels. Further detailed studies are required to compare the reproductive efficiency of the dromedary she-camels during the non–breeding season, under Egyptian environmental conditions.

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Ethnomedicinal Plant Diversity in Kumaun Himalaya of Uttarakhand, India

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Abstract: Kumaun Himalaya of Uttarakhand State is characterized by a rich diversity of ethnomedicinal plants as well as a rich heritage of traditional medicine system. The present study reveals the status of ethno-medicinal flora and their importance preserved by the local population in Kumaun region. During the study it was observed that 102 species of ethno-medicinal plants belonging to 48 families are being used in the folk-medicine system by the indigenous people of this region. For the present study, an intensive and extensive survey was made for four selected districts of Uttarakhand, viz. Almora, Champawat, Bageshwar and Pithoragarh. The neighboring villages of the study areas were also visited for identification of plant species and to explore the traditional knowledge about the use of indigenous medicinal plants. Therefore, the ethnobiological knowledge of people and listing of plants of particular region are important tools that may help in understanding human environment interactions. [Nature and Science. 2010;8(5):66-78]. (ISSN: 1545-0740).

Keywords: *e*thno-botany; folk medicines; Kumaun region; local communities

1. Introduction

Uttarakhand state encompasses an area of 53,485 sq. km., which accounts for nearly 15.5 per cent of the total geographical area of Western Himalayas. Most of the northern parts of the state are covered by the high Himalayan ranges and glaciers, while the lower reaches are densely forested. Due to these great altitudinal variation, wide array of climatic zones are available, which favors the luxuriant growth of diversified and rich vegetation which also has a number of raw drugs described in Ayurvedic texts. The value of biodiversity as a source of pharmaceutically important substances has been the subject of a number of studies, such as Farnsworth and Soejarto (1985), McNeely (1988), Principe (1991) and Pearce and Puroshothaman (1992), while documentation on ethno-botanical knowledge was done by Maikhuri et al. (2000), Nautiyal et al. (2001).While a comprehensive review has described a rich diversity and use of medicinal flora within Uttarakhand (Joshi, 2002), besides a study conducted on the medicinal plant diversity in riparian zone of River Ganga at Haridwar (Gangwar and Joshi, 2006) to understand the use of plant species from Himalayan region to cure various ailments.

Presently, 95% raw materials required by pharmaceuticals and drug manufactures are collected from the wild and remote areas (Kehimker, 2000). The pharmaceutical sector is using 280 medicinal plant species, out of which 175 are from the Indian Himalayan Region (Dhar et al, 2002). This region supports approximately 1748 plant species of known medicinal value (Samant et al, 1998). In India there exists over one million community based traditional workers and about 600,000 licensed medical practitioners of traditional systems like Ayurveda, Siddha and Unani. They diagnose and cure different diseases through their own traditional knowledge (Hafeel and Shankar, 1999). The health care system of 80% population of the developing world is still dependent on their surrounding vegetation/ forests and pastures. They rely on medicinal plants because of their effectiveness, lack of modern healthcare alternatives and cultural preferences (Caniago and Siebert, 1998). Mostly plant products are used by traditional healers as traditional medicine usually collected from the wild and hilly remote areas to accomplish the increasing demand of herbal medicines. The Indian Himalayan Region (IHR) is also the habitat of major tribal communities like Bhotias, Boaxas, Tharus, Rajis, Jaunsaries, Shaukas, Kharvar and Mahigiri. From ancient period these communities mainly rely heavily and directly on the endemic vegetation for their daily needs such as food, fodder and medicines for their illness and various types of ailments. Lack of alternate income sources; push them to over-exploit natural resources of the region. Non-sustainable collection methods cause threat from harvesting and many valuable medicinal herbs are becoming rare due to their continuous utilization (Swe and Win, 2005). Further, we are witnessing a sharp decrease in the biological species all across the globe, especially in the Kumaun region, perhaps as it forms one of the major hotspots and the conservation of high-altitude medicinal plants is of great concern throughout the Himalayan region, because they are important for traditional health care and in large scale collection for

trade. Hence, there is an immediate need to conserve this natural resource.

1.1 Significances of ethno-medicinal plants

Himalayan herbal medicine and their traditional knowledge is a good illustration of poor communities living in the remote areas, fighting even incurable diseases through the traditional methods, and even for their livestock, through these traditional herbal medicines. Medicinal plants are natural resources for new drugs. Plants parts are directly used as medicines by a majority of community in all over world and have no side effect like allopathic medicines. Most of the modern medicines are produced indirectly from medicinal plants.

2. Study Area

For the present study of medicinal plant diversity of Kumaun region of Uttarakhand State, four districts viz. Almora, Bageshwar, Champawat and Pithoragarh, were selected and a total number of 29 spots were identified. The study area varies from 1615 msl to 1646 msl. Geographical description of the study areas is given in Table 1.

Table 1. Districts Wise Description of Studied Sites for Survey of Ethno-medicinal Plant Diversity

S.NO	Districts	Study Sites
1.	**Almora** Located between 29° 36' North Latitude and 79° 30' East Longitude at an altitude of 1638 meter sea level (msl).	1.Danya, 2.Panwanaula 3.Valachiana 4.Kausani 5.Jageshwar
2.	**Bageshewar** Located between 29°42'40" to 30°18'56" North Latitude and 79°23' to 80.9°East Longitude. The district is lies at an altitude of 1646 msl.	6.Nadi Gaon, 7.Shishakhani, 8.Chhatina, 9.Chandika
3.	**Champawat** Located between 29^0 5'and 29^0 30' in Northern Latitude and $79^0$59' and 80^0 3'at the center of Eastern Longitude with an altitude of 1615 msl.	11.Maneshwar, 12.Loha Ghat, 13.Ghat, 14.Marodapur
4.	**Pithoragarh** Located between 29.4° to 30.3° North Latitude and 80° to 81° East Longitude at a height of 1645 msl.	15. Dharamgarh, 16.Dhamara, 17.Kanalicheena, 18.Ogla, 19.Jauljeevi, 20.Baluakot, 21.Dharchula, 22.Tapovan, 23.Tawaghat, 24.Chirgala, 25.Sovla. 26. Around Swaminarayan Temple at Chhota Kailash, 27. Aincholi, 28. Dhamora and 29. Dhari villages

Fig.1. Map of the Uttarakhand State in India Showing Study Sites

3. Methodology

Present study is based on extensive and intensive field surveys made during 2006-08. The neighboring villages were visited for identification of medicinal plant species collected during the survey and to explore the more information about the traditional knowledge with the help of indigenous peoples of the concern areas who have knowledge about the uses of these medicinal plant species. The collected information was re-examined by consulting important works pertaining to medicinal plants and ethno-botany and identification of medicinal plant species was made with the help of available literature (Nair and Mohanan, 1998; Brahmvarchaswa, 2003; Kanjilal, 2004) and local experts.

4. Results

The present study investigates the medicinal uses of plant species and the associated indigenous knowledge preserved by the indigenous community in Kumaun region. The data of medicinal plants were collected from twenty nine selected sites of four districts *i.e.* Almora, Bageshwar, Champawat and Pithoragarh (Table 1). The documentation of 102 plant species belonging to 48 families collected from study sites and their medicinal use against various ailments are presented in Table 2. The families and the species within a family are arranged in alphabetical order. Species names are followed by vernacular names, local names, habit of plant and plant parts used. The reported species are presented with a highest representative of Asteraceae, Limiaceae and Rosaceae (9 species each) followed by Solanaceae and Poaceae (4 species); Araceae, Euphorbiaceae, Polygonaceae, Ranunculaceae, Scrophulariaceae and Valerianaceae (3 species each); Apiaceae, Apocynaceae, Liliaceae, Meliaceae, Moraceae, Pinaceae, Plantaginaceae, Rutaceae, Saxiferagaceae, Verbenaceae and Zingiberaceae (2 species each); and besides these 25 families (1 species each) were found to be used by the local communities for medicinal purposes.

On the behalf of the qualitative analysis, the maximum species were herbs (50) followed by shrubs (24), trees (22) and under shrubs and climbers (3 each) as depicted in Fig 2., while on the basis of plant parts used by the local people, it was observed that whole plants of 21 species, various plant parts used (<1 parts of plant such as leaves, twigs and roots; stems, roots and bark etc.) of 43 species, roots of 14 species, leaves of 08 species, fruits of 04 species, bark, rhizome and seeds of 03 species each, stem of 02 species, and flowers of 01 species used to cure various ailments (Fig. 3).

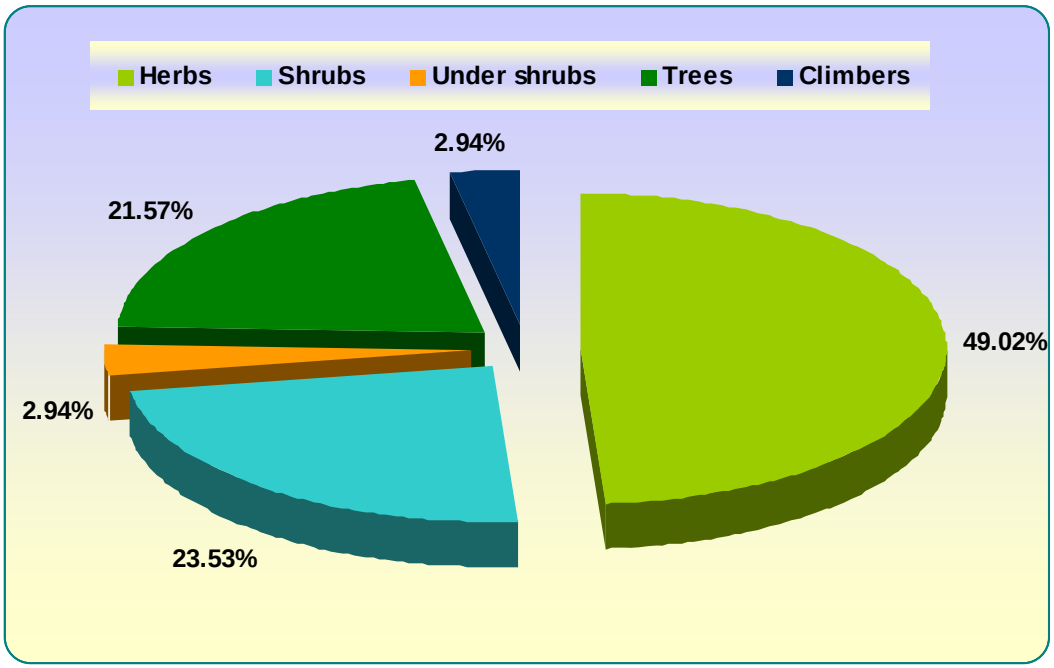

Fig. 2. Distribution of plant species according to habitat type, used to cure various ailments

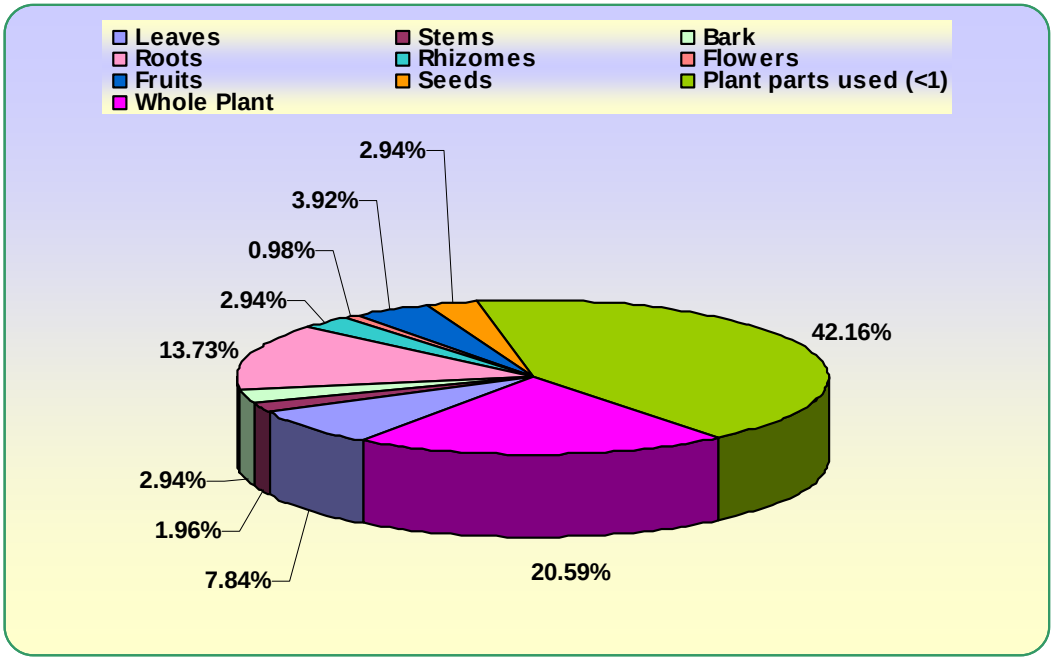

Fig. 3. Plant parts used to cure various ailments

Table 2. Ethno-medicinal plants species used by the indigenous people of Almora Bageshwar, Champawat and Pithoragarh districts of Kumaun Himalaya

Species name are followed by vernacular/ local names, habitat of the plant and plant parts used as medicine.

4.1. Acanthaceae

4.1.1. Justicia adhatoda Linn./ Basa/Shrub/ Leaves

The plant is used for treatment of various ailments of respiratory tract, cough and bleeding piles Inflorescence and leaf liquid is used for fever (Ahmad et al., 2008).

4.2. Agavaceae

4.2.1. Agave Americana Linn./ Rambans /Shrub/ Leaves

The sap of agaves is antiseptic, diaphoretic, diuretic and laxative and used internally for the treatment of diarrhea and dysentery (Chevallier, 1996). An infusion of the chopped leaf is purgative and the juice of the leaves is applied to bruises (Duke and Ayensu, 1985).

4.3. Anacardiaceae

4.3.1. Mangifera indica Linn./ Aam /Tree /Fruits

Seeds are used to cure asthma, fruits are diuretic, and bark used to cure hemorrhages (Sharma et al., 2006).

4.4. Apiaceae

4.4.1. Centella asiatica Linn./ Mandukparni/Herb / Whole herb

The herb is used to treat inflammatory infections, surgical lesions, damaged skin, slow healing wounds and leg ulcers (Gangwar and Joshi, 2008).

4.4.2. Pleurospermum angelicoides DC. */ Chhipi /Herb / Roots*

*Roots used to cure f*ever, stomach pain, body pain, dysentery and spice. Root grounded into powder with seed of jeera and black piper to cure the typhoid fever, stomach pain (Nautiyal et al., 2004).

4.5. Apocynaceae

4.5.1. Holarrhena antidysenterica Linn. / Koraya, kura /Tree/Stems, bark roots & seeds

The plant parts are used to remove constipation and to stimulate discharge of urine. This also controls diarrhoea, dysentery, hemorrhoids, rheumatic arthritis, and skin diseases (Singh et al., 2002).

4.5.2. Carissa spinarum, A. DC / Jungli karonda/Shrub/ Leaves, fruits, bark and roots

The fruit is used as astringent and antiscorbutic and remedy for biliousness. The leaf decoction is used to cure of intermittent fever, diarrhea, oral inflammation and ear ache. The root is employed as a bitter stomachic and vermifuge (Parmar and Kaushal, 1982).

4.6. Araceae

4.6.1. Acorus calamus Linn. / Buch /Herb/ Roots

Small dose reduces stomach acidity whilst larger doses increase stomach secretions (Chevallier, 1996), and also recommended in the treatment of anorexia nervosa (Phillips and Foy, 1990). An infusion of the root can bring about an abortion, whilst chewing the root alleviates toothache (Weiner, 1980). It is a folk remedy for arthritis, cancer, convulsions, diarrhoea, dyspepsia, epilepsy (Duke and Ayensu, 1985).

4.6.2. Arisaema tortuosum Wall Schott / Baank/ Herb/ Whole herb
Herb used to cure various ailments related to digestive tract like constipation, indigestion, abdominal pain and dysentery. It showed anti-nematodal activities and also used treat bone fracture (Choudhary et al., 2008)

4.6.3. Arnebia benthamii Wallich Ex G. Don / Balsamjari/Herb / Roots
Plant having antibacterial, antifungal, anti-inflammatory and wound-healing properties (Manjkhola and Dhar, 2002). The roots yield a red pigment, Shikonin, which has several medicinal properties and is marketed under the trade name Ratanjot (Kirtikar and Basu, 1984). The plant is considered to be useful in the treatment of diseases of the tongue and throat (Singh and Kachroo, 1976).

4.7. Asclepiadaceae
4.7.1. Calotropis procera (Ait.) R.Br. / Akha, Madar/Shrub/ Leaves & flowers
Roots and bark are used as tonic, surdorific, antispasmodic and expectorant. Flowers digestive, stomachic. Milky juice is used in leprosy, asthma, fever with enlarged liver, cough and skin diseases. (Qureshi et al., 2001). Plant latex has cytotoxic, procoagulant, anti-inflammatory and abortifacient activity. Root extract have been reported anti-tumorus and anti cancerous (Mathur et al., 2009).

4.8. Asteraceae
4.8.1. Artemisia annua L./ Quin-ghaosu, Sweet Worm wood/Herb/ Leaves & plant oil
Used as anti-malarial medicine, lowers fevers and checks bleeding (Chevallier, 1996). The leaves are antiperiodic, antiseptic, digestive, febrifuge (Yeung, 1985). An infusion of the leaves is used internally to treat fevers, colds and diarrhoea (Foster and Duke, 1990).

4.8.2. Artimisia nilagirica (Clarke) Pamp./ Nagdona/Shrub/ Whole plant
Plant having antimicrobial and antifungal properties. Used in skin diseases, burns cuts, wounds and inflammations.

4.8.3. Aster flaccidus Bung. / Alpine aster/Herb/ Whole plant
Used in bronchitis, cramps, common cold and relieves pain (Wangchuk, 2004).

4.8.4. Cichorium intybus Linn / Kasni /Herb/Seeds, root and leaves
Herb is taken internally to cure liver disorders, spleen problems; decoction of the powdered seeds is used in obstructed or disordered menstruation (Gangwar and Joshi, 2008). The root and the leaves are appetizer, cholagogue, depurative, digestive, diuretic, hypoglycaemic and laxative (Foster and Duke, 1990).

4.8.5. Centipedia minima Linn / Nakh chhikni, Spreading sneezeweed/Herb/Whole herb
Sandy blight , a kind of eye inflammation, in which the eye feels as if it was full of sand, purulent opthalmia, as well as other eye infections, have been reportedly treated by Aborigines and white alike by bathing the eyes in infusion of decoctions of the plant (Lassak and McCarthy, 2001).

4.8.6. Erigeron asteroids Roxb. / Bangua/ Herb/ Seeds & roots
Herb is used as a stimulating diuretic in febrile condition (Pullaiah, 2006; Gangwar and Joshi, 2008).

4.8.7. Eupatorium odoratum Linn. /Tivra gandha/Under shrub/ Leaves and plant extract
Extract of plant is used to cure cuts and wounds. Decoction of leaves is used to cure soft tissue wounds, burn wounds and skin infections due to anti-inflammatory, anti-microbial and wound healing properties.

4.8.8. Sonchus olereaceous L./ Dudhi /Herb/ Leaves and stem
Leaves and stems are used to control lever disorders (Gangwar and Joshi, 2008).

4.8.9. Xanthium strumarium Linn / Gokhru, Chhota datura/ Under shrub/ Whole plant
Whole plant is used for malarial fever, renal complaints. The infusion of the plant is used to treat rheumatism, diseased kidneys and tuberculosis (Moerman, 1998).

4.9. Berberidaceae
4.9.1. Berberis asiatica Roxb.ex D.C /Rasanjana, Daruhaldi, Kilmora /Shrub/Root bark, stem, wood and fruits
The roots are used for curing diabetes and jaundice. Fresh roots are used to cure diabetes and jaundice (Uniyal et al., 2006).

4.9.2. Podophyllum hexandrum Royle/ Ban Kakri/Herb /Whole plant
The whole plant, but especially the root, is cholagogue, cytostatic and purgative. It is used for treatment of cancer and especially in case of ovarian cancer. The plant has an antimiotic effect and thus prevents the growth of cells. (Uphof, 1959; Polunin and Stainton, 1984; Phillips and Foy, 1990).

4.10. Cupressaceae
4.10.1. Cupressus torulosa D.Don / Surai /Tree/ Oil of cones
Oil shows antimicrobial activity (Sellappan et al., 2007).

4.11. Dioscoreaceae
4.11.1.Dioscorea deltoidea Wall / Ban tarur /Climber/ Tuber
The extract of the root tuber is taken in the treatment of urino-genital disorders (Gangwar and Joshi, 2008), control of roundworm and to alleviate constipation (Manandhar, 2002).

4.12. Dipsacaceae
4.12.1. Morina longifolia Wall. ex DC. */ Kandru/Herb / Shoot & roots*
Used to cure worm infected wounds in animals (Pande et al., 2007).Juice of the root is used to treat dysentery and diarrhea (Malla and Chhetri, 2009).

4.13. Ephedraceae
4.13.1. Ephedra gerardiana Wall. ex Stapf / Tut gatha/ Herb/ Stem
The herb is widely used in preparations for the treatment of asthma and catarrh, reduces swellings of the mucous membranes and has antispasmodic properties (Bown, 1995). The herb also used to treat fever wounds, injuries, bleeding and heals every fever including malaria (Wangchuk, 2004).

4.14. Ericaceae
4.14.1. Rhododendron arboretum Smith / Buransh, brash/ Tree/ Flower extract
Flower extract is used to cure stomach diseases (Brahmverchas , 2003) and snuffed to stop nasal bleeding (Uniyal *et al.*, 2006).

4.15. Euphorbiaceae
4.15.1. Emblica officinalis Gaerth /Aonla, Aonwala /Tree/ Bark and Fruits
Bark decoction is used for treating diarrhea, dysentery, cholera and jaundice. Fruits are used in the Ayurvedic medicine 'triphala' as one of the ingredient (Brahmverchas, 2003).

4.15.2. Euphorbia royleana Boiss / Sulu/ Shrub/ Latex
Latex showed antiseptic and germicidal activity, stop bleeding, ear complaints and hollow cavities of tooth (Gangwar and Joshi, 2008).

4.15.3. Ricinus communis L./ Arand /Shrub/ Leaves, seeds and oil
The leaves are used to cure pain, wounds, disuria, cough and worm infestations. Fruit used to cure epilepsy, piles, asthma, bronchitis, skin diseases, jaundice, nervous diseases; rheumatism and bacterial infections (Katewa et al., 2004; Luseba et al., 2007).the seed oil is given to the children in case of constipation. The decoction of leaves is applied to the breasts of women, act as glactagogue, i.e. increase milk secretion (Gorsi and Shahzad, 2002).

4.16. Fabaceae
4.16.1. Bauhinia variegata Linn./ Kachnar/ Tree/ Roots & bark
Roots are carminative, decoction prevent obesity. Bark is anathematic and used in scrofula and coetaneous troubles (Sharma et al., 2006).

4.17. Fagaceae
4.17.1. Quercus leucotrichophora Cam/ Banj, rein/Tree/ Rhizome and wood
Corm is used as astringent and diuretic. It is also given in diarrhea, indigestion, asthma and gonorrhea (Gorsi and Shahzad, 2002).

4.18. Gentianaceae
4.18.1. Swertia chirata Roxb.Ex Flem/ Bhucharitta, Kariyata, Chirata/ Shrub/Whole plant
Roots are used to cure malarial fever (Ahmed et al., 2004), leprosy, leucoderma, scabies, menstrual disorders, urinary and heart disorders.Flowers, stem and roots are used in asthma, jaundice and anemia (Gangwar and Joshi, 2008).

4.19. Guttiferae
4.19.1. Hypericum podocarpoides N. Robson/ Tikua/Shrub*/ Whole plant*
Used as a wound healing agent, prepared ointments from dried extracts of the leaves and stems (Butola, et al., 2007).

4.20. Hippocastanacaeae
4.20.1. Aesculus indica (Wall Ex. Camb) Hook. f./ Pangar/ Tree/ Fruits
Oil extracted from fresh fruits is externally used against wounds and bruises (Ahmed et al., 2004).

4.21. Lamiaceae

4.21.1. Ajuga bracteosa Wall ex Bent/ Ratpatia, kori booti/ Herb/ Leaves & roots
A bitter astringent given in the treatment of fevers and is also regarded as diuretic (Gorsi and Shahzad, 2002).

4.21.2. Colebrookea oppositifolia Smith/ Binda/ Shrub/ Leaves & roots
Root juice is given to treat in epilepsy. Leaf juice is used to relieve fever, headaches and wounds. The juice of the young inflorescence is given to treat gastric problems and is also put in the nose for sinusitis. The plant is lopped for fodder to cattle (Malla and Chhetri, 2009).

4.21.3. Mentha longofolia Linn. / Wild pudina /Herb/ Whole herb
The herb is used for its antiseptic properties and it is beneficial for antifertility, antiovulatory, gastrointestinal disorders, cough, cold and chronic fever. The leaves and flowering stems are antiasthmatic, antispasmodic, carminative and stimulant (Brahmverchas, 2003).

4.21.4. Mentha piperita Linn./Peppermint, Hortela, Mentha/Herb/ Whole herb
Used to treat dryness, dysentery and haematuria in animals (Pande et al., 2007).

4.21.5. Mentha sylvestris L./ Pudina /Herb/ Leaves
The herb is used for digestive disorders, particularly for flatulence, all kind of pain, headache in particular (Pullaiah, 2006).

4.21.6. Micromeria biflora Benth./ Lemon scented thyme/Herb/ Whole herb
The plant is used as a relief from pain of joints in human (Gorai and Shahzad, 2002) and treat worm infested wounds, shoulder wounds and lock jaws (tetanus) in animals (Pande et al., 2007).

4.21.7. Ocimum kilimand-scharicum Guerke./ kilimanjaro basil, Kapoor tulsi /Herb/ Leaves & oil
Oil showed significant protection efficiency against *Anopheles gambiae* ss (Kweka et al., 2009).

4.21.8. Ocimum sanctum Linn. / Ram tulsi /Herb/ Leaves, twigs & oil
Plant has antibacterial, anti-inflammatory and wound healing properties and also used in diarrhea, astringent and rheumatism (Brahmverchas, 2003). Leaf paste applied on skin to treat infections cuts and wounds (Gangwar and Joshi, 2008). Oil used to treat pains and sprains.

4.21.9. Thymus serpyllum Linn./ Ajwain/Herb/ Leaves & floral shoots
The plant has sharp pleasant taste; the leaves are used as laxative, stomachic, and useful in purifying the blood (Gorsi and Shahzad, 2002). The oil is a remedy in toothache. The herb is given in weak vision, complaints of liver and stomach, suppression of urine and menstruation (Qureshi et al., 2007).

4.22. Liliaceae
4.22.1. Asparagus filicinus Buch.-Ham. Ex D. Don /Sharanoi/Climber/ Roots
The roots of herb are used to cure diarrhoea, dysentery and diabetes (Dhiman, 2005).

4.22.2 Asparagus recemosus Willd./ Satawar, Sahasmuli / Climber/ Flashy roots & cladodes
Roots and cladodes are useful in leucorrhoea, seminal debility, general debility, headache, hysteria, reduced blood pressure also useful in acidity and ulcer, extract of cladode is anticancerous. Dried root powder is used to cure liver disorders and to enhance lactation of cattle and women (Singh et al., 2002; Brahmverchas , 2003).

4.23. Lythraceae
4.23.1. Woodfordia fruiticosa (L.) Kurtz/ Dhaula, Phooldhawai/Shrub/ Flowers & fruits
Flower paste is used in skin diseases and leucorrhoea. Flower and fruit paste/decoction are used to cure bowel complaints, menorrhazia, haemorehage, seminal weakness and for cooling (Singh et al., 2002; Sharma et al., 2006).

4.24. Malvaceae
4.24.1. Malvastrum coromandelianum Garcke. / Bala/Herb/ Leaves and stem
Ulceroprotective and antipyretic plant (Dahanukar *et al.,* 2002). Emollient and decoction are given in dysentery (Sharma et al., 2006).

4.25. Meliaceae
4.25.1. Toona ciliate M.Roem. /Toon /Tree/ Stem bark
The extract of stem bark have antibacterial and antifungal activity (Chowdhury, et al., 2003), and is used to cure infantile dysentery, cough, bronchitis, intermittent fever, verminosis, leprosy and ulcer (Sharma *et al.,* 2006)

4.25.2. Albizia lebbek Benth./Siris/ Tree/Seeds & leaves
The seed are reported to be used as tonic to the brain. The leaves are used in relieving tooth ache and strengthen the gum and teeth (Gorsi and Shahzad, 2002).

4.26. Moraceae
4.26.1. Ficus carica L./ Anjir/ Tree/ Fruits and bark

Fruits and bark powder are used to control diabetes by reducing blood sugar (Chakraborty, 2004).The root is useful in leucoderma and ring worm. The fruit is useful in inflammation, weakness, paralysis, thirst diseases of liver and spleen, cure piles and stimulate growth of hair (Gorsi and Miraj, 2002).

4.26.2. Ficus religiosa L./ Peepal /Tree/ Leaves, fruits, seeds & bark

Fruit powder is laxative and is used to cure asthma and bark powder is used to cure gonorrhea and scabies (Singh et al., 2002). Plant showed anti-protozoal properties and plants part used to control diabetes by reducing blood sugar (Chakraborty, 2004).

4.27. Myricaceae

4.27.1. Myrica esculanta Buch Ham./Kaphal/Tree/ Bark

Used in chronic cough, asthma, painful dental gin and ear ache, external application in healing of chronic and malignant ulcers (Gangwar and Joshi, 2008).

4.28. Orchidaceae

4.28.1. Dactylorhiza hatagirea (D. Don) Soo/ Hathajari Herb/ Roots

Root paste is used to treat burns and cuts. Provides supplements to the body and builds tissues (Wangchuk, 2004).

4.29. Paeoniaceae

4.29.1. Paeonia emodi Wall ex Hooker.f. / Hilto, Himalayan Peony */Herb/ Rhizome*

The powdered rhizome is used to cure backache, general weakness, headache, dizziness, vomiting and to aid pregnancy (Khan et al., 2007).

4.30. Parnassiaceae

4.30.1. Parnassia nubicola Wallich ex Royle/ Mamira, Nirbansi */Herb/ Roots*

Root paste is taken to get relief from cuts & wounds. Leaf juice is applied to treat eye problems and inflammation (Kunwar and Adhikari, 2005).

4.31. Pinaceae

4.31.1. Cedrus deodara Roxb.Loud./ Deodar, Dyar/Tree/ Bark wood- oil

Bark wood- oil is used as aphrodisiac(Ahmed et al., 2004).

4.31.2. Pinus roxburghii Roxb/ Chir, Sarala/Tree/ Wood & resin

Wood is used to cool burning sensation of the body. Wood and Resin wood used in snake bite and scorpion sting. Water with a small amount of resin in it is used as antiseptic (Ahmed et al., 2004). The green needles are ground and sap is extracted. It is taken to increase the flow of urine (Uniyal et al., 2006).

4.32. Plantaginaceae

4.32.1. Plantago ovata Forsk/ Isabgoal/ Herb/ Husk of seeds

The seeds are cooling, demulcent, useful in inflammatory and applied as poultice to rheumatic and gouty swelling, good in dysentery and decoction useful in cough and chronic diarrhea and constipation (Gorsi and Miraj, 2002).

4.32.2. Plantago lanceolata Linn/Jangli isabgoal/Herb/ whole herb

Seeds are chewed as carminative and used against dyspepsia (Ahmed *et al.,* 2004) and the herb is used to cure sore wounds, dysentery, purgative mouth disease and chicks (Matin et al., 2001).

4.33. Poaceae

4.32.1 Cenchrus biflorus Roxb./ Chirchitta, Kutta ghash, Bur grass/Shrub/ Stem & seeds

Fresh crushed stem and seed powder are used for easy child birth and abortion (Bozzini, 1991).

4.33.2. Cynodon dactylon (Linn.)Pers./ Doov/ Herb/ Whole plant

Entire aboveground parts are crushed with water. Two to three drops of this extract are poured in the nostril to cure nasal bleeding (Uniyal et al., 2006).

4.33.3. Dendrocalamus hamiltonii Nees et Arn. ex Munro /Phulrua/Herb/ Leaves and roots

Leaves and roots used to reduce blood sugar level (Kar et al., 2003).

4.33.4. Eulaliopsis binata (Retz.) Hubb./Bhabhar ghas/Herb/ Roots

The herb is used to treat papillae and internal injuries (Pande et al., 2007).

4.34. Polygonaceae

4.34.1. Rheum australe D. Don/ Chhirchey/Tree/ Aerial parts

Whole plant is crushed and poultice is made in a cotton cloth. This is then heated and applied to cure swelling, which has developed as a result of fractured bone (Uniyal et al., 2006).Plants parts are also used to cure alimentary disorders, cuts, wounds, bone fracture, indigestion, cough, dysentery, haematuria, eye disease, skin disease, sprain, constipation, mastitis, hoof diseases, broken horn and internal injuries in animals (Pande et al., 2007).

4.34.2. Rumex histatus D. Don/ Chalmori, almoru/ Herb/ Leaves

Leaves are believed to have cooling properties and help in stopping nasal bleeding (Uniyal et al., 2006).

4.34.3. Rumex nepalensis D. Don./ Khatura, jungali palak/ Herb/ Leaves
Leaf extract is antiseptic and used to stop bleeding. It is also used against allergy caused by leaves of *Acacia nilotica* (Ahmed et al., 2004).

4.35. Punicaceae
4.35.1. Punica granatam Linn./ Anar/ Shrub/ Juice, fruit bark & flower
Pulp is used as cardiac and stomachache (Ahmed *et al.,* 2004).Fruit juice used in piles, flower juice used in nose bleeding ,bark and flowers in diarrhea and dysentery, decoction of flower buds used in bronchitis and vaginal discharges (Gangwar and Joshi, 2008).

4.36. Ranunculaceae
4.36.1. Aconitum heterophyllum Wall ex Royle/ Ativisha, atees / Herb/ Bark & roots
Dried roots are powdered and taken orally to cure stomach ache and fever (Uniyal et al., 2006), good in periodic and intermittent fevers, useful in diarrhea and vomiting (Gorsi and Miraj, 2002).

4.36.2. Ranunculus sceleratus L. / Celery-leaved buttercup, Jaldhania/Herb/ Whole herb
Herb is used to treat dysuria, asthma and pneumonia (Gangwar and Joshi, 2008).

4.36.3. Thalictrum foliolosum DC./ Mamira/ Herb/ Roots
Herb used to control external parasites (Pande et al., 2007). Dried root powder mixed with *Thymus linearis* in equal proportion is taken regularly to cure stomach pain and gastric trouble (Uniyal et al., 2006).

4.37. Rosaceae
4.37.1. Cotoneaster microphyllus Wall. Ex. Lindn/ Little leaf cotoneaster/ Under shrub/ Fruits, wood & stolons
The stolons are used as an astringent (Qureshi et al., 2007).

4.37.2. Fragaria nubicola Lindley /Bhi kaphal, bud mava/ Herb/ Fruits and leaves
Powdered leaves with leaves of *Berberis lyceum* are used against gastric ulcer, as antiseptic and against wounds (Ahmed *et al.,* 2004). Decoction of plant is consumed to cure fever. (Uniyal et al., 2006).

4.37.3. Geum urbanum Linn/ Bohay, clove wort / Herb / Roots
Roots are used to control fever (Matin et al., 2001).

4.37.4. Potentilla fulgens Wall Ex Hook/ Bazra danti / Herb/ Roots
Leaves are chewed for strengthening the tooth (Singh, 2008).

4.36.5. Prinsepia utilis Royle*./Jhatalu /Shrub/Roots*
Root extract is taken orally as an antidote to neutralize the effect of poison intake. Root paste after heating at low temperature in an earthen pot is applied on wounds (Uniyal *et al.,* 2006).

4.37.6. Prunus persica Stokes./ Aru / Tree/ Leaves
The fruit is antipyretic, tonic to the brain, enriches the blood, flowers are said to be used as laxative (Gorsi and Shahzad, 2002).

4.37.7. Pyracantha crenulata D.Don/ Ghigharu/ Shrub/ Fruits
Used to cure burns (Pande et al., 2007)

4.37.8 Pyrus malus L. /Seb / Tree/ Fruits and barks
The poultice made of rotten apple is used for weak eye and brain tonic (Gorsi and Shahzad, 2002). An infusion of apple tree bark is given in intermittent, remittent and bilious fevers (Gorsi and Miraj, 2002).

4.37.9. Rubus ellipticus Smith./ Hisal, hisalu / Shrub/ Fruits & roots
Roots used to control blood pressure and diarrhea in human (Dhiman, 2005) and haematuria in animals (Pande et al., 2007) and fruits purifies blood and very effective for heart patients (Matin et al., 2001).

4.38. Rutaceae
4.38.1. Murraya koenigii (L.) Spreng./ Kadli nimb, Karwil, Curry leaves /Tree/ Leaves & fruits
Leaves used to cure diarrhea, dysentery and vomiting (Sharma et al., 2006) and are also known to be good for hair, for keeping them healthy and long (Palanisamy and Pillai, 2007).

4.38.2. Zanthoxylum armatum DC./ Timur/Shrub/ Stem & fruits
Used to control gastric disorders, constipation and external parasites (Pande et al., 2007).

4.39. Sapindaceae
4.39.1. Sapindus mukorossi Gaertn./ Reetha/ Tree/ Seeds
Used to control external parasites, hair and skin diseases and to expel leach (Pande *et al*., 2007)

4.40. Saxiferagaceae
4.40.1. Berginia ligulata (Wall.)Engl./ Pashanbheda/ Herb/ Leaves & rhizome
The plant has been recognized for its role in dissolving kidney and bladder stone. Rhizome is useful in cough and cold, cardiac problems, fever, ulcer, swelling, old

wounds, cuts and burns, septic, laizi, gastrointestinal problems, colitis and eye ailments (Chowdhary et al., 2009).

4.40.2. Berginia strachyi Hook f. & Thoms. Engl. /Pashanbheda/ Herb/ Rhizome and bark
The herb is used in curing several ailments like old wounds, kidney stones, opthalmia, cough and colds, tonsils etc (Chowdhary et al., 2009).

4.41. Scrophulariaceae
4.41.1. Bocopa monieri Linn./ Brahammi, Jal neem/Herb/ Whole herb
Used to control dyspepsia, cough, fever, insomnia, epilepsy, debility after heart attack, hoarseness of voice, less memory tension and blood purifier (Brahmverchas, 2003; Dhiman, 2005).

4.41.2. Picrorihza kurroa Royle/ Kutki/ Shrub/ Roots
Roots used to cure dyspepsia, asthma, biliousness, fever, piles, blood troubles, burning sensation, inflammation, ring worm, jaundice, anemia, heart disease, malarial fever, worms infestation in children, indigestion (Brahmverchas ,2003; Dhiman, 2005).

4.41.3. Verbascum thapsus Linn./ Ekalveer/Herb/ Whole herb
Leaves are useful in fever. Leaves and flowers are useful in pulmonary diseases, cough, bleeding of lungs and bowels. Dried corolla of the flower is used in gout and rheumatism (Gorsi and Miraj, 2002).

4.42. Solanaceae
4.42.1. Datura stramonium Linn./ Datura/ Herb/ Whole plant
The seeds have an acid and bitter taste, used as tonic, febrifuge. The leaves after roasting are applied locally to relive pain (Gorsi and Shahzad, 2002).

4.42.2. Solanum indicum Linn Syn.S.ferox Linn./ Bhata katari/Shrub/ Fruits
Fruits and roots used to cure asthma, dry cough, colic, disuria, chronic fever, alopecia, dropsy and toothache (Dhiman, 2005).

4.42.3. Solanum nigrum Linn/ Makoi/Herb/Whole herb
Decoction of leaves is used for liver and skin diseases. Fruits are used to treat eye diseases, dysentery and fever. Seeds and roots are used to treat liver related problems, (Chakraborty, 2004; Dhiman, 2005).

4.42.4. Withania somnifera Dunal./ Ashwaganda, aksun/ Herb/Roots
To improve memory and weakness in humans (Brahmverchas, 2003).The leaves are applied to tumors. The roots are regarded as useful in rheumatism and dyspepsia. The fruits are diuretic (Gorsi and Shahzad, 2002).The tuberous roots are also effective in treating leucoderma, constipation, insomnia, tissue-building and nervous breakdown. Leaves are recommended for fever, painful swellings and ophthalmitis (Sharma et al., 2006).

4.43. Taxaceae
4.43.1. Taxus baccata Linn/Thuner/Tree/ Bark
Decoction of the stem is used early morning to cure tuberculosis (Ahmed *et al*., 2004).

4.44. Theaceae
4.44.1. Camella sinensis (L.) Kunize / Chahua, Chai/Shrub/Leaves and seeds
Leaves and seeds used to treat asthma, angina pectoris peripheral vascular disease and coronary artery diseases. Tea extract have antibacterial activities (Gangwar and Joshi, 2008).

4.45. Urticaceae
4.45.1. Urtica dioica Linn/ Bichchhua, Bichhu ghas /Herb/ Leaves
The leaves are used to cure uterine hemorrhages, bleeding from nose and blood vomiting (Dhiman, 2005; Gangwar and Joshi, 2008), regulate menstrual period (Matin et al., 2001).

4.46. Valerianaceae
4.46.1. Nardostachys grandiflora DC./Masi, / Herb/ Leaves
The herb is used in chronic fevers and heart disorders (Wangchuk, 2004), stem used as a contraceptive and combat stress condition (Chakraborty, 2004).

4.46.2. Valeriana wallichii DC / Samoy/Herb/ Whole herb
The plant used in treatment of inflammatory disease habitual constipation, insomnia, epilepsy, neurosis, anxiety and as a diuretic, hepatoprotective, analgesic and cytotoxic (Subhan et al., 2007).

4.46.3. Valeriana jatamansi DC / Sugandhbala, jatamasi / Herb/Rhizome & roots
This wild herb is being exploited for its roots and rhizomes which contain valepotriates, which are highly effective against leprosy (Kaur et al., 1999).used in hysteria, epilepsy, cholera, dysentery (Matin et al., 2001).

4.47. Verbenaceae
4.47.1. Vitex negundo Linn./ Nirgundi/ Shrub/Leaves and bark
Used in asthma and urinary diseases. Leaves yield a tonic and febrifuge, smoked for relief in catarrh and

headache. Flowers astringent, used in diarrhea, fever and liver complaints (Sharma et al., 2006)

4.47.2. Lantana camera L.var. aculeate/ Baramasi, Phoolwari/Shrub/Whole plant

Whole plant is used for the treatment of bronchitis; leaf decoction is used in treating constipation (Singh et al., 2002).

4.48. Zingiberaceae

4.48.1. Roscoea alpina Royle / Safed musli,kakoli /Herb/ Roots

Roots of herb are used to cure rheumatism (Gangwar and Joshi, 2008).

4.48.2. Hedychium spicatum Buch. Ham Ex. Smith./ Kapoor kachri, Van Haldi/Shrub/ rhizome

The powder as well as decoction of root is carminative and digestive. Decoction is expectorant; stimulant and stomachic. The powder of root is useful in the treatment of liver complaints, and is also used in treating fevers, vomiting, diarrhea, inflammation, pains and snake bite. The root is given for heating potency to the female. It is used in the treatment of indigestion and poor circulation due to thickening of the blood (Bhatt et al., 2007).

5. Discussion

Traditional knowledge of Himalayan medicine is a good illustration of poor communities, fighting even incurable diseases through the traditional methods and even for their livestock, through these traditional herbal medicines. The indigenous traditional knowledge of medicinal plants and therapies of various local communities has been transmitted orally for centuries is becoming extinct, due to changes in traditional culture and introduction of modern technologies. Hence, these traditional practices need proper documentation and the present study is an attempt to collect/ explore, preserve and proper documentation of medicinal plants which are being used traditionally. The investigation revealed that the local people, herbalist and vaidyas have explored a number of plant species to cure various ailments. Qualitative analysis of present study reveals that a total of 102 plant species were identified, of which 49.02 % were herbs; 23.53 % shrubs; 21.57% trees and 2.94 % climbers and under shrubs each (Fig. 2).While on the basis of the plant parts used, it was observed that the maximum plant species (42.16%) were found to be used as various parts such as leaves, roots, stems and bark etc. followed by whole plants (20.59%), roots (13.73%), leaves (7.84%),fruits (3.92%), bark, rhizome and seeds (2.94% each) and flowers (0.98%) to cure various diseases (Fig. 3.).

6. Conclusion

Despite the development of rural health services, villagers still use medicinal herbs to a large extinct for treatment of common ailment like cough, cold and fever, headache and body ache, constipation, dysentery, cuts and burns, boils, ulcer, skin and respiratory diseases etc. Further herbal medicines have no side effects, easily available and economically viable Hence; there is an urgent need of detailed investigation and documentation of indigenous knowledge about medicinal plants and therapies which were being passed orally from generation to generation.

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ENVIRONMENTAL STUDIES ON THE MUDSKIPPERS IN THE INTERTIDAL ZONE OF KUWAIT BAY

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Abstract: This work deals with monitoring mudskippers in their natural environment (intertidal zone) along the Kuwait Bay muddy shores in the State of Kuwait. This is to provide information is concerning the environmental factors effecting mudskipper diversity in Kuwait Bay. Kuwait Bay is a large mud-flat with a fascinating associated fauna of mud-skippers and crabs provide rewarding feeding-grounds for many birds. A number of fifty mudskipper samples are collected during the hot summer season (July and August) of the year 2009 and examined for parasites and to evaluate the different environmental factors controlling the biodiversity in this marine environment. The results of the present study indicate the abundance of the mudskippers allover the intertidal mud flat of the Bay and the total absence of either external and/or internal parasites in the mudskipper tissues and organs. Mudskippers are found to be completely amphibious fish that are adapted to live in the intertidal environment. Mudskippers are very active when they are outside the water, feeding and interacting with one another. The mud in the Kuwait Bay environment is very good for burrowing in, since the particles are very sticky, unlike sand. Often, the mudskipper form mixed colonies with digging crabs (Fiddler crabs-Caidae). Specific physiological and behavioural changes in bioindicators are used to detect changes in environmental health, so Mudskippers can be considered as bioindicators of marine pollution in Kuwait Bay, this needs further studies. [Nature and Science. 2010;8(5):79-89]. (ISSN: 1545-0740).

Key words: Mudskippers, Intertidal Zone, Kuwait Bay

1. Introduction

Mudskippers are members of the subfamily Oxudercinae (tribe Periophthalmini, Murdy, 1989), within the family Gobiidae (Gobies). They are completely amphibious fish that can use their pectoral fins to "walk" on land (Swanson, and Gibb, 2004; Harris, 1960). Being amphibious, they are uniquely adapted to intertidal habitats, unlike most fish in such habitats which survive the retreat of the tide by hiding under wet seaweed or in tidal pools. Mudskippers are quite active when they are out of water, feeding and interacting with one another. Mudskippers constitute a group of 25 air-breathing species in four genera (Periophthalmodon, Periophthalmus, Boleophthalmus and Scartelaos) that are the most derived and the most amphibious of the ten genera of the teleost subfamily Oxudercinae (Gobiidae: Murdy, 1989; Clayton, 1993; Graham, 1997; Aguilar, 2000). These fishes spend extensive periods of time out of water and have numerous physiological, morphological and behavioral specializations for amphibious life (Gordon et al., 1969; Clayton, 1993; Graham, 1997; Lee and Graham, 2002). These fishes present a range of peculiar behavioural and physiological adaptations to an amphibious lifestyle. These include: Anatomical and behavioural adaptations that allow them to move effectively on land as well as in the water (Harris, 1960). As their name implies these fish use their fins to move around in a series of skips. They can also flip their muscular body to catapult themselves up to 2 feet (60 cm) into the air (Piper, 2007) .They have ability to breathe through their skin and the lining of their mouth (the mucosa) and throat (the pharynx). This is only possible when the mudskipper is wet, limiting mudskippers to humid habitats and requiring that they keep themselves moist. This mode of breathing, similar to that employed by amphibians, is known as cutaneous air breathing (Graham, 1997). Another important adaptation that aids breathing while out of water is their enlarged gill chambers, where they retain a bubble of air. These large gill chambers close tightly when the fish is above water, keeping the gills moist, and allowing them to function. They supply oxygen for respiration also while on land (Graham, 1997). Digging of deep burrows in soft sediments that allow the fish to thermo regulate (Tytler and Vaughan, 1983), avoid marine predators during the high tide when the fish and burrow are submerged (Sasekumar et al.. 1994) and for laying their eggs (Brillet, 1969).

Even when their burrow is submerged, mudskippers maintain an air pocket inside it, which allows them to breathe in conditions of very low oxygen concentration (Ishimatsu et al., 1998; Ishimatsu et al., 2000; Lee et al., 2005). Eighteen species of the genus Periophthalmus have been described (Larson and Takita, 2004; Jaafar et al., 2009; Jaafar and Larson, 2008). Periophthalmus argentilineatus grows to a length of about 9.5 cm and is it feeds on small prey such as small crabs and other

arthropods (Milward, 1974). Another species, Periophthalmus barbarus, is the only oxudercine goby that inhabits the coastal areas of western Africa (Murdy, 1989).

Mudskipper colonies are reported from the Bay of Kuwait in the Arabian Gulf area. Each fish digs his own deep burrow where it hides from disturbances and during the high tide. Under certain conditions, the single fishes (most probably Boleophthalmus boddarti) build polygonal territories of a size of about one meter, surrounded by dams, defended against rivals, and large enough to provide food (Microphytobenthos) (Höpner, 1999). Often, the mudskipper form mixed colonies with digging crabs (Fiddler crabs -Ucaidae). Sayer and Davenport (1991) stated that amphibious behaviour in fish has resulted in the colonization and eventual domination by vertebrates of the terrestrial habitat. It is generally proposed that aquatic hypoxia, owing to metabolic oxygen consumption and organic decay, was the most important selective force in the evolution of air-breathing vertebrates (Randall et al., 1981). There is evidently scope for detailed examination of emersion in a number of amphibious fishes, testing a matrix of environmental and biotic stimuli, in an attempt to determine in more detail the reasons for such behaviour (Sayer and Davenport, 1991). Tytler and Vaughan (1983) reported that the annual range of body temperatures (14–35°C) of emergent mudskippers are substantially less than that of air temperatures (10–42°C) as a result of behavioural thermoregulation. Body temperatures generally match those of wet mud, which can be 7°C lower than air shade temperatures.

Colombini et al. (1996) stated that activity patterns and zonation of the mudskippers were directly influenced by the synodic and tidal cycles and depended more on environmental factors such as air temperature and relative humidity than on the diel light cycle.

Chen et al. (2007) reported that the mudskipper, Boleophthalmus pectinirostris forms a territory during the cold season to keep a pool of water that encourages diatom growth and enables the fish to engage in surface activity. Mudskippers have eyes at the top of the head for an all-round view, while their mouth faces downwards to feed on the mud surface. Their pectoral fins are used like crutches to crawl over mud. Mudskippers are a carnivorous opportunist feeder. Mudskippers dig deep burrows to escape predators and raise their young. They maintain an air pocket in their burrows to breathe. Even when their burrow is submerged, mudskippers are seen to maintain an air pocket inside it, which allows them to breathe in conditions of very low oxygen concentration (Ishimatsu et al., 1998; Ishimatsu et al., 2000; Lee et al., 2005). Ishimatsu et al. (1998) reported that mudskipper fishes can maintain their metabolism while they are confined in mudflat burrows filled with oxygen-depleted water, and their eggs, which are deposited in the burrows, can develop under severely hypoxic conditions. During the mating season, the males become much more active, and their colors become more intense. Jaafar et al. (2006) recorded the mudskipper, Periophthalmus walailakae from Singapore. This species most closely resembles Pn. schlosseri but with only one row of teeth on the upper jaw, scales on the isthmus, and a different upper lip and jaw morphology. The authors (ibid) added that contrary to an earlier report, scales are present on the snout, intertidal, and isthmus of Ps. walailakae. The two species can also be distinguished by size, external morphology, and body color patterns. As stated by Harris (1961), these fishes present a range of peculiar behavioral and physiological adaptations to an amphibious lifestyle. He (ibid) reported that these include anatomical and behavioral adaptations that allow them to move effectively on land as well as in the water. Piper (2007) reported that they can also flip their muscular body to jump up to 60 cm in the air. They have ability to breathe through their skin and the lining of their mouth and throat .This is only possible when the mudskipper body is wet, limiting mudskippers to humid habitats and requiring that they keep themselves moist. This mode of breathing, similar to that employed by amphibians, is known as "cutaneous air breathing" (Graham, 1997). Another important adaptation that aids breathing while out of water is their enlarged gill chambers, where they retain a bubble of air. These large gill chambers close tightly when the fish is above water, keeping the gills moist, and allowing them to function. They supply oxygen for respiration also while on land (Graham, 1997). As reported in the study of Tytler and Vaughan (1983) diggings of deep burrows in soft sediments that allow the fish to thermo- regulate avoid marine predators during the high tide when the fish and burrow are submerged (Sasekumar, et al., 1994) and for laying their eggs (Brillet, 1969). Periophthalmus argentilineatus is one of the most widespread and well-known species. It can be found in mangrove ecosystems and mudflats of East Africa and Madagascar east through the Sundarbans of Bengal, South East Asia to Northern Australia, Southeast China and Southern Japan, up to Samoa and Tonga Islands .Another species, Periophthalmus barbarus, is the only oxudercine goby that inhabits the coastal areas of Western Africa (Murdy, 1989). Both of these amphibious habits are completely unsuited for normal fish tanks. Clayton (1987), Clayton and Vaughan (1988) and Clayton and Wright (1989) stated that the proximate mechanisms of territorial behaviour in Boleophthalmus boddarti, an amphibious gobiid mudskipper that builds and maintains polygonal mud-walled territories provide a good example of the elastic disc concept of territories. Clayton and Wright (1989) stated that at high population densities, the amphibious and herbivorous

mudskipper Boleophthalmus boddarti construct mud walls around their territories as a means of reducing aggression between neighbours. The authors (ibid) stated there were no significant differences in diatom density between territorial and non-territorial areas or between grazed and non-grazed areas within territories. They concluded that the mud walls are considered to play a secondary, indirect role in maintaining populations of diatoms within territories. The mudflats are subject to intense bioturbation, and that for the Arabian Gulf area, mudskipper colonies are reported from the Bay of Kuwait. Each fish digs his own deep burrow where it hides from disturbances and during high tide (Höpner, 1999). Under certain conditions, the single fishes build polygonal territories of a size of about one meter, surrounded by dams, defended against rivals, and large enough to provide food (Microphytobenthos). This scenery is rare even in Kuwait Bay and difficult to be accessed; it is widespread on lower situated mudflats of the Khowre Musa area where it covers many km2. Even in the higher situated zone, mudskippers contribute decisively to the bioturbation power but do not form territories. Often, they form mixed colonies with digging crabs, e.g. Fiddler crabs (Ucaidae). In Kuwait, Clayton and Wells (1994) distinguished four main species of mudskippers around the mudflats of Kuwait Bay and the Northern coast of Kuwait. The authors stated that the mudskippers are also separated into zones. The mudskipper found on the high shore is called Periophthalmus, a carnivorous mudskipper up to 15 centimeters long which feeds on little crabs at low tide .Periophthalmus' eyes are well-adapted to vision in air, and when he's lying waiting for his prey only his eyes stick up out of the muddy water. In order to keep his eyes wet, Periophthalmus has little cups underneath the eyes and when he blinks, the eyes roll down into his skull and get remoistened by the water held in these little cups.

Slightly further down the shore the second species of mudskipper, called Boleophthalmus is the largest, measuring up to 25 cm and, like larger animals, is an herbivore. Boleophthalmus can be easily recognized by their feeding action, a side-to-side head movement that collects the fine surface film of diatoms and algae on which they feed. Even in the hot summer, this species of mudskipper can remain active for several minutes at a time out of water, and it can be recognized by its characteristic mode of locomotion. The pelvic fins have moved forward and fused together to form a little cup which he uses as a sort of crutch to balance on. His very strong and well-muscled pectoral fins have moved down the body and are, allowing him to swing along on them, using the life between the tides rear fin as a stabilizer. Boleophthalmus build and maintain these polygonal walled territories by carrying mouthfuls of mud from their burrows and depositing them on their walls. As far as is known, this population in Sulaibikhat Bay is unique throughout the Indo-Pacific area (Clayton and Wells, 1994). Boleophthalmus stays always within its mud walls, which enclose an area rather like a pasture that has enough food for each individual to survive. Inside each walled com- pound, Boleophthalmus digs a burrow up to one and a half metres deep. The burrow may be anywhere in the territory, maybe right beside the wall (Clayton and Wells, 1994).

The mud is very good for burrowing in, since the particles are very sticky, unlike sand. In all except the very softest ooze, burrows will last for quite a long time. Some burrows have a little chimney sticking up above the surface, others have small water-filled pools beside the openings, and the reason for these variations is unknown (Clayton and Wells, 1994). Boleophthalmus is very quick to dive into his burrow, and constantly alert to danger. If they suspect a predator is around, Boleophthalmus will merely raise his bulbous eyes above the surface of the mud to check out the situation, ducking down again at the slightest sign of trouble.

Clayton and Wells (1994) reported that all mudskippers are sexually monomorphic, which means that the males and females look exactly alike. However, during the breeding season, which extends from March until late August, the males go through an elaborate display that advertises their sex quite unmistakably. After hatching, the larvae swim off with the tide and spend some time simply floating around with the other types of plankton. Then they turn into very miniature mudskippers and return to the mudflats where they congregate in the soft mud areas. Here, as stated by Clayton and Wells (1994), they will remain until they reach maturity and manage to gain a territory of their own. Out of thousands of eggs, predators such as crabs will take the vast majority of larvae and only a handful will survive. The large xanthid crab, with its dark-tipped claws, feeds on mudskipper larvae. Clayton and Wells (1994) stated that the last two species of mudskippers found in Kuwait are both found much further out and so are much harder to observe. They are also much less amphibious than their inshore cousins and spend their time in permanently wet mud. The authors (ibid) stated that the larger of the two is called Scartelaos, and, although he is about as long as Boleophthalmus, is much thinner. Clayton and Wells (1994) reported that the final species of mudskipper is called Apocryptes and is much smaller than any of the others. Since both Scartelaos and Apocryptes inhabit areas of oozing wet mud, it is impossible for them to build burrows, so they hide from predators by simply squirming down into the soft mud out of sight.

Clayton and Snowden (2000) observed the surface activity of the carnivorous mudskipper Periophthalmus waltoni Koumans 1941 was on mudflat in Sulaibikhat

Bay, a muddy shore embayment in Kuwait Bay, at the north-western head of the Arabian Gulf. They reported that Each adult fish had a home range of between 2-3 m2 in which were located the main and subsidiary burrows, the main one simply being the most frequently used. The burrows were of two types such that the entrance was either a double- turreted one (the 'Y' shaped burrow) or was in a water-filled, saucer-like depression. While some fish had a single burrow system of either type, oth- utilise between two and six different ones. During the period of observation no other P. waltoni were seen to use them.

Mhaisen and Al-Maliki (1996) stated that the mudskipper Periophthalmus waltoni (Perciformes: Gobiidae) in the Khor Al-Zubair estuary (Iraq) are infected with Myxobolus pfeifferi (Sporozoa), Diplozoon sp. (Monogenea) and Neoechinorhynchus sp. (Acanthocephala). The State of Kuwait occupies approximately 17,800 km^2 of the Northwestern part of the Arabian Gulf, between 28º30' and 30º05'N, and 46º33' and 48º30'E. It comprises the mainland and nine offshore islands. Kuwait's climate is characterized by hot summers and mild winters. Temperature extremes are high, with means during the warmest and coolest months ranging between 46.2ºC and 6.9ºC. Winter brings occasional frost. Rainfall is minimal, not exceeding 115 mm/year, but evaporation is very high, averaging 14.1 mm/d. The relative humidity is low, and strong, dry and hot, Northwesterly winds prevail during summer, particularly in June and July (Al Nafisi et al., 2009).

"Kuwait bay" is considered as one of the characterized features of the Kuwaiti marine environment, which is an elliptically shaped bay that protrudes from the Arabian Gulf in Westward direction at its Northwestern corner. Kuwait Bay is of a moderate size (850 km²) with an average water depth of 5 m and a maximum depth of 20 m at the entrance (Al-Ghadban, 2004). The Northern shoreline is a pristine shore and in contrast, the Southern part the bay hosts urban activities, major ports such as Shuwaikh and Doha Ports and three major power and desalination plants (Doha East, Doha West and Subiya). The Bay of Kuwait presents a unique ecosystem and a significant nursery ground for many fishes and shrimp species (Al-Yamani et al., 2004). Although biological and ecological data on the marine biota of the region is limited, with some coastal areas receiving more attention than others at least four critical marine habitats, coral reefs, intertidal marshes, mangrove and sea grass beds, and kept forest, have been recognized in the region (Price et. al., 1993).

In recent years, increased developmental activities and misuse of native vegetation have greatly degraded the coastal environment and marine ecosystems. In addition, harsh weather conditions have accelerated the disappearance of vegetation cover in both inland and coastal areas. A forestation of intertidal zones with mangrove plants is considered a viable option to improve coastal environment and enrich marine biodiversity. Mangrove plantations also protect the coastline from strong currents and support the accumulation of sediments and organic matter in the intertidal zones. These changes would improve the quality of mudflats and promote the survival and growth of marine fauna (Sbandar et al., 20; Emabi, 1993; Ogino, 1993; Al Nafisi et a., 2009). The coastal zone of Kuwait, depending on the sediment nature and morphology, is classified into two main parts: Northern muddy province and Southern rocky/sible for them to build burrows, so they hide from predators by simply squirming down into the soft mud out of sight.

Clayton and Snowden (2000) observed the surface activity of the carnivorous mudskipper Periophthalmus waltoni Koumans 1941 was on mudflatal and growth of marine fauna (Sbandar et al., 20; Emabi, 1993; Ogino, 1993; Al Nafisi et a., 2009). The coastal zone of Kuwait, depending on the sediment nature and morphology, is classified into two main parts: Northern muddy province and Southern rocky/sandy part. These parts are subdivided into several zones (Abou-Seida and Al-Sarawi, 1990). The bay supports a thriving fishing industry and contains a site of an aquaculture facility of 80 net pen cages with a production of roughly 500 – 600 metric tons ole and normal values for this region of the world. Kuwait Bay is an exposed area and stressed as a result of the extensive man-made activities, such as dredging, indiscriminate solid and liquid waste disposal and over fishing. Al-Bakri et al., (1985) have concluded that the alteration of the coastal zone has resulted in more impact to the ecosystem compared to the harsh environmental condition. The environmental condition of the coastal area became more critical as a result of the war-related activities (Al-Ghadban et al., 1992).

El-Sammak et al., (2005) compared the levels of heavy metals in bottom sediments of Kuwait Bay and Sulaibikhat embayment with the reported values of Dubai, Greek and Canada and highlighted higher values of cadmium and nickel. Al-Majed et al. (2004) identified certain concentration of methyl mercury in the embayment and attributed such concentration due to the power plant and also due to the pervious industrial outfall and also due to the shipping activities and the discharged into the area from the emergency outlets. Al-Ghadban et al. (1994) reported higher values of total organic carbon content (more than 3%) in the area. Coastal pollution has been increasing significantly over the recent years and found expanding environmental problems in many developing countries. The discharges of industrial wastes have resulted in high metal concentrations in the local marine environment, especially in the coastal sediments (Saad et al., 1981; Mance, 1987; Ni et al., 2005). Al-Sarawi et al. (2002)

reported high levels of metal discharges from power, thermal, desalination and water treatment plants and leakage from oil wells in Kuwait marine environment Glibert et al. (2004) stated that the bay supports a thriving fishing industry and contains a site of an aquaculture facility of 80 net pen cages with a production of roughly 500 – 600 metric tons of sea bream (Sparus auratus L.) per year. Mudskipper, Periophthalmus waltoni is distributed in the northern region of Kuwait Bay's tidal mudflats. They are the prey for many predators and thus, it is essential to evaluate the bioaccumulation of metals toxicants in this fish. The gills, skin and food are the main routes of accumulation of metals by fish (Hein et al., 1993; Ni et al., 2005). The objectives of the present study were to study the ecological behavior of the mudskippers and to determine the relationships between the gobies fish behavior and the Kuwait Bay's mudflats habitat.

2. Materials and methods

The studied Mudskippers in the present study are monitored along the Kuwait Bay shores (Fig. 1) during the very hot summer season, near delivery hospital, Shuwaikh area, Kuwait Governorate. They are particularly abundant in the muddy shore. However, a number of fifty mudskipper samples are collected randomly to be examined for parasites.

Field observations:

The procedure followed for monitoring the mud skippers is described by Clayton and Wells (1994), that comprises using a sheet of plywood or a big stone to help to distribute our weights more evenly across the mud, as a surface crust of dried mud may seem quite hard, but if it cracks one, and one will sink right down into the soft ooze below. But if one chooses the right type of shore he should be able to observe the mudskippers from the safety of dry land. Fifty specimens of the amphibious gobies (2.17œ5.20 cm standard length (SL); 0.16œ2.70 g body wet weight) were collected using hand-nets during low tides in Kuwait Bay. Species are abundant and successfully surviving in the mud flat pool during low tides. The caught specimens were placed in small plastic containers, and kept alive in aerated sea-water prior to further studies in the College laboratory. Taxonomic identification and confirmation of the species were based on description given in Clayton and Wells (1994). Amphibious goby was identified as Periophthalmus sp.

Laboratory observations:

Collected specimens were examined for infestation with external parasites, and then dissected for detecting internal parasites in the body cavity and all other organs.

Results and Discussion

The Mudskippers observed in the present study appear to be quite active when they are out of water, feeding and interacting with each other (Fig. 2). The results of the present work are presented in the following items:

1- Description and habitat

In the present study, the mudskipper that are found in and around the mudflats of Kuwait Bay, are amphibious fishes that are called mudskippers. The results agree with those of Clayton and Wells (1994), who are separated the mudskippers into zones. The high shore mudskippers are called *Periophthalmus*, which are carnivorous mudskippers up to 15 centimeters long and feeds on little crabs at low tide. The genus *Periophthalmus* is by far the most diverse and widespread genus of mudskippers that contains eighteen described species (Larson and Takita, 2004).

They are uniquely adapted to intertidal habitats, unlike most fish in such habitats, which survive the retreat of the tide by hiding in tidal pools. Mudskippers need to live in habitats that are hot and humid in order to breathe, where air and water temperature range from 75 to 86 °F and humidity from 60 to 80 %. Mudskippers are only active when the temperature is above 55 °F. This result agrees with the results of Clayton and Well (1994).

Other adaptation of mudskippers to life on land is their huge goggly eyes at the top of their heads for an all-round view, while their mouth faces downwards to feed on the mud surface. These eyes sit on stalks while the rest of their bodies remain safely underwater. Unlike other fishes, mudskippers prefer to swim with their heads above water, their eyes giving them a good 360-degree view. To keep their eyes moist when they are on land, the eyes can be retracted to dip them into water that collects at the bottom of the eye socket. Mudskippers are probably the only fish with movable eyelids (Fig. 3). The pectoral fins of the mudskippers are used like crutches to crawl over mud.

Fig. 1. Kuwait Bay shoreline and mudflat, State of Ku[illegible]K

Figs. 2A &2B. The observed Mudskippers appear to be quite active when they are out of water.

Fig. 4. Walled territories.

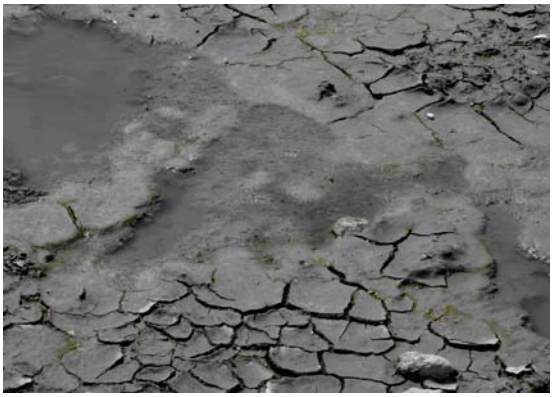

Fig. 3. Mudskippers are probably the only fish with huge eyes for an all-over view.

2- Territorial behaviour

Mudskippers are noticed to dig deep burrows to escape predators and raise their young. They maintain an air pocket in their burrows to breathe. Chen *et al.* (2007) reported that the mudskipper, *Boleophthalmus pectinirostris*, maintained territories in farming ponds during the cold season between November and February. Two types of territory are found, one is surrounded by mud-walls, and the other is without mud-walls. Both types of territory are entirely covered by shallow water (Fig. 4).

The area of walled territories was significantly bigger than that of non-walled ones. The nearest neighbour's distance of walled territories was significantly shorter than that of non-walled territories. This description is detected in the present study.

Fish surface activity occurred between 1200 and 1500 hours at which time the territorial water temperature reached a maximum, being significantly higher than that of the air, mud surface or deep burrow. The territorial sediments exhibited a significantly greater benthic micro- algal biomass as compared to the non-territorial sediments. The present study indicates that *B. pectinirostris* forms a territory a pool of water that encourages diatom growth and enables the fish to engage in surface activity (Fig. 4).

Clayton (1987), Clayton and Vaughan (1988) and Clayton and Wright (1989) stated that the proximate mechanisms of territorial behaviour in *Boleophthalmus boddarti*, an amphibious gobiid mudskipper that builds and maintains polygonal mud-walled territories provides a good example of the elastic disc concept of territories. The occurrence of wall building is density dependent and a contiguous mosaic of territories is only produced at high fish densities. Wall removal and replacement experiments show that the mud-wall acts as a visual barrier and reduces aggression between neighboring territorial fish.

Clayton and Wright (1989) stated that at high population densities, the amphibious and herbivorous mudskipper *Boleophthalmus boddarti* construct mud walls around their territories as a means of reducing aggression between neighbours. Because of the walls, territories contain pools of water and exposed mud slopes. Whilst the density of benthic diatom prey was highly variable, the highest was found on the exposed mud slopes and the lowest on the boundary walls. Fish grazed mainly on the mud slopes. No significant differences were found in diatom density between territorial and non-territorial areas or between grazed and non-grazed areas within territories. The variation of the diatom density, however, was reduced within territories. The mud walls play an indirect role in maintaining populations of diatoms within territories.

Höpner (1999) reported that the mudflats are subject to intense bioturbation, and that for the

Arabian Gulf area, mudskipper colonies are reported from the Bay of Kuwait. Each fish digs his own deep burrow where it hides from disturbances and during high tide. The author (ibid) stated that under certain conditions, the single fishes (most probably *Boleophthalmus boddarti*) build polygonal territories of a size of about one meter, surrounded by dams, defended against rivals, and large enough to provide food(microphytobenthos). Clayton and Wells (1994) stated that while this scenery is rare even in Kuwait Bay and difficult to be accessed, it is widespread on lower situated mudflats of the Khowr-e Musa area where it covers many km2. Even in the higher situated zone, mudskippers contribute decisively to the bioturbation power but do not form territories. Often, they form mixed colonies with digging crabs (Ucaidae). This result agrees with the results of the present study.

Boleophthalmus build and maintain these polygonal walled territories by carrying mouthfuls of mud from their burrows and depositing them on their walls. As far as is known, this population in Sulaibikhat Bay is unique throughout the Indo-Pacific area (Clayton and Wells, 1994)!

Although they are found all over Kuwait Bay, the most interesting population of *Boleophthalmus* can be seen in Sulaibikhat Bay where they construct and maintain and elaborate and continuous network of polygonal walled territories (see Fig. 3 and 4). Most of these territories are five-sided with an average area of 1.69 square metres. The ones with only four sides tend to be smaller, while those with six or seven sides are a little bigger. These territories are unusual in that each is occupied by a single fish of either sex.

Boleophthalmus stays always within its mud walls which enclose an area that has enough food for each individual to survive. They build the walls with mud from their burrows which they carry up in their mouths and spit out onto the section in need of repair. If part of the wall is destroyed, they will work hard to repair it within a matter of hours during low tide. The best time to see these walled territories is at low tide when the sun is low in the sky. If there is a mild winter, some of the walls will last for more than a year .Inside each walled compound, *Boleophthalmus* digs a burrow up to one and a half metres deep. The burrow may be anywhere in the territory, maybe right beside the wall (Clayton and Wells, 1994).

Young *Boleophthalmus* don't have their own territories, due to lack of suitable space. Instead, they are forced to live in areas where the mud is too soft for either walls or burrows, burying themselves in soft ooze whenever a predator threatens. Obviously, this is not as safe as a nice deep burrow, so the young mudskippers are live between the tides constantly on the lookout for a vacant territory and constantly trespass both in order to feed and to investigate the chances of finding an empty lot.

Naturally, the owners do not take kindly to this and will chase the intruder out, into a neighboring territory, where upon the neighbours will take up the pursuit and a splendid chain reaction of indignant outrage will follow the poor trespasser all the way back to the soft muddy area where belongs.. The main factor in ensuring a constant supply of vacant territories is predation and this is especially prevalent during the spring when the weather is cold and the fish are rather sluggish as a result. Shore birds are their worst enemies.

Clayton and Wells (1994) stated that most of the fish found in the intertidal zone come there at high tide to feed. However, to see the fish, which have best adapted to the amphibious life between the tides we have to go to the third main coastal region of Kuwait: the mudflats. Even when their burrow is submerged, mudskippers are seen to maintain an air pocket inside it, which allows them to breathe in conditions of very low oxygen concentration. This result accommodates with the results of (Ishimatsu *et al*., 1998; Ishimatsu *et al.*, 2000; Lee *et al*., 2005).

Clayton and Wells (1994) observed mudskippers dig deep burrows to escape predators and raise their young and that they maintain an air pocket in their burrows to breathe. During the mating season, the male's colors become more intense, and they become much more active. They (ibid) stated that males leap and flip in the air and even stand on their tails, all to attract the females. The male becomes more aggressive forward other males with biting and raising its dorsal fins. If the female is attracted to the male, she does her own mating ritual with distinctive movements. She then goes into the male's burrow where she lays her eggs in a special part of the burrow the male builds her. The males then fertilize the eggs and takes over responsibility for the eggs. The above descriptions are noticed in the present study. These fishes present a range of peculiar behavioral and physiological adaptations to an amphibious lifestyle. He reported that these include: Anatomical and behavioral adaptations that allow them to move effectively on land as well as in the water (Harris, 1961). They can also flip their muscular body to catapult themselves up to 60 cm into the air. This observation is noticed in the present study (Piper, 2007).

Another important adaptation that helps the mudskippers to breath while they are out of water is their gill chambers, where they keep a bubble of air. These large gill chambers close tightly when the mudskipper is above the water, allowing the gills to be kept moist, and make them work. They supply oxygen for respiration also while the gobies are on the land (Graham, 1997).

As reported in the study of Tytler and Vaughan (1983), digging of deep burrows in soft sediments that allow the fish to thermoregulate , avoid marine

predators during the high tide when the fish and burrow are submerged (Sasekumar *et al.*, 1994) and for laying their eggs (Brillet,1969).

Tytler and Vaughan (1983) stated that in winter, mudskippers avoid low surface temperatures by remaining in their burrows, in the present study, no mudskippers were detected during winter months. In summer, body temperatures are kept lower than ambient by selecting areas where evaporative cooling is high. Body temperatures generally match those of wet mud, which can be 7°C lower than air shade temperatures.

Chen et al. (2007) stated that the mudskipper *Boleophthalmus pectinirostris* maintained territories in farming ponds during the cold season between November and February.

The present study results accommodate with the above observations.

Fig. 5. Periophthalmus' eyes are well-adapted to see in air and out of the muddy water.

Periophthalmus' eyes are well-adapted to see in air, and when they are lying in wait for their preys only their eyes stick up out of the muddy water (Fig. 5). In order to keep their eyes wet, *Periophthalmus* have little cups underneath their eyes and when they blinks, the eyes roll down into their skull and get remoistened by the water held in these little cups. Even in the heat of summer, this species of mudskipper can remain active for several minutes at a time out of water. They are seen periodically rolling on their sides to help to keep their skins wet.

Every five minutes or so, *Periophthalmus* will dash back to its burrow to cool off and refresh itself. This species of mudskipper can be recognized by its characteristic way of locomotion. It lifts his tail, and then arches his back. It has two sets of paired fins, pectorals near his chest, and pelvic fins at the hips. The pelvic fins have moved forward and fused together to form a little cup, which is used as a sort of crutch to balance on. This description agrees with the results of the present study.

Periophthalmus, was observed sitting on the mudflats splashing water over his body with his pectoral fins for about several minutes before moving back to his burrow. This agrees with the description of Clayton and Wells (1994). These burrows are quite remarkable; they are y -shaped, with two entrances and are more than a meter deep in order to get down to permanent water level. The mud is suitable habitat for burrowing in, since the particles are very sticky, unlike sand. In all except the very softest ooze, burrows will last for quite a long time (Clayton and Wells, 1994).

3- Feeding

Periophthalmus needs to be out of water to feed and it needs to keep his skin wet, it can absorbs oxygen through its skin as well as through its gills. Some times, it will fill its mouth with water to help him to survive for a longer time out side the water (Fig. 6), since the inside of its mouth has lots of tiny blood vessels that absorb oxygen. However, this water isn't 'essential; the mudskipper loses it anyway when it eats his prey (Clayton and Wells, 1994).

Ishimatsu *et al.*, (1998) reported that mudskipper fishes can maintain their metabolism while they are confined in mudflat burrows filled with oxygen-depleted water, and their eggs, deposited in the burrows, can develop under severely hypoxic conditions.

Their feeding action, a side-to-side head movement that collects the fine surface film of diatoms and algae on which they feed can easily recognize Boleophthalmus. Terns are another ménage to the mudskipper that they catch by flying along about three to five metres up, then dive-bombing their prey with their bayonet-like beaks. Sea gulls use a craftier approach, skimming very low, just above the mud walls and trying to catch the *Boleophthalmus* unawares.

But, *Boleophthalmus* like all the other mudskippers, is very quick to dive into his burrow, and constantly alert to danger. If they suspect a predator is around, *Boleophthalmus* will merely raise his bulbous eyes above the surface of the mud to check out the situation.

4- Infection of the mudskippers with parasites:

External examination and dissection of 50 samples of the mudskipper collected from the studied area indicates no parasitic infections.

Fig. 6. The mudskipper needs to be out of water to feed and keep his skin wet.

CONCLUSIONS

Mudskippers have been monitored in the intertidal zone along the Kuwait Bay muddy shores during the very hot summer season of the year 2009. However, a number of fifty random mudskipper samples are examined externally and internally for parasites. These examinations are to evaluate the different factors controlling the biodiversity in that area. The results indicate the abundance of the Mudskippers allover the muddy flat of the Bay and the total absence of either external and/or internal parasites in the mudskipper.

A forestation of intertidal zones with mangrove plantations is viable options to improve coastal environment and enrich marine biodiversity. These factors also protect the coastline from strong currents and support the accumulation of sediments and organic matter in the intertidal zones. These changes would improve the quality of mudflats and promote the survival and growth of intertidal zone fauna.

The results indicated, also, that mudskippers are free from parasitic infection. However, important results concerning the controlling the parasitic infection of mudskippers is needed.

The above findings deduce that the mudskippers caught from Kuwait bay tidal mud flats have the capability to live in the Kuwait Bay, although the studies reported the toxicity by metals and the trace metals in the habitat

RECOMMENDATION

Further environmental studies on the Bay area and its fauna are recommended to evaluate the probable reasons of absence of either external and/or internal parasites in the mudskipper. Also, toxicological investigations that use the gobies as bioindicator are recommended

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ENVIRONMENTAL STUDIES ON THE MUDSKIPPERS IN THE INTERTIDAL ZONE OF KUWAIT BAY

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Abstract: This work deals with monitoring mudskippers in their natural environment (intertidal zone) along the Kuwait Bay muddy shores in the State of Kuwait. This is to provide information is concerning the environmental factors effecting mudskipper diversity in Kuwait Bay. Kuwait Bay is a large mud-flat with a fascinating associated fauna of mud-skippers and crabs provide rewarding feeding-grounds for many birds. A number of fifty mudskipper samples are collected during the hot summer season (July and August) of the year 2009 and examined for parasites and to evaluate the different environmental factors controlling the biodiversity in this marine environment. The results of the present study indicate the abundance of the mudskippers allover the intertidal mud flat of the Bay and the total absence of either external and/or internal parasites in the mudskipper tissues and organs. Mudskippers are found to be completely amphibious fish that are adapted to live in the intertidal environment. Mudskippers are very active when they are outside the water, feeding and interacting with one another. The mud in the Kuwait Bay environment is very good for burrowing in, since the particles are very sticky, unlike sand. Often, the mudskipper form mixed colonies with digging crabs (Fiddler crabs-Caidae). Specific physiological and behavioural changes in bioindicators are used to detect changes in environmental health, so Mudskippers can be considered as bioindicators of marine pollution in Kuwait Bay, this needs further studies. [Nature and Science. 2010;8(5):79-89]. (ISSN: 1545-0740).

Key words: Mudskippers, Intertidal Zone, Kuwait Bay

1. Introduction

Mudskippers are members of the subfamily Oxudercinae (tribe Periophthalmini, Murdy, 1989), within the family Gobiidae (Gobies). They are completely amphibious fish that can use their pectoral fins to "walk" on land (Swanson, and Gibb, 2004; Harris, 1960). Being amphibious, they are uniquely adapted to intertidal habitats, unlike most fish in such habitats which survive the retreat of the tide by hiding under wet seaweed or in tidal pools. Mudskippers are quite active when they are out of water, feeding and interacting with one another. Mudskippers constitute a group of 25 air-breathing species in four genera (Periophthalmodon, Periophthalmus, Boleophthalmus and Scartelaos) that are the most derived and the most amphibious of the ten genera of the teleost subfamily Oxudercinae (Gobiidae: Murdy, 1989; Clayton, 1993; Graham, 1997; Aguilar, 2000). These fishes spend extensive periods of time out of water and have numerous physiological, morphological and behavioral specializations for amphibious life (Gordon et al., 1969; Clayton, 1993; Graham, 1997; Lee and Graham, 2002). These fishes present a range of peculiar behavioural and physiological adaptations to an amphibious lifestyle. These include: Anatomical and behavioural adaptations that allow them to move effectively on land as well as in the water (Harris, 1960). As their name implies these fish use their fins to move around in a series of skips. They can also flip their muscular body to catapult themselves up to 2 feet (60 cm) into the air (Piper, 2007) .They have ability to breathe through their skin and the lining of their mouth (the mucosa) and throat (the pharynx). This is only possible when the mudskipper is wet, limiting mudskippers to humid habitats and requiring that they keep themselves moist. This mode of breathing, similar to that employed by amphibians, is known as cutaneous air breathing (Graham, 1997). Another important adaptation that aids breathing while out of water is their enlarged gill chambers, where they retain a bubble of air. These large gill chambers close tightly when the fish is above water, keeping the gills moist, and allowing them to function. They supply oxygen for respiration also while on land (Graham, 1997). Digging of deep burrows in soft sediments that allow the fish to thermo regulate (Tytler and Vaughan, 1983), avoid marine predators during the high tide when the fish and burrow are submerged (Sasekumar et al.. 1994) and for laying their eggs (Brillet, 1969).

Even when their burrow is submerged, mudskippers maintain an air pocket inside it, which allows them to breathe in conditions of very low oxygen concentration (Ishimatsu et al., 1998; Ishimatsu et al., 2000; Lee et al., 2005). Eighteen species of the genus Periophthalmus have been described (Larson and Takita, 2004; Jaafar et al., 2009; Jaafar and Larson, 2008). Periophthalmus argentilineatus grows to a length of about 9.5 cm and is it feeds on small prey such as small crabs and other

arthropods (Milward, 1974). Another species, Periophthalmus barbarus, is the only oxudercine goby that inhabits the coastal areas of western Africa (Murdy, 1989).

Mudskipper colonies are reported from the Bay of Kuwait in the Arabian Gulf area. Each fish digs his own deep burrow where it hides from disturbances and during the high tide. Under certain conditions, the single fishes (most probably Boleophthalmus boddarti) build polygonal territories of a size of about one meter, surrounded by dams, defended against rivals, and large enough to provide food (Microphytobenthos) (Höpner, 1999). Often, the mudskipper form mixed colonies with digging crabs (Fiddler crabs -Ucaidae). Sayer and Davenport (1991) stated that amphibious behaviour in fish has resulted in the colonization and eventual domination by vertebrates of the terrestrial habitat. It is generally proposed that aquatic hypoxia, owing to metabolic oxygen consumption and organic decay, was the most important selective force in the evolution of air-breathing vertebrates (Randall et al., 1981). There is evidently scope for detailed examination of emersion in a number of amphibious fishes, testing a matrix of environmental and biotic stimuli, in an attempt to determine in more detail the reasons for such behaviour (Sayer and Davenport, 1991). Tytler and Vaughan (1983) reported that the annual range of body temperatures (14–35°C) of emergent mudskippers are substantially less than that of air temperatures (10–42°C) as a result of behavioural thermoregulation. Body temperatures generally match those of wet mud, which can be 7°C lower than air shade temperatures.

Colombini et al. (1996) stated that activity patterns and zonation of the mudskippers were directly influenced by the synodic and tidal cycles and depended more on environmental factors such as air temperature and relative humidity than on the diel light cycle.

Chen et al. (2007) reported that the mudskipper, Boleophthalmus pectinirostris forms a territory during the cold season to keep a pool of water that encourages diatom growth and enables the fish to engage in surface activity. Mudskippers have eyes at the top of the head for an all-round view, while their mouth faces downwards to feed on the mud surface. Their pectoral fins are used like crutches to crawl over mud. Mudskippers are a carnivorous opportunist feeder. Mudskippers dig deep burrows to escape predators and raise their young. They maintain an air pocket in their burrows to breathe. Even when their burrow is submerged, mudskippers are seen to maintain an air pocket inside it, which allows them to breathe in conditions of very low oxygen concentration (Ishimatsu et al., 1998; Ishimatsu et al., 2000; Lee et al., 2005). Ishimatsu et al. (1998) reported that mudskipper fishes can maintain their metabolism while they are confined in mudflat burrows filled with oxygen-depleted water, and their eggs, which are deposited in the burrows, can develop under severely hypoxic conditions. During the mating season, the males become much more active, and their colors become more intense. Jaafar et al. (2006) recorded the mudskipper, Periophthalmus walailakae from Singapore. This species most closely resembles Pn. schlosseri but with only one row of teeth on the upper jaw, scales on the isthmus, and a different upper lip and jaw morphology. The authors (ibid) added that contrary to an earlier report, scales are present on the snout, intertidal, and isthmus of Ps. walailakae. The two species can also be distinguished by size, external morphology, and body color patterns. As stated by Harris (1961), these fishes present a range of peculiar behavioral and physiological adaptations to an amphibious lifestyle. He (ibid) reported that these include anatomical and behavioral adaptations that allow them to move effectively on land as well as in the water. Piper (2007) reported that they can also flip their muscular body to jump up to 60 cm in the air. They have ability to breathe through their skin and the lining of their mouth and throat .This is only possible when the mudskipper body is wet, limiting mudskippers to humid habitats and requiring that they keep themselves moist. This mode of breathing, similar to that employed by amphibians, is known as "cutaneous air breathing" (Graham, 1997). Another important adaptation that aids breathing while out of water is their enlarged gill chambers, where they retain a bubble of air. These large gill chambers close tightly when the fish is above water, keeping the gills moist, and allowing them to function. They supply oxygen for respiration also while on land (Graham, 1997). As reported in the study of Tytler and Vaughan (1983) diggings of deep burrows in soft sediments that allow the fish to thermo- regulate avoid marine predators during the high tide when the fish and burrow are submerged (Sasekumar, et al., 1994) and for laying their eggs (Brillet, 1969). Periophthalmus argentilineatus is one of the most widespread and well-known species. It can be found in mangrove ecosystems and mudflats of East Africa and Madagascar east through the Sundarbans of Bengal, South East Asia to Northern Australia, Southeast China and Southern Japan, up to Samoa and Tonga Islands .Another species, Periophthalmus barbarus, is the only oxudercine goby that inhabits the coastal areas of Western Africa (Murdy, 1989). Both of these amphibious habits are completely unsuited for normal fish tanks. Clayton (1987), Clayton and Vaughan (1988) and Clayton and Wright (1989) stated that the proximate mechanisms of territorial behaviour in Boleophthalmus boddarti, an amphibious gobiid mudskipper that builds and maintains polygonal mud-walled territories provide a good example of the elastic disc concept of territories. Clayton and Wright (1989) stated that at high population densities, the amphibious and herbivorous

mudskipper Boleophthalmus boddarti construct mud walls around their territories as a means of reducing aggression between neighbours. The authors (ibid) stated there were no significant differences in diatom density between territorial and non-territorial areas or between grazed and non-grazed areas within territories. They concluded that the mud walls are considered to play a secondary, indirect role in maintaining populations of diatoms within territories. The mudflats are subject to intense bioturbation, and that for the Arabian Gulf area, mudskipper colonies are reported from the Bay of Kuwait. Each fish digs his own deep burrow where it hides from disturbances and during high tide (Höpner, 1999). Under certain conditions, the single fishes build polygonal territories of a size of about one meter, surrounded by dams, defended against rivals, and large enough to provide food (Microphytobenthos). This scenery is rare even in Kuwait Bay and difficult to be accessed; it is widespread on lower situated mudflats of the Khowre Musa area where it covers many km2. Even in the higher situated zone, mudskippers contribute decisively to the bioturbation power but do not form territories. Often, they form mixed colonies with digging crabs, e.g. Fiddler crabs (Ucaidae). In Kuwait, Clayton and Wells (1994) distinguished four main species of mudskippers around the mudflats of Kuwait Bay and the Northern coast of Kuwait. The authors stated that the mudskippers are also separated into zones. The mudskipper found on the high shore is called Periophthalmus, a carnivorous mudskipper up to 15 centimeters long which feeds on little crabs at low tide .Periophthalmus' eyes are well-adapted to vision in air, and when he's lying waiting for his prey only his eyes stick up out of the muddy water. In order to keep his eyes wet, Periophthalmus has little cups underneath the eyes and when he blinks, the eyes roll down into his skull and get remoistened by the water held in these little cups.

Slightly further down the shore the second species of mudskipper, called Boleophthalmus is the largest, measuring up to 25 cm and, like larger animals, is an herbivore. Boleophthalmus can be easily recognized by their feeding action, a side-to-side head movement that collects the fine surface film of diatoms and algae on which they feed. Even in the hot summer, this species of mudskipper can remain active for several minutes at a time out of water, and it can be recognized by its characteristic mode of locomotion. The pelvic fins have moved forward and fused together to form a little cup which he uses as a sort of crutch to balance on. His very strong and well-muscled pectoral fins have moved down the body and are, allowing him to swing along on them, using the life between the tides rear fin as a stabilizer. Boleophthalmus build and maintain these polygonal walled territories by carrying mouthfuls of mud from their burrows and depositing them on their walls. As far as is known, this population in Sulaibikhat Bay is unique throughout the Indo-Pacific area (Clayton and Wells, 1994). Boleophthalmus stays always within its mud walls, which enclose an area rather like a pasture that has enough food for each individual to survive. Inside each walled com- pound, Boleophthalmus digs a burrow up to one and a half metres deep. The burrow may be anywhere in the territory, maybe right beside the wall (Clayton and Wells, 1994).

The mud is very good for burrowing in, since the particles are very sticky, unlike sand. In all except the very softest ooze, burrows will last for quite a long time. Some burrows have a little chimney sticking up above the surface, others have small water-filled pools beside the openings, and the reason for these variations is unknown (Clayton and Wells, 1994). Boleophthalmus is very quick to dive into his burrow, and constantly alert to danger. If they suspect a predator is around, Boleophthalmus will merely raise his bulbous eyes above the surface of the mud to check out the situation, ducking down again at the slightest sign of trouble.

Clayton and Wells (1994) reported that all mudskippers are sexually monomorphic, which means that the males and females look exactly alike. However, during the breeding season, which extends from March until late August, the males go through an elaborate display that advertises their sex quite unmistakably. After hatching, the larvae swim off with the tide and spend some time simply floating around with the other types of plankton. Then they turn into very miniature mudskippers and return to the mudflats where they congregate in the soft mud areas. Here, as stated by Clayton and Wells (1994), they will remain until they reach maturity and manage to gain a territory of their own. Out of thousands of eggs, predators such as crabs will take the vast majority of larvae and only a handful will survive. The large xanthid crab, with its dark-tipped claws, feeds on mudskipper larvae. Clayton and Wells (1994) stated that the last two species of mudskippers found in Kuwait are both found much further out and so are much harder to observe. They are also much less amphibious than their inshore cousins and spend their time in permanently wet mud. The authors (ibid) stated that the larger of the two is called Scartelaos, and, although he is about as long as Boleophthalmus, is much thinner. Clayton and Wells (1994) reported that the final species of mudskipper is called Apocryptes and is much smaller than any of the others. Since both Scartelaos and Apocryptes inhabit areas of oozing wet mud, it is impossible for them to build burrows, so they hide from predators by simply squirming down into the soft mud out of sight.

Clayton and Snowden (2000) observed the surface activity of the carnivorous mudskipper Periophthalmus waltoni Koumans 1941 was on mudflat in Sulaibikhat

Bay, a muddy shore embayment in Kuwait Bay, at the north-western head of the Arabian Gulf. They reported that Each adult fish had a home range of between 2-3 m2 in which were located the main and subsidiary burrows, the main one simply being the most frequently used. The burrows were of two types such that the entrance was either a double- turreted one (the 'Y' shaped burrow) or was in a water-filled, saucer-like depression. While some fish had a single burrow system of either type, oth- utilise between two and six different ones. During the period of observation no other P. waltoni were seen to use them.

Mhaisen and Al-Maliki (1996) stated that the mudskipper Periophthalmus waltoni (Perciformes: Gobiidae) in the Khor Al-Zubair estuary (Iraq) are infected with Myxobolus pfeifferi (Sporozoa), Diplozoon sp. (Monogenea) and Neoechinorhynchus sp. (Acanthocephala). The State of Kuwait occupies approximately 17,800 km^2 of the Northwestern part of the Arabian Gulf, between 28º30' and 30º05'N, and 46º33' and 48º30'E. It comprises the mainland and nine offshore islands. Kuwait's climate is characterized by hot summers and mild winters. Temperature extremes are high, with means during the warmest and coolest months ranging between 46.2ºC and 6.9ºC. Winter brings occasional frost. Rainfall is minimal, not exceeding 115 mm/year, but evaporation is very high, averaging 14.1 mm/d. The relative humidity is low, and strong, dry and hot, Northwesterly winds prevail during summer, particularly in June and July (Al Nafisi et al., 2009).

"Kuwait bay" is considered as one of the characterized features of the Kuwaiti marine environment, which is an elliptically shaped bay that protrudes from the Arabian Gulf in Westward direction at its Northwestern corner. Kuwait Bay is of a moderate size (850 km²) with an average water depth of 5 m and a maximum depth of 20 m at the entrance (Al-Ghadban, 2004). The Northern shoreline is a pristine shore and in contrast, the Southern part the bay hosts urban activities, major ports such as Shuwaikh and Doha Ports and three major power and desalination plants (Doha East, Doha West and Subiya). The Bay of Kuwait presents a unique ecosystem and a significant nursery ground for many fishes and shrimp species (Al-Yamani et al., 2004). Although biological and ecological data on the marine biota of the region is limited, with some coastal areas receiving more attention than others at least four critical marine habitats, coral reefs, intertidal marshes, mangrove and sea grass beds, and kept forest, have been recognized in the region (Price et. al., 1993).

In recent years, increased developmental activities and misuse of native vegetation have greatly degraded the coastal environment and marine ecosystems. In addition, harsh weather conditions have accelerated the disappearance of vegetation cover in both inland and coastal areas. A forestation of intertidal zones with mangrove plants is considered a viable option to improve coastal environment and enrich marine biodiversity. Mangrove plantations also protect the coastline from strong currents and support the accumulation of sediments and organic matter in the intertidal zones. These changes would improve the quality of mudflats and promote the survival and growth of marine fauna (Sbandar et al., 20; Emabi, 1993; Ogino, 1993; Al Nafisi et a., 2009). The coastal zone of Kuwait, depending on the sediment nature and morphology, is classified into two main parts: Northern muddy province and Southern rocky/sible for them to build burrows, so they hide from predators by simply squirming down into the soft mud out of sight.

Clayton and Snowden (2000) observed the surface activity of the carnivorous mudskipper Periophthalmus waltoni Koumans 1941 was on mudflatal and growth of marine fauna (Sbandar et al., 20; Emabi, 1993; Ogino, 1993; Al Nafisi et a., 2009). The coastal zone of Kuwait, depending on the sediment nature and morphology, is classified into two main parts: Northern muddy province and Southern rocky/sandy part. These parts are subdivided into several zones (Abou-Seida and Al-Sarawi, 1990). The bay supports a thriving fishing industry and contains a site of an aquaculture facility of 80 net pen cages with a production of roughly 500 – 600 metric tons ole and normal values for this region of the world. Kuwait Bay is an exposed area and stressed as a result of the extensive man-made activities, such as dredging, indiscriminate solid and liquid waste disposal and over fishing. Al-Bakri et al., (1985) have concluded that the alteration of the coastal zone has resulted in more impact to the ecosystem compared to the harsh environmental condition. The environmental condition of the coastal area became more critical as a result of the war-related activities (Al-Ghadban et al., 1992).

El-Sammak et al., (2005) compared the levels of heavy metals in bottom sediments of Kuwait Bay and Sulaibikhat embayment with the reported values of Dubai, Greek and Canada and highlighted higher values of cadmium and nickel. Al-Majed et al. (2004) identified certain concentration of methyl mercury in the embayment and attributed such concentration due to the power plant and also due to the pervious industrial outfall and also due to the shipping activities and the discharged into the area from the emergency outlets. Al-Ghadban et al. (1994) reported higher values of total organic carbon content (more than 3%) in the area. Coastal pollution has been increasing significantly over the recent years and found expanding environmental problems in many developing countries. The discharges of industrial wastes have resulted in high metal concentrations in the local marine environment, especially in the coastal sediments (Saad et al., 1981; Mance, 1987; Ni et al., 2005). Al-Sarawi et al. (2002)

reported high levels of metal discharges from power, thermal, desalination and water treatment plants and leakage from oil wells in Kuwait marine environment Glibert et al. (2004) stated that the bay supports a thriving fishing industry and contains a site of an aquaculture facility of 80 net pen cages with a production of roughly 500 – 600 metric tons of sea bream (Sparus auratus L.) per year. Mudskipper, Periophthalmus waltoni is distributed in the northern region of Kuwait Bay's tidal mudflats. They are the prey for many predators and thus, it is essential to evaluate the bioaccumulation of metals toxicants in this fish. The gills, skin and food are the main routes of accumulation of metals by fish (Hein et al., 1993; Ni et al., 2005). The objectives of the present study were to study the ecological behavior of the mudskippers and to determine the relationships between the gobies fish behavior and the Kuwait Bay's mudflats habitat.

2. Materials and methods

The studied Mudskippers in the present study are monitored along the Kuwait Bay shores (Fig. 1) during the very hot summer season, near delivery hospital, Shuwaikh area, Kuwait Governorate. They are particularly abundant in the muddy shore. However, a number of fifty mudskipper samples are collected randomly to be examined for parasites.

Field observations:

The procedure followed for monitoring the mud skippers is described by Clayton and Wells (1994), that comprises using a sheet of plywood or a big stone to help to distribute our weights more evenly across the mud, as a surface crust of dried mud may seem quite hard, but if it cracks one, and one will sink right down into the soft ooze below. But if one chooses the right type of shore he should be able to observe the mudskippers from the safety of dry land. Fifty specimens of the amphibious gobies (2.17œ5.20 cm standard length (SL); 0.16œ2.70 g body wet weight) were collected using hand-nets during low tides in Kuwait Bay. Species are abundant and successfully surviving in the mud flat pool during low tides. The caught specimens were placed in small plastic containers, and kept alive in aerated sea-water prior to further studies in the College laboratory. Taxonomic identification and confirmation of the species were based on description given in Clayton and Wells (1994). Amphibious goby was identified as Periophthalmus sp.

Laboratory observations:

Collected specimens were examined for infestation with external parasites, and then dissected for detecting internal parasites in the body cavity and all other organs.

Results and Discussion

The Mudskippers observed in the present study appear to be quite active when they are out of water, feeding and interacting with each other (Fig. 2). The results of the present work are presented in the following items:

1- Description and habitat

In the present study, the mudskipper that are found in and around the mudflats of Kuwait Bay, are amphibious fishes that are called mudskippers. The results agree with those of Clayton and Wells (1994), who are separated the mudskippers into zones. The high shore mudskippers are called *Periophthalmus*, which are carnivorous mudskippers up to 15 centimeters long and feeds on little crabs at low tide. The genus *Periophthalmus* is by far the most diverse and widespread genus of mudskippers that contains eighteen described species (Larson and Takita, 2004).

They are uniquely adapted to intertidal habitats, unlike most fish in such habitats, which survive the retreat of the tide by hiding in tidal pools. Mudskippers need to live in habitats that are hot and humid in order to breathe, where air and water temperature range from 75 to 86 °F and humidity from 60 to 80 %. Mudskippers are only active when the temperature is above 55 °F. This result agrees with the results of Clayton and Well (1994).

Other adaptation of mudskippers to life on land is their huge goggly eyes at the top of their heads for an all-round view, while their mouth faces downwards to feed on the mud surface. These eyes sit on stalks while the rest of their bodies remain safely underwater. Unlike other fishes, mudskippers prefer to swim with their heads above water, their eyes giving them a good 360-degree view. To keep their eyes moist when they are on land, the eyes can be retracted to dip them into water that collects at the bottom of the eye socket. Mudskippers are probably the only fish with movable eyelids (Fig. 3). The pectoral fins of the mudskippers are used like crutches to crawl over mud.

Fig. 1. Kuwait Bay shoreline and mudflat, State of Ku[illegible]K

Figs. 2A &2B. The observed Mudskippers appear to be quite active when they are out of water.

Fig. 4. Walled territories.

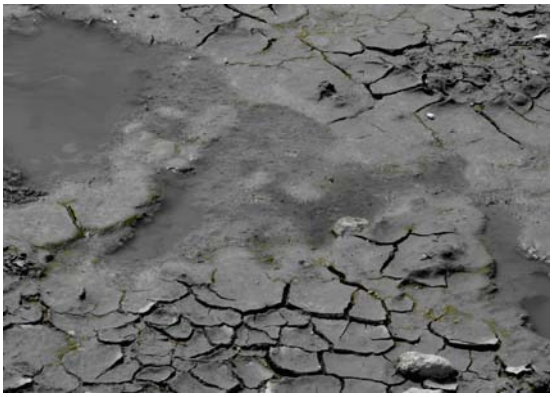

Fig. 3. Mudskippers are probably the only fish with huge eyes for an all-over view.

2- Territorial behaviour

Mudskippers are noticed to dig deep burrows to escape predators and raise their young. They maintain an air pocket in their burrows to breathe. Chen *et al.* (2007) reported that the mudskipper, *Boleophthalmus pectinirostris*, maintained territories in farming ponds during the cold season between November and February. Two types of territory are found, one is surrounded by mud-walls, and the other is without mud-walls. Both types of territory are entirely covered by shallow water (Fig. 4).

The area of walled territories was significantly bigger than that of non-walled ones. The nearest neighbour's distance of walled territories was significantly shorter than that of non-walled territories. This description is detected in the present study.

Fish surface activity occurred between 1200 and 1500 hours at which time the territorial water temperature reached a maximum, being significantly higher than that of the air, mud surface or deep burrow. The territorial sediments exhibited a significantly greater benthic micro- algal biomass as compared to the non-territorial sediments. The present study indicates that *B. pectinirostris* forms a territory a pool of water that encourages diatom growth and enables the fish to engage in surface activity (Fig. 4).

Clayton (1987), Clayton and Vaughan (1988) and Clayton and Wright (1989) stated that the proximate mechanisms of territorial behaviour in *Boleophthalmus boddarti*, an amphibious gobiid mudskipper that builds and maintains polygonal mud-walled territories provides a good example of the elastic disc concept of territories. The occurrence of wall building is density dependent and a contiguous mosaic of territories is only produced at high fish densities. Wall removal and replacement experiments show that the mud-wall acts as a visual barrier and reduces aggression between neighboring territorial fish.

Clayton and Wright (1989) stated that at high population densities, the amphibious and herbivorous mudskipper *Boleophthalmus boddarti* construct mud walls around their territories as a means of reducing aggression between neighbours. Because of the walls, territories contain pools of water and exposed mud slopes. Whilst the density of benthic diatom prey was highly variable, the highest was found on the exposed mud slopes and the lowest on the boundary walls. Fish grazed mainly on the mud slopes. No significant differences were found in diatom density between territorial and non-territorial areas or between grazed and non-grazed areas within territories. The variation of the diatom density, however, was reduced within territories. The mud walls play an indirect role in maintaining populations of diatoms within territories.

Höpner (1999) reported that the mudflats are subject to intense bioturbation, and that for the

Arabian Gulf area, mudskipper colonies are reported from the Bay of Kuwait. Each fish digs his own deep burrow where it hides from disturbances and during high tide. The author (ibid) stated that under certain conditions, the single fishes (most probably *Boleophthalmus boddarti*) build polygonal territories of a size of about one meter, surrounded by dams, defended against rivals, and large enough to provide food(microphytobenthos). Clayton and Wells (1994) stated that while this scenery is rare even in Kuwait Bay and difficult to be accessed, it is widespread on lower situated mudflats of the Khowr-e Musa area where it covers many km2. Even in the higher situated zone, mudskippers contribute decisively to the bioturbation power but do not form territories. Often, they form mixed colonies with digging crabs (Ucaidae). This result agrees with the results of the present study.

Boleophthalmus build and maintain these polygonal walled territories by carrying mouthfuls of mud from their burrows and depositing them on their walls. As far as is known, this population in Sulaibikhat Bay is unique throughout the Indo-Pacific area (Clayton and Wells, 1994)!

Although they are found all over Kuwait Bay, the most interesting population of *Boleophthalmus* can be seen in Sulaibikhat Bay where they construct and maintain and elaborate and continuous network of polygonal walled territories (see Fig. 3 and 4). Most of these territories are five-sided with an average area of 1.69 square metres. The ones with only four sides tend to be smaller, while those with six or seven sides are a little bigger. These territories are unusual in that each is occupied by a single fish of either sex.

Boleophthalmus stays always within its mud walls which enclose an area that has enough food for each individual to survive. They build the walls with mud from their burrows which they carry up in their mouths and spit out onto the section in need of repair. If part of the wall is destroyed, they will work hard to repair it within a matter of hours during low tide. The best time to see these walled territories is at low tide when the sun is low in the sky. If there is a mild winter, some of the walls will last for more than a year .Inside each walled compound, *Boleophthalmus* digs a burrow up to one and a half metres deep. The burrow may be anywhere in the territory, maybe right beside the wall (Clayton and Wells, 1994).

Young *Boleophthalmus* don't have their own territories, due to lack of suitable space. Instead, they are forced to live in areas where the mud is too soft for either walls or burrows, burying themselves in soft ooze whenever a predator threatens. Obviously, this is not as safe as a nice deep burrow, so the young mudskippers are live between the tides constantly on the lookout for a vacant territory and constantly trespass both in order to feed and to investigate the chances of finding an empty lot.

Naturally, the owners do not take kindly to this and will chase the intruder out, into a neighboring territory, where upon the neighbours will take up the pursuit and a splendid chain reaction of indignant outrage will follow the poor trespasser all the way back to the soft muddy area where belongs.. The main factor in ensuring a constant supply of vacant territories is predation and this is especially prevalent during the spring when the weather is cold and the fish are rather sluggish as a result. Shore birds are their worst enemies.

Clayton and Wells (1994) stated that most of the fish found in the intertidal zone come there at high tide to feed. However, to see the fish, which have best adapted to the amphibious life between the tides we have to go to the third main coastal region of Kuwait: the mudflats. Even when their burrow is submerged, mudskippers are seen to maintain an air pocket inside it, which allows them to breathe in conditions of very low oxygen concentration. This result accommodates with the results of (Ishimatsu *et al*., 1998; Ishimatsu *et al.*, 2000; Lee *et al*., 2005).

Clayton and Wells (1994) observed mudskippers dig deep burrows to escape predators and raise their young and that they maintain an air pocket in their burrows to breathe. During the mating season, the male's colors become more intense, and they become much more active. They (ibid) stated that males leap and flip in the air and even stand on their tails, all to attract the females. The male becomes more aggressive forward other males with biting and raising its dorsal fins. If the female is attracted to the male, she does her own mating ritual with distinctive movements. She then goes into the male's burrow where she lays her eggs in a special part of the burrow the male builds her. The males then fertilize the eggs and takes over responsibility for the eggs. The above descriptions are noticed in the present study. These fishes present a range of peculiar behavioral and physiological adaptations to an amphibious lifestyle. He reported that these include: Anatomical and behavioral adaptations that allow them to move effectively on land as well as in the water (Harris, 1961). They can also flip their muscular body to catapult themselves up to 60 cm into the air. This observation is noticed in the present study (Piper, 2007).

Another important adaptation that helps the mudskippers to breath while they are out of water is their gill chambers, where they keep a bubble of air. These large gill chambers close tightly when the mudskipper is above the water, allowing the gills to be kept moist, and make them work. They supply oxygen for respiration also while the gobies are on the land (Graham, 1997).

As reported in the study of Tytler and Vaughan (1983), digging of deep burrows in soft sediments that allow the fish to thermoregulate , avoid marine

predators during the high tide when the fish and burrow are submerged (Sasekumar *et al.*, 1994) and for laying their eggs (Brillet,1969).

Tytler and Vaughan (1983) stated that in winter, mudskippers avoid low surface temperatures by remaining in their burrows, in the present study, no mudskippers were detected during winter months. In summer, body temperatures are kept lower than ambient by selecting areas where evaporative cooling is high. Body temperatures generally match those of wet mud, which can be 7°C lower than air shade temperatures.

Chen et al. (2007) stated that the mudskipper *Boleophthalmus pectinirostris* maintained territories in farming ponds during the cold season between November and February.

The present study results accommodate with the above observations.

Fig. 5. Periophthalmus' eyes are well-adapted to see in air and out of the muddy water.

Periophthalmus' eyes are well-adapted to see in air, and when they are lying in wait for their preys only their eyes stick up out of the muddy water (Fig. 5). In order to keep their eyes wet, *Periophthalmus* have little cups underneath their eyes and when they blinks, the eyes roll down into their skull and get remoistened by the water held in these little cups. Even in the heat of summer, this species of mudskipper can remain active for several minutes at a time out of water. They are seen periodically rolling on their sides to help to keep their skins wet.

Every five minutes or so, *Periophthalmus* will dash back to its burrow to cool off and refresh itself. This species of mudskipper can be recognized by its characteristic way of locomotion. It lifts his tail, and then arches his back. It has two sets of paired fins, pectorals near his chest, and pelvic fins at the hips. The pelvic fins have moved forward and fused together to form a little cup, which is used as a sort of crutch to balance on. This description agrees with the results of the present study.

Periophthalmus, was observed sitting on the mudflats splashing water over his body with his pectoral fins for about several minutes before moving back to his burrow. This agrees with the description of Clayton and Wells (1994). These burrows are quite remarkable; they are y -shaped, with two entrances and are more than a meter deep in order to get down to permanent water level. The mud is suitable habitat for burrowing in, since the particles are very sticky, unlike sand. In all except the very softest ooze, burrows will last for quite a long time (Clayton and Wells, 1994).

3- Feeding

Periophthalmus needs to be out of water to feed and it needs to keep his skin wet, it can absorbs oxygen through its skin as well as through its gills. Some times, it will fill its mouth with water to help him to survive for a longer time out side the water (Fig. 6), since the inside of its mouth has lots of tiny blood vessels that absorb oxygen. However, this water isn't 'essential; the mudskipper loses it anyway when it eats his prey (Clayton and Wells, 1994).

Ishimatsu *et al.*, (1998) reported that mudskipper fishes can maintain their metabolism while they are confined in mudflat burrows filled with oxygen-depleted water, and their eggs, deposited in the burrows, can develop under severely hypoxic conditions.

Their feeding action, a side-to-side head movement that collects the fine surface film of diatoms and algae on which they feed can easily recognize Boleophthalmus. Terns are another ménage to the mudskipper that they catch by flying along about three to five metres up, then dive-bombing their prey with their bayonet-like beaks. Sea gulls use a craftier approach, skimming very low, just above the mud walls and trying to catch the *Boleophthalmus* unawares.

But, *Boleophthalmus* like all the other mudskippers, is very quick to dive into his burrow, and constantly alert to danger. If they suspect a predator is around, *Boleophthalmus* will merely raise his bulbous eyes above the surface of the mud to check out the situation.

4- Infection of the mudskippers with parasites:

External examination and dissection of 50 samples of the mudskipper collected from the studied area indicates no parasitic infections.

Fig. 6. The mudskipper needs to be out of water to feed and keep his skin wet.

CONCLUSIONS

Mudskippers have been monitored in the intertidal zone along the Kuwait Bay muddy shores during the very hot summer season of the year 2009. However, a number of fifty random mudskipper samples are examined externally and internally for parasites. These examinations are to evaluate the different factors controlling the biodiversity in that area. The results indicate the abundance of the Mudskippers allover the muddy flat of the Bay and the total absence of either external and/or internal parasites in the mudskipper.

A forestation of intertidal zones with mangrove plantations is viable options to improve coastal environment and enrich marine biodiversity. These factors also protect the coastline from strong currents and support the accumulation of sediments and organic matter in the intertidal zones. These changes would improve the quality of mudflats and promote the survival and growth of intertidal zone fauna.

The results indicated, also, that mudskippers are free from parasitic infection. However, important results concerning the controlling the parasitic infection of mudskippers is needed.

The above findings deduce that the mudskippers caught from Kuwait bay tidal mud flats have the capability to live in the Kuwait Bay, although the studies reported the toxicity by metals and the trace metals in the habitat

RECOMMENDATION

Further environmental studies on the Bay area and its fauna are recommended to evaluate the probable reasons of absence of either external and/or internal parasites in the mudskipper. Also, toxicological investigations that use the gobies as bioindicator are recommended

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Genetic Variations Between Horse Breeds Using RAPD Markers

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Abstract: Genetic diversity is the basis for present day diversified living systems and future genetic improvement needs. Within the framework of breed conservation, genetic characterization is important in guarding breeds and is a prerequisite for managing genetic resources. The objective of the present study is to estimate the genetic diversity and phylogenetic relationships among Egyptian horse breeds (Native and Arabian) and an exotic breed (Thoroughbred) using Random Amplified Polymorphic DNA – Polymerase Chain Reaction (RAPD) technique. Initially, 25 primers were screened among all the breeds of which 14 primers amplified the genomic DNA. Four primers generated reproducible and distinct RAPD profiles and were used for further analysis. A total of 40 loci were amplified, of which 37 were found polymorphic (92.5%), useful for genetic variation study between breeds. The genetic diversity had the highest value (0.2048) in Arabian and the lowest value (0.0116) in Native breed. The genetic distance was found highest between Arabian and Thoroughbred (D=0.5442) and lowest between Native and Arabian (D=0.3280). However, genetic identity was highest (I=0.7204) between Native and Arabian and lowest (I=0.5803) between Arabian and Thoroughbred. UPGMA dendrogram based on Nei's genetic distance grouped the investigated horse breeds genotypes into two clusters. The first cluster includes Egyptian breeds (Native and Arabian) where as the second cluster include Thoroughbred which appeared to be most distant from the other breeds. In conclusion, these results indicated the effectiveness of RAPD in detecting polymorphism between horse populations and their applicability in population studies and establishing genetic relationships among the horse populations. [Nature and Science. 2010;8(5):90-99]. (ISSN: 1545-0740).

KeyWords: Genetic diversity, Horse breeds, RAPD-PCR.

1. INTRODUCTION

Horses have shared an intimate association with human civilization as in both historic and current societies they have fulfilled key agricultural, economic and cultural roles. Horses are members of the Equidae family. In Egypt, there are two horse breeds (Native and Arabian). The Egyptian Native horses are referred as Baladi horses. According to Mason (1996), it is a light riding animal of the Arab type found in Egypt. The herd book for the breed was formed in 1900. The Egyptian Arabian horse is one of the outstanding and most expensive horses in the world. It is also considered the most beautiful of its kinds that exist worldwide. It is an ancient breed which originated on the Arabian Peninsula (Bailey and Lear, 1994; Andrade, 1954).

Farm animal genetic diversity is required to meet current production needs in various environments, to allow sustained genetic improvement and to facilitate rapid adaptation to changing breeding objectives (Notter, 1999). Genetic diversity may be measured through genetic markers. These have been used to estimate the genetic diversity of species, breeds and populations, as well as decisions related to selection of breeds/ populations to be conserved (Zhang *et al*., 2006). However, breeders tend to concentrate on specific genotypes for determination of genetic diversity which combine traits of interest and may be used as progenitors in several breeding programmes in order to introduce agronomical important traits (Rahman *et al*., 2006). In an attempt to solve the problem of maintaining pure breeds using the observed morphological characteristics that require a lot of time and effort, the use of molecular markers in maintaining horse breeds is more suitable and less time consuming.

Many researchers employed the Random amplified polymorphic DNA markers or RAPD technique to characterize and estimate genetic distances between breeds (Williams *et al*., 1990; Welsh and McClelland, 1990), in the study of genetic diversity within breeds (Apostolidis *et al*., 2001; El-Seoudy *et al*., 2005; Eroglu and Arica, 2009) and in determination of gene mapping in farm animals (genetic linkage maps are now available for horse, Shiue *et al*.,1999).The RAPD technology has provided a quick and efficient screen for DNA sequence-based polymorphisms at a very large number of loci. The major advantage is that no prior DNA sequence information is required. The vast range of potential primers that can be used gives the technique great diagnostic power. Reproducible RAPD bands can be found by a careful selection of primers, optimization of PCR conditions for the target species and replication

to ensure that only the reproducible bands are scored (Rahman *et al*., 2006).

RAPD markers have been used successfully in estimating genetic relatedness among various populations of sheep, cattle , goat, buffalo and chicken (Mahfouz *et al*., 2008; Hassan *et al*., 2007; Rahman *et al*., 2006; Abdel-Rahman and Hafez ,2007 ; Okumus and Kaya, 2005, respectively). Moreover, the effectiveness of RAPD in detecting polymorphism between horse populations and their applicability in population studies and establishing genetic relationships among horse populations has been reported by Bailey and Lear (1994), Shiue *et al*. (1999), Apostolidis (2001) and Egito *et al*. (2007).

The objective of this study was to use the RAPD technique to evaluate genetic diversity and relatedness within and among three horse breeds, Native, Arabian (Egyptian horses) and Thoroughbred (exotic breed). To our knowledge there is currently no information about RAPD genetic markers that detect genetic polymorphism in Egyptian horse breeds and its relation to other horse breed. Information from this work provides basic genetic knowledge that is critical for conservation and breeding programs.

2. MATERIALS AND METHODS

Sample Collection

Blood samples of about 10 ML were collected from 30 horse animals belonging to three different breeds, Egyptian breeds (Native and Arabian) and exotic breed (Thoroughbred). They were collected from the faculty of veterinary - Cairo University, El Zahra Station and El -Gizera club, respectively.

Genomic DNA extraction

High quality genomic DNA was extracted from blood according to the established protocol of Miller *et al*. (1988). Briefly, Buffy coats of nucleated cells obtained from anticoagulated blood (EDTA) were resuspended in 15 ml polypropylene centrifugation tubes with 3 ml of nuclei lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM Na_2EDTA). The cell lysates were digested overnight at 37°C with 0.2 ml of 10% SDS (sodium dodecyl sulphate) and 0.5 ml of a protease K solution (1 mg protease K in 1% SDSand and 2 mM Na_2EDTA). Nucleic acid was extracted by addition of 1 ml saturated NaCl and shake vigorously for 15 sec, centrifuge at 2500 rpm for 15 min. The supernatant was transferred to another tube and 2 volumes of ice cold absolute ethanol were added and gently invert several times (until DNA precipitates). Precipitated DNA strands were removed with a plastic spatula or pipette and place in a 1.5 ml microfuge tube that contains 100-200µl TE buffer (10mM Tris-HCl, 1mM Na_2EDTA).

The quality of isolated genomic DNA was checked by agarose gel electrophoresis. For this purpose, well dissolved DNA samples were resolved on 0.8% agarose (w/v) gel. The concentration of the DNA and its purity were determined by spectrophotometry based on the absorbance at 260 and 280 nm, respectively. The DNA samples were adjusted to a final concentration of 50 ng\ul.

RAPD-PCR analysis

A set of 25 decamer oligonucleotide primers (Operon Technologies Inc., Alameda, Calif.: A, B and C) (Table 1) were screened using unique samples of DNA from distinct animals and breeds. Primers were designated as useful if they yielded well-amplified, distinguishable polymorphic bands. Finally four primers (OPA07, OPA09, OPA11, and OPA18) were selected and used to amplify DNA from all individuals. RAPD-PCR reactions were carried out as described by William *et al*. (1990). The total reaction volume of 50 ul contained100 ng genomic DNA, 200 µM (each) dNTPs, 2 µM of a random primer , 3.5 mM $Mgcl_2$, 0.75Uof DyNAcymeII DNA polymerase (finnezymes Oy) and 5 µl 10X DNA polymerase buffer. The PCR reactions were carried out in DNA thermocycler (Perkin-Elmer 9700) programmed with a first denaturation of 5 min at 94°C, followed by 45 cycles of 1 min denaturation at 95°C, 1 min annealing at 36°C and 2 min extension at 72°C. Final extension at 72°C for 5 min was allowed before holding the reaction at 4°C for 10 min. Reaction products were stored at 4°C prior to electrophoresis. A volume of 10 µl of each sample was mixed with 6× gel loading buffer (2 µL) (analytical grade water containing 25% ficoll, 0.25% bromophenol blue and 0.25% xylene cyanol) and used for electrophoresis on 1.2% agarose gel run at a constant voltage of 10V/cm. RAPD patterns were visualised and documented using the Gel Documentation system, Gel-Pro Analyzer (Media Cybernetics). A Φx174 DNA digested with HaeIII were used as known molecular size DNA markers.

Recording of data and Statistical analysis

The RAPD patterns were scored for the presence and absence of amplicons. In a binary matrix the presence of a band was recorded as one and the absence as zero. The scores obtained using all primers in the RAPD analysis were then combined to create a single data matrix. This was used for estimating polymorphic loci, Nei's (1973) gene diversity (h), allele frequencies, genetic distance (D) and genetic identity (I). All calculations were carried out using the population genetic analysis software, PopGene version 1.31 (Yeh *et al*., 1999). Dendrogram was constructed using Unweighted Pair Group Method using Arithmetic Averages (UPGMA) based on Nei's (1972) genetic distances.

Table 1. Sequence, operon codes and GC content of random primers used to study variation in horse species.

Primers	Sequence (5'- 3')	GC%	Primers	Sequence (5'- 3')	GC%
OPA02	TGC CGA GCTG	70	OPB18	CCA CAG CAGT	60
OPA06	GGT CCC TGAC	70	OPB20	GGA CCC TTAC	60
OPA07	GAA ACG GGTG	60	OPC02	GTG AGG CGTC	70
OPA08	GTG ACG TAGG	60	OPC03	GGG GGT CTTT	60
OPA09	GGG TAA CGCC	70	OPC05	GAT GAC CGCC	70
OPA10	GTG ATC GCAG	60	OPC06	GAA CGG ACTC	60
OPA11	CAA TCG CCGT	60	OPC07	GTC CCG ACGA	70
OPA12	TCG GCG ATAG	60	OPC08	TGG ACC GGTG	70
OPA15	TTC CGA ACCC	60	OPC09	CTC ACC GTC C	70
OPA17	GAC CGC TTGT	60	OPC12	TGT CAT CCCC	60
OPA18	AGG TGA CCGT	60	OPC16	CAC ACT CCAG	60
OPB13	TTC CCC CGCT	70	OPC20	ACT TCG CCAC	60
OPB15	GGA GGG TGTT	60			

3. RESULTS AND DISCUSSION

The ability to detect polymorphisms at the DNA level has led to new approaches for the genetic analysis of livestock species. The RAPD assay has the potential to make useful contributions to genetic analysis of livestock, especially in terms of relatedness among either breeds or species (Cushwa and Medrano, 1996). A major application has been to compare the genomes of closely related species in order to determine the extent of genetic divergence (Bowditch *et al*., 1993).

As a result of an initial RAPD analysis of pooled DNA, a total of 25 random primers (decamers) were screened, out of which 6 (OPA02, OPA12, OPA17, OPB13, OPB15 and OPC16) yielded monomorphic bands (24%), and 8 (OPA07, OPA09, OPA11, OPA18, OPB18, OPB20, OPC02 and OPC09) (32%) yielded polymorphic bands. Primers were selected based on number of polymorphic bands and amplification quality. Out of the 8 primers that produced polymorphic bands only 4 primers generated reproducible and distinct RAPD profiles and were retained to investigate polymorphism within and between individuals of all the studied horse breeds (Native, Arabian and Thoroughbred). An overall of 40 loci were amplified, 37 of them were polymorphic (92.5%). In another study, Egito *et al*. (2007) reported that 13 out of 146 primers generated 44 polymorphic bands between different breeds of horses (Pantaneiro, Arabian and Thoroughbred). In the present study, the number of polymorphic bands per primer ranged from 2 to 15 with an average 9.25 bands/primer. The molecular size of the amplicons was in the range of 76 to 2358 bp. Bailey and Lear (1994) studying Arabian and Thoroughbred breeds

found an average of 3.6 polymorphic bands/primer in a RAPD assay. Martins (1996) studying three Brazilian breeds (Lavradeiro, Crioulo and Campolina) found 2.9 bands/primer, using 29 markers. Apostolidis *et al*. (2001) found 10.2 bands/primer and 51 polymorphic bands in Greek horses (Thessalian, Skyros Pony, Pinia, Cretan and Andravida). Egito *et al*. (2007) studying the genetic variability of Pantaneiro, Arabian and Thoroughbred horses using RAPD-PCR markers found 3.38 bands/primer. Thus, the proportion of primers capable of detecting polymorphism among the breeds evaluated depends upon the genetic background of the breeds, genetic distance between the breeds and complexity of the genome (Ahlawat *et al*., 2004). The present study also revealed that primer OPA07 produced maximum number of bands (16), while minimum number of bands (4) was recorded in primer OPA18 in all the breeds (Table 2). It has been suggested that the sequence of primer OPA07 may occur frequently in all breeds and scored the maximum number of polymorphic bands whereas primer OPA18 was found less polymorphic within and between breeds. Sharma *et al*. (2001) found that RAPD technique detects sufficient polymorphism within and between breeds.

The present and earlier studies (Wei *et al*., 1994; Bailey and Lear, 1994; Smith *et al*., 1996; Egito *et al*., 2007) indicate that RAPD analysis requires screening of a large number of random primers in order to detect polymorphism, because the amplification from the arbitrary primers depends on the presence or absence of the corresponding primer binding sites in the genome. Hence comparatively large numbers of random primers are required to detect sufficient polymorphism to be utilized for genetic analysis. Figures 1, 2, 3 and 4 show the amplification pattern of genomic DNA of different breeds with various random decamer primers. The RAPD profile generated from these primers was utilized to estimate genetic diversity and relatedness within and among horse breeds based on the band frequency. Table (3) show the estimated allele frequencies at RAPD-encoding loci varied among breeds. The number and proportion of polymorphic bands and the values of genetic diversity in different horse breeds are shown in Table (4). The percentage of the polymorphic loci ranged from 52.5% in Arabian horses to 5 % in Native horses. According to Nei (1987) the proportion of polymorphic loci is not a good measure of genetic variation. A more appropriate measure of genetic variation is average heterozygosity or gene diversity. Higher heterozygosity values indicate broader genetic diversity. Accordingly, the estimated genetic diversity had the highest value (0.2048) in Arabian and the lowest value (0.0116) in Native. Also, Shannon's diversity (Lewontin , 1972) was calculated to provide a relative estimate of the degree of variation within each breed. The Shannon index ranged from 0.0196 (Native) to 0.3011 (Arabian).The greatest index of genetic diversity was observed when the three breeds were considered as one (0.3026). These results are inconsistent with the findings of Egito *et al*. (2007), who found that the genetic diversity in Arabian breed was (0.2814). In addition they recorded greatest diversity index when the five populations of Pantaneiro horses were considered as one, but when considered separately each Pantaneiro population showed lower diversity index than that of the breed.

In the present study, the Egyptian Native breed had low genetic diversity or variation among its individuals indicating low heterozygosity. Similar results were obtained in another Egyptian Native breeds, Ali (2003) showed closer proximity in Egyptian Native sheep breeds Barki to Rahmani and Baladi (95.7 and 91.3%, respectively) that was detected by random amplified polymorphic DNA markers. Also, Abdel-Rahman and Hafez (2007) found a high genetic similarity among three Egyptian water buffalo flocks using RAPD-PCR technique. Our results also indicated that the Thoroughbred had low genic diversity index (0.0391) compared to the Arabian breed. Egito *et al*. (2007) reported that Thoroughbred has the lowest genic diversity index compared with Arabian and Pantaneiro horses. Also, Ouragh (2005) was studying the DNA polymorphism in Thoroughbred, Arabian and Anglo-Arabian horses in Morocco using microsatellite marker analysis. They found that heterozygosity value was lower in Thoroughbred (0.7036), but relatively high in Arabian and Anglo-Arab (0.7216 and 0.7232, respectively). Guérin *et al*. (1993) Observed that the lowest polymorphism information content value is found in the Thoroughbred, which confirmed the well known homogeneity of this breed (Ellegren *et al*., 1992), conversely Arab horse is the most heterogeneous of the all studied breeds (Thoroughbred, Sell Français, Trotteur Français). Moreover, in the study of Bowling and Clark (1985) in Thoroughbred, Arabian, Standardbred, Morgan, Quarter Horse, *Paso Fino* and *Peruvian Paso*, they observed a lower biochemical marker variation in Thoroughbred and concluded that this was due to a Stud Book that has been closed for more than 200 years, as well as intense selection for a single trait: speed in flat races.

The genetic distance (D) and genetic identity (I) among the three horse breeds are shown in Table (5). The genetic identity between the populations from the amplified patterns of four random primers was (I=0.7204) between Native and Arabian, (I=0.6460) between Thoroughbred and Native, and (I=0.5803) between Arabian and Thoroughbred. The genetic distance expresses the degree of divergence between populations. Higher values were found for the pairs formed between Arabian and Thoroughbred (D=0.5442) and the lower value found for distances between Native

Table 2. Total number of bands, polymorphic bands, % of polymorphic loci and their size ranges from the random primers.

Primer	Total No. of bands	No. of polymorphic bands	% of polymorphic loci	Size range (bp)	
				Max.	Min.
OPA07	16	15	93.75	2358	76
OPA09	9	9	100	1227	194
OPA11	11	11	100	1416	310
OPA18	4	2	50	1050	280

and Arabian (D=0.3280). The UPGMA dendrogram, based on genetic distance, was constructed to show phylogenetic relationships among the horse breeds Figure (5). The Thoroughbred appeared to be most distant from the other breeds whereas the Native and Arabian breeds were closely related with the highest genetic similarity. The close identity between the Native and Arabian breeds suggests that all the indigenous breeds are more or less comparatively similar and branched from the same line of evolutionary tree. In a study of genetic variability by microsatellites and protein polymorphisms alleles, Kelly *et al*. (2002) found that American horse breeds together with Barb and Arabian horses clearly formed a separate cluster from the Spanish pure-bred and Thoroughbred breeds, as shown by an UPGMA dendrogram based on Nei's standard genetic distance.

In conclusion, the present study indicated the effectiveness of RAPD markers in detecting the polymorphism and estimating the genetic relationship within the horse breeds. Though the Egyptian horse breeds showed the least genetic distance with each other while Thoroughbred appeared to be most distant from the Egyptian breeds.

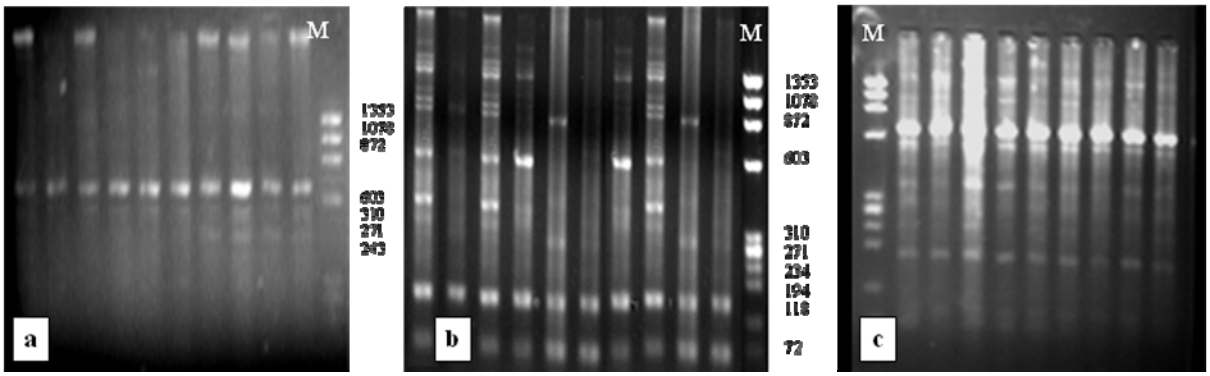

Figure1. Random amplified polymorphic DNA (RAPD) profile generated by primer OPA07 in individual horses of different breeds, a: Native, b: Arabian, c: Thoroughbred, Lane M = molecular marker (Φx174 DNA HaeIII digest).

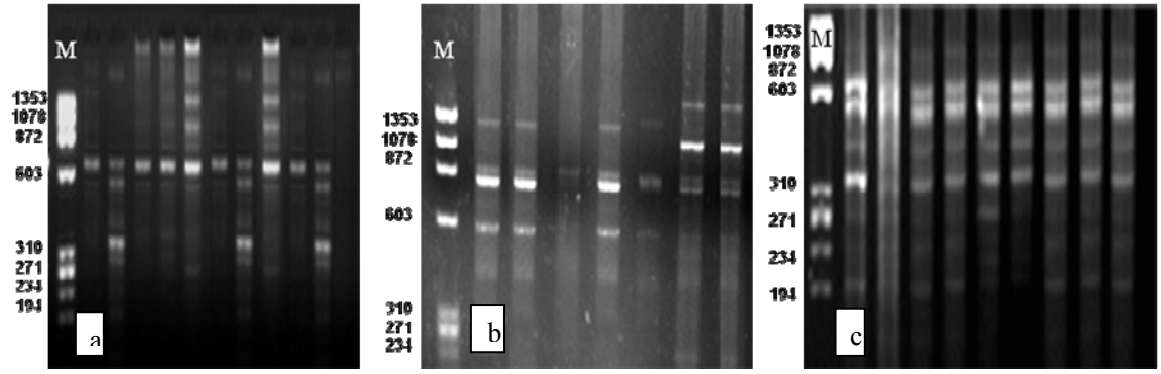

Figure2. Random amplified polymorphic DNA (RAPD) profile generated by primer OPA09 in individual horses of different breeds, a: Native, b: Arabian, c: Thoroughbred, Lane M = molecular marker (Φx174 DNA HaeIII digest).

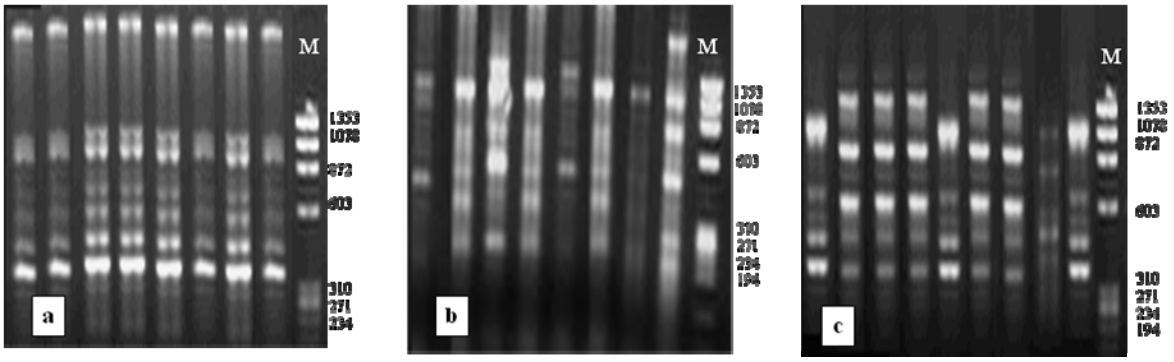

Figure 3. Random amplified polymorphic DNA (RAPD) profile generated by primer OPA11 in individual horses of different breeds, a: Native, b: Arabian, c: Thoroughbred, Lane M = molecular marker (Φx174 DNA HaeIII digest).

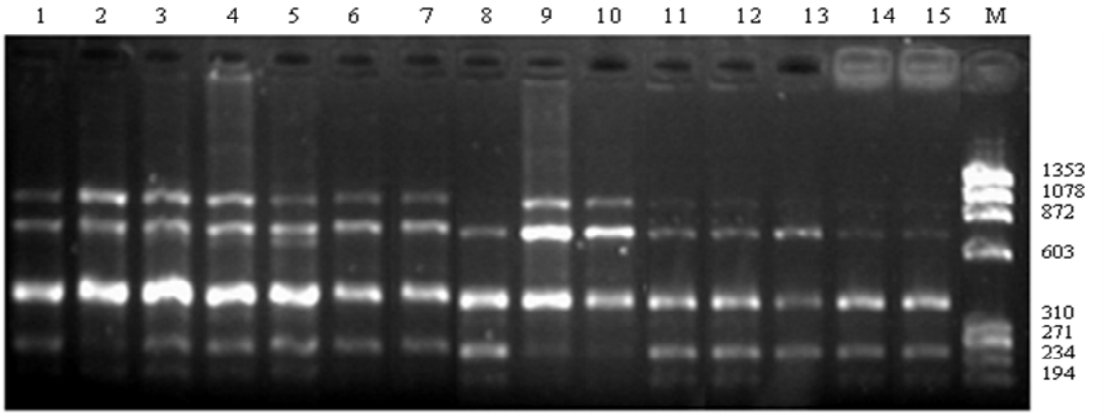

Figure 4. Random amplified polymorphic DNA (RAPD) profile generated by primer OPA18 in individual horses of different breeds, Lane 1-5: Arabian, Lane 6-10: Thoroughbred, Lane 11-15: Native, Lane M = molecular marker (Φx174 DNA HaeIII digest).

Table 3. Estimation of gene frequency (Allele \ Locus) of the 37 polymorphic RAPD markers scored in three breeds, each marker is assigned by the name of the primer and its approximate size in base pairs.

RAPD markers	Native		Arabian		Thoroughbred	
	Allele0	Allele1	Allele0	Allele 1	Allele 0	Allele 1
$OPA07_{2358}$	1	0	0.5477	0.4533	1	0
$OPA07_{1879}$	1	0	0.7746	0.2254	1	0
$OPA07_{1459}$	1	0	0.6325	0.3675	1	0
$OPA07_{1353}$	1	0	1	0	0	1
$OPA07_{1134}$	1	0	0.7746	0.2254	1	0
$OPA07_{1046}$	1	0	0.8367	0.1633	1	0
$OPA07_{955}$	1	0	0.8367	0.1633	1	0
$OPA07_{771}$	1	0	0.7746	0.2254	1	0
$OPA07_{542}$	1	0	0.7071	0.2929	1	0
$OPA07_{438}$	1	0	0.3162	0.6836	1	0
$OPA07_{390}$	1	0	1	0	0	1
$OPA07_{303}$	1	0	0.8367	0.1633	1	0
$OPA07_{219}$	1	0	1	0	0	1
$OPA07_{171}$	1	0	0	1	1	0
$OPA07_{76}$	1	0	0	1	1	0
$OPA09_{1227}$	0.8944	0.1056	0.5477	0.4523	1	0
$OPA09_{1060}$	1	0	0.8367	0.1633	0.7071	0.2929
$OPA09_{825}$	1	0	0	1	1	0
$OPA09_{766}$	1	0	0.4472	0.5528	1	0
$OPA09_{740}$	0	1	1	0	0	1
$OPA09_{569}$	1	0	0.5477	0.4523	0	1
$OPA09_{369}$	1	0	1	0	0	1
$OPA09_{348}$	0.8367	0.1633	1	0	1	0
$OPA09_{194}$	1	0	1	0	0	1
$OPA11_{1416}$	1	0	1	0	0.8944	0.1056
$OPA11_{1076}$	0	1	0	1	1	0
$OPA11_{936}$	0	1	1	0	1	0
$OPA11_{903}$	1	0	1	0	0.8944	0.1056
$OPA11_{785}$	1	0	0.5477	0.4523	1	0
$OPA11_{667}$	0.8944	0.1056	0.8944	0.1056	1	0
$OPA11_{569}$	0	1	0.8944	0.1056	1	0
$OPA11_{465}$	0	1	0.3162	0.6838	0	1
$OPA11_{387}$	1	0	1	0	0	1
$OPA11_{369}$	0	1	1	0	1	0
$OPA11_{310}$	0	1	0.5477	0.4523	1	0
$OPA18_{1050}$	1	0	0	1	0.4472	0.5528
$OPA18_{280}$	0	1	0.4472	0.5528	0.6325	0.3675

Table 4. Genetic diversity in investigated horse breeds based on RAPD markers.

Horse breeds	No. of polymorphic loci	% of polymorphic loci	Genetic diversity Nei's (1973)	Shannon's diversity (Lewontin 1972)
Native	2	5	0.0116	0.0196
Arabian	21	52.5	0.2048	0.3011
Thoroughbred	4	10	0.0391	0.0572
All	37	92.5	0.3026	0.4557

Table 5. Genetic identity (above diagonal) and genetic distance (below diagonal) between the investigated horse breeds (Nei's 1972).

Breeds	Native	Arabian	Thoroughbred
Native	****	0.7204	0.6460
Arabian	0.3280	****	0.5803
Thoroughbred	0.4370	0.5442	****

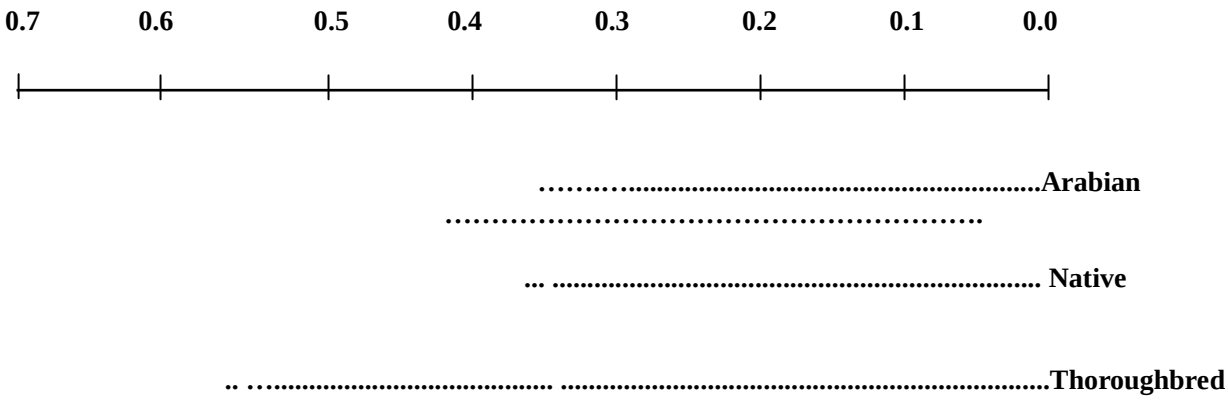

Figure 5. UPGMA dendrogram of three horse breeds, based on Nei's (1972) genetic distance.

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The Role of Hyperthermia in Potentiation of Chemotherapy and Radiotherapy in Mice Bearing Solid Tumor

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Abstract: Hyperthermia is procedure in which body tissue is exposed to a high temperature up to 41°C and is an effective tool in cancer treatment. Hyperthermia also is a therapy applied together with other modalities in the treatment of cancer. The aim of this study was to determine if there was a change in immunological and biochemical parameters after using each of hyperthermia, radiotherapy or chemotherapy separately and the combined treatments in mice bearing solid tumor. Seventy females Albino mice weighing (20-25g) were used in the current study. The animals were divided into five groups. Group I: served as a control animals. Group II: animals were cancered by solid tumor and were untreated. Group III: animals exposed to WBH alone. Group IV: animals administered doxorubicin (Dox) 3mg/kg body weight i.p. once a week. Group V: animals were exposed to fractionated whole body gamma rays (WB-γ) at a dose level of 0.5 Gy once a week. Group VI: animals were exposed to WBH and administered doxorubicin (Dox) 3mg/kg body weight (i.p.) once a week. Group VII: animals were exposed to WBH then fractionated whole body gamma rays (WB-γ) at a dose level of 0.5 Gy once a week. After four weeks (the end of treatments), blood samples were collected from orbital venous plexus in heparinized tubes from all animal groups. The results of the present study indicated that WBH with or without radio- and chemotherapy induced significant increase in TNF-α, IL-2 and HSP70 values as compared to cancered group. As well as WBH with or without radio- and chemotherapy induce significant increases of phagocytosis and killing cells percent as compared to untreated cancered group. On the other hand WBH alone or with radiotherapy and (Dox) induced significant decrease of α -FP as compared to cancered group. Also, the results revealed that WBH with or without radio- or chemotherapy induced apoptosis for cancer cells. It could be concluded that, WBH enhances the response of tumor cells to radiation and chemotherapy and it has an important role in potentiation of radio- and chemotherapy in solid tumor treatment. [Nature and Science. 2010;8(5):100-108]. (ISSN: 1545-0740).

Key Words: Hyperthermia, mice bearing solid tumors, doxorubicin, whole body gamma irradiation, apoptosis, immune responses.

1. Introduction

Hyperthermia is the use of therapeutic heat to treat various cancers on and inside the body. The purpose of this anticancer therapy is to shrink and hopefully destroy cancer without harming noncancerous cells. It can be used to treat cancer in many areas of the body, including brain [1] , thyroid [2], lungs [3], breast [4], and prostate [5]. It is thought that high temperatures, up to 40 °C, can help shrink cancerous tumors. It is applied alone or as an adjunctive with various established cancer treatment modalities such as radiotherapy and chemotherapy [6]. Hyperthermia is now being used more widely, because it does not have as many negative side effects as conventional forms of cancer treatment such as radiation or chemotherapy [7]. It's characters seemed to be mostly pronounced in the non proliferating tumor cells situated in the central area of solid tumors *in vivo* [8]. Moreover, **Chang et al.** [9] suggested that hyperthermia may augment vaccines delivery to tumors after systemic injection, as hyperthermia increases the permeability of the endothelial vasculature to nanoparticles. Also**,** they have demonstrated that the tumors that were treated with systemic vaccines under conditions of hyperthermia (41.5°C for 30 min) had significantly higher levels of vaccines marker gene activity and the (>100-fold) than those treated under normothermic conditions ($p < 0.05$) and that this effect was specific to tumor. Recent studies have confirmed and extended the old observation that heat may cause complete and selective tumor destruction of malignant cells [10] by activating of the immune system [11] .Radiation induces tumor cell apoptosis and necrosis, resulting in the release of tumor antigen and danger signals. Combined treatment with radiotherapy and Hyperthermia could induce a potent antitumor immune response, resulting in a significant decrease in the rate of local tumor relapse and might be associated with the production of apoptotic and necrotic tumor antigens and heat shock proteins after irradiation, phagocytosis and induction of more efficient tumor-specific cytotoxic T lymphocyte activity through a cross-presentation pathway [12].

Doxorubicin (DOX) is the most widely chemotherapeutic agent in the ultrasound-mediated drug delivery studies. This is because DOX is an intercalating drug that stacks between paired bases in DNA. However, like other anticancer drugs of anthracycline family, DOX is cardio toxic due to the induced production of active oxygen radicals [13].

Radiation therapy is the treatment of cancer with ionizing radiation. Radiation works by damaging the DNA (genetic material) within the tumor cells, making them unable to divide and grow. Radiation is often given with the intent of destroying the tumor and curing the disease (curative treatment). However, although radiation is directed at the tumor, it is inevitable that the normal, non-cancerous tissues surrounding the tumor will also be affected by the radiation and therefore damaged [14]. In general, cells are most radiosensitive in M and G2 phases and most radioresistant in S phase [15].

Material and Methods:

Chemicals:

Adriamycin: (Doxorubicin Hydrochloride)

Doxorubicin is a cytotoxic anthracycline antibiotic isolated from cultures of *Streptomyces peucetius* var. *caesius*. Doxorubicin consists of a naphthacenequinone nucleus linked through a glycosidic bond at ring atom 7 to an amino sugar, daunosamine

Chemically, doxorubicin hydrochloride is: 5,12-Naphthacenedione,10-[(3-amino-2,3,6-trideoxy-alpha-L-*lyx*o-hexopyranosyl)oxy]-7,8,9,10-tetrahydro -6,8,11-trihydroxy-8-(hydroxylacetyl)-1-methoxy-, hydrochloride (8S-*cis*). Doxorubicin hydrochloride was used in the form of an injectable commercial product (Adriablastina, from Pharmacia Italy).

Irradiation Technique:

The animals exposed to whole body irradiation with a total dose level of 2 Gy fractionated into four doses. The animals received 0.5 Gy once a week. Gamma irradiation source was from ^{60}Co, in the Middle Eastern Regional Radioisotope Center for the Arab Countries in Dokki, Giza, Egypt, at a dose rate (15.26/ min.)

Induction of solid tumor:

The mice had been inoculated with syngeneic tumor cells (Ehrlich carcinoma) obtained from the National Cancer Institute – Egypt. Ehrlich carcinoma cells ($2x10^6$) were injected into interapritoneal cavity of the animal using a hypodermic syringe with a 18 gauge needle. 7-8 days following tumor transplantation, subcutaneous tumors had reached a palpable size.

Experimental Animals:

Seventy female Swiss Albino mice weighting 20-25gm were purchased from the National Cancer Institute Cairo University, Egypt. The animals were kept at constant environmental and nutritional conditions throughout the experimental period with room temperature ($21 \pm 2°C$) with a 12h light / 12h dark cycle. They were fed standred pellet rat diet and water *ad- libitum* throughout the experiment. Mice were divided into seven equal groups each of 10 mice. First group served as control.

As soon as solid tumor was palpable, the animals were classified into 6 groups, (each of 10 mice).

Second group: the animals carried with solid tumor without any treatment.

Third group: the animals carried solid tumor exposed to WBH 41-5°C once a week for four weeks.

Fourth group: animals administered doxorubicin (Dox) 3mg/kg body weight iteraperitoneally (i.p) once a week.

Fifth group: animals were exposed to fractionated whole body gamma rays (WB-γ) at a dose level of 0.5 Gy once a week

Sixth group: the solid tumor animals were exposed to WBH (41.5°C) and injected (i.p) with 3mg /kg body weight Doxorubicin (Dox) once a week for four weeks.

Seventh group: the solid tumor animals were exposed to WBH (41.5°C) and whole body gamma irradiation (0.5 Gy) once a week for four weeks.

The relative temperature of the mice was recorded using a thermocouple (Cole Parmer type T. thermocouple thermometer) connected with a rectal probe, which was inserted 2 cm beyond the anal sphincter.

After one month of the last treatment, the blood samples were withdrew from orbital venous plexus of experimental animals into fresh heparinized tubes. The blood samples divided into two parts, the first part was used for assaying the phagocytosis, killing .[16], interleukin 2 (IL2).[17] and heat shock protein 70 (HSP70) was assayed by an Hsp70 EIA Kit (StressGen Biotechnologies, British Columbia, Canada), which can detect and quantitate inducible HSP70 in samples originating from both human and mouse. ELISA was performed according to the manufacturer's instructions.The second part was centrifuged at 3000 rpm for 20 min. The plasma was separated and stored for tumor necrosis factor (TNF-α). [18] and alpha fetoprotein (α- FP) [19] determination · As well as apoptosis of cancer tissues can be detected. [20]

Statistical Analysis:

All results are expressed as mean ± SEM. The statistical analysis was carried out with Duncan's

multiple range test. A $P < 0.05$ was considered the level of statistical significance.

RESULTS:

WBH induced significant increase (P< 0.05) of phagocytosis % in cancered mice. The mean values of phagocytosis % were 51.70 ± 2.98 and 69.30 ± 1.77 before and after WBH treatment respectively. The increase was 20.52% Table (1&2). Combined treatments of WBH and Dox administration or whole body gamma rays induced highly significant increase amounting to 33.74% and 35.18%respectively. As well as there was a significant increase of percentage of blood concentration of killing cells after WBH combined with Dox or whole body γ- rays representing 43.41% and 51.11% respectively.

As apparent from table 1 the level of HSP70 which was increased remarkably after WBH alone as compared to untreated cancered mice. The mean values of HSP70 were 6.53 ± 1.01 and 3.65 ± 0.69 respectively. The increase was 78.90% Table (1&2). Furthermore, it shows a significant increase of HSP70 after WBH with Dox or with whole body γ-rays but the last combined was higher than that the first as compared to untreated cancered group 50.96% Table 2.

It is shown in table 1 that there was a significant increase of TNF-α after WBH with or without Dox or γ- rays as compared to untreated group. The maximum increase was detected in the cancer mice treated with combined WBH with Dox by 130.80 % table 2.

It can be noted from table 1 the level of serum tumor marker α-FP significantly decreased after WBH alone. This decrease was 64.80%. As shown as from table 1 the combined treatment of WBH and Dox or with γ-rays induced highly significant decrease of α-FP amounting to 74.18% and 75.84% respectively
Table (1) shows that after WBH combined with Dox or with γ- rays there was highly significant increase of IL-2 representing 38.15% and 41.38% respectively as compared to untreated group (table 2). Table (3) demonstrates a significant increase of apoptosis in the tumor cells (Ehrlich carcinoma) after WBH either as alone or combined with γ rays or (Dox) . DNA ladder and hallmarks of apoptosis were observed post three treatments (Fig. 1a lanes 3-8 &b lanes 1-3). Hyperthermia increased the sensitivity of cancer cells to chemotherapy or radiotherapy and affected the mode of cancer cells death then DNA ladder formation was observed. These results suggest that heat is required for the induction of apoptosis.

Table (3) demonstrates a significant increase of apoptosis in the tumor cells (Ehrlich carcinoma) after WBH either as alone or combined with γ rays or (Dox) . DNA ladder and hallmarks of apoptosis were observed post three treatments (Fig. 1a lanes 3-8 &b lanes 1-3). Hyperthermia increased the sensitivity of cancer cells to chemotherapy or radiotherapy and affected the mode of cancer cells death then DNA ladder formation was observed. These results suggest that heat is required for the induction of apoptosis.

Table (1): Effect of WBH, Dox, whole body γ- rays alone or combined on various biological parameters in mice bearing solid tumor.

Groups / Parameters	Control mice	Cancered mice	Cancered mice+ WBH	Cancered mice+ Dox	Cancered mice Rad.	Cancered mice WBH+ Dox	Cancered mice WBH+ Rad.
Phagocytosis%	82.02 ± 1.62 [a]	57.7 ± 2.98 [bc]	69.30 ± 1.77 [b]	55.70 ± 2.16 [c]	47.1 ± 1.44 [c]	76.90 ± 1.19 [a]	77.70 ± 1.64 [a]
Killing cells%	78.6 ± 1.84 [a]	49.50 ± 2.17 [g]	63.80 ± 2.09 [d]	53.3 ± 2.70 [f]	56.25 ± 2.10 [e]	70.99 ± 2.32 [c]	74.80 ± 2.10 [b]
HSP70 (ng/ml)	2.59 ± 0.28 [e]	3.65 ± 0.69 [cd]	6.53 ± 1.01 [a]	3.48 ± 0.40 [d]	1.79 ± 0.48 [f]	4.26 ± 1.28 [b]	4.60 ± 0.44 [b]
TNF-α (pg/ml)	354.40 ± 7.11 [a]	135.70 ± 11.10 [f]	289.78 ± 8.13 [c]	265.46 [bc] ± 7.30	238.8 ± 6.64 [d]	313.06 ± 9.10 [c]	280.54 [c] ± 7.93
α -FP(ng/ml)	1.11 ± 0.48 [d]	28.10 ± 1.86 [a]	9.89 ± 2.69 [d]	13.40 ± 0.63 [b]	12.20 ± 1.40 [bc]	5.85 ± 0.82 [c]	6.79 ± 1.38 [c]
IL2 (pg/ml)	651.86[a] ± 19.07	433.45 [c] ± 13.85	554.30 ± 7.74 [b]	446.8 ± 15.20 [c]	466.78 [c] ± 17.21	598.79 [ab] ± 11.30	612.80 ± 14.304[a]

Means with different small letters within each column are significant at α 0.05

Dox: doxorubicin, WBH: whole body hyperthermia, Rad: radiation

Table (2): Effect of WBH, Dox, whole body γ- rays alone or combined on various biological parameters in mice bearing solid tumor (% change).

Groups / Parameters	Cancered mice+ WBH	Cancered mice+ Dox	Cancered mice WBH+Dox	Cancered mice Rad.	Cancered mice+ WBH
Phagocytosis %	20.10	3.47	33.28	18.37	34.66
Killing cells%	28.89	8.08	43.41	13.64	51.11
HSP70 (ng/ml)	78.90	4.66	16.71	50.96	26.03
TNF-α (pg/ml)	113.54	95.62	130.80	75.98	106.74
α -FP(ng/ml)	-64.80	-52.31	-74.18	-56.58	-75.84
IL2 (pg/ml)	27.88	3.08	38.15	7.69	41.38

Table (3) Apoptosis in the tumor cells (Ehrlich carcinoma) after WBH either as alone or combined with γ rays or (Dox)

Lanes:	M		Cancer tissues				Radiation only				Dox only				WBH only			
			3		15		1		5		6		11		2		4	
Bands	Bp	%	bp	%	bp	%	bp	%	bp	%	bp	%	bp	%	bp	%	bp	%
1	1000	32.15	1782	61.3	1845	85.8	1824	59.41	1890	53	1824	48.58	1815	45.11	1755	50.4	1755	43.4
2	700	12.7	540	21.2	540	4.5	540	15.24	540	18.93	450	25.68	540	18.4	540	16.9	540	43.4
3	500	13.2	360	13.2	360	4.5	360	15.24	360	14.54	360	11.79	360	26.9	540	16.9	540	15.3
4	300	13.8	180	4.29	180	4.28	180	10.12	180	13.54	180	13.98	180	9.6	360	17.3	360	23.60
5	200	11				5.4									180	15.5	180	17.7
6	100	17.1																
Sum		100.0				100.0		100.0						100.0				
In Lane		100				100		100						100				

Lanes:	M		WBH only 10		WBH + Dox 8		9		12		WBH + Rad 7		13		14	
Bands	Bp	%	bp	%	bp	%	bp	%	bp	%	bp	%	bp	%	bp	%
1	1000	32.1	1754	51.28	1854	49.9	1805	43.7	1769	45.26	1854	48.18	1828	44.88	1750	46.3
2	700	12.7	540	17.58	540	8.724	540	14.88	540	19.28	540	24.78	540	13.6	540	27.31
3	500	13.2	360	15.215	360	11.72	360	22.1	360	15.74	360	11.59	360	15.8	360	12.21
4	300	13.8	180	16.251	180	29.62	180	19.3	180	19.73	180	15.48	180	15.7	180	14.16
5	200	11.07												15.7		
6	100	17.1														
Sum		100.0								100.0				90.0		100.0
In Lane		100								100				100		100

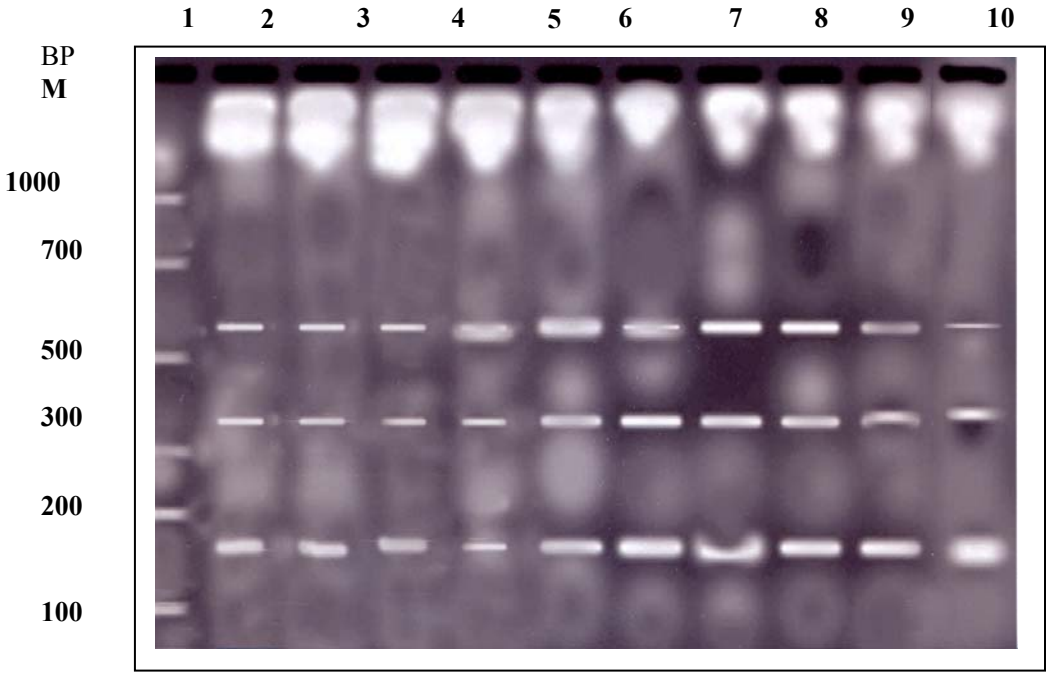

Fig. 1 a
The effect of WBH alone (41.5^{O}C) and combined with Dox or γ rays treatments on DNA ladder formation.

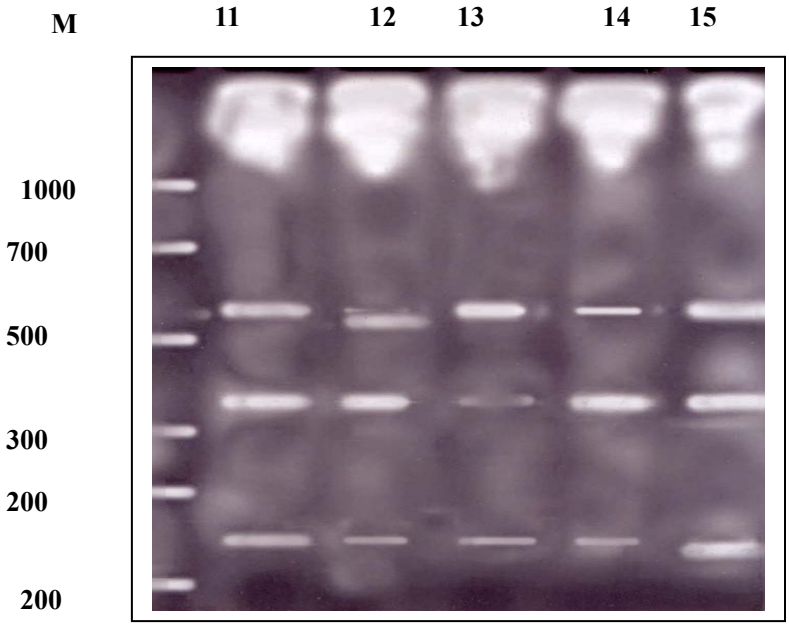

Fig. 1 b
Fig 1 a& b: The effect of WBH alone (41.5^{O}C) and combined with Dox or γ rays treatments on DNA ladder formation. Lane 1,5: γ rays alone. Lane 3&15: untreated cancer. Lane 6& 11: Dox alone. Lane 2, 4& 10: WBH treatment alone. Lane 8, 9&12: WBH treatment combined with Dox. Lane 7, 13&14: WBH treatment combined with γ rays.

Discussion:

Hyperthermia, a therapeutic method by increasing tissue temperature (range 40- 43C°), potentially induces tumor cell death by a spectrum of molecular, metabolic, cellular and tumor tissue changes [21] .Moreover, a raised body temperature raises the metabolic rate and makes the immune response more efficient [22] .

In the present study showed that WBH 41.5°C given in combination with doxorubicin or whole body-γ irradiation showed decrease significantly of FP-α,and increased phagocytosis and killing cells. Reflecting the frequency of cure mice bearing solid tumors these results were supported by the published data of other authors [23, 24] , who found that tumor cells increase the accumulation of doxorubicin under hyperthermic conditions. In general, mild hyperthermia (HT) at 39-42^{o}C induces an increase in tumor tissue blood flow volume [25]. Thus, the effect of HT on tumor tissue and tumor drug accumulation varies with thermal dose. Furthermore, it is supposed to be applicable in the development of less toxic regimens to reverse drug resistance by using relatively low concentrators of reversal agents combined with HT in order to reduce their side effects [26] . Tumor necrosis factor alpha (TNF- α), is a potent mediator of inflammatory and metabolic function and it was originally detected as a highly cytotoxic cytokine for tumor cells, it causes tumor necrosis *in vivo* [27] . One of the causes of cancer progression is the failure of tumor cells recognition by the immune system [28]. Thus, in the present study WBH alone or combined with γ irradiation or Dox induced significant increase of IL-2 and TNF-α as compared to cancer group. This result in accordance with **Tarner el al**. [29] showed that HT caused induction of all cytokines by 40-50% such as TNF-α & IL-2.

In the present study, the elevated level of HSP 70 level after WBH alone and combined with radiation or Dox treatments, studies of other authors have shown that hyperthermia (HT) can be applied successfully in an antitumor protocol based on TNF and HSP70 leading to a significant inhibition of lethality but not to a reduction of antitumoral capacity [30] .Stress induced HSP70 effectively protects cells against apoptosis. In the present study HSP70 released from solid tumor, this result is in a harmony with those obtained by **Salamatu et al.** [31] suggested that HSP70 can be released from tumor cells and stimulate a potent antitumor immune response. Up-regulation of HSPs in various cancers suggests that they might be involved in tumorigenesis. [32] Enhancement of tumorigenesis by overexpression of HSP70 has been implicated in a rodent model [33]. HSPs are known to be essential for the survival of cancer cells in different cancers [34, 35]. Also, HSPs as molecular chaperones might sustain cancer cells by modulating the activity of different proteins involved in cell cycle and apoptosis. . As well as, **Park et al**. [36] suggested that a subsequent accumulation of heat shock proteins (HSP) is likely to contribute to the malignant progression of hypoxic tumor cells. However, HSP70 does not contain a consensuses secretory signal and thus can not transverse the plasma membrane by conventional mechanisms [37]. The data of the present study shows statistically highly significant higher mean of HSP70 in the group treated with WBH alone or combined with WB-γ rays or (Dox) than in normal. It has reported that many HSPs are known to regulate apoptosis and even prevent apoptosis induced by anticancer drugs [32]. Also, they protects cellular elements from injury by reducing oxidation inflammation and apoptosis and by refolding damage proteins [38]. Moreover, it is also well established that heat shock or elevated HSP70 alters the regulation of signaling cascades and transcription factors and potently sensitizes tumors to radiation. [39]

The results reveal that the mechanism of enhancement of WBH induced apoptosis by Dox or γ irradiation .A biochemical hallmark of apoptosis is characteristic from of DNA degradation in which the genome is cleaved at inter-nucleosomal sites, generating a ladder of DNA fragments [40]. The present study observed that WBH alone induced apoptosis in solid tumor cells *in vivo* in accordance with previous studies indicated that relatively mild heat shock induces apoptosis [41]. The sensitizing effect on killing the tumor cells can be related to an enhanced inhibition of nucleic acid synthesis, as both modalities are known to have that effect [42,43] An inhibited repair of DNA damage, which is known to be one of the factors in the heat-sensitizing effect of irradiation and doxorubicin [44] , may also be a possible mechanism.. Heat induced apoptosis has been studied in normal cells or tissues and in a variety of tumor cells or tissues. These studies demonstrate that cellular ATP level may be involved in determining the mode of heat – induced cell death i.e. apoptosis or necrosis, since cellular ATP levels are indicated to be an important determinant of apoptosis [45]. Also DNA Ladders and hall marks of apoptosis were observed post WBH combined with Dox or radiation. The appearance of DNA fragmentation was increased post WBH combined radiation treatment. Hyperthermia can modulate the action of many anticancer drugs and lead to temperature dependent DNA damage [28]. Apoptosis may be due to change in expression of some genes in the cancer cells transplanted into nude mice such as p53, Bcl-2, and Bax [46]. As well as, the mechanisms of cell killing are explained that the influences of tumor

cells may be due to the impairment of a DNA, RNA and proteins synthesis [46, 47]. Moreover, the promotion of apoptosis is an important component of the antitumor activity of traditional anticancer therapies, including chemotherapeutic drugs and radiotherapy [48]. Furthermore, the creation of a functional blood supply from the normal tissue vasculature via the process of angiogenesis is critical for the continued growth and development of solid tumors [49]. Hyperthermia has been identified as angiogenesis inhibitor [50, 51] . However, it has been proposed that when massive apoptosis occurs, the normally efficient phagocytic system is overwhelmed, resulting in secondary necrosis in vivo, release of proinflammatory mediators [52]. In other studies reported that, apoptosis was manifested by classical changes in cell morphology and activation of caspase -3, both considered the hall marks of apoptotic mode of cell death [40]. **Sosman and Puzanov** [53] reported that ,in MCF-7 breast cancer cells, tumor necrosis factor α(TNFα) and TNF-related apoptosis-inducing ligand inhibit overall translation by a mechanism that requires caspase (but not necessarily caspase-3) activity.

Conclusion:

WBH enhances the response of tumor cells to radiation and chemotherapy and it has an important role in potentiation of radio- and chemotherapy in solid tumor treatment.

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Effect of Calcium and Some Antioxidants treatments on Storability of Le Conte Pear Fruits and its Volatile Components

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Abstract: The possibility of calcium nitrate and / or some antioxidants i.e. citric acid and ascorbic acid as preharvest treatment alone or in combination to control decay and its role in improvement the quality of Le Conte pear fruits as well as volatile components under cold storage condition and marketing period during to successive seasons 2007 and 2008.Le Conte pear trees were foliar spraying twice with calcium nitrate at concentration of (0.0 and 1700 ppm), citric acid at concentration of (0.0,50 and 100 ppm) and ascorbic acid at concentration of (0.0,50 and 100 ppm), ten treatments were used including control. All treated and untreated pear fruit were stored at 0 ± 1 ºC and 85 – 90% relative humidity (RH) for 75 days and additional one week at room temperature (20-25ºC) as stimulated marketing period. Fruit quality assessments i.e. weight loss and decay percentage, fruit firmness, total soluble solids %, total acidity %, total sugars, fruit calcium content and volatile components were evaluated. Results showed that treated and control fruits withstand free from chilling injury and pathogenic rot up to 45 days of cold storage. While, almost treatments prevented chilling injury symptoms and fruit deterioration up to 60 days of cold storage. Moreover, all treatments alone or in combination decreased the weight loss (%), total acidity % and fruit softening, while increasing fruits content of TSS %, total sugars and calcium (%) as a good keeping fruit conditions for along time. Furthermore, the same trend was observed during marketing period. Therefore, it can be concluded that prolonging storage period of the Le Conte pear fruits by using the considered treatments. However, the combined treatments with calcium nitrate + citric acid, calcium nitrate + ascorbic acid or /and the single treatment of calcium nitrate could be recommended because its gave the best results for keeping fruits and their volatile components under cold storage and marketing period extinction. The headspace volatiles of fresh and stored Le Conte Pear were collected and subjected to GC and GC-MS analysis. 27 volatile components were identified: 15 esters, 8 alcohols, 3 aldehydes and one terpene. Volatile components varied considerably both quantitatively and qualitatively between fresh and stored samples. The best treated samples at fresh were (Ca + CA1) and (Ca + CA2) compared to the control treatments. Although all samples retain a good quality during storage period, Ca(NO3)2, AsA1, (Ca + CA1), (Ca + CA2), and (Ca + AsA1) treated samples were the best compared to the control samples because of the highest content of esters which exhibit it more fruity aroma and cause it more acceptable for consumer. [Nature and Science. 2010;8(5):109-126]. (ISSN: 1545-0740).

Keywords: Le Conte pear, Calcium, Citric Acid, Ascorbic Acid, Volatile Components, Storage, Quality Assessments.

1. Introduction

Le Conte pear is one of the most important deciduous fruit that shows great success and is widespread in the newly reclaimed areas in Egypt.

Calcium is the most important mineral element determining fruit quality. The multiple roles of Ca associated with the plant cell. Soluble Ca is involved in protein phosphorylation via Ca-Cal- modulin binding. A large portion of the Ca in plant cells is located in the cell wall and plasma membrane where it plays a major role in senescence and ripening. Concentrations of 1-5 mm Ca^{2+} occur in the cell wall region (Poovaian *et al*., 1988). Cell wall – bounded Ca is involved in maintaining cell wall integrity by binding carboxyl groups of polygalacturonate chains, which are mainly present in the middle lamella and primary cell wall (Chardonnet *et al*., 2003). Preharvest Ca treatments used to increase Ca content of the cell wall were effective in delaying senescence, resulting in firmer, higher quality fruit (Serrano *et al*., 2004; Kluter et al., 2006 and Raese and Drake, 2006) that were less susceptible to disease during storage (Hafez and Haggag, 2007).

In recent years there has been a growing interest in all classes of flavonoids as integral antioxidants in the human diet, due in part to their demonstrated ant carcinogenic activity, inhibition of tumor cell proliferation, antioxidant and free radical scavenging capabilities, as well as their effectiveness as metal chelators (Harborne and Williams, 2000). A group of antioxidants, including ascorbic acid (AsA) and citric acid (CA) were screened as possible chemical inhibitors for the reaction (Wang and Mellenthin,

1974). Lin *et al*., 2007 suggested that the effects of AsA treatment on inhibiting core browning and improving post harvest quality in pear cv. Yali may be due to a reduction membrane lipid peroxidation by enhancing the capacity of cells to scavenge reactive oxygen species. Also, Lin *et al*., 2008 found that application of chitosan combined with AsA was more effective than chitosan alone in decreased weight of losses, delayed softening, decreased respiration rate and improved total soluble solids in pear fruits as well as inhibited the incidence core browning throughout storage.

The purpose of this study was to investigate the ability of calcium nitrate and some antioxidant agents i.e. citric acid and ascorbic acid as pre harvest treatments separately or in mixture to control decay and its role in improvement the quality of Le Conte pear fruits as well as their volatile components under cold storage condition and during marketing period.

Volatile components of pear have been investigated with many authors (Kahle *et al*., 2005, Chen *et al*., 2006 (a, b) and Diban, *et al*., 2007).

Material and Methods:

Pear orchard: Pear trees cv. Le Conte (*Pyrus communis*, L.) in a private orchard at El-Tall El-Kepeer, Ismaalia Governorate. Fruit were picked from five years growen in sandy soil, speased 4x4m, under drip irrigation system, similar in growth and received common horticulture practices, were selected for this investigation. Fertilization, irrigation and other agriculture practices were applied as recommended. The soil texture of the experimental site was used with organic matter 0.36%, pH 8.9, E.C 0.18 dsm^{-1} and $CaCO_3$ 3.6%, P 0.26 mg/100g, K 18.2 mg/100g, Ca 420 mg/100g, Mg 10.2 mg/100g, Na 32 mg/100g, Fe 3.5 ppm , Mn 4.o ppm, Zn 1.6 ppm, and Cu o.4 ppm.

Treatments: Preharvest treatments of calcium as form calcium nitrate at 1700 ppm, citric acid (CA) at 50 or 100 ppm and ascorbic acid (AsA) at 50 or 100 ppm were sprayed alone or in combination, ten treatments used including control, on pear trees during 2007 and 2008 seasons. In each season, the foliar spraying treatments were applied at two times. The 1^{ST} spraying was at the second week of July. While, the 2^{nd} one was at after the first with ten days. All spray solutions contained 0.1% Triton B as a wetting agent and sprayed till run off.

Storage fruits: Undamaged mature pear fruits, free from apparent pathogen infection, uniform in shape, weight and color picked separately from each treated pear trees groups. Fruits were harvested at the last week of August during each growing seasons and transported to the laboratory of Agriculture Development System (ADS) Project, Faculty of Agriculture, Cairo University, Egypt. The initial quality measurements were determined.

Fruit keeping: The selected fruits were washed with tap water; air dried and then packed in perforated carton boxes in three replicates for each treatment (about 120 fruit/treatment, with 20 fruit/replicates). Each treatment classified into two groups. The first group contains fruits for periodical determination of loss in weight of fruit and fruit decay percentage. The other contained fruits were used for the determination of fruit quality characteristics. Fruit stored at 0 ± 1 °C with relative humidity (RH) 85 – 90 % for 75 days. Assay of the stored fruits was made at 15 days intervals.

Marketing period: A sample of 10 fruits of each replicate was taken out at the end of cold storage period and left at room temperature (20 – 24 °C) for one week. Pear fruits quality assessment and fruit decay were assessed.

Quality assessments:

A- Physical characteristics:

Weight losses: Pear fruits were periodically weighted and the losses were recorded for each replicate. Date of weight losses were calculated as percentage from the initial weight.

Fruit decay percentage: Evaluated by type, as skin appearance, shriveling, chilling injury and pathogenic rots. In every inspection, decayed fruits were discarded and the number of fruits per replicate was used to express decay percentage.

Fruit firmness: Pear fruit firmness was determined as $Lb/inch^2$ by using fruit pressure tester mode. FT 327 (3 – 27 Lbs).

B- Chemical characteristics:

Total Soluble Solids (TSS): were determined in pear fruit juice using a hand refract meter model (10430 Brix reading 0 – 30 ranges Bausch & lomb Co. Calif., USA) according to A.O.A.C., 1995).

Total acidity (TA %): Was estimated as malic acid by titrating 5 ml juice with 0.1Nsodium hydroxide using phenolphthalein as an indicator (A.O.A.C., 1995).

Total sugars (g/100g fresh weight "F.W"): Were determined in pear fruits by method described by Smith *et al*.; 1956 using the phenol and sulphoric acid.

Fruit calcium content: Samples of fruits pulp were randomly taken from all treatments of each replicate after harvest time and 15 days intervals during storage of periods to determined calcium (Ca %) as described by Shapman and Pratt, 1978.

C- Volatile components:

Isolation and analysis of headspace volatiles:

The volatiles in the headspace of each sample under investigation were isolated by using a dynamic headspace system according to Fadel et al., 2006.

Gas chromatographic (GC) analysis:
GC analysis was performed by using Hewlett-Packard model 5890 equipped with a flame ionization detector (FID). A fused silica capillary column DB5 (60m x 0.32 mm id) was used. The oven temperature was maintained initially at 50°C for 5 min, and then programmed from 50 to 250°C at a rate of 4°C/min. Helium was used as the carrier gas, at flow rate 1.1 ml/min. The injector and detector temperatures were 220 and 250°C, respectively. The retention indices (Kovats index) of the separated volatile components were calculated using hydrocarbon (C8-C22, Aldrich CO.) as references.

Gas chromatographic-mass spectrometric (GC-MS) analysis:
The analysis was carried out by using a coupled gas chromatography Hewlett-Packard (5890)/mass spectrometry Hewlett-Packard-MS (5970). The ionization voltage was 70 eV, mass range m/z 39-400amu. The GC condition was carried out as mentioned above. The isolated peaks were identified by matching with data from the library of mass spectra (N1ST) and compared with those of authentic compounds and published data (Adams, 2001). The quantitative determination was carried out based on peak area integration.

Statistical analysis: The data were subjected to analysis of variance and the method of Duncan was used to differentiate means**,** Duncan (1955).

RESULTS AND DISCUSSION

Fruit quality characteristics as affected by calcium and some antioxidant agents treatments of pear cv. Le Conte during cold storage periods:

Weight loss percentage:
Effect of calcium and some antioxidant agents treatments on weight loss (%) of Le Conte pear fruits stored at 0 ± 1 º C are listed in table (1). Data showed that the percentage of weight loss was ranged from 1.4 to 7.8 % and from 1.3 to 7.3 % with the nutrition treatments comparing with control ranged from 1.8 to 9.1 % and from 1.8 to 9.4 % in both seasons. It obvious that the fruit weight loss was significant increased gradually with the progress of storage period up to 75 days. The lowest significant values of weight losses percentages were recorded by the combined spray Ca + CA2 (3.4 & 3.8%) respectively in 2007 and 2008 seasons. Followed by the combined spray of Ca + CA1 (4.1%) in the 1st season, while, a single treatment of calcium nitrate (4.0%) in the 2nd season. Came next the alone treatment of calcium nitrate (4.2%) in the first season, but the combined spray with Ca + CA1 and Ca + AsA2 (4.2 & 4.3 %) consecutively in the second season, without significant between them.
The loss in fruit weight is mainly due to water loss as a result of evaporation and transpiration and the amount of dry matter was lost by respiration. Our results are in agreement with Serrano *et al.,* 2004 on peaches and nectarines as well as Hafez & Haggag, 2007 on apple concerning in the effect of calcium treatment, they found that during cold storage, lower levels of weight loss were recorded in treated fruits compared with control fruits. As for the effect of antioxidants treatments, The present result are in agreement with that obtained by Lin *et al.,* 2008, who found that "Yali" pear fruits coating with ascorbic acid and stored deceased respiration rate and decreased weight of loss percentage.

Decay percentage:
Data in table (2) clearly revealed that all preharvest treatment with calcium nitrate, citric acid and ascorbic acid either alone or combination reduced decay percent and Le Conte fruits deterioration up to 75 days of cold storage at 0 ± 1 º C compared with untreated fruits (control). In general, to identify the classification of decay injuries influenced by pre harvest treatments, it can be stated that the physiological disorders as chilling injury (CI) and shriveling symptoms were higher percent than pathological rots in all treatments in both seasons. Moreover, it can be noticed from data in Table (2) all treatments including control prevented CI symptoms and pear fruit determination for 45 days at 0 ± 1 º C. However, the preharvest treatments alone or in combination prevented CI symptoms up to 60 days except the alone treatment of AsA1 in the 2nd season, as well as prevented the pear fruit determination up to 60 days except the alone treatment of AsA1 in the 1st season and combined treatment of Ca + AsA2 in the 2nd season. The best treatment prevented CI symptoms and pear fruit determination, as a good keeping fruits for along time (up to 75 days), obtained with the alone treatment of calcium nitrate and the combined treatment of Ca + CA2 in the 1st season, they recorded 100% total healthy. Meanwhile, in the 2nd season the two prevented treatments recorded 100% total healthy fruits after 60 days of cold storage. The alone treatment of Ca superior on this respect, it recorded the lowest significant CI symptoms 4.8% at 75 days. Followed by the combined treatment of Ca + CA2 gave 9.53%.
The results of preharvest treatments of calcium and some antioxidants study confirmed the previous finding of Guy *et al*., 2003 they reported that the pre harvest calcium sulfate application as bud sprays reduced both the progress and severity of gray mould

and increasing vase life of the rose flowers. Also, Richardson & Lombard**,** 1979 revealed that cork spot of pear Cv. "d" Anjou fruits was reduced 20% to 80% with orchard application of calcium at rates ranging from 325 to 350 ppm as chloride or nitrate spray containing surfactant. Late season sprays were more effective than early season sprays. Fruit calcium spray increased fruit calcium concentrations by 15 – 30%, sufficient to decrease the incidence of the disorders. Moreover, Hafez & Haggag, 2007 found that preharvest calcium application resistance to pathological disorders and keeping fruit quality. As for the effect antioxidants in the respect, Lin *et al*., 2007 & 2008 suggested that AsA treatment inhibiting core browning and improving postharvest quality in "Yali" pears may be due to a reduction membrane lipid peroxidation by enhancing the capacity of cells to scavenge reactive oxygen species.

Fruit firmness (Lb/inch^2):

Fruit firmness as affected by nutrition treatments during 2007 and 2008 seasons are listed in Table (3). Resulted showed that the fruit firmness were 7.8 to 13.8 Lb/inch^2 during 2007 season and 8.3 to 14.8 Lb/inch^2 during 2008 season compared with 7.5 to 12.4 Lb/inch^2 and 7.9 to 12.0 Lb/inch^2 in control treatment, respectively, within the storage days. It is clear that fruit firmness was decreased as storage period advanced. Also, it can be noticed from data obtained that all tested treatments had the highest effects on firmness comparing with control, but without any significant differences between them in the 1st season. However, in the 2nd season the highest significant values were obtained from all treatments in this connection. A combined treatment of Ca + CA2 and a single treatment of Ca were significantly increased the fruit firmness (12.2 & 11.62 Lb/inch^2) consecutively, but with no significant differ them. Meanwhile, the other treatment with antioxidants alone or with combined with calcium gave the same effect in reducing the rate of fruit softening without significant differences between them. On the other side, the untreated fruits were the lowest significant rate of fruit firmness in 2008 season.

These results might be due to the positive of applying calcium, citric acid and ascorbic acid alone or in combination on treated fruits. The obtained results could be explained by statement of Lin *et al*., 2007 which showed that antioxidants application improving postharvest quality and inhibiting core browning in Yali pears may be due to a reduction membrane lipid peroxidation by enhancing the capacity of cells to scavenge reactive oxygen species. The favorable effect of calcium obtained by Siddiqui and Bangerth, 1995 on Golden Delicious apple, it suggested that the observed effects of $CaCl_2$ on fruit firmness are likely to be associated with the calcium content of the covalently-bound pectin fraction. Also, Benavides *et al*., 2002 on Golden Smoothee apple found that the fruit firmness increased when calcium was applied. Similar results were obtained by Casero *et al*., 2004 on Golden Smoothee apple who indicated that fruit firmness shows positive correlation with fruit Ca content and bitter pit incidence correlates negatively with this nutrient concentration. Further more, Saure (2005) "on fleshes fruit" reported that Ca is known to stabilize cell membranes and in this way may prevent physiological disorders attributed to Ca deficiency. AS only very limited quantities of Ca can be directly supplied to the fruit, reducing excessive gibberellins levels by various means may be the better way to control such disorders. AS well as Montanaro *et al*., 2006 on "Kiwifruit" suggests that transpiration is not the only factor controlling Ca transport, and light also influenced the Ca concentration in xylem sap. Taking into account that auxin is able to stimulate Ca uptake and light promotes the biosynthesis of auxin protecting phenols (hydroxycinnamic acid). So, a new working hypothesis is proposed that light induces the biosynthesis of such phenols, which in directly decreases auxin degradation, and therefore, increases Ca accumulation.

Table (1): Effect 0f calcium and some antioxidant agents treatments on weight loss percentage of Le Conte pear fruits stored for 75 days at 0º C during 2007 and 2008 seasons.

Treatments	Storage period in days					
	15	30	45	60	75	Means
	Season 2007					
Control (water)	1.8	3.6	5.1	7.3	9.1	5.4 a
$Ca(NO_3)_2$ (1700ppm)	1.5	3.0	4.2	5.5	6.7	4.2 cd
CA1 (50 ppm)	1.6	3.2	5.0	6.8	7.6	4.8 b
CA2 (100 ppm)	1.6	3.1	4.4	5.7	7.3	4.4 bc
AsA1 (50 ppm)	1.6	3.1	4.6	6.0	7.6	4.6 b
AsA2 (100ppm)	1.5	3.1	4.5	6.1	7.8	4.6 b
Ca + CA1	1.5	2.8	4.0	5.4	6.8	4.1 d
Ca + CA2	1.4	2.8	3.9	5.1	6.3	3.9 e

Ca + AsA1	1.4	2.9	4.3	5.7	7.2	4.3 c
Ca + AsA2	1.6	3.2	3.5	5.9	7.5	4.3 c
Means	1.6 e	3.1 d	4.4 c	6.0 b	7.4 a	
	Season 2008					
Control (water)	1.8	3.8	5.6	7.3	9.4	5.6 a
$Ca(NO_3)_2$ (1700ppm)	1.5	2.8	4.0	5.1	6.4	4.0 e
CA1 (50 ppm)	1.6	3.3	5.5	6.4	7.3	4.8 b
CA2 (100 ppm)	1.4	2.9	4.3	5.7	7.1	4.3 d
AsA1 (50 ppm)	1.7	3.2	4.5	5.7	7.3	4.5 c
AsA2 (100ppm)	1.6	3.1	4.4	6.3	7.3	4.5 c
Ca + CA1	1.4	3.0	4.2	5.5	6.8	4.2 d
Ca + CA2	1.3	2.7	3.9	5.1	6.2	3.8 f
Ca + AsA1	1.4	3.0	4.4	5.8	7.3	4.4 cd
Ca + AsA2	1.5	3.0	4.4	5.8	7.0	4.3 d
Means	1.5 e	3.1 d	4.5 c	5.9 b	7.2 a	

Table (2): Effect 0f calcium and some antioxidant agents treatments on decay percentage and types of Le Conte pear fruits stored for 75 days at 0 ºC during 2007 and 2008 seasons.

Treatments	Storage period in days								
	Season 2007								
	Chilling injury (shriveling)			Pathogenic (soft rots)			Total healthy fruits		
	60	75	Means	60	75	Means	60	75	Means
Control (water)	9.53	14.3	4.77 a	4.8	19.1	4.78 a	85.7	66.6	90.5 b
$Ca(NO_3)_2$(1700ppm)	0.0	0.0	0.0 a	0.0	0.0	0.0 b	100.0	100.0	100.0 a
CA1 (50 ppm)	0.0	23.8	4.76 a	4.8	9.53	2.87 a	95.2	66.7	92.4 ab
CA2 (100 ppm)	0.0	19.1	3.82 a	0.0	4.8	0.96 b	100.0	76.1	95.2 a
AsA1 (50 ppm)	0.0	23.8	4.76 a	4.8	4.8	1.92 b	95.2	71.4	93.3 ab
AsA2 (100ppm)	0.0	23.8	4.76 a	0.0	9.5	1.90 b	100.0	66.7	93.3 ab
Ca + CA1	0.0	14.3	2.86 a	0.0	0.0	0.0 b	100.0	85.7	97.1 a
Ca + CA2	0.0	0.0	0.0 a	0.0	0.0	0.0 b	100.0	100.0	100.0 a
Ca + AsA1	0.0	23.6	4.76 a	0.0	0.0	0.0 b	100.0	76.2	95.2 a
Ca + AsA2	0.0	19.1	3.82 a	0.0	0.0	0.0 b	100.0	80.9	96.2 a
Means	0.95 b	15.72 a		1.44 b	4.77 a		97.61 a	79.03 b	
	Season 2008								
Control (water)	9.53	19.1	13.32 a	14.3	14.3	5.72	76.2	66.6	88.6 c
$Ca(NO_3)_2$(1700ppm)	0.0	4.8	0.96 d	0.0	0.0	0.0	100.0	95.2	99.04 a
CA1 (50 ppm)	0.0	23.83	4.77 c	0.0	4.8	0.96	100.0	71.4	94.3 b
CA2 (100 ppm)	0.0	4.8	0.96 d	0.0	14.3	2.86	100.0	80.9	96.2 a
AsA1 (50 ppm)	4.8	33.3	7.62 b	0.0	9.5	1.9	95.2	60.5	91.1 b
AsA2 (100ppm)	0.0	23.8	4.76 c	0.0	0.0	0.0	100.0	76.2	95.2 ab
Ca + CA1	0.0	19.1	3.82 c	0.0	0.0	0.0	100.0	80.9	96.2 a
Ca + CA2	0.0	9.53	1.91 d	0.0	4.8	0.96	100.0	85.7	97.1 a
Ca + AsA1	0.0	14.3	2.86 c	0.0	4.8	0.96	100.0	80.9	96.2 a
Ca + AsA2	0.0	14.3	2.86 c	4.8	0.0	0.96	95.2	85.7	96.1 a
Means	1.43 b	16.7 a		1.91 b	5.3a		96.7	78.4 b	

Decay (%) and types in all treatments up to 45 days = 0.0 in both studied seasons.

Table (3): Effect 0f calcium and some antioxidant agents treatments on fruit firmness (Lb/inch^2) of Le Conte pear fruits stored for 75 days at 0º C during 2007 and 2008 seasons.

Treatments	Storage period in days					
	15	30	45	60	75	Means
	Season 2007					
Control (water)	12.4	11.5	10.3	9.9	7.5	10.3 a
$Ca(NO_3)_2$(1700ppm)	13.3	12.5	10.7	10.1	8.6	11.04 a
CA1 (50 ppm)	12.4	11.6	10.3	10.0	8.3	10.52 a
CA2 (100 ppm)	12.6	11.9	10.4	10.0	8.3	10.64 a
AsA1 (50 ppm)	12.4	11.5	10.5	9.9	7.8	10.42 a
AsA2 (100ppm)	12.7	11.8	10.6	10.0	7.9	10.60 a
Ca + CA1	13.4	12.3	11.5	10.1	8.9	11.30 a
Ca + CA2	13.8	13.0	12.0	10.4	9.0	11.64 a
Ca + AsA1	12.7	12.0	11.0	10.0	8.6	11.90 a
Ca + AsA2	13.0	12.7	11.1	10.2	8.8	11.20 a
Means	12.9 a	12.1 b	10.84 c	10.1 c	8.4 d	
	Season 2008					
Control (water)	12.0	10.9	9.7	8.9	7.9	9.90 d
$Ca(NO_3)_2$(1700ppm)	13.7	13.5	11.2	10.7	9.0	11.62 a
CA1 (50 ppm)	13.0	12.4	10.1	9.7	8.9	10.82 B
CA2 (100 ppm)	13.6	12.8	10.9	10.0	9.0	11.30 abc
AsA1 (50 ppm)	12.8	11.7	10.3	9.5	8.3	10.52 c
AsA2 (100ppm)	13.0	12.3	11.4	10.2	9.5	11.30 abc
Ca + CA1	13.3	12.5	12.0	10.0	9.2	11.40 abc
Ca + CA2	14.8	13.0	12.3	10.9	10.0	12.20 a
Ca + AsA1	13.0	11.0	10.8	9.6	9.0	10.70 bc
Ca + AsA2	13.6	12.0	11.1	10.7	9.6	11.40 abc
Means	13.3 a	12.2 b	11.0 c	10.02 d	9.04 e	

Table (4): Effect 0f calcium and some antioxidant agents treatments on total Soluble solids (TSS %) of Le Conte pear fruits stored for 75 days at 0 ºC during 2007 and 2008 seasons.

Treatments	Storage period in days					
	15	30	45	60	75	Means
	Season 2007					
Control (water)	13.5	14.7	15.3	15.5	15.9	15.0 c
$Ca(NO_3)_2$(1700ppm)	14.7	15.2	15.5	15.6	17.0	15.6 ab
CA1 (50 ppm)	14.3	14.6	15.0	15.5	16.0	15.1 ab
CA2 (100 ppm)	14.5	15.0	15.2	16.5	17.7	15.8 a
AsA1 (50 ppm)	13.8	14.6	15.3	15.8	16.5	15.2 bc
AsA2 (100ppm)	14.2	14.9	15.0	15.6	16.7	15.3 bc
Ca + CA1	15.0	15.4	15.6	16.0	17.5	15.9 a
Ca + CA2	15.4	15.6	16.3	17.0	17.5	16.4 a
Ca + AsA1	13.4	14.5	15.0	15.7	16.9	15.1 ab
Ca + AsA2	13.8	14.7	15.3	16.5	19.3	15.5 ab
Means	14.3 e	14.9 d	15.4 c	16.0 b	16.9 a	
	Season 2008					
Control (water)	13.8	14.5	14.8	15.0	15.7	14.8 d
$Ca(NO_3)_2$(1700ppm)	14.9	15.2	15.7	16.0	17.2	15.8 a
CA1 (50 ppm)	14.9	15.0	15.4	15.7	16.0	15.4 bc
CA2 (100 ppm)	14.9	15.0	15.5	15.7	16.2	15.5 b
AsA1 (50 ppm)	13.9	14.0	14.7	15.5	15.9	14.8 d
AsA2 (100ppm)	14.0	15.0	15.2	15.7	16.0	15.2 bcd
Ca + CA1	14.8	15.5	15.8	159	16.0	15.6 ab
Ca + CA2	14.9	15.7	16.0	16.4	17.3	16.1 a
Ca + AsA1	14.0	14.5	14.9	15.3	15.8	14.9 cd
Ca + AsA2	14.5	14.7	15.0	15.8	16.4	15.3 bcd
Means	14.5d	15.0 c	15.3c	15.7 bc	16.3 a	

Table (5): Effect of calcium and some antioxidant agents treatments on total Acidity (TA %) of Le Conte pear fruits stored for 75 days at 0ºC during 2007 and 2008 seasons.

Treatments	Storage period in days					
	15	30	45	60	75	Means
	Season 2007					
Control (water)	0.32	0.31	0.30	0.23	0.20	0.272 a
$Ca(NO_3)_2$(1700ppm)	0.30	0.30	0.30	0.20	20.0	0.260 b
CA1 (50 ppm)	0.31	0.30	0.30	0.30	0.20	0.282 a
CA2 (100 ppm)	0.30	0.30	0.30	0.30	0.20	0.280 a
AsA1 (50 ppm)	0.31	0.31	0.30	0.23	0.20	0.270 ab
AsA2 (100ppm)	0.31	0.30	0.30	0.30	0.20	0.282 a
Ca + CA1	0.30	0.23	0.21	0.20	0.20	0.228 d
Ca + CA2	0.31	0.30	0.30	0.30	0.20	0.282 a
Ca + AsA1	0.30	0.30	0.23	0.20	0.20	0.264 c
Ca + AsA2	0.23	0,23	0.20	0.20	0.13	0.198 e
Means	0.299 a	0.288 b	0.274 ab	0.246 b	0.193 c	
	Season 2008					
Control (water)	0.32	0.31	0.30	0.23	0.20	0.272 a
$Ca(NO_3)_2$(1700ppm)	0.30	0.30	0.30	0.20	20.0	0.260 b
CA1 (50 ppm)	0.31	0.30	0.30	0.30	0.20	0.282 a
CA2 (100 ppm)	0.30	0.30	0.30	0.30	0.20	0.280 a
AsA1 (50 ppm)	0.31	0.31	0.30	0.23	0.20	0.270 ab
AsA2 (100ppm)	0.31	0.30	0.30	0.30	0.20	0.282 a
Ca + CA1	0.30	0.23	0.21	0.20	0.20	0.228 d
Ca + CA2	0.31	0.30	0.30	0.30	0.20	0.282 a
Ca + AsA1	0.30	0.30	0.23	0.20	0.20	0.264 c
Ca + AsA2	0.23	0,23	0.20	0.20	0.13	0.198 e
Means	0.299 a	0.288 b	0.274 ab	0.246 b	0.193 c	

Table (6): Effect of calcium and some antioxidant agents treatments on total sugars (g/100gFW) of Le Conte pear fruits stored for 75 days at 0º C during 2007 and 2008seasons.

Treatments	Storage period in days					
	15	30	45	60	75	Means
	Season 2007					
Control (water)	8.0	8.6	9.0	9.5	9.6	8.94 f
$Ca(NO_3)_2$(1700ppm)	9.9	10.0	10.2	10.3	11.4	10.4 b
CA1 (50 ppm)	8.5	9.0	9.5	9.9	10.6	9.5 e
CA2 (100 ppm)	8.8	9.6	9.9	10.0	10.8	9.82 d
AsA1 (50 ppm)	8.5	8.6	9.9	10.0	10.5	9.5 e
AsA2 (100ppm)	8.6	8.8	9.5	10.3	10.7	9.6 e
Ca + CA1	8.9	9.3	10.0	10.5	11.4	10.02 c
Ca + CA2	9.4	9.7	10.3	11.6	12.0	10.72 a
Ca + AsA1	8.7	9.5	9.8	10.3	11.0	9.9 d
Ca + AsA2	9.3	9.3	10.9	11.2	11.9	10.52 b
Means	8.9 e	9.24 d	10.0 c	10.4 b	11.01 a	
	Season 2008					
Control (water)	8.0	8.2	8.7	9.6	10.1	8.92 a
$Ca(NO_3)_2$(1700ppm)	8.7	9.0	10.6	11.1	11.2	10.12 b
CA1 (50 ppm)	8.3	8.8	9.9	10.2	11.5	9.74 d
CA2 (100 ppm)	8.5	8.9	10.3	10.6	11.7	10.0 bc
AsA1 (50 ppm)	8.4	8.5	9.3	10.3	11.3	9.6 d
AsA2 (100ppm)	8.6	8.8	9.5	10.6	11.6	9.82 cd
Ca + CA1	8.7	9.0	10.5	11.8	12.5	10.5 a
Ca + CA2	8.9	9.5	10.7	11.9	12.9	10.8 a
Ca + AsA1	8.7	8.9	10.4	11.2	11.8	10.2 b
Ca + AsA2	8.8	9.3	10.6	11.9	12.7	10.7 a
Means	8.6 e	8.9 d	10.1 c	10.92 b	11.72 a	

Table (7): Effect of calcium and some antioxidant agents treatments on fruit Ca content (%) of Le Conte pear fruits stored for 75 days at 0º C during 2007 and 2008 seasons.

Treatments	Storage period in days at					
	15	30	45	60	75	Means
	Season 2007					
Control (water)	0.024	0.026	0.026	0.028	0.029	0.0270 c
$Ca(NO_3)_2$(1700ppm)	0.027	0.028	0.029	0.030	O.O32	0.0292 ab
CA1 (50 ppm)	0.024	0.025	0.026	0.027	0.027	0.0260 d
CA2 (100 ppm)	0.025	0.025	0.026	0.027	0.028	0.0260 d
AsA1 (50 ppm)	0.024	0,026	0.027	0.028	0.029	0.0270 c
AsA2 (100ppm)	0.025	0.026	0.027	0.028	0.029	0.0270 c
Ca + CA1	0.026	0.028	0.029	0.030	0.032	0.0290 d
Ca + CA2	0.027	0.029	0.029	0.030	0.032	0.0294 ab
Ca + AsA1	0.027	0.028	0.028	0.029	0.033	0.0290 b
Ca + AsA2	0.027	0.028	0.030	0.031	O.033	0.0300 a
Means	0.026 e	0.029 d	0.028 c	0.029 b	0.030 a	
	Season 2008					
Control (water)	0.025	0.026	0.027	0.028	0.029	0.0270 e
$Ca(NO_3)_2$(1700ppm)	0.028	0.028	0.029	0.031	0.033	0.0300 b
CA1 (50 ppm)	0.025	0.026	0.027	0.028	0.029	0.0272 d
CA2 (100 ppm)	0.025	0.026	0.027	0.029	0.029	0.0272 d
AsA1 (50 ppm)	0.025	0.026	0.028	0.028	0.029	0.0272 d
AsA2 (100ppm)	0.026	0.026	0.027	0.028	0.029	0.0272 d
Ca + CA1	0.027	0.028	0.029	0.030	0.031	0.0290 c
Ca + CA2	0.028	0.029	0.030	0.031	0.032	0.0300 b
Ca + AsA1	0.028	0.028	0.029	0.031	0.033	0.0300 b
Ca + AsA2	0.028	0.029	0.030	0.031	0.033	0.0302 a
Means	0.027 d	0.027 d	0.028 c	0.030 b	0.031 a	

Table (8): Effect of calcium and some antioxidant agents treatments on decay (%) and types of Le Conte pear fruits after marketing period during 2007 and 2008 seasons.

Treatments	For 75 days cold stored fruits +7 days at room temperature					
	Season 2007			Season 2008		
	Chilling injury (shriveling)	Pathogenic (soft rot)	Total healthy fruits	Chilling injury (shriveling)	Pathogenic (soft rot)	Total healthy fruits
Control (water)	13.33 a	26.70 a	60.00 a	20.00 a	33.33 a	46.70 b
$Ca(NO_3)_2$(1700ppm)	13.33 a	6.70 ab	80.00 a	20.00 a	6.70 ab	73.33 ab
CA1 (50 ppm)	26.70 a	13.33 ab	60.00 a	13.33 a	20.00 ab	66.70 ab
CA2 (100 ppm)	13.33 a	6.70 ab	80.00 a	20.00 a	0.00 b	80.00 a
AsA1 (50 ppm)	20.00 a	6.70 ab	73.33 a	20.00 a	6.70 ab	73.33 ab
AsA2 (100ppm)	6.70 a	6.70 ab	86.70 a	13.33 a	6.70 ab	80.00 a
Ca + CA1	13.33 a	6.70 ab	80.00 a	13.33 a	20.00 ab	73.33 ab
Ca + CA2	13.33 a	0.00 b	86.90 a	6.70 a	0.00 b	93.33 a
Ca + AsA1	26.70 a	0.00 b	73.33 a	13.33 a	13.33 ab	73.33 ab
Ca + AsA2	6.70 a	6.70 ab	86.70 a	6.70 a	6.70 ab	86.90 a

Table (9): Effect of calcium and some antioxidant agents treatments on physical characteristics of Le Conte pear fruits stored for 75 days + 7 days at (20 - 24^{0}C) during 2007 and 2008 seasons.

Treatments	For 75 days cold stored fruits +7 days at room temperature					
	Season 2007			Season 2008		
	Weight loss (%)	Firmness (Lb/inch^2)	TSS (%)	Weight loss (%)	Firmness (Lb/inch^2)	TSS (%)
Control (water)	2.9 a	7.7 a	14.20 a	3.1 a	6.3 c	14.33 c
$Ca(NO_3)_2$(1700ppm)	2.6 bc	8.6 a	15.90 a	2.9 ab	8.0 ab	16.00 a
CA1 (50 ppm)	2.7 b	8.0 a	15.50 a	2.8 ab	7.8 b	15.33ab
CA2 (100 ppm)	2.6 bc	8.3 a	15.70 a	2.7 bc	8.3 a	16.00 a
AsA1 (50 ppm)	2.8 a	7.4 a	15.00 a	2.9 ab	7.5 b	15.40ab
AsA2 (100ppm)	2.7 b	7.8 a	15.73 a	2.8 ab	8.0 ab	15.90 a
Ca + CA1	2.5 c	8.4 a	16.00 a	2.6 bc	8.4 a	15.70 a
Ca + CA2	2.3 d	8.8 a	16.00 a	2.5c	9.0 a	16.00 a
Ca + AsA1	2.6 bc	7.8 a	14.90 a	2.8 ab	7.6 b	15.00 b
Ca + AsA2	2.5 c	8.3 a	15.20 a	2.7 bc	8.3 a	15.33ab

Table (10): Effect of calcium and some antioxidants treatments on chemicals characteristics of Le Conte pear fruits stored For 75 days + 7 days at (20 - 24^{0}C) during 2007 and 2008 seasons.

Treatments	For 75 days cold stored fruits +7 days at room temperature					
	Season 2007			Season 2008		
	TA (%)	Total sugars (g/100gFW)	Fruit calcium content	TA (%)	Total sugars (g/100gFW)	Fruit calcium content
Control (water)	0.30 a	9.9 g	0.029 c	0.30 a	11.0 f	0.029 c
$Ca(NO_3)_2$(1700ppm)	0.31 a	11.7 c	0.033 b	0.31 a	12.1 c	0.033 b
CA1 (50 ppm)	0.30 a	10.7 f	0.028 d	0.30 a	11.3 e	0.028 d
CA2 (100 ppm)	0.30 a	11.4 d	0.029 c	0.30 a	11.3 e	0.029 c
AsA1 (50 ppm)	0.30 a	9.9 g	0.029 c	0.30 a	11.1 f	0.029 c
AsA2 (100ppm)	0.30 a	11.0 e	0.029 c	0.30 a	11.3 e	0.029 c
Ca + CA1	0.31 a	11.3 d	0.033 b	0.31 a	12.0 c	0.033 b
Ca + CA2	0.31 a	14.4 a	0.034 a	0.31 a	14.0 a	0.034 a
Ca + AsA1	0.30 a	11.1 e	0.033 b	0.30 a	11.6 d	0.033 b
Ca + AsA2	0.30 a	12.4 b	0.034 a	0.30 a	13.4 b	0.034 a

Table (11): Volatile Compounds Identified in Headspace of Le Conte Pear Fruits in Fresh (Zero time) as affected by pre harvest treatments with calcium, citric acid and ascorbic acid alone or in combination. (*values expressed as relative area percentages to total identified compounds)

Peak No	KI[a]	Components	Fresh Treated Samples										Methods of identification[b]
			Control (water)	Ca $(No_3)_2$	CA_1 (50ppm)	CA_2 (100ppm)	A_sA_1 (50ppm)	A_sA_2 (100ppm)	Ca + CA_1	Ca + CA_2	Ca + A_sA_1	Ca + A_sA_2	
1	614	Ethanol	–	0.22	0.22	–	0.35	0.87	–	–	–	0.17	MS, KI, St
2	646	Ethyl acetate	*15.01	0.47	0.17	13.25	16.35	1.51	10.15	3.28	0.33	12.04	MS, KI, St
3	655	Methyl propanoate	1.43	1.93	1.68	1.37	24.85	2.50	4.58	16.92	–	1.07	MS, KI, St
4	695	1-Butanol	0.82	0.97	12.00	42.00	14.75	4.21	3.55	–	19.35	9.51	MS, KI, St
5	686	Methyl-2-methyl propanoate	0.71	0.16	0.25	1.13	2.49	0.66	0.35	0.19	–	6.07	MS, KI
6	716	Ethyl propanoate	0.69	0.11	0.66	1.44	0.55	2.44	0.63	0.10	–	1.00	MS, KI
7	722	Methyl butanoate	0.46	–	0.76	1.61	6.85	1.29	0.25	0.51	0.67	0.51	MS, KI
8	737	1-Penten-3-ol	1.36	1.80	1.72	0.84	6.70	9.56	1.56	0.87	1.07	0.86	MS, KI
9	744	Ethyl-2-methyl propanoate	4.59	0.46	0.99	1.47	–	1.50	0.19	–	–	1.96	MS, KI
10	748	1-Pentanol	1.38	–	0.47	7.29	6.59	4.84	1.36	0.36	0.16	–	MS, KI
11	772	(E)-2-hexenal	0.67	0.70	1.52	1.56	6.13	6.76	1.08	0.84	0.56	2.07	MS, KI
12	797	(Z)-3-hexen-1-ol	1.40	0.26	0.55	0.71	–	0.88	0.08	0.18	–	–	MS, KI
13	826	Butyl acetate	0.78	–	0.38	0.36	0.57	2.99	0.12	–	0.11	0.27	MS, KI
14	842	Ethyl butanoate	33.06	51.40	42.65	1.32	0.87	19.47	38.40	40.28	41.00	32.27	MS, KI
15	851	Ethyl-2-methyl butanoate	1.59	–	0.97	15.48	2.19	1.93	–	–	–	1.55	MS, KI
16	862	1-Hexanol	1.26	1.45	0.81	1.47	1.05	1.57	–	2.16	–	2.46	MS, KI
17	873	2-methyl-1-buty acetat	0.43	0.2	2.32	1.33	2.89	1.76	0.16	1.31	0.68	2.11	MS, KI
18	930	Methyl hexanoate	0.50	0.35	0.28	0.34	0.63	2.36	0.09	0.18	0.47	0.39	MS, KI
19	955	(E) -2-heptenal	0.11	0.16	0.33	0.06	0.16	1.28	–	0.06	0.49	0.16	MS, KI
20	977	1-heptanol	1.04	2.80	3.24	0.55	0.96	3.37	–	3.16	5.83	4.01	MS, KI

21	999	Ethyl hexanoate	16.04	19.46	17.97	1.66	0.23	5.77	27.01	18.75	18.13	11.36	MS, KI
22	1011	Hexyl acetate	9.15	11.93	8.58	0.13	–	3.06	9.23	9.40	8.49	4.63	MS, KI
23	1022	Octanol	0.81	0.20	0.17	0.22	0.23	0.34	0.12	0.08	0.17	0.16	MS, KI
24	1353	(E,E) 2,4-Decadienal	0.83	0.30	0.08	0.45	0.77	1.40	0.16	0.08	0.03	0.17	MS, KI
25	1372	Methyl E,Z-2,4-decadienoate	1.50	0.09	0.34	1.38	0.13	2.42	0.08	0.14	0.40	1.31	MS, KI
26	1449	Ethyl E,Z-2,4-decadienoate	2.33	1.80	0.03	1.44	2.23	12.99	0.05	0.06	1.00	1.86	MS, KI
27	1500	α-Farnesene	2.04	2.77	0.85	1.13	1.47	2.26	0.79	1.08	1.05	2.02	MS, KI

– Not detected.

Compounds listed according to their elution on DB5 column.

[a] Kovats index.

compound identified by GC-MS (MS) and / or by kovats index on DB5 (KI) and / or by comparison of MS and KI of standard compound (St) run under similar

GC-MS conditions.

Table (12): Volatile Compounds Identified in Headspace of Le Conte Pear Fruits cold stored for 75 days +7 days at room temperature as affected by pre harvest treatments with calcium, citric acid and ascorbic acid alone or in combination. (*values expressed as relative area percentages to total identified compounds)

Peak No	KI[a]	Components	Cold Stored Treated Samples after 75 days + 7 days at room temperature										Methods of identification
			Control (water)	Ca $(No_3)_2$	CA_1 (50ppm)	CA_2 (100ppm)	As A_1 (50ppm)	As A_2 (100ppm)	Ca + CA_1	Ca + CA_2	Ca+AsA_1	Ca+AsA_2	
1	614	Ethanol	27.11	22.51	19.17	22.30	2.34	15.64	23.42	30.99	26.85	17.81	MS, KI, St
2	646	Ethyl acetate	–	–	–	–	1.37	–	–	–	–	–	MS, KI, St
3	655	Methyl propanoate	–	–	–	–	5.51	–	–	–	1.40	2.24	MS, KI, St
4	695	1-Butanol	–	–	7.23	–	–	0.19	–	–	0.67	16.60	MS, KI, St
5	686	Methyl-2-methyl propanoate	–	–	–	–	0.37	1.22	–	–	1.61	3.83	MS, KI
6	716	Ethyl propanoate	0.59	–	–	–	1.42	0.55	–	–	–	–	MS, KI
7	722	Methyl butanoate	0.29	–	–	15.43	–	–	–	10.82	–	4.07	MS, KI
8	737	1-Penten-3-ol	10.84	5.89	6.39	-	0.90	1.78	16.79	4.46	3.79	6.44	MS, KI
9	744	Ethyl-2-methyl propanoate	59.52	69.71	32.86	34.25	4.84	10.73	43.12	47.04	21.72	23.69	MS, KI
10	748	1-Pentanol	0.91	1.05	8.41	18.41	7.08	35.39	–	–	–	–	MS, KI
11	772	(E)-2-hexenal	–	–	–	–	0.34	–	–	–	–	0.79	MS, KI
12	797	(Z)-3-hexen-1-ol	–	–	–	–	0.35	–	0.57	0.22	0.47	5.82	MS, KI
13	826	Butyl acetate	–	–	–	–	–	–	0.17	–	0.75	0.45	MS, KI
14	842	Ethyl butanoate	0.29	0.50	1.17	1.32	1.94	0.27	–	–	1.70	1.22	MS, KI
15	851	Ethyl-2-methyl butanoate	–	–	–	–	29.17	25.54	5.88	2.91	2.28	0.34	MS, KI
16	862	1-Hexanol	–	–	–	–	0.60	0.27	–	–	0.51	1.35	MS, KI
17	873	2-methyl-1-buty acetate	–	–	–	–	–	–	–	–	0.49	–	MS, KI
18	930	Methyl hexanoate	–	–	–	–	–	–	–	–	–	0.30	MS, KI
19	955	(E) -2-heptenal	–	–	–	–	2.16	–	–	–	–	–	MS, KI
20	977	1-heptanol	–	–	–	–	17.28	–	0.58	0.20	–	0.78	MS, KI
21	999	Ethyl hexanoate	–	–	11.12	–	18.80	6.75	6.16	2.65	33.68	5.59	MS, KI
22	1011	Hexyl acetate	–	0.33	–	–	1.09	0.27	–	–	2.76	3.09	MS, KI
23	1022	Octanol	0.44	–	–	1.35	0.81	0.23	3.30	0.7	–	0.59	MS, KI
24	1353	(E,E) 2,4-Decadienal	–	–	–	1.42	0.79	0.11	–	–	–	1.97	MS, KI
25	1372	Methyl E,Z-2,4-decadienoate	–	–	–	0.88	0.96	0.8	–	–	–	0.41	MS, KI
26	1449	Ethyl E,Z-2,4-decadienoate	–	–	6.89	1.53	2.21	0.14	–	–	1.31	1.64	MS, KI
27	1500	α-Farnesene	–	–	6.75	4.61	0.84	0.63	–	–	–	0.37	MS, KI

– Not detected.
Compounds listed according to their elution on DB5 column.
[a] Kovats index.
[b] compound identified by GC-MS (MS) and / or by kovats index on DB5 (KI) and / or by comparison of MS and KI of standard compound (St) run under similar
GC-MS conditions.s

Fig. 1. The total area percentages of the main chemical classes of volatile components in the headspace of Le Conte fruits fresh (zero time) as affected by pre harvest treatments with calcium, citric acid and ascorbic acid alone or in combination.

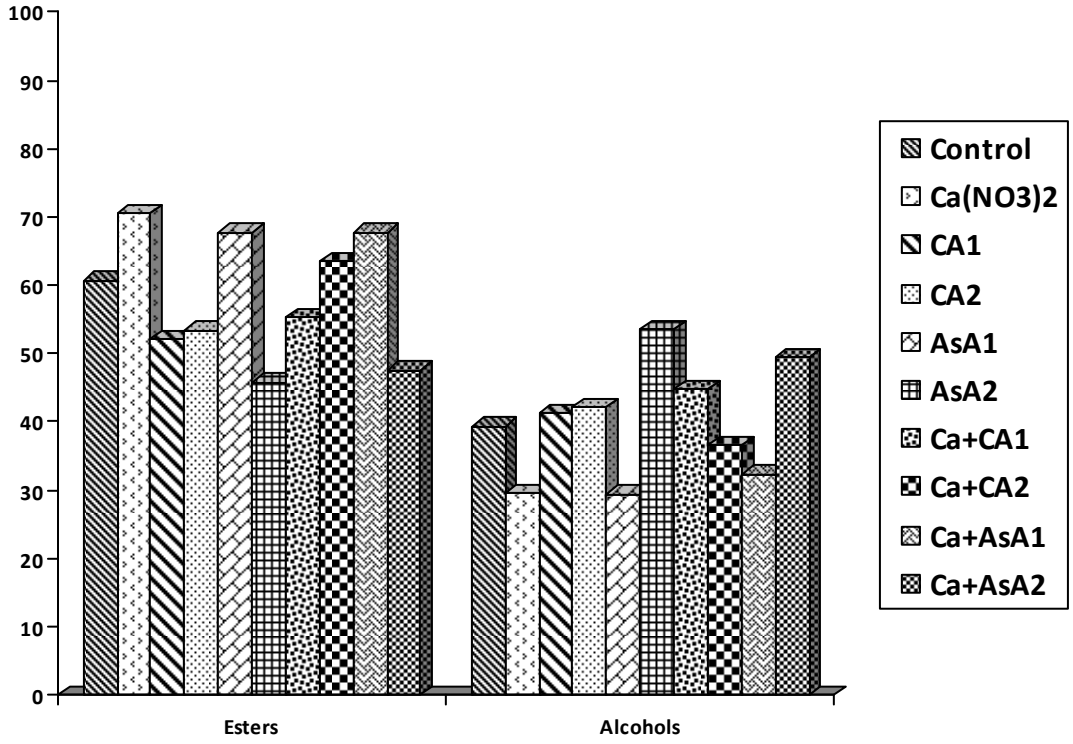

Fig. 2. The total area percentages of the main chemical classes of volatile components in the headspace of Le Conte pear Fruits cold stored for 75 days +7 days at room temperature as affected by pre harvest treatments with calcium, citric acid and ascorbic acid alone or in combination.

Total soluble solids percentage (TSS %):

Total soluble solids percentage were 13.4 to 17.7% and 13.9 to 17.3% developed by nutrition treatments, during 2007 and 2008 respectively comparing with 13.5 to 15.9 % and 13.8 to 15.7 % in the control (Table,4). It is obvious that TSS % was significant increased with all treatment throughout the progress of the storage periods in both seasons. Data presented in Table (4) indicated that all conductive treatments more effective statistically in increasing TSS % at initial or at end of storage, when compared with untreated fruits. These results are true in both studied seasons except the Ca + AsA1treatment in the 1st season after 15 days of storage only. The best results were obtained with Ca + AC2 treatment it recorded the highest significant values of TSS % (16.4 & 16.1 %, consecutively, in both seasons. Followed by Ca + AC1 treatment (15.9 %), CA2 treatment (15.8 %), Ca $(NO_3)_2$ treatment (15.6 %) and Ca + AsA2 treatment (15.5 %) in the 1st season. However, the Ca $(NO_3)_2$ treatment (15.8 %) and the Ca + CA2 treatment (15.6) in the 2nd season. But without any significant differences between them. The other treatments were next. The lowest significant levels of TSS % was detected by the control treatment (15.o & 14.8 %) respectively, in 2007 and 2008 seasons. Also, the AsA1 treatment (14.8 %) in the 2nd season.

These results are in line to those achieved by Nomier, 2000, Montanaro *et al*., 2006 and Lin *et al*., 2008.

Total Acidity percentage (TA %):

Data in Table (5) show the effect of calcium nitrate, citric acid and ascorbic acid treatment alone or in combination on the Le Conte pear fruits content of TA %. It can be noticed the same trend and values were recorded from all treatments in both studied seasons. The values of TA % in both seasons varied from 0.13 to 0.31 %, while in the control was varied from 0.2o to o.32 %. The total acidity % of pear fruits showed a slight reduction up to 45 days of cold storage and a gradual statistically decrease as storage period advanced for treated and untreated fruits. The significant reduction in fruits acidity reached maximum with Ca + AsA2 treatment (0.198 %). Followed by Ca + CA1 treatment (0.228 %). The combined treatment at Ca + AsA1 (0.264 %) came next, but with significant among them. On the other hand, the highest statistical value were recorded by CA1 (0.282 %), AsA2 (0.282 %), Ca + CA2 (0.282 %) CA2 (0.280 %) and the control treatment (0.272 %).

The previously results are in agreement with these found by Hafez and Haggag, 2007 mentioned that on Anna apple fruits spraying with calcium decrease the acidity during storage. Also, Mansour *et al.,* 2000 on Tomsson seedless grapevines treated with ascorbic acid reduced the total acidity percentage in berries. Moreover, Lin *et al*., 2008 who found that pear coating with AsA and stored helped maintain titratable acidity compared with control.

Total Soluble Sugars (g/100g FW):

The effect spraying of calcium, citric acid and ascorbic acid preharvest application on pear fruit content of total sugars listed in Table (6). Data showed that the nutrition treatments gave the values of total sugars ranged from 8.5 to 12.0 (g/100g FW) and from 8.3 to 12.9 (g/100g FW) during the different storage period in 2007 and 2008 seasons, consecutively as compared with values from 8.0 to 9.6 (g/100g FW) and from 8.0 to 10.1(g/100g FW) in the control treatment. The present results indicated that a continuous steady increased total soluble sugars content of Le Conte fruit during storage at 0 ± 1 º C up to 75 days. This increase took place in all treatments as well as control. Also, this increment was gradually by extension of storage period. At the beginning or the end of storage period, all treatment resulted in higher total sugars than the control for both investigate seasons. The best results had more effective in increasing the total sugars were obtained from the combined treatments of Ca + CA2 (10.72 & 10.8 g/100g FW) respectively, in the two seasons. Also, the Ca + AsA2 and Ca + CA1 treatments (10.7 & 10.5 g/100g FW) consecutively, in the 2nd season. But, without significant differences differ between them. The single treatment of Ca $(NO_3)_2$ (10.4 & 10.12 g/100g FW, respectively in both seasons, as well as Ca + AsA2 (10.52) in 2007 season and Ca + AsA1 (10.2 g/100g FW) in 2008 season come next, with no statistical found them. The signal treatment of antioxidants indicated that the moderately higher sugars contents in both studied seasons. The lowest significant values of fruit total sugars recorded by untreated fruits (8.94 & 8.92 g/100g FW, consecutively) in both seasons.

Effect of application antioxidant agents instead of using auxin for improving yield and fruit quality of various fruit crops is considered important tasks for pomologists. Antioxidants have synergistic action of flowering and productively of fruit trees as well as controlling the incidence of most fungi on fruit trees (Nomeir, 2000 and Mansour, 2000). Furthermore, to prolong storage quality of pears fruit (Lin *et al*., 2007 & 2008).

The great benefits of using antioxidants were observed when it incorporated with macro or micronutrients which were responsible for improving yield and fruit quality of different grapevine (Mansour,

2000). As for the effect of calcium nutrition on increasing the total sugars could be attributed to the balance in the nutrition status of the tree which advanced fruit maturity and ripening. The obtained results are in line with the findings with Hafez & Haggag, 2007 they found that the spraying with $CaCl_2$ treatment increased total sugars of Anna apple fruits at harvest and at end of the storage period.

Fruit Calcium Content (%):

Data presented in Table (7) show the effect of calcium, citric acid and ascorbic acid spray alone or in combination on fruit calcium contents during storage periods at 0 ± 1 º C up to 75 days. The rates of fruit calcium content in treated fruits ranged from 0.024 to 0.033% and from 0.025 to 0.033 % in 2007 and 2008 seasons, while in untreated fruit were 0.024 to 0.029 % and from 0.025 to 0.029 %, consecutively. It can be observed that Le Conte pear fruits contents of calcium were significant increased gradually with progress in the period of exposure to cold storage. Moreover, the treated fruits recorded a more concentrate in this respect as compared with control treatment. Also, it can be noticed all combined treatments gave the highest significant values of fruits calcium content. Followed by the single treatment of calcium. The alone treatments of antioxidants came next. These results were true in both investigated seasons.

The previously results are in line with those Tobias *et al*., 1993, found that calcium applied to fruit penetrates primarily through lenticels and increase Ca content of the tissues, mainly in the middle lamella region. Also, Chardonnet *et al*., 2003 mentioned that on "Golden Delicious" apple the effect of calcium infiltration after harvest and throughout storage at 0 ± 1 º C up to 6 months, resulted in an increase in both total and cell wall – bound Ca of the apple tissue during storage, with a maximum reached at 2 % $CaCl_2$ for fruit stored 4 or 6 months. In addition, Richardson and Lombard, 1979 found that fruit calcium sprays increased fruit calcium concentrations by 15 – 30 %, sufficient to decrease the incidence of the disorders.

Fruit quality assessments after marketing period (MP) as shelf life:

Effect of calcium and some antioxidant agents treatments alone or in combination on decay percentage & types, physical and chemical characteristics of Le Conte pear fruits after MP during 2007 and 2008 seasons.

On the other side, MP indicator of pear fruit for decay (%) was inspected after 7 days at 20 – 24 º C (Table, 8). The same trend of decay (%) and types of pear fruits were found after MP in all treatments but with slight increase than storage at 0 ± 1 º C in chilling injury symptoms. The pathogenic rots had the opposite trend. The best results were remarkable this respect, the combined treatments of Ca + CA2 and Ca + AsA1 in the 1st season while, Ca + CA2 in the 2nd season.

Concerning physical properties of pear fruits after MP for one week as shown in Table, 9. It can be detected that the lowest significant values of weight loss (%) was recorded with all treatments in stimulate marketing period comparing with control. These results are confirmed in both investigated seasons.

Data in Table (9) also, showed that although all conductive treatments recorded the highest values in fruit firmness after MP, but this increment without significant between them as compared with control in the 2007 season. However, the almost treatments gave higher significant effect in reducing the rate of fruit softening in 2008 season. On the other side, the untreated fruit were soft after MP in 2008 season.

TSS % of pear fruits in MP (Table, 9) revealed that the highest significant values of TSS% were recorded with all treatments when comparing with control in both studied seasons. In general, the alone or combined treatments had great role in increasing TSS% of pear fruits in MP after cold storage. While untreated fruits recorded the lowest rate of TSS% in 2007 and 2008 seasons.

As for the chemical properties of pear fruits after cold storage at 0 ± 1 º C up to 75 days and then 7 days at 20 – 24 º C (MP) as shown in Table (10). Total acidity percentage, no developed significant differences between all treatments in the two seasons.

The sugars content were 9.9 to 14.4g and 11.1 to 14.0g, while in the control treatment were 9.9 and 11.0g, respectively in 2007 and 2008 seasons. The highest significant values were obtained from a combined treatment with Ca + CA 2 (14.4 and 14.0g) consecutively in both studied seasons. Followed by the combined treatment with Ca + AsA2 (12.4 and 13.4g) respectively, in the both seasons. The single treatment with Ca $(NO_3)_2$ come next, it recorded 11.7 and 12.19 respectively, in both seasons, also Ca +CA 1 treatment 12.0g in the 2nd season. Afterwards, the single treatments of antioxidants had high dose then low dose. The CA remarkable on AsA in this respect. On the other hand, the lowest significant value was obtained from untreated fruits 9.9 and 11.0 g, consecutively in both seasons.

Fruit calcium content (%) as show in Table (10) it cleared that all combined treatments and the single treatments of Ca had a great role in increasing the average of fruit calcium content. The highest significant values were recorded from the combined treatments with Ca + CA2 (0.034 %) and Ca + AsA2 (0.034 %). Followed by the single treatment with Ca $(NO_3)_2$ (0.033%) and the combined treatment of Ca +CA1 (0.033 %). The other treatments including control came next (0.29 %). The single treatment of

CA1 had the lowest significant value in fruit calcium content (0.028%). These results are true and steady in both investigated seasons.

The above results are in line with findings found by Hafez and Haggag, 2007 who suggested that spraying Anna apple trees with calcium alone or in combination with boron for improving the fresh quality assessments after cold storage at S0C and after two weeks of marketing period at 20 - 25 ^{0}C. Also, Lin *et al*., 2008 found that ability of chitosan coatings with ascorbic acid (AsA) to prolong storage quality of Chinese pear fruits because AsA decreased weight loss, delayed softening, decreased respiration rate and membrane permeability and helped maintaining TSS and titratable acidity compared with controls. Incidence of core browning maintaining quality and reducing core browning and also enhanced fruit AsA contents and antioxidants defense mechanisms (superoxide dismutase, catalase and ascorbate peroxidase activity).

Volatile components in headspace of fresh (zero time) Le Conte pear fruits as affected by pre harvest treatments with calcium, citric acid and ascorbic acid alone or in combination:

Twenty seven volatile compounds were identified by using high resolution gas chromatographic (HRGC) and GC-MS analysis listed with their area percentages in Table (11). The majority of compounds were 15 esters, 8 alcohols, 3 aldehydes and one terpene. The total area percentages of the main chemical classes of volatile components in the headspace of fresh (zero time) control sample and fresh treated pear fruits samples with calcium; citric acid and ascorbic acid at different ratios are shown in Fig. (1). Esters of aliphatic acids were the predominant class of constituents in headspace volatiles of pear in all samples under investigation, it comprised 88.27% in control sample; 88.36% in $Ca(No_3)_2$ sample; 78.03% in citric acid 50ppm (CA1); 43.71% in citric acid 100ppm (CA2); 60.83% in ascorbic acid 50ppm (AsA1); 62-65% in ascorbic acid 100ppm. sample (AsA2); 91.29% in calcium and citric acid 50ppm sample (Ca + CA1); 91.12% in calcium and citric acid 100ppm (Ca + CA2); 71.28% in calcium and ascorbic acid 50ppm sample (Ca + AsA1) and 78.4% in calcium and ascorbic acid 100ppm sample (Ca + AsA2) Fig. (1). As shown from Table (11), the major esters which comprised high concentrations in most samples were ethyl butanoate, ethyl hexanoate, ethyl acetate, hexyl acetate, methyl propanoate; ethyl-2-methyl butanoate and ethyl (E, Z) - 2,4-decadienoate. These results are in accordance with those previously reported by Chen *et al*., 2006 (a, b). The most odour active esters were ethyl butanoate, ethyl hexanoate, hexyl acetate and ethyl-2-methyl butanoate. The odour quality of these compounds is described as an apple, pear and fruit type (Acree and Arn, 2006). Also, we can found that methyl and ethyl (E, Z)-2,4-decadienoate comprised remarkable concentrations in all samples under investigation since ethyl (E, Z)-2,4-decadienoate reached 12.99% in ascorbic acid treated sample 100ppm (AsA2),which responsible for the typical flavour impact of pears (Kahle *et al*.,2005 and Diban *et al*., 2007). Esters are important for the sensory impression because of their type of smell and their low odour thresholds (Pohjanheimo and Sandell 2009).

Alcohols were the second major compounds in headspace volatiles of pear fruits. Their total yield was 8.07% in control sample; 7.7% in Ca(NO3)2 treated sample; 19.18% in CA1 treated sample; 53.08% in CA2 treated sample; 30.63% in AsA1 treated sample; 25.64% in AsA2 treated sample; 26.58% in (Ca + AsA1) and 17.17% in (Ca + AsA2) treated sample Fig (1). These high increases in concentrations of later six samples was attributed to the high increase in major alcohol 1-butanol which comprised 12%, 42.00%, 14.75%, 4.21%, 19.35%, 9.5% in these six treated samples, respectively Table (11), also 1-penten-3-ol comprised a high concentration in AsA1 and AsA2 treated samples since it recorded 6.7% and 9.56% respectively, whereas 1-Pentanol comprised a high concentration in CA2; AsA1 and AsA2 treated samples (7.29%, 6.59% and 4.84% respectively) Table (11). The drop in concentrations of total alcohols in both (Ca + CA1) and (Ca + CA2) to 6.67% and 6.81% the respectively (Fig. 1) is due to the very sharp decrease in concentrations of butanol; 1-Penten-3-ol and 1-Pentanol Table (11). These results are in accordance with Abd El Mageed and Ragheb (2006) who found that butanol was the predominate alcohol and the major compound in headspace volatiles of fresh apple juice (31.31%) and it was considered responsible for the characteristic flavour of fresh apple. 1-Hexanol and (Z)-3-hexen-1-ol comprised considerable concentrations in all samples under investigation Table (11). These two compounds have a typical resinous and green grass aroma, in fresh fruit flavours, they considered as degradation products of lipid (Roberts *et al*., 2004).

(E)-2-hexenal, (E)-2-heptenal and (E, E)-2, 4-decadienal were the three aldehydes identified in headspace volatiles of fresh (control) and in all fresh treated samples Table (1). Their total yield were 1.61% in control sample; 1.16% in Ca $(NO_3)_2$ treated sample; 1.93% in CA1 treated sample; 2.07% in CA2 treated sample; 7.06% in AsA1 treated sample; 9.44% in AsA2 treated sample; 1.24% in (Ca + CA1) treated sample; 0.98% in (Ca + CA2) treated sample; 1.08% in (Ca + AsA1) treated sample and 2.4% in (Ca + AsA2) treated sample Fig (1). The major aldehyde was (E)-2-hexenal

which comprised high concentrations (6.16% and 6.76%) in AsA1 and AsA2 treated samples respectively, (Table 1), It has leaf-like, apple like, green unrip-fruit (concentration dependent) note (Rychlik *et al*., 1998). α- Farnesene was the only sesquiterpene found in headspace volatiles of Le Cont pear with considerable concentration in fresh control and in all fresh treated samples, Table (11). It was the main volatile compound of Japanese pear peel, Shiota *et al.*, 1981.

Volatile components in headspace of Le Conte pear fruits cold stored for 75 days and 7 days at room temperature (marketing period) as affected by pre harvest treatments with calcium, citric acid and ascorbic acid alone or in combination:

The volatile components in headspace of Le Cont pear fruits after marketing period were identified and listed with their area percentages in Table (2).The total area percentages of the main chemical classes of volatile components in the headspace their fruits are shown in Fig. (2).

As shown from Table (12) volatile components varied considerably both quantitatively and qualitatively as effect of storage. Storage of Le Conte pear fruits for 75 days at 0°C + 7 days at 20 – 24 ° C cause a sharp decrease in both number of esters in most samples Table (12) and on their total yield Fig (2), but still esters constitute the predominant ratio of headspace volatiles of stored samples. These results are in accordance with that previously reported by Chen *et al*., 2006 a, b. Although the major esters in all fresh samples were ethyl butanoate, ethyl hexanoate, ethyl acetate and hexyl acetate we found that a very sharp decrease in ethyl butanoate and hexyl acetate and approximately absent for ethyl acetate (Table 12) which meaning a decrease in odour quality (Abd El-Mageed & Ragheb 2005 and Acree & Arn, 2006). Where as at the same time, as shown from (Table 12) we found that ethyl-2-methyl propanoate. Became the major ester in all stored samples also ethyl hexanoate and ethyl-2-methyl butanoate comprised a remarkable increase in most stored samples which compensate the decrease in the above mentioned esters. Takeoka *et al*., 1992 reported that ethyl-2-methyl butanoate, ethyl hexanoate and ethyl-2-methyl propanoate are important contributors to pear aroma. The importance ethyl-2-methyl butanoate is due to its particularly low odour threshold of 0.006 ppb.

Concerning alcohols their total concentration increased in all treated samples including control sample after storage period Fig (2). This increase is due to the high increase in ethanol (which is the major alcohol in most stored samples) and in 1-penten-3-ol in control sample (27.11% and 1.84%) respectively; in $Ca(NO_3)_2$ treated sample; (22.51% and 5.89%) respectively; in (Ca + CA1) treated sample (23.42% and 16.79%) respectively; in (Ca + CA2) treated sample (30.42% and 16.79% respectively; in (Ca + AsA1) treated sample (26.85% and 3.79%) respectively concerning the other samples the increase in total alcohols were due to ethanol, 1-penten-3-ol and 1-pentanol like CA1 treated sample (19.17%, 6.39% and 8.41%) respectively; whereas CA2 sample the increase in alcohols is due to ethanol and 1-pentanol (22.30% and 18.41%); also in AsA2 treated sample (15.64% and 35.39%) respectively. Whereas in (Ca + AsA2) treated sample the increase was due to increase in ethanol, butanol and 1-penten-3-ol (17.81%, 16.6% and 6.44%) respectively Table (12). Aldehydes and α-Farnesene showed remarkable decrease after storage in most samples Table (12). These results are in agreement with previously reported by (Zhang, 1990 and Chen *et al.*, 2006 (a, b)) who found that the volatiles of climacteric fruit accumulated after the respiratory climacteric, but decreased during storage. The major alcohols were ethanol, 1-penten-3-ol, and 1-pentonal. All samples retain good quality during storage period and the best ones storage were AsA1 treated sample and (Ca + AsA1) treated sample and Ca $(No_3)_2$ treated sample which have a highest content of esters which exhipt it more fruity aroma and cause it more acceptable for consumer.

CONCLUSION

As a conclusion from the results obtained in this work, spraying Le Conte pear trees with the combined treatments of Ca $(NO_3)_2$ + Citric acid at 100 ppm or Ca $(NO_3)_2$ + ascorbic acid at 100 ppm or the single treatment of calcium nitrate are suggested to be a good recommendation for keeping fruit quality under cold storage and in stimulate marketing period as well as the highest content of esters which exhibit it more fruity aroma and cause it more acceptable for consumer.

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2/1/2010

A comparative Study On Different Carbon Source Concentrations And Gelling A Gent On In Vitro Proliferation Of Pineapple (Ananas colossus)

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Abstract: The shoots regenerated from shoot tip of Pineapple Ananas Comosus Cv. Smooth cayenne) plantlets from the establishment stage were cultured individually on Ms medium supplemented with 200 mg/L 6- benzylamin opurine (BAP). Sucrose, fructose and mannitole with concentrations (20, 30 and 40 gl/L) were tested. Various kinds of gelling agent i.e. Agar and Gerlited were tested. Data indicated that all sucrose treatment (20, 30 and 40 g/L) enhanced the proliferation percentage and shoot number compared with other treatment and control except treatment of mannitol at 40g/L improved the shoot length only. Moreover, agarasa gelling agent was better than Gelrite at proliferation stage. The best shoot length, shoot number and growth percentage were obtained when 2.0 mgL Gelrite was added to the medium. [Nature and Science. 2010;8(5):127-130]. (ISSN: 1545-0740).

Key words: Carbon Source-gelling-pineapple

1. Introduction

Pineapple (Ananas Comosus) isatroical fruit crop which originated in south America, then was transferred to many countries. In Egypt, Pineapple is a promising new crop to be grown in plastic green house, particularly, in the newly reclaimed land, pineapple can not be propageated by seeds because most varieties show strong selfin comp atibility white the others is complete parthenocarpy. Also. Buds (which are produced limited number per plant) may beused in propagation (Wakasa 1989). Sorbitol or mannitol in combination with sucrose was found beneficial for inducing differentiation in long term culture. In case of the combination of two hexoses (glucose and fructose) at different concentrations, the best proliferation of cell was obtained at the combination of 30 g/L glucose and 30g/L fructose (Duong et al 2006). Gelrite as an alternative gelling a gent is clearly non toxic but results in hyperhydric (vitrified) tissues.

Inan effort to overcome these problems, the controlling mechanism found in agar was examines. Hydric soluble was shown to be affected by a non – gelling, cold water soluble constituent of a commercial agar, rather than by physical properties of the gel (**Nairn et al 1995).** This study was determine the effect of different carbon sucrose concentrations and gelling agent on in vitro proliferation of pineapple plant lets.

2 - Materials and Methods

This study was carried out at the tissue culture laboratory of pamology Dept., National research Center during the period from 2008 to 2009.

Shoot tip of pineapple (Ananas Comosus Cv. Smooth Cayenne) plantlets from the establishment stage were cultured individually on Murashige and skoog medium (1962) as a basal medium supplemented with 2.00 mg/L 6- Benzylamin opurine (BAP), during the proliferation stage. The pH of the media was adjusted to 5.7 and outclaved at 121 ^{0}C and 151 b/ In^2 for 15 minutes. The culture explants were incubated under 16 hours of artificial light (Fluorescent light at 30 uM/ sec) and 8 hours of darkness at average temperature of 28 ± 2^{0}C. Thus, the following experiments were carried out.

1- Effect of carbon source and its concentration

Sucrose, fructose and mannitol were added at 20, 30 and 40 g/L Ms medium supplemented with BAP and 2.0 mg/L. shoot number , shoot length, proliferation percentage and leaf number parameters were determined.

2- Effect of type of gelling agents

Agar at 7.0 g/L or Gelrite at 2.0g/L were used in Ms medium supplemented with BAP at 2.0 mg/L. Necrosis, shootnumber, shoot length , proliferation percentage, growth percentage, leaf number, and greening were determined.

Data and Calculations

Scores were given for necrosis and greening as follows: Negative results =1 , below average = 2, average = 3, above average = 4 and excellent =5 according to pottino (1981) .

Also, shoot number (numbers), shoot length (cm), proliferation as percentage, growth (as

percentage) and number of leaves /plantlet.
Treatment were arranged in a completely randomized design , each treatment was replicated three times according to snedecrodand and cochran (1980), each replicate involved 5 Jars, Pach contained a single explant. The obtained data were statistically analyzed and the means were differentiated according to Duncan multiple range test 1% level (**Duncan, 1955).**

RESULT AND DISCUSSION

1- Effect of carbon source and its concentration

Table (1) and Photo (1) show the effect of carbon source concentrations and growth and proliferation percentage of pineapple plantlets after 6 weeks. It is clear that all sucrose treatment (20, 30 and 40 g/L) gavev the maximum proliferation percentage and shoot number as compared with the other treatment and the control. However, mannitol gave significantly the highest leaf number followed by 40g/L fructose as compared with the other carbon sucrose concentrations and the control. Meanwhile, supplementation of the culture medium with mannitol at 40g/L level maximized shoot length in comparison to the other carbon source concentrations and the control under study. In general, summarizing the above results indicated that all sucrose treatments (20,30 and 40g/L) enhanced the proliferation percentage ad shoot number compared with other treatment and control except treatment of mannitol at 40g/L improved the shoot length only. In addition, both (30, and 40 g/L) sucrose surpassed others improving leaf number. This may be due to sucrose is geneally regarded as the best carbon source and is universally used as the principal energy source although in certain cases glucose and fructose may be substituted, but most other sugars are poor carbohydrate sources for the plant. These results are in coordination with the finding of Duong et al (2006) and Khafagy (2007 on Grand Naine Banana, they found that as a signle carbohydrate source in medium fructose exhibited a better growth when compared with sucrose or glucose. In case of the combination of two hexoses (glucose and fructose) at different concentrations the best proliferation of cell was obtained at the combination of 30 g/L glucose and 30g/L fructose.

2- Effect of gelling agent:

Data in table (1) and Photo (1) shoe the effect of using (celrite of agar on parameters of pineapple shoots at multiplication stage. It is obvious that using Gelrite was significantly more superior than using agar in increasing shoot number, shoot length, greening and growth percentage and decreasing necrosis. Meanwhile, suing agar was more effective in increasing proliferation percentage as compared with Gelrite. On the other han, statistical differences were nil between Gelrite and agar when leaf number parameter was considered.

Generally, the above results can recommended that Gelrite gave the highest shoot length, shoot number and growth percentage in pineapple plantlets. Meanwhile, agars as a gelling agent was better than Gelrite at proliferation stage. These results agree with the findings of Arrequi et al (2003) found that tuberizalion was higher when phytogel(TM) was used rather than Difco Bacto agar for all cultivars. Also Taha (2009) who found that Gelrite gave the highest average shoot number and shoot length compared with agar.

Table (1) : Effect of carbon sucrose concentration on growth and proliferation of pineapple plantlets after 6 weeks.

Measurement	Shoot number	Shoot length	Proliferation (%)	Leaf number
Control	$26.00^{\pm}$	10.33^{b}	40.33^{f}	4.92C
20 g/L sucrose	29.76^{C}	11.36^{ab}	97.63^{a}	3.67E
30 g/L sucrose	31.00^{b}	11.67^{ab}	99.00^{a}	4.30C
40 g/L sucrose	32.00^{a}	11.33^{ab}	100.00^{a}	4.30C
20 g/L fructose	25356^{f}	11.00^{ab}	73.30^{cb}	4.00D
30 g/L fructose	25.00^{g}	10.36^{b}	87.33^{b}	4.67C
40 g/L fructose	24.00^{h}	10.67^{b}	89.63^{b}	5.20B
20 g/L Mannitol	26.67^{f}	11.67^{ab}	54.67^{e}	5.67B
30 g/L Mannitol	26.99^{e}	11.67^{ab}	66.33^{d}	6.30A
40 g/L Mannitol	29.0^{d}	12.63^{a}	81.36^{b}	6.32A

Means followed by the same letter are not significantly different from each other at 1% level.

Photo (1): Effect of carbon source concentrations on grow th and proliferation of pineapple plantlets after 6 weeks.
A = 30 g/L sucrose
B = 30 g/L fructose
C = 30 g/L mannitol

Table (2): Effect of gelling agent on growth and proliferation of pineapple plantlets after 6 weeks

Measurement	Necrosis (secores)	Shoot number (N)	Shoot length (cm)	Proliferation (%)	Growth (%)	Leaf number (N)	Greening (secores)
Gelrite	1.00A	6.50A	1.37A	86.73B	88.33A	4.33A	5.00A
Agar	1.33B	4.36B	0.89B	99.80A	73.00B	4.00A	3.67B

Means followed by the dame letter are not significantly different from each other at 1% level.

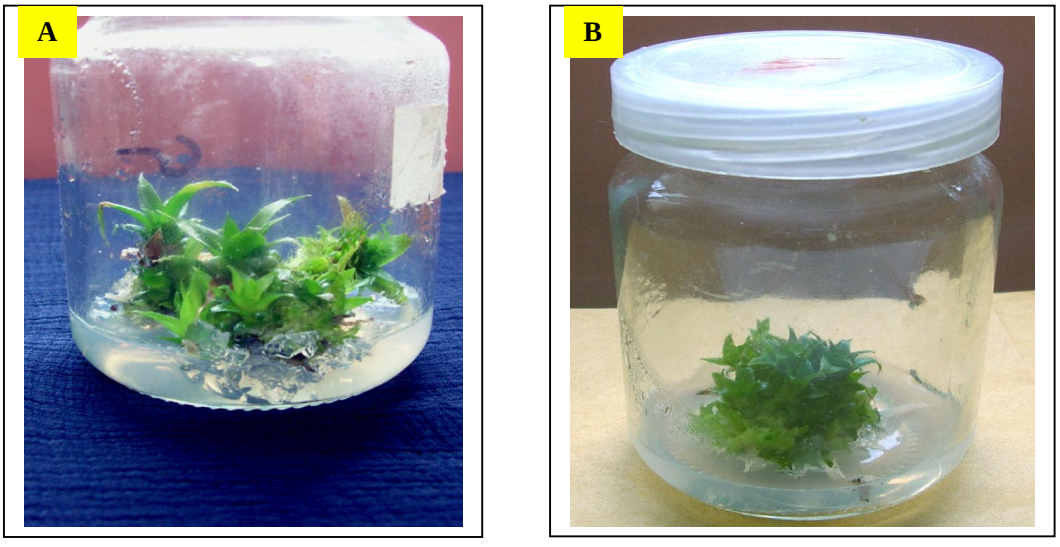

Photo (2): Effect of gelling agent on growth and proliferation of pineapple plantlets after 6 weeks.
A = 2g/L Gelrite.
B = 7 g /L Agar.

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3/3/2010

Relation between Glycoprotein and EA4 – Time Mechanism in *Secamia creatica*

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Abstract: ATPase (EA4) seems to measure time- interval as a diapause – duration timer in the seasonal cycle of the Sesamia cretica. A peptide named peptidyl – inhibitory needle (PIN) seems to regulate the time measurement of EA4. We characterize the EA4 in the first step to analyse its interaction with PIN. Matrix – assisted laser desorption/ ionization – time of flight- mass spectrometry shows EA4 of an equimolar complex with PIN. The binding affinity of EA4 for PIN is about 460nM, measured by surface plasmon resonance. Western blot analysis of EA4 with a variety of biotynylated lectins suggest that EA4 is a glycoprotein containing N- linked oligosaccharide. By enzymatic cleavage of the glycosyl chain the carbohydrate is revealed to be essential for the regulation of EA4- time measurement through the interaction with PIN. PIN holds the timer by binding to EA4, and the dissociation of the complex could constitute the cue for the time measurement. [Nature and Science. 2010;8(5):131-138]. (ISSN: 1545-0740).

Key words: Timer protein, Glycoprotein, Time – EA4, ATPase

1. Introduction

Biological systems to measure and mark elapsed time may be involved in the accurate timing of developmental events in cells (/). In principle, the systems could measure time in two very different ways. One possibility is that each cellular event is dependent on the previous events, and all events are linked together in a fixed order. Alternatively, cells may have an independent internal clock, together with devices to ensure that key reactions occur at certain times, like an alarm clock. Many important contributions have been made to identify such time-measuring mechanisms. In a self-sustaining clock that regulates daily rhythm, some key insights have led to the identification of putative clock components. Among the best candidates at present is the period protein (PER). PER is required for the proper manifestation of circadian rhythms (2. J). But we have comparatively few clues as to how cells are able to measure elapsed time in a long-term interval timer type of a biological clock. Recently, an ATPase called EA4 was found to have a possible capability of measuring a time interval as a diapause-duration timer in the seasonal cycle of the sesamia cretica (4.5).

The EA4 of sesamia cretica diapauses eggs exhibits a one- time transitory burst activation during the chilling of eggs to terminate diapauses. It is noteworthy that the activation is also observed in vitro. The sudden elevation of EA4 activity hi vitro was equivalent to that observed in vivo and was coincident with the chilling period that is known to be indispensable for diapauses termination. The in vivo and in vitro combination experiment demonstrated that the in vitro activation of EA4 occurred at the same rate as in viva activation (4, 5). EA4 is likely to possess some sort of inherent time-measuring activity.

The possible timer function of EA4 may comprise a built-in mechanism in the EA4 protein structure, and it may undergo a series of conformational changes with time (4). A peptide named PIN (Peptidyl Inhibitory Needle) may inhibit the conformational change of EA4 (6, 7). When EA4 was mixed with PIN, not only was the activation of the enzyme inhibited but also the clock-run of EA4 delayed to the period equivalent to that of PIN inclusion. It is conceivable that the interaction between EA4 and PIN is involved in the regulatory mechanism of the timer. Although these observations reinforce the validity of EA4 as an interval timer-type biological clock, the mechanism by which EA4 measures the time-interval and the question of how EA4 activation is related to the resumption of embryogenesis are still unresolved. In this context, it is crucial that the time-interval activation of EA4 be accomplished after Sephadex G-25 filtration in the EA4 purification (4, 5). Certain considerations about the Sephadex-results form the basis for the design of the following experiment.

EA4 is eluted later than the void volume fraction through the Sephadex G-25 column, even though its

Mr is about 20kDa (4). Thus, the Sephadex column works not as a gel-filtration but as an affinity chromatography.

This suggests a significant proportion of the EA4-mass may be composed of carbohydrate structures, which contribute to the delayed elution from the Sephadex column. This also suggests the EA4 carbohydrate moiety is involved in the mechanism of time measurement. Therefore, the glycoprotein characterization of EA4 is of importance in the research to address the regulatory EA4-timer mechanism. The present experiment is carried out in the first attempt in that direction. Our principal conclusions are that the carbohydrate is essential for the regulation of EA4-time measurement through the interaction with PIN and that the EA4-PIN into reaction may play a central role in the EA4 timer.

2 - Materials and Methods

Preparation of EA4 and PIN - C108(old) Sesamia cretica diapuase eggs were used. Eggs laid within 3h were collected at 25°C to obtain synchronous egg batches. Two days (50h) after oviposition, the eggs were washed with cold acetone and then EA4 was prepared. The preparation procedures are described in details in a previous paper (5), and included acetone powder from the eggs, heat treatment at 85°C, precipitation from 80% saturated ammonium sulfate and gel filtration through a Sephadex G-25 column with EA4 being eluted later than the void volume fraction. The collected EA4 fractions were removed from contaminated PIN by Centricon-10™ centrification (Amicon, Lexington, USA). PIN removal was accomplished by repeated dilutions and filtrations basically by the method of Kai et al. (5), the exception being that the final filtration Was achieved with HEPES buffer (25 mM HEPES, 12.5 mM Trizma Base, pH 7.4) containing 50 mM NaCI, 20 mM KC1, 1.0 mM EDTA, and 100 µ g/ml salmon testes DNA. Unless otherwise noted, all preparation procedures were conducted in a cold room (4°C) or in an ice-water bath.

The Sephadex G-25 filtration is the critical step in the purification of EA4 (4, 5). Therefore, the first part of EA4 preparation was carried to the step of the ammonium sulfate precipitation in one day. The last part of EA4 preparation from the Sephadex filtration was completed another day when enzyme activities were determined.

In the Sephadex G-25 gel filtration, PIN was contained mainly in the void volume fractions, and only in low concentration in fractions after the void volume. As suggested previously, EA4 was retained while PIN was filtered and recovered in the filtrate through Centricon- 10™ (Amicon, USA) centrification (7). PIN was obtained from the filtrate and concentrated by repeated centrification according to the method of Kai et al.(5, 7).

Incubation of EA4 and AT Pase assay - ATPase assay of the EA4 preparation was run at 25°C for 30 min or 3h in sterilized silicon-coated test tubes (5); the reaction HEPES buffer contained 50 mM NaCI, 20 mM KC1, 1.0 mM EDTA, and 100 µ g/ml salmon testes DNA. The time of EA4 activity-appearance was expressed as the time elapsed after the Sephadex G-25 step of the EA4 preparation as done previously (5).

Purification of EA4 for lectln-binding - The EA4 was further purified for lectin binding. First, the EA4 preparation as described above was separated and desalted utilizing disposable cartridges packed with silica bonded to C_{18} hydrocarbon (Sep-Pak C_{18} cartridges, Welters Associates). The cartridge was developed sequentially with Milli Q (Millipore) water containing 0.1% trifluoroacetic acid (TFA) (5 ml), 20% acetonitrile in 0.1% TFA (2 ml), 50% acetonitrile in 0.1% TFA (4 ml) and acetonitrile (6 ml). The 50% acetonitrile fraction was lyophilized and then dissolved in 100µ Milli Q water containing 0.05% TFA to apply to a 4.6mm x 25cm (5 µ m) YMC-Pack PROTEIN-RP (YMC, Japan) reverse-phase column in 0.05% TFA in Milli Q water (solvent A). The column was eluted with a stepwise gradient generated from solvent A and B (0.05% TFA in 100% acetonitile) at a flow rate of 1 ml/min, using the following time course: solvent A (5 min), 0 à 30% B (5 min), 30% B (5 min) and 30 à 45% B (15 min). The absorbance of the column eluate was monitored at 215 nm. The absorbance peak fraction with a retention time of 23 min was lyophilized and then dissolved in 100µ 25% acetonitrile in 0.05% TFA to reapply to YMC-Pack PROTE1N-RP. The second column was eluted at a flow rate of 0.8 ml/ml, using a gradient of 25% B (5 min) and 25 à 35% B (40 min). The absorbance peak of 33 min was collected and used for the following lectin blot analysis. All HPLC procedures were done at room temperature.

Electrophoretic transfer of proteins to PVDF sheets and lectin-binding for glycoprotein detection - SDS-PAGE was performed in Imm-thick slabs with a RAPIDAS Mini-Slab Electrophoresis Cell (ATTO, Japan) by the procedure of Laemmli (5). Briefly, the separating gels containing 12.5% (w/v) acrylamide, 0.5% NN"- methylenebisacrylamide, 0.1% SDS and 375mM-Tris/HCl, pH 8.8; the stacking gels contained 4.5% (w/v) acrylamide, 0.18% NN" methylenebisacrylamide, 0.1% SDS and 125mM-Tris/HCl, pH6.8. The running buffer was 25mM-Tris/19.2mM-glycine/0.1% SDS, pH8.3. After the run, the proteins were either transferred to a

polyvinylidene difluoride membrane (see below) or fixed and stained with silver (Silver Stain Kit Wako, Wako, Japan).

For the trophoelecretic transfer of proteins to polyvinylidene difluoride membrane (Immobilon PVDF Transfer Membrane, Millipore, USA) after completion of the run, the gels and identically sized membranes were placed in a Horiz-Blot Electrophoresis Apparatus (ATTO, Japan) containing electrode buffer (20% methanol/100mM-Tris/192mM-glycine, pH8.3). Transfer was performed at 120 mA for 100 min. After transfer, the membranes were cut longitudinally at 1 cm intervals. One of the sample strips and the strip containing the molecluar-weight makers were then stained in colloidal gold (LECTIN SENSOR Honen, Honen, Japan). In every experiment, the acrylamide gel was also stained with silver (Silver Stain Kit Wako) to assess the efficiency of transfer.

The protein on PVDF strips was detected with lectins. First, the strips were soaked in blocking buffer (10mM Tris- HCI, 0.053% Tween 20, 1% NaCI, pH 7.4) four times for every 15 min and then incubated with biotynylated lections (LECTIN SENSOR Honen) for 80 min (only for ConA, 90 min). After washing four times with blocking buffer I, the bound biotinyl conjugates were introduced to complexes of avidin-biotinyl-peroxidase (ABC) by incubation with horseradish peroxidase-avidin solution (HRP-avidin, Honen). The treated sheet was washed four times with blocking buffer I, then reacted with 3,3'-diaminobenzidine tetrahydrocholoride for visualization. After staining, the sheet was washed several times with water and dried.

Enzymatic deglycosylation of EA4 using N-glycanase – PNGase F (N-glycanase (EC 3.2.2.18), 25,000 units/mg of enzyme protein) was obtained from Boehringer-Mannheim (Germany). The enzyme of 10 units (10μ 1) was added to 1 nmol of EA4 (about 1 ml) in the HEPES buffer containing 50 mM NaCI, 20 mM KC1, 1.0 mM EDTA, 2.5% Triton X-100, 0,2% SDS and 1% 2-mercaptoethanol in final concentration. The mixture was incubated first at 5°C for 1 week and then transferred to 25°C to incubate for additional 1h. At appropriate intervals during the incubation, an aliquot was assayed for ATPase or ligand blots.

MALDI-TOF-MS - MALDI-TOF-MS spectra of EA4 were obtained using 2,5-dihydroxybenzoic acid as matrix and those for the mixtures of EA4 and PIN were obtained using a-cyano 4-hydroxycinnamic acid or 3,5-dimethoxy-4-hydroxy cinnapinic acid as matrix. Each μof sample (EA4, about 10 pmol; or the mixture, about 5 pmol EA4 and about 50 pmol PIN) was placed in a plastic bottle to which was added a two μ1 of matrix solution (saturated in acetone). The resultant solution was quickly transferred on a target (sample plate) and then allowed to crystallize in a refrigerator with spontaneous evaporation. Finally, the target was attached to a mass spectrometer. The MS spectra were measured on a TofSpec E mass spectrometer (MicroMass Co. Ltd., United Kingdom) in reflectron mode. Positive ions were generated by a pulsed nitrogen laser beam. The data were processed with a MassLynx program.

Surface plasmon resonance measurements - A carboxymethyl dextran lAsys cuvette (Affinity Sensors) was activated with 200 mM 1-ethy 1-3 (3-dimethylaminopropyi) carbodiimide (Pierce), 50 mM N-hydroxysuccinimide (Pierce) for 7 min and washed extensively with PBS (9). PIN at 27.38 μ.M was coupled to the activated cuvette in 10 mM sodium acetate buffer, pH 5.0, for 12 min at room temperature (330 arc seconds, 1.65 ng/mm^2). Uncoupled PIN was washed away with PBS and free amines blocked with 1 M ethanolamine (pH 8.5) for 3 min. A stable baseline was established for 10 min before data collection. All binding experiments were performed with EA4 over a range of concentrations from 2.2 to 8.8 ^m in volumes of 70 μ1. The binding surface was regenerated between binding measurements using 30 mM HC1 with no decrease in extent measurements over the duration of an experiment. Data were collected over 3-s intervals and analyzed using the FASTfit™ software provided with the lAsys instrument.

3. Result

Purification of EA4 and detection of glycoprotein by lectin binding -EA4 obtained by the rechoromatography on the YMC-Pack PROTEIN- RP column (the absorbance peak fraction with a retention time of 33 min) showed a single band by silver staining on SDS-PAGE (Fig. 1). The MALDI-TOF-MS spectrum of the EA4 contained a single peak at m/z 17,387 (Fig. 2). A smaller m/z peak on the MS spectrum (8,694) is probably a double charged ionized species of EA4. Thus, EA4 was applicable to lectin binding analysis.

We tested five different lectins to bind to the EA4 (Fig. 3). All five, SSA, DSA and Con A, and to a lesser extent AAL and RCA120, led to detection of the band. AAL and RCA120 are known to bind fucose and galactose residues, respectively. The latter RCA120 reaction is reduced by Sia α (2-6) Gal, the presence of which being indicated by SSA reaction.

The high intensity of the DSA and Con A reactions indicated that EA4 contained Gal β(l-4)GlcNAc units and trimannosyi core structures of Man α l-6(Man *a*

1-3) Man. Essentially the same lectin reactions were observed with the EA4 preparation obtained from the Sephadex G-25 filtration (data not shown).
Although the precise structure of the carbohydrate chains is uncertain until further analysis, EA4 was characterized as a glycoprotein.

Enzymatic deglycosylation of EA4- We conducted deglycosylation experiments to address the function of the carbohydrate moiety involved in the mechanism of time measurement. To cleave glycosyi chains, enzymes such as Endo H, Endo F and PNGase F were first applied to EA4 obtained by Sephadex G-25 filtration. Treatment of EA4 with the former two enzymes, however, resulted in no decrease in molecular mass and no decrease in Con A binding (data not shown). In contrast, treatment with PNGase F at 37°C for 20h completely abolished Con A binding to EA4 and resulted in a decrease in the EA4 molecular mass by approximately 2.5 kDa (Fig. 4). Since PNGase F is known to release proximal N-acetylglucosamine linked to Asn of peptides, the whole glycosyi chain may be cleaved. Irrespecitve of this effectiveness, the incubation condition for the deglycosylation was not applicable to the present timer-investigation. As discussed below, some modification of the deglycosylation condition was necessary.
The one time-transitory burst activation of EA4 shows a temperature dependency in solution (5, 10*)*. Without PIN, EA4 suddenly increased reaching maximal activity in about 7h at 25℃ (Fig. 5 and Ref.5) instead of taking about 2 weeks at 5°C (4, 7). Therefore, the temperature of 37℃ was too high and the duration of 20h was too long for the present experiment. Accordingly, various concentrations of deglycosylation by PNGase F were tested at various temperatures and with varying incubation times. Consequently, a temperature-incubation procedure was established: at 5°C for 1 week followed by additional 25°C for 1h in a reaction mixture of 1 nmol EA4 and 10 units PNGase F. By the incubation, EA4 abolished Con A binding and decreased molecular mass by 2.5 kDa (Fig. 4). Furthermore, EA4 did not complete the time measurement in that incubation period as mentioned in detail below.

Effect of enzymatic deglycosylation on EA4-timer - We studied whether the deglycosylated EA4 could measure the time interval. We had to carry the experiment with special care to the following two points. One of them is that the activation of PIN free-EA4 takes place virtually instantaneously at higher temperatures. For the time-run of EA4, as suggested previously, a one-day incubation at 5°C is equivalent to 30 min at 25°C (5). Therefore, an incubation for 1 week at 5°C followed by 1 h at 25°C was equivalent to one of 4.5h at 25°C. Additionally, about half a day at 5°C, equivalent to 0.25h at 25oC, was required for PIN removal and EA4 concentration by Centr icon-spin. Therefore, about 5h elapsed before the ATPase assay of deglycosylated EA4. Another point is that the coordination of clock-run in each EA4 molecule is disordered by the somewhat complicated treatments for deglycosylation. It was difficult to obtain data points to define the peak of EA4 appearance. Because of these two points, the ATPase activity of EA4 was measured by the method of one-time integration (5) instead of a time-course assay at intervals of 30 min. In the integration assay, amounts of liberated phosphate were detected during successive 3h intervals after 5h, 5-8h and 8-llh incubation. The 5-8h time span was expected to coincide with EA4 appearance and the 8-llh time span was expected to coincide with EA4 disappearance (Fig. 5).
As shown in Figure 6, while the amount of liberated phosphate was low during the incubation done between the last 8-llh, high amounts were detected between 5-8h. EA4 exhibited activity only between 5-8h after the onset of incubation, even though the activation period of each EA4 protein molecule should be very short, and the detected amount of phosphate was the integrated amount liberated during that period. It is worth noting that the high amounts were detected with both native and deglycosylated EA4 during the same 5-8h time span. The carbohydrate moiety may not directly contribute to the time measurement of EA4.

Significance of carbohydrate moiety on the PIN-regulation - To examine the function of the carbohydrates, in the next experiments PIN was added to EA4. A 76 µ1 PIN fraction of Centricon-3™ retentate (about 150 µg protein equivalent) was mixed with 40 *a* \ EA4 (about 0.8 µg protein) and assayed for ATPase activity. As shown in Figure 7, the activity of native EA4 during the 5-8h time span after incubation was strongly inhibited by PIN. This result is in accord with the previous finding that PIN inhibits EA4 (5, 7). On the other hand, no inhibition was observed when deglycosylated EA4 was mixed with PIN; deglycosylated EA4 exhibited essentially the same activity as without PIN. It is apparent that the carbohydrate moiety is indispensable for the execution of inhibitory activity by PIN.

Interaction between EA4 and PIN - PIN peptide was synthesized chemically and the mixture of the PIN and EA4 was analysed by MALDI-TOF-MS (Fig. 8). Since the Mr of PIN is 4,618 Da (6), Figure 8

demonstrates that EA4 forms an equimolar compex with PIN. A smaller m/z peak on the MS spectrum (14,266) is probably a by-product in the chemical synthesis of PIN. This is the first direct binding datum, and it has become appear ent that 1 mol of PIN is bound to 1 mol of EA4. To investigate further the interaction between EA4 and PIN, the binding affinity was measured by surface plasmon resonance. Binding of EA4 to immobilized PIN was measured over a range of EA4 concentrations (2.2-8.8 μM).

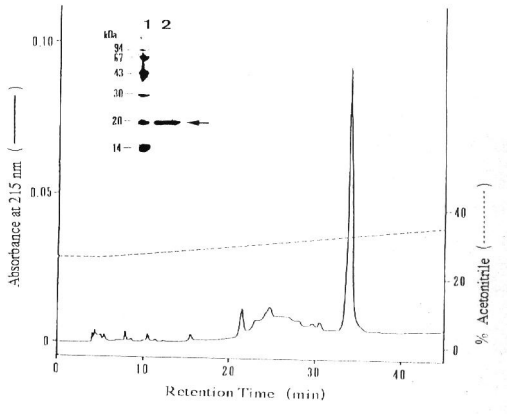

Fig. 1. Isolation of EA4 by RPC on YMC-Pack PROTEIN-RP column. Chromatographic conditions; 25% B for 5 min, 25-35% B linear gradient for 40 min at a flow rate of O.Sml/min, where solvent A was aqueous containing 0.05%(v/v) trifluoroacetic acid and solvent B was 100% acetonitrile containing 0.05% trifluoroacetic acid. Inset; SDS-PAGEof the isolated EA4 on 12.5% gel (lane 1, molecular mass standards; lane 2, isolated EA4; the arrow indicates the position of EA4; numbers indicate molecular mass on kDa).

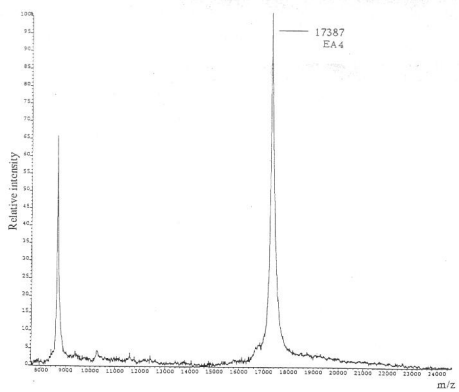

Fig. 2. MALDI-TOF-MS spectrum of EA4 isolated by the rechromatography on YMC-Pack PROTEIN-RP column. The spectrum was acquired in the reflectron mode using 2,5-dihydroxybenzoic acid crystals as matrix.

The association rate constant (K_a $4.370x10^3M^{-1}s^{-1}$) was obtained by plotting the measured K_{on} versus EA4 concentration (Fig. 9). The dissociation rate constant ($K_d 2.003x10^{-3}s^{-1}$) was obtained directly from dissociation experiments. These values were used to calculate a K_D of 460nM for the EA4-PIN interaction. The equilibrium constant in the order of 10^{-7}M provides significant information regarding the timer regulation as discussed below.

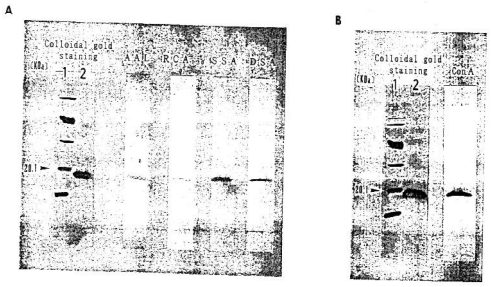

Fig. 3. Lectin blot analysis of EA4. EA4 was subjected to SDS-PAGE and electrophoretically transferred to PVDF membrane, which was then cut into strips. The strips were exposed to biotinyl-AAL, RCA120, SSA, DSA (panel A) and Con A (panel B), followed by reaction with avidin-biotinyl-peroxidase complexes and staining with 3,3-diaminobenzidine. The strip containing the moled uar-weight makers (1) and one of the sample strips (2) were stained in colloidal gold (protein standards, the same molecular masses as shown in the Fig. 1).

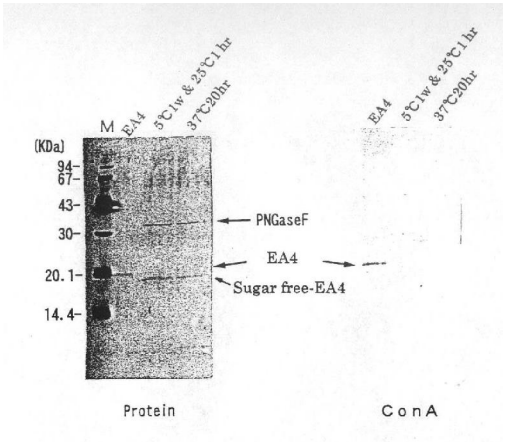

Fig. 4. Western blots of native and deglycosylated EA4 after treatment with PNGase F at 37°C for 20 hr or 5°C for 1 week followed by additional 25°C for 1h. Proteins were stained by colloidal gold. M, protein standards. Numbers indicate molecular mass on kDa. Glycoprotein was detected with biotinyl-Con A, followed by reaction with avidin-biotinyl-peroxidase complexes and staining with 3,3'-diaminobenzidine.

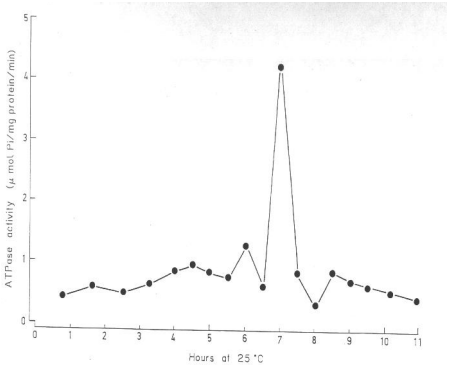

Fig. 5. Time-interval activation of EA4 in ATPase activities during incubation at 25°C for 11h. The EA4 preparation was freed from PIN fraction by Centr icon-spin (see details in the text) and the ATPase activities were determined at about 30 min intervals during the incubation. The numbers on the abscisa indicate the time elapsed after the Sephadex G-25 step of the EA4 preparation. (From reference 5.)

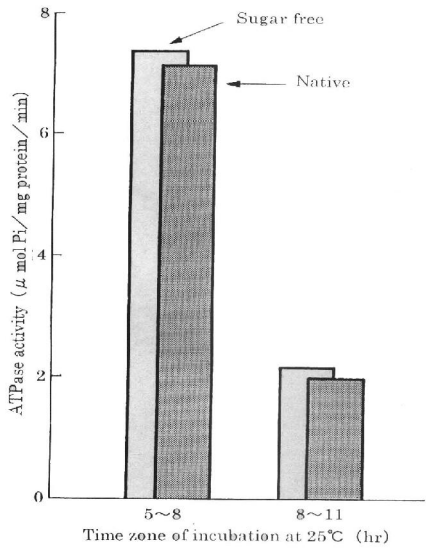

Fig. 6. Changes in ATPase activities measured by the method of one-time integration during incubation of sugar-free EA4 at 25°C. Sugar-free EA4 was obtained by PNGase F treatment. ATPase assays were carried at 25°C during successive 3h time intervals. The time intervals were expressed by the equivalent time elapsed after the Sephadex G-25 step of the EA4 preparation (the same applies to the Fig. 7).

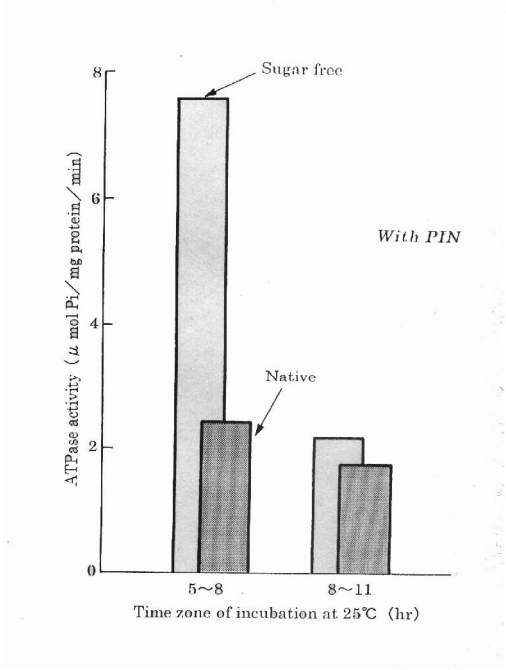

Fig. 7. Significance of sugar chain in PIN-effect on the time-interval activation of EA4. EA4 was mixed with PIN and the ATPase activity was assayed by the method of one-time integration.

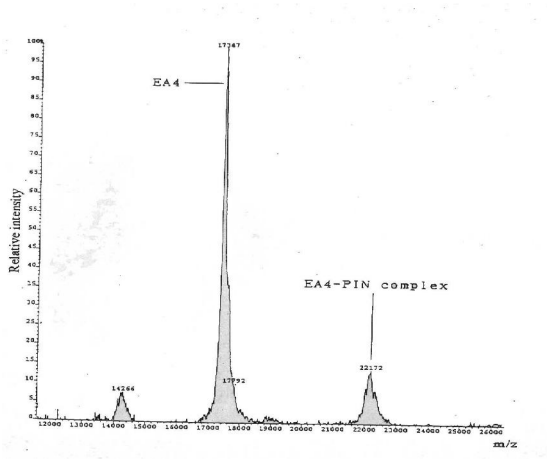

Fig. 8. MALDI-TOF-MS spectrum used for the determination of EA4-PIN complex. Mixture of EA4 and PIN was deposited on a thin layer of α-cyano 4-hydroxycinnamic acid crystals and the spectrum of them was acquired in the reflectron mode.

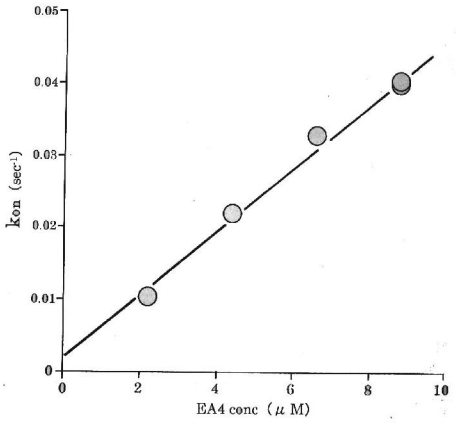

Fig. 9. Affinity measurement of EA4 and PIN. The binding affinity of EA4 and PIN was measured by surface plasmon resonance. The PIN was immobilized on the surface of the lAsys cuvette, and the immobilized PIN was incubated with EA4 over a range of concentrations (2.2-8.8 μM).Shown is a representative plot from three experiments.

4. Discussion

EA4 may have the possible capability of measuring a time interval in accordance with development (4). PIN seems to regulate time measurement (6, 7). PIN may also be involved in the mechanism by which the EA4 timer operates only in cold. The interaction between EA4 and PIN is important in the timer-mechanism, and the results of this study provide evidence that 1) PIN binds to EA4 forming an equimolar complex, 2) EA4 has carbohydrate moieties and 3) the carbohydrate is essential for the assembly of a high affinity PIN-binding site within the timer motif of the EA4 structure. Although the exact mechanisms of time-mesurement and time-regulation must be considered speculative until further analysis is carried out, it is revealed that formation of a regulatory substructure is involved in those mechanisms.

The equilibrium constant for the EA4-PIN interaction provides significant information regarding the mechanisms. The equilibrium constant is within the physiological concentration range for these peptides. Besides, the constant in the 10^7 molar range means that the affinity is not too high and, at the same time, not too low. These are consistent with the hypothesis that the association-dissociation inter conversion may be involved in the regulatory mechanism in the cell. One possible explanation is that EA4 in eggs may originally be in complex with PIN and that environmental signals may induce the dissociation of the complex to localize EA4 and PIN. The dissociation could constitute the cue for time measurement to EA4 activity burst, which in turn initiates new developmental programs. Whether or not the association and dissociation is involved in the regulatory mechanism is now under investigation in this laboratory.

The result of lectin binding analysis indicates that N-linked oligosaccharides may be involved. Based on the detected carbohydrate compositions, the lowest molecular mass of the oligosaccharides is estimated to be about 2.5 kDa, although no definitive strucuture has been established. PNGase F is an endoglycosidase known to release proximal N-acetylglucosamine linked to Asn of peptide. Treatment of EA4 with the PNGase F resulted in a decrease in molecular mass of about 2.5 kDa. In addition, only one glycosylation site (Asn-Ile-Thr) has been detected in EA4 (unpublishied results). Taken together, these findings suggest EA4 may contain a single oligosaccharide chain. Determination of the precise structure of the oligosaccharide and EA4, itself, is critical in the timer investigation and are now in progress.

Eggs of animals seem to undergo carefully timed sequence of events controlled by endogenous timing mechanisms. To control when genes act, cells must be able to measure time. Examples of a timed event in development are provided by the regulated apoptosis at the onset of gastrulation'in *Xenopiis* embryos (77, 72, 73, 14). The timing and execution of the maternal cell death program is set at fertilization and is independent of the types of stress applied on cell cycle progression or on *denovo* protein synthesis. There are long-term mechanisms which trigger the events at the proper time. EA4 might be one of candidates for the timer-protein as a Time Interval Measuring Enzyme (TIME).Although many scientists working to understand diapauses by using molecular biology techniques Gkouvitsqs, et al (2009)in Secamia creatica ,also Qiu, et al (2007) in crustacean, Kion andDenlinger (2009) in Culex pipiens , but this system of analysis is still the cheaper and saving time, to understand the programmers of diapouse, Also with emphases on Egyptian secamia creatica races in Egyptian phona.

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Flavone-5-O-Glycosides from *Cheilanthes dalhousiae* (Hook)

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Abstract: Fern fronds (about 500gm) of Cheilanthes dalhousiae Hook. Vouch. Sp. No. 21 was collected from Pindari glacier routes (2200-2800m above sea level) of Almora district of Uttarakhand state. It was extracted with acetone-water (1:1, V/V) and extract was concentrated under reduced pressure until H_2O layer (up to 50ml) remained. The H_2O layer was partitioned with CH_2Cl_2, EtOAc and BuOH Successively. The CH_2Cl_2 fraction gave antibacterial tests against Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus epidermidis and Escherichia coli by the standard method of disc-diffusion using DMSO-d_6 solution of CH_2Cl_2 residue impregnated on Whatman No. 3, paper disc (6 nm) and base plates containing 10ml MH agar. Antibacterial activity was expressed as the ratio of the inhibition zone produced by CH_2Cl_2 extract and the inhibition zone caused by the reference, neomycin (2µg). No antibacterial activity was observed in ethyl-acetate and n-butanol fractions. EtOAc fraction was evaporated to dryness and residue obtained was dissolved in MeOH. The MeOH soluble of EtOAc fraction was fractionated on Whatman No. 3 chromatographic papers using BAW (n- BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer) as an eluent. Two blue UV fluorescent flavone-5-O-glycosides: Quercetin-3-methyl ether-5-O-glycoside and Kaempferol-5-O-(6"-O-malonyl)-glycoside were isolated by RPPC from EtOAc fraction of acetone-H_2O (1:1) extract of fern fronds of Cheilanthes dalhousiae. The structural elucidation of the compounds was carried out by UV, ^{1}HNMR and MS spectral studies. [Nature and Science 2010;8(5):139-143]. (ISSN: 1545-0740).

Keywords: Kumaun Himalaya, *Cheilanthes dalhousiae* (Hook), Medicinal plants

1. Introduction

Cheilanthes dalhousiae Hook, (family Sinopteridaceae), is widely distributed in Kumaun Himalaya from 2000-3000 m. Genus *Cheilanthes*, a member of leptosporangiate group of highly advanced ferns, comprises 130 species with cosmopolitan in distribution and its 9 species have been reported from Kumaun Himalaya (Pande, 1990; Pande *et al.*, 1997). In morphological point of view, *Cheilanthes dalhousiae* is characterized by the presence of deltoid-lanceolate and tripinnafied lamina (15x5) and 2, 3 fronds arise from a single rhizome.

Since Vedic period, ferns have been recognized as a medicinal plants in Ayurvedic, Unani and Chinese systems of medicines (Kritikar and Basu, 1935). A number of ferns have been used for curing diseases like cough, bronchitis, asthma, tuberculosis, typhoid and ulcers (Chopra *et al.*, 1958). Therefore various fern species have previously been screened for antimicrobial, antimalarial, antitumoral and anticancer activities (Banerjee and Sen, 1980). Some high altitude species of *Cheilanthes* have been identified as a traditional medicinal ferns (Lal *et al.*, 1944; Chopra *et al.*, 1956; pande *et al.*, 1989).

Literature survey revealed that high altitude species of genus *Cheilanthes* are still awaited for the screening of antibiotic activities and active constituents. Although, flavonoidal constituents have been reported (Erdtman *et al.*, 1966; sunder *et al.*, 1974; Wollenweber *et al.*, 1980; Scheele *et al.*, 1987; Imperato, 1989; Wollenweber and Roitman, 1991; Tandon *et al.*, 1991; Khetwal and Verma, 1983, 1984, 1986, 1990; Khetwal *et al.*, 1985, 1986, Mishra, 2008, Mishra and Verma, 2009[a-e]) from medicinal plants. Present communication reveals the screening of antibacterial activity, isolation and structural elucidation of two flavonoid compounds from *Cheilanthes dalhousiae*.

2. Material and Methods

2.1 Plant Material

Fern fronds of *Cheilanthes dalhosiae* were collected from Pindari glacier routes (2200-2800 m.) of Almora district (Uttarakhand state). Its authentification was made by the help of taxonomist of Botany Department, Faculty of science, DSB Campus, Nainital and vouchers specimen No. 21, was deposited in the Botany Department of Kumaun University, SSJ Campus, Almora (India).

2.2 Extraction of Plant Material

About 1kg air dried fern fronds were extracted with Acetone: Water (1:1) by cold percolation methods for three days. The aqueous-acetone extract was decanted and concentrated under reduced pressure until only H_2O layer (75 ml) remained. It was partitioned with CH_2Cl_2, EtOAc and BuOH successively.

The CH_2Cl_2 fraction was evaporated to dryness in Rota-evaporator at 30°C. The residue was adsorbed on cellulose column (Merck grade) and eluted initially with H_2O and then increasing polarities with HOAc. On eluting column with 10% HOAc, three dark purple

fluorescent bands were observed on column with UV light (360 nm). All the purple fluorescing bands on column were eluted and combined.

The combined fraction was concentrated and residue obtained was used for the characterization of flavonoidal constituents by the help of 2DPC and screening of antibacterial activity. A part of residue was dissolved in MeOH and examined for flavonoidal compounds on 2DPC using BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer) and 15% HOAC, as a developing solvent. The dried and developed chromatogram was inspected with UV light (360nm). Eight UV fluorescent spots were discernible on PC. Out of eight spots, five purple UV fluorescent spots were identified as a flavonoids on the basis of their colour reaction with NH_3, UV+NA+PES, UV+$AlCl_3$ and UV+$ZrOCl_2$ (Mabry *et al*., 1970; Homberg and Geiger,1 980, 1983; Markham, 1982, 1989). Using BAW and 15%HOAc as developing solvents on 2DPC, the high motilities of the purple UV fluorescent flavonoidal compounds were observed. On the basis of colour reactions and Rf values in BAW and 15% HOAc solvent systems, these flavonoidal constituents were characterized as a 3-O- methoxylated flavonols (Mabry *et al*., 1970; Fang *et al*., 1985a, 1985b, 1986; Markham, 1989; Liu *et al*., 1997; Mousallami *et al.*, 2002).

The major portion of the residue which comprises five purple UV fluorescent compounds on 2DPC was dissolve in MeOH and methanolic solution was used for the isolation of flavonoidal compounds. On RPPC of the methanolic solution using BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer) as an eluent, five purple fluorescent bands were observed on PC with UV light (360nm). Each band was cut and eluted with 70% MeOH. The isolate of each compound was finally purified on Sephadex LH-20 column eluting initially with H_2O and then decreasing polarity with MeOH. Each isolate was examined for antibacterial activities by the standard disc-diffusion method (Rahalison *et al*., 1991, 1994; Saxsena *et al.*, 1995). From these isolates, five compounds representing structure (A), (B), (C), (D) and (E) were isolated. Using BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer) as an eluent the compound (A), Rf 90 and compound (E), Rf 72, were identified as a faster moving and slower moving component respectively on paper chromatogram.

3. Antibacterial Screening of Each Isolate

Each isolate was screened for antibacterial activities against three different strains of bacteria *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus epidermis* obtained from CMI, London. Bacteria were maintained on Mueller-Hinton (MH) Nutrient Agar (NA) at 4°C. Molten MH Agar (10 ml) was inoculated with a broth culture (1 ml containing 10^6-10^8 bacteria, ml) of the respective bacterial strains and poured over base plates containing 10 ml MH Agar in sterile 9cm Petri-dishes. Whatman No. 3 chromatographic paper was cut in a disc shape. The residue of each isolate was dissolved in DMSO solution. The paper disc was impregnated with the DMSO solution of sample. The impregnated paper disc was hot air dried. The sample impregnated discs were placed into the seeded top layer of the agar plates. Each plate contained four paper discs with each isolate and a disc with a neomycin control (2 mg). Each isolate was tested in quadruplicate. The base plates were incubated at 37°C for 12hours, where after inhibition zones were recorded. After incubation of the base plates were inspected with visible and UV light. The antibacterial activity of these flavonoidal compounds is being summarized as follows:

Compound (A): The isolate of compound (A) gave zones of inhibition with the *Staphylococcus epidermis, Pseudomonas aeruginosa* and zones of inhibition was not detected with *Bacillus subtitles*.

Compound (B): No any zones of inhibition were observed with *Staphylococcus epidermis, Pseudomonas aeruginosa* and *Bacillus subtitles* strains.

Compound (C): No zones of inhibition were observed with *Staphylococcus epidermis, Pseudomonas aeruginosa* and *Bacillus subtitles.*

Compound (D): It gave zones of inhibition with the bacterial strains *Bacillus subtitles* but no zones of inhibition were detected with the *Staphylococcus epidermis, Pseudomonas aeruginosa.*

Compound (E): Compound (E) did not give any zones of inhibition with these three bacterial strains *Bacillus subtitles, Staphylococcus epidermis* and *Pseudomonas aeruginosa.*

Thus, out of the five purple UV fluorescent compounds isolated from 10% HOAc fractionation of dichloromethane extract on cellulose CC, only two compounds (A) and (D) gave zones of inhibition with the tested bacterial strains.

The EtOAc fraction of aqueous-acetone extract of fern fronds of *Cheilanthes dalhousiae* was evaporated to dryness in rotatory evaporator at 35°C. The residue was dissolve in MeOH and chromatographed on Whatman No. 3 strips (10). On RPPC of the fraction using BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer) as an eluent, a blue fluorescent band observed on PC with UV light at Rf 60 was eluted with 70% MeOH. The aqueous methanolic elute was concentrated and residue was adsorbed on Sephadex LH-20 column and eluted with H_2O and then decreasing polarity with MeOH. On eluting column with 20% MeOH, two fluorescent blue compounds observed on column with UV light were eluted separately. The compound (I) and (II) were isolated. The structural elucidation of these two compounds is being summarized as follows:

The compound (I) and (II) appeared as a blue fluorescent on PC under UV light and changed to yellow-green with NH_3 vapors, indicating the presence of 4'-hydroxyl group and substituted 5-OH group (Mabry *et al.*, 1970). When a cellulose TLC plate was sprayed with Naturstoffreagent and 5% PEG solution, the compound (I) turned orange and compound (II) turned yellow, indicating presence of orth-di-hydroxyl group in the B-ring of compound (A), 4'-hydroxyl group in B-ring of compound (I) and (II).

4. Structural determination

4.1 Structural determination of compound (I): The compound (I) gave positive Feigl spot test for sugar. It was hydrolyzed with 12% HCl for1hour at 60°C, gave a dull purple UV fluorescent aglycone in the organic layer and a sugar component was present in the aqueous layer. The aglycone was isolated by paper chromatographic method. The MS of aglycone exhibited a molecular ion at m/z 316 (100%) for $C_{16}H_{12}O_7$ in accord a flavone containing four hydroxyl and one methoxyl group. Flavone appeared as a dark purple fluorescent on paper chromatogram with UV light and changed to lemon yellow with NH_3 vapors indicating the presence of 5- and 4'-hydroxyl groups. When a cellulose TLC plate was sprayed with Naturstoffreagent (NA) reagent, the spot turned orange, indicating the presence of orth-di-hydroxyl group in B-ring. The dark purple fluorescent spot of the compound on PC when sprayed with 2% $ZrOCl_2$, gave a bright yellow colour which disappeared on addition of 2% citric acid and H_2O, indicating 4'-oxygenated flavonol bearing a free hydroxyl at the 5-position and the substituted one at the 3- position (Liu *et al.*, 1997; Mousallami *et al.*, 2002). The dark purple fluorescence of the compound was turned to dull yellow fluorescence, when the alcoholic solution of the compound treated with HI reagent, indicating the OCH_3 group at C-3 position.

The dull yellow UV fluorescent compound, which obtained after the treatment of aglycone with HI reagent was identified as a quercetin by its CoPC using four solvent systems, BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer), 30% HOAc, 50% HOAc and t-BAW (t-BuOH-AcOH-H_2O, 3:1:1). Thus, the aglycone was identified as 3-methoxy-quercetin.

The aqueous hydrolysate was repeatedly evaporated to dryness and residue was dissolved in isopropanol and chrmatographed on Whatman No. 1 PC, using BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer) as a developing solvent. The dried and developed chromatogram was sprayed with benzidine reagent, a brown spot at Rf 21 appeared was identified as a glucose by its CoPC using two solvent systems, BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer) and BuOH saturated water. Thus, an acid hydrolysis of compound (I), gave an aglycone, quercetin-3-methyl ether and a sugar, glucose. The compound (I) appeared as a blue fluorescent on PC under UV light while its aglycone gave dark purple fluorescent spot on PC with UV light, indicating the glucose moiety is attached with 5-OH group (Mabry *et al.*, 1970; Markham, 1989).

Thus, on the basis of colour reactions, acid hydrolysis with 12% HCl and HI, the compound was identified as a quercetin-3-methyl ether-5-O-glucoside (Fig. 1).

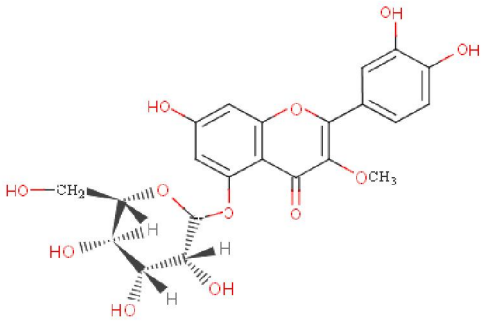

Fig. 1

Further, the compound (I) was identified on the basis of its UV spectral datas in MeOH and shifts obtained with various diagnostic reagents (Table 1) and 1

HNMR spectra in DMSO solution (Table 2).

Table 1**:** UV spectra of compound [I] in MeOH (λ_{max}, nm)

Shift Reagent	Shift (λ_{max}, nm)		
	band II	band I	
MeOH	252	351	
NaOH	263	361	402
$AlCl_3$	260	375	
$AlCl_3$+HCl	251sh	350	
NaOAc	268	320	
NaOAc+H_3BO_3	255	373	

4.2 Structural Determination of Compound (II): The compound appeared as a dull blue fluorescent spot on PC under UV light and changed to lemon yellow with NH_3 vapours and NA reagent indicating a 4'-hydroxyl group but no ortho-di-hydroxyl group in the B-ring and presence of a substituted 5-OH group in the A-ring (Mabry *et al.*, 1970). Acid hydrolysis of the compound gave a dark purple UV fluorescent aglycone which was identified as kaempferol-3-methyl ether and a sugar, glucose. Alkaline hydrolysis of the compound with 2N NaOH at room temperature for 120 minutes, gave kaempferol-3-methyl ether-5-O-glucoside and malonic acid. Both the constituents were identified by their respective authentic by CoPC using three solvent systems, BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer), t- BAW (t-BuOH-AcOH-H_2O, 3:1:1) and BEW (n-BuOH-EtOH-H_2O, 4:1:2.2, upper layer).

Table 2: ^{1}HNMR of compound [I] in DMSO-d_6, 400MHz

Shift (δ)	Multiplicity	Identification
7.53	1H, d, J=2.0Hz	H-2'
7.40	1H, dd, J=2.3Hz and 2.0Hz	H-6'
6.89	1H, d, J=8.0 Hz	H-5'
6.75	1H, d, J=2.0 Hz	H-8
6.63	1H, d, J=2.0 Hz	H-6
4.76	1H, d, J=7.8Hz	glucose anomeric proton
3.2 to 3.7	6H, m	glycosyl proton

^{1}HNMR of the compound (II) gave two symmetrical doublets each with J=8.0 Hz, at δ 8.08 (2H, d, for H-2' and H-6') and at δ 6.89 (2H, d, for H-3' and H-5') and two down field meta coupled protons each with J=2.0 Hz, at δ 6.85 (1H, d, J = 2.0, +1.8) and δ 6.49 (1H, d, H-6) appeared in aromatic region. In aliphatic region, a singlet appeared at δ 3.15, is identified for methylene protons of malonic acid and anomeric proton singlet appeared at δ 4.77 while remaining protons of sugar appeared as multiplet between δ 3.2 to 3.9. On comparing the ^{1}HNMR of kaempferol-5-O-glycoside with the ^{1}HNMR of compound (II) in sugar region, the down field shift of H-6' and H-6'' proton (H-6', 3.9 and H-6", 4.2) of compound (II) clearly indicated that the malonyl group substitutes C-6 OH of glucose sugar. Thus, the compound (II) was identified as kaempferol-3-methyl ether -5-O-β-(6'-malonyl) glycoside (Fig.2).

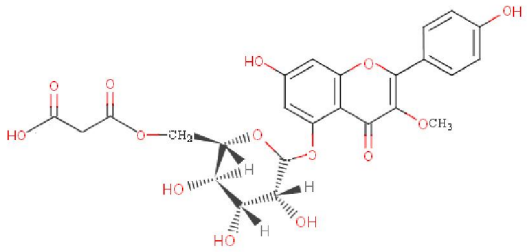

Fig.2

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Physiological Studies on the Risk Factors Responsible for Atherosclerosis in Rats

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Abstract: Despite the well established correlation between hypercholesterolemia and coronary artery disease (CAD), a substantial body of evidence challenge this relationship. The study aimed to examine whether hyperlipidemia per se constitutes the principal risk factor for atherosclerosis or just a coordinator to other critical mediators. Hyperlipidemia was produced by feeding rats with high-fat diet for two months. The occurrence of hyperlipidemia was determined by measuring lipid profile. The hyperlipidemic rats were subdivided into two groups i) hyperlipidemic rats ii) hyperlipidemic rats injected with single dose of *Escherichia coli* (*E. Coli)* (and kept for two weeks to develop bacteremia and its subsequent effects. Result showed that hyperlipidemia significantly increased total cholesterol, triglycerides, low density lipoprotein (LDL) and homocysteine levels, whereas decreased high density lipoprotein cholesterol (HDL) levels. Moreover, hyperlipidemia induced mild oxidative stress in terms of elevated levels of malondialdehyde (MDA) and nitric oxide (NO) and decreased level of reduced glutathione (GSH) in blood. In addition, hyperlipidemic rats exhibited high plasma viscosity, altered hematological indices and caused histological abnormalities manifested as perivascular hemorrhage, vacuolation of the tunica media and minor thickening in aorta wall. Bacteremia provoked inflammatory reactions and oxidative stress, elevated plasma homocysteine and caused noticeable considerable thickening of media-intema layer suggesting the commencement of atherosclerosis. Hyperlipidemic-bacteremic rats showed an additive effect. The study indicated that although hyperlipidemia is an apparent risk factor, homocysteinmia, the inflammatory component and the oxidative stress emerge to be the underlying mechanisms of atherosclerosis pathogenesis. [Nature and Science 2010;8(5):144-151]. (ISSN: 1545-0740).

Key words: Hyperlipidemia- Bacteremia- inflammation- Atherosclerosis

1- Introduction

Clinical studies indicated that hypercholesterolemia is an essential risk factor for coronary artery disease (CAD), where low-density lipoprotein (LDL) cholesterol plays a major role in the atherosclerosis and pathogenesis of CAD and other vascular diseases (Trubelja et al, 2005). Furthermore, several studies showed that hyperlipidemia induces oxidative stress and the oxidative modification of lipoproteins in vessel walls might play a key role in atherogenesis (Wittenstein et al., 2002). Noteworthy, there is a substantial body of evidence challenging the theoretical relationship between dietary cholesterol and CAD. In addition, the relationship between cholesterol in foods and cholesterol in the blood has never been conclusively established and remains a topic of considerable debate. Moreover, hypercholesterolemia has been shown to have a protective effect against atherosclerosis (Ravnskov 2003).

On the other side, elevated C-reactive protein level, an important marker of inflammation, has been acknowledged as an independent risk factor for the development of atherosclerosis and ischemia even in normal cholesterol levels (Collins et al., 2004). In addition, the observation that lipid lowering agents other than statins, such as fibrates, resins, or diet has no impact on stroke incidence (Collins et al., 2004), might indicate that in absence of the inflammatory reactions and oxidative stress, hyperlipidemia is not the prominent risk factor for stroke (Ridker et al., 2000). However, it seems that the interaction between the hemorheological variables (plasma and whole blood viscosities, hematocrit, red blood cell aggregation) and hematological parameters [plasma fibrinogen and von Willebrand factor (vWf)], and platelet aggregation are acknowledged to play roles in atherosclerotic heart diseases (Kesmarky et al., 2006).

Escherichia coli (E. coli), a Gram negative bacteria, is a common cause of infections in all populations and countries of the world (Al-Hasan et al., 2009). E. coli escape the intestinal tract and enter the abdomen through an ulcer, a ruptured appendix, or a surgical error. This leads to peritonitis and elicits a vigorous immune system response and consequently cause bacteremia, sepsis (blood poisoning) and septic shock, which has a relatively high mortality rate (Ruthrauff et

al., 2009). Endotoxin or LPS, a component of the wall of Gram-negative bacteria with significant proinflammatory properties, is a primary initiator of inflammatory and hemodynamic consequences of sepsis [Wang et al., 2007].

The aim of the present study was to evaluate the hematological, biochemical, histopathological effect of hyperlipidemia and inflammation alone or in combination in rat. This was achieved by measuring the lipid profile, the inflammatory parameters (C-reactive protein, erythrocyte sedimentation rate, (ESR); and the differential count of the white blood cells and platelets) and the plasma viscosity. In addition, the oxidative stress parameters (nitric oxide, NO; reduced glutathione (GSH); malondialdehyde (MDA) and homocysteine in plasma of different groups were determined. Moreover, the histopathological studying and measuring the thickness of aorta in different treatment were carried out.

2- Materials and Methods

Male adult Sprague Dawley rats (150-200 g) were kindly provided from our breeding center of National Organization for Drug Control and Research (NODCAR) and kept for a week for acclimatization under normal conditions and constant temperature (25±1C°) with *ad libitum* of water and food. All experiments were carried out in accordance with research protocols established by the animal care committee of the National Organization for Drug Control and Research, Egypt.

All chemicals used were of analytical grade. Solution of pathogenic strain of *Escherichia coli* (*E. coli*) bacteria was kindly provided by Mrs. Eqbal Abdel Hafez, microbiology department, NODCAR. The solution was diluted with saline to colony forming units $2x10^7$ CFU/ml. Each rat intraperitoneally administered 0.5 ml containing 10^7CFU.

2.1. Induction of hyperlipidemia:

Hyperlipidemia in rats was done according to the method of Gröne et al. [1989]. In briefly, hyperlipidemia was induced by feeding the animals high-fat diet [(40%) fat / cholesterol (5%0)] for two months. The high-fat diet contained cholic acid (0.35%) to enhance the enteral absorption of lipids. The occurrence of hyperlipidemia was determined by measuring lipid profile(total cholesterol, triglycerides and HDL). The hyperlipidemic animals were only used.

2.2. Experimental Design

A number of thirty hyperlipidemic rats were divided into two groups as follows.

- Positive control.(n=10)
- Hyperlipidemic- bacteremic group: twenty hyperlipidemic rats injected with single dose of (10^7 CFU/rat) E.Coli and kept for two weeks. The group is comprised of twenty rat, because from previous studied the mortality rate of bacteremia amounted to 50%. In addition, a number of 28 normal diet- fed animals were divided into two groups as follows:
- Bacteremic group (normal diet-fed animals were injected with single dose of E.Coli (10^7 CFU/rat) and kept for two weeks (n=20). The group is comprised of twenty rat, because from previous studied the mortality rate of bacteremia amounted to 50%.
- A group of animals fed normal diet served as normal control group (n=8).

The animals were sacrificed by decapitation; the blood samples were collected into heparinized tubes and centrifuged at 3000 r.p.m for 10 min. for plasma separation.

2.3. Methods

Determination of total cholesterol, triglycerides, and high density lipoprotein (HDL) were analyzed using commercial available kits (STANBIO Lab. TX, USA). Low density lipoprotein was calculated mathematically by Friedwald's formula (1972). Determination of reduced glutathione, homocysteine, malondialdehyde and nitric oxide (as total nitrite and nitrate) levels were determined by HPLC methods according to the Jayatilleke and Shaw (1993), Or-Rashid et al. (2000), Karatepe (2004) and Everett et al. (1995) respectively. Erythrocyte sedimentation rate, leucocytes differential and platelets count were carried out using the method adapted by Simmons and Bernard (1997). Plasma viscosity was determined using BROOK FIELD DV-III ULTRA Programmable Rheometer-USA. CRP was detected with ELISA kit for rat (Genway Biotech, Inc., CA, USA), with the normal level being less than 0.5 mg/ml.

2.4. Histopathological examination and Morphometric Measurements:

Histopathology was carried out according to Carleton and Drury (1973). Cuts were made at a right angle to aorta and fixed in 10% buffered formalin. Sections of 4 µm thickness were stained with hematoxylin and eosin, aorta with uniform throughout its circumference have been selected for morphometric measurements. Morphometric measurements of thickness of cross-sectionally cut aorta were obtained under x 450 magnification with a calibrated filar micrometer.

2.5. Statistical analysis

Data presented as means ± SE. One-way ANOVA followed by LSD test were used to evaluate significant differences from the control and hyperlipidemic groups. Statistical processor system support (SPSS) for Windows software, release 10.0 (SPSS, Inc, Chicago, IL) was used.

3. Results

3.1. Biochemical investigation.

Feeding of animals with high fat-diet for two month significantly increased the levels of blood total cholesterol, triglycerides and LDL and decreased the level of HDL in both the hyperlipidemic group and hyperlipidemic-bacteremic group in comparison to control group. Bacteremic group didn't differ from control group (Table 1). Hyperlipidemic, bacteremic, and hyperlipidemic- bacteremic groups exhibited low count of blood platelets, and total and differential (lymphocyte and monocyte) white blood corpuscles (WBC) in comparison to control (Table 2).

Table 1 Effect of high fat- diet and *Escherichia coli* (10^7 CFU/ rat i.p) treatment alone and in combination on plasma lipid profile in rat.

Parameter	Group			
	Control	**Hyperlipidemic**	**Hyperlipidemic - Bacteremic**	**Bacteremic**
Total Cholesterol (Mg/dl)	71.000 ± 1.852	105.750 ± 3.081*	93.000 ± 2.847*,+	71.375 ± 1.679+
Triglyceride (mg/dl)	62.375 ± 2.828	82.375 ± 2.095*	73.750 ± 4.092*,+	65.625 ± 1.133+
HDL (mg/dl)	42.875 ± 2.158	35.625 ± 1.752*	36.500 ± 1.439*	40.625 ± 1.475
LDL (mg/dl)	15.650 ± 1.487	53.650 ± 3.659*	41.750 ± 3.760*,+	17.625 ± 2.884+
* Significant different from control group at P< 0.05.				
+ Significant different from hyperlipidemic group at P< 0.05				

Table 2 Effect of high fat- diet and *Escherichia coli* (10^7 CFU/ rat i.p) treatment alone and in combination on total and differential (lymphocytes and monocytes) leucocytic and platelets count in rat.

Parameter	Group			
	Control	**Hyperlipidemic**	**Hyperlipidemic-Bacteremic**	**Bacteremic**
WBCs ($x10^3/mm^3$)	11.179 ± 0.328	9.778 ± 0.289 *	6.330 ± 0.219 *,+	4.534 ± 0.245 *,+
Lymphocyte ($x10^3/mm^3$)	6.077 ± 0.297	4.245 ± 0.244 *	2.116 ± 0.165 *,+	1.069 ± 0.712 *,+
Monocyte ($x10^3/mm^3$)	0.742 ± 0.034	0.722 ± 0.024	0.438 ± 0.018 *,+	0.257 ± 0.121 *,+
Blood Platelets ($x10^3/mm^3$)	0.381 ± 0.01	0.350 ± 0.009 *	0.299 ± 0.006 *,+	0.243 ± 0.005 *,+
* Significant different from control group at P< 0.05.				
+ Significant different from hyperlipidemic group at P< 0.05				

Hyperlipidemic, bacteremic and hyperlipidemic-bacteremic groups exhibited heightened plasma viscosity in comparison to control. In addition, the ESR was higher in hyperlipidemic, hyperlipidemic- bacteremic and bacteremic groups in an ascending order respectively in comparison to control. On the other hand, bacteremic and hyperlipidemic- bacteremic groups gave strong positive indication for C-reactive protein, while hyperlipidemic rats gave weak positive indication (Table 3). Data in Table 4 showed that the hyperlipidemic, bacteremic and hyperlipidemic- bacteremic groups showed elevated level of homocysteine, NO and MDA and decreased GSH level in comparison to control. The hyperlipidemic-bacteremic group showed an additive effect.

Table 3 Effect of high fat- diet and *Escherichia coli* (10^7 CFU/ rat i.p) treatment alone and in combination on plasma viscosity, erythrocyte sedimentation rate, and C-reactive protein in rat.

Parameter	Group			
	Control	**Hyperlipidemic**	**Hyperlipidemic-Bacteremic**	**Bacteremic**
Viscosity (CPS)	1.188 ± 0.295	1.625 ± 0.590 *	1.300 ± 0.267 $^{+}$	1.138 ± 0.324 $^{+}$
Erythrocyte sedimentation Rate (ESR)-1 hr (mm/hr)	6.625 ± 0.420	13.250 ± 0.366 $^{*,+}$	24.250 ± 1.750 $^{*,+}$	35.000 ± 1.336 $^{*,+}$
Erythrocyte sedimentation Rate (ESR)-2 hr (mm/hr)	12.250 ± 0.526	24.625 ± 0.925 $^{*,+}$	43.125 ± 1.043 $^{*,+}$	59.750 ± 1.359 $^{*,+}$
C-reactive protein (mg/ml)	0.475 ± 0.033	0.520 ± 0.041	0.790 ± 0.053 $^{*,+}$	0.850 ± 0.061 $^{*,+}$
* Significant different from control group at P< 0.05.				
+ Significant different from hyperlipidemic group at P< 0.05.				

Table 4 Effect of high fat- diet and *Escherichia coli* (10^7 CFU/ rat i.p) treatment alone and in combination on plasma reduced glutathione, nitric oxide, homocysteine and malondialdehyde in rat.

Parameter	Group			
	Control	**Hyperlipidemic**	**Hyperlipidemic-Bacteremic**	**Bacteremic**
Reduced glutathione (mg/ml)	41.583 ± 2.160	33.438 ± 1.594*	27.813 ± 1.008*,+	32.083 ± 0.932*
Nitric oxide (nmol/ml)	24.750 ± 0.491	28.750 ± 0.701*	37.000 ± 0.655$^{*,+}$	30.125 ± 0.693*
Homocysteine (µmol/L)	6.831 ± 0.236	13.005 ± 0.443 *	11.954 ± 0.229 *,+	9.301 ± 0.325 $^{*,+}$
Malondialdehyde (nmol/L)	61.344 ± 3.417	103.454 ± 2.182*	133.550 ± 2.877*,+	113.000 ±. 3.600*
* Significant different from control group at P< 0.05.				
+ Significant different from hyperlipidemic group at P< 0.05.				

3.1. *Histopathological and morphometric investigations.*

Figure 1 and Table 5 showed normal architecture of aorta in normal control animals and normal aorta thickness. Aorta of hyperlipidemic animals showed hemorrhage in perivascular tissue (Fig. 2), with vacuolation in the cells of the tunica media (Fig. 3), and minor increase in the thickness of aorta wall (Table 5). Bacteremic group showed desquamation in the lining endothelium while the tunica media was hyalinized (Fig. 4) and exhibited remarkable increase in the thickness of aorta wall (Table 5). Histopathological examination of hyperlipidemic- bacteremic animals showed hemorrhage in the perivascular tissue (Fig. 5), and oedema in the tunica adventitia (Fig. 6), accompanied with thickening of aorta (Table 5).

Table 5 Effect of high fat- diet and *Escherichia coli* (10^7CFU/ rat i.p) treatment alone and in combination on aorta wall thickness in rat.

Parameter	Group			
	Control	**Hyperlipidemic**	**Hyperlipidemic-Bacteremic**	**Bacteremic**
Aorta Wall Thickness (mm)	0.071 ± 0.002	0.078 ± 0.002*	0.096 ± 0.003*,+	0.094 ± 0.003 *,+
% of control	100.00%	109.9%	135.2%	132.4%
* Significant different from control group at P< 0.05. n=6				
+ Significant different from hyperlipidemic group at P< 0.05				

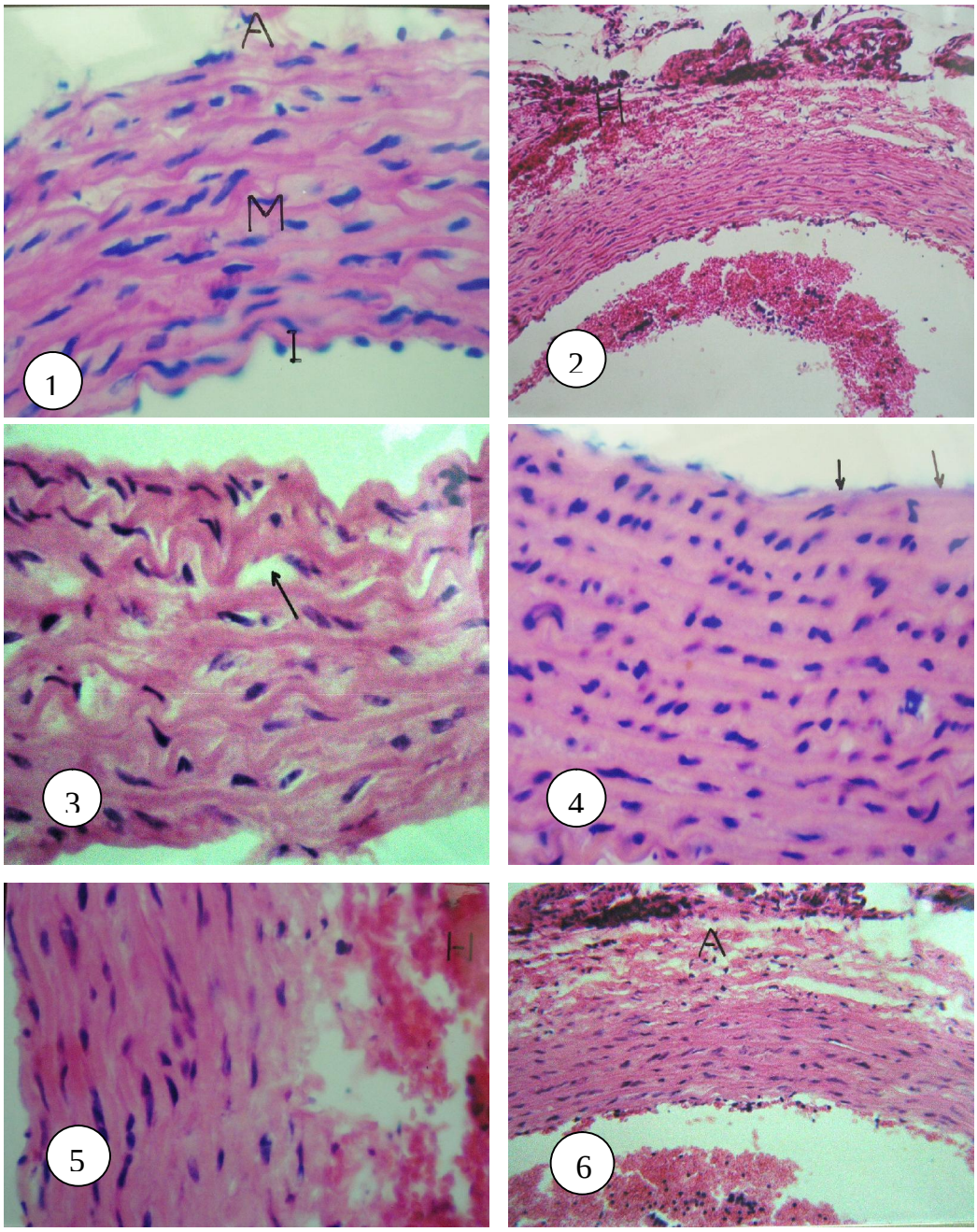

Fig. (1): Transverse section of aorta of control rat showing normal histological structure of the tunica intima (I), media (M) and adventitia (A). (H & E.X 160)
Fig. (2): Transverse section of aorta of hyperlipidemic rat showing perivascular hemorrhage (H). (H&E X 40).
Fig. (3): Transverse section of aorta of hyperlipidemic rat showing vaculation in the cells of tunica media (arrow). (H & E X 160
Fig. (4): Transverse section of aorta of *Escherichia coli* (10^7CFU/ rat, i.p) treated rat showing focal desquamation of the endothelial cells lining the intima (arrow) with hyalinization in the tunica media (M). (H & E X 160).
Fig.(5): Transverse section of aorta of hyperlipidemic and *Escherichia coli* (10^7CFU/ rat, i.p) treated rat showing the perivascular hemorrhage (H). (H & E X 160).
Fig. (6): Transverse section of aorta of hyperlipidemic and *Escherichia coli* (10^7CFU/ rat, i.p) treated rat showing oedema in the adventitia (A) (H & E X 64).

4. Discussion

4.1. Biochemical, hematological and histological effects of hyperlipidemia

The present data showed that feeding rats with high-fat diet for two months induced hyperlipidemia. Hyperlipidemic animals exhibited high plasma viscosity, elevated plasma homocysteine level, mild oxidative stress, altered hematological indices and thickening in media-intema layer. In agreement to the present results, a recent study showed that feeding of albino rats with high fat diet increased atherogenic indices and induced vascular endothelial dysfunction in isolated aorta of atherogenic-diet rats (Nakagawa et al., 2009). Furthermore, feeding of rats with high fat diet and a single dose of vitamin D (3) produced atherosclerosis in Sprague-Dawley rats, and induced hemorrheological and histopathological abnormalities in the atherogenic diet fed rat model. (Wu et al., 2009). Moreover, Chen et al. (2009] studied the mechanical properties of aortic artery in rats with atherosclerosis (AS), where the relationship between mechanical measurements and collagen concentration was evaluated. A close relationship between the mechanical constants and the percentage of elastin and collagen content was observed. It was concluded that mechanical remodeling in aortic artery of AS might be related with histological remodeling.

Besides, it is likely that the elevated plasma viscosity might constitute a risk factor in hyperlipidemic subjects (Cecchi et al., 2006). In accordance to present finding, previous studies indicated that hyperlipidemia increased the levels of lipid parameters and induces oxidative stress and initiated atherosclerosis (Wittenstein et al., 2002, Collins et al., 2004). On the other hand, severe hyperlipidemia in patients with glycogen storage disease type Ia (GSD Ia) failed to provoke premature atherosclerosis (Wittenstein et al., 2002), which might indicate that in absence of the inflammatory reactions and oxidative stress, hyperlipidemia was not the prominent risk factor for stroke. In accordance to the present findings, hyperhomocysteinemia (hHcys) per se has been recognized as a new risk factor for cardiovascular diseases, independent of plasma lipid levels or other factors (Li et al., 2002). Moreover, hyperhomocystinemia has been found to increase carotid intima-media thickness which is a marker of early atherosclerosis (Spence 2002).

The present histopathological findings indicated that hyperlipidemia causes mild structural abnormalities manifested as thickened media-intema layer in comparison to control group; this effect might be due to accumulation of fatty vacuoles cells in the tunica media which resulted into narrowing of the aorta diameter. In accordance, a previous study indicated that hyperlipidemia causes accumulation of fatty plaque deposits in the arteries and aggravate narrowing of the arterial diameter, which restricts blood flow to vital organs (Rioufol & Finet 2002).

4.2. Biochemical, hematological and histological effects of bacteremia

Bacteremic group exhibited normal lipid profile and manifested oxidative stress status and noticeable inflammatory reaction in terms of positive C-reactive protein, increased ESR and increased lymphocyte count and noticeable thickened media-intema layer of aorta. In accordance to our finding, Ross (1999) indicated that inflammation has a pivotal role in the development of atherosclerosis. Moreover, a previous study suggested that measurement of the inflammatory marker C-reactive protein, may provide a useful method of assessing the risk of cardiovascular disease in apparently healthy persons, particularly when lipid levels are low (Ridker et al., 2000). In rats, CRP is not a typical acute- phase protein and exists in plasma under basal condition in a concentration which is 100 times higher than that in humans (Diaz Padilla et al., 2003).

In addition, it's suggested that the inflammatory effect of bacterial endotoxin induced oxidative stress which oxidizes LDL. Oxidized LDL, in turn, activates further inflammatory processes at the level of gene transcription such as up-regulation of nuclear factor kappa-B, expression of adhesion molecules, and recruitment of monocytes/macrophages and the generation of blood C-reactive protein (Ipatova et al., 2003). These activated macrophages produce numerous factors that are injurious to the endothelium (Kolodgie et al., 2003). Consequently, denudation of the overlying endothelium or rupture of the protective fibrous cap may result in exposure of the thrombogenic contents of the core of the plaque to the circulating blood and increased blood viscosity and coagulation (Cuthbertson & Christophi 2006). Moreover, it is likely that reactive oxygen species formation by phagocytes and subsequent modifications of vascular wall are involved in the early step of atherogenesis (Delbosc et al., 2002).

Observation that bacteremic rats exhibited structural abnormalities and considerable thickening of the aorta wall, nearly equals hyperlipidemia-bacteremia group might indicate that hyperlipidemia may have minor effect. Also, this might suggest that inflammation plays an essential role in the initiation and progression of

atherogenesis in presence or in absence of hyperlipidemia. Consistently, several studies indicated that inflammation causes abrasion of the overlying endothelium of the blood vessels through the exposure to the immune cells monocytes/macrophages and deposition of LDL-cholesterol leading to arteries stenosis even in normal lipid profile individuals (Delbosc et al., 2002).

4.3. Biochemical, hematological and histological effects of hyperlipidemia-bacteremia

It's worthy to note that the hyperlipidemic-bacteremic rats exhibited an additive effect regarding the oxidative stress parameters and the inflammatory reactions. This might indicate the pathophysiological effect is principally originated from the inflammation and that hyperlipidemia is a coordinator component. Alternatively, atherosclerosis could be recognized in part an inflammatory disease and that the lowering of lipid levels may represent an anti-inflammatory process. Consistently, oxidized low-density lipoprotein (LDL) cholesterol; infectious agents; toxins, the byproducts of cigarette smoking; hyperglycemia; and hyperhomocystinemia are the probable causes of endothelial injury (Kesmarky et al., 2006).

5. Conclusion:

The study might indicate that there is a correlation between cholesterol level and heart disease but does not prove causation. Moreover, inflammation emerges to be independent risk factor for the development of atherosclerosis even in normal cholesterol level. In absence of the inflammatory reactions and oxidative stress, hyperlipidemia alone is not the principal risk factor for atherosclerosis.

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Comparative study of endo-parasitic infestation in *Channa punctatus* (Bloch, 1793) collected from Hatchery and Sewage lagoon

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Abstract: The study was conducted to collect and identify endoparasites of *Channa punctatus* (Bloch,1793) from different water bodies of varying water quality in Bangladesh and to determine the prevalence and intensity of infestation brought about by the endoparasites in the hosts. The host fishes were collected from polluted water at sewage treatment lagoon in Narayangong and fresh water at Tongi Hatchery in Gazipur, Bangladesh. The prevalence of endoparasites in the host fish *Channa punctatus* was 91.30% in female and 88.88% in male fishes, among them in polluted water fishes the prevalence was 85.71% in female and 86.66% in male and in fresh water fishes the prevalence was 100% in both the male and female fishes. The intensities of infestation in *Channa punctatus* was 6.78 in female and 6.55 in male fish collected from hatchery; and in sewage water fishes the intensity was 3.50 in females and 1.15 in males respectively. Six parasite species were found from polluted water fishes and seven species of parasites were recorded from fresh water fishes. The parasite groups were trematodes (*Genarchopsis bangladensis, Allogomtiotrema attu, Phyllodistomum* sp., *Neopecoelina saharanpuriensis*), nematodes (*Ascaridia* sp., *Procamallanus* sp.) and Acanthocephalan (*Pallisentis nandai*). Acanthocephalans were found in the fishes collected from sewage lagoon. Liver, stomach, intestine and body cavity of the host fishes were examined for parasites. Females were more infected than the males. The intensity and the prevalence were higher in host fishes collected from hatchery than the sewage water host fishes. The hosts of intermediate length and weight group were found to be more infected than smaller and larger length groups. [Nature and Science 2010;8(5):152-156]. (ISSN: 1545-0740).

Key words: Endoparasite, *Channa punctatu*, Hatchery, Sewage Lagoon

1. Introduction

Parasitic diseases, either alone or in conjunction with other environmental stresses, may influence weight or reproduction of the host, alter its population characteristics, and affect its economic importance (Rhode 1993). Parasites occupy a definite position in the animal kingdom for their remarkable adaptations and damaging activities to host. The importance of parasite is related directly to the fish that may affect the general public health (Hoffman 1967).

Every parasite living in or on a fish extents some degree of harmful influence on its host. The normal growth of fish is interrupted or inhibited if they are heavily infected with parasites. The composition of the parasites of fish depends on various environmental factors such as geographical location of the habitat, season of the year, physico-chemical factors of the water, the fauna present in and around the habitat etc. Dogiel (1964) suggested factors that directly influence parasitic fauna of fish include age, diet, abundance of fish, interdependence of members of parasitic fauna within the fish and the season. The normal growth of the fishes is impeded if they are heavily infested with endoparasites.

According to Gupta (1983) injury of fishes can carry heavy infection of parasites that cause deterioration in the food of fish and may even result in their mortality. Besides there are a number of "helminth parasites" which are transmitted to man only through fishes. The similarity in parasitic fauna between species utilizing similar food was also noted (Dogiel 1964). The difference in feeding habits has considerable impact on intestinal parasites, but related species living together are likely to share a similar array of ectoparasites, in spite of their differences.

Study of parasites is scant and recent in Bangladesh. Attempts have been taken to explore the parasitic fauna of fishes of this country (Rahman 1989, Khan 1985, Ahmed and Rouf 1981). Elahi (1969) studied in some endo-parasites of fresh water fishes of the family channidae in Dhaka. He described *crowcrocacum pakistanensis* from the intestine of *channa marulius*. Bashirullah (1973) listed some helminth parasites in *Channa striatus* and *Channa marulius*. Chowdhury (1992) studied on the helminth parasites infestation and

histopathological changes in snake head fishes. She described cestode *Gangesia bengalensis* from the intestine of *Channa marulius;* nematode *Camallanus ophiocephali* from the intestine of *Channa striatus* and *Spirocamallanus* sp. in the intestine of *Channa striatus;* acanthocephalan *Pallisentis nandi* from the intestine, stomach and body cavity of *Channa striatus* collected from different fish markets of Dhaka city. Nahar (1993) reported incidence of endoparasites of *Channa striatus* and *Channa marulius* from Dhaka, Bangladesh.

The aquatic environment encompasses a wide variety of biological, chemical and physical parameters, which if altered beyond acceptable limits, such as under culture conditions, may weaken the fish leading to disease outbreaks (Roberts 1989). Fish play an important role in economy of Bangladesh. Mortality of fishes occurs due to heavy infestation of parasites. For that reason the present work was undertaken to investigate the prevalence and intensity of endoparasites of the host fish *Channa punctatus* (Bloch, 1793) in two different water bodies of varying water quality.

2. Materials and methods

The host fish, *Channa punctatus* (Bloch, 1793) were collected using traps and gill nets from Tongi Hatchery, Gazipur and from sewage treatment lagoon at Narayangonj, Bangladesh which were selected based on availability of laboratory space (Aloo *et al*. 2004). In the lagoon the sewage water is kept for oxygenation and treatment before disposal into the river (Hasan *et al*. 2006). Before dissection length, weight and sex of each fish were recorded. Then an incision was made along the mid-ventral line of the fish. The surfaces of the visceral organs and body cavities and serous membranes were examined for encysted larvae and parasites by using hand lens. All the organs were removed intact and carefully from the body and put into formalin solution in petridishes. After separating, the internal organs (stomach, intestine, liver and body cavity) were examined individually for parasite in separate petridishes under compound microscope. The stomach and intestine were carefully opened by an incision and then were shaken to dislodge the parasites that might remain attached to the lining of the epithelium by their head ends. The epithelial layers of the stomach and intestine were scrapped with a scalpel to remove any parasite that might remain attached to the layers, and the liver and body cavity were shredded with a pair of forceps and needles. The collected parasites were then washed in fresh saline solution.

The contents of each petridish were then stirred well and allowed to settle in the bottom of the petridish. The sediment was then examined with a dissecting microscope. The collected parasites were washed in fresh water to clean any debris before making temporary mounts or permanent slides. For the purpose of fixation of nematode and acanthocephalan parasites hot glacial acetic acid and AFA (Alcohol Formol Acetic) were used respectively. The collected parasites were placed in hot fixative and left there for a few minutes. After fixation the parasites were preserved in 70% ethyl alcohol in vials for prolonged storage. Lactophenol was used in order to clean the nematodes and acanthocephalan parasites. The nematodes were kept in lactophenol for five to seven days for visibility of the internal organs. The acanthocephalans required four to five days to be cleaned of in lactophenol. The cleaned parasites (nematodes and acanthocephalans) were mounted on slides temporarily in lactophenol. To make permanent slides of acanthocephalan the parasites were stained with borax carmine for one and half to two hours and then after dehydrating in alcohol graded series, the parasites were cleaned with xylene and mounted in Canada balsam. Collected parasites were identified with the help of Yamaguchi 1963 and Soota 1983.

3. Results

In the sewage lagoon the temperature varied from 20.5° to 32.3°C, pH 7.7 to 8.5, and dissolved oxygen 7.0 to 8.5 mg/l (Hasan *et al*. 2006). Seven species of parasites were found among them three were identified to genus level. Among all of those four were trematodes, two nematodes and one acanthocephalan. In *C. punctatus* four trematodes (*Genarchopsis bangladensis, Allogomtiotrema attu, Phyllodistomum* sp., *Neopecoelina saharanpuriensis)*, two nematodes (*Ascaridia* sp., *Procamallanus* sp.), one acanthocephalan (*Pallisentis nandai*) were recovered.

In this study, the parasites were found in the different internal organs of the collected host species (Figure 1 and 2) from both sampling points. The number of parasites varied in the different organs of the hosts. The intensity and the prevalence were highest in fishes of the hatchery than the sewage lagoon. Basirullah (1972) worked on some fresh water fishes of Dhaka and showed that *Encredium dacci, Camallanus adamsia, Camallanus ophicephali, Pallisentis* sp. were located in the intestine and *Genarchopsis* sp. in the stomach of *Channa marulius, Channa striatus, Channa punctatus, Channa gachu*. Chowdhury (1992) found all helminths except *Genarchopsis* sp. in the medle region and posterior region of the intestine; a few nematodes were also found in the body cavity of their host fishes.

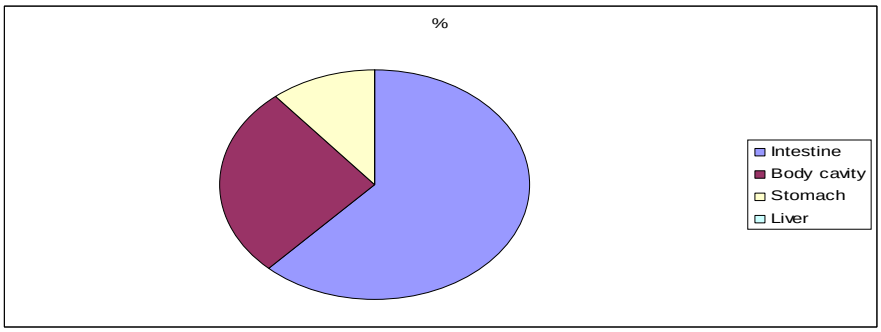

Figure 1: Parasites present in different organs of *Channa punctatus* collected from hatchery

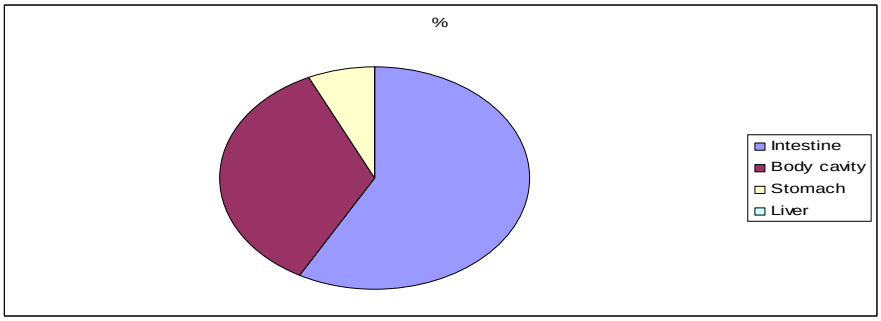

Figure 2: Parasites present in different organs of *Channa punctatus* collected from sewage lagoon

4. Discussion

The prevalence of endoparasites in the host fish *C. punctatus* was 91.30% in females and 88.88% in males, among them in polluted water fishes the prevalence was 85.71% in female and 86.66% in male and in fresh water fishes the prevalence was 100% in both the male and female fishes. The intensities of infestation in *C. punctatus* was 6.78 in female and 6.55 in male fish collected from hatchery; and in fishes of the sewage lagoon the intensity was 3.50 in females and 1.15 in males (Table 1). The intensities varied significantly in the different water bodies. It was observed that female fishes were more infected than the male fishes in this study.

Table 1: Prevalence and intensity of infestation in the fishes of hatchery and the sewage lagoon

Sex of fish	Hatchery		Sewage lagoon	
	Prevalence (%)	Intensity (± SD)	Prevalence (%)	Intensity (± SD)
Male	100	6.55 (± 4.91)	86.66	1.15 (± 2.33)
Female	100	6.78 (± 5.54)	85.71	3.50 (± 4.01)

The cause of higher intensity in female fishes may be ecological habitat and sex hormones responsible for depressing the level of infestation. According Aloo *et al*. (2004) the main reason for the differences in parasitic load with sex is physiological. In the present study, it was found that the prevalence and intensity of parasites of different groups varied for water quality and sex of hosts (Table 2).

Table 2: Prevalence and intensity of different groups of parasites in host fishes

Name of parasites	Hatchery				Sewage lagoon			
	Prevalence (%)		Intensity		Prevalence (%)		Intensity	
	Male	Female	Male	Female	Male	Female	Male	Female
Genarchopsis bangladensis	45.45	55.55	1	1	12.50	0	1.5	0
Allogomtiotrema attu	45.45	55.55	1.8	1.5	6.25	7.14	2	2
Phyllodistomum sp.	18.18	44.44	3	1.5	0	7.14	0	2
Neopecoelina saharanpuriensis	36.36	33.33	2	2	12.50	14.28	1	1
Ascaridia sp	100	100	1.27	1.22	43.75	57.14	1.14	1.5
Procamallanus sp.	90.90	100	2.4	2.4	50	64.28	1.12	1.66
Pallisentis nandai	36.36	22.22	1.75	2	0	0	0	0

5. Conclusion

Polyanski (1961) reported that the major factors determining the fish parasite fauna as well as intensity and prevalence of infestation in aquatic environments can be summarized as being: the diet of the host, lifespan of the host, the mobility of the host throughout its life including the variety of habitats it encounters, its population density and the size attained, with large hosts providing more habitats suitable for parasites than small ones. In most cases intensity does not differ for sex in same habitat, but it is observable that intensity differs strongly for habitat. It also differs from species to species. In this study, the hosts of intermediate length and weight were found to be more infected than the hosts of smaller and larger length. One major reason is that as the fish grows, the amount of food it consumes, which includes the larval stages of the parasites, increases (Paling 1965, Mashego 1989, Davey and Gee 1976). Intestinal parasites inhibit the digestive activity of the host and indirectly inhibit vitamin and blood sugar metabolism and growth; parasites in the liver affect glycogen metabolism and growth (Rhode 1993). Since fish play vital role in the economy of Bangladesh, more emphasis should be given on such type of negative interactions that can cause huge damage to the fish population and more importance should also be given to the water quality because the prevalence of parasites can vary for different water bodies.

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Comparison of digesting capacity of nitric acid and nitric acid-perchloric acid mixture and the effect of lanthanum chloride on potassium measurement

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Abstract: Nitric acid-perchloric acid mixture is the renowned digesting reagent in the scientific world of plant nutrition. Beside this, some other inorganic acids can be used as the digester of plant samples. Therefore, this experiment was conducted to find out if there is any difference between the digesting capacity of nitric acid (HNO_3) and nitric acid-perchloric acid mixture (HNO_3-$HClO_4$) or not. The hydroponic experiments were conducted with barley (*Hordeum vulgare* L. cv. Minorimugi) and rice (*Oryza sativa* L. cv. Akihikari) seedlings. At suitable stage, the plant samples were collected, washed with deionized water, separated into shoot and root, dried, grinded and then divided into two groups for shoot and root individually for two types of seedlings. One group was for only HNO_3 acid and the other group was for HNO_3-$HClO_4$ acid mixture. Phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu) were measured after digesting the samples. There was no significant difference between the digesting capacity of HNO_3 acid and HNO_3-$HClO_4$ acid mixture. Potassium was measured by diluting the samples (200-600 times) containing lanthanum chloride ($LaCl_3$) or without $LaCl_3$. Lanthanum chloride did not have any significant effect on K measurement in this dilution system. [Nature and Science 2010;8(5):157-162]. (ISSN: 1545-0740).

***Key words**: Concentration, Lanthanum chloride, Nitric acid and nitric acid-perchloric acid mixture*

1. INTRODUCTION

Before determination of inorganic elements from plant tissues it is necessary to destroy the organic matter in plant substances. However, such a destructive method is not necessary for some inorganic ions like K, Na, Ca and Mg, which do not form any organic complex in plant tissues (Imamul Huq and Didar-ul-Alam, 2005). The methods which are used to bring about the destruction of organic matter fall into two main groups such as "wet oxidation and dry ashing". The wet oxidation includes those methods in which the destruction of organic matter is brought about by oxidation in a liquid medium, while dry ashing refers to processes in which the sample is ignited. Generally the methods of wet oxidation include the digestion of the samples with aqua regia (HCl: HNO_3 = 3:1), ternary acid mixture (HNO_3: H_2SO_4: $HClO_4$= 5:1:2), nitric acid-perchloric acid mixture (HNO_3-$HClO_4$), sulfuric acid-nitric acid mixture (H_2SO_4-HNO_3), sulfuric acid-perchloric acid mixture (H_2SO_4-$HClO_4$), nitric acid-hydrogen peroxide mixture (HNO_3-H_2O_2) and/or sulfuric acid-hydrogen peroxide mixture (H_2SO_4-H_2O_2). In the wet oxidation methods, the greater part of the oxygen (O) is required for the oxidation is supplied by the nitric acid (HNO_3). For the most efficient use of HNO_3, digestion must be carried out at a low temperature, which helps to minimize the excessive losses of the elements by evaporation in the early stages. In wet oxidation methods, $HClO_4$ acid prevents the excessive frothing which occurs frequently when HNO_3 or H_2SO_4 acid is used singly. Wet oxidation results in the conversion of the elements P, Na, K, Ca, Mg, Fe, Mn, Zn, Cu and others to proper forms for analytical determination. Some elements like chlorine (Cl) and P may be lost at the time of wet ashing as volatile compounds (Imamul Huq and Didar-ul-Alam, 2005). From the historical period, HNO_3-$HClO_4$ mixture is being used as the suitable digester in wet oxidation method for the determination of inorganic nutrient elements of plant tissues. But there are some elements [e.g. - arsenic (As)] which determination procedure may be affected by chloride (Cl^-), because of antagonistic effect between arsenite (AsO_2^-) or arsenate (AsO_4^{3-}) and Cl^-. Carbonell-Barrachina et al. (1998) found that the increasing level of As in the nutrient solution significantly decreased the Cl concentration and uptake in the root system. Therefore, at the time of As determination Cl may show antagonistic effect on As if the solution contains excess Cl. The antagonisms

between Cl^- and NO_3^-; and Cl^- and SO_4^{2-} are well known phenomenon (Mengel and Kirkby, 1987; Marschner, 1995). It seems, Cl^- reduces the uptake of different anionic ions. Therefore, there is a possibility to interfere As determination if the solution contains Cl^-. In the digested solution As may exist as arsenate (AsO_4^{3-}) anion. In this study the samples were digested with HNO_3 acid and HNO_3-$HClO_4$ mixture to find out if there is any difference between the digesting capacities of these two. This study will provide some basic information for the future to find out the effect of $HClO_4$ on As determination, because loss of As can occur in presence of high halide concentration (Frankenberger, 2002) or during determination, halide may interferes As determination. The other objective of this report was to show the effect of $LaCl_3$ on K determination.

2. MATERIALS AND METHODS

Two experiments were conducted in this study. Experiment 1 (barley plants in minus iron [-Fe] condition) was conducted to compare the digesting capacity of HNO_3 acid with HNO_3-$HClO_4$ acid mixture and to determine the effect of $LaCl_3$ on K determination. Experiment 2 (rice in plus [+Fe] condition) was conducted to verify the effect of $LaCl_3$ (purity 98% and 2% in final solution) on K determination that was obtained from the first experiment.

(1) Experiment 1:

Seed germination (Barley in -Fe condition)

Barley seeds were (*Hordeum vulgare* L. cv. Minorimugi) surface sterilized with 2% chlorinated lime [$Ca(OCl)_2$] for 45 minutes rinsed with tap water continuously for 1 hour and sandwiched between moistened towels covered with wrapping paper at 25˚C for 24 hours in electric incubator. Germinated seeds were placed on plastic net in the seed box containing 2 mM $CaCl_2$ solution in the greenhouse. After 7 days, the solution in the seed box was replaced with 1/5-strength modified Hoagland-Arnon solution containing 4.0 µM Fe^{3+}-EDTA. The plants were allowed to grow (another 7 days) until the length of the second leaf was about 20% of that of the first leaf.

Plant culture (Barley in -Fe condition)

The seedlings were transplanted after 14 days of germination (7 days in $CaCl_2$ solution and 7 days in 1/5-streangth modified Hoagland-Arnon solution) in bunches (5 plants were wrapped with sponge rubber to make one bunch). 50 bunches were placed in one bucket (capacity 35 liters) filled with ½-strength modified Hoagland-Arnon solution containing 10 µM Fe^{3+}-EDTA in the greenhouse for two days. Then the treatment was started (-Fe condition). The full-strength of modified Hoagland-Arnon solution contained 6.0 mM KNO_3; 4.0 mM $Ca(NO_3)_2$; 1.0 mM $NH_4H_2PO_4$; 2.0 mM $MgSO_4$; 20.0 µM Fe^{3+}-EDTA; 3 µM H_3BO_3; 0.5 µM $MnSO_4$; 0.2 µM $CuSO_4$; 0.4 µM $ZnSO_4$ and 0.05 µM H_2MoO_4, (Kawai et al., 1993). The –Fe medium was prepared by removing Fe^{3+}-EDTA and substituting $NH_4H_2PO_4$ with the same concentration of NaH_2PO_4 (Takagi 1993). The pH (6.5) of the solutions was adjusted daily with 1 M HCl and/or 1 M NaOH. The solutions were aerated throughout the experiment and the water level was maintained by adding deionized water. The nutrient solutions were renewed every week. The plants were grown up to 28 DAT (days after treatments). In this experiment the treatment was only –Fe modified solution of Takagi (1993). The typical photograph of –Fe experimental plants was presented in the Plate 1.

Plate 1: Typical photograph of barley seedlings at 28 DAT grown hydroponically in -Fe condition.

Heating procedure (Barley in -Fe condition)

For shoot, around 0.5 g and for root around 0.3 g samples were taken in 100 mL acid washed glass beaker. Almost 20 times HNO_3 was added for each sample and was heated at 100ºC continuously for 10 hours on electric hot plate (National Electronics Company, Japan, Model-NF-HG 59). After cooling (over night 7 hours), the samples were again heated at 140ºC for 7 hours. After cooling (over night 7 hours), additional 5 mL HNO_3 was added with one group and for other group 5 mL $HClO_4$ was added and again heated for 11 hours at 140ºC. Then the digested samples were cooled and were volume in 50 mL volumetric flask and stored in 50 mL acid washed plastic bottle.

Sample collection and preparation (Barley in -Fe condition)

The plant samples were collected and washed with deionized water three times. Shoot and root were separated with sterilized scissor and dried at 55-60˚C for 48 hours in electric oven. The oven dried plant samples were grinded properly in mortar with pestle to make it homogenous and then the samples were divided into two groups. One group was treated with HNO_3-$HClO_4$ acid

mixture (Piper, 1942) and the other group was treated with HNO_3 acid only. For each group 4 replications were used.

(2) Experiment 2:

Seed germination (Rice in +Fe condition)

Rice (*Oryza sativa* L. cv. Akihikari) seeds were surface sterilized with 2% chlorinated lime [$Ca(OCl)_2$] for 45 minutes and rinsed with tap water continuously for 1 hour. After washing, the seeds were wrapped between moistened towels and were kept in a seed growth chamber at 25˚C for 72 hours. Then the seeds were transferred on a net in a plastic seed box containing 2% $CaCl_2$ for 9 days in the greenhouse. Then the seedlings were transferred in ½-strength nutrient solution for another 9 days.

Plant culture (Rice in +Fe condition)

When the seedlings were suitable for transplantation (18 days after germination, at 3rd leaf stage of the seedlings), the treatments were started with full-strength rice solution containing 1 mM NH_4NO_3, 1 mM K_2SO_4, 0.8 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.5 mM NaH_2PO_4, 10 µM $MnSO_4$, 1 µM $CuSO_4$, 1 µM $ZnSO_4$, 3 µM H_3BO_3, 0.05 µM H_2MoO_4 and 10 µM Fe^{3+}-citrate. 5 plants were taken in one bunch and each bucket (10 liter) containing 16 bunches. The treatments were T_1 (control, containing full-strength solution), T_2 (control + aeration), T_3 (control + 13.4 µM As), and T_4 (control + 13.4 µM As + aeration). The pH (5.5) was adjusted daily with 1 M HCl and/or 1 M NaOH at around 16.00 hours during the experiment (22.09.2004 to 01.11.2004). The solution was renewed every weak and was not aerated. Basically experiment 2 was conducted to observe the effect of aeration on As in hydroponic culture up to 21 DAT but we collected the samples to verify the result of Experiment 1. The typical photograph of +Fe rice seedlings was presented in the Plate 2.

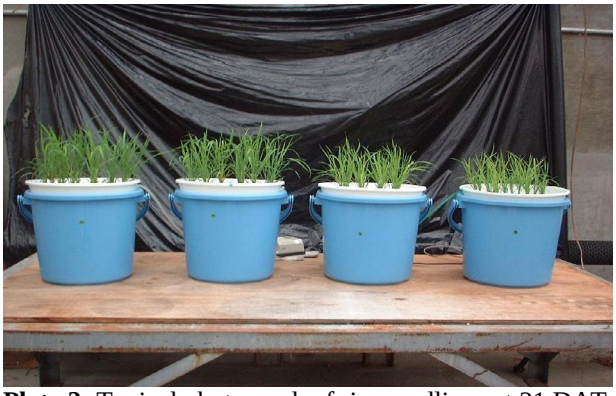

Plate 2: Typical photograph of rice seedlings at 21 DAT grown hydroponically in +Fe condition.

Sample collection and preparation (Rice in +Fe condition):

After 21 DAT, 3 bunch were taken and the plants were washed with deionized water properly and separated into shoot and root with sterilized scissor and were dried for 48 hours at 55-60˚C in electric oven. The samples were cut into small pieces suitable for digestion.

Heating procedure (Rice in +Fe condition): The individual sample was taken in acid washed 100 mL glass beaker, 3 mL analytical grade H_2SO_4 was added and covered with glass coverer and heated at 100˚C for 1.5 hours, at 140˚C for 1.5 hours and at 180˚C for 2 hours on an electric hot plate (National Electronics Company, Japan, Model NF-HG 59). After that the samples were cooled and 2 mL analytical grade H_2O_2 was added to the each sample and heated at 180˚C for 5 hours. The samples were kept for over night for cooling. In the following day another 3 mL H_2SO_4 and 2 mL H_2O_2 was added to the each samples and heated at 180˚C for 9 hours continuously. At the last stage of the digestion, all the samples were clear. After cooling, the samples were volumes at 50 mL were transferred in 50 mL acid washed plastic bottle. This extract was used for mineral elements determination.

Chemical analysis (Experiment 1 & 2):

Potassium, Ca, Mg, Fe, Mn, Zn and Cu were determined with atomic absorption spectroscopy (AAS) (Hitachi 170-30, Japan) from the digested solution. Phosphorus was determined colorimetrically using a UV-visible spectrophotometer (model UV mini 1240, Shimadzu Corporation, Kyoto, Japan) at 420 nm wavelength after developing the yellow color with vanadomolybdate as described by Barton (1948) and Jackson (1962).

Experimental design (Experiment 1 & 2):

The experiments were a completely randomized block design with 4/3 replications. Data were subjected to ANOVA. Differences between means were evaluated using a Ryan-Einot-Gabriel-Welsch multiple range test (P = 0.05) (SAS Institute, 1988) using computer origin 5 at Iwate University, Morioka, Japan.

3. RESULTS AND DISCUSSION

(1) Digesting capacity of HNO_3 acid and HNO_3-$HClO_4$ acid mixture

The concentration data of K, Ca, Mg, Fe, Mn, Zn and Cu were slightly higher or almost no change in shoot and root samples of HNO_3-$HClO_4$ digested samples as compared to only HNO_3 acid digested samples (**Fig. 1bcd and 2abcd**). However, the concentration data of P in HNO_3-$HClO_4$ digested samples were slightly lower as compared to only HNO_3 acid digested samples (**Fig. 1a**). These higher or lower values were not significant

($p<0.05$). In the case of K, Ca, Mg, Fe, Mn, Zn and Cu it could be said that, HNO_3 acid could digest the samples in better way in presence of $HClO_4$ acid as compared to without $HClO_4$ acid and gave the higher concentration data. In this experiment, slightly lower concentration of P was found in HNO_3-$HClO_4$ acid digested samples as compared to only HNO_3 acid digested samples. This may be due to the fact that in presence of $HClO_4$, oxidation of P might be little higher and evaporation loss of P might be occurred as compared to only HNO_3 acid digested samples. Phosphorus may be lost as a volatile compound at the time wet digestion (Imamul Huq and Didar-ul-Alam, 2005).

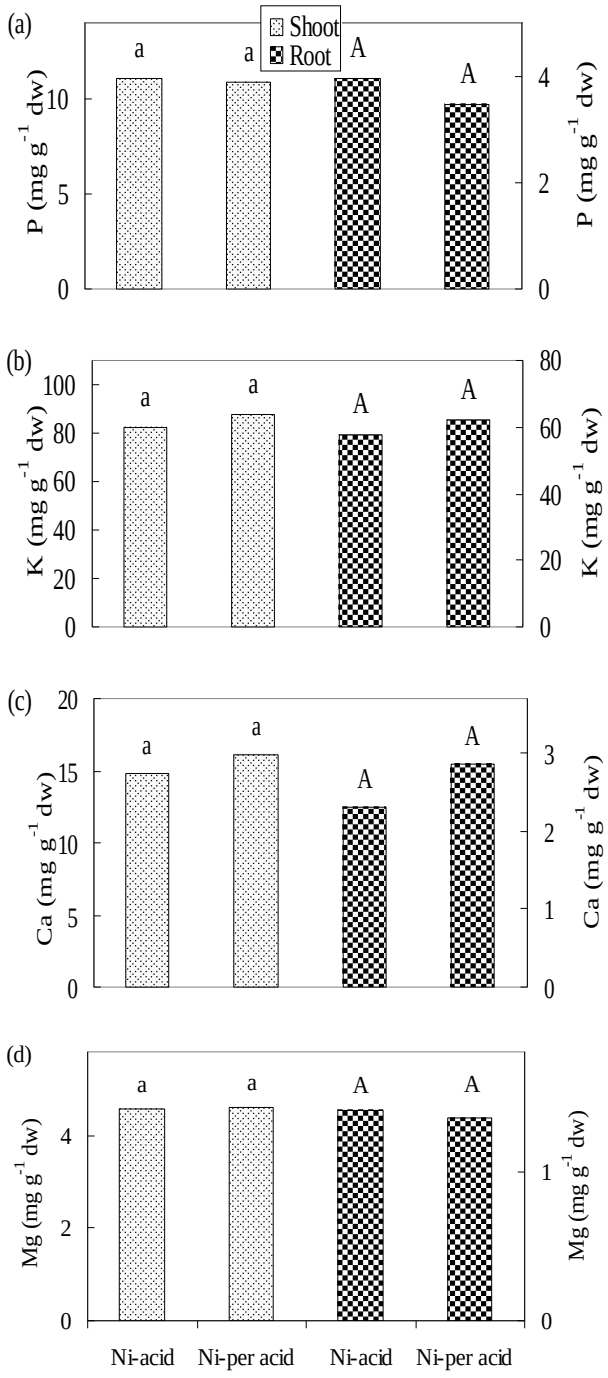

Fig. 1 Comparison of concentration of (a) P, (b) K, (c) Ca and (d) Mg of barley seedlings digested with HNO_3 acid and HNO_3-$HClO_4$ acid mixture. Bars with same letters are not are not significantly different ($p < 0.05$). Ni-acid indicates HNO_3 acid and Ni-per acid indicate HNO_3-$HClO_4$ acid mixture.

(2) Effect of $LaCl_3$ on K measurement

Potassium concentration was determined by diluting the samples 200 to 600 times with or without $LaCl_3$. Magnesium was also determined from the samples containing $LaCl_3$ both for shoot and root (data were not presented). It was observed that there was no significant difference of K concentration data between the samples of with or without $LaCl_3$ solution (**Table 1**). The concentration of K was also measured to verify the result of the first experiment from the digested samples of As containing solution. It was also found that $LaCl_3$ did not have any significant effect on K measurement (**Table 2**) and confirming the result of the first experiment.

Table 1 Concentration of K (mg g^{-1}) in presence or in absence of $LaCl_3$ of –Fe barley seedlings

	Shoot		Root	
Solution	HNO_3	HNO_3+ $HClO_4$	HNO_3	HNO_3+ $HClO_4$
Without $LaCl_3$	82.42 A	87.97 a	58.73 B	62.25 b
With $LaCl_3$	83.66 A	86.06 a	60.63 B	61.39 b

Note: Means followed by same letters in each column are not significant (p = 0.05) according to Ryan-Einot-Gabriel-Welsch Multiple Range Test.

Table 2 Concentration of K (mg g^{-1}) in presence or absence of $LaCl_3$ of +Fe rice plants

Treat.	Shoot		Root	
$LaCl_3$	without	with	without	with
T_1	47.32 a	48.85 a	32.94 A	33.09 A
T_2	49.94 b	48.37 b	34.13 B	35.55 B
T_3	41.96 c	44.38 c	29.21 C	29.48 C
T_4	45.96 d	45.97 d	34.28 D	34.32 D

Note: Means followed by same letters in each row (for shoot or root individually) are not significantly different (p = 0.05) according to a Ryan-Einot-Gabriel-Welsch Multiple Range test. T_1 (control, containing full-strength nutrient solution), T_2 (control + aeration), T_3 (control + 13.4 µM As), T_4 (control + 13.4 µM As + aeration).

(3) Effect of $LaCl_3$ on Ca measurement

The Ca concentration was also measured from the solution with or without $LaCl_3$. Higher amount of Ca was found in $LaCl_3$ containing solution both for shoot and root (**Table 3**). Shoot solution was diluted 20 times but the root solution was diluted 4 times in this case.

Table 3 Concentration of Ca (mg g^{-1}) in presence or absence of $LaCl_3$ of +Fe rice seedlings

Treat.	Shoot		Root	
DT	1	20	1	4
T_1	0.64 a	1.09 b	0.15 a	0.20 b
T_2	0.63 á	1.11 b́	0.10 á	0.22 b́
T_3	0.64 A	1.04 B	0.21 A	0.25 B
T_4	0.52 Á	1.31 B́	0.13 Á	0.30 B́

Note: Means followed by same letters in each row (for shoot or root individually) are not significantly different (p = 0.05) according to a Ryan-Einot-Gabriel-Welsch Multiple Range test. DT (Dilution times), T_1 (control, containing full-strength nutrient solution), T_2 (control + aeration), T_3 (control + 13.4 µM As), T_4 (control + 13.4 µM As + aeration).

It is well known that when the plant samples are digested with a concentrated acid digester, the inorganic elements in plants tissues are available in solution like PO_4^{3-}, K^+, SO_4^{2-}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, $Zn^{2+}$and Cu^{2+}. At this condition the pH of the solution is as below as 2 and there is no precipitation of nutrient elements (Koshino, 1988). Above pH 2 (after dilution), the minerals in the solution may form complex with each other (Koshino, 1988) like Ca and Mg with P. When the diluted samples are passed through the tube of AAS, it is spilled up in presence of air pressure and reaches at the burner chamber where mist is formed in presence of acetylene gas and is burnt into flame (Koshino, 1988). In the flame, ion is transferred into atom. The samples containing Ca^{2+}, Mg^{2+}, PO_4^{3-} and SO_4^{2-} may form complexes like- $CaSO_4$, $Ca_3(PO_4)_2$, $MgSO_4$ and $Mg_3(PO_4)_2$. Thermal dissociation is one of the complex phenomenon occurred in the flame of atomic absorption without $CaSO_4$, $Ca_3(PO_4)_2$, $MgSO_4$ or $Mg_3(PO_4)_2$. After adding 2% $LaCl_3$ with the solution, $LaCl_3$ may react with SO_4^{2-} and PO_4^{3-} of Ca and/or Mg and may form insoluble complex like $La_2(SO_4)_3$ and $LaPO_4$. As a result Ca and or Mg may be free from those anions and the atoms could be measured. May be K does not form any complex with SO_4^{2-} or PO_4^{3-} in the flame and therefore, $LaCl_3$ may not have any effect on K measurement. May be $LaCl_3$ could free Ca from the insoluble complex as a result; higher Ca concentration was recorded in $LaCl_3$ treated samples (**Table 3**).

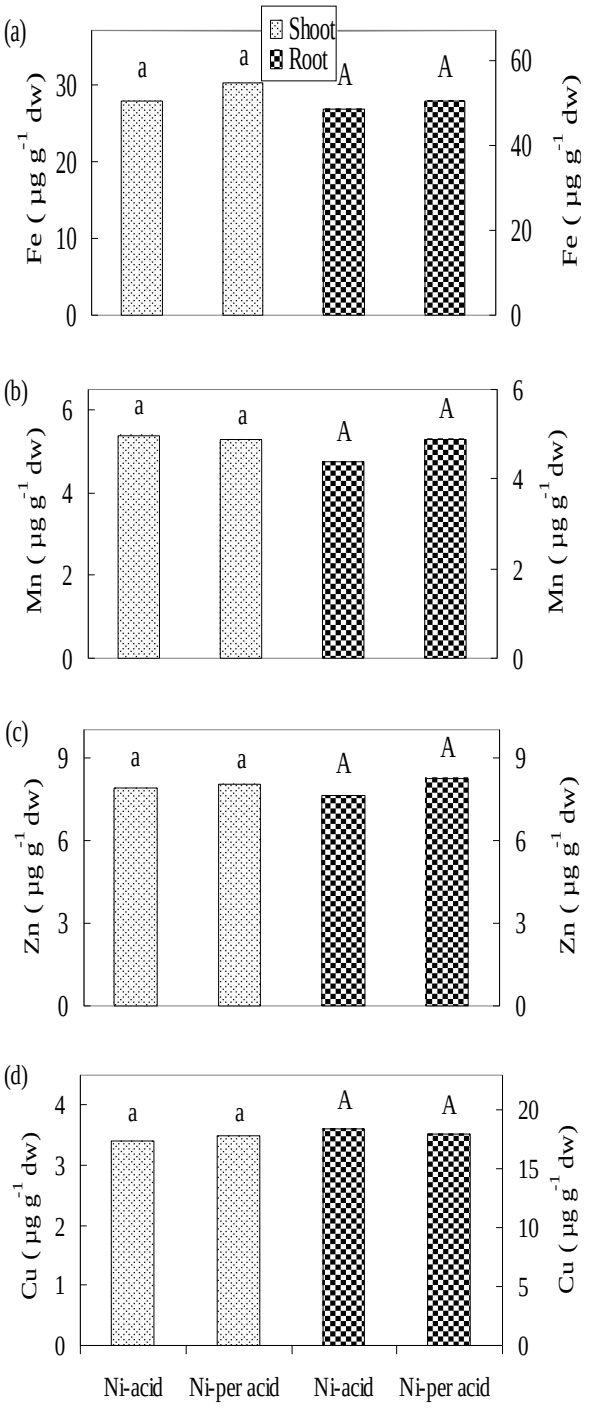

Fig. 2 Concentration of (a) Fe, (b) Mn, (c) Zn and (d) Cu of barley seedlings digested with HNO_3 acid and HNO_3-$HClO_4$ acid mixture. Bars with same letters are not are not significantly different (p < 0.05). Ni-acid indicates HNO_3 acid and Ni-per acid indicate HNO_3-$HClO_4$ acid mixture.

After adding $LaCl_3$ with the acid extract the probable reaction may be as follow

$Ca_3(PO_4)_2 + 2LaCl_3 ====== 2LaPO_4 + 3CaCl_2$
$Mg_3(PO_4)_2 + 2LaCl_3 ====== 2LaPO_4 + 3MgCl_2$
$3CaSO_4 + 2LaCl_3 ====== La_2(SO_4)_3 + 3CaCl_2$
$3MgSO_4 + 2LaCl_3 ====== La_2(SO_4)_3 + 3MgCl_2$
(Insoluble) (Soluble) (Insoluble) (Soluble)

4. CONCLUSION

The results of the present study showed that there were no significant differences between the digesting capacities of HNO_3 acid and HNO_3-$HClO_4$ acid mixtures. With the present heating procedure only HNO_3 acid could be used for the digestion of plant samples especially which contains elements, which determination could be affected by $HClO_4$. Moreover, we found less P in HNO_3-$HClO_4$ acid digested samples as compared to only HNO_3 acid digested samples. Therefore, it was suggested that only HNO_3 acid is a good digester for P and may be for As containing samples also. It was also revealed that there was no significant difference of K concentration data between the solutions containing $LaCl_3$ or without $LaCl_3$ for both experiments. Therefore, it is suggested that the K can be measured from the $LaCl_3$ containing solution especially when the mother solution was diluted enough in this experiment. In this laboratory, K and Mg could be measured from the same solution if the solution is diluted appropriately.

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Study on Seed Germination and Growth Behavior of Brinjal *Solanum melongena* var.BR 112 in Admiration to Effect of C.M.L. (Country Made Liquor)

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Abstract: In India a leading News paper Times of India published an unconfirmed report citing the use of Country made Liquor by the farmers in the National Capital Region Gurgaon for the cultivation of Brinjal crop. It was reported that use of CML increased the production of Brinjal by 06to 08 times. The brinjal thus produced were reported to be of good quality and appearance. An experiment was conducted to study the seed germination and growth behavior of brinjal (*Solanum melongena* L.) with country made liquor under environmental conditions. Seeds of *Solanum melongena* L. var. BR 112, were sown at the depth of 2.5 cm. with different treatments i.e. S1 (Control- Without Country made liquor 36 %V/V), S2 (Soil + 10% solution Spray of Country made liquor at intervals of 03 days with original Concentration 36% V/V). 1000 replicates of each treatment were used for the study. Total numbers of germinated plants were counted from each set of all treatments, at the interval period of 5 days after sowing, and reported as emergence count. For growth study plant height, number of leaves, length and width of leaves and root length were measured from all the treatments. Result revealed that CML treated batch showed maximum germination% i.e. 700 plantlets from 1000 seeds then control i.e. 500 plantlets. After 20 days of Growth plantlets also showed maximum plant height (7.1 cm.), number of leaves (4.5) length of leaves (2.5 cm.), width of leaves (2.6 cm.) and root length (4.3 cm.) in S2 treatment then control S1 plant height(6.5 cm), number of leaves(3.8), length of leaves(2.0 cm), width of leaves(1.0 cm), root length(4.7 cm). [Nature and Science 2010;8(5):163-166]. (ISSN: 1545-0740).

Key Words: *Solanum melongena* BR 112, Country Made Liquor, Tharra.

Introduction

The eggplant, aubergine, begun, or brinjal, or baygan (Solanum melongena), is a plant of the family Solanaceae (also known as the nightshades) and genus Solanum. It bears a fruit of the same name, commonly used as a vegetable in cooking. As a nightshade, it is closely related to the tomato and potato and is native to India. One of the Sanskrit names of brinjal, vartaku, is considered to be a pre-Sanskrit word, derived from an ancient Indian language spoken by the Mundas or Austrics (one of the oldest inhabitants of India), who now live mostly in the state of Jharkhand.

Brinjal is used in all over the world as an edible vegetable crop. Brinjal or egg-plant (*Solanum melongena* L) is one of the most commonly grown vegetable crops of solanaceae family in India. India, China, Turkey, Japan, Philippines are the major brinjal production countries. India contributes 6,44,3062 MT to the global production of brinjal and ranks 2nd to China (Thamburaj and Singh, 2003). In Uttarakhand hilly regions it is grown only in summer. As we know the population of India increases day by day and by this region the scarcity of food also increases. To fulfill all human needs or to meet the demand of today's peoples, farmers generally used inorganic fertilizers to increases the quality and quantity of the crop. Although fertilizers increase the yield of crop but this create an adverse effect on consumer health and as well as on environment (Biswas and Mukharjee, 1994).

In this study BR 112 variety was chosen to see the effect of country made liquor on its productivity. This variety of Brinjal is grown widely in the National Capital Region of Delhi. Recently an Article in a leading News Paper (Times of India, Dated: Feb 22, 2010) published an unconfirmed report that farmers in and around Gurgaon are using C.M.L to increase the yield of brinjal crop. Farmers of every village growing the crop spray country-made as well as India-made foreign liquor (IMFL) on the soil and they claim that this practice not only results in better shape of the crop but also leads to increased yields. Farmers said that this practice of using alcohol had been going on for the past several years and that it ensured that there were more flowers on a brinjal plant thus boosting production.

We sprinkle alcohol in the soil right from the time we sow the seeds. In the last few years, it was observed that the brinjal crop yield had increased manifold. This study is focused on this aspect and to verify the effects of CML on seed germination and growth behavior of *Solanum melongena* BR 112 variety.

This variety is largely grown in the National Capital Region of India and well adapted to the environmental conditions of this region.

MATERIAL AND METHODS:

The present study was carried out with the objective to evaluate the effect of Country made Liquor on seed germination, growth of Brinjal cultivar BR 112. Following treatments were used for the study:

Control (Only Soil): S1

Soil + Spray 10 % solution of Country made Liquor (Original Concentration 36 % V/V) at intervals of 03 days: S2

1000 replicates for each treatment were used for the study. Some important descriptions of the layout are given below:

Total number of seeds used in the experiment: 2000
Number of plants used for each treatment: 1000
Country made liquor used in S2 treatment: 36 % v/v

The country made Liquor was sprayed before sowing of seed in S 2 treatment as well after every 03 days of interval. There was no use of country made Liquor in S 1 treatment. Total numbers of germinated plants were counted from all treatments at interval period of 5 days after sowing and reported as emergence count. Plant growths were observed with different parameters i.e. plant height, number of leaves, length and width of leaves and root length. Ten normal seedlings were randomly taken at the end of the germination count for the study of plant height (shoot length); length and width of leaves were measured in cm. The no. of leaves was counted after 20 days of germination. Three plants of each treatment were randomly selected to measure the root length, which were already used to measure the other growth parameter, and the mean values were arrived at different growth stages.

- Country Made Liquor (CML) **Tharra** is locally brewed alcoholic drink, or moonshine; from yeast fermentation of sugarcane, or wheat husk; in regions of northern India especially Bihar, Uttar Pradesh, Punjab, Nasik (Maharashtra) and Haryana. Due to the pungent smell of the distilling process, ***Tharra*** is often prepared in remote fields, away from human settlements. It recycles some of the waste products of agricultural economy of the region. It is often consumed by poor or landless tillers as well as migrant laborers. It is typically distilled with connivance of officials and police officers, generating huge profit margins.

RESULT:

Germination Study: The germination was influenced by different treatments. Result shows that the maximum number of seedling emergence was in S2 treatment, which contains CML, in contrast to followed by S1 (Table-1).

Growth Study:

Plant height: Maximum plant height was recorded in S2 treatment, at 20 days after sowing which are higher than S1.

Number of leaves per plant: Number of leaves was recorded higher in S2 treatment in contrast to S1. (At 27 days after sowing).

Length & Width of leaves per plant: The leaf length and width were recorded up to 27 days after sowing of seeds in all the treatments. The length and width of green active leaves in S2 treatment is much higher than S1.

Root length: Three plants of each treatment were randomly selected to measure the root length, which were already used for other growth parameter. The root length in S2 treatment was recorded higher than all other treatments i.e. S1.

DISCUSSION:

The result of this investigation shows that the effect of the CML has been very much pronounced. The number of germinated seeds is 20% more in CML treated soil, plant height, Number of leaves, leave width is also more in CML treated soil.

Day by day the use of CML has increased rapidly, in all Brinjal growing fields of National Capital Region of Delhi, India. Farmers are using CML in more quantity to increase the yield and economy, now the use of these is 6 to 8 times more than the time was first reported and growing with every passing season of Brinjal. The use of the CML, affect the soil as well as the crop characteristics and the product from the crop is also influenced. How CML influences the soil, seed germination and growth of the Brinjal plant is a matter of extensive research.

The main advantage of CML is that it doesn't pollute the soil and not give any negative effect to environment because of its biodegradable nature. Also the effect of CML on other crops can be studied. Because of the use of CML is very cost effective. The farmers reported that though the cost of production has increased a little, the high yield makes up for it as far as Brinjal is concerned.

Conclusion:

The results are quite promising and we are very much optimistic about future of this study.

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Table: 1. Effect of different treatments on germination:

Treatments	Germination counts (%)				
	1-5 DAYS	5-10 DAYS	10-15 DAYS	15-20 DAYS	TOTAL
S1	0	200	100	200	500
S2	0	200	300	**200**	**700**

Table: 2. Effect of different treatment on Seedling growth:

S.N	Treatment	Plant height	No. of leaves	Length of leaves	Width of leaves	Root length
1.	S1	6.0 ±0.5	3.0 ±0.8	1.53 ±0.5	0.9 ±0.1	4.05 ±0.7
2.	S2	7.0 ±1.0	4.0 ±0.5	2.0 ±0.5	2.0 ±0.6	4,0 ±0.3

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ON THE REALIZATION OF FLOATING INDUCTORS

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ABSTRACT: Floating inductor circuits using minimum number of passive elements namely two resistors and one capacitor is reviewed in this paper. All the circuits considered in this paper are floating. Previously reported non-floating circuits are modified to be floating and new floating circuits are introduced as well. The active elements used in this paper are floating conveyor building blocks as well as pairs of non-floating conveyor blocks acting as a floating pair. Simulation results of second order lowpass filters realized using different types of floating inductors are included. [Nature and Science 2010;8(5):167-180]. (ISSN: 1545-0740).

Keywords: Floating inductors, current conveyors, gyrator, DVCC, FDVCC.

1. INTRODUCTION

The classification of active RC circuits simulating floating inductors was given in [1]. The floating inductor circuits reported in [1] employ the operational amplifier (Op Amp) or the nullor element [2] known also as the operational floating amplifier (OFA) [3] as the active building block. Detailed derivation of the admittance matrix equation of different types of gyrators was given in [1]. A single Op Amp gyrator realization was introduced in [4]. Gyrator realization using two second generation current conveyors (CCII) with opposite Z polarities was introduced in [5].The use of single CCII- in realizing non-ideal inductor was first introduced in [6] followed by a single CCII+ gyrator circuit [7].

Simulated ideal floating inductors using CCII and transconductance amplifiers (TA) was classified and reviewed in [8] and new CCII floating gyrator circuits were given.

Most recently generation method of floating ideal inductors based on using nodal admittance matrix (NAM) expansion [9-10] was introduced in [11].

This paper concentrates on the realization of floating inductors using floating gyrator circuits and a single capacitor. The total number of resistors in each of the gyrator circuits considered is limited to two resistors.

2. GENERALIZED INDUCTOR CONFIGURATIONS

Figures 1(a) and 1(b) represent the two generalized configurations defined as types A and B realizing floating inductors in accordance with the classification given in [1]. The circuit shown in Figure 1(a) includes two general cases depending on the summation of the four currents.

If the summation $I_1+ I_2+ I_3+ I_4$ is not zero the circuit is not floating. On the other hand a necessary condition that the circuit is floating is given by [1]:

$$I_1+ I_2+ I_3+ I_4 = 0 \qquad (1)$$

Most of the floating inductor circuits that belong to the generalized configuration shown in Figure 1(a) in which the capacitor is grounded are not floating. Table 3 in [8] includes nine gyrator circuits that belong to this case.

Six generalized configurations that belong to type A, and realize floating inductors satisfying the condition of equation (1) are given in this paper. Six generalized configurations that belong to type B are also given in this paper.

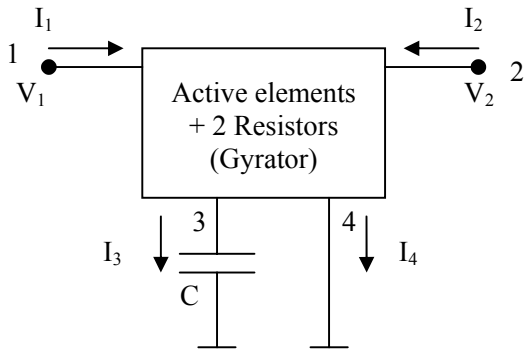


Figure 1(a) Generalized Type A configuration

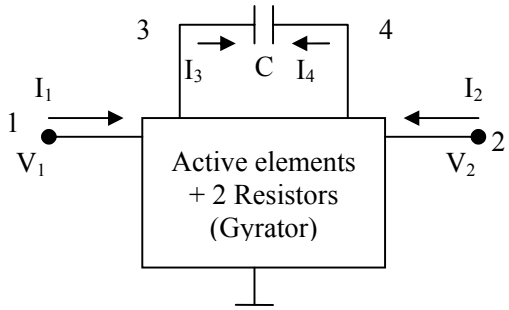


Figure 1(b) Generalized Type B configuration

The first active building block that is used in the paper is the generalized conveyor (GC) defined by the following matrix equation:

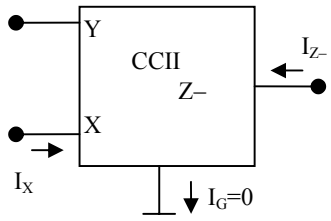
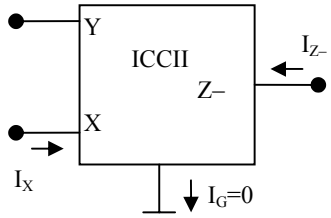
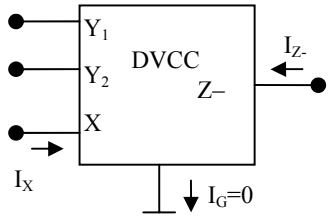
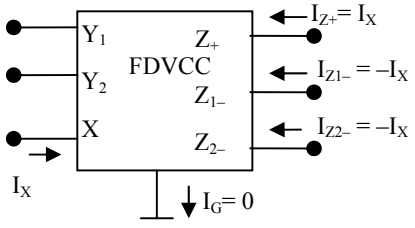
$$\begin{bmatrix} I_Y \\ V_X \\ I_Z \end{bmatrix} = \begin{bmatrix} 0 & 0 & 0 \\ a & 0 & 0 \\ 0 & K & 0 \end{bmatrix} \begin{bmatrix} V_Y \\ I_X \\ V_Z \end{bmatrix} \quad (2)$$

The parameter a determines the type of the GC, a CCII is realized if a =1 and ICCII is obtained if a = –1. The parameter K determines the Z polarity of the GC, for Z+ the parameter K =1 and for Z– the parameter K = –1.
The GC includes four different types; the CCII– and the ICCII– are floating whereas the CCII+ and ICCII+ are not floating. The CCII+ and ICCII+ although non-floating can also be used in realizing floating circuits provided they are used in pairs as will be demonstrated in the next section.
Table 1 includes a summary of the floating conveyor building blocks that will be used in this paper.

3. TYPE A INDUCTOR CIRCUITS

In this section six floating circuits realizing floating inductors satisfying the condition of equation (1) and using two resistors and one capacitor are given.

Table 1 Floating building blocks used in the paper

Conveyor	Definition	Floating Building Block Symbol
CCII- [5]	$\begin{bmatrix} I_Y \\ V_X \\ I_Z \end{bmatrix} = \begin{bmatrix} 0 & 0 & 0 \\ 1 & 0 & 0 \\ 0 & -1 & 0 \end{bmatrix} \begin{bmatrix} V_Y \\ I_X \\ V_Z \end{bmatrix}$	
ICCII-[12]	$\begin{bmatrix} I_Y \\ V_X \\ I_Z \end{bmatrix} = \begin{bmatrix} 0 & 0 & 0 \\ -1 & 0 & 0 \\ 0 & -1 & 0 \end{bmatrix} \begin{bmatrix} V_Y \\ I_X \\ V_Z \end{bmatrix}$	
DVCC- [13]	$\begin{bmatrix} Vx \\ IY1 \\ IY2 \\ I\,Z- \end{bmatrix} = \begin{bmatrix} 0 & 1 & -1 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ -1 & 0 & 0 & 0 \end{bmatrix} \begin{bmatrix} Ix \\ VY1 \\ VY2 \\ VZ- \end{bmatrix}$	
FDVCC [New]	$\begin{bmatrix} VX \\ IY1 \\ IY2 \\ IZ+ \\ IZ1- \\ IZ2- \end{bmatrix} = \begin{bmatrix} 0 & 1 & -1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 \\ 1 & 0 & 0 & 0 & 0 & 0 \\ -1 & 0 & 0 & 0 & 0 & 0 \\ -1 & 0 & 0 & 0 & 0 & 0 \end{bmatrix} \begin{bmatrix} IX \\ VY1 \\ VY2 \\ VZ+ \\ VZ1- \\ VZ2- \end{bmatrix}$	

3.1. Four Generalized Conveyor Circuit

The configuration shown in Figure 2 using four nullors was first reported in [1]. The generalized configuration using four CCII+ realized from operational amplifiers (Op Amps) together with current mirrors was first reported in [14] and republished in [15] with a comment given in [16]. The generalized configuration using four CCII– (equivalent to four nullors) was reported in [8]. The same generalized configuration using four ICCII– was reported in [17].

The circuit shown in Figure 2 employs four GC and a necessary condition for V_1 and V_2 to appear in a subtraction form is that $a_1 = a_2$. A necessary condition for the circuit to be floating is that $K_1 = K_2$.These two conditions imply that GC1 and GC2 must be matched.

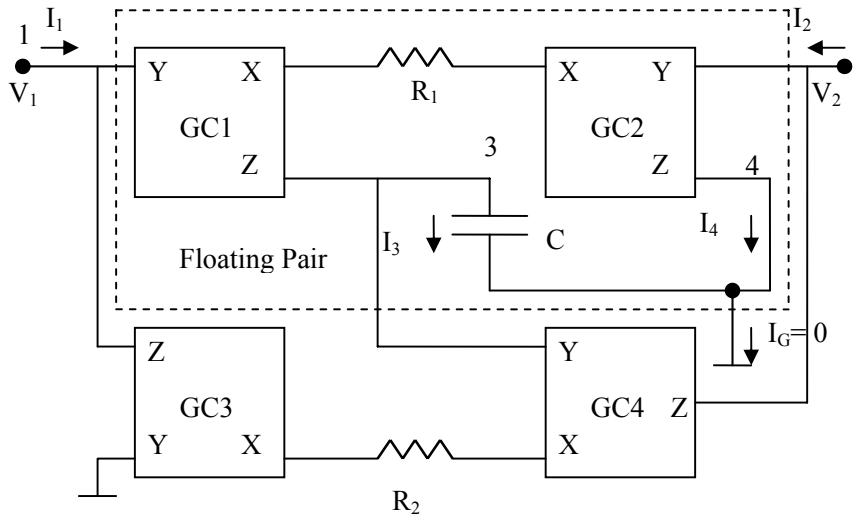


Figure 2. A floating inductor using four generalized conveyor

A necessary condition for the current I_1 to equal to $-I_2$ is that $K_3 = K_4$.
Although a_3 can be either 1 or –1 and has no effect on circuit operation it is taken equal to a_4 for a symmetrical circuit. Therefore GC3 and GC4 are taken to be matched.
By direct analysis it can be shown that:

$$I1 = -I2 = \frac{V1 - V2}{sCR1R2}\,[a_1K_1a_3K_3] \qquad (3)$$

A necessary condition for a floating inductor realization is that $a_1K_1a_3K_3$ must be +1. Eight possible conveyor realizations are given in Table 2 that satisfies this coefficient condition. It is seen that this approach of analysis resulted in five new conveyor circuits.

3.2 DVCC– and Two CCII– Or Two ICCII– Circuits

The circuit shown in Figure 3(a) is a modified version of the newly reported DVCC and two CCII+ circuit shown in Figure 14 of [11] by replacing the two CCII+ by two CCII– .The circuit is floating since I_G is zero and it realizes a floating inductor given by CR_1R_2.
Figure 3(b) represents a second equivalent floating circuit which uses two ICCII– instead of the two CCII– in Figure 3(a).

3.3 Two Floating DVCC Circuits

The two floating circuits shown in Figure 4 are new and they are obtained from the newly reported circuit shown in Figure 16 of [11]. The circuits employ the DVCC– and the newly defined floating DVCC (FDVCC) given in Table 1.
The third circuit is shown in Figure 5 is a modified version of the floating inductor circuit proposed in [18] by using the FDVCC instead of the DVCC+ in [18].
The three circuits shown in Figures 4 and 5 are floating since I_G is zero and each realizes a floating inductor given by CR_1R_2.

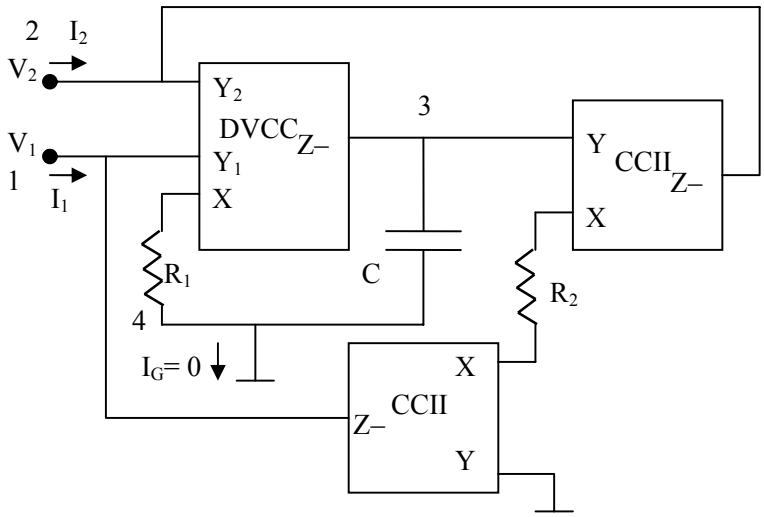


Figure 3(a) A floating inductor using a DVCC and two CCII- [11]

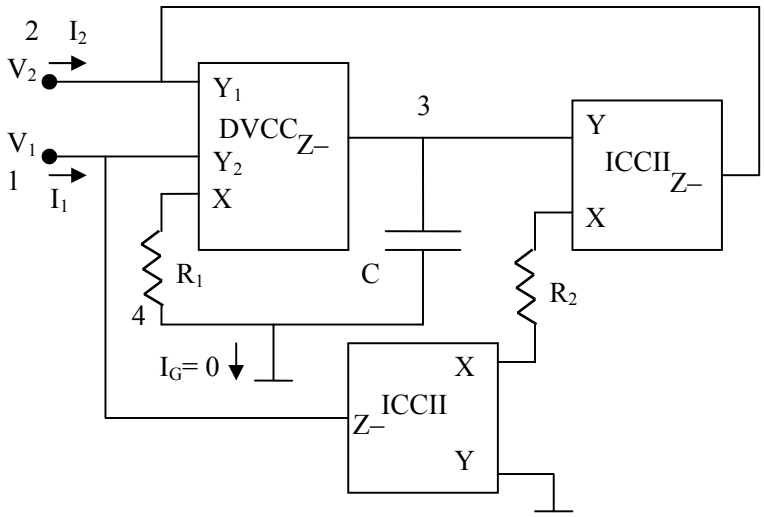


Figure 3(b) Alternative floating inductor using a DVCC and two CCII-

4. TYPE B INDUCTOR CIRCUITS

The circuit shown in Figure 6(a) is a modified version of the circuit of Figure 2. It was introduced in [1] using four nullors and in [19] using four CCII– or four CCII+, it was also reported in [8] using four CCII–.The GC1 and GC2 must be identical types; also GC3 and GC4 must be identical types. The types of conveyors given in Table 2 apply to this floating circuit also.

An alternative generalized configuration realizing a floating inductor is shown in Figure 6(b). The GC1 and GC2 must be identical types; also GC3 and GC4 must be identical types. By direct analysis it can be shown that:

$$I1 = -I2 = -\frac{V1 - V2}{sCR1R2}\ [a_1K_1a_3K_3] \qquad (4)$$

A necessary condition for a floating inductor realization is that $a_1K_1a_3K_3$ must be -1. Eight possible conveyors realizations are given in Table 3 that satisfies this coefficient condition. It is seen that seven new circuits are generated based on this circuit topology.

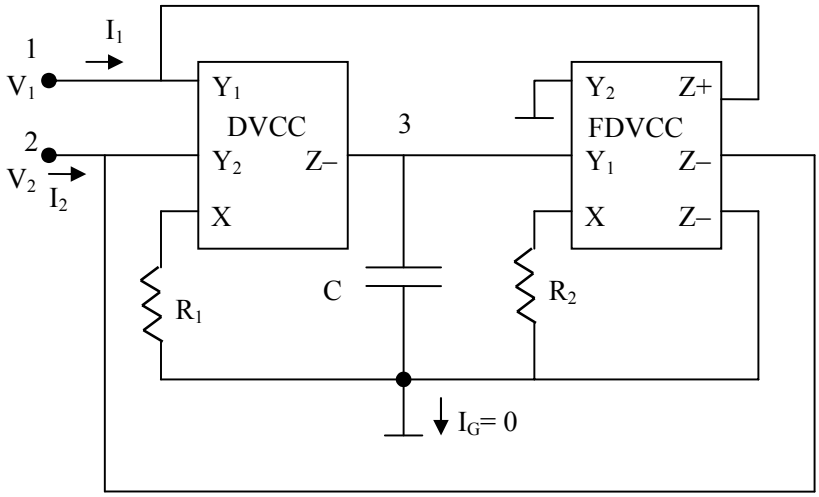
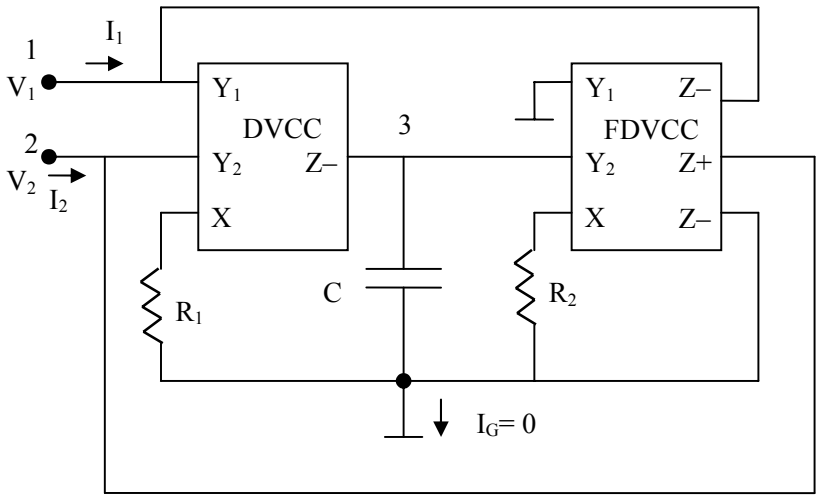


Figure 4(a)



4(b)

Figure 4 Two floating inductor circuits using DVCC and FDVCC [11]

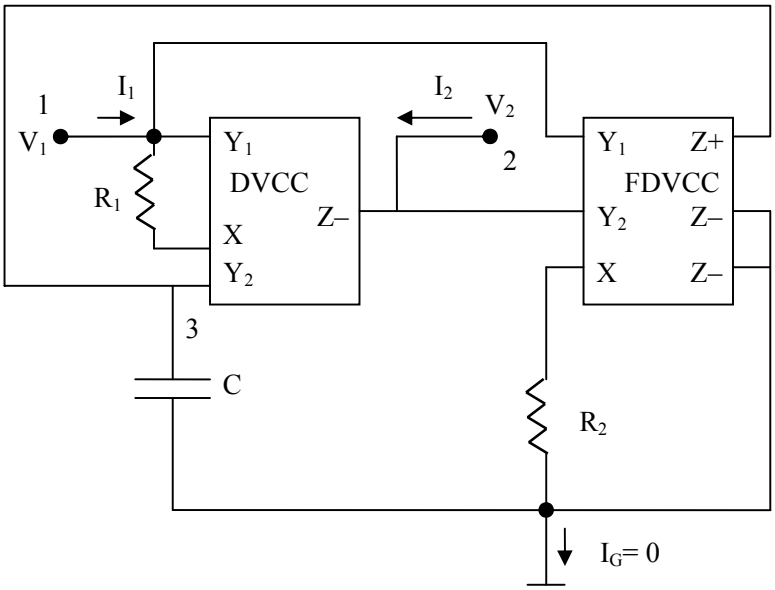


Figure5 A modified floating inductor using DVCC and FDVCC [18]

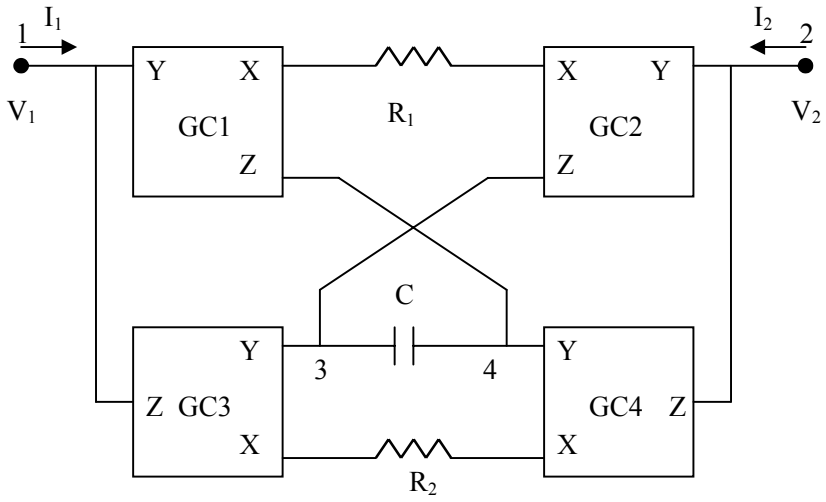


Figure 6(a) A floating inductor using four generalized conveyors [19]

Figure 7(a) represents a floating inductor circuit using two CCII+ and one CCII– [20]. This is among the four floating gyrator circuits reported in [8]. A new modified floating inductor circuit using two ICCII– and one CCII–is shown in Figure 7(b).

Figure 8(a) represents a modified floating inductor circuit to the two DVCC circuit reported in [21] using two FDVCC in order to have a floating circuit with I_G equal to zero. An alternative new equivalent circuit is shown in Figure 8(b). Both circuits realize an inductor of magnitude CR_1R_2.

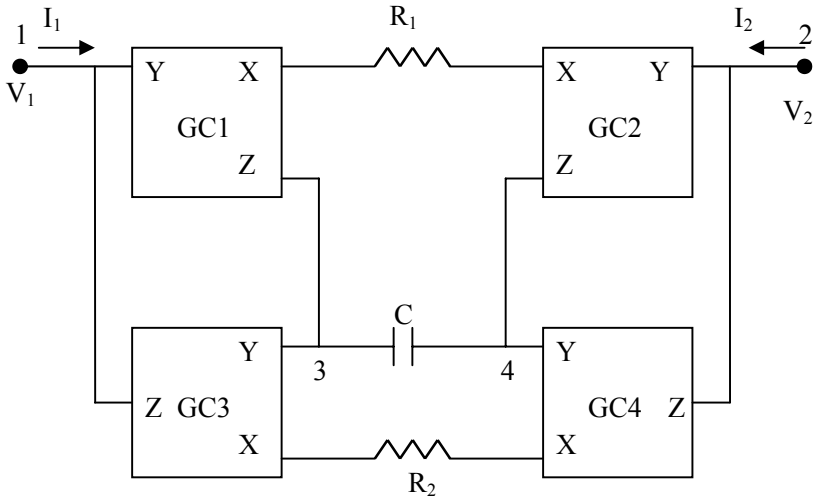


Figure 6(b) Alternative floating inductor using four generalized conveyors

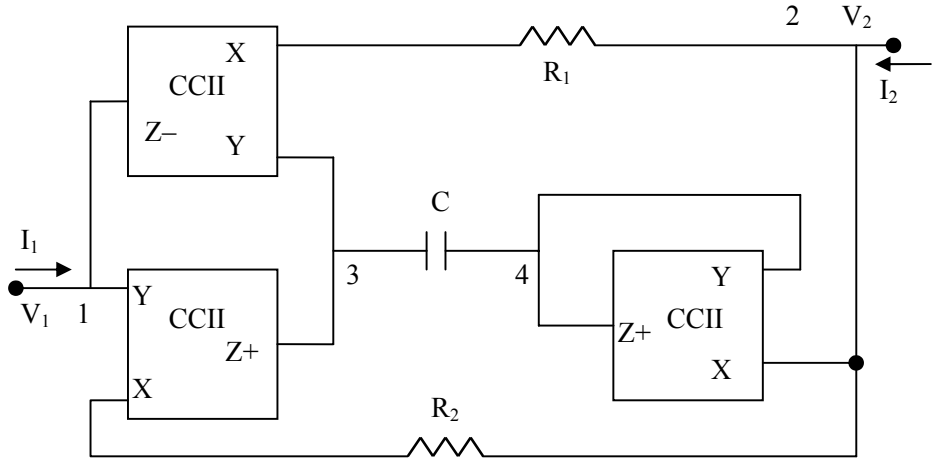


Figure 7(a) A floating inductor using two CCII+ and one CCII– [20]

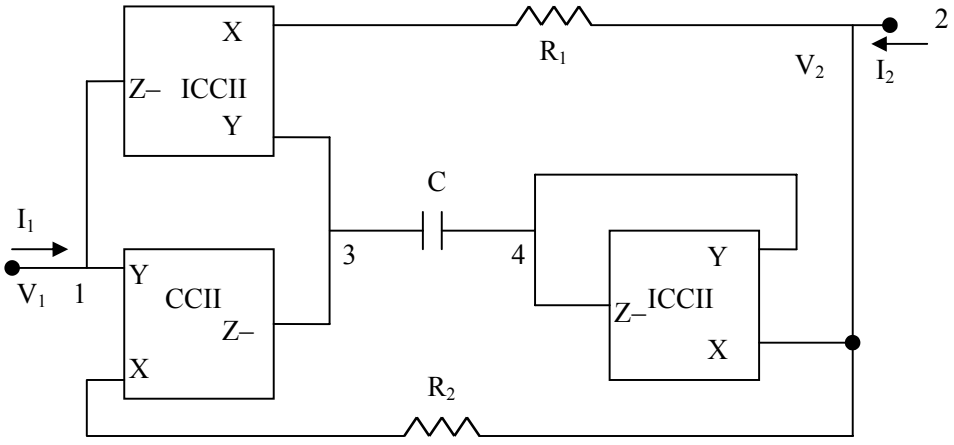


Figure 7(b) A new floating inductor using two ICCII– and one CCII–

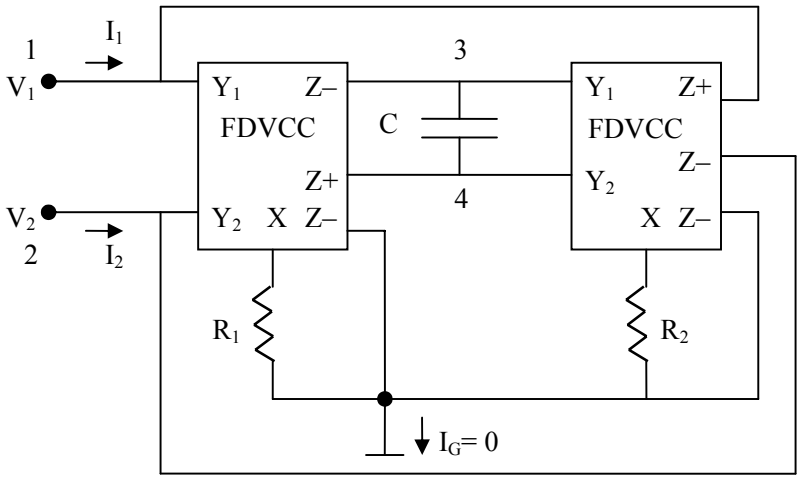


Figure 8(a) A modified floating inductor using two DVCC [21].

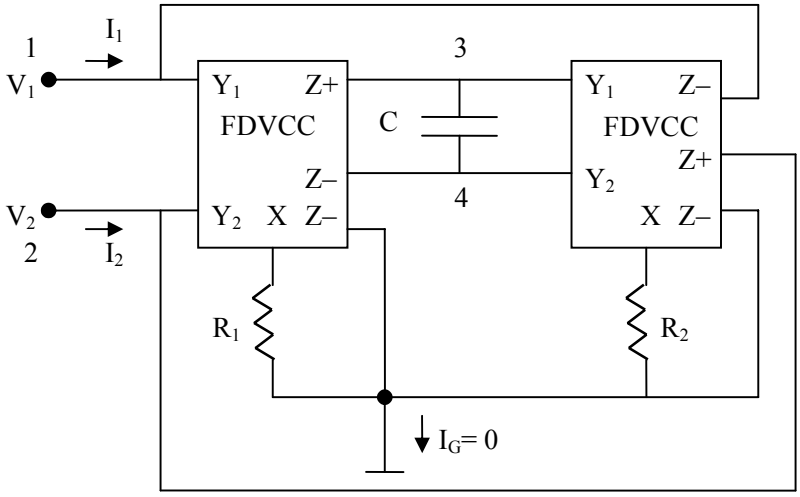


Figure 8(b) Alternative modified floating inductor using two DVCC.

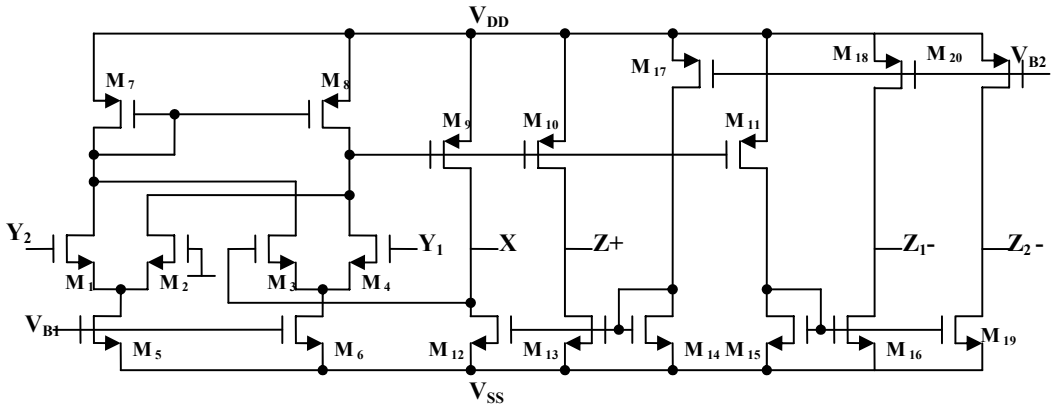


Figure 9 CMOS circuit of the floating DVCC [21]

5. SIMULATION RESULTS

The CMOS circuit realizing the FDVCC is obtained directly from the well known DVCC [21] by adding the two MOS transistors M_{19} and M_{20} as shown in Figure 9.
The transistor aspect ratios are given in Table 4 based on the 0.5μm CMOS model from MOSIS. The supply voltages used are ± 1.5 V and V_{B1}= –0.52 V and V_{B2} = 0.33 V.
This circuit will be used in the following simulations to realize different types of conveyors.
As an application of some of the floating circuits reported, a floating inductor of magnitude 0.253 m. H is realized taking the capacitor C =100pFand $R_1 = R_2 = 1.59$ kΩ. The floating inductor is used to realize a maximally flat (Q=0.707) second order low-pass filter with cutoff frequency of 1MHz using a series resistor of $R_S = 2.25$ kΩ and C_S of 100pF.
Figure 10(a) represents the simulated magnitude and phase responses together with the ideal responses using the inductor circuit of Figure 2 with four ICCII+.
Figure 10(b) represents the simulated magnitude and phase responses together with the ideal responses using the inductor circuit of Figure 3(a).
Figure 10(c) represents the simulated magnitude and phase responses together with the ideal responses using the inductor circuit of Figure 5.
Figure 11(a) represents the simulated magnitude and phase responses together with the ideal responses using the inductor circuit of Figure 6(b) with four ICCII+.
Figure 11(b) represents the simulated magnitude and phase responses together with the ideal responses using the inductor circuit of Figure 7(b).
Figure 11(c) represents the simulated magnitude and phase responses together with the ideal responses using the inductor circuit of Figure 8(b).

Table 2 Eight alternative conveyor circuits based on Figure 2

Circuit	a_1, a_2	K_1, K_2	a_3, a_4	K_3, K_4	GC_1,GC_2	GC_3,GC_4	Ref
1	+	+	+	+	CCII+	CCII+	14–16
2	+	–	+	–	CCII–	CCII–	1, 8
3	–	–	+	+	ICCII–	CCII+	New
4	+	+	–	–	CCII+	ICCII–	New
5	–	+	–	+	ICCII+	ICCII+	New
6	–	+	+	–	ICCII+	CCII–	New
7	+	–	–	+	CCII–	ICCII+	New
8	–	–	–	–	ICCII–	ICCII–	17

Table 3 Eight alternative conveyor circuits based on Figure 6(b)

Circuit	a_1, a_2	K_1, K_2	a_3, a_4	K_3, K_4	GC_1,GC_2	GC_3,GC_4	Ref
1	+	–	+	+	CCII–	CCII+	8
2	+	+	+	–	CCII+	CCII–	New
3	–	+	+	+	ICCII+	CCII+	New
4	+	+	–	+	CCII+	ICCII+	New
5	+	–	–	–	CCII–	ICCII–	New
6	–	+	–	–	ICCII+	ICCII–	New
7	–	–	+	–	ICCII–	CCII–	New
8	–	–	–	+	ICCII–	ICCII+	New

6. CONCLUSIONS

Realization of ideal inductor circuits using different types of conveyor building blocks is reviewed. Two types of inductor circuits are defined as was originally classified in [1]. The FDVCC is defined and is used in several circuits in this paper. Spice simulation results are given. Although this paper is partially a review paper it includes several new floating circuits

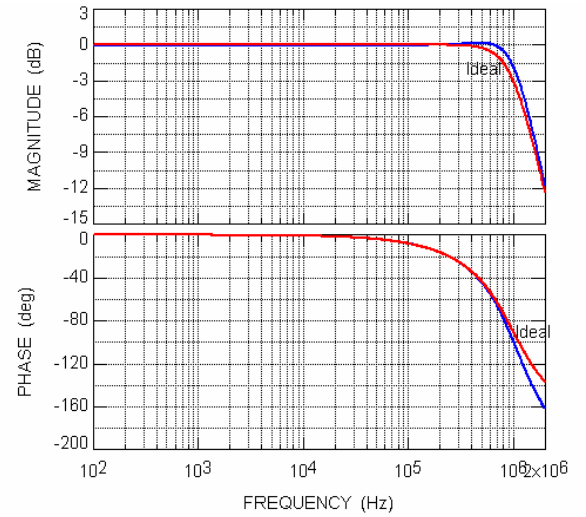


Figure 10(a) Simulation results of a lowpass filter using L of Figure 2

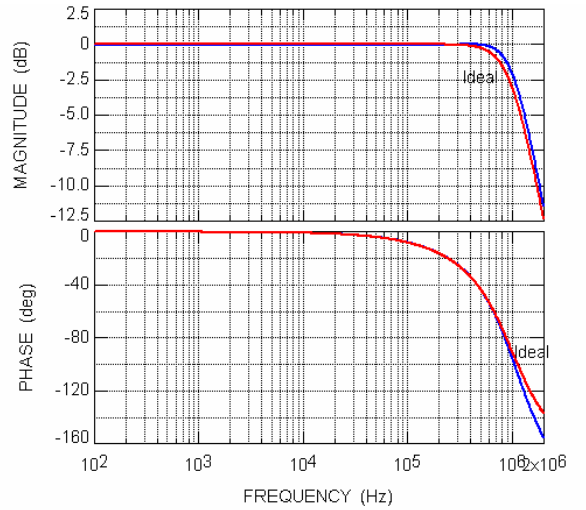


Fig 10(b) Simulation results of a lowpass filter using L of Figure 3(a)

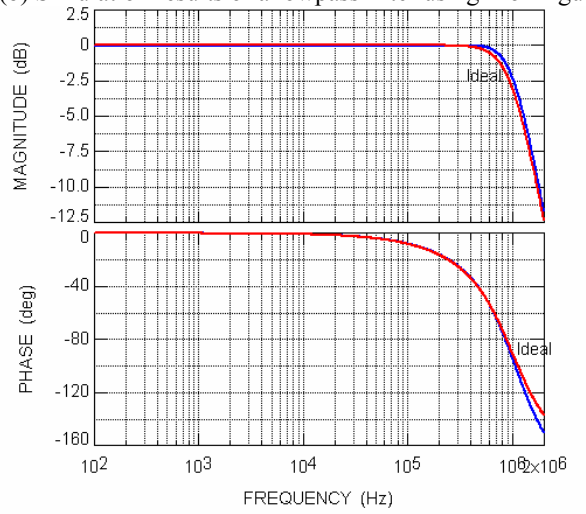


Fig 10(c) Simulation results of a lowpass filter using L of Figure 5

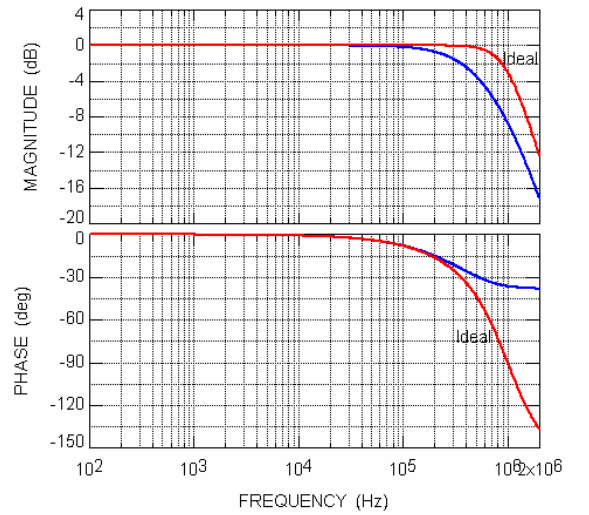


Fig 11(a) Simulation results of a lowpass filter using L of Figure 6(b)

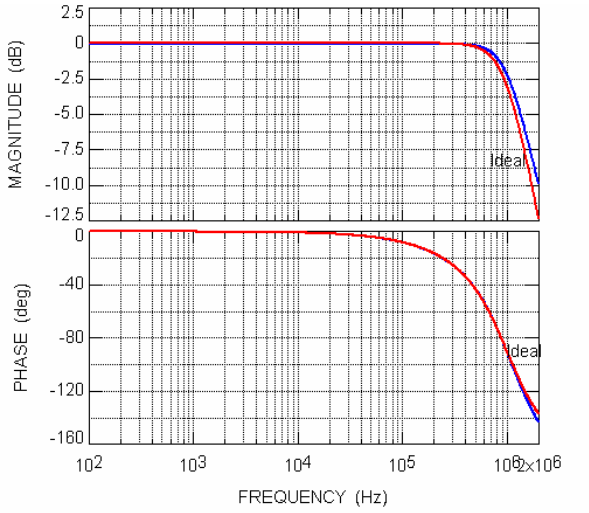


Fig 11(b) Simulation results of a lowpass filter using L of Figure 7(b)

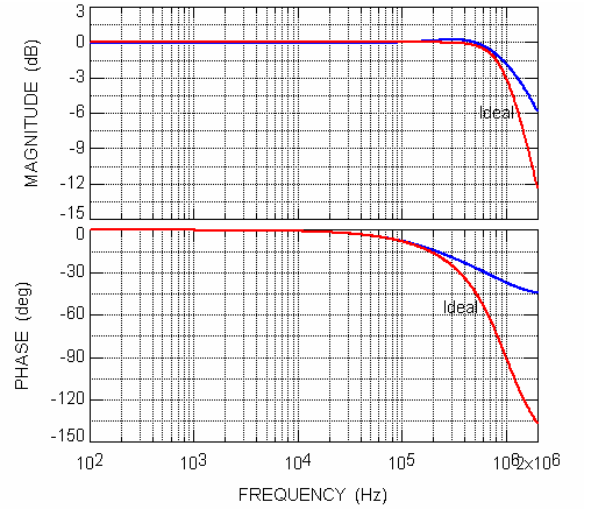


Fig 11(c) Simulation results of a lowpass filter using L of Figure 8(b)

Table 4 Transistor aspect ratios of the FDVCC of Figure 9

MOS Transistors	W(μm)/L(μm)
M_1, M_2, M_3,M_4	8/1
M_5 , M_6	8/1
M_{12}, M_{13}, M_{14}, M_{15}, M_{16}, M_{17}	20/2.5
M_7, M_8	10/1
M_9, M_{10}, M_{11}, M_{18}, M_{19}, M_{20}	40/2

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BIOGRAPHY:

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He was a visiting scholar at Bochum University, Germany (Summer 1985) and with the Technical University of Wien, Austria (Summer 1987).
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Dr Soliman is a Member of the Editorial Board of Analog Integrated Circuits and Signal Processing.
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3/9/2010

Functional-Food Supplementation and Health of Broilers

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Abstract: This study was conducted to evaluate the effect of a probiotic mixture and chromium as food supplementation on broiler chicks' performance. The experiment was conducted, to determine the effect of a probiotic mixture (BiovetYC) and chromium chloride supplementation on growth performance, carcass traits and immune response against Avian Influenza virus from 0- to 42-d-old broiler chicks as well as to determine the anti-stress effect of the dietary probiotic mixture and chromium chloride supplementation on broiler chicks (0-42days old) when subjected to high stocking density (15 birds/m^2 in open-system) as a stress factor. Growth performance, carcass traits and Avian Influenza immune response were recorded. At 42-d of age, 50 birds were randomly selected from each group for blood samples collection and slaughtered for carcass traits. Stress indicators in blood (cortisol and L/H ratio) were measured. The current results revealed: (1) The activation effect of the probiotic mixture on growth performance (2) Chromium chloride supplementation improves growth performance, carcass traits, and immune response and had a strong anti-stress effect. [Nature and Science 2010;8(5):181-189]. (ISSN: 1545-0740).

Keywords: Functional food; Performance; Immune response.

1. Introduction

The microbial populations in the gastrointestinal tracts of poultry play a key role in normal digestive processes and in maintaining animal health. In Greek probiotic means "for life" and can be defined as a live microbial feed supplement, which beneficially affect the host animal by improving its intestinal balance (Huang *et al.*, 2004). The inclusion of probiotics in foods is designed to encourage certain strains of bacteria in the gut at the expense of less desirable ones. The use of probiotics, yeast cultures and acidifiers in poultry feeds generated because of increased public awareness and objection to the use antibiotics as growth promotant feed additive.

The combine use of *lactobacillus* and yeast cultures in the feed and water has been shown to be effective in reducing morbidity and mortality and improving growth performance and production. (Choudhari, *et al*; 2008). Live yeast culture (*S. cerevisiae*) plus lactic acid producing bacteria (*L. acidophilus* and *S. faecium*) was supplemented in broiler (1 kg/ton) and the results showed improved weight gain and feed conversion. With laying hens *lactobacilli* resulted in an improvement in egg production and feed efficiency (Mohan *et al*., 1996). In commercial broilers the inclusion of *L.sporogens* @100 mg/kg feed resulted into increased body weight gain, improved FCR and humoral immune response in broiler chicks during 0-6 weeks of age (Panda *et al*., 2005). Over the last several years considerable attention has been given. The mechanism of action of probiotics had not been fully explained although there are several hypotheses. The health-promoting effect of probiotics in the gastrointestinal tract had been mainly associated with their capacity to stimulate the immune response and to inhibit the growth of pathogenic bacteria. (Barnes *et al.*, 1972 and Gérard *et al.*, 2008), as well as to modulate the immune markers (Dekker *et al.*, 2007). *Lactobacillus* administration had been shown to enhance nutrient absorption, improve growth rates and feed conversion in broiler chickens (Kalavathy *et al.*, 2003).

On the other hand, with the success of the Human Genome Project and the advances in molecular biology, a new discipline, namely nutrigenomics, in the field of nutrition research has emerged (Kaput *et al.*, 2007). The goal of nutrigenomics -- short for nutritional genomics -- is to develop foods and feeds that can be matched to genotypes of animals to benefit health and enhance normal physiological processes. Chromium has been demonstrated to enhance the expression of plasmalemmal calcium-ATPase in smooth muscle cells; it is possible that chromium may influence calcium homeostasis by increasing the calcium storage capacity through up-regulation of calsequestrin (Moore *et al*., 1998).

The intriguing possibility that supplementation chromium increased longevity and retarded aging by improving immune function and enhancing resistance to infectious diseases is being investigated (Burton *et al.*, 1996). Currently, the predominant hypothesis on

the Cr (III) action is the chromodulin-mediated role on the insulin-activated glucose uptake by cells. (Hamilton and Wetterhahn, 1986 and EFSA, 2009). Besides the effects on blood glucose clearance, Cr(III) via insulin action is thought to participate also in the protein metabolism by stimulating the amino acids uptake by cells (Evans and Bowman, 1992).

In poultry, studies using supplementation with Cr-picolinate, Cr-yeast and Cr-nicotinate at doses up to 0.8 mg Cr kg-1feed did not show consistent effects on performance and carcass traits in chickens or turkeys for fattening (Lee *et al*., 2003 and Debski *et al.*, 2004). Piva *et al*. (2003), could not show adverse effects in laying hens fed with a basal diet containing 3.4 mg Cr kg^{-1} feed after a five-week supplementation with 24.1 mg Cr kg^{-1} feed from Crchloride,36.3 mg Cr kg-1 feed from yeast and 47.5 mg Cr kg^{-1} feed from aminoniacinate,respectively.

Similarly to cattle, birds under stress conditions tend to show reduced mortality. The only relatively consistent effects of Cr (III) supplements occur in serum lipid profile and reduced egg cholesterol. (Lindemann, 2007).

The aim of current study was to examine the effects of a probiotic mixture as functional-food and a trivalent chromium chloride as nutrigenomics additives on growth performance (feed intake, body weight gain , food conversion rate), carcass traits (dressing %, liver somatic indices), immune response (against AIV) and stress conditions(high stocking density) in broiler chickens.

2 - Materials and Methods

These experiments were conducted from January to March of 2009. A total 2700 of commercial one day-old Hubbard chicks of both sexes were used. All pens contained litter top-dressed with 10 cm of clean wood shavings. The broiler chicks had ad libitum access to feed in mash form and water for the duration of the experiment .The chicks were brooded between 31 and 32°C on wk 1, and the temperature was lowered gradually each week until 24 to 27°C was achieved. The 2-phase feeding program consisted of starter, and finisher diets (Table 1) fed from 0 to 21, and 22 to 42.

The experiment was conducted to determine the effect of a probiotic mixture (Biovet) and chromium chloride supplementation on growth performance, carcass traits and AI immune response of broiler chicks (0-42-d-old) also, to determine the anti-stress effect of the dietary probiotic mixture and chromium chloride supplementation on broiler chicks when subjected to high stocking density (15 birds/m2 in open-system) as a stress factor. The experiment was consisted of 6 treatments x 3 replication, each 150 broiler chicks of both sexes per pen.

Table 1. *Composition of basal diets (%)*

Ingredients	Starter (0 – 21 d)	Finisher (22 – 42 d)
Yellow corn	50.86	58.66
Corn gluten	5.00	3.00
Soybean meal	35.0	30.00
Soy oil	5.80	5.00
Dicalcium phosphate	2.50	2.50
Lime stone	0.13	0.13
Common salt	0.33	0.33
DL-Methionine	0.05	0.05
L-lysine	0.03	0.03
Broiler premix[1]	0.30	0.30
Nutrient Profile:		
ME (kcal/kg)	3184.21	3187.81
Crude protein%	22.82	20.00
C / P ratio	139.5	159.4
Crude fat%	7.3	6.1
Crude fibre%	4.3	4.8
Total ash%	6.1	6.2
Calcium%	0.9	0.9
Non-phytate phosphorus%	0.46	0.48

[1] Supplied per kilogram of diet: vitamin A, 10000 IU; vitamin D_3 , 9790 IU; vitamin E, 121 IU; B_{12}, 20 µg; riboflavin, 4.4 mg; calcium pantothenate, 40 mg; niacin, 22 mg; choline, 840 mg; biotin, 30 µg; thiamin, 4 mg; zinc sulfate, 60 mg; manganese oxide, 60.

On 0-d, chicks were randomly divided into 18 floor pen groups containing 150 birds each. Mortalities were recorded daily, and the weights of the dead birds were recorded weekly to adjust gain, feed consumption (feed intake, and gain: feed) and body weights .At 42-d of age, 50 birds were randomly selected from each group for blood sample collection and slaughtered for evaluation of carcass traits, stress indicators in blood (cortisol and L/H ratio). All chicks were s\c immunized with killed vaccine of Avian Influenza (H5N1) virus at age of 7 days (each chick was given 0.05 mL of vaccine). On day 21, 28, 35 and 42, blood samples were collected from the wing vein of 15 chicks per the two replicates and serum antibody titer against Influenza (H5N1) virus were determined by Haemagglutination Inhibition (HI) test and were expressed as logarithm base 2 (OIE,2005).

Experiment

This experiment was conducted to:

1- Evaluate the effect of a probiotic mixture and chromium chloride supplementation on growth

performance, carcass traits and AI immune response of broiler chicks in 0-42-d-old commercial broilers. The supplemented and non-supplemented diets were assigned to be isocaloric and isonitrogenous. All groups were stocked at 10 birds/m², group A consumed non-supplemented diets and served as a negative control group. Group B fed on probiotic mixture supplemented diets and served as a positive control group. Group C fed on chromium chloride supplemented diets with 10 birds/m^2 and served as a positive control group. Probiotic mixture was used in the diet to provide 5 g/kg. Chromium chloride was included in the diet to provide 200 ppm (0.2 g/kg) supplemental.

2- To determine the anti-stress effect of the dietary probiotic mixture (Biovet) and chromium chloride supplementation on broiler chicks in 0- to 42-d-old broilers and stocked at 15 birds/m^2 as a stress factor. Group D consumed non-supplemented diets with 15 birds/m^2 and served as a negative control group. Group E fed on probiotic mixture supplemented diets. Group F fed on chromium chloride supplemented diets with 15 birds/m^2.

Probiotic

A commercially available probiotic mixture (Biovet) was used. Each kg of the probiotic mixture contains a combination of Lactobacillus sporogenes ($75x10^8$ c.f.u.), Lactobacillus acidophilus ($330x10^9$ c.f.u.), Saccharomyces cerevisiae $125x10^9$ c.f.u.), alpha-amylase 5 g and sea weed powder 100g. Biovet is a commercial feed additive used by an inclusion rate of 500 g/tone and marketed by WOCKHARDT Limited, Mumbai, India.

Chromium chloride

Chromium chloride (anhydrous Merck-Germany) was used in the diet in the rate of 200 mg/kg in basal diet (NRC, 1997 and Ahmadi et al., 2004).

Basal diets

Corn-soybean meal based diets (Table 1) as mash form were formulated to meet the nutrient requirements of broilers (NRC, 1994). Feed and water were provided *ad-libitum* during the entire experimental period (42 d).

Growth performance

Feed consumption (FC) throughout the experimental period (6 wks), feed efficiency (FE), and final Live body weight (LBW) as well as body weight gain were determined for each group separately (Feddes et al., 2002).

Immune response

Hemagglutination (HA) and Hemagglutination Inhibition (HI) tests. The recommended method use V-bottomed micro well plastic plates were applied. In which the final volume for both types of HA and HI test was 0.075 ml. The reagents required for these tests are isotonic PBS (0.1 M), pH 7.0-7.2 and RBCs. Positive and negative control antigens and antisera should be run with each test. The test was applied to quantify AIV antibodies in chicken sera according to OIE (2005).

Carcass traits

Carcass yield (dressing %) was obtained by expressing the dressed carcass weight as a percentage of live body weight (Brake *et al.*, 1993). The birds were then processed by removing the head, neck, shanks and feet, and eviscerated by cutting around the vent then carefully removing the viscera.

Haematological analysis

Blood samples were collected from randomly selected 50 birds from each group at the end of the experimental period and the number of heterophils and lymphocytes were determined using the haemocytometer method. Also the plasma cortisol level was determined (Gross and Siegel, 1983)

Statistics analysis

All data were statistically analyzed using SPSS® version 11.0 software for personal computer (2005). Means were compared by the Post-Hoc test.

3- Results

Growth performance

Results in group A,B and C, showed that addition of the probiotic mixture and chromium chloride supplementation improved significantly *(P<0.05)* performance (feed gain and feed conversion), increase immune response and carcass traits in group B and C compared with group A. Broiler chicks in group C recorded highest performance, immune response and carcass traits compared with group B.

The highest performance (growth rate and feed efficiency) noticed in group C post Cr supplementation. Results in group D, F and E (Anti-stress) showed that, the lowest performance parameters and highest feed conversion (*P<0.05*) were recorded in the high stocking density group D, which consumed non supplemented diets. Furthermore, BiovetYC affected positively the chicken performance under the stress of high stocking density in group E and the bird performance parameters recorded in that group were significantly different from those in the control group D.

Immune response

Antibody titers against Influenza virus (H5N1)

are shown in Table 4, at age 21 days, antibody titer was significantly affected by supplemental chromium and probiotic mixture (groups C and B respectively) in comparison with group A *(P < 0.05)*. At age 28 and 35 days, chicks in group C were showed a highly significant improvement of antibody titers against Influenza virus (H5N1) *(P < 0.05)*.

Carcass traits

Table 5 demonstrated the results of dressing, liver weights and their percentages of live body weights (somatic index). Dressing weights were improved significantly (*P<0.05*) versus group A with chromium and probiotic supplementation in groups B and C. The highest carcass traits (*P<0.05*) were recorded with chromium supplementation in group C. These results confirmed that chromium supplementation had a benefit health effect and enhanced normal physiological processes.

Dressing weights were improved significantly in groups B and C (*P<0.05*) versus group A with chromium and probiotic supplementation. Group E recorded significant difference (*P<0.05*) compared with the control negative group D.The highest carcass traits (*P<0.05*) were recorded in the high stocking density group F, which consumed chromium supplemented diets. These results revealed that the chromium used had strong anti-stress effects and thereby the overcrowded conditions in group F which did not negatively affect carcass traits of broiler chickens.

Stress indicators

Blood Stress indicators in (Table 6) significantly increased (*P<0.05*) in group D, while there was no significant difference between the other groups Conclusively; the present research showed: (1) the activation effect of the probiotic mixture on growth performance and stress (2) Cr3+ supplementation improved aspects of growth performance, carcass traits, immune response and has a strong anti-stress effect.

Table 2. *Effect of the dietary supplementation of probiotic mixture and chromium chloride on weekly body weight, feed intake, body weight gain and feed conversion factor (FCR) in broiler chickens (mean±SD)*

Age (d)		Group A	Group B	Group C
7	Body weight (g/bird)	138.50±2.7	142.33±3.9	148.8±3.3
	Feed Intake (g/bird)	127.21±4.5	126.51±6.40	127.14±5.2
	Body weight gain (g/bird)	98.5±1.87	100.34±1.68	100.12±1.82
	FCR	1.29	1.25	1.25
14	Body weight (g/bird)	385.87±3.5 [a]	404.1±4.8 [b]	407.1±4.8 [b]
	Feed Intake (g/bird)	365.4±6.7 [a]	374.3±7.50 [a]	374.3±7.50 [a]
	Body weight gain (g/bird)	247.37±7.5 [a]	263.76±5.13 [b]	263.76±5.13 [b]
	FCR	1.48	1.41	1.40
21	Body weight (g/bird)	720.45±4.65 [a]	823.43±6.12 [b]	840.42±4.5 [b]
	Feed Intake (g/bird)	557.2±8.84	563.81±2.93	564.90±2.14
	Body weight gain (g/bird)	334.58±2.18 [a]	423.33±4.14 [b]	434.84±5.82 [b]
	FCR	1.67 [a]	1.34 [b]	1.31 [b]
28	Body weight (g/bird)	1095.00±9.84 [a]	1124.50±5.72 [b]	1174.50±7.38 [b]
	Feed Intake (g/bird)	884.60±8.93 [a]	718.80±124.24 [b]	867.30±10.65 [a]
	Body weight gain (g/bird)	375.0±4.44 [a]	305.07±8.50 [b]	355.08±13.68 [c]
	FCR	2.36	2.39	2.32
35	Body weight (g/bird)	1654.20±6.80 [a]	1720.23±10.10 [b]	1835.78±6.45 [c]
	Feed Intake (g/bird)	877.10±6.65	864.9±8.82	876.68±10.43
	Body weight gain (g/bird)	559.20±9.76 [a]	609.73±12.90 [b]	582.29±4.19 [a]
	FCR	1.57	1.45	1.44
42	Body weight (g/bird)	2000.5±20.92 [a]	2160.45±14.00 [b]	2202.88±24.58 [b]
	Feed Intake (g/bird)	675.35±13.74 [a]	818.57±14.77 [b]	825.86±23.52 [b]
	Body weight gain (g/bird)	346.30±16.87 [a]	446.27±15.50 [b]	467.07±22.86 [b]
	FCR	1.95 [a]	1.85 [b]	1.81 [b]
Total	Body weight (g/bird)	2000.5±20.92 [a]	2160.45±14.00 [b]	2242.88±25.66 [c]
	Feed Intake (g/bird)	3486.86±17.89	3449.87±13.37	3492.13±24.08
	Body weight gain (g/bird)	1960.5±8.17 [a]	2118.48±16.24 [b]	2287.85±16.34 [c]
	FCR	1.78 [a]	1.61 [b]	1.58 [b]

Figures in the same row with different letters are statistically significantly different (*P<0.05*)

Table 3. Effect of the dietary supplementation of probiotic mixture on weekly body weight, feed intake, body weight gain and feed conversion factor (FCR) in broiler chickens under stress conditions (mean±SD)

Age (d)		Group D	Group E	Group F
7	Body weight (g/bird)	139.2±4.1	140.32±3.8	140.32±3.8
	Feed Intake (g/bird)	131.62±6.37	125.50±6.40	125.50±6.40
	Body weight gain (g/bird)	98.2±2.19	99.42±178	100.32±1.68
	FCR	1.32	1.28	1.25
14	Body weight (g/bird)	383.3±5.8 [a]	388.2±4.7 [a]	402.1±4.7 [b]
	Feed Intake (g/bird)	384.5±9.23 [a]	366.3±8.60 [b]	370.3±7.50 [b]
	Body weight gain (g/bird)	248.1±14.32 [a]	241.88±5.12 [b]	261.78±5.12 [b]
	FCR	1.59	1.47	1.41
21	Body weight (g/bird)	710.5±5.23 [a]	760.53±8.14 [b]	820.43±6.12 [c]
	Feed Intake (g/bird)	540.6±8.68	558.80±2.83	558.80±2.83
	Body weight gain (g/bird)	326.2±2.25 [a]	350.43±5.14 [b]	418.33±4.14 [c]
	FCR	1.66 [a]	1.64 [a]	1.34 [b]
28	Body weight (g/bird)	1001.60±10.48 [a]	1110.52±5.82 [b]	1120.50±5.72 [b]
	Feed Intake (g/bird)	887.20±7.22 [a]	875.80±124.24 [a]	715.80±124.24 [b]
	Body weight gain (g/bird)	291.10±17.02 [b]	380.17±7.56 [b]	300.07±8.50 [b]
	FCR	3.05 [a]	2.34 [b]	2.39 [b]
35	Body weight (g/bird)	1561.89±9.99 [a]	1710.23±10.10 [b]	1710.23±10.10 [b]
	Feed Intake (g/bird)	826.55±9.46	854.9±8.82	854.9±8.82
	Body weight gain (g/bird)	561.29±8.51 [a]	589.73±12.90 [b]	589.73±12.90 [b]
	FCR	1.47	1.5	1.45
42	Body weight (g/bird)	1803.35±24.52 [a]	2050.25±16.11 [b]	2150.45±14.00 [b]
	Feed Intake (g/bird)	484.92±13.77 [a]	714.76±13.87 [b]	814.57±14.77 [c]
	Body weight gain (g/bird)	242.46±26.86 [a]	350.44±14.60 [b]	440.22±14.50 [c]
	FCR	2.00 [a]	1.9 [a]	1.85 [b]
Total	Body weight (g/bird)	1804.35±24.52 [a]	2000.45±14.00 [b]	2150.45±14.00 [c]
	Feed Intake (g/bird)	3258.39±18.79 [a]	3439.87±13.37 [b]	3439.87±13.37 [b]
	Body weight gain (g/bird)	1764.35±28.22 [a]	2000.46±1 [a] 6.26 [b]	2110.45±15.24 [c]
	FCR	1.86 [a]	1.8	1.63 [b]

Figures in the same row with different letters are statistically significantly different ($P<0.05$)

Table 4. The immune response of broiler chickens vaccinated by AIV inactivated oil-emulsion vaccines H5N1 Immune response§ (log-2) of post-vaccination

Group	Titers(Log2)				
Group	At age14	At age21 d	At age 28 d	At age35 d	At age42
A	1.8 ±0.22 [a]	2.2±0.42 [a]	3.2±0.52 [a]	3.31±0.72 [a]	2.56±0.44 [a]
B	2.2±0.34 [a]	4.4±0.44 [b]	4.2±0.72 [b]	3.81±0.72 [a]	3.76±0.42 [b]
C	2.3±0.24 [a]	4.4±0.42 [b]	4.8 ±0.52 [b]	6.31±0.92 [b]	5.56±0.48 [b]
D	1.8 ±0.24 [a]	2.4±0.42 [a]	3.2±0.62 [a]	2.8±0.72 [a]	2.26±0.62 [a]
E	2 ±0.24 [a]	3.4±0.42 [b]	3.2±0.52 [a]	3.31±0.94 [a]	2.56±0.42
F	2.2±0. 48 [a]	4.4±0.72 [b]	6.2±0.82 [b]	4.31±0.62 [b]	4.56±0.42 [b]

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All chicks were S\c immunized with 0. 5 mL of killed vaccine of Avian Influenza (H5N1) virus at age of 7 days. On day 21, 28, 35 and 42, blood samples were collected from the wing vein of 15 chicks per the two replicates and serum antibody titres against Influenza (H5N1) virus were determined by Haemagglutination Inhibition (HI) test and were expressed as logarithm base 2. Figures in the same column with different letters are statistically significantly different ($P<0.05$).

Table 5. *Effect of the dietary supplementation of probiotic mixture and chromium chloride on dressing weight percentage, liver weight and percentage (mean±SD)*

Group	Group A	Group B	Group C	Group D	Group E	Group F
Dressing weight (%)	73.31	74.50	76.45	73.80	74.37	75.25
Liver weight (g)	45.8±2.19 [a]	53.5±3.11 [b]	55.8±2.19 [b]	42.95±4.21 [a]	44.5±3.02 [a]	48.5±3.11 [b]
Liver weight (%)	2.31	2.53	2.72	2.39	2.42	2.53

Figures in the same row with different letters are statistically significantly different ($P<0.05$)

Table 6. *Effect of the dietary supplementation of probiotic and chromium chloride on physiological stress indicators (mean±SD)*

Parameter	Group A	Group B	Group C	Group D	Group E	Group F
H/L Ratio	0.39 ± 0.04[a]	0.38 ± 0.05 [a]	0.37 ± 0.06 [a]	0.82 ± 0.09[b]	0.45 ± 0.06[a]	0.41 ± 0.06[a]
Cortisol level (ng/ml)	3.79 ± 0.17 [a]	3.69 ± 0.09 [a]	3.79 ± 0.09 [a]	8 ± 0.12[b]	4.82 ± 0.08[a]	4.12 ± 0.08[a]

Figures in the same row with different letters are statistically significantly different ($P<0.05$).

4-Discussion

The highest growth rate and feed efficiency were noticed in group C post Cr supplementation was confirmed by many researchers (Lien *et al*., 1999; Uyanik *et al*., 2002; Sahin *et al*., 2002; Amayta *et al*., 2004; Jackson *et al*., 2008; Samanta *et al*., 2008).

In addition, Lien *et al*., (1999) had demonstrated that a supplement of 1600 mg/kg of chromium picolinate in the ration influences the growth, carcass, serum traits and lipid metabolism of broilers, while Jackson *et al*., (2008) had used Cr as chromium propionate and recorded improved feed efficiency in the later phases of growth and decreased mortality as well as no effect on carcass traits.

Chromium supplementation in diet had been related to increased protein deposition (Seerley, 1993; Ward *et al*., 1995), with decrease in muscle fat (Ward *et al*., 1995). It also affects body mass and feed conversion ratio (Moore *et al*., 1998 and Hossain *et al.*, 1998). The enhancing effect of Chromium supplementation could attributed to its involvement in stimulating the biological activity of insulin by increasing the insulin-sensitive cell receptors or binding activity (Mertz *et al*.,1974;Lien *et al.*,1999) . Insulin can also stimulate anabolism and inhibit catabolism (Lien *et al*., 1999). The improved BW, FI, FE, in group A is confirmed by Yeo and Kim (1997).

Moreover, chromium is involved in protein metabolism and may have a role in nucleic acid metabolism (Lukaski, 1999). Its role in stress condition in animals and birds is more appreciated where it alleviate the negative influence of environmental and nutritional impacts.

The observed improvement in immune responses with chromium supplemented to broilers in groups B and C especially at 28 and 35 days old is coincide by results of (Luo *et al*., 1999 and Toghyani *et al*., 2007).As well previously elevated antibodies titer against Newcastle disease virus in broiler chicks with supplement of 2 or 10 mg\kg chromium either in the form of CrCl3 or yeast (Guo *et al*., 1999) and infectious bronchitis titer in broiler chicks fed on 400 ug\kg Cr picolinate (Lee *et al*., 2003). Beside Cr supplementation enhance the IFN-c m-RNA expression in response to Newcastle disease vaccine according to (Bahgat *et al*., (2008).

The highest carcass traits recorded in group F confirmed that the probiotic mixture used had anti-stress effects and thereby the overcrowded conditions in group E did not negatively affect carcass traits of broiler chickens. These data coincided with that findings reported by Yeo and Kim (1997), and may reflect the activation effect of the probiotic mixture on the liver cells which improved the overall growth performance.

The results also, showed that Cr supplementation improved aspects of growth performance, carcass traits, (Steel and Rosebrough, 1981; Hossain *et al.*, 1998; Lein *et al*., 1999; Uyanik *et al*.,2002;Sahin *et al*.,2002; Amayta *et al*.,2004; Jackson *et al*.,2008;Samanta *et al.*,2008) and immune response (Lou *et al*.,1999; Toghyani *et al*.,2007; Bahgat *et al*.,2008;Kheiri and Toghyani,2009) .The review by the NRC (NRC. 1997) regarding the variability of Cr supplementation agreed with some researchers (Kim, *et al*,1996; Lien *et al*.,1999;Sahin *et al*.,2002 and Kim *et al*.,2002). (Lee *et al*., 2003) reported that CrPic had no effect on growth, but feed efficiency was improved. Also, researchers (Chen et al., 2001; Sahin *et al*., 2002; Kroliczewska *et al*., 2004) had shown that Cr as CrPic improved growth performance of broilers during heat stress. Similarly, others (Kim *et al*., 2002) had reported that 1,600 ppb Cr as CrPic improved gain in broilers. However, not all research showed positive effects of Cr as CrPic on growth performance in broilers (Sahin *et al.*, 2002). The use of probiotic in broiler chick's diets significantly improved the daily body weight gain, feed intake and feed efficiency as reported by Yeo and Kim (1997).The obtained results confirmed that the chromium supplement had good anti-stress effects and thereby the overcrowded condition in group F did not negatively affect performance, immune response and carcass traits of broiler chickens. Although, probiotics as feed additive can act as an anti-stress factor and has positive effects on broiler chickens performance as well as carcass traits, chromium chloride supplementation significantly acted as a powerful anti-stress factor and improved performance (decreased mortality rate, feed intake, feed gain, feed conversion, and body weight), immune response and carcass traits as well as its economic nature. Similarly, CrPic and Cr yeast had been reported to decrease mortality in broilers (Kim *et al*., 1996a; Kim *et al*., 1996b; Jackson *et al*., 2008). These results confirmed that the probiotic mixture used had anti-stress effects and thereby the overcrowded conditions in group E which did not negatively affect carcass traits of broiler chickens. These results might reflect the activation effect of the probiotic mixture on the liver cells which improved the overall growth performance .This may be due to the anti-stress effect of: (1) the chromium supplement where, its role in stress condition in animals and birds is more appreciated as it helps to reduce the negative influence of environmental and nutritional stress (Seerley, 1993; Ward *et al.*, 1995), (2) the anti-stress effect (Gross and Siegel, 1983).

5-Conclusion

The present research confirmed: (1) the activation

effect of the probiotic mixture on growth performance (feed intake, body weight gain , food conversion rate), carcass traits (dressing %, liver somatic indices), and stress conditions (stocking density) in broiler chickens and stress (2) Cr3+ supplementation improved aspects of growth performance, carcass traits, immune response (against AIV) and has a strong anti-stress effect.

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Epidemiology of Brucellosis Among Farm Animals

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Abstract: : In this article we studied the epidemiology and the role of risk factors of *Brucella* infection in ruminants, besides the methods concerning the evaluation of biosecurity measures which are taken against the disease in farms. A cross sectional study was carried out on different Governorates representing allover Egypt to evaluate the potential major risk factors, mal- biosecurity practices and their role in the maintenance of the disease among farm animals. Serum samples (1670) were collected from 126 Herds / Flocks of sheep, goats and cattle and analyzed using Rose Bengal Plate test and iELISA test. A structured questionnaire was designed to identify and evaluate the role of risk factors for Brucellosis. .The results pointed out that, prevalence of brucellosis among herds/flocks of sheep, goats and cattle were; 26.66%, 18.88% and 17.22% respectively. And the seropositive percentages in blood samples were 21.20%, 14.5 % and 2.16% respectively. Major risk factors play a very important role in the prevention and maintenance of the disease among farm animals. The role and magnitude of risk factors varied but the presence of good sanitary measures in farms are considered as a protective factor, where R.R was less than 1 and the attributable risk was -0.01. [Nature and Science 2010;8(5):190-197]. (ISSN: 1545-0740).

Keywords: Seroprevalence, Questionnaires, Role and magnitude of risk factors, Relative and Attributable risk, Brucellosis. Abbreviations: R.R: Relative risk A.R: Attributable risk: Omega.

1. Introduction

Brucellosis is a contagions disease caused by bacteria of genus *Brucella;* it is an animal disease in origin and is a disease of the sexually mature animals with predilection of placenta, fetal fluids and tests of male animals. It has been recognized as a global problem of wild and domestic animals, especially cattle, sheep and goats (Rijpens, 1996).

Brucellosis is the most important zoonosis in terms of human suffering and is a true zoonosis in that almost all human cases are acquired from animals , in particular goats and sheep . In Egypt, brucellosis is still remaining one of the major disease problems that affect animal industry as well as human health and is still an endemic serious disease among domestic animals and humans in spite of attempts that were implemented to control the disease through bilateral projects with some agencies or international organization (Şahin et al., 2008). It has been recorded in Egypt since 1939 (Ahmed, 1939), and the estimated annual economic losses due to brucellosis were about 60 million Egyptian pounds yearly (AOAD, 1995).

In one hand, the annual incidence of brucellosis in people in the Mediterranean and Middle East countries varies from 1 to 78 cases per 100.000 (OIE, 2000, El Sherbini et al., 2007). In Egypt, many authors had reported the incidence of brucellosis among animals (Refai, 1994, and Abdel Hafeez et al., 2001) but only few reports concerning the role of risk factors and the epidemiologic evaluation of the disease (Al-Majali et al.,2007)

On the other hand animal brucellosis is well established in the Middle East and affect both cattle and small ruminants (Abdel- Ghani et al., 1983, Ismaily et al., 1988, Aldomy et al., 1992 and Darwish & Benkirane 2001) .Infection with *Brucella spp*. continues to pose a human health risk globally despite strides in eradicating the disease from domestic animals (Mantur et al.; 2007).

The main objectives of this study were to declare the role of risk factors in the maintenance of the disease among farm animals and to suggest a model of control of brucellosis in Egypt.

2. Materials and Methods

The study was carried out through the period from

March, 2006 to March, 2008 on different Governorates representing allover Egypt. A total of 126 herds/flocks of different species included 45 flocks of sheep, 55 flocks of goats and 26 herds of cattle were selected for this study. In each region, blood samples were taken from herds /flocks with no previous history of vaccination against *Brucella*. The number of samples was collected according to Thrusfield (1995) and Kaoud (2001) in simple and\or systemic random sampling as follows: Animals from each herd were randomly selected using a table of random digits. Only female cows older than 6 months of age were sampled. The herds were stratified into three herd sizes: small herds (≤ 50), medium herds (50-150) and large herds (> 150).

1-Questionnaire

A questionnaire, for each farm, was completed by the farm owner or manager to identify possible independent variables associated with the presence of seropositive cattle on the farm:

a- Precautions taken to visitors.
b- Addition of new animals
C-Multiple raising of animal species
d- Exogenous fertilizing system.
e- Lack of common sanitary measures which include routine or regularity of disinfection program, disposal of carcasses & wastes, control of traffic & vehicles and regular veterinary services).

Calculation:

•Biosecurity practices and mal- practices as a risk factor concerning seropositives:

Risk factors	Seroprevalence		
	Positive	Negative	
Exposed	A:Number of seroprevalence	B:Number of seroprevalence	A + B
Not-Exposed	C:Number of seroprevalence	D:Number of seroprevalence	C + D
	A + C	B + D	

$$RR = \frac{\text{Incidence in the exposed}}{\text{Incidence in the nonexposed}} = \frac{a/a + b}{c/c + d}$$

AR= Incidence of exposed – Incidence of non exposed

OR= a×d / c×b

Omega Risk Measure= a+c/b+d

2-Collection of blood samples

Approximately 7- 10 ml of blood was down from Jugular vein of apparently healthy animals using plain vacutainer tubes and needles. Samples were kept overnight at 4◦C to allow the separation of serum then centrifuged at 3000 rpm for 10 minutes. The collected sera were coded and kept at -20◦C up to the time of the test.

3-Serologial examination ofthe samples

The collected sera were screened for the presence of antibodies against *Brucella* antigens (Alton *et al.*, 1988) by using the Rose Bengal plate test "RBPT" and a commercially available indirect enzyme linked immunosorbent assay (iELISA)

3. Results

Prevalence of Brucellosis among herds/flocks of sheep, goats and cattle

Prevalence of *Brucella* among herds/flocks of sheep, goats and cattle in the studied Governorates using RBPT are shown in Figure (1a).

The results of RBPT pointed out that, prevalence of brucellosis among herds/flocks of sheep, goats and cattle were 26.66%, 18.88% and 21.6%, respectively. When RBPT positive samples were subjected to iELISA test, 21.20%, 14.5 % and 17.22%, respectively showed positive reactions to brucellosis (Figure 1-b).

Prevalence of Brucellosis of the seropositive blood samples in animals (sheep, goats and cattle).

The seropositive percentages in blood samples (subjected to iELISA test) of sheep, goats and cattle were 21.20%, 14.5 % and 2.16% respectively.

Risk factors and their role in the introduction and maintenance of *Brucella* infection in animal farms.

Figure(2) indicated that there was an association concerning the applied biosecurity practices in farms and seropositive reactors of brucellosis, where the relative risk of: farm visitors, addition of new animals, multiple raising of animal species, exogenous fertilizing system and presence of common sanitary measures were: 1.14 , 5 , 8.33 , 2.27 and 0.96, respectively. The presence of good sanitary measures in farms considered as a protective factor where R.R was less than 1 (negative association) and the attributable risk was - 0.01.

The magnitude of the risk factors.

Figures (3) declared the magnitude of the risk factors (Farm visitors, addition of new animals, multiple species rearing and exogenous fertilizing system) were 0.03, 0.32, 0.66 and 0.28,

respectively; in other meaning these risk factors are responsible for the introduction of *Brucella* infection in farms in percentages of 3 %, 32%, 66% and 28%, respectively. Multiple raising of different animal species is considered as highly risk factors.

Odds in favor of acquiring *Brucella* infection in animal farms and the risk of having risk factors.

The degree of association between risk factors and *Brucella* occurrence was calculated by omega measure as shown in Figure (4). According to this measure, risk factors were arranged as following: visitors, exogenous fertilization & lack of sanitary measures (0.31) addition of new animals (0.26) and rearing of multi-species (0.25).

Odds ratio (Psi) and the relative frequency of risk factors for Brucellosis.

Odds ratio (Psi) measures the relative frequency of risk factors for brucellosis to be occurred in farms. According to Odds ratio (Psi) measure, the frequency of risk factors was arranged as following: visitors (1.20), addition of new animals (7.77), rearing of multi-species (28.80) exogenous fertilization (3.2) and lack of sanitary measures (1.7) as shown in Figure (5).

Table 1. Distribution of the *Brucella* seropositive and seronegative herds/flocks of sheep, goats and cattle and the relevance with different investigated variables

		Brucella result		
Variable	Category	Number	Positive No. (%)	Negative No. (%)
Visitors biosecurity	Yes No	80 46	20 (25) 10 (21.74)	60 (75) 36 (78.26)
Addition of New animals	Yes No	50 76	20 (40) 6 (7.89)	30 (60) 70 (92.11)
Rearing of multiple-species	Yes No	20 106	15 (75) 10 (9.43)	5 (25) 96 (90.57)
Exogenous fertilizing	Yes No	10 116	5 (50) 25 (21.55)	5 (50) 91 (78.45)
Sanitary measures	Yes No	26 100	6 (23.08) 24 (24)	20 (76.92) 76 (76)

Yes: means the presence of the factor No: Means the absence of the factor

Table 2. The magnitude and association between different risk factors and seropositive reactors of brucellosis in Egypt

Biosecurity practices	Relative risk	Attributable risk	Omega measure	Odds ratio
-Farm visitors	1.14	0.03	0.31	1.20
-Addition of new animal	5.00	0.32	0.26	7.77
-Multiple raising of species	8.33	0.66	0.25	28.80
-Exogenous fertilizing system	2.27	0.28	0.31	3.2
-Lack of sanitary measures	0.96	- 0.01	0.31	1.7

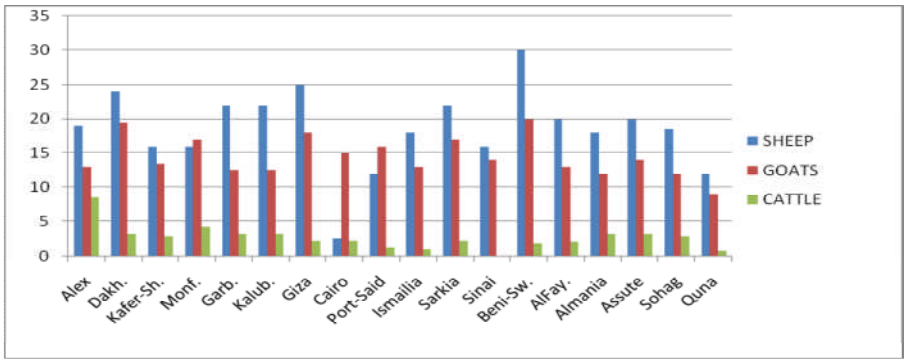

Figure (1-a)

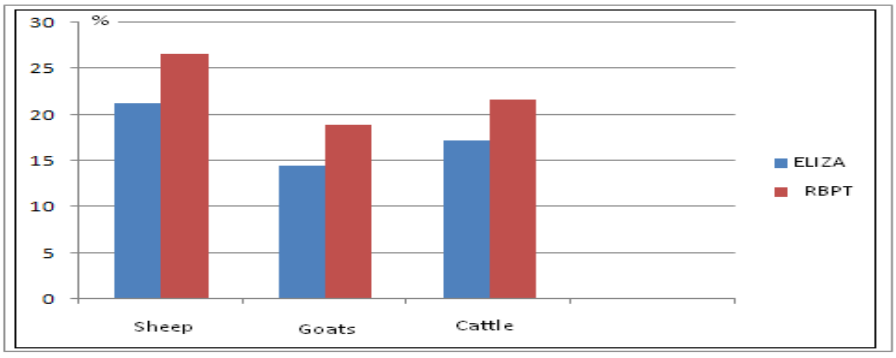

Figure (1-b)

Figure (1-a&1-b) Prevalence of *Brucella in Governorates and* among herds/flocks using iELISA and RBPT

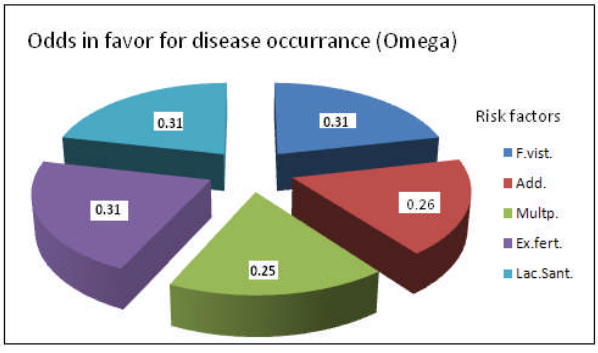

Figure (2) Relative Risk

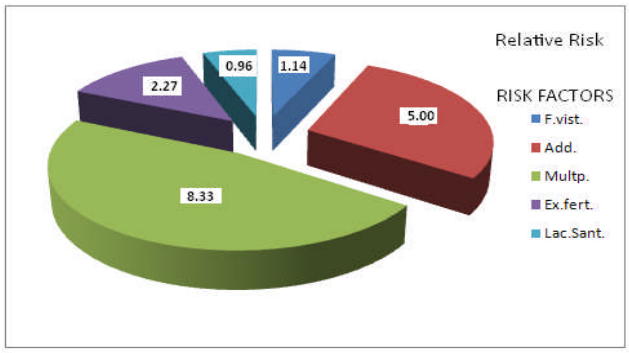

Figure (3) The magnitude of the risk factor

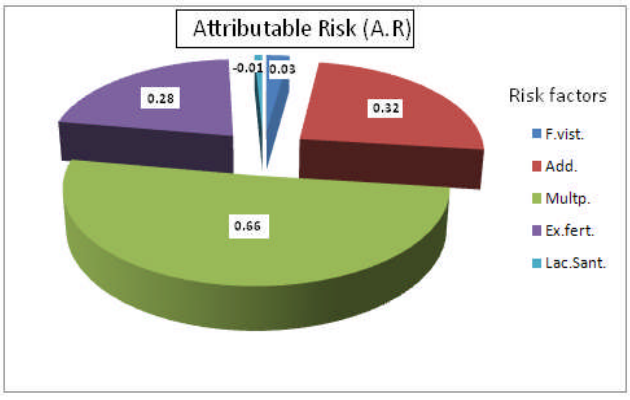

Figure (4) Odds in favor of acquiring *Brucella* infection

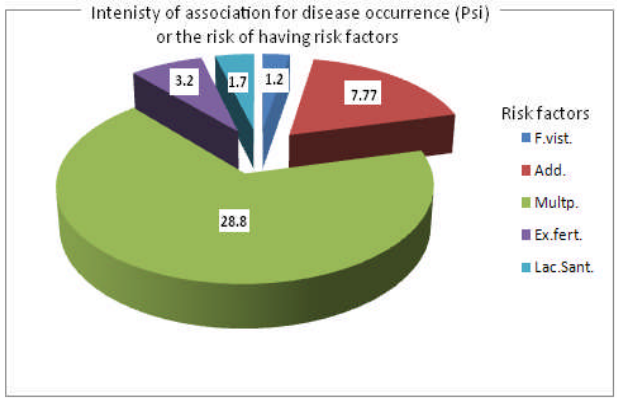

Figure (5) The association between the risk factors and occurrence of brucellosis in animal farms

4. Discussions

This study was conducted with the aim of identifying herd and biosecurity characteristics as potential risk factors associated with brucellosis seroprevalence in ruminants in Egypt. This information is required in order to outline measures to control zoonotic brucellosis (Lithg-Pereira, 2001).

Small ruminant brucellosis is still endemic in most countries of the Mediterranean basin, the Middle East and Central Asia (Aldomy *et al*., 1992 and Radostitis *et al.*, 2000). The public health and economic impact of brucellosis remains of particular concern in developing countries. The clinical manifestations of brucellosis in goats are similar to those in cattle in regard to abortion, stillbirth and reproductive failure. Thus this disease causes heavy economic losses in animal production resulting form abortions, sterility, decreased milk production, and the costs of replacer animals (Ariza *et al*., 1995 and Radostitis *et al.*, 2000). In addition, the disease is an impediment to free animal movement and export.

The prevalence of bovine brucellosis in Egypt was lower than those reported by Refai *et al*., (1990) and Ahmed *et al*., (2002) and it was higher than those reported by Darwesh and Benkiran (2001) in Syria, Amin *et al*., (2005) in Bangladesh and Silva *et al*.,(2000) in Srilanka. The prevalence of brucellosis in sheep and goats in Egypt was lower than that reported by AL-Majali (2005) in Jordan, Radwan *et al*., (1982) in Saudia Arabia, Jackson *et al*., (2004) in Kosovo and it was higher than those reported by Abdel-Hafeez (2001), Shalaby *et al*.(2002) & (2003) and Noha Oraby *et al*.*(* 2007).

The Seroprevalence of brucellosis in sheep, goats and cattle were widely but unevenly distributed throughout flocks and provinces (Jackson *et al.*, 2004, Al- Ani *et al*., 2004 and Muma *et al.*; 2006).

Risk factors and their role in the introduction and maintenance of Brucella infection in animal farms

Major risk factors play very important role in the maintenance and spreading of the disease among farm animals and their human contacts. Relative risk used as a measure to determine if an association exists, and whether there is an excess risk of the disease in populations who have been exposed to disease (Gordis, 2004). Figs (3) and (4) indicated that there was an association concerning the applied biosecurity practices in farms and seropositive reactors of brucellosis, where the relative risk of farm visitors, addition of new animals, multiple raising of animal species, exogenous fertilizing system and presence of common sanitary measures were 1.14, 5, 8.33, 2.72 and 0.96, respectively. The presence of good sanitary measures in farms considered as a protective factor where R.R was less than 1 (negative association) and the attributable risk was - 0.01, this means that the sanitary measures (routine farm disinfection, disposal of carcass & wastes, vehicle disinfection and the presence of veterinary service) play a minor role in preventing the introduction of infection, while its role in preventing the spread of the infection inside the farms or herds has a major property. These results indicated that farm visitors, addition of new animals, multiple species rearing and exogenous fertilizing system were considered as very important risk factors for the introduction and spread of *Brucella* infection among animal farms. Reviriego *et al.*, (2000),Al-Majali (2005) and Earhart *et al*.,(2009) suggested that the addition of new animals or contact with other small ruminants flocks might be a risk factor for brucellosis at the herds /flocks level, in addition the contact between animal herds/flocks will increase the chance for disease transmission to susceptible animals (Crespo, 1994). On contrary, Izquier and Villanueva (1996), Al-Majali *et al*., (2009) stated that the contact between small ruminant flocks had no impact on seropositivity to *Brucella*. Veterinary services play a minor role in preventing the introduction of infection, while its role in preventing the spread of the infection inside the farms or herds has a major role. These results are coincide with Crespo (1994), Mainar–Jaine & Vazquez- Boland (1999) and AL-Majali (2005), who suggested that the absence of disinfection programs in raising farms considered a risk factor for *Brucella* seropositivity in small ruminants. Proper disposal of aborted materials and highly hygienic procedures are extremely important steps in any successful *Brucella* control program. It is well known that delivering adequate animal health services results in a low incidence of diseases, and especially those diseases that have an infectious nature. In addition, controlling brucellosis in small ruminants (mainly by Rev-1 vaccination) will indirectly reduce the prevalence of this disease in other animal species especially cattle. Poor veterinary service has been identified as a risk factor for brucellosis in Argentina (Samartino, 2002) and Mexico (Luna-Martínez and Mejía-Terán ,2002).

Attributable risk is a measure of how much of the disease risk is attributable to a certain exposure, and is useful in answering the question of how much can be prevented. The magnitude of the

risk factors (Farm visitors, addition of new animals, exogenous fertilizing system and multiple species raising) were 0.03, 0.32, 0.28 and 0.66, respectively, in other meaning these risk factors are responsible for the introduction of *Brucella* infection in farms in percentages of 3 %, 32%, 28% and 66%, respectively. In this study, multiple species raising, addition of new animals and the exogenous fertilizing system were identified as the risk factors associated with seropositivity to *Brucella* antigen. Prevention or at least the reduction of brucellosis can be achieved through the satisfactory or strict application of biosecurity measures in animal farms. The use of disinfectants and the presence of adequate veterinary services were identified as the factors that protect against bovine brucellosis. Similar observations were reported for sheep, goats and camels (AL-Majali, 2005).

Odds in favor of acquiring *Brucella* infection in animal farms and the risk of having risk factors (Fig.5)

Odds are commonly used as measures in epidemiology (the odds of *Brucella* infection is the ratio of the number of ways the infection can occurred to the number of ways the infection cannot occurred). The chance and the probability of *Brucella* occurrence in animal farms and the degree of association between risk factors and *Brucella* occurrence were calculated by omega measure. According to the association by this measure, the risk factors were arranged as following: visitors, exogenous fertilization & lack of sanitary measures (0.31), addition of new animals (0.26) and rearing of multi-species (0.25).

Odds ratio measures the relative frequency of the risk factors for brucellosis to be occurred in farms or the degree of association between the risk factors with brucellosis. According to this measure, the frequency of risk factors was arranged as following: rearing of multi-species (28.80), addition of new animals (7.77), visitors (1.20), lack of sanitary measures (0.95) and exogenous fertilization (0.40). The OR value showed that the farms that rear multi-species, added continuously new animals and didn't made biosecurity measures against visitors were about 28.80, 7.77 & 1.20 times more at risk to brucellosis than those rear only one species, didn't add new animals from external sources and made a strict biosecurity measures against visitors. Mixed farming which raising sheep and/or goats along with cattle, was reported by many researchers to be a risk factor for *Brucella* transmission between different animal species (Abbas and Agab 2002, Al-Majali *et al*., 2007). In addition, producers introduced new animals into the herd on a more frequent basis, and stock interchange for breeding is most frequent when the herd is large. This increase the risk of introducing an infected animal into the herd. Practices that involve movement of animals between herds are also likely to carry risks (Kabagambe *et al*., 2001). Such husbandry practices, with animals of different species being herded together, increase the likelihood of animals being exposed to the disease. This factor should be taken into consideration in the planning and execution of control programs. Movements of animals should be controlled by appropriate legislation and regulations.

This study indicated high prevalence of *Brucella* seropositive reactors which pose a human health risk. Lack or mal-practices in the application of sanitary program in farms (such as rearing of multi-species, addition of new animals, and lack of sanitary measures) are highly risk factors which result in the maintenance and spread of brucellosis among animal farms and humans.

5 .Conclusions

Our results could make a useful contribution towards preventing brucellosis in small ruminants and decreasing losses in the livestock industry. More attention should be paid towards implementing a proper control program for brucellosis and more efforts should be directed towards improving the animal health biosecurity program in those Governorates that are large in size. In addition, controlling brucellosis in small ruminants (mainly by Rev-1 vaccination) will indirectly reduce the prevalence of this disease in other animal species especially cattle. Control progress should be monitored serologically and evaluated epidemiologically; Veterinary extension should be played a major role to guarantee the application of the sanitary procedures and measures in rearing, raising and breeding places and education of personnel and dissemination of awareness as well as veterinary public health culture through various multimedia.

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Physico-Chemical Evaluations And Trace Metals Distribution in Water-Surficial Sediment of Ismailia Canal, Egypt

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Abstract: Ismailia Canal is considered as one of the most important irrigation and drinking water resources. During drought period the water level decreased and the concentrations of the most physico-chemical parameters were increased. Physical parameters include (air and water temperatures EC,TS,TDS and TSS).Chemical parameters (pH, DO, BOD, COD, CO_3^{--}, HCO_3^-, Cl^-, SO_4^{--}, Ca^{2+}, Mg^{2+}, Na^+, K^+, NO_2^-, NO_3^-, NH_3, PO_4^{3-}, TP and SiO_2^-). In addition to some trace metals (Fe, Mn, Zn. Cu, Pb, Cd, Al, As, Ba, Co, Cr, Ni, Sb, Se, Sn, Sr, V and Mo) in water and surficial sediment of Ismailia Canal during drought period were studied. [Nature and Science 2010;8(5):198-206]. (ISSN: 1545-0740).

Keywords: Chemical evaluation, trace metals, water, sediment, Ismailia Canal.

1. Introduction

Ismailia Canal was constructed in 1862. It transports fresh water from River Nile in North Cairo at El-Mazalat square (mouth of canal) to El-Khanal Cities (Ismailia, Port Said and El-Suez) for water supply. The original canal dimensions average 2.1, 18 m depth and width respectively. It extends for about 128 km and has more than one regulator bridges constricted along the canal. The first is a head regulator at the mouth of the canal, the second at 13.8 km far from the canal mouth and the third at 75 km far from mouth of canal. The water canal discharge is 433.56 m^3/sec. The total area surrounded the canal is about 108.200 fedden and transported 5.000.000 m^3 per day for drinking, industrial and irrigation purposes. It is worthy to note that some factories are constructed on the two banks of the canal, discharging their wastes into the canal water, leading to change in the water quality of the canal El-Hadad, (2005) and El-Sayed, (2008).

Saad, (2003) reported that the current configuration of Nile Delta Lakes is changing rapidly, due to man's activities and natural processes. Most of their water supply comes from polluted agricultural drains several problems affect the conservation of the Nile Delta Lakes mainly pollution, land reclamation, intensive aquatic vegetation, over fishing and coastal erosion. Erection of Aswan High Dam accompanied by considerable increase in population and consequently in man's activities constitutes the main causes of pollution.

Water quality of Ismailia Canal from EL-Mazalat square to EL- Khosose city studied by Abdo (1998) and concluded that, there is an adverse effect of industrial wastes of different factories on two banks of canal on the distribution of the most physical and chemical parameters. Also, during drought period the values of some physical and chemical parameters were increased.

El-Hadad, (2005) studied the distribution and concentrations of Fe, Mn, Zn, Cu and Pb in water and sediment of Ismailia Canal and were found that the ranges of these metals in water are: Fe: 110 – 640, Mn: 40 – 360 µg/l, Zn: 1.8 – 54.8, Cu: 3.6 – 18.9 and Pb: 7.5 – 35.7 µg/l. In sediment: 7500 – 26900, 150 – 710, 31.1 – 78.5, 3.3 – 56.5 and 12.8 – 32.5 µg/g for the same metals respectively.

El-Sayed , (2008) determined that physical and chemical variables of Ismailia Canal water and recorded that the ranges of these variables were found to be water temperature: 16.5 – 34.6 ^{0}C, transparency: 50 – 140 cm, EC: 246 – 510 µmohs/cm. pH: 7.17 – 8.17, DO: 8.4 – 13.6 mg/l, BOD: 0.8 – 6.0 mg/l, COD: 4.2 – 35.6 mg/l, CO_3^{--}: nil – 15.1 mg/l, HCO_3^-: 172 – 250 mg/l, SO_4^{--}, 31.1 – 40.6 mg/l, NO_2^-: 7.77 – 10.22 µg/l, NO_3^-: 17.00 – 19.38 µg/l, NH_3: 1.03 – 1.94 mg/l and PO_4^{3-}: 30.6 – 90.2 µg/l.

The quality of water is now the concern of experts in all countries of the world. The water quality depends on the location of the source and the state environmental protection in a given area. Therefore, the quality and the nature of water may be determined by physical and chemical analysis (Abdo, 2005).

The contamination of soil, sediment, water resource and biota by heavy metals is one of the major concern especially in many industrialized countries because of their toxicity persistence and bioaccumulation (Iken et al., 2003).

The aim of the present study is to determine the spatial and temporal distribution of physical variables (air and water temperatures, EC, Transparency, TD, TDS and TSS) chemical variables (DO, BOD, COD, pH, CO_3^{--}, HCO_3^-, Cl^-, SO_4^{--}, NO_2^-, NO_3^-, NH_3, PO_4^{3-}, TP and SiO_2^-) in addition to Fe, Mn, Zn, Cu, Pb, Cd, Al, Co, Ni, Cr, Ba, As, Sr, V, Sb, Se, Sn and Mo) in water and sediment of Ismailia Canal during three months of drought period to assess the environmental status of the water-sediment of Ismailia Canal.

2 - Materials and Methods

2.1 water-sediment samples collection and analyses:-

The present study was done during drought period (January, February and March 2007). The water and sediment samples were collected during three successive months of drought period. Six stations were selected along Ismailia Canal extended from El-Mazalat square to Abu Za'baal City. These stations are represented in Fig (1). The locations of these stations are:

Station I: The mouth of Ismailia Canal branched from River Nile at El-Mazalat square.
Station II: In front of the water inlet of El-Amyria drinking water purification station.
Station III: In front of El-Delta company for iron and steel production.
Station IV: In front of El-Cablate company for electrical wire production.
Station V: In front of Shiny company for ceramics production.
Station VI: At Abu Za'baal company for fertilizer and chemical products.

Water samples were collected at 60 cm depth (sub surface layer), using polyvinyl chloride Van Dorn bottle and kept in plastic bottle, preserved in an ice-box then returned immediately to the laboratory for analysis. Surficial sediment samples were collected using Eckman Dredge and kept in plastic bags. Then dried at 105 ^{0}C and preserved for analysis.

2.2 Field measurements:-

Air and water temperatures, electrical conductivity, as well as pH values were measured, using Hydrolab, Model "Multi 340I/SET". The transparency of water was measured in the field, using Secchi-disc (diameter 25 cm).

2.3 Laboratory analyses:-

Water samples for all selected parameter, were analyzed according to procedures specified in APHA, (1998). Total solids (TS) were measured by evaporating a known volume of well mixed sample at 180 ^{0}C. TDS were determined by filtrating a known volume of water sample with glass microfiber filter (GF/C) and evaporating at 105 ^{0}C. TSS is direct obtain by subtracted of TS-TDS. Concentrations of CO_3^{--} and HCO_3^- were measured titrimetric against 0.025 N H_2SO_4, using phenolphthalein and methylorang indicator. Cl^- was determined by argenometric method. SO_4^{--} was determined using turbidimetric method. DO was determined by azide modification method. BOD by 5 days incubation method. COD was performed by potassium permanganate oxidation method. Ca^{2+} and Mg^{2+} were determined by EDTA titrimetric method. Na^+ and K^+ were measured directly using the flame photometer "Model Jenway PFP, U.K.". Concentrations of NO_2^-, NO_3^-, NH_3, PO_4^{3-} and SiO_3 were determined using the colorimetric techniques with formation of reddish purple azo-dye, Copper-Hydrazin salphat reduction, phenate, stannous chloride reduction and molybdosilicate methods respectively. Total phosphorus (TP) was measured as reactive phosphate after persulphate digestion.

Total Fe, Mn, Zn, Cu, Ni, Co, Pb, Cd, V, Cr, Al, Sr, As, Sn, Ba, Sb, Mo and Se in water after digestion by conc. HNO_3. And in sediment after digestion according to Jackwerth and Würfels, (1994), using Inductive Coupled Plasma Spectrometer "ICP, Model: Elan 9000, Perkin Elmer, USA".

2.4 Statistical Analysis

In order to study the different relationships among physical and chemical parameters of the area under investigation the correlation coefficient matrix (r) were calculated using the computer program EXCEL (Office XP).

3- Results and Discussion:

The physico-chemical parameters are considered as the most important principles in the identification of the nature, quality and type of the water (fresh, brackish or saline) for any aquatic ecosystem (Abdo, 2005).

3.1 Physical measurements:-

3.1.1 Air and water temperatures

Temperature was the water quality indicator that exhibited little variance between the sites and months of water collection. The highest values were recorded during March (31 – 35, 30 – 34 ^{0}C) for air and water respectively. The lowest in February (18.5 – 21.0, 20.0 – 26.0 ^{0}C).

3.1.2 Transparency

The lowest values of transparency were showed

at sites receiving the effluent discharge of the companies e.g. V, VI (40, 30 cm) respectively during January. Also, the changes in visibility of canal is generally low due to shallowness and the drought period effect, the water level decrease and suspend organic-inorganic particles increase. This is agree with that reported by Abdel-Satar, (2008) on Manzala Lake.

Electrical conductivity (EC)

EC is a good indicator parameter on the total dissolved ions in aquatic ecosystem. The EC values were ranged between 340 – 742 µmohs/cm during the investigation period. The maximum values 392, 742 and 369 µmohs/cm were recorded at VI which may be receiving the effluents of Abu Za'baal company. These results agree with that finding by **El-Sayed, (2008)** on the same area.

3.1.4 Solids (TS, TDS and TSS)

The distribution of TS, TDS and TSS in Ismailia Canal water are relatively increase during drought period due to the decay and degradation of the most microorganism species in the lower water level (Abdo, 1998). The present values of TS, TDS and TSS in Table (1) were found in the same ranges that recorded by El-Hadad, (2005) during winter on the same area. The highest values of TS were found at VI during three months, and reached to 742.630 and 112 mg/l in February. This mainly attributed to the waste water discharge of Abu Za'baal fertilizers company at this site.

3.2. Chemical variables:-

3.2.1 Hydrogen ion concentration (pH)

The pH of Ismailia Canal water at most sites are around to 8 and the lower values recorded during February Table (1). This may be due to fermentation of the organic matter which increase during this month of the drought period and coincident with Abdel-Satar, (2008) for Lake Manzalah. The minimum value of 3.9 and acidity 150 mg/l were recorded at stationVI representing good evidence on the effect of the effluent of Abu Za'baal fertilizers company at this site.

3.2.2 Carbonate and bicarbonate:-

CO_3^{--} and HCO_3^- results are present in Table (1). The CO_3^{--} were not detected during February at all sites and the HCO_3^- were increased at all sites during this month. This mainly attributed to the presence of high amount of organic matter during drought period, especially during February and its accessible to bacterial decomposition and fermentation, where the HCO_3^- is the final product of the decomposition. These results agree with that reported by Ali, (2008) on the Manzalah Lake. The ranges of CO_3^{--} and HCO_3^- were found to be ND – 14.2, 162 – 222 mg/l respectively.

3.2.3 Chloride and sulphate

Concentrations of chloride varied between 30 – 40 mg/l at all sites during three months, where site VI showed the highest levels 62,60 and 124 mg/l during January, February and March respectively. Also, sulphate concentrations was taken the same behavior of chloride. The sulphate concentrations ranged between 21.0 – 36.6 mg/l at all sites during three months, where the maximum values 39, 156 and 38 mg/l were recorded at site VI. The high values of SO_4^{--} and Cl^- at site VI are strongly attributed to effluent discharging from Abu Za'baal fertilizers company at this site. This results coincident with that finding by Elewa et al., (2001).

3.3 Calcium and magnesium

The monthly variations of Ca^{2+} and Mg^{2+} in Ismailia Canal water during drought period are present in Table (1). The ranges of Ca^{2+} and Mg^{2+} concentrations were found to be 31 – 36 and 11 – 20 mg/l respectively at different sites during three months. The highest values of Ca^{2+}: 44, 64, 38 and Mg^{2+}: 22, 58 and 18 mg/l at site VI opposite to the discharge point of Abu Za'baal fertilizer company. Comparison of the present results with the reported by El-Hadad, (2005) for the same area were showed similar levels during winter (2005) and the lower values were recorded during other seasons. This mainly attributed to the lower and staining of Ismailia Canal water during drought period, the death and decaying of most phyto-zooplanktons may be leading to facilitate in the liberation of different ions (Abdo, 1998 and El-Hadad, 2005).

3.4 Dissolved Oxygen (DO), Biochemical (BOD) and Chemical Oxygen Demand (COD):-

DO, BOD and COD were ranged between 6 – 10, 2.8 – 5.2 and 6 – 16 mg/l respectively. Generally the DO at all selected sites of canal water were within the guideline values cited by USEPA, (1999) for the protection of aquatic life [for warm water biota: early life stages = 6 mg/l, other life stages = 5.5 mg/l]. For cold water biota: carly life stages = 9.5 mg/l, other life stages = 6.5 mg/l. BOD and COD values were in agreement with these obtained by El-Hadad, (2005). He reported that the BOD and COD were varied in the ranges of 2.4 – 3.6 and 8.0 – 19.2 mg/l respectively during winter season.

3.5 Nutrient salts

Nitrogen and phosphorus limit the growth of terrestrial plants, phytoplankton, macroalgae and vascular plants in fresh water and marine ecosystem,

and silicon additionally limits the growth of diatoms (Rabalais, 2002).

3.5.1 Nitrite and Nitrate

NO_2^- and NO_3^- concentrations were found slightly variations at different locations and during three collection months, Table (1). NO_2^- and NO_3^- were ranged between 9 – 18 and 20 – 52 µg/l respectively. The maximum value of NO_3^- 516 µg/l at site VI during February, mainly related to the waste water of Abu Za'baal fertilizers company discharge at this site.

3.5.2 Ammonia

Ammonia-N accounted for the major proportion of total soluble inorganic nitrogen. It fluctuated in the ranges of 0.108 – 0.50 mg/l during drought period at different sites. The values of ammonia at different sites of Ismailia Canal (Table 1) were within chronic guide lines recommended by USEPA (1999) at pH 8, ammonia (1.27 – 8.41 mg/l).

3.5.3 Ortho and total phosphate

Ortho-P and Total-P values exhibited local variations with interrupted monthly trends and total-P increase during February, while ortho-P increase during January. The ranges of ortho and total-P were found to be 23 – 165.5 and 109 – 1478 µg/l respectively at different sites. The high values of TP at all sites of Ismailia Canal during February could be attributed to the lower and staining water during this month may be facility the death and decaying of the most microorganism in column water, which leading to liberation different ions especially ortho and total-P. This interpretation was supported by Nesbeda, (2004) showing that phosphorus enters the lakes through anthropogenic sources, such as fertilizer runoff into either inorganic or organic fractions. Also the maximum values of ortho-P were reached to 607, 3503, 605, 713, 4415 and 879 µg/l at site VI opposite to the discharge point of the Abu Za'baal fertilizers company. These results coincide with that reported by Abdel-Satar, (2005) on the River Nile at the Sugar Integrated Industries Company.

5.3.4 Reactive silicate

Silicate fluctuated between 2.45 – 7.70, 1.44 – 4.00 and 3.12 – 4.00 mg/l during January, February and March months respectively, (Table 1). The lower values of silicate were recorded during February compare with other months is related to the silicate consumption by diatoms which bloom at low temperature and water level (Wetzel, 2001).

3.6 Trace metals

Trace metals are considered harmful to environment and belong to one of the most toxic groups of water pollutants. There has been increasing concern and more stringent regulation standards pertaining to the discharge of heavy metals to the aquatic environment, due to their toxicity and determint to living species including humans. Heavy metals are non-degradable and can accumulate in living tissue (Ikem et al., 2003).

3.6.1 Trace metals in water

To assessment the water quality of Ismailia Canal water the eighteen trace elements were determined in water and sediment of the canal. There are some detected trace elements in Ismailia Canal water e.g. Fe, Mn, Zn, Cu, Cd, Pb, Al, As, Ba and not detected elements e.g. Co, Ni, Cr, Sr, V, Sb, Sn and Mo during three months at all collection sites,(Table 2).

The concentrations of studied trace metals in water are present in (Table 2). They declare that the ranges of detected trace elements were found to be Fe; 0.19 – 0.30, Mn; 0.10 – 0.18, Zn; 0.040 – 0.095, Cu; 0.005 – 0.0161, Pb; 0.0150 – 0.029, Cd; 0.001 – 0.003, Al; < 0.1 – 0.8, As; < 0.01 – 0.100 and Ba; < 0.0005 – 0.002 mg/l during investigation period at all sites.

The concentration levels of all studied heavy metals are within permissible limits of WHO, (1995) except for Al, Mn, and Zn up to 0.2, 0.1 and 0.05 mg/l respectively.

Generally, the main natural source of heavy metals in water is weathering of minerals (Klavins et al, 2000). Industrial effluents and non-point pollution sources, as well as changes in atmospheric precipitation can lead to local increase in heavy metals concentration water. Also, total heavy metals concentrations in aquatic ecosystem can mirror the present pollution status of these areas (Haiyan and Stuanes, 2003).

3.6.2 Trace metals in sediment

The analysis of heavy metals in the sediment permits detection of pollutants that may be either absent or in low concentrations in the water column (Binning and Baird, 2001). The accumulation of metals from the overlying water to the sediment is dependent on a number of external environmental factors such a pH, EC, the ionic strength, anthropogenic input, the type and concentration of organic and inorganic ligands and the available surface area for adsorption caused by variation in grain size distribution (Awfolu et al., 2005).

The determination of eighteen trace elements in Ismailia Canal sediment during three months of drought period revealed that Mo, Sb, Se and Sn were not detected, but Fe, Mn, Zn, Cu, Pb, Cd, Al, Ba, As,

Co, Cr, Ni, Sr and V were detected as present in Table (3).

The ranges of thirteen elements were detected in sediment varied between, Fe; 8112 – 26688, Al; 11496 – 47680, Mn; 169.6 – 780.00, As; 115 – 350, Ba; 109.2 – 332.0, Sr; 66 – 223.6, Zn; 28 – 193.2, V; 18 – 169.2, Cr; 12 – 118, Pb; 10 – 43.2, Co; 2 – 36.4, Cu; 16.2 – 73.2, Ni; 2.8 – 74 and Cd; 1.6 – 9.2 mg/kg during drought period at all sites, Table (3).

In view of the concentration levels of studied trace elements, the highest values were Fe, Al at all station. This may be attributed to the fact that Fe, Al are the most abundant elements in earth crust and is also confirmed with that reported by Abdel-Satar, (2005) on Nile sediment. The concentration levels of Fe, Mn, Zn, Cu and Pb were found in the same ranges determined by El-Hadad, (2005) on the same area. Also, the values of Ni, Co and Cd in Ismailia Canal sediment were taken in the same ranges of Nile sediment determined by Abdel-Satar, (2005). The other studied elements Ba, As, Sr, V and Cr were measured in canal sediment and can not compared with other studies because of the lack in determinations in aquatic ecosystem researches.

The heavy metals concentrations of Zn, Ni, Cu, Pb and Cd from the Ismailia Canal sediment compared with the probable-effects-level (PEL) guidelines for toxic biological effects established by USEPA, (1997), sediment-quality guidelines cited by Salomons & Förstner, (1984), and USPHS, (1997**)** in Table (4).

Table (4): Sediment- quality guidelines of trace elements (µg/g) measured in fresh water sediments.

Metal	USEPA (1997)		Salomons & Förstner, (1984), USPHS (1997)	Present trace metal levels
	TEL	PEL		
Zn	124	271	< 100	28 – 193.2
Ni	15.9	42.8	45 – 65	2.8 – 74
Cu	18.7	108	45 – 50	16.2 – 73.2
Pb	30.2	112	20 - 30	10 – 43.2
Cd	0.68	4.21	1	1.6 – 9.2

The previous table revealed that, these elements in Ismailia Canal sediments did not exceed the PEL guidelines for USEPA, (1997) except for Ni. On the other side the sediment concentrations of these elements showed higher level than acceptable guidelines cited by Salomons & Förstner, (1984), and USPHS, (1997).

4. Statistical analysis

Correlation coefficient "r" is one of the most important statistical test to evaluate the strength or weakness relationships among physical and chemical parameters as well as trace metal concentrations determined in the this study.

The obtained results of correlation coefficient "r" revealed that water temperature showed positive correlations (n = 10, P < 0.05) among different physical and chemical parameters e.g. EC, TS, TDS, Cl^-, SO_4^{2-}, Ca^{2+}, Mg^{2+}, NO_3^-, PO_4^{3-} and SiO_3^- (r = 0.56, 0.53, 0.57, 0.51, 0.52, 0.49, 0.60, 0.46, 0.52 and 0.63 respectively). It's showed that the important role of water temperature in the distribution of physical and chemical parameters in aquatic ecosystem. The negative correlation coefficient "r" between transparency with TS, TDS and TSS (r = -0.85, -0.84 and -0.79) represented the inverse relationship between transparency and different solids forms. TS was found positive correlations with TDS and TSS (r = 0.99 and 0.93) revealed that the strong interrelationship among these solids. pH was controlled in the distribution of CO_3^{2-} and HCO_3^- related to through the positive correlations of pH with CO_3^{2-} and HCO_3^- (r = 0.5 & 0.79). Both of (Cl^-, SO_4^{2-}) and (Ca^{2+}, Mg^{2+}) were similar behaviour through the high significant correlation "r" at (n = 10, P < 0.05) (r = 1.0 and 0.98 respectively). The obtained results coincide with that finding by Abdel-Satar, (2008) on Manzalah Lake. Cl^- & SO_4^{2-} fulfilled a significant positive relationship with Ca^{2+} and Mg^{2+} (r = 1.0, 0.98 and 1.0, 0.99) but CO_3^{2-} and HCO_3^- with two cations were negative correlations (r = -0.45, -0.59 and -0.83, -0.87), this means that Ca^{2+}, Mg^{2+} may be present in chloride or sulphat salts and not found as carbonate or bicarbonate salts in Ismailia Canal water. Also, the present results coincide with that reported by Elewa ***et al.,*** (2001) on River Nile. Nutrient salts; NO_2^-, NO_3^-, NH_3, PO_4^{3-}, TP and SiO_3^- through the statistical analysis "r" declared that high significant correlation between NO_3^- and PO_4^{3-}, TP and SiO_3^- (r = 0.99, 0.96 and 0.68) respectively, as well as PO_4^{3-} with TP (r = 0.98) revealed the strong relationship between ortho and total phosphate.

The correlation coefficient among eighteen trace elements showed a positive correlation between Fe and Mn in water and sediment (r = 0.57 and 0.83) (n = 10 at P < 0.05) indicating that the association of two elements originates from a common source during transportation and/or depositional reactions. The positive correlation between concentrations of Mn/Zn, Mn/Cu, Mn/Pb and Mn/Al (r = 0.97, 0.66, 0.36 and 0.49 respectively) indicates Mn oxide or hydroxide as part from the suspended matter plays an important role in the distribution dynamic of total trace metals in the canal water. These results agree with that reported by Abdel-Satar, (2008) on

Manzalah Lake. For other trace element concentrations in water and sediment were positively correlated with each other e.g. Fe, Mn, Cu and Pb (r = 0.62, 0.95, 0.58 and 0.72 respectively) revealing that the concentrations of these elements are dependent on each other. The other relationships among other trace elements (Ni, Co, Cr, Sr, V, Sb, Sn and Mo) were found not significant may be related to these elements were not detected in Ismailia Canal water and sediment.

4. Conclusion

From previous discussion mentioned we can concluded that, the water quality parameters were slightly increase especially PO_4^{3-} and TP during drought period. The point discharged of Abu Za'baal fertilizers company (VI) act as source of pollution in the Ismailia Canal. The order of detected trace elements in water and sediment were arranged from high to low concentrations as follows:

Al > Fe > Mn > As > Zn > Pb > Cu > Cd > Ba and Al > Fe > Mn > As > Ba > Sr > Zn > V > Cr > Pb > Co > Cu > Ni > Cd respectively.

The correlation coefficient showed that the strong inter-relationships among physical, chemical and trace metal concentrations measured in the Ismailia canal water and sediment.

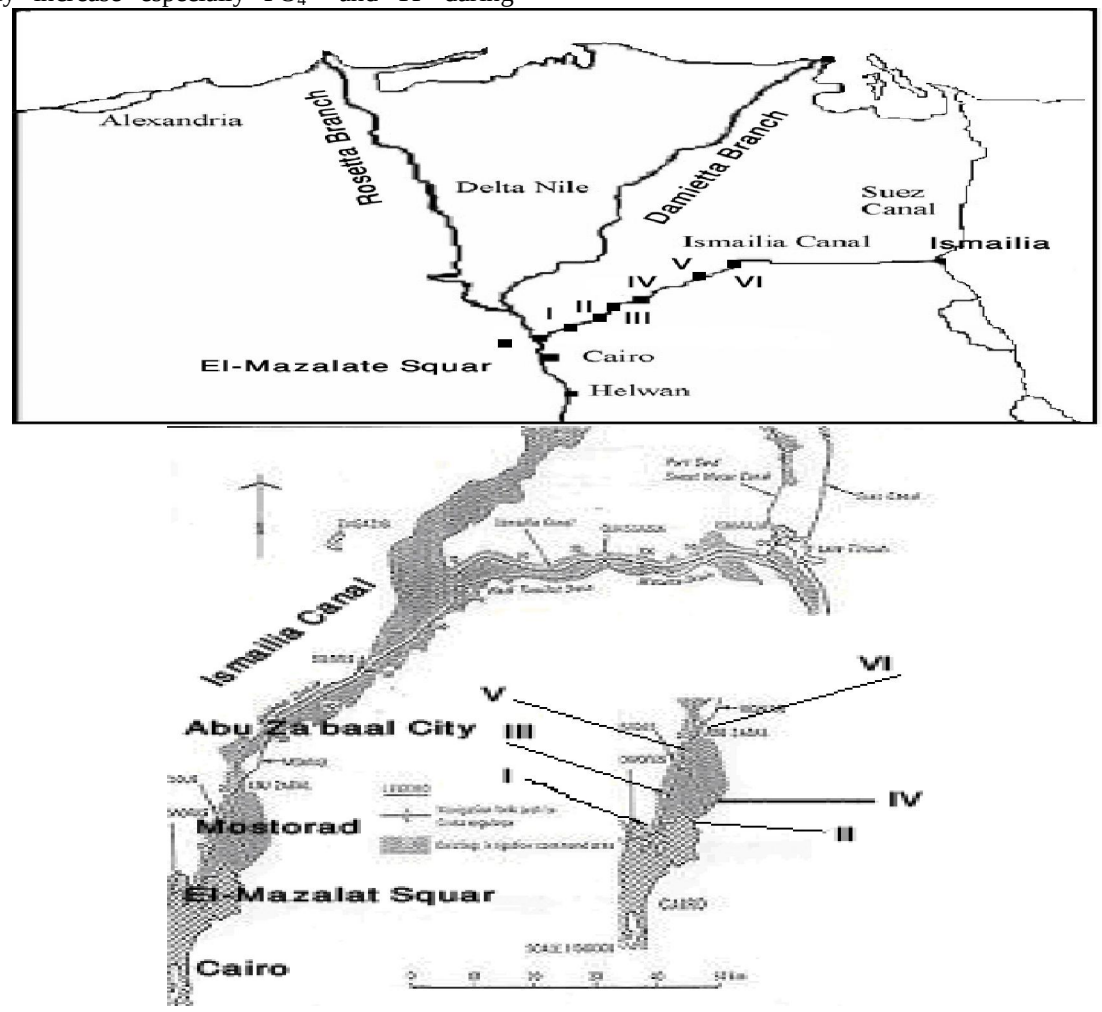

Fig. 1. The sampling locations of smailia Canal

Table (1): Physicochemical parameters of the Ismailia Canal water during January, February and March 2007.

Parameters	Month	January						February						March					
	Sites	I	II	III	IV	V	VI	I	II	III	IV	V	VI	I	II	III	IV	V	VI
Air Temp. (^{0}C)		19	23	20	20	25	24	19	20	19	18.5	18.5	21	35	35	32	32	35	31
Water Temp. (^{0}C)		22	24	23	23	24	23	19.5	20	20	20	20.5	26	32	33	31	31	35	30
Transparency (cm)		70	80	60	70	40	30	90	60	94	65	65	60	90	65	75	60	80	60
EC (µmohs/cm)		340	341	346	348	358	392	358	375	361	360	367	742	342	347	346	343	350	369
TS (mg/l)		252	222	222	288	232	398	272	300	256	270	262	742	260	276	232	260	290	300
TDS (mg/l)		208	212	218	230	208	304	234	266	224	219	216	630	240	220	200	220	260	264
TSS (mg/l)		44	10	4	58	24	94	38	34	32	51	46	112	20	56	32	40	30	36
pH		8.3	8.1	8.2	8.2	8.1	7.3	7.5	7.9	7.4	7.4	8.0	3.9	8.2	8.3	8.4	8.5	8.2	8.0
CO_3^{--} (mg/l)		ND	14.2	14.2	10.2	ND	ND	ND	ND	ND	ND	ND	ND	4.84	4.84	2.42	2.42	ND	ND
HCO_3^- (mg/l)		203	162	186	195	162	162	226	227	219	203	203	150 Acidity	220	180	180	180	196	211
Cl^- (mg/l)		38	40	38	40	38	62	36	36	34	34	32	60	30	30	30	30	30	124
SO_4^{--}(mg/l)		29.7	31.2	32.0	36.6	29.1	39	28.9	36.6	33.7	32	35	156	25	25	23	21	23	35
Ca^{2+} (mg/l)		32	33	34	32	32	44	32	34	36	36	34	64	32	32	31	31	31	38
Mg^{2+} (mg/l)		11	12	12	15	18	22	20	20	19	19	21	58	15	16	17	17	16	18
DO (mg/l)		10	9	8	8	8	7	8	8.4	7.2	8	7.2	6	8	8	8	8	7.6	8
BOD (mg/l)		3	4	4	4.4	4.6	3.3	2.8	5.6	3.6	3.6	5.2	2.4	4.4	4.4	4.4	4	4.4	4.4
COD (mg/l)		16	8	16	16	13	13	8	8	6	8	8	8	8	8	8	8	8	8
NO_2^- (µg/l)		12.2	18	16	15	9	12	16	17	10	11	12	15	12	11	10	9	10	11
NO_3^- (µg/l)		43	23	27	28	22	23	30	25	20	26	24	516	21	52	50	32	20	28
NH_3 (mg/l)		0.40	0.50	0.50	0.41	0.40	0.43	0.18	0.36	0.45	0.11	0.12	0.19	0.15	0.18	0.14	0.15	0.26	0.29
PO_4^{--} (µg/l)		76.6	99.1	118.5	165.5	131.2	607	30.6	36	37	23	43	3503	25	49	34	38	54	605
TP (µg/l)		160	109	207	427	167	713	147.8	1107	445	556	1177	4415	215	213	195	244	191	879
SiO_2^- (mg/l)		7.70	5.70	3.4	2.73	2.45	5.5	1.7	1.6	1.52	1.44	2.31	4.0	3.43	3.55	3.12	3.20	3.5	4.0

Table (2): Monthly variations of studied trace metals of Ismailia Canal water (mg/l) during January, February and March 2007.

Parameters	Month	January						February						March					
	Sites	I	II	III	IV	V	VI	I	II	III	IV	V	VI	I	II	III	IV	V	VI
Fe		0.26	0.28	0.37	0.25	0.19	0.24	0.25	0.27	0.28	0.23	0.29	0.26	0.30	0.25	0.21	0.20	0.27	0.25
Mn		0.12	0.18	0.17	0.15	0.100	0.14	0.15	0.17	0.16	0.11	0.13	0.12	0.10	0.15	0.13	0.12	0.11	0.16
Zn		0.06	0.09	0.085	0.075	0.05	0.07	0.07	0.095	0.095	0.050	0.040	0.040	0.05	0.075	0.065	0.06	0.055	0.08
Cu		0.018	0.019	0.009	0.008	0.005	0.007	0.017	0.019	0.019	0.015	0.014	0.014	0.015	0.017	0.0161	0.016	0.015	0.018
Pb		0.028	0.029	0.019	0.018	0.015	0.017	0.027	0.029	0.010	0.016	0.016	0.018	0.025	0.027	0.021	0.020	0.020	0.020
Cd		0.003	0.003	0.002	0.0018	0.0015	0.0018	0.003	0.003	0.001	0.0016	0.0016	0.0018	0.0025	0.0027	0.0016	0.0016	0.0015	0.0018
Al		0.8	0.6	< 0.1	< 0.1	< 0.1	0.6	0.209	0.409	0.312	0.29	0.396	0.682	< 0.1	0.4	0.2	< 0.1	< 0.1	< 0.1
Co		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ni		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cr		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ba		< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005	0.002	0.001	< 0.0005	< 0.0005	< 0.0005	< 0.0005	0.002	0.001	< 0.0005	< 0.0005	< 0.0005	< 0.0005
As		< 0.01	< 0.01	< 0.01	0.039	0.026	0.025	0.051	0.058	0.118	0.118	0.068	0.069	0.061	0.068	< 0.1	0.080	0.1	0.09
Sr		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
V		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sb		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Se		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sn		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mo		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table (3): Monthly variations of studied trace metals of Ismailia Canal sediment (mg/kg) during January, February and March 2007.

Parameters	Month	January						February						March					
	Sites	I	II	III	IV	V	VI	I	II	III	IV	V	VI	I	II	III	IV	V	VI
Fe		18348	25352	21764	17568	8112	16420	17340	23052	26688	15664	19296	18444	13900	20192	14640	14600	25432	20680
Mn		644.8	683.6	611.2	514.0	192.8	197.2	411.2	780.0	731.6	317.2	446.84	400.8	169.6	598.4	535.2	504.8	226.8	645.6
Zn		59.2	119.2	65.2	73.6	38.4	36.8	118.4	141.6	134.4	51.6	193.2	28.0	71.6	28.0	48.0	74.0	66.8	69.2
Cu		31	60	28.3	45	16.2	19.6	32.1	27.6	27.8	30.2	19.8	13.2	29.7	33.5	26.3	52.2	15.0	25.6
Pb		31.6	10.0	36.8	25.2	15.6	18.4	36.0	43.2	12.0	18.8	16.8	14.8	18.8	14.8	17.2	13.2	14.0	14.8
Cd		3.6	2.4	1.6	4.8	5.2	6.0	2.80	2.04	2.52	3.40	2.56	3.60	3.6	9.2	4.8	4.0	7.6	4.0
Al		16252	47680	31836	21032	18760	11496	18788	36096	36908	23412	33388	19740	11740	30300	18672	18924	17128	26140
Co		6.0	6.0	16.8	6.4	2.0	4.0	4.0	11.6	16.4	23.2	2.0	26.0	34.0	30.4	35.2	25.2	23.6	36.4
Ni		30.0	40.4	36.0	38.4	30.4	30.4	44.8	57.2	61.2	2.8	56.8	58.4	58.4	74.0	59.6	62.0	52.4	59.2
Cr		12.0	44.8	76.8	40.4	32.8	14.8	81.6	66.4	118.0	21.2	51.6	29.2	34.8	79.2	53.6	88.0	52.8	50.0
Ba		132	332	169.2	130.4	110.8	174.8	135.2	213.6	197.2	212.8	179.6	137.6	109.2	207.2	200.4	208.4	168.4	191.2
As		160	350	180	150	120	190	166	225	210	230	200	150	115	218	220	225	210	212
Sr		77.6	223.6	200.4	124.4	70.8	66.0	92.4	126.8	175.6	214.8	107.2	87.6	96.8	108.8	109.6	79.6	84.4	128.4
V		125.6	169.2	118.8	57.6	43.2	42.4	44.0	30.8	24.4	168.0	26.0	14.0	18.4	24.8	152.8	151.6	107.2	111.2
Sb		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Se		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sn		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mo		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

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