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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

3	**Response of vermi-compost on Growth and Yield of Pea (*pisum sativum* L.) cv. Arkel** Hakim Singh Chauhan*, Sunil Chandra Joshi[1] and D.K. Rana[2] GBPUAT Hill Campus Ranichauri- 249199 (Uttrakhand) India [1] Division of Seed Science and Technology, Indian Agricultural Research Institute, New Delhi-110012 India [2]Department of Horticulture, HNB Garhwal University Srinagar- 246174 (Uttrakhand) India. hakim_ag2007@rediffmail.com **Abstract:** The present investigation "Response of vermi-compost on growth and yield of pea (*Pisum sativum* L.) cv. Arkel'' comprised of seven treatments consisting of three level of vermi-compost, three level of vermi-compost with NPK, and one level of FYM + NPK. During the experimentation, growth character and yield characters were recorded. The germination of pea cv. Arkel, Seeds became faster with T_4 (vermi-compost-10 t/ha+NPK) treatment but there after the germination occurred at slower rate and days taken for completion of germination increased progressively. The T_4 (vermi-compost-10 t/ha + NPK) treatment exhibited the maximum nodule formation and yield. A comparative study of the present findings led to the conclusion that sowing of pea with the application of vermi-compost @ 10 t/ha and NPK @ 25:60:50 kg/ha was found most effective to best growth of pea crop under Srinagar valley condition of Garhwal region of Uttrakhand state. [Nature and Science 2010;8(4):18-21].ISSN:1545- 0740). **Keywords:** *Pisum sativum*, vermi-compost, FYM, NPK	Full Text
4	**In vitro antioxidative acitivity of *Azadirachta indica* and *Melia azedarach* Leaves by DPPH scavenging assay** Gayatri Nahak[1] and Rajani Kanta Sahu[1] 1Department of Botany, B.J.B. Autonomous College, Bhubaneswar751014, Orissa, India gayatri_bioteq@yahoo.co.in; sahurajani@yahoo.co.in **Abstract:** Medicinal plants are a major source of raw material for the traditional system like Ayurveda, Siddha & Unani. Even the modern system of medicine has more than 25 percent of drugs in use, which are either plant based or plant derived. Although several tree posses various medicinal properties, it has been ignored by indigenous & modern system of medicine. Among them *Azadirachta indica* & *Melia azedarach* belonging to family Meliaceae play a vital role in day to day usage of different indigenous communities due to its sacred and medicinal value. Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants. In the course of finding potential antioxidant from plant source, two medicinal tree species belonging to family Meliaceae has been selected. Leaves were dried and extracted with different solvent systems namely water, ethanol & methanol. Antioxidant activity using DPPH radical scavenging assay of six extracts from two genus of the family Meliaceae is reported & a comparison of the free radical scavenging ability of the extracts is emphasized. The result of the present study showed that the extract of *Melia azedarach*., which contains highest amount of phenolic compounds exhibited the greatest anti-oxidant activity in comparison to *Azadirachta indica*. The high scavenging property of may be due to hydroxyl groups existing in the phenolic compounds chemical structure that can provide the necessary components as a radical scavenger. [Nature and Science 2010; 8(4):22-28]. (ISSN: 1545-0740]. **Key words:** Antioxidant activity, *Azadirachta indica*, *Melia azedarach.*	Full Text
5	**Effect of Chromium on *Mucor* species and optimization of growth conditions** Bijay Kumar Sethi[1], Satyajit Kanungo[1,]*, Jyoti Ranjan Rout[1], Prativa kumari Nanda[2], Santi Lata Sahoo[1] [1] Microbiology Laboratory, P.G Department of Botany, Utkal University, Vani Vihar, Bhubaneswar, Pin-751004, Orissa, India. [2] Saila Bala Women's College, Cuttack, Orissa, India. satya_9bt@yahoo.com, santi_bot_uu@yahoo.co.in **Abstract:** Czapek Dox broth medium is frequently used for the culture of fungal species like *Mucor*. The influences of incubation period, pH, Cr (VI) concentration, temperature on the concentration of biomass were also evaluated. At pH 5.5, the fungi *Mucor* species yields maximum biomass and the fungus can be able to degrade chromium to a particular concentration but at higher concentration growth reduces. From a practical viewpoint, this abundant and inexpensive fungal biomass has potential application in the conversion of toxic Cr (VI) into less toxic or nontoxic Cr (III). Maximum biomass weight was observed which is about 0.33±0.01mg/20ml at a constant	Full Text

	temperature of 35^0C with an incubation period of 8 days. The protein content of the fungus was estimated and it was found that maximum yield of protein was recorded in the presence of 0.005 mM of chromium. [Nature and Science 2010;8(4):29-32]. (ISSN: 1545-0740). **Key words:** Biomass; *Mucor* species; Czapek Dox medium; incubation period.	
6	**Biochemical and neurological effects of obesity on primary school girls** Hanaa H. Ahmed[1]; Abla G. Khalifa[2]; Emad F. Eskander[1]; Alaa H. Sayed[1] and Ismail M. Abdel-Nabi[3] [1] Hormones Dept., National Research Centre, Dokki, Giza, Egypt [2] Child Health Dept., National Research Centre, Dokki, Giza, Egypt [3] Zoology Dept., Fac. Of Science, Suez Canal University, Ismailia, Egypt. alaasc@yahoo.com **Abstract:** The number of obese children worldwide has increased noticeably. As with adults, obesity in childhood is strongly related to hypertension, dyslipidemia, type II diabetes, and insulin resistance. Also, obese children are at increased risk of becoming obese adults. Therefore, obese children tend to develop serious medical and psychosocial complications, and have a greater risk of adult morbidity and mortality. The principal goal of this study was to investigate the effects of obesity on the levels of some biomarkers and their relation to the cognitive function in elementary school obese girls. The current study was conducted on 45 obese girls (mean age 10.53 ± 1.29 years; mean BMI 28.43 ± 4.62 kg/m^2) and 45 normal age-matched girls (mean age 10.36 ± 1.53 years; mean BMI 19.07 ± 3.47 kg/m^2). Estimation of serum adrenomedullin (AM) and substance P (SP), and plasma noradrenaline (NA) and acetylcholine (ACh) were carried out. Cognitive function tests (auditory vigilance, digit span, coding and visual memory) were done for all subjects. The levels of serum AM and SP as well as plasma NA were significantly increased (P<0.01) in the obese group as compared with the control group. Total wrong response to auditory vigilance test (TW) showed a significant increase (P<0.05) in the obese group as compared with the control group. Digit span and visual memory classification showed a significant decrease (P<0.01) while coding showed a significant increase (P<0.05). Our study showed that obesity, to some extent, has an adverse effect on cognitive function in primary school girls. The lack of effect of obesity on some cognitive tests may be due to different factors which may include increased levels of SP which has memory-promoting and reinforcing effects and higher levels of NA and the normal level of ACh, which also have important roles in memory processing. [Nature and Science 2010;8(4):33-43]. (ISSN: 1545-0740)] **Key words:** obesity- adrenomedullin -substance *P* - noradrenaline - acetylcholine – cognition – girls	Full Text
7	**NEAREST NEIGHBOUR PATTERN OF SPATIAL VARIATION IN EXPERIMENTAL FIELDS.** Dauda, T.O. Institute of Agricultural Research and Training, Obafemi Awolowo University, PMB 5029, Moor Plantation, Ibadan, Nigeria. taofik_biomet@fastmail.net **Abstract:** Evaluations of the nearest neighbour analysis in the study of spatial variation in experimental plot have been attempted for kenaf using a split plot experiment distributed in a complete randomized design. The experiment was carried out between June and September 2006 at Ilora and Ikenne outstation of the Institute of Agricultural Research and Training, Ibadan to evaluate nearest neighbourhood in experimental plots. The results of the cluster analyses of the stem girth at Ilora showed that 80% of the pairing plots were isotropic in nature while all other plot pairs are anisotropic in nature because their euclidean distances are not equal. For stem girth at Ikenne, isotropic property was exhibited between only $x_{4,1}$ vs $x_{3,1}$ and $x_{5,3}$ vs $x_{2,3}$ (0.032). All other plots pairs are anisotropic in nature. For plant height at both Ilora and Ikenne, none of the pairing plots exhibited isotropic property but anisotropic property. Also, the nearest neighbour indices are 0.00197 (for stem girth at Ilora), 0.00734 (for stem girth at Ikenne), 0.1831 (for plant height at Ilora) and 0.2456 (for plant height at Ikenne). From the study, the variogram is found to be related to the variance covariance using the model, $\gamma(h) = C(0) - C(h)$ (where $\gamma(h)$ = variogram, C(0) is the variance at the plot x_i and C(h) is the covariance at both plot x_i and x_j). Finally, low but positive nearest neighbour index obtained in this work implied that the neighbourhood pattern falls between cluster and randomness thereby reflecting patchiness of neighbourhood pattern. [Nature and Science 2010;8(4):44-53]. (ISSN: 1545-0740)] **Keywords:** Nearest neighbour, Euclidean distance, Clusters, initial soil nutrient deposition ((ISND).	Full Text
8	**QUANTITATIVE SPECIFICATION OF POTENTIALLY TOXIC METALS IN EXPIRED CANNED**	Full Text

	TOMATOES FOUND IN VILLAGE MARKETS [1] Itodo U. Adams and [2] Itodo U. Happiness [1]Department of Applied Chemistry, Kebbi State University of Science and Technology, Aliero, Nigeria. [2]Department of Chemistry, Benue State University, Makurdi. itodoson2002@yahoo.com **Abstract:** Varieties of expired canned tomatoes were pre-treated using standard digestion methods and were analysed for heavy metals. Cr, Pb, Cd, Fe, Ni, Co, Zn, Mg, Cu, AI and Mn were determined using Atomic absorption spectroscopy and photometry techniques. Mg, Mn, Co and Pb presented higher concentration values ranging from 32.18± 9.25; 4.35 ±1.60; 2.62 ±1.76 and 2.82 ±0.53 μgg^{-1} respectively. Unlike the Cd contents, Cr and Pb concentration were above the threshold limit values (TLV) of 2.0μgg^{-1}. The levels of metals for some of the canned foods exceed that of their corresponding uncanned products reported in literatures. Physicochemical variables of the brands were also estimated as 76.4 ±3.85 and 3.20± 1.09 % for moisture and ash contents respectively. The arrays of health implications of heavy metals computed in this work will at a glance access the roles of excessive and prolonged intake of such foods. [Nature and Science. 2010;8(4):54-59]. (ISSN: 1545-0740). **Key words:** canned tomato, toxic metals, AAS, Photometry.	
9	**THE EFFECT OF DIFFERENT CONCENTRATION OF GINGER ON THE QUALITY OF SMOKED DRIED CATFISH (*Clarias gariepinus*)** Idris, Garba Libata*, Omojowo, Funso Samuel.;* Omojasola Patricia Folake**, Adetunji Charles Oluwaseun***, and Ngwu Emmanuel onyebuchi* *NATIONAL INSTITUTE FOR FRESHWATER FISHERIES RESEARCH, (NIFFR) P.M.B. 6006, NEW-BUSSA, NIGER STATE. NIGERIA. ** DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF ILORIN, ILORIN, NIGERIA. ***NIGERIAN STORED PRODUCTS RESEARCH INSTITUTE, ILORIN, KWARA STATE, NIGERIA. **idrisgarbalibata@yahoo.com**; **jowosam@yahoo.com**; **folakejasola@yahoo.co.uk**, **charliguitar@yahoo.com** and **ngwuemma@yahoo.com** **Abstract:** Fresh live catfish (*Clarias gariepinus*) obtained from Private pond in NIFFR, New-Bussa. The samples were divided into five groups. Four groups were dressed and dipped in a solution of 2.5%, 5%, 7.5% and 10% of Ginger respectively for thirty (30) minutes and smoked dried. The fifth group acts as control. They were examined microbiologically, chemically and organoleptically. The Ginger reduced the free fatty acid (FFA) values, trimethylamine (TMA) values, and the fungi load of the processed fish. Ten percent of ginger had the best result in terms of reduction in fungi load, FFA and TMA values and followed by 7.5 and 5%. However, from the organoleptic results of overall acceptability, taste, colour and texture of the products, 5% ginger concentration had the best acceptance and significantly different ($P<0.05$) when compared to the non treated control after 8 weeks of storage. [Nature and Science. 2010; 8(4):59-63] (ISSN: 1545-0740). **Key words:** Catfish, Ginger, smoked, storage and Fungi load.	Full Text
10	Diagnostic Role Of Resistin In Nonalchoholic Fatty Liver Disease Engy Yousry Elsayed, Amal Shawky Mohamed, Hala Abd Elal* and Eman Hamed** Internal Medicine, Clinical Pathology* and Pathology** Departments Faculty Of Medicine, Ain Shams University, cairo, Egypt. ashorengy@yahoo.com, amalshawky-mb@hotmail.com, hala_abdelal@yahoo.com, imihewedi99@yahoo.com **Abstract:** Introduction: Nonalcoholic fatty liver disease (NAFLD) is a major cause of liver-related morbidity and mortality. Insulin resistance is believed to be a key factor in the development of fatty liver. Moreover, insulin resistance states characterized by elevated expression and production of several cytokines; of particular adiponectin, leptin, resistin. Leptin and adiponectin have been implicated in the pathogenesis and progression of NAFLD but direct evidence of the role of resistin in NAFLD is lacking. The aim of this study was to determine the circulating resistin level in patients affected by NAFLD and to correlate resistin level with insulin sensitivity, liver function and histologic feature. Subjects and methods: This study included 100 subjects divided in to: Forty patients with NAFLD, forty obese person with BMI >30 with normal transaminases and normal liver ultrasound and twenty controls with BMI <20, for all subjects serum resistin was measured, Homeostasis model assessment	Full Text

	(HOMA) was calculated and liver profile was assessed. Liver biopsy was done in NAFLD patients. Results: Serum resistin was higher in patients with NAFLD (16.2 ± 4) compared to obese and control groups (6.8 ± 4.1 and 3.4 ± 1.1) respectively (p <0.01), serum resistin was higher in advanced cases of NAFLD compared to mild cases (19.2±3.6 vs. 13.5±2.7) respectively (P < 0.01). Moreover serum resistin was positively correlated to BMI, HOMA , highly sensitive CRP, AST and ALT. Conclusion and recommendation: Resistin has a role in pathogenesis of NAFLD, resistin level is a predictive of histology in NAFLD, so the use of serum resistin assay as a simple diagnostic biomarker for NAFLD is recommended. [Nature and Science 2010;8(4):64-68]. (ISSN: 1545-0740). **Key word:** NAFLD, NASH, Obesity and Resistin.	
11	**Assessing Environmental Flow Modeling For Water Resources Management: A Case of Sg. (River) Pelus, Malaysia** Mohd Ekhwan Toriman School of Social Development and Environmental Study, FSSK. 43600. Universiti Kebangsaan Malaysia, Bangi Selangor Malaysia. ikhwan@ukm.my **Abstract:** In Detailed Environmental Impact Assessment (DEIA), modeling of environmental flows is one of the main studies that need to be delivered in the final DEIA report. The model is important to the project proponent to engage suitable designs that can be suited to environmental needs, particularly on future water resources management. In this respect, Environmental Flow Assessment (EFA) is used to estimate the quantity and timing of flows to sustain the ecosystem values. The proposed of hydropower projects in Sg Pelus, Perak was studied aimed to evaluate existing river flow characteristics and to model EFA due to river diversion of Sg Pelus. Daily river flow (m^3/s) recorded at Sg Pelus (Station No. 6035) and Sg. Yum (Station No. 6044) gauging stations were used to design the flow duration curve. The low flow then calculated using the 7Q10 equation to estimate the lowest 7-day average flow that occurred on average once every 10 years. The results indicate that the average daily flows for both stations (6035 and 6044) are 5.080 m^3/s and 11.391 m^3/s, respectively. The flow duration curve shows that 50 percent of 4 m^3/s of discharge will be exceeded/ equaled in Station 6044 while 8.2 m^3/s of discharge will be exceeded or equaled in Station 6035. The requirement environmental flows for both parameters are 0.613 and 0.426 m^3/s for Environmental Flow Assessment, respectively. The results obtained in this model are important to managing the river at least in Class II after river diversion project. [Nature and Science 2010;8(4):69-76]. (ISSN: 1545-0740). **Keywords:** Environmental Flow Assessment; Detailed Environmental Impact Assessment; Low flow; Flow duration curve.	Full Text
12	**Mutagenic and antimutagenic effects of some plant extracts in Drosophilla melanogaster** Ahmed, E.S.[1] ;Twaty,N.H[2]; Fakiha K.G[2]. and Bibars M.A.[1] 1-*Department of Cell Biology National Research Center Egypt.* *2-Department of Biology,Faculty of Science, King Abdelaziz University,Jeddah* ***Abstract.*** This study was designed to investigate the mutagenic potential of the anticancer drug vincristine and some plant extracts (fennel and parsley) on *Drosophilla melanogaster* using two test systems: the sex linked recessive lethal (SLRL) and the estimation of the activity of cholinesterase enzyme (ChE) in F1 and F2 bar eye females and F2 wild type males. A wild type strain Oregon-R (or-R) male flies of *D.melanogaster* were treated on a medium containing a concentration of only one of the three agents, followed by a combined treatment in an alternative way of fennel extract or parsley extract followed by vincristin, then vincristin followed by fennel extract or parsley extract and finally the three agents together. The results obtained, showed non significant increase in the percentage of the S.L.R.L in all stages of spermatogenesis in all treatments. Meanwhile, vincristine as a single treatment or combined with fennel or parsley extracts showed genotoxic effects in the three categories of the two generations of S.L.R.L: F1 females heterozygous F2 bar eye females and F2 wild type males on the genetic background of ChE in all treatments. [Nature and Science 2010;8(4):77-82]. (ISSN: 1545-0740]. **Keywords**: *Drosophilla melanogaster* - cholinesterase enzyme – vincristin – fennel – parsley.	Full Text
13	**Evaluation of Proximate and Phytochemical Compositions of Fermented Raw and Fermented *Napoleona Imperialis* Seed and Their Feeding Values on Finisher Broilers**	Full Text

	Martin Chukwudi Uchegbu, Cynthia Okere, Ifeanyi Princewill Ogbuewu*, Ifeanyi Charles Okoli, Chibuzor Hope Nwaodu, Chike Timothy Ezeokeke, George Akalefu Anyanwu Department of Animal Science and Technology, Federal University of Technology, P.M.B.1526, Owerri, Imo State, Nigeria. Princiano2001@yahoo.com **Abstract:** The high cost of feed in poultry enterprise is well established. This is blamed on limited availability of conventional feedstuff which is also in competition with man's dietary needs. This has necessitated the search for alternative protein sources such as *Napoleona imperialis* seed. Ripe *N. imperialis* seeds (NISs) were harvested in and around the Federal University of Technology, Owerri with the pods opened, the seeds extracted, and sun dried for 7 days. A portion of the sundried NIS was milled using hammer mill to produce the raw *N. imperialis* seed meal (NISM) while, the remaining portion was soaked in water for 4 days and sundried before milling to produce soaked NISM. Samples of raw and soaked NISMs were taken to the laboratory to determine its proximate and phytochemical compositions. Phytate, tannins, HCN, alkaloids, saponins and metabolisable energy value of the raw NISs were significantly ($p<0.05$) affected by the treatment. Birds on control diet performed significantly ($p<0.05$) better than those on 10% soaked NISM diet in terms of average daily feed intake and feed conversion ratio but similar ($p>0.05$) to those on 5% raw and 5% soaked NISMs. The average daily weight gain of birds on 5% raw and 10% soaked NISMs was significantly ($p<0.05$) lower than the control group. It is concluded that soaking for 4 days in water do not reduce the anti-nutritional content of *N. imperialis* seeds to a tolerable level for broilers. [Nature and Science 2010;8(4):83-88]. (ISSN: 1545-0740). **Keywords:** novel seeds, proximate composition, phytochemistry, performance, broilers.	
14	**Plasmid Associated Anthracene Degradation by *Pseudomonas* sp. Isolated from Filling Station Site** Gulshan Kumar[1], Rajesh Singla[2], Rakesh Kumar[1]* Biotechnology Department; 2. Microbiology Department, Dolphin PG College of Life Sciences, Chunni-Kalan-140307, Fatehgarh Sahib, Punjab, INDIA. rakesh_panchal1@yahoo.co.in **Abstract:** Bacterial strains were isolated from oil contaminated soil of 5 different filling stations of Himachal Pradesh, India and screened for their anthracene degradation ability. Enriched media was used to isolate the anthracene degrading bacteria with 0.5% peptone and 0.1% w/v anthracene in Basal Salt Mineral medium and during successive enrichment the peptone concentration was decreased to 0.25 g, 0.1 g and to 0.0 g. After one month of enrichment 5 strains were found to be potent anthracene degrader out of total 76 strains screened. These 5 strains were further subcultured for 10 days and on the basis of percent anthracene degradation strain E was found to degrade 74.8% anthracene supplemented in BSM medium at 0.1% as sole source of carbon and energy and identified as *Pseudomonas* sp. As evident by antibiotic sensitivity test, *Pseudomonas* sp. showed resistance against Cefadroxil and Ampicillin among tested 7 antibiotics. Acridine orange induced plasmid curing of isolate lead to complete loss of plasmid and anthracene degradation activity. The study suggests that the plasmid could have a role in anthracene degradation activity. [Nature and Science 2010;8(4):89-94]. (ISSN: 1545-0740). **Key words:** anthracene, *Pseudomomas* sp., plasmid curing, acridine orange, marker antibiotic	Full Text
15	**Anthelmintic comparative study of *Solanum lycocarpum* St. Hill extracts in mice naturally infected with *Aspiculuris tetraptera*.** Borba, H .R. [1] ,Freire, R. B. [1], Albuquerque, A. C. [3], Cardoso, M.E.O. [3], Braga, I.G. [3], Almeida, S. T. P. [3], Ferreira, M. J. C. [3], Fernandes, G. L. T [3], Camacho, A. C. L. F. [3], Lima, R. C. [3], Almeida, A. C. C. [3], Mattos, D. M. M. [3], Duarte, R. M.[3], Nascimento, S. F. [3], Framil R. A.[3], Diré, G. F. [1,2,3,4] [1]Universidade Federal Rural do Rio de Janeiro, Instituto de Biologia, Departamento de Biologia Animal, Laboratório de Atividade Anti-helmíntica de Plantas. Br 465; Km 7-Seropédica, Rio de Janeiro, RJ 23890.000, Brazil. Fax: +552126821763/ +552126821763. [2]Centro Universitário da Zona Oeste- UEZO, Avenida Manuel Caldeira de Alvarenga, 1203. Campo Grande, RJ 23070-200, Brazil. Telefone/Fax: 2415-8392; e-mail: gdire@hotmail.com [3]Universidade Estácio de Sá. Centro de Ciências da Saúde. Rio de Janeiro, RJ, Brazil. [4]Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro, Maracanã, Rio de Janeiro, RJ, Brazil. Gratitude: We thanks to Thiago de Azevedo Amorim, technician of herbarium of the Botanical Department of Rural Federal University from Rio de Janeiro, by the identification of the studied species. borba@ufrrj.br	Full Text

	Abstract: This study intends to add new data on the helminthes parasites of laboratory mice. It has been investigated the antihelmintic activity of *Solanum lycocarpum* (*Solanaceae*) extracts against *Aspiculuris tetraptera* in mice naturally infected. The extracts were applied for oral saw (intragastric), into the volume of 0.04mL/g , with the employing of a dead and bend probe during three consecutive days. The fecal material, collected 24 hours after each application, performing a total of four fecal collection, have been softened previously, transferred about to sieve of network of 125 micrometers and tested under microscope stereoscope, with the objective of behave the identification and counting from the worms eliminated of the second to the fifth day of the experimental. Tukey-Kramer Multiple Comparisons Test was applied to compare the results. This approach intends to add new data on the helminthes parasites of laboratory mice. According to the analysis of the results it was observed that there were differences ($p<0.001$) in the %of elimination between 20%TM and 20% 20%UR (from 2.24 ± 3.33 to 2.92 ± 3.33), 20%TM and Nit (from 2.24 ± 3.33 to 64.0 ± 2.89), 20%TM and Meb (from 2.24 ± 3.33 to 100.0 ± 3.16), 20%UR and Nit (from 2.92 ± 3.16 to 64.0 ± 2.89) and ($p<0.01$) 20%UR and C (from 2.92 ± 3.16 to 1.56 ± 3.16). It was published that medicinal plants which were reported as useful in the treatment of diabetes the *S. lycocarpum* was the sixth most frequently mentioned. According to the results obtained in the present study, we can speculate that the anthelmintic effect of *Solanum lycocarpum* was noticed due to the concentration of steroidal alkaloid oligoglycosides and short-chain fatty acids. [Nature and Science 2010;8(4):95-100]. (ISSN: 1545-0740). **Key words:** *Solanum lycocarpum* ; helminthes, mice; *Aspiculuris tetraptera* l;antihelmintic; medicinal plants	
16	**A Review on the Future of Ecotourism in the Valley of Flowers National Park: A Case Study of Garhwal Himalaya, India** GBG Shashi. K Tiwari[1], GBG Pananjay K. Tiwari[2] and S.C Tiwari[3] [1]Department of Tourism, Amity University, Noida, India. [2]Department of Natural Resource Management, Debre Markos University, Debre Markos, Ethiopia [3]Department of Botany, Ecology and Environment Laboratory, HNB Garhwal University, India pananjay_gbg@rediffmail.com; tiwariji_gbg@rediffmail.com; prof_sctiwari@rediffmail.com **Abstract:** This paper reports the future of Ecotourism in the Valley of Flowers national park in Garhwal Himalaya, Uttarakhand, India. The valley has an unusually rich flora of over 600 species with many rarities. Animals found are nationally rare or endangered. 13 species of mammals are recorded for the Park and its vicinity although only 9 species have been sighted directly. Other factors that are contributing to ecotourism are beautiful landscapes, peaks, lakes and tarns etc. But now-a-days the problem of Solid waste is increasing at an alarming rate because of the heavy influx of tourists and improper management practices. This paper reviews the various ecotourism resources of the area and their future prospects. [Nature and Science. 2010;8(4):101-106]. (ISSN: 1545-0740). **Keywords:** Fauna Flora, Glaciers, Tarns	Full Text
17	**Diabetogenic Effect of Pregnancy in Sprague-Dawley (SPD) Rats: Potential use as Experimental Model of Human Gestational Diabetes** Idowu Adewunmi Taiwo[1*], Olusoji Olurotimi Adewumi[1], Albert Kolawole Odunlade[2], Liasu Adebayo Ogunkanmi[1], Peter Godwin Chikwenye Odeigah[1] [1]Department of Cell Biology and Genetics, Faculty of Science, University of Lagos, Lagos 101017. Nigeria. [2]Department of Biological Science, Yaba College of Technology, Yaba, Lagos. Nigeria tai_dex@yahoo.com sojiadewumi@yahoo.com **Abstract:** The effect of pregnancy on the pattern of oral glucose tolerance was investigated using Sprague-Dawley (SPD) rats. Adult virgin, timed-pregnant and non-pregnant rats were subjected to brief ether anaesthesia after 18-hour overnight fasting period to allow for oro-gastric administration of glucose load at 3.0g/kg body weight (b. wt.) as 30% solution. Glucose concentration determined from the tail blood shows that the starting glucose concentration of the pregnant rats was 6.9±0.4 mmol/l, a significantly higher ($P<0.05$) value than 5.8 mmol/l, the starting blood glucose concentration of the non-pregnant animals (Controls). The peak blood glucose level attained at the 60th minute was significantly higher ($p<0.05$) in the pregnant rats (13.5±0.3 mmol/l) as compared to that of the non-pregnant rats (8.5±0.3 mmol/l). After 120minutes, the blood glucose level of the non-pregnant rats dropped to a near starting level while the corresponding value in the pregnant rats remained comparatively higher ($P<0.05$). Assessment of the rate of appearance and disappearance of glucose in the blood and the determination of	Full Text

	glucose response and glucose tolerance indexes (GRI and GTI) respectively showed that pregnancy caused poor glucose utilization in the rats. The results of this short-term study suggest that pregnancy is largely diabetogenic in Sprague-Dawley (SPD) rats. The diabetogenic effect of pregnancy did not necessitate administration of any other diabetogenic agent such as streptozotocin or fructose. Thus, pregnancy induced diabetes in this strain of rats may have potential value as model of gestational diabetes in human. [Nature and Science 2010; 8(4):107-111]. (ISSN: 1545-0740). **Keywords:** Gestational diabetes; glucose response index; glucose tolerance index; insulin resistance	
18	**Studies on growth, nutritional and microbiological status of citrus seedlings infested with root-rot disease** 1Elham Z. Abd El-Motty; 2 Selim, El-Metwally; 2 Youssef, Rifaat Abou and 3 Sahab, Ahmed Farahat.1 Pomology Dept., National Research Centre, Giza, Egypt 2 Soils and Water Use Dept., National Research Centre, Giza, Egypt. 3 Plant Pathology Dept., National Research Centre, Giza, Egypt. ahmedsahab2002@yahoo.co.uk **Abstract:** This research aims to evaluate the suppressive effects of compost fortified with Trichoderma harzianum and Top.Zn formulations on citrus root-rot and plant growth. Pathogenicity test proved that isolate no.1 of Fusarium solani and Macrophomina phaseolina were the most frequently causing infection of all orange plants with 87.5 and 93.75% disease severity respectively. Soil infested with F. solani or M. phaseolinea decreased plant growth and N, P and K contents in the orange leaf tissues compared to the control. Meanwhile, application of Top.Zn compound alone raised up N, P and K contents (%) in leaves of orange and mandarin survived in soil infested with F. solani and M. phaseolina. Use of compost with T. harzianum and Top.Zn simultaneously with a pathogen inoculation caused a significant increase in plant growth, chlorophyll a and b, macronutrients (N, P and K) content, micronutrient (Fe, Zn, Mn and Cu) contents orange and mandarin seedlings. The total fungal and bacterial counts in the orange and mandarin rhizosphere were increased progressively as the plant grew up reaching their maximum at the last count which was taken after 90 days (seedlings were 1-year old). In soil infested with F. solani and M. phaseolina, treatment with compost fortified with T. harzianum increased the total fungal count 3.34 and 28.98 times, respectively in orange and 2.60 and 21.99 times, respectively in mandarin compared with non-treated control. In soil infested with F. solani and M. phaseolina, the treatment with compost fortified with T. harzianum in combination with Top.Zn decreased the average number of total bacterial counts in the rhizosphere of orange 85.04 and 78.92% respectively and 59.32 and 92.74 % respectively in the rhizosphere of mandarin. [Nature and Science. 2010;8(4):112-121]. (ISSN: 1545-0740). **Key words:** Citrus root rot, rhizosphere, compost, Trichoderma harzianum, Top.Zn formulation.	Full Text
19	***Scanning Electron Microcopy Studies on Mango Malformation*** *[1] Wahab M-Abd El ,[1]Sehab A ,[3] ,Hazza M ,*[1] Wafaa Haggag M* -Department of Plant Pathology National Research Center, Dokki, Cairo, Egypt. -Science Faculty, Botany Department, Banha University, Egypt. Wafaa_haggag@yahoo.com **bstract:** Mango malformation disease (MMD) is an economically important disease of *Mangifera indica* lobally. *Fusarium subglutinans* has been associated with mango floral and vegetative malformation although onfusion still remains regarding the etiology of the disease. In order to determine the *Fusarium ubglutinans* penetration site, artificial inoculation of mango seedlings variety Alfonso were conducted. When oil was infested with *F. subglutinans*, the malformation was detected in the buds, three months post inoculation. ymptoms of the disease include loss of the apical dominance and swelling of vegetative buds, proliferation of eaves and flowers, phyllody and hypertrophy of panicle axes. Using scanning electron microscope (SEM), ymptoms of vegetative and floral malformation appeared where mycelium of *Fusarium subglutinans* were resent in the tissue at high concentrations compared to that of the untreated controls. Studies also revealed the resence of, pin-sized to large holes, disorganised cells and fungal mycelial infection at the base of the malformed uds during bud-inception stages. Moreover, *Fusarium* isolate colonized seedling root systems and became ystemic, spreading to above-ground plant tissues include apical and lateral buds. *Fusarium subglutinans* proved o be the dominant fungus. [Nature and Science. 2010;8(4):122-127]. (ISSN: 1545-0740). **Key words:** Egypt, *F. subglutinans*, Mango Malformation, *Mangifera indica*.	Full Text
20	**Epidemiology and the Association of the *Fusarium* Species with the Mango Malformation Disease in Egypt** *[1] Wahab M-Abd El ,[1]Sehab A ,[3] ,Hazza M ,*[1] Wafaa Haggag M*	Full Text

	1-Department of Plant Pathology National Research Center, Dokki, Cairo, Egypt. 2-Science Faculty, Botany Department, Banha University, Egypt. Wafaa_haggag@yahoo.com **Abstract:** Mango malformation disease (MMD) is an economically important disease of *Mangifera indica* globally. This disease is caused by a complex of fungal pathogens, of which various *Fusarium* spp. dominate. This study was conducted to assess the epidemiology and its pathogenesis of mango malformation disease in Egypt. In three main Governorates of mango production, El Giza, Esamalîa and El-Bohera, disease incidence reached up to 80%. Maximum infection of traditional cultivars was observed in Hindi Sennara, Alfonso, Timour and Zebda. Exotic Tomy , Keet and Kent cultivars appeared to be moderate infection . Nine additional taxa have been isolated, i.e., *F. subglutinans, F. oxysporum, F. sterilihyphosum, F. proliferatum, F. culmorum, F. nygamai, F. pseudonygamai, F. nelsonii* and *F. verticilioides* from Egypt. *Fusarium subglutinans* proved to have the high frequency in all mango cultivars in tested area, while, *F. oxysporum, F. sterilihyphosum, F. proliferatum* frequently were less. To date, Koch's postulates have been applied with *Fusarium* for their pathogenic potential on mango cultivars seedlings under greenhouse conditions. Apparently, not all isolates of this *Fusarium* species are equally virulent on mango seedlings. *Fusarium subglutinans* proved to be the dominant fungus in all varieties. At the same time, *F. oxysporum , F. sterilihyphosum, F. proliferatum*, displayed also moderate virulence. Moreover, isolates colonized seedling root systems and became systemic, spreading to above-ground plant tissues include apical and lateral buds. *Fusarium subglutinans* proved to be the dominant fungus. Complex Strains of *F. subglutinans, F. oxysporum, F. sterilihyphosum* and *F. proliferatum* induced typical malformation symptoms on mango seedlings and trees in Egypt [Nature and Science. 2010;8(4):128-135]. (ISSN: 1545-0740). **Key words**: Egypt, *F. subglutinans, F.oxysporum , Fusarium sterilihyphosum* and *F. proliferatu*, Mango Malformation, *Mangifera indica.*	
21	**Biochemical evaluation of the effect of *Rhazya stricta aqueous* leaves extract in liver and kidney functions in Rats** Nabih A. Baeshen [1]; Sahira A. Lari [2]; Huda A. Aldoghaither [1] and Ayman I. Elkady [1,3] [1]Department of Biological sciences, Faculty of Science, King Abdulaziz University, Jeddah [2]Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah [3]permenant address: Zoology Department, Faculty of Science, Alexandria University, Alexandria, Egypt. Nabih_Baeshen@hotmail.com **Abstract:** Rhazya *stricta* (*R. stricta*) is an important medicinal species used in indigenous medicinal herbal drugs to cure various diseases in South Asia and Middle East Countries. Over 100 alkaloids have been isolated, from *R. stricta* leaves, stems, roots and legumes and mixtures of aerial parts. The aim of this study was evaluatation of the beneficial effects of oral administration of extracts of the *R. stricta* leaves on serum lipid profile concentrations, the activity of liver enzymes and the kidney functions, using doses comparable to those applied by humans in the folkloric medicine. To achieve this goal, fifty five male Wistar rats were divided into four groups as follows: group 1 (control, n= 10) received a daily single oral dose of 0.5 ml of distilled water, groups 2, 3 and 4 (each of 15), each animal received a daily single oral dose of 0.5 ml of distilled water containing 0.1 gm/ml (group 2), 0.125 gm/ml (group 3) and 0.150 gm/ml (group 4) of the *Rhazya* leaf aqueous extract, for 18 weeks. Blood samples were collected, after an overnight fast, 1, 2, 4, 8, 12 and 18 weeks post-treatment. The aqueous extract of the *R. stricta* leaves significantly decreased concentrations of TGs, LDL-c, cholesterol, uric acid and creatinin, but increased concentration of HDL-c. It triggered all these activities without affecting liver enzyme activities or kidney functions. These findings may have a positive impact on the cardiovascular patients and may provide a new therapeutic strategy to reduce hypertriglyceridemia. [Nature and Science. 2010;8(4):136-142]. (ISSN: 1545-0740). **Key words**: *Rhazya stricta*; lipid profile; liver enzymes; aqueous extracts; uric acid	Full Text
22	**Some biochemical Studies on Friesian Suffering from Subclinical Mastitis** Mona S. Zaki1 and Nabila El-Battrawy2 & Susan, O. Mostafa3, Olfat M. Fawzi3 Iziz Awad3 1 Department of Hydrobiology - National Research Center. 2 Department of Hydrobiology - animal Institute of Reproduction, El-Haram, Cairo, Egypt. 3 Department of Biochemistry National Research Center. dr_mona_zaki@yahoo.co.uk **Abstract:** The present study was conducted to investigate the effect of subclinical mastitis on clinicopathological changes in Mastitic friesian. A total of 400 individual milk samples from clinically normal udder quarters of 100	Full Text

	diary friesians were examined microbiologically as well as by using California mastitis test (C.M.T.) for detection of subclinical mastitis and designing rapid diagnostic tests for other infection. Blood samples were analysed for hemogram, cortisol, alanine aminotransferase, asparate aminotransferase, total protein, inorganic phosphorous and calcium. Also L.DH in milk was detected. The results indicated that there is a significant elevation of cortisol, Sgot, p.cv, L.DH activity in milk while a notable decrease in total protein, serum calcium and Hemogram. was observed. However; Serum phosphorous level did not exhibit obvious changes. [Nature and Science. 2010;8(4):143-146]. (ISSN: 1545-0740). **Key words**: Microbiology of mastitis, Pathology of mastitis, Enzymes in mastitis, changes in blood	
23	**Bioaccumulation and histopathological alterations of the heavy metals in *Oreochromis niloticus* fish** H.A. Kaoud* and A.R. El-Dahshan Department of Veterinary Hygiene and Environmental Pollution, Faculty of Veterinary Medicine, Cairo University, Egypt. ka-oud@link.net **Abstract:** Copper, lead, cadmium and mercury concentrations were recorded in water and tissues of *Oreochromis niloticus* from Egyptian fish farms in 2007-2009. Histopathological alterations in fish tissues were also studied. Bioconcentration factors of copper, lead, mercury and cadmium in liver and muscle tissue were (3.93 & 3.87), (8.10 & 7.60), (0.79 & 50.0) & (38.25 & 30.25), respectively. Mercury was the most bioaccumulated and biomagnified metal in the muscles, while Cu was the least. The concentration of cadmium**,** lead and copper were highest in liver and lowest in kidney tissue, while mercury (Hg) concentrations were highest in muscles, lowest in kidney tissue. Several histopathological changes were noted in muscles, liver, gills, kidney and intestine tissue attributable to heavy metals exposure. [Nature and Science. 2010;8(4):147-156]. (ISSN: 1545-0740). **Key words**: Bioconcentration, copper, lead, cadmium, mercury, Tilapia, Pollution, histopathology	Full Text
24	**Bioaccumulation of cadmium in the freshwater prawn *Macrobrachium rosenbergii*** H.A. Kaoud* and A. Rezk Department of Veterinary Hygiene and Environmental Pollution, Faculty of Veterinary Medicine, Cairo University, Egypt. ka-oud@link.net **Abstract:** The effects of Cd on mortality, resistance and bioaccumulation in giant freshwater prawn *Macrobrachium rosenbergii* in Egypt were studied. Survival of prawns exposed to cadmium doses over 60 µg $/l^{-1}$ were significantly lower than of those exposed to lower doses. After 96 hours prawns exposed to >40 µg $/l^{-1}$of cadmium had a greater reduction in total haemocyte count and phagocytic activity than those exposed to lower concentrations. Bioaccumulation of Cd in the gills, hepatopancreas and muscleswas variable. Cadmium accumulated in gills and hepatopancreas, but muscles had a moderately significant Cd level increase. *Macrobrachium rosenbergii* manifested histopathological alterations in gills, hepatopancreas and muscles when exposed to different concentrations of cadmium. [Nature and Science 2010; 8(4):157-168]. (ISSN: 1545-0740). **Keywords:** toxicity, survival, haemocyte count	Full Text

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Plant growth pattern, tiller dynamics and dry matter accumulation of wetland rice (*Oryza sativa* L.) as influenced by application of different manures

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Abstract: To observe the comparative performance of different organic manures with inorganic fertilizers on the growth rate, tillering and dry matter accumulation of rice an experiment was conducted in the Research Farm of Sher-e-Bangla Agricultural University, Dhaka, Bangladesh during June to November, 2008. The 10 treatments comprised viz. T_1 (Control), T_2 (Green manure @ 15 t ha^{-1}), T_3 (Green manure @ 15 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ i.e.50% NPK), T_4 (Poultry manure @ 4 t ha^{-1}), T_5 (Poultry manure @ 4 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ i.e. 50% NPK), T_6 (Cowdung @ 12 t ha^{-1}), T_7 (Cowdung @ 12 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ i.e. 50% NPK), T_8 (Vermicompost @ 8 t ha^{-1}), T_9 (Vermiconpost @ 8 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ i.e. 50% NPK) and T_{10} ($N_{80}P_{12}K_{72}S_{10}$ i.e.100% NPK). Plant height, number of tillers $hill^{-1}$, total dry weight of plants, crop growth rate and relative growth rate were significantly influenced by different treatments. Except plant height and total tiller per hill all the parameters were found to be the highest with the treatment T_5 (Poultry manure @ 4 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ i.e. 50% NPK). The dry matter production showed a significant relationship with grain yield of rice.

[Nature and Science 2010;8(4):1-10]. (ISSN: 1545-0740).

Key words: Rice, Organic manures, CGR, RGR, Dry matter partitioning, Yield

1. Introduction

After the industrial revolution widespread introduction of inorganic fertilizers led to a decline in the use of organic material in the cropping systems (Rosegrant and Roumasset, 1987). The impact of increased fertilizer use on crop production has been large and important (Hossain and Singh, 2000). More recently, attention is focused on the global environmental problems. The world elite society is giving emphasize on utilization of organic wastes, FYM, compost, vermicompost and poultry manures as the most effective measure to save the environment to some extent. Organic materials are the safer sources of plant nutrient which have no detrimental effect to crops and soil. Cowdung, farm yard manure, poultry manure and also green manure are excellent sources of organic matter as well as primary plant nutrients (Pieters, 2005).

Rice production in Asia increased upto 25% between 1965 and 1980 due to fertilizer use (Barker et al., 1985). In recent years there has been serious concern about long-term adverse effect of continuous and indiscriminate use of inorganic fertilizers on deterioration of soil structure, soil health and environmental pollution (Ghosh and Bhat, 1998; Shukla et al., 1998; Singh, 2000). In contrast to inorganic fertilizer the use of green manures and other organic matter can improve soil structure, improve nutrient exchange and maintain soil health and that is why interests have been raising in organic farming (Ayoub, 1999; Becker et al., 1995). Poultry manure is an excellent organic fertilizer, as it contains high nitrogen, phosphorus, potassium and other essential nutrients. Poultry manure supplies phosphorus more readily to plants than other organic manure sources (Garg and Bahla, 2008). Vermicompost has been shown to have high levels of total and available nitrogen, phosphorous, potassium (NPK) and micro nutrients, microbial and enzyme activities and growth regulators (Parthasarathi and Ranganathan 1999; Chaoui et al., 2003) and continuous and adequate use with proper management can increase soil organic carbon, soil water retention and transmission and improvement in other physical properties of soil like bulk density, penetration resistance and aggregation (Zebarth et al., 1999) as well as beneficial effect on the growth of a variety of plants (Atiyeh et al., 2002).

Most of the cultivated soils of Bangladesh have less than 1.5% organic matter while a good agricultural

soil should contain at least 2% organic matter. In last 20 years in the content of organic matter decreased by 15 to 30% (Miah, 1994). The reasons for declining organic matters with time is intensive cropping and use of higher dose of chemical fertilizers with little or no addition of organic manure.

Rice is the staple food of Bangladesh and majority of food grain comes from rice. About 80% of cropped area of this country is used for rice production, with annual production of 4,37,29,000 metric tons (IRRI, 2006) in total acreage of 1,10,59,000 ha. The average yield of rice in Bangladesh is 3.90 t ha^{-1} (BRRI, 2007) which is almost less than 50% of the world average yield. Due to declining factor of productivity under increased intensification the production level of rice is maintaining the same level for years. Therefore, farmers are compelled to apply increasing rates of fertilizers to maintain current yield levels (Pagiola, 1995). But it is more detrimental for the soil health. The reasons for low yield of rice are manifold; some are varietals, others are technological and rests are climatic. The yield can be increased by using improved cultural practices like use of quality seed, high yielding varieties, adopting plant protection measures, judicious application of fertilizers, etc. Among them integrated nutrient management can be one of the most effective means to increase the productivity of rice.

Guowei et al. (1998) reported that rice (*Oryza sativa* L.) crop functions as a population of tillers produced at different times and possessing specific growth characteristics. They showed significant contribution of cultivar tillering ability to dry matter accumulation, yield components, and grain yield. Singh et al. (2003) reported that crop growth rate and relative growth rate was significantly influenced by NPK. The tiller number and total dry matter production are closely correlated with yield depending on the rice cultivar (Tanaka, 1968) which can be greatly enhanced by applying proper nutrient. Prasad (1981) observed the increase of TDM due to increased N application.

A good amount of plant nutrients are supplied by organic manure that contribute to crop growth and yields. To maintain the present levels of crop productivity of high yielding varieties the use of organic manures single-handedly, as a substitute to chemical inorganic fertilizer is not economic and sufficient (Garrity and Flinn, 1988). Therefore, integrated nutrient management in which both organic manures and inorganic fertilizers are used simultaneously is probably the most effective method to maintain healthy sustainable soil system while increasing crop productivity (Janssen, 1993). Organic manures and chemical fertilizers should be used combined to get higher yield, to maintain soil health as well as a cost effective production system. Thus it is necessary to carry out studies by using fertilizers and manures in an integrated way to find out the appropriate dose or proportion of chemical fertilizers and manures use to maintain a desirable yield level. Considering these facts the present study was undertaken to determine the suitable manure and fertilizer combination for optimum growth, tillering and dry matter production of transplanted rice.

2. Materials and Methods

2.1 Experimental site:

The experiment was conducted at the Agronomy field of Sher-e-Bangla Agricultural University, Dhaka, Bangladesh during June to November, 2008. Geographically, the experimental area is located at 23^0 77′ N latitude and 90^0 33′ E longitude at the elevation of above 18 m of the sea level. The soil of the experimental field belongs to the Shallow Red Brown Terrace Soils. Physical and chemical properties of initial soil is presented in Table 1.

Table 1. Physical and chemical characteristics of the initial soil (0-15 cm depth)

Characteristics	Value
Mechanical fractions:	
% Sand (0.2-0.02 mm)	22.26
% Silt (0.02-0.002 mm)	56.72
% Clay (<0.002 mm)	20.72
Textural class	Silt Loam
pH (1: 2.5 soil: water)	6.2
CEC (cmol kg^{-1})	17.9
Organic C (%)	0.686
Organic Matter (%)	1.187
Total N (%)	0.032
Exchangeable K (cmol kg^{-1})	0.12
Available P (mg kg^{-1})	19.85
Available S (mg kg^{-1})	14.40

2.2 Experimental treatments and design:

The experiment was carried out with 10 different treatments were as follows:

T_1= Control

T_2= Green manure @ 15 t ha^{-1}

T_3= Green manure @ 15 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK)
T_4= Poultry manure @ 4 t ha^{-1}
T_5= Poultry manure @ 4 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK)
T_6= Cowdung @ 12 t ha^{-1}
T_7= Cowdung @ 12 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK)
T_8= Vermicompost @ 8 t ha^{-1}
T_9= Vermicompost @ 8 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK)
T_{10}= $N_{80}P_{12}K_{72}S_{10}$ (100% NPK)

The experiment was laid out in a randomized completely block design (RCBD) with 3 replications. The unit plot size was 12 m^2.

Table 2. Chemical compositions of the organic manures used for the experiment (oven dry basis)

Organic manure	Nutrient content				
	C (%)	N (%)	P (%)	K (%)	C: N
Cowdung	36.0	1.48	0.29	0.75	24.0
Poultry manure	29.0	2.19	1.98	0.81	8.0
Sesbania	46.0	2.95	0.26	1.56	15.0
Vermicompost	11.5	1.66	1.25	0.25	9.6

2.3 Crop establishment and application of treatments:

The experiment was carried out with rice variety 'BRRI dhan40'. A common procedure was followed in raising of seedlings in seed bed. Seedlings of 25 days old were uprooted from the nursery beds carefully. Seedlings were transplanted according to the treatments in the well-puddled experimental plots. Spacings were given as 20 cm × 15 cm. Organic manures were applied before land preparation as per treatments. The nutrient compositions of the manures used in this experiment are presented in Table 2. Thirty-days-old *Sesbania rostrata* green plants were incorporated as green manure. Others manures were used as decomposed. Chemical fertilizers were applied as per treatments during final land preparation. Urea, triple superphosphate, muriate of potash and gypsum were applied as sources of N, P, K and S. In case of N one-third urea was applied as basal dose at the time of final land preparation and incorporated well into the soil. Rest two-third of urea was applied in two equal splits at 30 and 60 days after transplanting (DAT). All intercultural operations were done carefully. The first weeding was done at 15 days after transplanting (DAT) followed by second and third weeding was done at 15 days interval after first and second weeding. Irrigation was applied by alternate wetting and drying from transplanting to maximum tillering stage. From panicle initiation (PI) to hard dough stage, a thin layer of water (2-3 cm) was kept on the plots. Water was removed from the plots during ripening stage. The crop of each plot was harvested separately on different dates when 90% of the grains become golden yellow in colour.

2.4 Data collection and analysis:

The first plant height was measured at 30 DAT and continued up to harvesting period with 20 days interval. Plant height was determined by measuring the distance from the soil surface to the tip of the leaf before heading and to the tip of the flag leaf after heading. The collected data were finally averaged. Number of tillers hill^{-1} was counted at 20 days interval starting from 30 DAT and continued up to harvest from 10 pre-selected hills and finally averaged them to have tiller number hill^{-1}. Ten hills from each plot were uprooted and oven dried at 85 ± 5^{0}C for 72 hours from which the dry matter weight was recorded at 20 days interval up to 90 days.

The dry matter accumulation of the crop per unit land area in unit of time is referred to crop growth rate (CGR), expressed as g m^{-2} d^{-1}. The mean CGR values for the crop during the sampling intervals were computed using the formula of Brown, (1984).

$$CGR = \frac{W_2 - W_1}{SA(t_2 - t_1)} \text{ g m}^{-2} \text{ d}^{-1}$$

Where,

SA= Ground area occupied by the plant at each sampling. W_1 and W_2 are the total dry matter production in grams at the time t_1 and t_2, respectively.

The relative growth rate at which a plant incorporates new material into its sink is measured by Relative Growth Rate of dry matter accumulation and is expressed in g g^{-1}d^{-1}. Relative growth rate was worked out by following the formula of Radford (1967).

$$RGR = \frac{L_n W_2 - L_n W_1}{T_2 - T_1} \quad \text{g g}^{-1} \text{ d}^{-1}$$

Where, W_1 and W_2 is initial and final dry matter weight at the time T_1 and T_2, respectively. L_n refers to Natural Logarithm.

The grain weights for each plot were recorded after proper sun drying and then converted into t ha^{-1}. The grain yield was adjusted at 12% moisture level.

The data was analyzed using CoStat software (CoHort, 2008) programme. The mean differences among the treatments were compared by multiple comparison tests using Duncan's Multiple Range Test

(DMRT). Regression analysis was done by using SPSS software package (SPSS, 2009).

3. Results and Discussion

3.1 Plant height

From the study it was observed that plant height of rice cv. BRRI dhan40 was significantly affected by the manure treatments regardless the crop duration (Fig. 1). The increase rate of plant height was more between 50 DAT and 70 DAT as it was the maximum vegetative stage in rice plant. At 110 DAT the plants were about to maturity and hence the plant height was increased further very slightly. Regarding the treatments T_{10} (full dose of NPK) produced the tallest plants in each stage of growth. At maximum vegetative stage 50% NPK and *Sesbania* green manure incorporation (T_3) also gave significantly taller plants than others manures. Significant roles of *Sesbania* green manures to plant height might be due to its high N content which influenced the vegetative growth at the earlier stage of plant growth. Any organic manure applied in combination with 50% NPK gave identical results in this study (Fig. 1). In case of rice vegetative growth is greatly mediated by N fertilizers. In this study treatment T_{10} produced the tallest plant because it provided sufficient N available for plant. The amount of N released by *Sesbania* with 50% NPK was also sufficient to supply the required amount of N. However, control treatment (without fertilizer) produced the shortest plants in this experiment. The variation in plant height due to nutrient sources was considered to be due to variation in the availability of major nutrients. Chemical fertilizer offers nutrients which are readily soluble in soil solution and thereby instantaneously available to plants. Nutrient availability from organic sources is due to microbial action and improved physical condition of soil. These results were supported by Sarker et al. (2004).

3.2 Number of tillers

Tillering is an important trait for grain production and is thereby an important aspect of rice growth improvement. Production of tillers in rice plant was also influenced by different fertilizer combination at all the growth stages (Fig. 2).

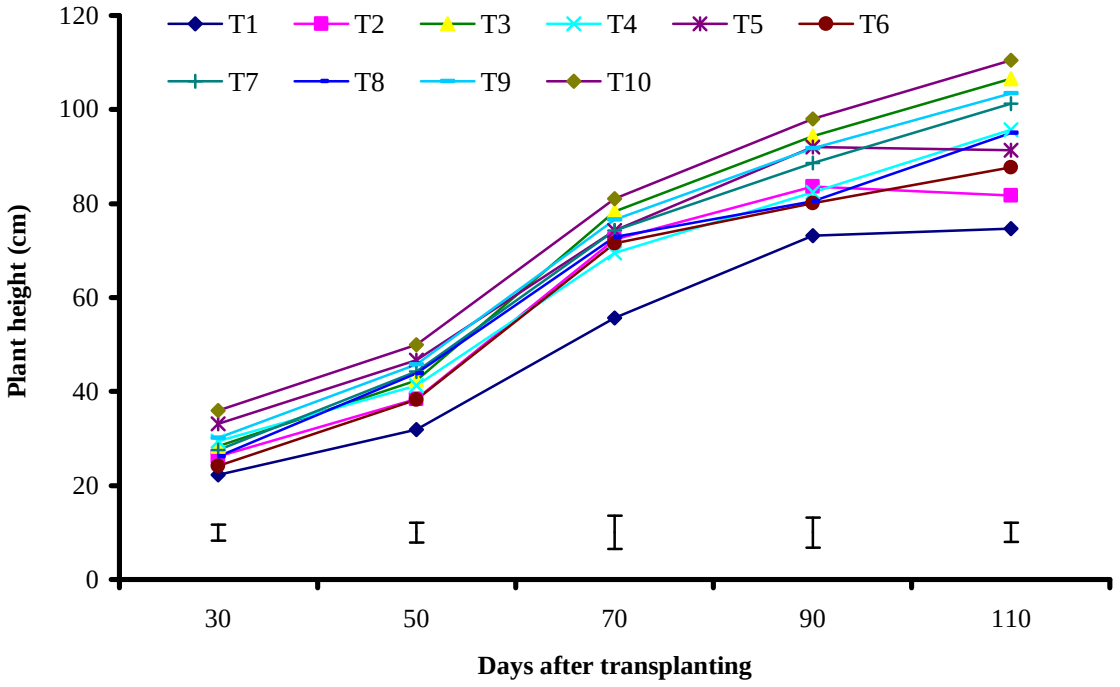

Figure 1. Plant height of transplanted rice cv. BRRI dhan40 at different days after transplanting as affected by different manuring treatments (vertical error bars represents the LSD values at $P < 0.05$)

[T_1= Control; T_2= Green manure @ 15 t ha^{-1}; T_3= Green manure @ 15 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_4= Poultry manure @ 4 t ha^{-1}; T_5= Poultry manure @ 4 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_6= Cowdung @ 12 t ha^{-1}; T_7= Cowdung @ 12 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_8= Vermicompost @ 8 t ha^{-1}; T_9= Vermicompost @ 8 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK) and T_{10}= $N_{80}P_{12}K_{72}S_{10}$ (100% NPK)]

At initial sages tiller number was not remarkably influenced by the treatments because of the slower activity of nutrients. After 30 DAT tiller numbers were linearly increased up to 70 DAT. But after the counting from 90 DAT tiller number was found decreased. It was due to the tiller mortality and the senescence of plants. In the present study up to 50 DAT (just before maximum tillering stage) the highest number of tillers was produced by the treatment T_{10} (100% NPK) but at 70 DAT and 90 DAT T_5 (Poultry manure @ 4 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$) produced the highest number of tillers per hill which was statistically identical with T_{10}. Just before harvest (110 DAT) maximum number of tillers (13.4 per hill) was produced with T_{10} which was followed by T_5 and T_9. Tiller productions with these treatments were 90.14%, 80.28% and 70.42% higher than control (T_1) treatment. In case of control treatment there was deficiency of N and other essential nutrients which was required for tiller production while the other treatments supplied it which rendered the higher number of tillers. Similar findings were reported by Tanaka (1968). The productivity of rice plant is greatly dependent on the number of productive tiller (tillers which bears panicle) rather than the total tiller numbers. Hence we observed the maximum number of effective tillers (10.4 per hill) with T_5 which was similar to T_{10} and T9. However, application of cowdung with 50% NPK (T_7) also gave higher number of effective tillers than any organic manures alone (Table 3). The number non-effective tillers were also lower with proper fertilization. From this study it was observed that excess application of inorganic fertilizers is not necessary to produce effective tillers if we can supplement it with organic manures. However, organic sources offer more balanced nutrition to the plants, especially micro nutrients which has caused better affectivity of tiller in plants grown with poultry manure and vermicompost (Miller, 2007). This result was also supported by Rakshit et al. (2008), Ayoub (1999) and Uddin et al. (2002).

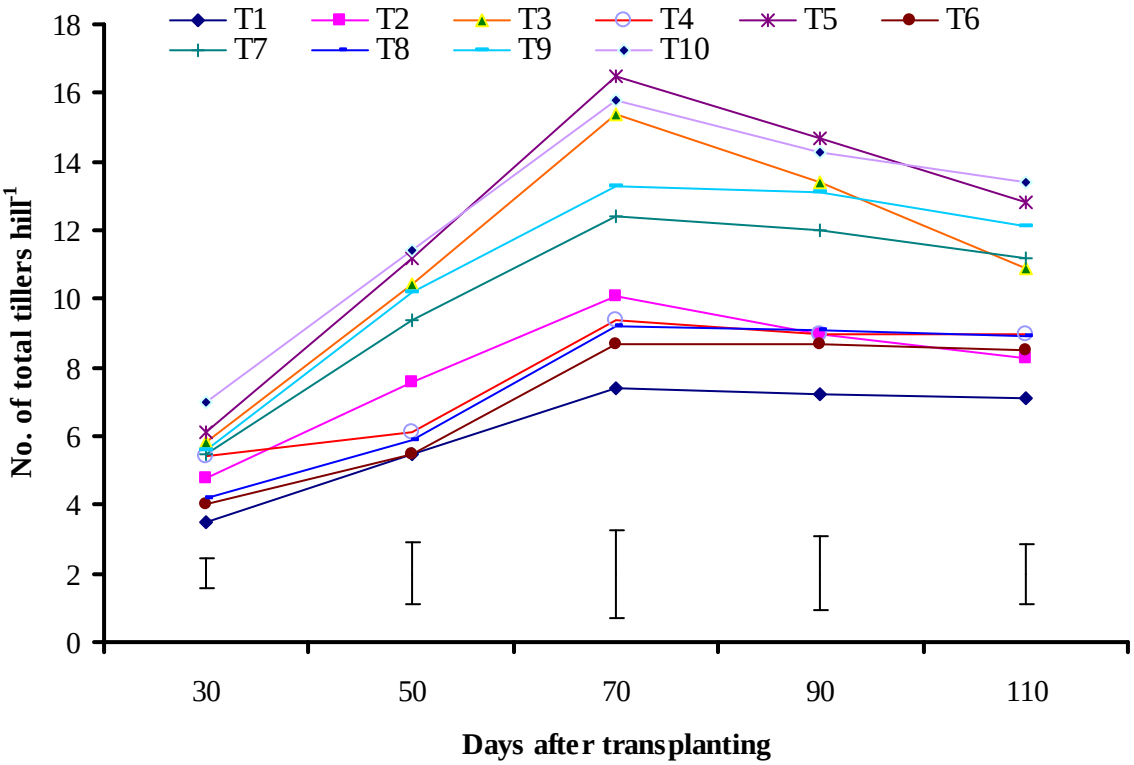

Figure 2. No. of total tillers hill^{-1} of transplanted rice cv. BRRI dhan40 at different days after transplanting as affected by different manuring treatments (vertical error bars represents the LSD values at $P < 0.05$)

[T_1= Control; T_2= Green manure @ 15 t ha^{-1}; T_3= Green manure @ 15 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_4= Poultry manure @ 4 t ha^{-1}; T_5= Poultry manure @ 4 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_6= Cowdung @ 12 t ha^{-1}; T_7= Cowdung @ 12 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_8= Vermicompost @ 8 t ha^{-1}; T_9= Vermicompost @ 8 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK) and T_{10}= $N_{80}P_{12}K_{72}S_{10}$ (100% NPK)]

Table 3. No. of total tillers hill^{-1} of transplanted rice cv. BRRI dhan40 at different days after transplanting as affected by different manuring treatments

Treatments	Effective tillers hill^{-1}	Non-effective tillers hill^{-1}
T_1	4.2	2.9
T_2	5.6	2.7
T_3	7.1	3.8
T_4	6.3	2.7
T_5	10.4	2.4
T_6	5.8	2.7
T_7	8.3	2.9
T_8	5.3	3.6
T_9	10.1	2
T_{10}	10.3	3.1
$LSD_{0.05}$	1.1	0.88

[T_1= Control; T_2= Green manure @ 15 t ha^{-1}; T_3= Green manure @ 15 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_4= Poultry manure @ 4 t ha^{-1}; T_5= Poultry manure @ 4 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_6= Cowdung @ 12 t ha^{-1}; T_7= Cowdung @ 12 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_8= Vermicompost @ 8 t ha^{-1}; T_9= Vermicompost @ 8 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK) and T_{10}= $N_{80}P_{12}K_{72}S_{10}$ (100% NPK)]

3.3 Total dry weight:

Dry matter production is the ultimate goal of application of any inputs in crop because it is directly related to the yield. The data presented in Fig. 3 revealed a statistically significant increase due to different manures combination (Fig. 3). At initial stages the differences of dry weight of plant was not influenced greatly. However, the treatments T_5 produced the highest dry weight in rice plant compared to other treatments at any growth stages except 30 DAT. At 30 DAT the basal application of inorganic fertilizer with T_{10} made the rapid availability of NPK for plant which rendered the maximum dry matter production at initial stages. The rapid increase of dry matter was observed between 50 DAT and 70 DAT (Fig. 3). It was due to the maximum growth and tillering of plant. After 70 DAT although tillers mortality and senescence occurred but reproductive parts contributed a considerable amount of dry matter in plant. Application of poultry manure combined with half of NPK enhances the nutrient availability and suitable soil condition for proper plant growth by reducing the losses of nutrient and hence produced the maximum dry weight. The production of maximum dry matter with proper manuring might be accounted for the luxuriant growth of plant as well as higher number of tillers plant^{-1} (Rahman et al., 2007). Total dry matter production increased due to nitrogen application at active tillering stage and panicle initiation stage. This result was also supported by Zhang et al. (2009).

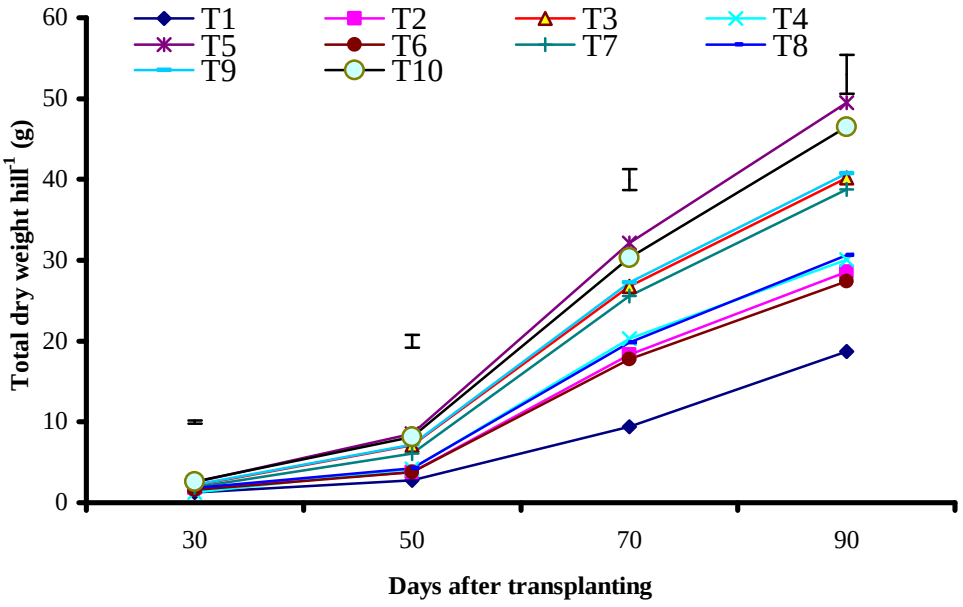

Figure 3. Dry weight of transplanted rice cv. BRRI dhan40 at different days after transplanting as affected by different manuring treatments (vertical error bars represents the LSD values at $P < 0.05$)

[T_1= Control; T_2= Green manure @ 15 t ha^{-1}; T_3= Green manure @ 15 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_4= Poultry manure @ 4 t ha^{-1}; T_5= Poultry manure @ 4 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_6= Cowdung @ 12 t ha^{-1}; T_7= Cowdung @ 12 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_8= Vermicompost @ 8 t ha^{-1}; T_9= Vermicompost @ 8 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK) and T_{10}= $N_{80}P_{12}K_{72}S_{10}$ (100% NPK)]

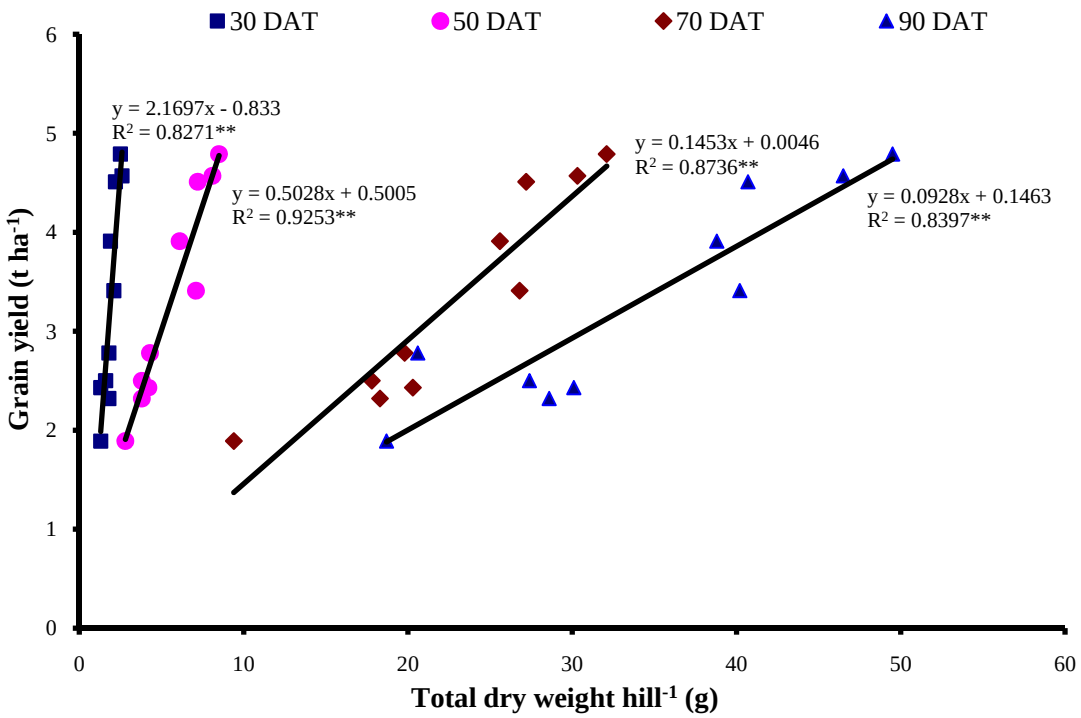

Figure 4. Relationship between dry matter production at different days after transplanting and grain yield of transplanted rice cv. BRRI dhan40 as affected by different manuring treatments (** indicates significant at $P < 0.01$)

From regression analysis we observed the contribution of the dry matter accumulation to the yield and found a very significant relationship among them (Fig. 4). Ibeawuchi et al. (2008) also showed the positive relationship between dry matter accumulation and yield in maize.

3.4 Growth rate

Crop growth rate (CGR) as well as relative growth rate (RGR) of BRRI dhan40 was significantly affected by manuring at different stages (Fig. 5 and 6). Growth rate was lowest during 30-50 DAT while the maximum growth occurred at 30-70 DAT. After maximum vegetative stage (70-90 DAT) the growth rate decreased. The treatments T_5 enhanced the highest CGR and RGR in the present study. It might be due to maximum tillering and vegetative growth facilitated by proper nutrient supply. The higher growth rate achieved by using poultry manure and NPK fertilizer treated plants which would be associated with the positive effect of nitrogen, phosphorus and potassium. Singh et al. (2003) reported that crop growth rate, averaged across treatments, was highest at 45-60 days after transplanting of rice and significantly influenced by NPK fertilizers.

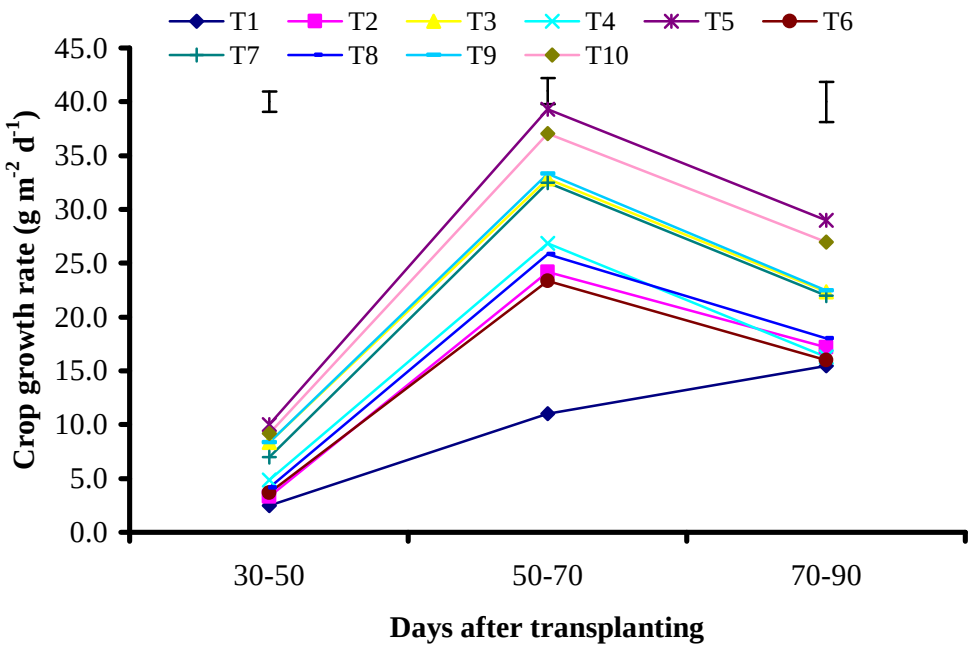

Figure 5. Crop growth rate (CGR) of transplanted rice cv. BRRI dhan40 at different days after transplanting as affected by different manuring treatments (vertical error bars represents the LSD values at $P < 0.05$)

[T_1= Control; T_2= Green manure @ 15 t ha^{-1}; T_3= Green manure @ 15 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_4= Poultry manure @ 4 t ha^{-1}; T_5= Poultry manure @ 4 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_6= Cowdung @ 12 t ha^{-1}; T_7= Cowdung @ 12 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_8= Vermicompost @ 8 t ha^{-1}; T_9= Vermicompost @ 8 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK) and T_{10}= $N_{80}P_{12}K_{72}S_{10}$ (100% NPK)]

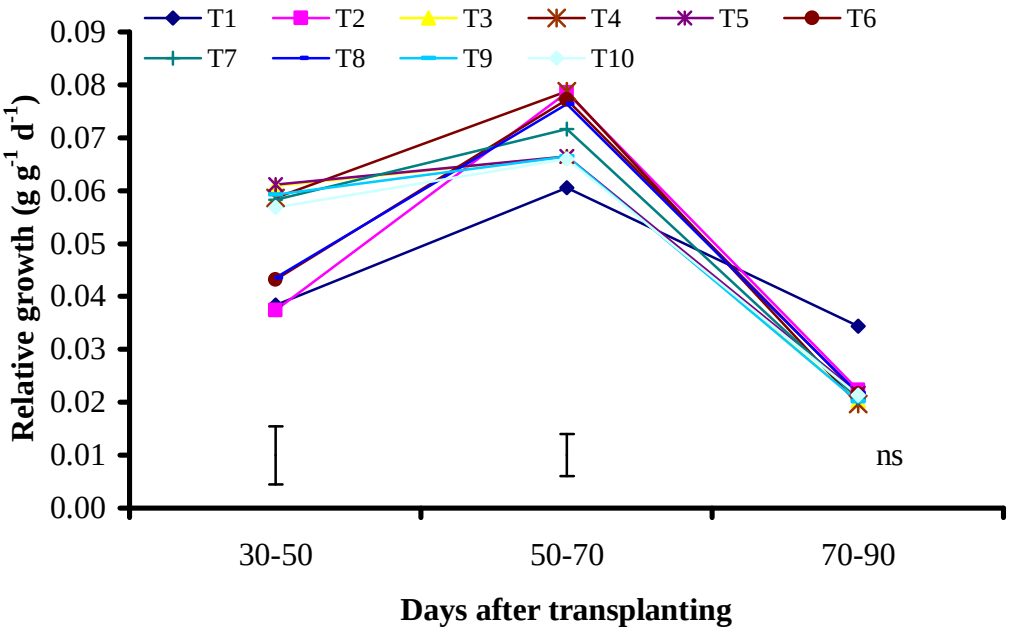

Figure 6. Relative growth rate (RGR) of transplanted rice cv. BRRI dhan40 at different days after transplanting as affected by different manuring treatments (vertical error bars represents the LSD values at $P < 0.05$; ns=non-significant)

[T_1= Control; T_2= Green manure @ 15 t ha^{-1}; T_3= Green manure @ 15 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_4= Poultry manure @ 4 t ha^{-1}; T_5= Poultry manure @ 4 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_6= Cowdung @ 12 t ha^{-1}; T_7= Cowdung @ 12 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK); T_8= Vermicompost @ 8 t ha^{-1}; T_9= Vermicompost @ 8 t ha^{-1} + $N_{40}P_6K_{36}S_{10}$ (50% NPK) and T_{10}= $N_{80}P_{12}K_{72}S_{10}$ (100% NPK)]

4. Conclusions

From the above discussion it is clear that organic manures have significant influence on the growth pattern and tillering of transplanted rice. Organic manure can be a better supplement of inorganic fertilizer to achieve better growth. From the present study it was observed that poultry manures combined with 50% of the recommended NPK fertilizers gave the best results compared to the other combinations. Organic manure alone could not enhance the better growth and dry matter production. For farmers practice, the full doses of commercial inorganic NPK fertilizers can be replaced with poultry manures combined with 50% NPK.

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References

[1]. Atiyeh, RM, Lee S, Edwards CA, Arancon NQ, Metzger JD. The influence of humic acids derived from earthworms- processed organic wastes on plant growth. Biores. Technol 2002; 84: 7-14.

[2]. Ayoub AT. Fertilizer and environment. Nutr. Cycl. Agroecosys. 1999; 55: 117-121.

[3]. Becker M, Ladha JK, Ali M Green manure technology: potential usage, limitations: a case study for lowland rice. Plant Soil. 1995; 174: 181-194.

[4]. Brown RH. In: Teasar, M. B (ed). Growth of the green plant. Physiological Basis of Crop Growth and Development. ASA. CSSA. Madison, Wisconsin, USA. 1984; pp.153-173.

[5]. BRRI (Bangladesh Rice Research Institute). Bangladesh Rice Knowledge Bank. http://riceknowledgebank.brri.org. Accessed on January, 2007.

[6]. Chaoui I, Zibiliske M, Ohno T. Effects of earthworm casts and compost on soil microbial activity and plant nutrient availability. Soil Biol. Biochem 2003; 35: 295-302.

[7]. CoHort. CoStat Statistical software version 6.400. CoHort Software, 798 Lighthouse Ave. PMB 320, Monterey, CA, 93940, USA; 2008.

[8]. Garg S, Bahla GS. Phosphorus availability to maize as influenced by organic manures and fertilizer P associated phosphatase activity in soils. Biores. Technol. 2008; 99:5773-5777.

[9]. Garrity DP, Flinn JC. Farm-level management systems for green manure crop in Asian rice environment. In: Green Manures in Rice Farming: Proc. Symp. the Role of Green Manures in Rice Farming Systems, IRRI, Manila, Philippines, May 25-29, 1987, pp. 111-129.

[10]. Ghosh BC, Bhat R. Environmental hazards of nitrogen loading in wetland rice fields. Environ. Pollut. 1998; 102: 123-126.

[11]. Guowei W, Wilson LT, McClung AM. Contribution of rice tillers to dry matter accumulation and yield. Agron J 1998; 90:317-323.

[12]. Hossain M, Singh VP. Fertilizer use in Asian agriculture: implications for sustaining food security and the environment. Nutr. Cycl. Agroecosys. 2000; 57:155-169.

[13]. Ibeawuchi II, Matthews N E, Ofor MO, Anyanwu CP and Onyia VN . Plant Spacing, Dry Matter Accumulation and Yield of Local and Improved Maize Cultivars. The J. of American Sci. 2008; 4(1): 11-19.

[14]. Janssen BH. Integrated nutrient management: the use of organic and mineral fertilizer. In: van Reuler, H., Prins, W.H. (Eds.), The Role of Plant Nutrients for Sustainable Food Crop Production in Sub-Saharan Africa. VKP, Ledschendam, The Netherlands 1993; pp. 89-105.

[15]. Miah, MMU. Prospects and problems of organic farming in Bangladesh. Paper presented at the workshop on Integrated Nutrient Management for Sustainable Agriculture. Soil Resource Dev. Inst., Dhaka, June 26-28, 1994.

[16]. Pagiola S. Environmental and Natural Resource Degradation in Intensive Agriculture in Bangladesh: Environment Economics Series Paper No. 15. The World Bank, Environ. Depart., Washington DC, USA. 1995.

[17]. Parthasarathi K, Ranganathan LS. Longevity of microbial and enzyme activities and their influence on NPK content in pressmud vermicasts. Europ. J. Soil Biol. 1999; 35: 107-113.

[18]. Pieters AJ. Green Manuring: Principles and Practice. Agrobios, Jodhpur. 2005; 356pp.

[19]. Prasad M. Biological yield and harvest index of rice. Oryza 1981; 18(1):31-34.

[20]. Radford PJ. Growth analysis formulae. Their use and abuse. Crop Sci. 1967; 7:171-175.

[21]. Rahman MH, Ali MH, Ali MM, Khatun MM. Effect of Different Level of Nitrogen on Growth and Yield of Transplant *Aman* Rice cv BRRI dhan32. Int. J. Sustain. Crop Prod 2007; 2(1): 28-34.

[22]. Rosegrant MW, Roumasset JA. Economic feasibility of green manure in rice-based cropping systems. In: Green Manure in Rice Farming: Proc. Symp. Sustainable Agriculture-The Role Green Manures Crops in Rice Farming Systems, IRRI, Manila, Philippines, May 25-29, 1987. 1988; pp. 11-27.

[23]. Shukla BD, Misra AK, Gupta R.K. Application of nitrogen in production and post-production systems of agriculture and its effect on environment in India. Environ. Pollut. 1998. 102: 115-122.

[24]. Singh RB. Environmental consequences of agricultural development: a case study for the Green Revolution State of Haryana, India. Agr. Ecosys. Environ. 2000; 82: 97-103.

[25]. Singh VK, Dwivedi BS, Shukla AK, Yadav RL. Effects of nitrogen and phosphorus fertilization on the growth and yield of rice (*Oryza sativa*) and wheat (*Triticum aestivum*) as influenced by the inclusion of forage cowpea (*Vigna unguiculata*) in rice-wheat system. Indian J. of Agril. Sci. 2003; 73 (9): 482-489.

[26]. SPSS (Statistical Package for Social Sciences) SPSS (Computer based software package) v. 17. SPSS Inc., New York. 2009

[27]. Tanaka A. Historical changes in plant types of rice varieties in Hokkaido. J. Sci. Soil manure 1968; 39: 526-534.

[28]. Zebarth BJ, Neilsen GH, Hogue E, Neilsen D. Influence of organic waste amendments on selected soil physical and chemical properties. Can. J. Soil Sci. 1999; 79: 501-504.

[29]. Zhang L, Lin S, Bouman BAM, Xue C, Wei F, Tao H, Yang X, Wang H, Zhao D, Dittert K. Response of aerobic rice growth and grain yield to N fertilizer at two contrasting sites near Beijing, China. Field Crop Res 2009; 114(1): 45-53.

1/12/2010

Comparative Study of Seasonal Variation in Physico - Chemical Characteristics in Drinking Water Quality of Kanpur, India With Reference To 200 MLD Filteration Plant and Ground Water

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Abstract: In the present work various physico chemical parameters i.e. Turbidity, temperature, pH, total hardness , Iron, Chlorides, Dissolved Solids, Calcium, Sulphate, Nitrate, Fluoride, Chromium, total alkalinity are analyzed for various seasons; Summer, Monsoon, Autumn,Winter, Spring for the period (April-December-2008 and (January- March-2009) in the surface water, ground water and filtration plant treated water of Kanpur city. Significant variation of physico - chemical parameters of surface water were observed; various physico-chemical parameters for the water samples were within highest desirable limit (HDL) prescribed by WHO for drinking purposes for all seasons except for pH in summer, Total alkalinity and Fe contents in spring, autumn and winter; Total dissolved solids in winter, Turbidity in all seasons. The observations imply that Ganga water in monsoon is better than winter seasons, where as the ground water was found better in winter compared to that of summer season. The results suggest that the quality of surface water improved after treatment in filtration plant as compared to ground water.

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Keywords: Physico-chemical Parameters, Ganga water,Canal Ganga Water, Treated water, Ground water.

1. Introduction

Water is the principal need of life on earth, and is an essential component for all forms of lives, from micro-organism to man. The unplanned urbanization and industrialization (Singh et al., 2002)[1] has resulted in over use of environment (Petak, 1980)[2] in particular of water resource. A kind of crises situation has made getting clean water a serious problem. It is a known fact that when pure water is polluted its normal functioning and properties are affected. Ganges is a sacred river of India. The increased anthropogenic activities due to industrialization have contributed to decline in water quality of Ganges. Several works have been reported on water quality of river Ganges at Kanpur (Sinha et al., 2000[3a]; Pandey and Pandey, 1980[3b] and Tare et al., 2003[3c]) and other parts of country (Pahwa, and Mehrotra, 1966)[4]. The authors studied river Ganges from Kanpur city, west state UP, to Rajmahal city east state Jharkhand, covering total length of about 1090 kms. The maximum turbidity (1100-2170 ppm) was observed in monsoon and minimum (less than100 ppm) during January to June. The minimum value Ph of the river water ranged between 7.45 (minimum) observed during June to August and 8.30 (maximum) during January to May. A comprehensive study of physico-chemical properties of Ganga water at Buxar (Unnao) UP (Sinha,1986)[5], Narora and Kannauj,U.P (Khan et. al ,1984)[6], in and around Haridwar (Kaur and Joshi, 2003)[7] has also been reported. The seasonal analysis of Kanpur (Zafer and Sultana, 2007)[8] water showed that extent of pollution varied in different seasons. The steep growth in population due to rapid urbanization and industrial development of Kanpur city has increased the demand of water manifold. At present drinking water demand of the city is 650 MLD which is partially met by Kanpur Jalsansthan Benajhawar having the capacity of 200 MLD filtration plant. In the present work we report the drinking water quality of the filtration plant and other sources.

2. Study Area

Kanpur Jalsansthan Benajhawar's filteration plant gets 200 MLD water from Bhairav Ghat (Ganga), Panki (lower canal Ganga). The water samples were collected from the following sites; Ganga (GW), lower canal Ganga (CGW) coming for treatment to filtration plant (TW), close by hand

pumps, and from points close to the point where water come out after treatment from filtration plant for the period during April-December, 2008 and January – March, 2009.

3. Sampling

In the present work we report quality of water taken from 200 MLD filtration plant site and ground water resources. The sites are GW, CGW, TW and ground water sampling site is named as PSP i.e. postal station pumps (hand pumps). These PSP samples are collected from six different zones of Kanpur during Monsoon, Autumn, Winter, Spring and Summer seasons for the period the years April, 2008 - March, 2009.

4. Methods and Materials

The laboratory analysis of samples was done using standard methods (APHA, 1998)[9]. Analytical method used for determination of different physico-chemical parameters for surface waters of Ganga river, CGW and TW at 200MLD and ground water at PSP Kanpur are listed in Table-1.[10] The water samples were collected from different sites in plastic bottles and transported to the laboratory in an icebox jars to avoid unpredictable changes in different physico- chemical parameters. The selected parameters including Water Temperature (WT), pH, Turbidity, Total alkalinity (TA), Total dissolved solids (TDS), Total hardness (TH), Ca^{+2}, Mg^{+2}, Cl^{-}, NO_3^{-}, SO_4^{-2}, Cr^{+6}, Fe, F^{-} were analyzed at regular intervals. The observed values of various physico-chemical parameters of water samples were compared with standard values recommended by World Health Organization(WHO)[11] for drinking purposes.

Table-1 : Water quality parameters and analytical methods used in analysis of water samples.

Parameter	Analytical method
WT (°C)	Mercury thermometer
Tu (NTU)	Turbidimeter[10b]
pH	pH-meter
TA (CaCO3 mg/l)	Titrimetric
Cl^{-} (mg/l)	Argentometric Method [10a](Silver nitrate method)
NO_3^{-} (mg/l)	Colorimetric method
TH (CaCO3 mg/l)	EDTA Titrimetric Method[10c]
Ca^{+2}(mg/l)	EDTA Titrimetric Method[10c]
Mg^{+2}(mg/l)	EDTA Titrimetric Method[10c]
TDS (mg/[)	Gravimetric method
SO_4^{-2} (mg/l)	Aplab turbidity meter[10b]
Cr (mg/l)	Atomic absorption spectrophotometer
Fe (mg/l)	Atomic absorption spectrophotometer
F^{-} (mg/l)	Colorimetric method
* WT: temperature; Tu: turbidity; TA: alkalinity; Cl: chloride;NO_3^{-}: nitrate; TH: total hardness; TDS :Total dissolved Solids,	

The analysis period was divided in to 5 seasons i.e. monsoon (July and August), autumn (September and October), winter (November to January) spring (February and March) and summer (April to June). Experimental values of various physico-chemical parameters in different seasons are reported in Table- 2, 3, 4, 5 and 6 respectively. Data provided the extent of pollution removed by treatment of water in filtration plant and proved to be an indicator to evaluate the suitability for drinking the treated samples.

Tables- 2: Physico-chemical parameters in Monsoon season.

	GW	CGW	TW	PSP
WT (°C)	30	31	29	28
pH	8.4	7.8	7.7	8.6
Turbidity	470	75	3.0	4.0
TA	140	96	96	224
TDS	256	181	202	442
TH	108	80	94	140
Ca^{+2}	28	25.6	30.4	32.8
Mg^{+2}	9.234	3.888	4.374	14.094
Cl^{-}	8	5	12	78
NO_3^{-}	1.772	1.772	Nil	0.886
SO_4^{-2}	91	45	36	44
Cr	Nil	Nil	Nil	Nil
Fe	0.8	0.4	0.3	0.2
F^{-}	Nil	Nil	Nil	Nil

Tables-3: Physico-chemical parameters in Autumn season.

	GW	CGW	TW	PSP
WT (°C)	29	30	29	27
pH	8.5	7.9	8.2	8.5
Turbidity	100	8.0	1.0	1.0
TA	216	118	180	340
TDS	440	250	380	550
TH	190	116	178	70
Ca^{+2}	45.6	32	40.8	20
Mg^{+2}	18.468	8.748	18.468	4.86
Cl^{-}	14	8	20	135
NO_3^{-}	Nil	Nil	Nil	Nil
SO_4^{-2}	50	43	43	89
Cr	Nil	Nil	Nil	Nil
Fe	0.8	0.4	0.6	0.2
F^{-}	0.2	Nil	Nil	Nil

Table- 4: Physico-chemical parameters in Winter season.

	GW	CGW	TW	PSP
WT (°C)	16	18	14	12
pH	8.5	7.8	7.7	8.0
Turbidity	15	12	1.0	2.0
TA	260	134	180	140
TDS	540	290	430	330
TH	246	146	216	160
Ca^{+2}	80	32	44	24
Mg^{+2}	11.178	16.038	25.758	29.3
Cl^{-}	26	9.0	30	30
NO_3^{-}	3.544	Nil	1.772	Nil
SO_4^{-2}	47	38	55	48
Cr	Nil	Nil	Nil	Nil
Fe	0.4	0.4	0.3	Nil
F^{-}	0.4	0.4	Nil	Nil

Table- 5: Physico- chemical parameters in Spring season.

	GW	CGW	TW	PSP
WT (°C)	20	22	20	19
pH	8.5	7.8	7.9	8.5
Turbidity	22	15	1.0	2.0
TA	232	120	196	364
TDS	485	250	440	610
TH	216	116	206	120
Ca^{+2}	49.6	27.2	33.6	23.2
Mg^{+2}	22.356	11.664	29.646	15.066
Cl^{-}	25	10	32	118
NO_3^{-}	0.443	Nil	Nil	1.772
SO_4^{-2}	58	11	38	40
Cr	Nil	Nil	Nil	Nil
Fe	0.6	0.2	0.2	0.2
F^{-}	0.4	0.2	Nil	Nil

Table- 6: Physico - chemical parameters in summer season

	GW	CGW	TW	PSP
WT (°C)	30	32	31	28
pH	8.9	8.0	7.8	8.6
Turbidity	20	9.0	1.0	2.0
TA	104	114	132	284
TDS	400	260	352	684
TH	170	134	174	114
Ca^{+2}	24	28	28.8	32
Mg^{+2}	26.73	15.552	24.786	8.262
Cl^{-}	30	11	45	280
NO_3^{-}	1.772	0.443	Nil	Nil
SO_4^{-2}	50	36	56	61
Cr	Nil	Nil	Nil	Nil
Fe	0.4	0.4	0.2	0.6
F^{-}	0.4	0.4	Nil	Nil

5. Result and Discussion

The water quality analysis of different raw water, TW, Ground water samples has been carried out for fourteen physico-chemical parameters i.e; Temperature, pH, turbidity, TA, TDS, TH, Ca^{+2}, Mg^{+2}, Cl^{-}, NO_3^{-}, SO_4^{-2}, Cr, Fe and F^{-}.

Temperature:

In present study the temperature varied from 14 °C to 32 °C. The variation in the water temperature may be due to different timing of collection and influence of season (Jayraman et al.,2003)[12] In 2002 and 2003 (Zafer and Sultana), monsoon temperature was 25.8 °C, 26.1 °C respectively, however, for same seasons in 2008 - 09 temperature is found to be 30 °C. In spring and summer of years 2003 and 2004 (Zafer and Sultana, 2008) temperature was found to be 20.6 °C, 33.8 °C : 20.7 °C, 34 °C respectively, we found temperature of 20 °C and 30 °C for the same season. It indicates that over passage of time from 2002 to 2008 -09 the monsoon season temperature has increased significantly.

Hydrogen Ion Concentration (pH):

The pH of water is important because many biological activities can occur only within a narrow range. Thus, any variation beyond an acceptable range could be fatal to a particular organism. In 2002 and 2003, Zafer and Sultana, 2007 reported pH of 7.6 and 7.55 respectively of GW sample for monsoon season. In present study the same is found to be 8.4. However, for spring and summer seasons it is 8.5, 8.9 respectively compared to 7.8,7.7; 8.0,7.7 respectively for same period of 2003 and 2004 (Zafer and Sultana) [8]. It indicates that with passage of time the pH of water for a specific season has increased.

Present study also shows pH is alkaline in most of samples and it ranges from 7.7 to 8.9. pH value of different studied samples in different season is with in HDL prescribed by WHO which is 6.5 to 8.5 except during summer the pH of surface water of GW exceeded HDL prescribed by WHO .

Total Alkalinity:

We measured TA of 140 mg/l in surface GW for monsoon season compared to 102.0 and 75 mg/l respectively for same period of 2002 and 2003 (Zafer and Sultana-8, 2007) . However, for five seasons TA varied from 104 mg/l- 260 mg/l. The variation of TA is in accordance with fluctuation in pollution load (Parashar et al., 2006)[13] Total alkalinity for all seasons for treated water and GW is within permissible limit of WHO which is 200 mg/l except in winter season for GW samples TA is greater than HDL prescribed by WHO. TA for GW is lowest during summer and highest during winter

Total Hardness:

Hardness is an important parameter in decreasing the toxic effect of poisonous element. The measured value of TH for monsoon season increased to 108 mg/l compared to 81.70, 97.45 mg/l respectively of GW samples in 2002 and 2003 (Zafer and Sultana, 2007). The TH of surface water in GW and CGW and ground water samples at PSP and treated water was found to be in range of 80 mg/l - 246 mg/l, within prescribed limit of 300 mg/l by WHO .

Turbidity:

The turbidity is a major problem in the river water of all states. The turbidity value was found higher during monsoon season. In 2002 and 2003 in monsoon season turbidity was 66.2, and 56.4 NTU. Present study results show that turbidity of GW sample in monsoon season has increased tremendously to 470 NTU. Values of turbidity for TW samples and a ground water samples at PSP for all seasons were found to be lower than HDL prescribed by WHO, but surface water samples in

GW and CGW show higher values than HDL. During festival season immersion of idols in urban water bodies have grown in number and size over the years and therefore urban water bodies are facing an increasing nutrient load (Vyas et al, 2006)[14]. This could be the reason of high value of turbidity shown by surface GW samples during festival season in autumn (September and October) in our study.

Total Dissolved Solids:

TDS indicate the total amount of inorganic chemicals in solution. TDS of GW, CGW and PSP showed seasonal fluctuation for the study period. TDS values of ground water samples at PSP in spring, autumn and summer are higher than HDL 500 mg/l prescribed by WHO. Samples of surface water in GW in winter season showed values of TDS within Maximum desirable limit (MDL) prescribed by WHO of 600 mg/l.

Chloride:

Chloride concentration in water indicates presence of organic waste particularly of animal origin (Thresh et. al, 1949)[15]. Increase in chloride concentration on discharge of municipal and industrial waste has been reported (Ownby and Kee, 1967)[16]. In river Ganga at Varanasi (Chaudhary and Ojha, 1985)[17] it was found that chloride value ranged from 5.9 to 7.9 mg/l. However, in Allahabad region the rivers do not show chloride beyond 42.0 mg/l. In monsoon of 2002 and 2003 Cl^- contents were 9.75 and 9.9 mg/l respectively (Zafer and Sultana, 2007). In our present study maximum Cl^- contents are found to be 280 mg/l in summer season in PSP water samples. In the present study chloride contents of GW samples in monsoon season are found to be 8 mg/l. In TW water samples for monsoon, spring, autumn winter and summer seasons the Cl^- contents are 12, 20, 30, 32 and 45 mg/l. respectively. This indicates there is no appreciable seasonal variation in chloride concentration of TW although it is slightly higher in summer (Table- 6). Least and maximum Cl^- contents in sample ground water at PSP are present in winter and summer seasons respectively

For surface water samples in GW, Cl^- concentration increases from monsoon, autumn, spring, winter to summer season in the range of 8-30 mg/l. The high Chloride content in drinking water may indicate possible pollution from human sewage, animal manure or industrial waste. Present study results show that in summer ground water samples at PSP, the chloride concentration exceed HDL prescribed by WHO which is 250 mg/l.This High chloride contents in PSP water makes it taste salty and also promote pipe corrosion .

Nitrate:

In present study in PSP samples NO_3^- levels are below 1mg/l in monsoon, autumn, winter and summer season, but in spring season it is 1.772 mg/l. In GW samples in autumn and spring seasons it is less than 1mg/l, but in monsoon, winter and summer it is more than 1mg/l and its highest value is in winter season of 3.544 mg/l. In CGW samples it is less than 1mgl/l in all seasons except in monsoon. In TW, NO_3^- contents are nil in all seasons except for winter season. This shows that TW, surface water and PSP samples have nitrate contents less than 50 mg/l prescribed HDL of WHO for safe drinking water.

Fluoride:

Fluoride contents are nil in PSP and TW samples in all seasons. In GW and CGW samples fluoride contents ranged from 0 to 0.4 mg/l, less than 1 mg/l prescribed HDL of WHO for good health.

Sulfate:

Value of SO_4^{-2} contents for surface water in GW, CGW , ground water at PSP and for TW is far below the maximum allowable concentration for sulfate ions in drinking water prescribed by WHO which is 250 mg/l.

Iron:

Water containing iron does not show deleterious effect on human health, its presence in drinking water is not desirable for various reasons. Excessive iron content makes the water turbid, discolored and imparts an astringent taste to water. Present study shows that in monsoon, spring and autumn seasons iron contents of CGW is greater than GW and values are greater than HDL prescribed by limit of WHO which is 0.3 mg/l. Fe contents of TW samples in autumn season and ground water samples at PSP in summer are higher than prescribed limit of WHO. It indicates that filteration plant is not effective for reducing iron contents of surface water during autumn season

Ca^{+2} and Mg^{+2} contents:

Ca^{+2} and Mg^{+2} are important contributors to water hardness. For all season surface water at GW, CGW and ground water at PSP and for TW, contents of Ca^{+2} is greater than Mg^{+2} except for summer season surface water samples at GW contents of Mg^{+2} is greater than Ca^{+2}. The values of Ca^{+2} and Mg^{+2} obtained from surface water samples in GW, CGW and ground water samples at PSP and for TW for all seasons except winter are with in HDL

prescribed by WHO which is 75mg/l and 30mg/l respectively. But the Ca^{+2} contents in surface water of GW in winter was detected just above the drinking water permissible level of 75 mg/l.

6. Conclusion

Significant seasonal variation in the physico – chemical parameters of surface water of Kanpur city were observed during study period April – December, 2008 and January – March, 2009. With passage of time from 2002 to 2008-09 the values of some physico -chemical parameters like TH, turbidity, TA, pH of sample water for GW in monsoon season has increased considerably, yet within HDL prescribed WHO value except turbidity which is on higher side.

For all seasons the surface water samples in GW show higher values of pH, turbidity, TA, TDS, TH, Ca^{+2}, Mg^{+2}, Cl^{-}, NO_3^{-}, SO_4^{-2} than values of respective parameters for CGW and ground water samples at PSP and for TW. This quality deterioration in GW is due to various reasons like extent of pollution occurring due to urbanization and anthropogenic activities.

In present study pH, Turbidity, TH and Cl^{-} values for TW samples for all five seasons is less than or equal to ground water samples at PSP. In winter season surface water samples from CGW and ground water samples at PSP were free from all contamination. It indicates better quality of water at PSP in winter compared to summer season.

In GW samples for all seasons the values of eight parameters TH, Mg^{+2}, Cl^{-}, NO_3^{-}, SO_4^{-2}, and F^{-} ,Ca^{+2}, SO_4^{-2} were found to be within HDL prescribed WHO for drinking. After treatment of GW in filtration plant for all seasons the water quality is improved as for GW water samples pH in summer; TA and iron contents in spring and autumn ,winter season; TDS contents in winter season reduced to within HDL prescribed by WHO. It also indicates that GW in monsoon is better than winter season.

PSP ground water source was found contaminated with Cl^{-} in summer, TA in monsoon and summer, TDS in autumn, spring and summer season respectively as their values were found to be higher than HDL prescribed by WHO meant for drinking purposes.

Thus present study reveals that for all seasons the quality of surface water is highly improved and is free from all contamination after treatment in filtration plant and it is better than ground water at PSP in monsoon, autumn, spring and summer season. More studies are required at different sites of GW, CGW and PSP to compare the water quality of drinking water of Kanpur, India with reference to 200 MLD filteration plant and ground water at different time and places. To create increasing awareness among people that to maintain the Kanpur Ganga river water at its highest quality and purity filteration plant plays a crucial role, the present study may prove to be useful in achieving this goal.

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References:

[1] Singh S.P., Pathak D. and Singh R.Eco. Env. and Cons., 2002,8(3),:289-292.
[2] Petak W.J. Environ. Managem.1980, 4, 287-295.
[3](a) Sinha A.K. , Singh V. P. and Srivastava K., Physico –chemical studies on river Ganga and its tributaries in Uttar Pradesh –the present status. Pollution and Biomonitoring of Indian Rivers.(ed.) Dr. R.K. Trivedi.(Ed.), ABD publishers, Jaipur. 2000:1-29
(b) Pandey P.K and Pandey ,G.N. , J.Inst.Engr. India 1980,60,27-34
(c.)Tare,V.,Yadav,A.V.S and Bose,P. Water Research 2003,37 :67-77
[4] Pahwa, D.V. and Mehrotra, S.N. Proc. Nat. Acad. Sci., India,1966, Sec. 368 (2):157-189.
[5] Sinha, U.K., Ganga pollution and health hazard. Inter-India Publication, New Delhi. 1986
[6.] Khan A.A, Haque N, Instisar A.S and Narayanan K, : J. Freshwater Biol.1984, 6(4**),** 295-304
[7] Kaur S and Joshi B.D, -Him. J.Zool., 2003, 17(1*)* :45-55
[8] Zafer A and Sultana N., Seasonal Analysis in the Water Quality of River Ganga Disaster Ecology and Environment Arivnd Kumar (Ed.)Daya publishing House, India. 2007, 57- 62

[9]. Standard Methods for the Examination of Water and Waste Water, 20th Ed., APHA, AWWA, WEF. Washington DC, 1998
[10] Vogel A.I, A text book of Quantitative Inorganic Analysis Including Elementary Instrumental Analysis 4th Edition. The English Language Book Society and Langman.Co 1978 (a) 837 (b) 328-329 (c) 504-506
[11] World Health Organization, Guidelines for drinking water quality-I, Recommendations, 2nd Ed. Geneva WHO,1993 www.lenntech.com/drinking-water-standards.htm.
[12] Jayaraman P.R., Ganga Devi T. and Vasuena Naya T Poll. Res.,2003, 32(1),:89-100.
[13] Parashar C., Dixit S.. Srivastva R.. :Seasonal variations in Physico-chemical characteristics in upper lake of Bhopal, Asian .J. Exp. Sci., 2006, 20(2):297-302
[14] Vyas A., Mishra D. D. , Bajapai A., Dixit S. and Verma N. .Asian J. Exp. Sci.,2006, 20(2): 289-296
[15]. Thresh J.C., Beale J.F. and Suckling E.V. :The examination of water and water supplies B.W. Taylor, London 1949.
[16] .Ownby C.R. and Kee ,D.A.:Chloride inlake Eric.Proc. Cong. Grat Lakes 1967,:382-389
[17] Chaudhary U.K and Ojha C.S.P, Environmental impact of the river Ganga at Varanasi Proc. Env. Impact Assessment of Water Resources: Project Univ, Roorkee:1985: 760-771

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Response of vermi-compost on Growth and Yield of Pea (*pisum sativum* L.) cv. Arkel

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Abstract: The present investigation "Response of vermi-compost on growth and yield of pea (*Pisum sativum* L.) cv. Arkel'' comprised of seven treatments consisting of three level of vermi-compost, three level of vermi-compost with NPK, and one level of FYM + NPK. During the experimentation, growth character and yield characters were recorded. The germination of pea cv. Arkel, Seeds became faster with T_4 (vermi-compost-10 t/ha+NPK) treatment but there after the germination occurred at slower rate and days taken for completion of germination increased progressively. The T_4 (vermi-compost-10 t/ha + NPK) treatment exhibited the maximum nodule formation and yield. A comparative study of the present findings led to the conclusion that sowing of pea with the application of vermi-compost @ 10 t/ha and NPK @ 25:60:50 kg/ha was found most effective to best growth of pea crop under Srinagar valley condition of Garhwal region of Uttrakhand state. [Nature and Science 2010;8(4):18-21].ISSN:1545-0740).

Keywords: *Pisum sativum,* vermi-compost, FYM, NPK

1. Introduction

Pea (*Pisum sativum* L.) is one of the most important ancient vegetable and belongs to the family Leguminaceae. It ranks third or fourth in world wide production, amongst the grain legumes (Farrington, 1974). The pea generally called as legumes (pod bearing plants). Because, they are characterized by the pods with a single cavity ovary which splits along two margins when dry, legumes thus have ability to improve the soil fertility and structure.

The plants of pea are 35-60 cm tall. The plant is short leaved, herbaceous annual, glaucous which climbs by leaf let tendrils. The stem is slender, circular and weak. The root system is not strongly developed except taproot. Peas are grown particularly on all types of soil from light sandy to heavy clay. Frequent irrigation tends to increase vegetative growth at the expense of pod formation (Singh and Joshi, 1970). Pea have specific requirement in respect of seasonal changes in temperature during their growth cycle.

The "Vermi-compost" (The compost made from organic matter with the use of earth worm) has gained impetus in organic farming to boost agricultural production to its important multifarious features such as being rich in nutrients, vitamins, growth regulators, free from pathogen and containing immobilized micro flora.

2. Material and Methods

The present investigation entitled "Response of vermi-compost on growth and yield of pea (*Pisum sativum* L.) cv. Arkel'' was carried out during 2006-2007 at Horticultural Research Centre, Chauras Campus, HNB Garhwal University, Srinagar (Garhwal) to standardize the optimum dose of vermi-compost for obtaining best growth, flowering and production. The Horticultural Research Centre of HNB Garhwal University, Srinagar (Garhwal) is situated in the Alaknanda valley which lies between 78^0 47′ 30" E longitude and 30^0 13' 0" N latitude, right in the heart of Garhwal region at an elevation of 540 m above MSL, in the lesser Himalayan region. The minimum and maximum temperature, relative humidity and rainfall vary between 6.7 to 36.5^0 C, 55.45 to 95.23% and 3.05 to 324.28 mm, respectively.

The experiment comprised of seven treatments consisting of three level of vermi-compost, three level of vermi-compost with NPK, and one level of FYM + NPK was laid out in randomized block design with three replication. The treatments are as, 10 t/ha Vermi-compost (T_1), 15 t/ha Vermi-compost (T_2), 20 t/ha Vermi-compost (T_3), 10 t/ha Vermi-compost + 25kg/ha N +60kg/ha P +50kg/ha K (T_4), 15 t/ha Vermi-compost + 25kg/ha N + 60kg/ha P + 50kg/ha K (T_5), 20 t/ha Vermi-compost + 25kg/ha N + 60kg/ha P + 50kg/ha K (T_6), 20 t/ha FYM+25 Kg/ha N + 60kg/ha P + 50kg/ha K (T_7). Basal application of ½ dose of N in the form of urea, full dose of P in the form of single super phosphate (S.S.P.) and K in the form of murate of potash, and vermi-compost with broad cast method was done.

Rest half dose of N was applied 30 days after germination.

During the experimentation, Five plants under each treatment combination were randomly selected and tagged for recording the observation on growth and yield characters (whenever required).

Growth Characters

1. Days taken to germination
2. Number of nodules per plant
3. Fresh weight of nodules per plant
4. Dry weight of nodules per plant
5. Plant height

Yield Characters

1. Days taken to first flowering
2. Number of pods per plant
3. Number of grains per pod
4. Fresh weight of 100 grains
5. Yield per plant
6. Yield per plot
7. Yield per hectare

After sowing, each plot was regularly watched to record the number of days taken for full germination (about 75% germination was considered full germination). Five plants were randomly uprooted carefully along with soil from each treatment at flower bud initiation. The roots of plants were dipped in water for some time to facilitate the removal of soil from the roots without damage. The number of nodules present on the roots counted under each treatment and then averaged. The nodules of five plants under each treatment were carefully removed with the help of forceps. Weighted on the electronic top loading balance for getting their accurate fresh weighs and finally averaged. Freshly weighted nodules from five plants were put in oven for drying at 60^0C for 72 hrs. The nodules were again weighted on electronic top loading balance and averaged. Height of the selected plants were recorded from ground level to the tip of apical buds at last picking stage and then averaged to get mean heights.

Days taken for appearance of first flower from the date of sowing were recorded on randomly selected and tagged plants under each treatment. Then average days required for flowering were calculated. Number of pods from randomly selected plants under each treatment were counted after each picking and then summed up to get average. After each picking, ten pods were randomly collected from each treatment and grains inside per pod were counted. The average number of grains per pod was worked out. A composite sample of seeds was drawn from five tagged plants and then weight of 100 seeds were recorded for the purpose. After each picking green pods per plant, pods per bed were weighed under each treatment to work out cumulative total which was then converted into yield q/ha.

3. Results and Discussion

The germination of pea cv. arkel. Seeds became faster with T_4 (vermi-compost-10 t/ha+NPK) treatment but there after the germination occurred at slower rate and days taken for completion of germination increased progressively. The T_4 (vermi-compost-10 t/ha) treatment exhibited the maximum nodule formation. The performance of pea with respect to germination and nodulation was influenced by the temperature, rainfall, humidity etc. Lopes *et al*.(1996) reported that an increase in levels of vermi-compost upto 10 t/ha significantly increased nodulation and dry matter yield of cowpea over rest of the treatments.

Here, under Srinagar valley condition, the vermi-compost-10 t/ha showed the best growth of pea cv. Arkel. These findings were agreed with Lopes *et. al.* Plant height of pea was significantly increased by different Vermi-compost levels over control. Under the present investigation, the maximum, significant plant height was recorded under T_4 (vermi-compost-10 t/ha+NPK) treatment. The maximum growth of pea with the application of Vermi-compost 10 t/ha. Reddy *et. al.* (1998) also recorded maximum plant height at harvest, days to first flowering and branches per plant with the application of Vermi-compost – 10 t/ha and recommended dose of NPK 27.5:60:50 kg/ha in garden pea.

Data recorded on Growth characters have been presented in table-1.

Table 1: Effect of vermi-compost on days taken to germination, No. of nodules/plant, Fresh weight of Nodules and Dry weight of nodules.

T	DTG	N/P	FWN (in gm)	DWN (in gm)
T_1	14.66	4.55	0.040	0.131
T_2	12.66	4.52	0.056	0.202
T_3	11.33	4.24	0.060	0.171
T_4	11.26	4.25	0.023	0.073
T_5	14.00	4.36	0.051	0.158
T_6	13.66	3.37	0.031	0.111
T_7	15.00	4.10	0.046	0.144

Acronym used: T = Treatment, DTG=Days taken to Germination, N/P= No. of Nodules/Plants, FWN=

Fresh weight of nodules, DWN= Dry weight of nodules

Data recorded on plant height have been presented in table-2. Data indicate that the maximum significant height (47.42cm) was produced by T_4 (vermi-compost-10 t/ha+NPK) treatment.

Table 2:-Effect of vermi-compost on plant height

T	15 Days	30 Days	45 Days	P
T_1	19.56	31.91	46.29	47.33
T_2	20.32	26.39	39.46	41.43
T_3	18.86	29.27	43.77	46.07
T_4	17.93	31.10	40.97	47.42
T_5	18.94	27.73	40.11	45.58
T_6	16.52	27.38	39.54	46.05
T_7	19.62	31.36	38.95	41.06

Acronym used: T = Treatment, P = Picking

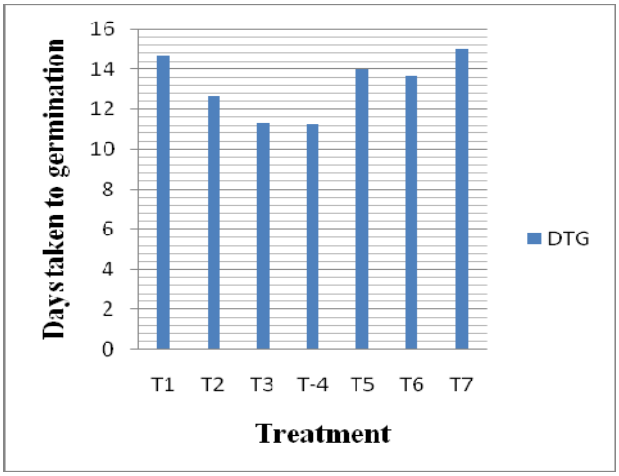

Fig 1:- Effect of vermi-compost on days taken to germination

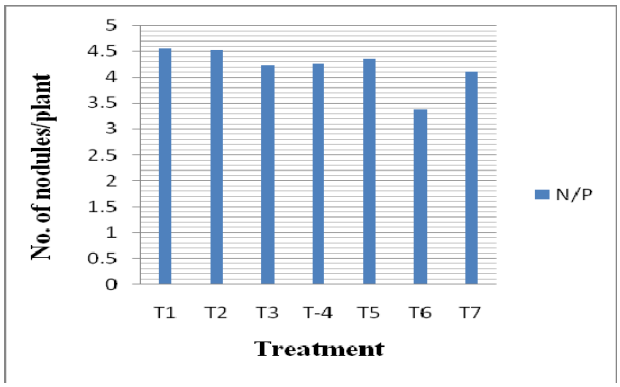

Fig 2:- Effect of vermi-compost on no. of nodules per plant

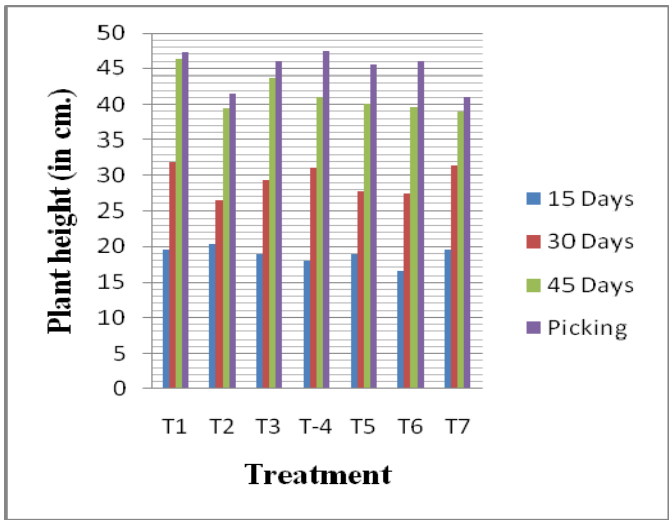

Fig3:- Effect of vermi-compost on plant height

Table3:-Effect of vermi-compost on yield characters of Pea

T	DTF	No. P/P	No. G/P	F W 100 G (in gm)	Y/P	Y/Pt (kg)	Y q/ha
T_1	16.46	6.00	6.36	50.33	2.10	14.94	81.11
T_2	17.80	6.46	6.33	46.00	2.76	12.25	64.48
T_3	19.00	7.00	6.33	40.33	3.95	9.70	75.41
T_4	19.40	6.46	6.73	50.66	7.55	15.21	85.73
T_5	18.53	7.46	6.63	41.00	2.40	14.24	62.98
T_6	18.86	5.66	6.03	47.00	3.70	13.21	63.45
T_7	18.93	6.93	6.72	41.33	2.30	11.20	60.16

Acronym used: T = Treatment, DTF = Days taken to first flowering, No. P/P = Number of pods per plant, No. G/P = Number of grains per pod, F W 100 G =

Fresh weight of 100 grains, Y/P = Yield per plant, Y / Pt= Yield per plot, Y = Yield per hectare

Data recorded on yield characters are presented on table- 3 data indicate that, Vermi-compost levels also influenced the number of pods per plant. The highest pod number was obtained with T_5 (Vermi-compost- 15 t/ha + NPK 25:60:50 kg/ha) treatment under the present study. An increasing trend in number of grains per pod was observed in pea with increasing levels of Vermi-compost, and the maximum being under T_4 (Vermi-compost- 10 t/ha+NPK, 25:60:50 kg/ha) level. Vermi-compost doses significantly increased the yield per hectare over control. Having produced the maximum yield per hectare found under T_4 (Vermi-compost- 10 t/ha+NPK, 25:60:50 kg/ha) treatment obtained the top rank.

A comparative study of the present findings led to the conclusion that sowing of pea with the application of vermi-compost @ 10 t/ha and NPK @ 25:60:50 kg/ha was found most effective to best growth of pea crop under Srinagar valley condition of Garhwal region of Uttrakhand state.

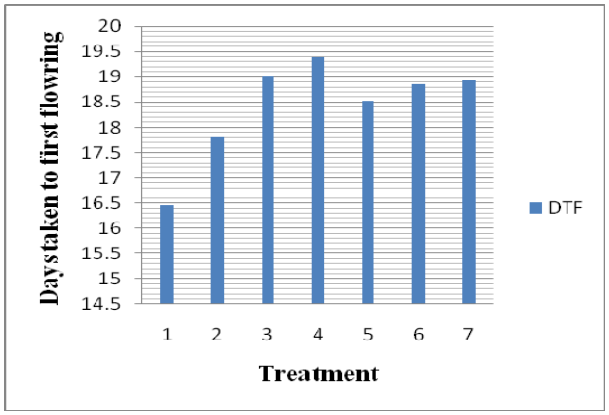

Fig4:- Effect of vermi-compost on days taken to first flowering

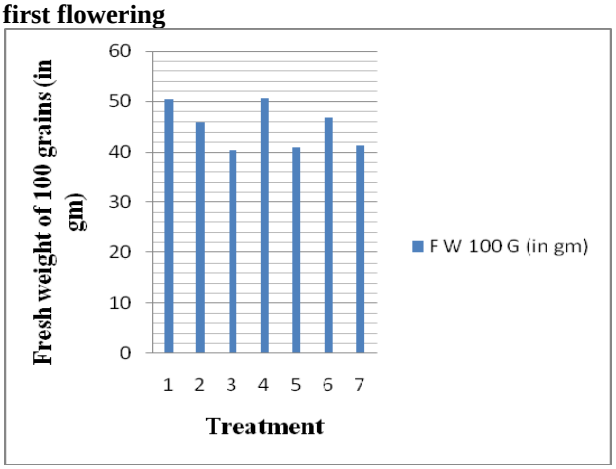

Fig 5:- Effect of vermi-compost on fresh weight of 100 grains

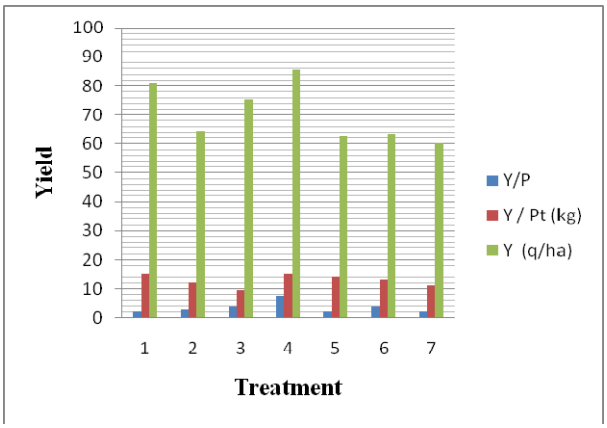

Fig. 6:- Effect of vermi-compost on yield per plant, yield per plot & yield per hectare

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References

1. Farrington, P. 1974. Pea and beans. *In*: Vegetables crop in India, (eds.: Bose, T.K. and Som, M. G.), Naya prakosh, Calcutta, p. 469. growth of tomatoes. *J. Plant Nutrition*, 27: 1107-1123.
2. Singh, H.B. and Joshi, B.S. (1970) Pulse crops in India, Indian council of Agricultural Research, New Delhi.
3. Lopes, A. J.; Stamford, N.P.; Figueired, M.V.B.; Burity, N.A. and Ferraz, E.B.(1996). Effect of vermicompost, mineral nitrogen and mineralizing agent on N fixation and yield in cowpea. *Revista - Brasileira - de-ciencia-do-solo*, 20 : 55-62.
4. Reddy, Ramchandra; Reddy, M.A. Narayana and Reddy, Y.T. Narayana (1998). Effect of organic and inorganic sources of NPK on growth and yield of pea. *Legume Res.* 21 (1) : 57-60.

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In vitro antioxidative acitivity of *Azadirachta indica* and *Melia azedarach* Leaves by DPPH scavenging assay

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Abstract: Medicinal plants are a major source of raw material for the traditional system like Ayurveda, Siddha & Unani. Even the modern system of medicine has more than 25 percent of drugs in use, which are either plant based or plant derived. Although several tree posses various medicinal properties, it has been ignored by indigenous & modern system of medicine. Among them *Azadirachta indica* & *Melia azedarach* belonging to family Meliaceae play a vital role in day to day usage of different indigenous communities due to its sacred and medicinal value. Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants. In the course of finding potential antioxidant from plant source, two medicinal tree species belonging to family Meliaceae has been selected. Leaves were dried and extracted with different solvent systems namely water, ethanol & methanol. Antioxidant activity using DPPH radical scavenging assay of six extracts from two genus of the family Meliaceae is reported & a comparison of the free radical scavenging ability of the extracts is emphasized. The result of the present study showed that the extract of *Melia azedarach*., which contains highest amount of phenolic compounds exhibited the greatest anti-oxidant activity in comparison to *Azadirachta indica*. The high scavenging property of may be due to hydroxyl groups existing in the phenolic compounds chemical structure that can provide the necessary components as a radical scavenger. [Nature and Science 2010; 8(4):22-28]. (ISSN: 1545-0740].

Key words: Antioxidant activity, *Azadirachta indica*, *Melia azedarach.*

1. Introduction

India has a wealth of medicinal plants most of which have been traditionally used in Ayurveda, Unani systems of medicine and by tribal healers for generation. In ancient Indian literature, it is mentioned that every plant on this earth is useful for human beings, animals and other plants. Medicinal plants constitute the major constituents of most indigenous medicines and a large number of Western medical preparations contain one or more ingredients of plant origin. Medicines that are used today are not definitely the same as those that were used in ancient times or even in the recent past. Several modifications, improvement, sophistication and newer discoveries contribute continuously to the type, quality, presentation and concept of medicinal preparation. The therapeutic use of development of human knowledge, scientists endeavored to isolate different chemical constituents from plant, put them to biological and pharmacological tests and thus have been used to prepare modern medicines.

There is an increasing interest in the measurement and use of plant antioxidant for scientific research as well as industrial (e.g., dietary, pharmaceutical and cosmetics) purposes. This is mainly due to their strong biological activity, excluding those of many synthetic antioxidants which have possible activity as promoters of carcinogenesis. Therefore, the need exists for safe, economic, powerful and natural antioxidants to replace these synthetic ones. Obviously, there has been an increasing demand to evaluate the antioxidant properties of direct plant extracts. (McClements, 2000; Decker, 2000). Many antioxidant compounds, naturally occurring in plant sources, have been identified as a free radical or active oxygen scavengers (Zheng, 2001; Wang, 2001). A number of plants have been investigated for their biological activities and antioxidant principles Baris et al. (2006). Saleem et al. (2001). Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants. Ito et al. (1983). In addition, naturally antioxidants have the capacity to improve food quality and stability and also act as nutraceuticals to terminate free radical chain reaction in biological systems, and thus may provide additional health benefits to consumers.

Recent works have highlighted the role of polyphenolic compounds of the higher plants. Hertog et al. (1993) such as flavonols. Salah et al, (1995) anthaquines Yen et al. (2000), Xanthaninsms that contribute to their anticarcinogen or cardioprotective effects. Increasing experimental evidence has suggested that these compounds can affect a wide range of cell biological function by virtue of their radical scavenging properties. (Aruoma, 1998). The

intake of antioxidants such as polyphenols has been effective in the prevention of diseases. Cao et al. (1997). Vinson et al. (1995). In the search of plants as a source of natural antioxidants, some medicinal plants and fruits have been extensively studied for their antioxidant activity and radical scavenging in the last few decades. Singh et al. (2002). Some antioxidant compounds are extracted from easy sources, such as agricultural and horticultural crops, or medicinal plants. Among them the medicinal plants are taking the main role for providing a large number of pure antioxidants.

It is an established fact that polyphenolic compounds possess remarkable antioxidant activities which are present quite commonly in the plant family Meliaceae. *A.indica* is well known in India and its neighboring countries for more than 2000 years as one of the most versatile medicinal plants having a wide spectrum of biological activity. *A. indica* and *M. azedarach* are two closely related species of Meliaceae family. The former is popularly known as Indian Neem (Margosa tree) or India lilac, and the latter as Mahaneem or Persian lilac. All parts of the plant have been used for medicinal purposes including fruits, seeds, leaves, roots and barks (Anon, 1985). Neem has been extensively used in Ayurveda,Unani and homoepathic medicine and has become a Synonym of modern medicine. The Neem tree contains more than 100 bioactive ingredients. The most important bioactive compound is azadirichtin. *Melia azedarach*, the Persian Lilac is popularly known as Maha neem tree and cultivated in all stations. It is a large evergreen tree found throughout India and very similar to Neem. It is native to upper Burmah region. It's Flowering time is May-June and Fruiting time is Nov-Dec. The inner bark contains a resinous alkaloid substance and is used as an anthelmintic. Various scientific studies reported the analgesic, anticancer, antiviral, antimalarial, antibacterial, and antifungal, antifeedent and antifertility activity of this plant. (Vishnukanta, 2008).

Leaf & bark extract of *A. indica* has been studied for its anti-oxidant activity. Ghimeray et al. (2009). Sultane et al. (2007). However anti-oxidant activity of *M.azedarach* another very important medicine plant has not been investigated. In present work leaves, extracted in water, ethanol & methanol of two trees**,** *A. indica* & *M. azedarach* belonging to family Meliaceae were investigated for the presence of phenol content & antioxidant activity in a comparative way.

2. Material and Methods

2.1 Chemicals and Reagents

Folin-Ciocalteu reagent (Merck Pvt. Ltd, India), Sodium chloride (S.D. Fine Chem, India), Sodium carbonet (Merck Pvt. Ltd, India), Catechol (Himedia Lab., India), 2, 2-Diphenyle-2-picryl hydrazyl (DPPH) and Ascorbic acid are obtained from (Himedia Lab., India). All solutions, including freshly prepared doubled distilled water. Stock solutions of the test extracts were prepared in ethanol. Appropriate blanks were used for individual assays.

2.1.2 Plant Materials

The leaves of the two species i.e. *A. indica* and *Melia azedarach* of Meliaceae family were collected from the Medicinal Garden of B.J.B (A) College, Bhubaneswar, Orissa. Fresh plant leaves were rinsed severally with clean tap water to make it dust and debris free. Then the leaves were spread evenly and dried in the shady condition for 3to4 days until they become crispy while still retaining the greenish coloration. Dried leaves were ground in electric chopper to get fine powder form for further use.

2.1.3 Instrumentations

Collection of multi-solvent extract was done by Soxhlet apparatus (J.S.G.W) with varying temperatures according to the B.P. of the solvents. The samples were evaporated through the Rotary vacuum evaporator at 60-100^{0}C according to the B.P. of supplied solvents. Absorbance spectrophotometery was carried out using a UV-vis spectrophotometer (EI, model-1371).Wavelength scans and absorbance measurements were in 1ml quartz cells of 1cm path length.

2.1.4 Preparation of plant extracts

The dried and powdered Neem and Maha-neem leaves (each 50g) were extracted successively with double distilled water, ethanol and methanol (each 400ml.) for 10-12 hrs.,using a Soxhlet apparatus. Then collected solutions were filtered through Whatman No-1 filer paper. The extracts were evaporated to dryness under reduced pressure at 90^{0}C by Rotary vacuum evaporator to obtain the respective extracts and stored in a freeze condition at −18^{0}C until used for further analysis.

2.2 Phenolic Estimation

The total phenolic content of plant extracts were determined by using Folin-Ciocalteu Spectrophotometric method according to the method described. Kim et al. (2007). Reading samples on a UV-vis spectrophotometer at 650 nm. Results were expressed as catechol equivalents (µg/mg).

2.3 Antioxidative activity

The antioxidant activity of the Neem and Mahaneem (Leaves) on the basis of the scavenging activity of the stable 2, 2- diphenyl-2-picrylhydrazyl (DPPH) free

radical was determined according to the method described. Brand-Williams et al. (1995). with slight modification. The following concentrations of extracts were prepared 0.02mg/mL, 0.04mg/mL,0.06mg/mL, 0.08mg/mL and 0.1mg/mL. All the solutions were prepared with methanol. 5 ml of each prepared concentration was mixed with 0.5mL of 1mM DPPH solution in methanol. Experiment was done in triplicate. The test tubes were incubated for 30 min. at room temperature and the absorbance measured at 517nm. Lower the absorbance of the reaction mixture indicates higher free radical scavenging activity. Ascorbic acid was used as a standard and the same concentrations were prepared as the test solutions. The different in absorbance between the test and the control (DPPH in ethanol) was calculated and expressed as % scavenging of DPPH radical. The capability to scavenge the DPPH radical was calculated by using the following equation.

Scavenging effect (%) = (1-As/Ac) ×100

As is the absorbance of the sample at t =0 min.

Ac is the absorbance of the control at t=30 min.

3. Results and Discussion

3.1 The effect of different solvents on the yields of Azadirachta and Melia leaf extracts.

The significant variation in the yields of Azadirachta and Melia extracts were shown using various fraction solvents. The yield of extracts using Water, Methanol and Ethanol in case of Azadirachta were 4.93gm, 4.34gm and 6.36gm respectively. Likewise the Melia leaf extract also followed the same order as the Azadirachta extracts, and they were 5.92gm, 5.62gm and 5.95gm. The variation in yield may be due to the polarity of the solvents used in the extraction process. (Table-1)

3.1.2 Free radical and antioxidative activity

Table-2 shows the results of the free radical (DPPH) scavenging activity in % inhibition. The result revealed that the ethanol fraction of Melia exhibited the highest radical scavenging activity with 68.23±0.03 followed by its aqueous extract with 64.34±0.04 and methanol extract with 61.17±0.05. In comparison to Melia the Azadirachta extract shows less scavenging activity. The Azadirachta extract of obtained from ethanol shows 50.48±0.03. i.e. highest scavenging activity followed by its aqueous extract with 49.48±0.03 and methanolic extract with 41.17±0.04. In overall comparison the ethanolic extract of both Azadirachta and Melia show the highest scavenging activity followed by the aqueous and then methanol. Methanol and ethanol has been proven as effective solvent to extract phenolic compounds. Siddhuraju and Becher (2003). In the present study, the values of ethanolic and aqueous extracts were higher than those of methanolic ones. Among solvents used in this study ethanol has showed the best effectiveness extracting phenolic components.

Ethanol is preferred for the extraction of antioxidant compounds mainly because its lowers toxicity. Karadeniz et al. (2005). Fig. 1. Shows the comparative study of radical scavenging activity between Melia and Azadirachta with respect to ascorbic acid as standard.

3.1.3 Phenol content & antioxidant activity

It is reported that phenols are responsible for the variation in the antioxidant activity of the plant. Cai et al. (2004). They exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals. (Pokorney, 2001) Pitchaon et al. (2007). The total phenolic content varied significantly between the two species of Maliaceae family i.e. *Azadirachta indica* and *Melia azedarach*. The contents of total phenolic compounds in crude ethanolic extracts obtained from these two Azadirachta plants are presented in Table-1. The results were reported as catechol equivalents (µg/mg). The highest concentration of total phenol was 360µg/mg present in the ethanolic extract of Melia plant where as lowest in aqueous extract of Azadirachta plant i.e. 120µg/mg. The aqueous and methanolic fractions of Melia showed 140µg/mg and 268µg/mg of phenol contents respectively. Similarly the Melia ethanolic extract and Azadirachta methanolic extract exhibited highest phenol contents of i.e.300µg/mg and 258µg/mg.

3.1.4 IC_{50} value

IC_{50} value is defined as the concentration of substrate that causes 50% loss of the DPPH activity and was calculated by linear regression mentioned of plots of the percentage of antiradical activity against the concentration of the tested compounds. Results showed in table-1 reports no IC_{50} value in water and methanol extraction of *Azadirachta indica*. Only ethanolic extract of Azadirachta showed an IC_{50} value of 0.008µg/mg. In comparison of Azadirachta, all extracts of Melia showed lower IC_{50} value, however ethanolic extract of Melia being the lowest (Figure 2).The ethanolic extract of Mahaneem exhibited significant activity with low IC_{50} value in comparison to Azadirachta. The antioxidant activity of Azadirachta and Melia extracts rise with the rising of polyphenol content of the extract. A linear relationship between the reciprocal of IC_{50} value and the total polyphenol content of Azadirachta and Melia was observed in this study, indicating that increasing the polyphenol content strengths the antioxidant activity. This finding is similar to that reported by Katsube et al. (2004).

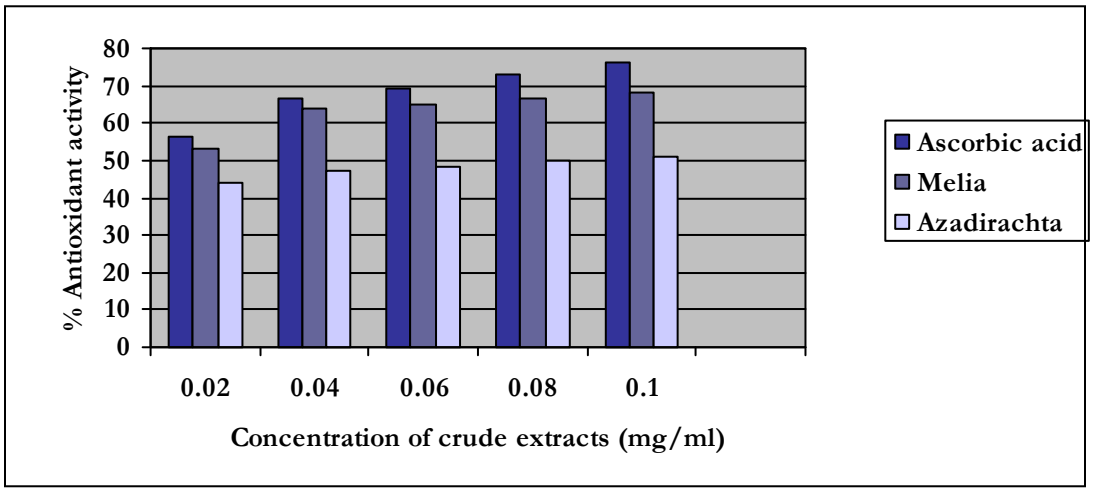

Figure. 1.Antioxidant activity of Melia **and Azadirchta in comparision to Ascorbic acid**

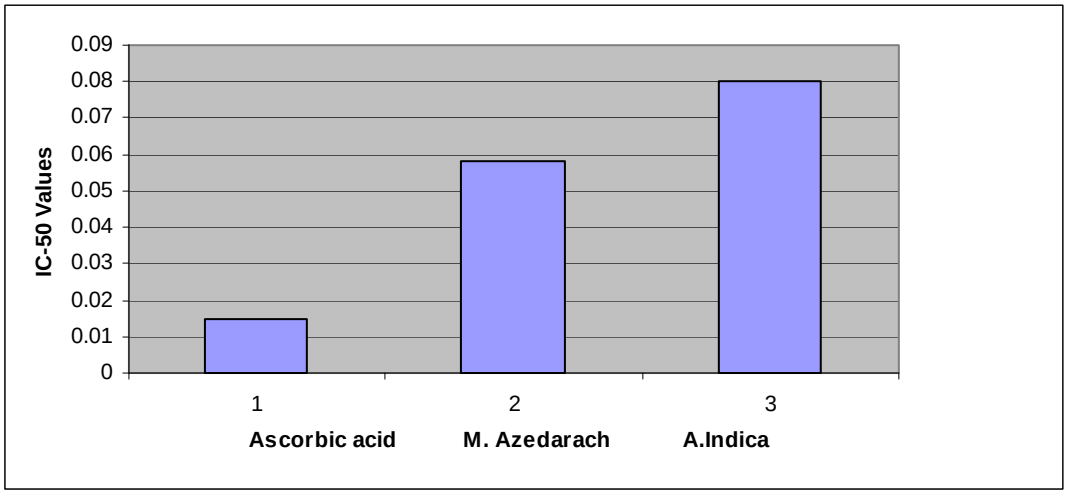

Figure. 2. IC_{50} value of Melia and Azadirachta leaf extracted in ethanol in comparison to Ascorbic acid.

Table-1-Crude extracts, phenol contents & IC_{50} Value in Melia and Azadirachta leaves

	Azadirachta indica			*Melia azedarach*		
Solvent used	Crude Extracts (gm)	Phenol content (mg/mg)	IC_{50} Value (mg/ml)	Crude Extracts (gm)	Phenol content (mg/g)	IC_{50} Value (mg/ml)
Water	4.93	120	<50%	5.92	140	0.062
Methanol	4.34	258	<50%	5.62	268	0.066
Ethanol	6.36	300	0.080	5.95	360	0.058

Table-2-Antioxidant activities of Melia and Azadirachta in different solvents

Concentration of extracts (mg/ml)	Antioxidant activity (%) Water		Antioxidant activity (%) Methanol		Antioxidant activity (%) Ethanol	
	Azadirachta	**Melia**	**Azadirachta**	**Melia**	**Azadirachta**	**Melia**
0.02	45.24±0.04	53.69±0.03	34.11±0.04	55.29±0.03	44.10±0.01	52.94±0.05
0.04	44.18±0.03	55.75±0.05	35.84±0.06	56.47±0.06	47.05±0.03	64.11±0.03
0.06	47.48±0.02	60.43±0.03	39.43±0.06	59.98±0.09	48.20±0.06	64.70±0.04
0.08	48.47±0.05	63.14±0.04	40.00±0.10	60.59±0.04	50.02±0.13	66.47±0.03
0.1	49.48±0.03	64.34±0.04	41.17±0.04	61.17±0.05	50.48±0.03	68.23±0.03

4. Conclusion

The result of the present study showed that the extract of *Melia azedarach*. which contains highest amount of phenolic compounds exhibited the greatest anti-oxidant activity in comparison to *Azadirachta indica*. The high scavenging property of Melia may be due to hydroxyl groups existing in the phenolic compounds chemical structure that can provide the necessary components as a radical scavenger.

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References

1) Anon. The wealth of India. A dictionary of Indian raw material and industrial products, revised edition. CSIR, New Delhi 1985;1(A):401-6.

2) Aruoma OI. Free radicals, oxidative stress and antioxidants in human health and

diseases. Journal of American Oil Chemists' Society 1998; 75(2): 199-212.

3) Baris O, Golloce M, Sahin R, Ozer H, Killic H, Ozkan H, et al. Biological activities of essential oil and methanol extract of Achillea bickersteinii Afan. (Asteraceae). Tukish Journal of Biology 2006, 30: 65-73.

4) Brand-Williams, W., Cuvelier,M.E., and Berset, C. Use of free radical method to evaluate antioxidant activity. Lebensmittel Wissenschaft and Technologie 1995; 28(1):25-30.

5) Cai Y, Luo Q, Sun M, Corke H Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life science 2004;74:2157-84.

6) Cao G, Sofic E, Prior. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationship. Free redical Biology and Medicine 1997; 22:749-60.

7) Ghimeray A k, Jin CW, Ghimire B K, Cho DH. Antioxidant activity quantitative estimation of azadirachtin and nimbin in Azadirachta indica. A. juss grown in foothills of Nepal. African Journal of Bio-technology 2009;8(33):3084-91.

8) Hertog MGL, Feskeens EJM, Hollman CH, Katan MB, Kromhout D. Dietary Antioxidant flavanoid and risk of coronary heart disease: The Zutphen elderly. Lancet 1993; 342:1007-11.

9) Ito N, Fukushima S, Hasegawa A, Shibata M, Ogiso T. Carcinogenicity of butalated hydroxyanisole in F344 rats. Journal of National Cancer Institute 1983;70:343-47.

10) Karadeniz F, Burdurulu HS, Koca N, Soyer Y. Antioxidant activity of selected fruits and vegetables grown in Turkey. Journal of Agriculture and Food Chemistry 2005; 29, 297-303.

11) Katsube T, Tabata H, Ohta Y, Yamasaki Y, Anuurad E, Shiwaku K, YamaneY. Screening for antioxidant activity in edible plant products: Comparision of low density lipoprotein oxidation assay. Journal of Agriculture and Food Chemistry 2004;52(8):2391-6.

12) Kim KT, Yoo KM, Lee JW, Eom SH, Hwang IK, Lee CY. Protective effect of steamed American ginseng (Panax quinquefolius L.) on V79-4 cells induced by oxidative stress. J. Ethnopharm 2007;111:443-445.

13) McClements J, Decker EA. Lipid oxidation in oil-water emulsions: Impact of molecular environment or chemical reactions in heterogeneous food system. Journal of Food Science 2000;65(8):1270-82.

14) Pitchaon M, Suttajit M, Pongsawatmani R. Assessment of phenolic content and free radical scavenging capacity of some Thai indigenous plants. Food Chem 2007,100:1409-18.

15) Pokorny J, Yanishlieva N, Gordon M. Antioxidants in food, Practical Applications,Cambridge Woodhead publishing limited 2001;72(5):145-71.

16) Salah N, Miller N J, Pagana G, Tijburg L, Bolwell G P, Rice Evans C. Polyphenolic flavonols as scavenger of aqueous phase radicals and as chain- braking Antioxidants. Archieves of Biochemistry and Biophysics 1995;2:339-46.

17) Saleem A, Ahotupa M, Pihlaja K. Total phenolic concentration and antioxidant potential of extracts of medicinal plants of pakistan, zeitschrift fur Natureforschung 2000;56:973-78.

18) Siddhuraju P, Becker K.Antioxidant properties of various extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (Moringa oleifera Lam.) leaves. Journal of Agriculture and Food Chemistry 2003;51:2144-55.

19) Singh RP, Murthy KNC, Jayaprakasha GK. Studies on the antioxidant activity of pomegranate (Punica granatum) peel and seed extracts using in vitro models. Journal of Agricultural and Food Chemistry 2002;50(1):81-6.

20) Sultana B, Anwar F, Przybylski R. Antioxidant activity of phenolic components present in barks of Azadirachta indica, Terminalia arjuna, Acacia nilotica, and Eugenia jambolana Lam.trees.Food Chemistry 2007;104(3):1106-14.

21) Vinson JA, Dabbagh YA, Serry MM, Jang J.Plant flavonoids, especially tea flavonoids, are powerful antioxidants using an in vitro oxidation model for heart disease. Journal of Agricultural and Food Chemistry 1995; 43(11):2800-02.

22) Vishnukanta AC, Rana. Melia azedarach: A phytopharmacological review. Journal of pharmacogenosy reviews 2008; 2:173-79.

23) Yen GC, Duh PD, Chuang DY.. Antioxidant properties water extracts from peanut hull. Journal of the American Oil Chemist's Society 2000;70:383-86.

24) Zheng W, Wang SY. Antioxidant activity and phenolic compounds in selected herbs. Journal of Agricultural and Food Chemistry 2001;49(11):5165-70.

1/ 19/ 2010

Effect of Chromium on *Mucor* species and optimization of growth conditions

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Abstract: Czapek Dox broth medium is frequently used for the culture of fungal species like *Mucor*. The influences of incubation period, pH, Cr (VI) concentration, temperature on the concentration of biomass were also evaluated. At pH 5.5, the fungi *Mucor* species yields maximum biomass and the fungus can be able to degrade chromium to a particular concentration but at higher concentration growth reduces. From a practical viewpoint, this abundant and inexpensive fungal biomass has potential application in the conversion of toxic Cr (VI) into less toxic or nontoxic Cr (III). Maximum biomass weight was observed which is about 0.33±0.01mg/20ml at a constant temperature of 35^0C with an incubation period of 8 days. The protein content of the fungus was estimated and it was found that maximum yield of protein was recorded in the presence of 0.005 mM of chromium. [Nature and Science 2010;8(4):29-32]. (ISSN: 1545-0740).

Key words: Biomass; *Mucor* species; Czapek Dox medium; incubation period.

1. Introduction

The majority of toxic metal pollutants are waste products of industrial and metallurgical processes. Their concentrations have to be reduced to meet ever increasing legislative standards according to World Health Organisation (WHO). The metals of most immediate concern are cadmium, chromium, cobalt, copper, lead, nickel, mercury and zinc. The effluent from metal finishing processes may contain up to 10 mg/L of metal dusts. Usually, when using methods such as chemical precipitation, reverse osmosis for the removal of metal ions from dilute aqueous stream, incomplete metal removal can be obtained. Furthermore, these processes have high reagent or energy requirements and generate toxic sludge that requires careful disposal (Wild et al, 1987). The need for cost-effective process and safe method for removing heavy metals from discharging effluents has resulted in search for other unconventional materials such as organic or inorganic sorbents (Allen et al, 1998). The use of microbial biomass of fungi (Kapoor and Viraraghavan, 1995) and bacteria for removal of heavy metals from aqueous solutions is gaining increasing attention. It has been found that both living and death microbial cells adsorb metal ions. Chromium is a transition metal most commonly found in the environment in its trivalent Cr (III) and hexavalent Cr (VI) forms (Anderson, 1997). Naturally occurring Cr is almost exclusively in the trivalent state, as the energy required for its oxidation is high. Hence, the hexavalent form is usually considered to be a man-made product (Bai and Abraham, 2001). The toxicities of the two forms of chromium are vastly different. Trivalent chromium is generally a nontoxic, non-mobile micronutrient (Bai and Abraham, 2002). Hexavalent chromium is water soluble, toxic, and carcinogenic, and is considered a pollutant by the United States Environmental Protection Agency (EPA) (Bai and Abraham, 2003). Chromium is the second most common inorganic contaminant of groundwater at hazardous waste sites (Baral and Engelken, 2002). The solubility and negative charge of it's more common forms, chromate and dichromate lead to limited adsorption in aquifer minerals, and results in high mobility of Cr(VI) in aquifers (Barnhart, 1997). The historical and present day contamination of groundwater and soils by Cr (VI) is a result of its industrial uses, including metal plating (for corrosion resistance), pigment production, and lumber and wood products (for preservation) (Clesceri, 1998).

Metal contaminants are commonly found in soils, sediments, and water. Metal pollutants can be produced through industrial processes such as mining, refining, and electroplating. A key factor to the remediation of metals is that metals are non-biodegradable, but can be transformed through sorption, methylation, and complexation, and changes in valence state. These transformations affect the mobility and bioavailability

of metals. At low concentrations, metals can serve as important components in life processes, often serving important functions in enzyme productivity. However, above certain threshold concentrations, metals can become toxic to many species. Fortunately, microorganisms can affect the reactivity and mobility of metals. Microorganisms that affect the reactivity and mobility of metals can be used to detoxify some metals and prevent further metal contamination. Of the various toxic heavy metals discharged into the environment hrough various industrial wastewaters, constituting one of the major causes of environmental pollution, chromium is one of the most toxic and has become a serious health concern. Extensive use of chromium, e.g., in electroplating, tanning, textile dyeing and as a biocide in power plant cooling water, results in discharge of chromium-containing effluents (Barnhart, 1997). The effluents from these industries contain Cr (VI) and Cr (III) at concentrations ranging from tenths to hundreds of milligrams/litre. While Cr (VI) is known to be toxic to both plants and animals, a strong oxidizing agent and a potential carcinogen, Cr (III) is generally only toxic to plants at very high concentrations and is less toxic or nontoxic to animals (Anderson, 1997).

However, none of these methods are completely satisfactory and all feature due to the following disadvantages: (1) generation of a large amount of secondary waste products due to various reagents used in a series of treatments such as reduction of Cr (VI), neutralization of acidic solution and precipitation, and (2) instability of ion-exchange resins due to serious oxidation by hexavalent chromium. Thus, the development of new, cost-effective, more environmentally friendly methods is needed. Biosorption of heavy metals by biomaterials has been suggested as a potential alternative to the existing physicochemical technologies for detoxification and recovery of toxic and valuable metals from wastewaters. Many biomaterials such as seaweed (Kratochvil et al, 1998; Yun et al, 2001), micro-algae (Gupta et al, 2001), fungi (Kapoor and Viraraghavan, 1995) and various other plant materials (Raji and Anirudhan, 1998; Sharma and Forster, 1993) have been studied for their chromium binding abilities. Particularly, fungal biomass can be cheaply and easily procured in rather substantial quantities, as a by product from established industrial fermentation processes. Furthermore, since such abundant dead fungal biomass is of little use, it has been identified as a potential source of biomaterial for the removal of chromium from wastewaters. The objective of the present investigation is to clarify the mechanism that governs Cr (VI) removal by fungal biomass and to evaluate the effects of various parameters such as contact time, pH, initial Cr (VI) concentration, biomass concentration and temperature. Furthermore, the potential of fungal biomass for the detoxification of Cr (VI) is discussed.

2. Materials and Methods:

2.1 Collection of soil sample:

Soil sample was collected from the garden of P.G Dept of Botany, Utkal University, near the waste dumping site since this soil may contain enormous number of saprophytic fungi.

2.2 Isolation of Organism:

A local isolate of *Mucor* species as used in this study was isolated from the soil using serial dilution technique. It was maintained on Potato Dextrose Agar medium (PDA) (Hi-media, Mumbai). The slants were grown as 30^0C for 7 days and stored at 4^0C for further study.

2.3 Inoculum Preparation:

10 ml of sterile distilled water containing 0.01% triton-X 100 was transferred to a sporulated (7 days old) PDA slant culture. The spores were dislodged using the sterile inoculation needle under aseptic condition and the suspension with appropriate dilution ($1X10^7$spores/ml) was used as inoculum throughout the experiment.

2.4 Establishment of medium and growth conditions:

The isolated *Mucor* species was grown in sterilized Czapek Dox broth medium for establishment of the optimum temperature, pH and incubation period that supports the exuberant growth of *Mucor* species. Hence, *Mucor* species was grown in Czapek Dox broth medium in temperature ranging from 20^0C-40^0C, pH ranging from 4 -12 and incubation period from 1day-10 days.

2.5 Determination of biomass:

The biomass formation was determined by cell dry weight measurement of 20 ml culture samples. The samples were filtered through dried and pre- weighed filter paper (Whatman No. 1), followed by washing twice with distilled water and then drying at 80^0C to constant weight. The growth responses ware measured in the form of the biomass produced under certain conditions.

2.6 Determination of soluble protein:

The concentration of soluble protein was estimated by Lowry et al. (1951) using Bovine serum albumin as the reference standard.

2.7 Statistical analysis:

Each experiment was carried out in five replicates. From this, arithmetic means, standard errors of mean

(SEM) were calculated and graphs were plotted using MS-Excel.

3. Results and Discussion:

From the mixed culture, *Mucor* was identified by its specific colony characteristics, colour and microscopic features. The optimum pH for the proper growth of *Mucor* was finally found to be at pH-5.5 where the biomass weight was about 0.33±0.01mg/20ml at a constant temperature of 35^{0}C (Table 1). Maximum biomass (0.35±0.02 mg/20ml) was obtained at 35^{0}C in an incubation of eight days. (Table 2). The fungus Mucor species showed maximum biomass (0.65±0.01mg/20ml) when incubated for 8 days. (Table 3).

pH Range	Biomass in (mg/20ml)
4.0	0.22±0.01
4.5	0.26±0.02
5.0	0.31±0.01
5.5	0.33±0.01
6.0	0.29±0.03
6.5	0.28±0.04
7.0	0.24±0.03
8.0	0.23±0.02
9.0	0.21±0.03
10.0	0.19±0.01
11.0	0.18±0.01
12.0	0.16±0.02

Table 1. Effect of various pH on biomass (mg/20ml) growth of *Mucor* species.

Temperature	pH	Biomass in (mg)
20^{0}C	5.5	0.19±0.01
25^{0}C	5.5	0.24±0.01
30^{0}C	5.5	0.29±0.03
35^{0}C	5.5	0.35±0.02
40^{0}C	5.5	0.25±0.04

Table 2. Effect of various Temperatures on growth of *Mucor* species at pH 5.5.

Incubation period (days)	Biomass in mg./20ml
1	0.04±0.01
2	0.12±0.01
3	0.26±0.02
4	0.37±0.03
5	0.44±0.02
6	0.52±0.01
7	0.58±0.03
8	0.65±0.01
9	0.61±0.01
10	0.59±0.03

Table 3. Effect of various incubation periods on growth of *Mucor sp.* at 35^{0}C and pH 5.5

Further investigation on *Mucor* species was carried out in the presence of chromium in the medium such as when the pure culture of *Mucor* was treated with various concentration of chromium, it was noticed that growth of *Mucor* biomass was obtained up to 0.020 mM concentration of chromium (VI) in the medium and further increase in the concentration reduced the biomass. This may be due to the tolerance of toxicity up to 0.020mM concentration of chromium and further addition of chromium may be acting as toxic for the growth of the organism (Table 4).

Concentration of Chromium (VI) in mM	Duration in days	Biomass in (mg)
Control	8	0.15±0.01
0.005	8	0.23±0.06
0.010	8	0.27±0.04
0.015	8	0.35±0.02
0.020	8	0.42±0.01
0.025	8	0.31±0.01
0.030	8	0.26±0.05
0.035	8	0.19±0.02
0.040	8	0.16±0.04

Table 4. Effect of different Chromium (VI) concentration in the culture media on biomass (mg/20ml) of *Mucor* species in 8 days of incubation.

Analysis of protein content showed 0.78 mg/ml protein at a concentration of 0.005 mM of chromium in the sample and gradually protein content was reduced when organism was grown in higher concentration of chromium as shown in the figure given bellow.

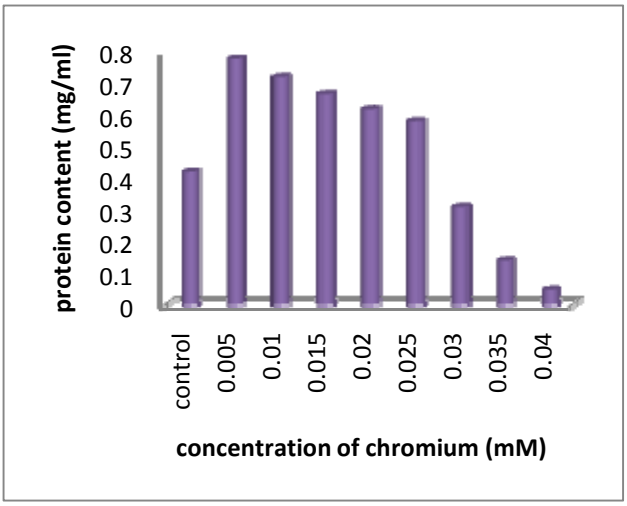

Figure 1. Effect of chromium on protein content of *Mucor* species on 8th day of incubation.

Chromium contamination of the environment has

become an important issue due to the potential health threat it poses. Conventional technologies to clean up heavy metal ions from contaminated waters have been utilized, but they remain cost-ineffective. Therefore, the use of dead fungal biomass for the detoxification of Chromium (VI) from contaminated waters may be a novel and cost-effective alternative. The use of dead fungal biomass has the following advantages: it is abundant and very cheap, the process does not require a continuous nutrient supply for maintaining the cells in good physiological conditions, and dead cells are not subjected to physiological constraints such as metals toxicity. Mucor was able to bioremediate the chromium present in the medium at lower concentration. Hence it can be used to treat the effluents containing chromium at a lower cost and it is also eco-friendly so it will not hamper to the environment.

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References:

[1] Allen HE, Garrison AW, Luther GW. Metals in Surface Waters. Chelsea, Michigan, Ann Arbor Press: 1998: 262-267.

[2] Anderson RA. Chromium as an essential nutrient for humans. Regul. Toxicol. Pharmacol. 1997: 26 (1): S35–S41.

[3] Bai RS, Abraham TE. Biosorption of Cr (VI) from aqueous solution by Rhizopus nigricans. Bioresour. Technol. 2001:79 (1): 73–81.

[4] Bai RS, Abraham TE. Studies on chromium (VI) adsorption-desorption using immobilized fungal biomass. Bioresour. Technol. 2003: 87 (1): 17–26.

[5] Bai RS, Abraham TE. Studies on enhancement of Cr (VI) biosorption by chemically modified biomass of Rhizopus nigricans. Water Res. 2002: 36 (5): 1224–1236.

[6] Baral A, Engelken RD. Chromium-based regulations and greening in metal finishing industries in the USA. Environ. Sci. Policy 2002: 5 (2): 121–133.

[7] Barnhart J. Occurrences, uses, and properties of chromium. Regul. Toxicol. Pharmacol. 1997:26 (1): S3–S7.

[8] Clesceri LS, Greenberg AE and Eaton AD. *Standard methods for the examination of water and wastewater.* 20th ed. American Public Health Association, Washington, 1998; 1325 p.

[9] Clesceri LS, Greenberg AE, Eaton, AD. Standard Methods for the Examination of Water and Wastewater, 20th ed. American Public Health Association, 1998: pp 366–368.

[10] Gupta VK, Shrivastava AK, Jain N. Biosorption of chromium (VI) from aqueous solutions by green algae Spirogyra species. Water Res. 2001: 35 (17): 4079–4085.

[11] Kapoor A, Viraraghavan T. Fungal biosorption-an alternative treatment option for heavy metal bearing wastewaters: a review. Bioresour. Technol. 1995: 53 (3): 195–206.

[12] Kratochvil D, Pimentel P, Volesky B. Removal of trivalent and hexavalent chromium by seaweed biosorbent. Environ. Sci. Technol. 1998: 32 (18): 2693–2698.

[13] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951:193:265-75.

[14] Raji C, Anirudhan TS. Batch Cr (VI) removal by polyacrylamide-grafted sawdust: kinetics and thermodynamics. Water Res. 1998: 32 (12): 3772–3780.

[15] Sharma DC, Forster CF. Removal of hexavalent chromium usingsphag num moss peat. Water Res. 1993: 27 (7): 1201–1208.

[16] Wild J, Barnes D, Forster CF, Hrudey SE. Liquid Wastes from the Metal Finishing Industry. John Wiley and Sons, New York 1987: pp. 21–62.

[17] Yun, YS, Park D, Park JM, Volesky, B. Biosorption of trivalent chromium on the brown seaweed biomass. Environ. Sci. Technol. 2001: 35 (21): 4353–4358.

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Biochemical and neurological effects of obesity on primary school girls

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The prevalence of childhood obesity has increased considerably worldwide. As with adults, obesity in childhood is strongly related to hypertension, dyslipidemia, type II diabetes, and insulin resistance. Also, obese children are at increased risk of becoming obese adults. Therefore, obese children tend to develop serious medical and psychosocial complications, and have a greater risk of adult morbidity and mortality. The principal goal of this study was to investigate the effects of obesity on the levels of some biomarkers and their relation to the cognitive function in elementary school obese girls. The current study was conducted on 45 obese girls (mean age 10.53 ± 1.29 years; mean BMI 28.43 ± 4.62 kg/m^2) and 45 normal age-matched girls (mean age 10.36 ± 1.53 years; mean BMI 19.07 ± 3.47 kg/m^2). Estimation of serum adrenomedullin (AM) and substance P (SP), and plasma noradrenaline (NA) and acetylcholine (ACh) were carried out. Cognitive function tests (auditory vigilance, digit span, coding and visual memory) were done for all subjects. The levels of serum AM and SP as well as plasma NA were highly significantly increased ($P<0.01$) in the obese group as compared with the control group. The total right response of auditory vigilance (TR) showed insignificant decrease while the total wrong response to auditory vigilance test (TW) showed a significant increase ($P<0.05$) in the obese group as compared with the control group. Digit span and visual memory classification showed a highly significant decrease ($P<0.01$) while coding showed a significant increase ($P<0.05$). Our study showed that obesity affected the measured biomarkers and, to some extent, has an adverse effect on cognitive function in primary school girls. [Nature and Science 2010;8(4):33-43]. (ISSN: 1545-0740)]

Key words: obesity- adrenomedullin -substance *P* - noradrenaline - acetylcholine – cognition – girls

Introduction

Obesity is well-known to result from the disturbance of the homeostasis between food intake and energy expenditure (Gura, 2003). It is a major risk factor for the development of type II diabetes and its complications such as the metabolic syndrome, coronary heart disease and peripheral neuropathy (Lazar, 2005). It also increases the risk for insulin resistance (Formiguera and Canton, 2004), high blood pressure, and other medical problems (Sothern *et al*., 2000). Obesity may also disturb cognition, as deficits in learning, memory, and executive functioning were reported in obese when compared to non-obese subjects (Waldstein and Katzel, 2006). A recent study suggested that obesity and its consequences, including midlife hypertension, diabetes, and cerebrovascular disease, contribute significantly to cognitive decline and accelerate the development of dementia (Qiu *et al*., 2007).

Adrenomedullin (AM) belongs to the family of adipokines (Nambu *et al*., 2005). This 52-amino acid peptide was first isolated from phaeochromocytoma tissue as a vasoactive and cardioprotective factor (Shimosawa *et al*., 2002). AM has been also found in the hypothalamus-pituitary-adrenal axis (Letizia *et al*., 2003) and produced largely by mature adipocytes and stromal vascular cells (Fukai *et al*., 2005). AM has local paracrine or autocrine effects on the tissue so that it can trigger many physiologic events by remaining in the plasma like the circulating hormone (Letizia *et al*., 2005). AM elicits a long-lasting vasodilatation and diuresis. Its action is mainly mediated by the intracellular adenylate cyclase coupled with cyclic adenosine monophosphate (cAMP) and nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathway through its specific receptor (Eto, 2001).

Substance P (SP), an undecapeptide, was first discovered in crude form in 1931 by von Euler and Gaddum (von Euler and Gaddum, 1931) but its study was limited until its isolation by Leeman and colleagues in 1971 (Hokfelt *et al*., 2001). SP belongs to the tachykinin family, which also includes neurokinin A and neurokinin B (Stout *et al*., 2001). Upon its release, SP binds to a family of neurokinin (NK) receptors, preferentially acting on the metabotropic NK1 receptor (Harrison and Geppetti, 2001).

SP is abundant in the stomach, duodenum, and jejunum, all important areas for digestion and nutrient uptake, as well as in hypothalamic areas concerned with feeding behavior, such as the arcuate and ventromedial nuclei, along with the presence of NK-1R in the hypothalamus (Mantyh *et al*., 1984) and in adipose tissue (Karagiannides *et al*., 2006). The orexigenic effect of SP has been demonstrated in mice and the administration of the NK-1R pharmacologic inhibitor (CJ 012,255) counteracts the increase in feeding, ameliorates the weight gain induced by feeding with high fat/high caloric content diets, and improves their ability to remove glucose from the blood and respond to insulin (Karagiannides *et al*., 2008). It has been found that SP has also neurotrophic as well as memory-promoting effects and when applied peripherally (i.p.), it promotes memory and is reinforcing. These effects of SP seemed to be encoded by different SP-sequences, since the N-terminal SP1-7 enhanced memory, whereas C-terminal hepta- and hexapeptide sequences of SP proved to be reinforcing in a dose equimolar to SP. Also, direct application of SP into the region of the nucleus basalis magnocellularis (NBM) has memory-promoting and reinforcing effects (Huston and Hasenöhrl, 1995).

The sympathetic nervous system (SNS) is an important contributor to energy expenditure, and is widely assumed to play a major role in the pathophysiology of obesity (Reaven *et al.*, 1996). Noradrenaline (NA) is synthesized and stored in sympathetic nerve endings and is the neurotransmitter involved in SNS signal transmission. Although most of NA released from sympathetic postganglionic neurons is cleared locally by neuronal reuptake and effector cell metabolism, a portion of released NA spills over into the bloodstream. Therefore, whole body NA spillover rate into plasma has been used as an index of SNS activity (Coppack *et al*., 1998). The noradrenergic system plays a role in appetite regulation, with activation of α_1- and β_2-adrenergic receptors inhibiting food intake. Phentermine acts as an NA reuptake inhibitor, thereby increasing synaptic NA to reduce appetite and weight gain (Bays and Dujovne, 2002). In contrast, activation of α_2-adrenergic receptors increases food intake (Stanley *et al*., 2005).

Forebrain ACh modulates several cognitive functions (Sarter and Bruno, 2000). There's a considerable evidence suggesting that loss of cholinergic functions may be a major contributor to the severe cognitive deficits evident in individuals with Alzheimer's disease (Winkler *et al*., 1998).

Objective: The main goal of the current study is to investigate the effects of obesity on the levels of some biomarkers (SP, AM, NA and ACh) and their relation to the cognitive function in primary school obese girls.

SUBJECTS AND METHODS

Research Design and Methods:

Forty-five Egyptian girls with simple obesity and Forty-five lean age-matched girls, as controls, were recruited from 4 primary schools in Dokki region, Giza governorate, Egypt, between September 2008 to Jan 2009. Their ages ranged from 8 to 12 years. Anthropometric measurements, body composition, cognitive tests and biochemical analysis were done to every subject. A questionnaire for the social information was answered by parents.

1. Study Population

To determine whether subjects presented previous diseases, an appropriate questionnaire was administered. Also, clinical diagnosis was done in the National Research Centre Clinic to ensure that subjects recruited were in good health and with no known diseases. None was anemic and none had a chronic illness, such as hypertension, diabetes mellitus, heart failure or chronic hepatic failure (none of the girls had any overt disease other than obesity). None of the girls was taking medication. Informed consent was signed by parents before taking part in the study. The protocol was approved by the Ethical Committee of the National Research Centre, Egypt. All examinations were performed during fasting and after emptying the urinary bladder.

2- Anthropometry and Body Composition

Body mass index (BMI) was calculated as

weight (Wt) in kilograms divided by squared height (Ht^2) in meters squared (Kg/m^2). BMI for age and sex was calculated. Normal weight children were defined as having a BMI for age and sex < 85^{th} percentile and obese children as having BMI for age and sex ≥95^{th} percentile (Ogden et al., 2002).

Ht was measured to the nearest 0.5cm on a wall-mounted Harpenden's stadiometer. Wt was measured to the nearest 0.1kg on a standard medical balance scale, with the subject dressed only in light underwear and no shoes. Waist (midway between the 10^{th} rib and the iliac crest) and hip (greater femoral trochanter) circumferences (WC and HC) were measured using a non-stretchable tape measure in a standing position. Waist to hip ratio (WHR) and waist to Ht were calculated. Also, Wt for age [percent median (%median), Z-score and percentile] and Ht for age (%median, Z-score and percentile) were calculated (McCarthy *et* al., 2001). Body composition was determined by a bioelectrical impedance analyzer using a formula provided by the manufacturers and fat mass percent (FM%) was calculated.

3- Cognitive Tests:

a- The Digit Span Test

The digit span memory task is a verbal measure of immediate memory and working memory maintenance and manipulation (subtest of the WAIS-III, Wechsler, 1997). The subjects were asked to repeat a number of digits after having been presented orally by the examiner, and this measures immediate memory. The list length began with two digits and increased sequentially until recall errors were made on at least one of two trials. The increasing set of numbers' backward recall can assess working memory. Performance of participants was calculated from the numbers of digits they could repeat without mistakes (Cserjési *et al.*, 2007).

b- Coding

In the coding test, children had to substitute symbols for numbers as quickly as possible. The score represents the total number of correct symbols written during a fixed time. The coding test primarily assesses visual-motor coordination, visual encoding, and short-term memory, concentration, and sustained attention.

c- The Auditory Vigilance Test

This test measures the attention ability. It's a measure of the efficiency of identifying figural stimulation in the context of non-signal stimuli. The subjects were asked to pay attention while listening to many words from different categories like key, ball, school, etc., and they were asked to give a sign, like raising their hands, when they hear certain words, chosen by the administrator. The scores of the test were calculated as total right and total wrong.

d- The Visual Memory Test

This test is a measure of free recall of visual object. It also taps some aspects of classification ability. The subjects were shown a group of different photos like animals, cars, plants, etc., and then were asked to mention as many photos as they can. The results of the test are categorized into classification- of photos according to their groups- and recall of photos shown. The score is calculated from the right results.

4- Biochemical Measurements:

Fasting blood samples were withdrawn from patients and controls in the morning and plasma as well as sera were separated using cooling centrifuge (4˚C) and then stored at -80˚C till analysis. AM was measured by an enzyme-linked immunosorbent assay kit (ELISA kit) purchased from DRG International Inc., USA according to the method of Porstmann and Kiessig (1992). SP was measured by an ELISA kit purchased from Cayman Chemical Company, Ellsworth, Ann Arbor according to the method of Renzi *et al*. (1987). NA was measured by an ELISA kit, purchased from Labor Diagnostika Nord GmbH & Co. KG. ACh was measured colorimetrically using the kit purchased from BioVision Research Products, Linda Vista Avenue, Mountain View, USA.

Statistical Analysis:

All statistical analyses were performed using SPSS for PC version 14. Student *t*-test and Pearson's correlation were performed to compare groups and detect the possible relationships among measurements. Also, stepwise regression analysis was done considering BMI as the dependent variable.

RESULTS

Table (1) shows descriptive statistics with mean (±SE) and *P* values of the anthropometric measurements in the control and obese girls. All of these measurements revealed highly significant increase ($P<0.01$) in the obese group as compared

with the control group, except for Ht which showed significant increase ($P<0.05$) and both of Ht-for-age parameters (% median, Z-score, percentile) and WHR which showed insignificant increase ($P>0.05$).

Table (2) represents the levels of serum AM and SP and plasma NA and Ach of control and obese girls. The levels of serum AM and SP and plasma NA showed highly significant increase ($P<0.01$) in the obese group as compared with the control group.

The data in table (3) illustrate the cognitive tests for control and obese groups. In the obese group, TW showed significant increase ($P<0.05$). Digit span and visual memory classification showed highly significant decrease ($P<0.01$) while coding score showed significant increase ($P<0.05$) in the obese group as compared with the control group.

Table (4) depicts the results of Pearson's correlation between the biochemical markers and anthropometric measurements in the obese group. Serum AM showed highly significant positive correlation ($P<0.01$) with Wt, BMI, WC, HC, waist/Ht, and Z-score and percentile of Wt-for-age and significant positive correlation ($P<0.05$) with %median of Wt-for-age.

The results in table (5) represent Pearson's correlation among the biochemical markers under study in the control and obese groups. In the control group, only significant positive correlation was recorded between levels of NA and ACh ($P<0.05$). In the obese group, only SP showed significant positive correlation with NA ($P<0.05$).

Table (6) depicts Pearson's correlation between cognitive tests and anthropometric measurements in the control group. TR showed highly significant negative correlation ($P<0.01$) while TW showed highly significant positive correlation ($P<0.01$) with FM%. Also, coding score showed significant negative correlation ($P<0.05$) with Waist/Ht ($P<0.01$) and parameters of Wt-for-age (%median, Z-score and percentile). Visual memory recall showed highly significant positive correlation ($P<0.01$) with Wt, Ht, BMI, WC and HC, but highly significant negative correlation ($P<0.01$) with Ht-for-age parameters (%median, Z-score and percentile). Visual memory classification showed significant negative correlation ($P<0.05$) with Waist/Ht and highly (2Bdeleted) significant negative correlation ($P<0.01$) with Wt-for-age parameters (%median, Z-score and percentile).

The data in table (7) illustrate Pearson's correlation between cognitive tests and anthropometric measurements in the obese group. There was significant negative correlation between TW and Wt ($P<0.05$). Also, coding score showed highly significant negative correlation ($P<0.01$) with Wt, WC and HC and significant negative correlation ($P<0.05$) with Ht. Digit span showed highly significant positive correlation ($P<0.01$) with FM%.

Table (1): Anthropometric and body composition measurements for control and obese girls

Parameters	Control (n = 45) Mean ± SE	Obese (n = 45) Mean ± SE
Wt (Kg)	39.211 ± 1.414	61.333 ± 1.977**
Ht (cm)	142.618 ± 1.352	147.778 ± 1.554*
BMI (Kg/m^2)	19.065 ± 0.518	28.430 ± 0.689**
FM %	21.194 ± 1.520	33.144 ± 1.011**
Waist (cm)	69.711 ± 0.960	83.133 ± 1.325**
Hip (cm)	82.200 ± 0.829	98.231 ± 1.806**

Waist/Ht	0.490	±	0.007	0.563	±	0.008**
WHR	0.847	±	0.005	0.850	±	0.010
Wt for age (% median)	107.891	±	1.457	184.296	±	18.863**
Wt for age (z-score)	0.294	±	0.061	2.733	±	0.216**
Wt for age (percentile)	60.776	±	2.217	98.013	±	0.343**
Ht for age (% median)	99.642	±	0.663	101.296	±	0.580
Ht for age (z-score)	-0.091	±	0.146	0.263	±	0.126
Ht for age (percentile)	48.998	±	3.940	58.758	±	4.012

Asterisks indicate significant differences between the two groups (*) $P<0.05$, (**) $P<0.01$

Wt= weight, Ht= height, BMI= body mass index, FM%= fat mass percent, WC= waist circumference, HC= hip circumference, WHR= waist to hip ratio.

Table (2): Levels of adrenomedullin (AM), substance P (SP), noradrenaline (NA) and acetylcholine (ACh) in control and obese girls

Parameter	Control (n = 45) Mean ± SE			Obese (n = 45) Mean ± SE		
AM (ng/ml)	0.896	±	0.011	5.547	±	0.275**
SP (pg/ml)	35.311	±	0.784	40.9	±	0.596**
NA (ng/L)	189.72	±	7.209	237.159	±	6.104**
ACh (nmol/ml)	2.371	±	0.107	2.711	±	0.149

Asterisks indicate the significant differences between the two groups, (**) $p<0.01$

Table (3): Cognitive tests for control and obese groups

Parameters	Control (n = 45) Mean ± SE	Obese (n = 45) Mean ± SE
TR	40.489 ± 0.269	39.711 ± 0.421
TW	1.511 ± 0.269	2.511 ± 0.416*
Digit span	13.867 ± 0.689	10.822 ± 0.724**
Coding	12.089 ± 0.256	13.244 ± 0.372*
Recall	10.600 ± 0.630	10.489 ± 0.362
Classification	8.111 ± 0.264	6.222 ± 0.246**

Asterisks indicate the significant differences between the two groups (*) $p<0.05$, (**) $p<0.01$

TR= total right response to auditory vigilance test, TW= total wrong response to auditory vigilance test

Table (4): Pearson's correlation between biochemical markers and anthropometric measurements in the obese group

	Wt	BMI	WC	HC	Waste/Ht	Wt for age %median	Wt for age Z-score	Wt for age Percentile
AM	0.698**	0.771**	0.614**	0.647**	0.501**	0.294*	0.492**	0.509**
SP	0.016	0.151	-0.071	-0.042	0.046	0.222	0.160	-0.240
NA	0.034	0.170	0.077	0.023	0.186	0.109	0.125	-0.048
Ach	0.015	0.075	0.030	-0.107	0.045	0.107	0.037	-0.109

Data are expressed as correlation coefficient (r) values, asterisks indicate significant correlation (*) $P<0.05$, (**) $P<0.01$

Table (5): Pearson's correlation among the biochemical markers in the control and obese groups

	AM		SP		NA		Ach	
Group	Control	Obese	Control	Obese	Control	Obese	Control	Obese
AM	1.000	1.000	0.238	0.076	0.073	0.113	0.050	0.038

SP	0.238	0.076	1.000	1.000	0.053	0.331*	0.007	0.089
NA	0.073	0.113	0.053	0.331*	1.000	1.000	0.320*	0.058
Ach	0.050	0.038	0.007	0.089	0.320*	0.058	1.000	1.000

Data are expressed as r values, asterisks indicate significant correlation (*) *P<0.05*

Table (6): Pearson's correlation between cognitive tests and anthropometric measurements in the control group

	Wt	Ht	BMI	WC	HC	FM%	Waste / Ht	Wt for age % median	Wt for age Z-score	Wt for age Percentile	Ht for age % median	Ht for age Z-score	Ht for age Percentile
TR	-0.075	-0.094	-0.034	-0.166	-0.239	-0.500**	-0.088	-0.143	-0.091	-0.085	-0.068	-0.073	-0.013
TW	0.075	0.094	0.034	0.166	0.239	0.500**	0.088	0.143	0.091	0.085	0.068	0.073	0.013
Digit span	0.037	-0.019	0.068	0.069	0.017	0.058	0.068	0.201	0.200	0.205	0.052	0.044	0.094
Coding	0.035	0.234	-0.088	-0.250	-0.247	0.067	-0.388**	-0.307*	-0.355*	-0.364*	-0.074	-0.065	-0.042
Recall	0.600**	0.460**	0.528**	0.420**	0.424**	0.106	0.105	0.215	0.139	0.123	-0.460**	-0.446**	-0.438**
Classification	-0.076	0.190	-0.185	-0.290	-0.224	0.124	-0.377*	-0.522**	-0.559**	-0.560**	-0.062	-0.052	-0.065

Data are expressed as r values, asterisks indicate significant correlation (*) *P*<0.05, (**) *P*<0.01

Table (7): Pearson's correlation between cognitive tests and anthropometric measurements in the obese group

	Wt	Ht	BMI	WC	HC	FM%
TR	0.260	0.229	0.163	0.270	0.252	0.151
TW	-0.314*	-0.275	-0.204	-0.283	-0.276	-0.151
Digit span	0.189	0.261	0.237	0.167	0.287	0.447**
Coding	-0.484**	-0.304*	-0.290	-0.445**	-0.439**	-0.077
Recall	0.081	0.181	-0.023	-0.014	0.192	0.280
Classification	-0.006	0.062	-0.036	-0.115	-0.045	0.024

Data are expressed as r values, asterisks indicate significant correlation (*) $P<0.05$, (**) $P<0.01$

DISCUSSION

The results of the current study revealed that plasma AM level showed a significant increase in obese girls as compared to the controls. This finding is in agreement with those of previous studies (Letizia *et al*., 2001; Kato *et al*., 2002 and Fukai *et al*., 2005). Because AM expression in the adipose tissue is increased in obesity, the source of elevated plasma AM in obese subjects is likely to be the adipose tissue (Li *et al*., 2007). In addition, pancreatic islets may also be a source of AM (Letizia *et al*., 2001) as AM causes a decrease in insulin secretion and the increased level of plasma AM may be an adaptive mechanism to decrease hyperinsulinemia (Letizia *et al*., 2005). It has been found that endogenous AM acts against insulin resistance via its vasodilator and anti-oxidant actions (Li *et al*., 2007).

Although AM showed insignificant correlation with all of the anthropometric measurements in the control group, it showed significant positive correlation with Wt, BMI, WC and HC in the obese group. These results agree with those of Kato *et al*. (2002). The relationship between AM and BMI can reflect the dysfunction of glucose and lipid metabolism (Kato *et al*., 2002).

In the present study, serum SP level was significantly higher in obese girls as compared to the control. In the intestine, SP produced by several cell types may circulate in the blood as a hormone or act locally in a paracrine fashion (Severini *et al*., 2002). SP has been found to have a role in promoting appetite and weight gain (Karagiannides *et al*., 2008). Also, there is potentially a direct physiological effect of SP on fat cells, which is mediated by NK-1R, and this is supported by the expression of the functional NK-1R on the surface of human preadipocytes (Karagiannides *et al*., 2008). These authors discovered a role for SP in appetite regulation and metabolism, in addition to the already established effects of this peptide in gastric motility and digestion (Nicholl *et al*., 1985). Most importantly, the effects of NK-1R blockade on appetite, body weight and adiposity point to a novel approach for treating obesity and insulin resistance (Karagiannides *et al*., 2008). Because of the orexigenic effect of SP, its increase in obese subjects may be a factor contributing to increased appetite and weight gain.

Our results showed insignificant correlation between SP level and all of the cognitive tests. However, Krappmann *et al*. (1994) reported that there is evidence that SP plays a role in learning and reinforcement processes and that reinforcing effects of SP were found upon injection into several parts of the brain. Also, Tomaz and Nogueira (1997) stated that peripheral (i.p.) post-training SP administration in rats enhances memory in a dose- and time-dependent way. The memory-enhancing effects are long-lasting and are mediated, at least in part, via interactions with the endogenous opioid system. The mnemotropic effects of peripherally administered SP are sensitive to the functional integrity of the vagus, suggesting that the vagus nerve may be one pathway by which systemic SP influences memory storage processes in the brain. Furthermore, these effects seemed to be encoded by different SP sequences, the N-terminal SP1–7, but not the C-terminal hepta- and hexapeptide sequences being responsible for the memory -promoting effects. Moreover, SP showed memory-promoting, reinforcing and anxiolytic-like effects when administered systemically or into the nucleus basalis of the ventral pallidum. In addition, SP injection into the ventral pallidum can lead to increases of ACh in frontal cortex and dopamine in nucleus accumbens, suggesting that the hypermnestic, positively reinforcing and anxiolytic effects observed upon basal forebrain injection of SP are mediated by activation of the nucleus accumbens-ventral pallidum circuitry (Hasenöhrl *et al*., 2000). The lack of correlation between SP level and any of the cognitive tests may be due to the small number of cases studied.

Our study showed that plasma NA level was significantly increased in the obese group as compared with the control group. However, plasma NA level showed insignificant correlation with all of the anthropometric measurements and FM% in both the obese and control group. Studies based on catecholamine levels in obese individuals produced conflicting results (Peterson *et al.*, 1988 and Young *et al.*, 1992), some of the variability in these studies may be related to confounding variables that influence SNS activity (Coppack *et al*., 1998) but the consensus favors increased NA levels in obese humans (Goldstein, 1995). Our finding of increased NA levels in obese girls is supported by that of Søndergaard *et al*. (1999). More recently, other studies reported that trend (Eikelis *et al.* 2004), thereby supporting the hypotheses attributable to

Landsberg and Young (Landsberg and Young, 1978) which supposed sympathetic activation as an adaptive response to overeating that helps to stabilize body weight but contributes to complications of obesity such as hypertension (Eikelis and Esler, 2005). Several mechanisms have been proposed to explain the sympathetic activation in obesity. It has been suggested that increases in sympathetic tone are due to the state of insulin resistance, as it has been documented that high levels of insulin may increase sympathetic nerve traffic in man (Blum *et al.* 1997).

In the current study, plasma ACh level in the obese group showed an insignificant increase as compared with the control group. Also, there were insignificant correlation between ACh and the cognitive tests and this may be due to small sample size. Many evidences that supported an important role for ACh in modulating cognitive functions include findings from a host of pharmacological studies that showed that interfering with cholinergic function generally impairs learning and memory, and that augmenting cholinergic functions generally results in an enhancement (Warburton *et al*., 2003). It was found that direct injections of cholinergic agonists and antagonists into the amygdala, striatum, and hippocampus generally enhance and impair, respectively, learning and memory for tasks associated with those neural systems (Wallenstein and Vago, 2001).

Our results showed that obesity affected digit span adversely and this is in accord with Gunstad *et al*. (2006) and Malter-Cohen (2007). Also, total wrong response to auditory vigilance test (TW) and visual memory classification were adversely affected, however, obesity had no effect on either total right response to auditory vigilance test (TR) or visual memory recall. Although coding was better in obese than control, it showed significant negative correlation with Wt, WC, HC and Ht.

When applying BMI as dependent variable, stepwise multiple regression analysis showed that AM was the most significant independent determinant of obesity ($r^2 = 0.594$, $P > 0.001$).

In conclusion, our study showed that obesity affected the levels of the measured biomarkers and, to some extent, had an adverse effect on cognitive function in girls. The lack of effect of obesity on some cognitive tests may be a result of increased levels of SP which has memory-promoting and reinforcing effects and as a result of the high levels of NA and the normal level of ACh which have roles in memory processing.

REFERENCES

Bays H, Dujovne C. Pharmacotherapy of obesity: currently marketed and upcoming agents. Am J Cardiovasc Drugs 2002; 2: 245-253.

Blum WF, Englaro P, Hanitsch S, Juul A, Hertel NT, Muller J, Skakkebaek NE, Heiman ML, Birkett M, Attanasio AM, Kiess W, Rascher W. Plasma leptin levels in healthy children and adolescents: dependence on body mass index, body fat mass, gender, pubertal stage, and testosterone. J Clin Endocrinol Metab 1997; 82: 2904–2910.

Coppack SW, Horowitz JF, Paramore DS, Cryer PE, Royal HD, Klein S. Whole body, adipose tissue, and forearm norepinephrine kinetics in lean and obese women. Am J Physiol Endocrinol Metab 1998; 275: 830-834.

Cserjesi R, Luminet O, Molnar D, Lenard L. Is there any relationship between obesity and mental flexibilty in cildren? Appetite 2007; 49: 675-678.

Eikelis N and Esler M. The neurobiology of human obesity. Experimental Physiology 2005; 90: 673-682.

Eikelis N, Lambert G, Wiesner G, Kaye D, Schlaich M, Morris M, Hastings J, Socratous F, Esler M. Extra-adipocyte leptin release in human obesity and its relation to sympathoadrenal function. Am J Physiol Endocrinol Metab 2004; 286: E744–752.

Eto T. A review of the biological properties and clinical implications of adrenomedullin and proadrenomedullin N-terminal 20 peptide (PAMP), hypotensive and vasodilating peptides. Peptides 2001; 22: 1693–711.

Formiguera X and Canton A. Obesity: epidemiology and clinical aspects. Best Pract Res Clin Gastroenterol 2004; 18: 1125–1146.

Goldstein DS. Clinical assessment of catecholamine function. In: Stress, Catecholamines, and Cardiovascular Disease. New York: Oxford University, 1995; 234–286.

Gunstad J, Paul RH, Brickman AM et al. Patterns of cognitive performance in middle-aged and older adults: a cluster analytic examination. J Geriatr Psychiat Neurol 2006; 19: 59-64.

Gura T. Obesity drug pipeline not so fat. Science 2003; 299: 849–52.

Harrison S and Geppetti P. Substance p. International Journal of Biochemistry and Cell Biology 2001; 33: 555–576.

Hasenöhrl RU, Souza-Silva MA, Nikolaus S, Tomaz C, Brandão ML, Schwarting RK and Huston JP. Substance P, and its role in neural mechanisms governing learning, anxiety and functional recovery. Neuropeptides 2000; 34(5): 272–280.

Hokfelt T, Pernow B, Wahren J. Substance P: a

pioneer amongst neuropeptides. Journal of Internal Medicine 2001; 249: 27–40.

Huston JP and Hasenöhrl RU. The role of neuropeptides in learning: focus on the neurokinin substance P. Behav Brain Res. 1995; 23; 66(1-2):117-27.

Karagiannides I, Kokkotou E, Tansky M, Tchkonia T, Giorgadze N, O'Brien M, Leeman SE, Kirkland JL, Pothoulakis C. Induction of colitis causes inflammatory responses in fat depots: Evidence for substance P pathways in human mesenteric preadipocytes. Proc Natl Acad Sci U S A. 2006; 103: 5207–12.

Karagiannides I, Torres D, Tseng Y, Bowe1 C, Carvalho E, Espinoza D, Pothoulakis C, Kokkotou E. SP as a novel anti-obesity target. Gastroenterology 2008; 134(3): 747–755.

Kato J, Kitamura K, Uemura T, Kuwasako K, Toshihiro K, Kangawa K, et al. Plasma levels of adrenomedullin and atrial and brain natriuretic peptides in the general population: their relations to age and pulse pressure. Hypertens Res 2002; 25: 887–92.

Krappmann P, Hasenöhrl RU, Frisch C, Huston JP. Self-administration of neurokinin SP into the ventromedial caudate-putamen in rats. Neuroscience 1994; 62(4): 1093-1101.

Landsberg L and Young JB. Fasting, feeding and regulation of the sympathetic nervous system. N Engl J Med 1978; 298, 1295–1301.

Lazar MA. How obesity causes diabetes: not a tall tale. Science 2005; 307: 373–5.

Letizia C, Iacobellis G, Caliumi C, Leonetti F, Cotesta D, Ribaudo MC, et al. Acute hyperinsulinemia is associated with increased plasma adrenomedullin concentrations in uncomplicated obesity. Exp Clin Endocrinol Diabetes 2005; 113: 171–5.

Letizia C, Rossi G, Cerci S. Adrenomedullin and endocrine disorders. Panminerva Medica 2003; 45: 241–51.

Letizia C, Tamburrano G, Alo P, Paoloni A, Caliumi C, Marinoni E, et al. Adrenomedullin, a new peptide, in patients with insulinoma. Eur J Endocrinol 2001; 144: 517–20.

Li Y, Jiang C, Wang X, Zhang Y, Shibahara S and Takahashi K. AM is a novel adipokine: AM in adipocytes and adipose tissues. Peptides 2007; 28(5): 1129-1143.

Malter-Cohen M. Adolescent BMI in Relation to Depression, Self-Esteem, and Academic Achievement. Graduate Student Journal of Psychology 2007; 9: 10-15.

Mantyh PW, Hunt SP, Maggio JE. Substance P receptors: localization by light microscopic autoradiography in rat brain using [3H]SP as the radioligand. Brain Res. 1984; 307: 147–65.

McCarthy HD, Jarrett KV, Crawley HF. The development of waist circumference percentiles in British children aged 5.0 –16.9 y. Eur J Clin Nutr 2001; 55: 902–907

Nambu T, Arai H, Komatsu Y, Yasoda A, Moriyama K, Kanamoto N et al. Expression of the adrenomedullin gene in adipose tissue, Regul Pept. 2005; 132: 17–22.

Nicholl CG, Polak JM, Bloom SR. The hormonal regulation of food intake, digestion, and absorption. Annu Rev Nutr. 1985; 5: 213–39.

Ogden CL, Flegal KM, Carroll MD, Johnson CL. Prevalence and trends in overweight among US children and adolescents, 1999-2000. JAMA 2002; 288, 1728-1732.

Peterson HR, Rothschild M, Weinberg CR, Fell RD, McLeisch KR, Pfeifer MA. Body fat and the activity of the autonomic nervous system. N Engl J Med 1988, 318: 1077-1083.

Porstmann T and Kiessig ST. Enzyme immunoassay techniques, an overview. Journal of Immunological Methods 1992; 150: 5-21.

Qiu C, De Ronchi D, Ratiglioni L. The epidemiology of the dementias: an update. Curr Opin Psychiatry 2007; 4: 380-385.

Reaven GM, Lithell H, Landsberg L. Hypertension and associated metabolic abnormalities: the role of insulin resistance and the sympathoadrenal system. N Engl J Med. 1996; 334: 374 –381.

Renzi D, Couraud, JY, Frobert, Y, et al. Enzyme immunoassay for substance P using acetylcholenesterase as label, in Trends in Cluster Headache. Sicuteri F, editor. Elsevier Science Publishers 1987; 125-134.

Sarter M and Bruno JP. Cortical cholinergic inputs mediating arousal, attentional processing and dreaming: Differential afferent regulation of the basal forebrain by telencephalic and brainstem afferents. Neuroscience 2000; 95: 933–952.

Severini C, Improta G, Falconieri-Erspamer G, Salvadori S, Erspamer V. The tachykinin peptide family. Pharmacol Rev. 2002; 54: 285–322.

Shimosawa T, Shibagaki Y, Ishibashi K, Kitamura K, Kangawa K, Kato S, Ando K, Fujita T. Adrenomedullin, an endogenous peptide, counteracts cardiovascular damage. Circulation 2002; 105: 106 –111.

Søndergaard SB, Verdich C, Astrup A, Bratholm P, Christensen NJ. Obese male subjects show increased resting forearm venous plasma noradrenaline concentration but decreased 24-hour sympathetic activity as evaluated by thrombocyte noradrenaline measurements. International Journal of Obesity 1999; 23 (8): 810-815.

Sothern MS, Despinasse B, Brown R, Suskind RM,

Udall JN, Blecker U. Lipid profiles of obese children and adolescents before and after significant weight loss: differences according to sex. Southern Medical Journal 2000; 93: 278-282.

SPSS for Windows, Rel. 14. Chicago, IL: SPSS Inc., Chicago, IL; 2006.

Stanley S, Wynne K, McGowan B, Bloom S. Hormonal regulation of food intake. Physiol Rev. 2005; 85(4): 1131–58.

Stout SC, Owens MJ, Nemeroff CB. Neurokinin(1) receptor antagonists as potential antidepressants. Annual Review of Pharmacology and Toxicology 2001; 41: 877–906.

Tomaz C and Nogueira PJC. Facilitation of memory by peripheral administration of SP. Behavioural Brain Research 1997; 83(1-2): 143-145.

von Euler US and Gaddum JH. An unidentified depressor substance in certain tissue extracts. Journal of Physiology 1931; 72: 74–78.

Waldstein SR and Katzel LI. Interactive relations of central versus total obesity and blood pressure to cognitive function. Int J Obes. 2006; 30: 201–207.

Wallenstein GV and Vago, DR. Intrahippocampal scopolamine impairs both acqution and consolidation of contextual fear conditioning. Neurobiology of Learning and Memory 2001; 75: 245–252.

Warburton EC, Koder T, Cho K, Massey PV, Duguid G, Barker GRI, Aggleton JP, Bashir ZI, Brown MW. Cholinergic neurotransmission is essential for perirhinal cortical plasticity and recognition memory. Neuron 2003; 38: 987–996.

Wechsler D: Wechsler Adult Intelligence Scale. 3rd ed. San Antonio: Psychological; 1997.

Winkler J, Thal LJ, Gage FH, Fisher LJ. Cholinergic strategies for Alzheimer's disease. Journal of Molecular Medicine 1998; 76: 555–567.

Young JB, Tro RJ, Weiss ST, Parker DR, Sparrow D, Landsberg L. Relationship of catecholamine excretion to body size, obesity, and nutrient intake in middle-aged and elderly men. Am J Clin Nutr. 1992; 56: 872– 834.

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NEAREST NEIGHBOUR PATTERN OF SPATIAL VARIATION IN EXPERIMENTAL FIELDS.

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Abstract: Evaluations of the nearest neighbour analysis in the study of spatial variation in experimental plot have been attempted for kenaf using a split plot experiment distributed in a complete randomized design. The experiment was carried out between June and September 2006 at Ilora and Ikenne outstation of the Institute of Agricultural Research and Training, Ibadan to evaluate nearest neighbourhood in experimental plots. The results of the cluster analyses of the stem girth at Ilora showed that 80% of the pairing plots were isotropic in nature while all other plot pairs are anisotropic in nature because their euclidean distances are not equal. For stem girth at Ikenne, isotropic property was exhibited between only $x_{4,1}$ vs $x_{3,1}$ and $x_{5,3}$ vs $x_{2,3}$ (0.032). All other plots pairs are anisotropic in nature. For plant height at both Ilora and Ikenne, none of the pairing plots exhibited isotropic property but anisotropic property. Also, the nearest neighbour indices are 0.00197 (for stem girth at Ilora), 0.00734 (for stem girth at Ikenne), 0.1831 (for plant height at Ilora) and 0.2456 (for plant height at Ikenne). From the study, the variogram is found to be related to the variance covariance using the model, $\gamma(h) = C(0) - C(h)$ (where $\gamma(h)$ = variogram, C(0) is the variance at the plot x_i and C(h) is the covariance at both plot x_i and x_j). Finally, low but positive nearest neighbour index obtained in this work implied that the neighbourhood pattern falls between cluster and randomness thereby reflecting patchiness of neighbourhood pattern. [Nature and Science 2010;8(4):44-53]. (ISSN: 1545-0740)]

Keywords: Nearest neighbour, Euclidean distance, Clusters, initial soil nutrient deposition ((ISND).

Introduction

Treatment responses are expected to be cumulative of the initial soil nutrients deposition (*ISND*). These treatments' responses on any experimental plots are usually subjected to varying degree of statistical tools with the assumption that that the pattern of distribution of the *ISND* are random. Hence, most of the statistical tools presume randomness of these *ISND* consequent to the nature of the preliminary investigation carried out randomly on the experimental site. The studies of spatial statistics have included the employment of several statistical tools to analyze treatment responses relative to spatial variability in experimental plots. These methods include the variance covariance analysis, autocorrelation as well as autocovariance, correlelograme, the similarity matrices and the global and local indicator of spatial autocorrelations, (Hardy and Sonke, 2004, Christakos and Hirstopulos, 1998, Richard and Dean, 2002 and Schabenberger and Pierce, 2000). These various methods have been employed by different authors to assess spatial variability at different level of spatial statistical study. Nearest neighbour analysis is an important spatial variability measurement tool because it incorporates the similarities and the diversity of such experimental plots into the analysis of experimental plots' variation pattern. Nearest neighbour index have been defined as a measure of the amount of spatial dispersion in a set of point features based on the distance (linear) of any points to its nearest neighbour, (Benwell, *et.,al*, 2002). It explore the amount of spatial dispersion based on the ratio of average inter – point distance between nearest neighbour (Ad) to the expected value of the average inter – point distance between randomly dispersed nearest neighbour (Ed). That is if X, D are a metric space $x \in X$ and P is a positive real number then, the D – p- neighbourhood of x is defined to be the set of all points y of X such that D(x,y) < P.

Works on nearest neighbour include Weigelt and Jolliffe (2003)'s indices of plant competition, Purves *et.,al* (2003)'s Nearest neighbour for avalanche forecasting in Scotland and Singh and Ganju (2004)'s Supplement to nearest neighbour method for avalanche forecasting. These works notwithstanding, nearest neighbour analysis of spatial variation in experimental plots is crucial because of its uses in the preliminary assessment of experimental plots. It has been less focused because activities at agricultural field are usually artificially induced unlike in other field (such as geography, ecology and forestry) where natural phenomenon takes its course. Also, nearest neighbour analysis is one of the required regular and periodic updating of spatial statistics. It is one of the tools that can boost the estimation of

spatial variability in experimental plots. Nearest neighbour analysis seeks to answer question relating to the distribution of the treatment responses which are said to be cumulative. The objective of this study was therefore to evaluate spatial variation pattern in experimental plots using nearest neighbour analyses.

Materials and Methods.

Data sets for this project were from "evaluation of the effect of fertilizer and insecticides on Kenaf" set up each at Ikenne and Ilora out stations of the Institute of Agricultural Research and Training, Ibadan between June and September 2006. Ikenne falls within the forest zone ($27^0.48^lN$ and $3^0.52^l$) of the country while Ilora is located in the intermediate guinea savanna ($126^0.52^lN$ and $3^0.41^l$). Each of the experiment was carried out using split plot design. The main plot was the spraying regime (S_1 = 300kg NPK+100kg Furadan + 2 pre flowering insecticide sprays and S_2 = 600g NPK + 200kg Furadan + 4 pre flowering insecticide sprays) while the sub plot was the varieties (V_1 = Cuba 108, V_2 = Ifeken 400 and V_3 = local cultivar). Data on stem girth and plant height were collected at interval of 2 weeks commencing at 4 weeks after planting and relative to their spatial positions.

The data obtained from the experiment were subjected to descriptive statistics (such as means and variances) as well as variogram of the original data and spatial variability effects data. Also, spatial proximity Matrices were constructed to establish the measure of nearest between the plots. This is done through Matrix W (n ***x*** n) where W_{ij} represents the measure of nearest between O_i and O_j. It should be noted that n = 36.

$$W_{ij} = \begin{cases} 1, if \quad (o_i, o_j) < h \\ 0 \qquad\qquad o.w \end{cases}$$

Where h is the average distance of all O_i and O_j. O_i and O_j are respectively, reference and neighbouring plots. Lastly, nearest neighbour index was computed using $NNI = Ad/Ed$

where $Ad = (\sum_i d_i)/n$ *and* $Ed = 1/2\sqrt{A/n}$

and n = number of points, A = map area.

NNI according to Benwell *et.al.*, 2002 and Scharbenberger and Pierce, 2000 can range between zero (all points are at the same location) and 2.1419.

Results.

For variogram measurements, N (*h*) is the number of pairs of values $Z(x_i)$ and $Z(x_i + h)$ separated by vector *h* which suppose to be the separating distance. W_{ij} as contained in table 1 have been converted to their proportions.

Table 1. Means and Variances of Different Variables at Different Plots irrespective of the weeks.

Treatment	Plot Address	Stem girth – (cm) (IIora)	Vari-ance	Stem girth- (cm) (Ikenne)	Vari-ance	Height (cm) (Ilora)	Vari-ance	Height (cm) (Ikenne)	Vari-ance
V1S1	1,1	0.582	0.039	0.825	0.023	78.604	727.327	100.968	852.406
V1S1	1,2	0.660	0.063	0.824	0.042	92.388	928.969	73.893	797.328
V1S1	1,3	0.642	0.043	0.998	0.079	67.452	672.832	83.448	1448.335
V1S1	1,4	0.737	0.043	1.178	0.192	108.376	761.429	131.812	2236.439
V1S1	1,5	0.816	0.043	1.166	0.132	116.092	791.860	141.060	3577.834
V1S1	1,6	0.641	0.033	0.941	0.033	59.192	454.969	75.736	558.897
V2S1	2,1	0.518	0.043	0.758	0.041	43.640	177.289	64.028	851.239
V2S1	2,2	0.627	0.034	0.754	0.062	54.184	301.752	46.984	672.676
V2S1	2,3	0.708	0.031	1.256	0.162	96.872	911.496	126.168	2917.620
V2S1	2,4	0.744	0.032	1.232	0.176	99.304	545.690	136.256	2121.984
V2S1	2,5	0.987	0.057	1.118	0.125	133.704	770.340	139.672	2504.302
V2S1	2,6	0.762	0.025	1.026	0.054	98.316	535.321	118.052	2577.796
V3S1	3,1	0.534	0.031	0.844	0.034	74.060	549.246	89.316	723.825
V3S1	3,2	0.611	0.023	0.829	0.064	82.460	370.370	71.108	744.144
V3S1	3,3	0.791	0.057	1.225	0.292	113.108	656.282	122.820	2174.450

V3S1	3,4	0.809	0.103	1.033	0.117	72.168	880.843	99.068	2256.535
V3S1	3,5	0.627	0.019	0.965	0.097	44.920	302.203	97.464	1991.748
V3S1	3,6	0.638	0.017	0.963	0.047	54.336	454.670	125.520	2932.981
V2S2	4,1	0.695	0.036	0.856	0.047	95.556	437.521	84.032	556.016
V2S2	4,2	0.639	0.044	0.860	0.067	58.044	294.328	86.812	1380.568
V2S2	4,3	0.665	0.038	0.986	0.101	64.672	488.808	98.992	2479.741
V2S2	4,4	0.851	0.044	1.266	0.135	123.160	1510.566	155.940	3644.054
V2S2	4,5	0.581	0.056	0.896	0.023	44.920	302.203	74.508	642.724
V2S2	4,6	0.623	0.035	0.958	0.049	54.300	456.036	68.760	568.775
V3S2	5,1	0.676	0.035	0.766	0.046	52.260	302.273	66.172	854.861
V3S2	5,2	0.725	0.034	1.096	0.105	109.504	1138.860	139.328	3084.581
V3S2	5,3	0.777	0.030	1.256	0.170	118.012	435.807	134.156	2563.701
V3S2	5,4	0.797	0.060	1.194	0.109	108.952	702.939	148.384	2746.284
V3S2	5,5	0.688	0.042	1.032	0.062	102.164	1021.825	128.220	2735.813
V3S2	5,6	0.784	0.029	0.988	0.166	108.184	510.308	112.768	3516.609
V1S2	6,1	0.690	0.028	0.776	0.062	94.804	477.117	75.252	734.898
V1S2	6,2	0.798	0.031	1.187	0.134	117.920	1089.066	130.636	2046.241
V1S2	6,3	0.844	0.052	1.206	0.177	120.408	953.019	125.096	2373.249
V1S2	6,4	0.774	0.099	1.058	0.075	79.740	414.530	106.732	2325.458
V1S2	6,5	0.762	0.028	0.912	0.023	105.672	890.485	124.648	3102.044
V1S2	6,6	0.753	0.015	0.975	0.064	103.504	290.515	124.908	4438.358

This was because of the need to factorize out the large values of the plant height returned for plots at both sites and for the fact that $-1 \angle \gamma(h) \angle 1$. Z ($x_i$) now becomes $Z(x_i) \Big/ \sum_{i=1}^{n} Z(x_i)$ and Z (x_i + h) = Z (xi + h) $\Big/ \sum_{i=1}^{n} Z(x_i)$ $\cdot \sum_{i=1}^{n} Z(x_i)$ were 0.002260833 (Stem girth, Ilora), 0.004194 (Stem girth, Ikenne), 0.003316145 (Plant height, Ilora) and 0.003611258 (Plant height, Ikenne) while the variogram { γ (h)}for stem girth and plant height at Ilora and Ikenne were respectively, 0.0000314, 0.0000583, 0.0000461 and 0.0000502. For the different variables including the SVE, nugget effect was observed to be negligible because semivariogram at $\gamma(0) = 0$ or approximately zero. The semivariogram thus were said not to present the nugget effects. Also, the semivariogram of the variables showed that the practical range for the stem girth at Ilora and Ikenne were 16 and 14 while real sill were not obtained for the variables at Ilora and Ikenne. The semivariogram of the stem girth continually increased monotonically up till lag 16 (for Ilora) and lag 12 (for Ikenne). The reverse were the case for lag greater than these two lags implying the presence of "hole effects" (Table 2). For plant height however, the presence of hole effects is entire because the semivariogram lack monotonical increments.

The variogram for the SVE of the different variable showed that the data does not fulfill the assumption of stationarity. This is because the fitted line does not follow a linear sill semi variogram pattern (Figure 1). That is none of the fitted line presented real range, sill and a very low nugget effect. The nugget effect of stem girth at Ilora is -0.02 while stem girth at Ikenne has practically no nugget effects. The nugget effects for the plant height at Ilora are 0.06 while that of Ikenne is – 0.05. It is noteworthy that the variogram patterns of the raw data differ from that of SVE. Since the variogram of the SVE presented nugget effects (that is $\gamma(0) \neq 0$), hence physical phenomenon at smaller scale which must not have been well resolved existed.

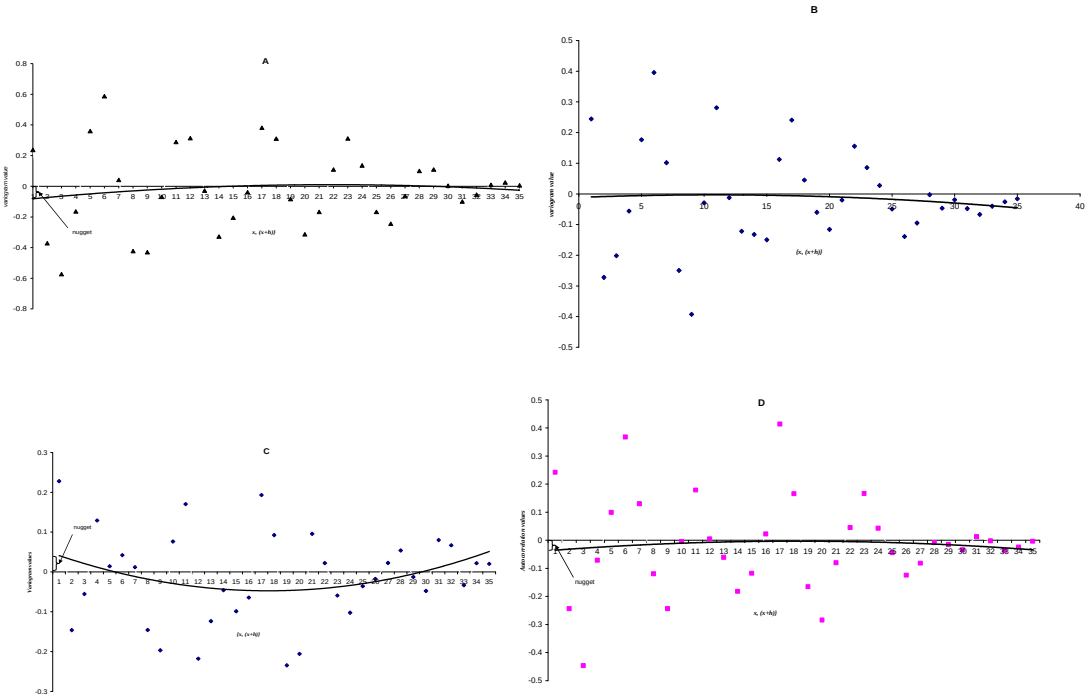

Figure 1. Variogram of the spatial variability effects for the Stem girth at Ilora (A) and Ikenne (B) as well as Plant height at Ilora (C) and Ikenne (D).

Table 2. Semivariogram, $Z(h_i)$ Values of stem girth and Plant height at Ilora and Ikenne.

Stem girth (Ilora)	Stem girth (Ikenne)	Plant height (Ilora)	Plant height (Ikenne)
5.55E-05	2.45E-05	1.61E-05	3.88E-07
3.48E-05	5.37E-05	2.69E-05	4.06E-05
1.97E-08	0.000223	3.57E-06	3.59E-05
1.97E-08	4.96E-05	3.88E-07	6.61E-05
1.44E-05	0.000297	6.04E-05	0.000551
1.33E-05	3.27E-06	8.37E-05	1.29E-05
9.56E-06	1.92E-05	2.14E-05	4.42E-06
1.97E-06	0.000215	0.000214	0.000332
1.78E-07	2.62E-06	6.08E-05	2.66E-05
7.11E-05	3.82E-05	2.52E-05	6.67E-06
0.000123	0.000133	2.78E-05	2.24E-07
6.4E-06	2.09E-05	1.16E-07	0.00024
1.23E-05	4.06E-05	2.29E-05	5.93E-08
0.000149	0.000751	5.29E-05	0.000158
0.000133	0.000355	2.15E-05	3.2E-07
0.000661	8.15E-06	0.000197	3.48E-06
9.67E-07	8.17E-05	2.02E-05	3.83E-05
6.64E-05	0	2.15E-07	0.000394
7.9E-06	1.6E-05	1.84E-05	7.77E-05
3.34E-06	3.15E-05	3.2E-05	6.73E-05
3.34E-06	2.17E-05	0.000366	4.71E-05
1.44E-05	0.000426	0.000601	0.000517
4.55E-05	4.56E-05	2.05E-05	9.52E-07

7.9E-08	4.44E-07	2.05E-05	1.22E-05
1.97E-08	0.00011	0.000349	0.000292
3.34E-06	7.01E-05	0.000216	1.01E-05
0.000102	6.09E-05	4.14E-05	1.32E-06
3E-05	5.87E-05	3.87E-05	4.21E-09
2.28E-05	0.000227	0.000115	2.06E-05
4.94E-07	0.000229	7.27E-07	0.000437
1.97E-06	0.000124	0.000162	0.000138
5.34E-05	2.74E-05	5.91E-06	5.11E-06
0.000149	0.000196	0.000144	1.02E-07
0.000432	0.000139	0.000117	2.35E-05
3.65E-05	9.6E-05	0.000213	5.03E-05

Spatial proximity matrices and nearest neighbour index

The spatial proximity matrix of the stem girth at Ilora station showed that magnificent percentage (31.5%) of the pairing neighbours were distinct from each other while the remaining 68.5% were similar to each other, (Appendix I). $x_{1,6}$, $x_{5,2}$ and $x_{5,6}$ were distinct from their neighbours because their proximity matrix returned zero values. However, these were not the case with the stem girth at Ikenne with 11.7% non similar plots. Also, there were some other non similar plots which include $x_{4,5}$ vs $x_{3,2}$, $x_{4,5}$ vs $x_{3,6}$, $x_{4,5}$ vs $x_{4,1,}$ $x_{5,5}$ vs $x_{5,2}$, $x_{5,6}$ vs $x_{4,5}$ and $x_{6,4}$ vs $x_{4,5.}$ Also not similar were $x_{6,5}$ vs $x_{2,2}$ and $x_{6,5}$ vs $x_{2,5}$ (Appendix II). Different trends were obtained for the similarity matrices of the plant height at Ilora where 17.4% of the pairing neighbours were not similar with each others, (Appendix III). There was no plot that was completely different from the others. More than 50% plots pairing with both plots $x_{1,2}$ and $x_{2,3}$ were dissimilar. All neighbouring plots of plant height at Ikenne station were similar and none was distinct from the others, (Appendix IV). The cluster analyses of the stem girth at Ilora showed that 0.8 of the pairing plots were isotropic in nature. This isotropic property was exhibited between the following pairs of plots;

Plots $x_{4,3}$ vs $x_{1,3}$ and $x_{6,2}$ vs $x_{5,3}$ = 0.044; $x_{1,4}$ vs $x_{4,3}$ and $x_{1,4}$ vs $x_{4,2}$ = 0.46; $x_{4,5}$ vs $x_{1,1}$ and $x_{5,6}$ vs $x_{6,2}$ = 0.051; $x_{6,6}$ vs $x_{5,6}$ and $x_{2,4}$ vs $x_{5,6}$ = 0.052; $x_{2,4}$ vs $x_{3,3}$ and $x_{3,2}$ vs $x_{2,4}$ = 0.053; $x_{3,2}$ vs $x_{5,1}$ and $x_{3,1}$ vs $x_{2,1}$ = 0.058; $x_{4,3}$ vs 14 and $x_{1,5}$ vs $x_{5,4}$ = 0.061. All other plot pairs are anisotropic in nature because their euclidean distances are not equal. For stem girth at Ikenne, isotropic property was exhibited between only $x_{4,1}$ vs $x_{3,1}$ and $x_{5,3}$ vs $x_{2,3}$ (0.032). All other plots pairs are anisotropic in nature. For plant height at both Ilora and Ikenne, none of the pairing plots exhibited isotropic property but anisotropic property. This is because none of the pairing plots cluster at the same Euclidean distances.

The nearest neighbour indices for the different parameters at both sites (Ilora and Ikenne) were calculated using the average interpoint distance-Ad between nearest neighbour, (Benwell, 2002). These inter point distances are 0.00411 (for stem girth at Ilora), 0.0153 (stem girth at Ikenne), 0.381 (plant height at Ilora) and 0.512 (plant height at Ikenne). The expected average inter point distance between nearest neighbour on the other hand is 2.083 for both sites and parameters.

The nearest neighbour indices are therefore;

Stem girth = 0.00197 (Ilora) and 0.00734 (Ikenne)

Plant height = 0.1831 (Ilora) and 0.2456 (Ikenne).

Appendix I. Similarity Matrix of Stem girth at Ilora Station.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1	1																																			
2	1	1																																		
3	1	1	0																																	
4	1	0	0	1																																
5	1	1	1	1	0																															
6	0	1	0	0	0	0																														
7	1	0	1	1	1	0	1																													
8	1	0	1	1	1	0	1	1																												
9	1	1	1	1	1	0	1	1	1																											
10	1	1	1	1	1	0	1	1	1	0																										
11	1	1	1	1	1	1	1	1	1	0	1																									
12	1	0	0	1	1	0	1	1	1	0	1	1																								
13	1	1	1	1	1	1	1	1	1	1	1	1	0																							
14	1	0	0	1	1	0	1	1	1	0	1	1	1	1																						
15	1	0	0	1	1	0	1	1	1	0	0	1	0	1	0																					
16	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1																				
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1																			
18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0																		
19	1	0	1	1	1	0	1	1	1	0	1	1	1	1	1	1	0	0	1																	
20	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	0																
21	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1															
22	1	0	0	1	1	0	1	1	1	1	0	0	0	1	0	1	0	0	0	1	1	0														
23	1	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1													
24	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	0	0	1	1	1	1	0	1	0												
25	1	0	1	1	1	0	1	1	1	0	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1											
26	1	0	0	1	1	0	1	1	1	1	0	1	1	1	1	1	0	0	0	1	0	1	1	0	0	0										
27	1	0	0	1	1	0	1	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	0	0	1	1									
28	1	0	0	1	1	0	1	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	1	0								
29	1	1	0	1	1	0	1	0	1	1	0	0	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	1	1							
30	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	0	1						
31	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	1	0					
32	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
33	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0	1	0	0	0	1	1	0	1	0	0	1	1	1	1	1	1	1	0			
34	0	0	0	1	1	0	1	0	1	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	1	0	1	0	1	1	1	1		
35	1	0	0	1	1	0	1	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	0	1	
36	1	0	1	1	1	0	1	1	0	0	1	1	1	1	1	0	0	0	1	1	1	0	1	1	1	0	0	0	0	1	0	0	0	0	1	1

Appendix II. Similarity Matrix of Stem girth at Ikenne Station.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1	1																																			
2	0	1																																		
3	1	0	1																																	
4	1	0	1	1																																
5	0	0	0	0	1																															
6	1	0	1	1	0	1																														

Appendix III. Similarity Matrix of Plant height at Ikenne Station.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1	1																																			
2	1	1																																		
3	1	1	1																																	
4	1	1	1	1																																
5	1	1	1	1	1																															
6	1	1	1	1	1	1																														
7	1	1	1	1	1	1	1																													
8	1	1	1	1	1	1	1	1																												
9	1	1	1	1	1	1	1	1	1																											
10	1	1	1	1	1	1	1	1	1	1																										
11	1	1	1	1	1	1	1	1	1	1	1																									
12	1	1	1	1	1	1	1	1	1	1	1	1																								
13	1	1	1	1	1	1	1	1	1	1	1	1	1																							
14	1	1	1	1	1	1	1	1	1	1	1	1	1	1																						
15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1																					
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1																				
17	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1																			
18	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1																		
19	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1																	
20	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1																
21	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1															
22	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1														
23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
24	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1												
25	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1											
26	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1										
27	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1									
28	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1								
29	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1							
30	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
31	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1					
32	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
33	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
34	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
35	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
36	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Appendix IV. Similarity Matrix of Plant height at Ikenne Station.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1	1																																			
2	0	1																																		
3	1	1	1																																	
4	1	1	1	1																																
5	1	1	1	1	1																															
6	1	1	1	1	1	1																														
7	1	1	1	1	1	1	1																													
8	1	0	1	1	1	0	1	1																												
9	0	0	0	1	0	0	0	0	1																											
10	1	1	1	1	1	1	1	0	0	1																										
11	1	0	1	1	1	1	1	1	0	1	1																									
12	1	0	0	1	0	1	0	1	0	0	1	1																								
13	1	1	1	1	1	1	1	0	0	1	1	0	1																							
14	1	0	0	1	1	1	1	0	1	0	1	1	0	1																						
15	1	0	0	1	0	0	1	0	1	0	1	1	0	1	1																					
16	1	0	0	1	0	1	1	1	1	0	1	1	0	1	1	1																				
17	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1																			
18	1	0	0	1	0	0	0	1	1	0	1	1	0	1	1	1	1	1																		
19	1	0	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1																	
20	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1																
21	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	1	1															
22	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1														
23	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1													
24	1	1	0	1	0	0	0	0	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1												
25	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1											
26	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1										
27	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1									
28	1	0	0	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1								
29	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1							
30	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1						
31	1	1	1	1	1	1	1	1	0	1	0	1	1	0	0	1	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1					
32	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
33	1	1	1	1	1	1	1	0	0	1	1	0	1	0	0	1	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1			
34	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
35	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
36	1	0	1	1	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	1	1

Discussion and conclusion.

It is obvious that the variogram does not follow a definite pattern hence, could be classified as zero nugget effects. Also, the variogram is noticeably not a function of the size of the values of the variable under consideration but may be function of both the number of the lag period returning negative as well as positive signs in addition to the size of the data. The practical range as obtained from the semivariogram implied that the spatially correlated data exist at almost around the same place regardless of the zones of the experimentation (that is Ilora or Ikenne). The variogram according to Doncker *et.al.*, (2006) can be related to the variance covariance using the model, $\gamma(h) = C(0) - C(h)$ (where $\gamma(h)$ = variogram, C(0) is the variance at the plot x_i and C(h) is the covariance at both plot x_i and x_j). In addition, pattern

of neighbourhood differs across sites. This might be a reflection of the spatial distribution of the soil or other site condition. Pattern of nearest neighbour from the nearest neighbour indices differs across the different growth variables. This may be hinged on the fact that the different growth variables are enhanced as well as inhibited by different nutrients and factor. The use of nearest neighbour analysis in the spatial variability measurements are often and widely discussed in geography and ecology due to dynamics of natural process. The original and formal idea of neighbourhood is restricted to object sharing common boundaries. This nearest neighbour has thus been incorporated into experimental agriculture where experimental activities are also based partly on natural phenomenon. Conceptually, the clear message is that randomness of spatial pattern does not imply lack of neighbourhood. Meanwhile, the three types of spatial pattern as identified by nearest neighbour index depicted different types of neighbours. These are: when similar plots share the same boundary; similar plots does not share the same boundary and when similar plots does not exist in the experimental plot. The low but positive nearest neighbour index as in this work implied that the neighbourhood pattern falls between cluster and randomness thereby reflecting patchiness.

Based on these therefore, nearest neighbour analysis is recommended to be carried out along with the preliminary investigation of the experimental plots. This would guide in the distribution of sampling points for preliminary investigation. The effects of plant type/plant species on the spatial variation in experimental plots is hereby recommended for further study.

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References

Benwell, G.L. McLennan, Grasberger, T. and Fryer, J. Spatial Data Analysis for Aboriginal Rock Extraction Sites at Brewarrina, NSW, Australia. Presented at the 14th Annual Colloquium of the Spatial Information Research Centre held at Victoria University of Wellinghton, Wellinghton, New Zealand between December 3rd – 5th 2002,pp 11.

Christakos, G., and Hristopulos, D.T. Spatio Temporal Environmental Health modeling: A Tractus Stochasticus. Dordrecht, the Netherland: Kluwer Academic Publisher, 1998; Pp 451.

Critoru, C.A. and Doytsher, D.Y. Random Field and (Marked) Point process: A Pratical Comparison of Two Stochastics Models – Proceedings of Joint Workshop on Multiscale Representations of spatial Data held in Ottawa, Canada, July, 7th -8th 2002;1-9.

Doncker, P.H., Dricot, J.M., Mey, R., Helier, M. and Tabbara, W. Electromagnetic Fields Estimation Using Spatial Statistics. Electromagnetcs, 2006; 26. 111 – 122.

Fagroud, M. and Meirvenne, M. V.. Accounting for Soil spatial Autocorrelation in the Design of Experimental Trials. Soil Science Society of Americal Journal. 2002; 66: 1134 – 1142.

Hardy, O.J. and Sonke, B.. Spatial Pattern analysis of Tree Species distribution in a Tropical Rainforest of Cameroon: Assessing the role of limited dispersal and niche differentiation in Forest Ecology and Management, 2004; 197; 191 – 202.

Purves, R.S., Morrison, K.W., Moss, G., and Wright, D.S.B. Nearest neighbours for avalanche forecasting in Scotland – development, verification and optimization of a model. In cold Region Science and Technology, 2003; 37 (3) 343-355.

Schanbenberger, O. and Pierce, F.J.. Contemporary Statistical Models for the Plant and Soil Science.Washington, D.C; CRC Press, 2002; Pp 738.

Singh, A., and Ganju, A. A Supplement to nearest-neighbour method for avalanche forecasting. In cold Region Science and Technology, 2004; 39, 2-3 pp 105-113.

Weigelt, A., and Jolliffe P.. Indices of Plant Competition. In Journal of Ecology, 2003; Vol 91 Pp 707-720.

Xin X, Shaw S. and Lin H. Developing a Spatio – Temporal Data Model for an exploratory Study on the Land use-Transportation interaction A temporal GIS Attemp- Being a report of a Project Sponsored by Florida Department of Transportation's University Research Programme (www. document URL http:/www.amc - on 23/02/2005. Pp 16.

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QUANTITATIVE SPECIFICATION OF POTENTIALLY TOXIC METALS IN EXPIRED CANNED TOMATOES FOUND IN VILLAGE MARKETS

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Abstract: Varieties of expired canned tomatoes were pre-treated using standard digestion methods and were analysed for heavy metals. Cr, Pb, Cd, Fe, Ni, Co, Zn, Mg, Cu, AI and Mn were determined using Atomic absorption spectroscopy and photometry techniques. Mg, Mn, Co and Pb presented higher concentration values ranging from 32.18± 9.25; 4.35 ±1.60; 2.62 ±1.76 and 2.82 ±0.53 μgg^{-1} respectively. Unlike the Cd contents, Cr and Pb concentration were above the threshold limit values (TLV) of 2.0μgg^{-1}. The levels of metals for some of the canned foods exceed that of their corresponding uncanned products reported in literatures. Physicochemical variables of the brands were also estimated as 76.4 ±3.85 and 3.20± 1.09 % for moisture and ash contents respectively. The arrays of health implications of heavy metals computed in this work will at a glance access the roles of excessive and prolonged intake of such foods.

Key words: canned tomato, toxic metals, AAS, Photometry. [Nature and Science. 2010;8(4):54-59]. (ISSN: 1545-0740).

1. Introduction.

Knowledge of transport and accumulation of potentially hazardous metals in the ecosystem forms the central theme of canned food contamination by heavy metals. Heavy metal is a term, given to the group of metals and metalloids with atomic density greater than 5g/cm^3, usually associated with pollution and toxicological problems (Abdulrahman and Itodo, 2006). ATSDR,(1993), stated that "heavy metals" are a group of metals and semimetals associated with contaminations and are potentially toxic. Based on these definitions and observations, heavy metals are therefore classified as essential (if they play basic role as components of vital biochemical or enzymatic activities in human body e.g Fe, Mn, Mo, Cr, V, Zn) and as non-essential (if the metals are classified as with no biological, chemical and physiological importance in man (Itodo *et al.*,2009). The role played by industrial processes and emissions cannot be over emphasized. The ingestion of accumulated trace metals from canned food by man poses health hazard such as skin irritation, damage to the liver, kidney, circulatory and nerve tissues, resulting from acute or chronic exposure (Adekunle *et al.,* 2003).

The soil is the main source of metals for plants. These metals readily occur in mining areas. Other sources of these metals are natural occurrence such as earthquake, volcanic eruption, and mans activities including dumping of wastes and agricultural activities (pesticides, herbicides etc) are contributing factors (Ademoroti, 1995). Pollution of streams and rivers through agricultural effluent discharges are sources of polluting the water bodies (Fodeke and Fisher, 1989). The neurological aspects of poisoning from many metals indicate the nervous system as target organ with respect to metal toxicity. Other target organs are gastrointestinal tract, respiratory tract, blood, and kidney, bone, nails, hair, endocrine (ATSDR, 1993). When the concentrations of the metals are beyond the tolerable limit, they become toxic. The tolerable limit of some metals in drinking water given in mg/l are 0.01, 0.001, 0.003, 3.00, 0.01, 0.05, 0.05, and 1.2 for Hg, Cd, Zn, Sn, Se, As, Cr(vi), and Cu respectively (WHO, 1971). Others are Fe 0.3 µg/g, Mn (0.1-0.5mg/l) WHO values (Ademoroti, 1995).

Table 1: Some heavy metals and their target organs in man.

Heavy Metals	Target Organs	Organ screened for Medical test
Aluminum	Bone, brain, Kidney and stomach	Blood, Urine, hair, faces and fingernail
Arsenic	Most organ of the body, especially Lungs, skin, gastrointestinal system	Urine, hair and finger nails
Cadmium	Brain, heart, blood vessel, kidney and lungs	Blood, urine.
Lead	Bones, brain	Blood, urine and hair
Copper	Lungs, skin and gastrointestinal tract, kidney and bones	Blood, urine, hair and finger nails
Mercury	Gastrointestinal tract, Brain, kidney and lung	Urine, blood, hair and finger nails

Sources: Abdulrahman and Itodo, 2006; ATSDR, 1993; Itodo *et al*., 2009.

Food canning implies the storing of food in airtight containers. The food is preheated to bring about the destruction of organisms .The lethal effect of heat on micro organism has been used for food preservation long before the microbial causes of food spoilage was discovered (Ngoddy and Ihekomonye, 1992).Canning of food product is the sealing of the food products after heat treatment. Canning became necessary for the following reasons: To free the food from micro organism capable of causing spoilage, or which is of public health significance e.g *Clostridium botulinum.*To keep the product clean and produce a barrier against dirty and other contaminants. It is a means of preserving and circulating excess food produced over a long period of time.Finally, perishable foods of economic values are better secured in sealed cans for import and export practices or distributions (Ngoddy and Ihekomonye, 1992).

Chemistry of Tin Plates Canning

Tin exists in two oxidation states, as divalent tin Sn^{2+}, tin (II) and as tetravalent tin (IV). Dissolution of metallic tin from a can body into the food contents will result in it, being present in the divalent form. One of the factors affecting its presence is pH. At pH>2, tin forms $Sn(OH)_2$ which has low solubility . Other chemicals which may also be present in food stuffs and known to complex with tin are alcohols and high fatty acids ,citric, tartaric and oxalic acids (Steve and Tony, 2003) Reactions involving the reducing properties of tin can also occur. Therefore, the bioavailability of potentially toxic tin in food depend on the quantity of food ingested and pH, oxidation state, extent of complexation or adsorption, and solubility. Tin is not absorbed after ingestion and its toxic responses may be due to gastrointestinal irritation and not systemic poisoning (Steve and Tony, 2003).Storage conditions such as temperature, affect the rate of dissolution of tin into canned food. After detinning, delaminating and leaching of alloying metals into the contents will then results (Itodo *et al*., 2009). This work was embarked upon to estimate the heavy metal concentration of various canned tomatoes marketed in villages within Nigeria for the purpose of: Providing data for future compilation of Nigerian food consumption table and for use by the Nigerian Standard Organizations and regulatory bodies.

2. Materials and Methods

Five varieties of canned tomato products were randomly bought from villages around Sokoto town. The considerations for sampling were manufacturing dates, place of manufacture, net weight of sample, company and brand names (not disclosed), NAFDAC registration numbers, ingredient and other information provided in Table 2 below.

Table 2: *Sampling and Nutritional Data of Various Canned Tomatoes Nov., 2005.*

Tomato Samples	Net wt.	Man. Date	Exp. date	Shelf life (months)	Food duration in cans	Ingredients	NAFDAC Number
C1	70g	June 2003	June 2005	24	5 month	Tomato, salt	01-5188
C2	70g	May 2004	April 2005	12	6 month	Tomato, salt	NS
C3	70g	May, 2003	May, 2005	24	6 month	Tomato, salt	NS
C4	70g	June, 2003	June 2005	24	5 month	Tomato, salt	NS
C5	70g	Dec. 2003	Dec. 2004	12	11 month	Tomato, salt	NS

Methods by AOAC, (1990); Abdulrahman and Itodo,(2006) and Fodeke and Fisher,1998 were modified and adopted as described below for sample treatment prior to analysis.

Nitric Acid – Hydrogen Peroxide (HNO_3/H_2O_2) Method

Already washed and dried digestion tubes were weighed and labeled to ± 0.5g. The samples were opened and the contents were homogenized after which 3 ± 0.01g samples were accurately weighed out into each digestion tube. The samples were first acidified with 2M HNO_3 and swirled to mix properly before evaporating to dryness. 25 cm^3 concentrated HNO_3 was added to the residue in the digestion tube and was heated to boiling. Addition of 25 cm^3 conc. HNO_3 was repeated with small quantity of H_2O_2. The digestion was completed by repeating the procedure until the content in the tubes became brown, pink or colorless. 5 cm^3 of water was added to the digest and was allowed to cool, followed by filtration using the Whatman filter paper No.42. (Ademoroti 1996).A Unicam 969 AAS was set up according to manufacturers' instruction with the wavelength corresponding to that of the element under investigation. The spectrometer was set to zero absorbance using the blank solution. The absorbance of each sample was read with an automatic calculation of the average (μgg^{-1})

Nitric Acid – Sulfuric Acid (HNO_3-H_2SO_4) Method

The homogenized samples were weighed into the digestion tubes. 10 cm^3 conc. H_2SO_4 and 5 cm^3 conc.HNO_3 were added. The sample was digested and its volume was reduced to 2cm^3. The digestion was continued until the solution was colorless. This ensured the removal of all HNO_3. Sample was allowed to cool and 15 cm^3 of water was added with gentle swirling. 1M NaOH was added dropwise until a pink tinge, brown or colorless solution was produced. The solution was filtered using a Whatman filter paper No.42 followed by dilution to the mark in a 25 cm^3. Volumetric flask.

Procedure for Photometric Analysis

The machine (Windaus L.F. 2400 photometer) was set up according to manufacturer's instruction with the wavelength corresponding to that of the element under analysis. The photometer was set to zero using the 0 mg /cm^3 solution. The absorbance of each sample in the sample cell was measured in duplicate.

3. Results and Discussions

Physicochemical Parameters: Table 3 presents the physicochemical variables for the sampled canned tomato. This is to appreciate the role of certain parameters that could possibly enhance the delaminating of tin coatings and subsequently, its detinning. Results on table 3 indicates the acidic nature of the products (mean pH value of 3.69 ± 0.32) and high moisture contents (76.4± 3.85 %),which could induce tin plate corrosion when combine with factors like storage temperature, air space in poorly sealed cans, and protein content (8.90 ± 2.17%) which could be responsible for content denaturing (Ngoddy and Ihekomonye, 1992)

Table 3. pH, Conductivity ,Protein, Moisture, Ash and Organic Matter Content of Canned Tomato Products

Canned Tomato Products	Physicochemical Variables					
	pH	Conductivity (µs/Cm)	Protein content (%)	Moisture content (%)	Ash Content %	Organic solid %
C1	4.10	1.00	10.38	74.00	2.00	24.00
C2	3.70	1.00	9.63	78.00	4.00	18.00
C3	3.40	1.00	9.56	76.00	4.00	20.00
C4	3.36	1.00	5.06	82.00	2.00	16.00
C5	3.92	1.00	9.87	72.00	4.00	24.00
Mean	3.69	1.00	8.90	76.4	3.20	20.40
±	±	±	±	±	±	±
S.D	0.32	0.00	2.17	3.85	1.09	3.58

Metal Analysis: The results of determination of metals (µg/g) are presented in Tables 4. Data generated were presented as mean ± standard deviation. These results were compared with those of selected canned products using histogram of the frequency distribution (Fig.1). From analysis, sample,C1 has the highest mean value of iron content of 4.30μgg^{-1} and 9.50 μgg^{-1} using AAS and photometric analysis respectively. Metals such as Cd and Pb in any concentration can pose health hazards. On the other hand, Cr, Cu, Fe, Zn and Mn are essential for human health. However for these

metals to be essential, there are allowed levels for adequate dietary intake. For adults, the intake can range from 0.50 – 2.00µg Cr(iii), from 1.2 – 3.0 mg (Cu) from 10.0 – 50.0mg (Fe), from 5.0 –22.00mg (Zn) and 2.0 – 20.0mg Mn. (WHO, 1996). Cr (iv) penetrate cell membrane while Cr (III) does not. Thus, Cr(IV) may cause genotoxic effect and cancer whereas Cr(III) cannot (WHO, 1996). The mean range for values of Cd contents ($0.04\pm 0.02 \mu g g^{-1}$ using AAS and $0.05 \pm 0.32 \mu g g^{-1}$,using photometer) is low compared to that of any other metals analysed. Cadmium concentration is usually low in canned food (Kent *et al.,* 2003). Cd concentration could be traced to contaminated waste water into river with subsequent flow through the food chain.

The lead content in canned food depends on the method used to seal the can. A better option is the use of welded or lacquered cans with low lead content (Kent *et al.,* 2003). Mean lead value ($2.82 \pm 0.53 \mu g g^{-1}$) was obtained for tomato products. Its availability could be linked to contamination of food by lead when tomato is cooked in casserole vessels before canning (Kent *et al.,* 2003). A blood lead level greater than $1.0 \mu g/cm^3$ is dangerous to health (Adekunle, 2003).

Table:4. Concentration of Heavy Metals in Various Canned Tomato Products

HEAVY METAL CONCENTRATION ($\mu g g^{-1}$)																
	(I) AAS								(II) Photometric Analysis							
Canned tomato	**Cr (i)**	**Pd (i)**	**Cd (i)**	**Fe (i)**	**Ni (i)**	**Co (i)**	**Zn (i)**	**Mg (i)**	**Cr (ii)**	**Pd (ii)**	**Cd (ii)**	**Fe (ii)**	**Ni (ii)**	**Cu (ii)**	**Al (ii)**	**Mn (ii)**
C1	3.15	3.35	0.03	4.30	4.40	3.90	0.90	32.59	1.25	4.00	0.85	9.50	1.85	5.25	0.85	6.00
C2	5.55	1.95	0.4	3.80	2.95	3.80	2.70	26.70	1.20	4.25	0.50	5.50	1.25	3.00	0.55	5.00
C3	3.90	2.80	0.09	3.85	4.10	7.00	3.45	47.00	0.85	6.25	0.85	5.50	1.15	2.50	0.95	5.25
C4	3.05	3.05	0.06	3.40	2.70	2.85	2.65	32.15	0.40	5.60	0.10	2.75	2.66	2.75	0.20	2.00
C5	3.50	2.95	0.06	1.95	3.70	2.55	5.70	22.55	0.50	6.88	0.40	4.00	1.35	3.50	0.55	3.50
Mean ± S.D	3.83 ± 1.01	2.82 ± 0.53	0.04 ± 0.02	3.46 ± 0.90	3.57 ± 0.73	2.62 ± 1.76	3.08 ± 1.74	32.18 ± 9.25	0.85 ± 0.40	5.40 ± 1.25	0.54 ± 0.32	4.81 ± 2.53	1.65 ± 0.62	2.09 ± 1.10	0.62 ± 0.29	4.35 ± 1.60

Comparative study.

Figure 1 presents the amount of Cr (in µg/g) for canned tomatoes as compared to those for various canned foods and drinks using AAS and photometric method. The AAS result obtained for tomato ($3.38 \pm 1:01 \mu g g^{-1}$) is by far higher than those of others, especially canned drinks (0.52 ± 0.25 to 0.86 ± 0.56 for juice and beer respectively

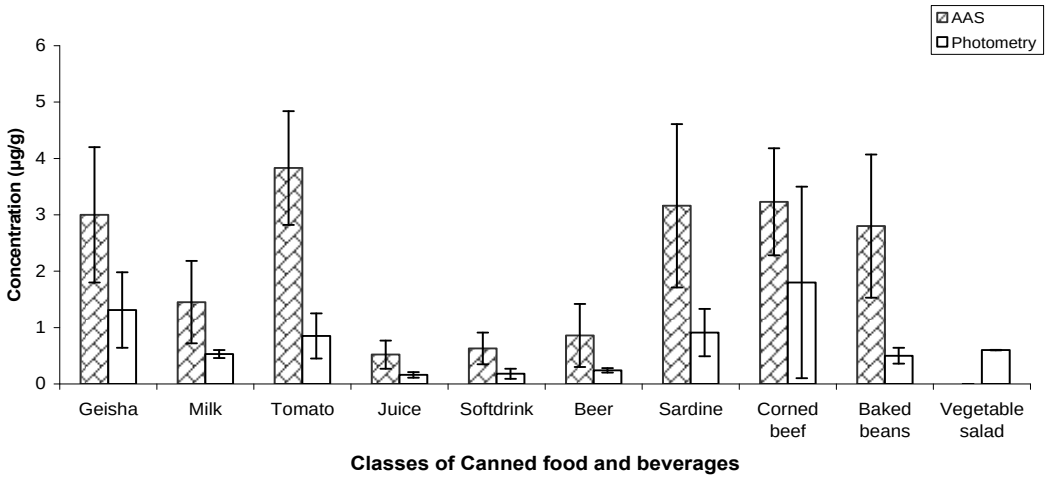

Figure 1: The concentration of Chromium in canned tomatoes as compared to various canned foods and beverages

The investigated products in this work have uncovered the fact that canning of semi – solid food and drinks is a menace, with an usually higher concentration of the toxic metals (Mn, Pb, Cd and Cr) especially when the cans are not lacquered. The researcher is uncomfortable with this high level. Therefore the contents of the containers need be checked by appropriate government regulatory bodies. Food manufacturers should avoid the use of acidic water with low pH, such foods are better bottled or paper packaged than canned. We also recommend that;

- Excessive heating and lead – soldering should be avoided.
- Shelf life should be reduced to avoid oxygen intake by corroded container,
- pH should be adjusted to values between 5.5 – 8.5 coupled with the use of internally lacquered containers or packaging material made up of glass, paper and polymers.

A critical observation of the results also revealed that metals originating from mined products and their corrosion and leaching into food is critical for assessing semi – liquid food and beverages. In the same fashion, the attenuation of heavy metals and their concentrations in canned food varies depending on the type and origin of food, pH of the medium, oxygen and carbon dioxide concentration in the headspace, quality of the inside lacquer coating of cans, storage place, temperature.

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REFERENCES

1.Abdulrahman F W , Itodo AU. Canned fish poisoning: High level of some toxic metals. Medical and Pharm .sciences 2006; 2(1):10-14.

2.Adekunle J, Ndahi J, Owolabi D. level of some Hazardous trace metal and simulated blood lead from high way soil of South – west Nigeria. International Journal on Environmental issue 2003;1 (1): 44-48.

3.Ademoroti CMA. .Environmental Chemistry and toxicology". 1st Edn. Fodulex Press Ltd Ibadan 1994; 61-67.

4.Alka S. Environmental Biochemistry A Textbook of Medical Biochemistry Brothers Med. Pub. Ltd New Delhi. 2000; 444-467.

5.AOAC.- Association of official Analytical Chemists. Official Methods of Analysis" 15th edn. Washing D.C, USA 1990; 69-88.

6.ATSDR - Agency for Toxic Substances and Disease Registry. Toxicological profile for Heavy Metals. US Department of health and Human Service 1993. Retrieved on 10/11/2004 from http//www.atsdr.com

7.Fodeke V, Fisher A. Studies on Heavy Metal Contamination of Sarotheradon (Ruppel from Lagos lagoon Journal Nigeria Natural science1989; 4 (1 and 2): 46-52.

8.Itodo .AU, Abdulrahman FW, Happiness UO, Abubakar MN. corrosion impact on the leacheability of heavy metal ions in canned juices and beverages marketed in Nigeria . Research in Sc.,Edu,Info. And Comm tech. 2009; 1 (1),164

9.Kent C. Canned Fruit. 2003.Rretrieved on 30/10/04 from www.tradingstrandard.gov.uk.

10.Steve B, Wallace T. Tin in Canned Food. Food and Chemical Toxicology 2003; 41: 1651- 1662.

11.WHO – World Health organization. "Health criteria other supporting information in: Guideline for drinking water quality, Geneva 1996 ; 2 (1):31- 388.

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THE EFFECT OF DIFFERENT CONCENTRATIONS OF GINGER ON THE QUALITY OF SMOKED DRIED CATFISH (*Clarias gariepinus*)

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Abstract: Fresh, live catfish (*Clarias gariepinus*) were obtained from Private pond in NIFFR, New-Bussa. The samples were divided into five groups. Four groups were dressed and dipped in a solution of 2.5%, 5%, 7.5% and 10% of Ginger respectively for thirty (30) minutes and smoked dried. The fifth group acts as control. They were examined microbiologically, chemically and organoleptically. The Ginger reduced the free fatty acid (FFA) values, trimethylamine (TMA) values, and the fungi load of the processed fish. Ten percent of ginger had the best result in terms of reduction in fungi load, FFA and TMA values and followed by 7.5 and 5%. However, from the organoleptic results of overall acceptability, taste, colour and texture of the products, 5% ginger concentration had the best acceptance and significantly different ($P<0.05$) when compared to the non treated control after 8 weeks of storage. [Nature and Science. 2010; 8(4), 59-63] (ISSN: 1545-0740)

Keywords: Catfish, Ginger, smoked, storage and Fungi load.

1. INTRODUCTION

World fish production was estimated at 100 million tons in 1989, 15% of which was cured in one or another way. One third of the cured fish was smoked and about 20% of the smoked fish goes into international trade. Smoking of fish and/or meat products is one of the most ancient processing technologies. It has been for centuries used for preservation, and is still widely used for this purpose among several communities in the third world where up to 70% of the catch is smoked for preservation (Ward, 1995). In industrialized countries, however, smoking of fish is done for enhancement of flavor and texture (Dillon *et al*., 1994); often producing value added products whose preservation is achieved by other means. Nutritionally, fish proteins are noted for a high degree of digestibility and as a rich source of lysine and sulfur containing amino acids. Therefore it is suitable for complementing high carbohydrate diets especially in developing countries (Kent, 1984). Much attention is being directed at fresh water fish because of its health benefits, as a result of the presence of omega-3-fatty acids in the fish oil (Vileg and Body, 1988; Negbenebor, 1990). The reduction of these losses can only be achieved by systematic improvements in handling, processing, storage and distribution (FAO, 1990).

Catfish live in both freshwater and saltwater; however, the species cultured by fish farmers are raised in fresh water. About 1,250 species of catfish exist; and attracts more value than other fish species of its size (Lee, 1991). The common species are usually differentiated by colour and arrangement of external features. The most prominent feature is the fins. *Clarias gariepinus*, one of the species of catfish is a highly nutritious fish that contain high amount of vitamins, proteins, minerals and a little or no saturated fat, and is low in carbohydrates. Smoked catfish may have some appeal as a special catfish product. Spices such as Ginger are grown locally and have been known to enhance aroma and flavor of foods (Purseglove et al., 1981). Such spices like ginger could also have anti-microbial properties. The concentration of the ginger to be used should be effective and acceptable by consumers by actually enhancing the quality of such processed fish products.

The objective of this study is therefore to investigate the percentage concentration of ginger that will be very effective microbiologically and at the same time acceptable by consumers.

2.0 MATERIALS AND METHODS

Collection of sample

Fresh live catfish samples *(Clarias gariepinus*) were obtained from Private fish pond in National Institute for Freshwater Fisheries Research (NIFFR) New Bussa, Niger-State. However, the garlic and ginger were obtained from Monday market, New-Bussa.

2.1 Preparation of the samples

The Ginger samples were ground using a hammer mill, wrapped in aluminum foil and autoclaved at a 121^{o}C for 15 min and plated out on Nutrient agar and Sabouraud dextrose agar to ensure there was no mould and bacterial growth.

Twenty-five fish samples with weight ranges from 170-210g each were selected for each of the seven groups. The fish samples were headed gutted and cleaned with water. The first, second, third and fourth group was soaked in 2.5%, 5%, 7.5% and 10% Ginger solution respectively. The fifth group however, was soaked in Sterile distilled water for 30mins at ambient temperature (29-35°C). The samples were smoked dried according to the methods described by Omojowo and Ibitoye (2005). The samples were submitted to microbiological, chemical and sensory scores following processing.

2.2 Microbiological Analyses

The Fungal count were evaluated according to the methods described by Harrigan and McCance 1976; Speck 1984 and Sneath *et. al*., 1986). All samples were done in triplicates. Sensory evaluation was carried out according to the method of Afolabi et. al. (1984).

2.3 Chemical Analyses

The trimethylamine (TMA) values were measured by the AOAC (1984) method, while the free fatty acid (FFA) was determined using the method of Pearson (1981). The pH values were determined directly by using the pH probe (Negbenebor *et al*., 1995).

2.4 Sensory Evaluation

A taste panel of ten members made of Staff of National institute For Freshwater Fisheries Research (NIFFR), New-Bussa. They rated the samples for color, texture, taste and overall acceptability using a hedonic scale of 1- 5 with 5 representing "like much" and 1 representing "dislike much"(Afolabi, *et al.,* 1984).

2.5 Statistical Analyses

Statistical analysis was according to SAS, Institute, Inc, (1992) at $P < 0.05$.

3.0 RESULTS AND DISCUSSION

The Fungi count of the Fresh treated samples ranged from mean log 1.65 to 2.28 Cfu/g while the control is 2.76 Cfu/g. Also the treated smoked samples ranged from 0.70 to 1.35 Cfu/g on Day 0 as against the 2.30 Cfu/g of the control (Table 1). The 5% ginger concentration is significantly higher than 2.5% at $P < 0.05$. Likewise, both 7.5% and 10% which are not significantly different from each other at $P > 0.05$ are both significantly different from 5% respectively. This result shows that the higher the concentration of ginger the higher the antifungal effects. This result agrees with earlier results of (Negbenebor, *et.al.,* 1996) where clove and ginger individually and in combination reduced the fungal loads of smoked fish. This results also indicates that the ginger which is natural spice clearly have anti fungal properties that can compare with synthetic antimicrobial agents like Potassium sorbate, Citric acid and Sodium metabisulphite which antifungal agents as reported earlier (Omojowo *et. al*., 2008, Omojowo *et. al.*, 2009a, Omojowo *et. al.*, 2009b). Samples treated with both 7.5% and 10% ginger had no detectable mould growth after four weeks of storage. Mould rather than bacterial growth is the major problem in this type of product because of its low water activity (Negbenebor et al., 1995; FAO, 1992). The ability of the ginger to inhibit mould growth would in a way enhance the over-all quality of the product.

Table 1. The Effect of Different Concentration of Ginger on the Fungi count (Cfu/g in Log10) of smoked Catfish during storage.

Duration (Week)	Samples	TREATMENTS				
		Control	2.5% Ginger	5% Ginger	7.5% Ginger	10% Ginger
	Fresh	2.76±0.05a	2.28± 0.04b	2.10± 0.04b	1.92± 0.04c	1.65± 0.04c
Day 0	Smoked	2.30±0.03 a	1.35± 0.03b	1.21± 0.04c	0.84± 0.03d	0.72± 0.10d
Week 2	Smoked	2.18±0.03 a	1.38± 0.04b	1.14± 0.06c	0.45± 0.01d	0.32± 0.03d
Week 4	Smoked	2.24±0.07 a	1.50± 0.03b	1.08± 0.02c	0.42± 0.05d	0.36± 0.03d
Week 6	Smoked	4.28±0.04 a	1.64± 0.01b	1.30± 0.03c	ND	ND
Week 8	Smoked	6.02±0.06 a	1.83± 0.12b	1.46± 0.21c	ND	ND

Means in the same rows with different letters are significantly different ($p < 0.05$).
ND = Not Detected

3.1Trimethylamine Value

The trimethylamine (TMA) value of the fresh fish sample was 15.43 mg N/100 g. following processing the TMA values of the treated samples were significantly (P<0.05) lower than that of the non-treated controls, and remained so after 2 months of storage at ambient temperature (29-35°C) (Table 2).

Table 2. The Effect of Different Concentration of Ginger on Trimethylamine (mg N/100g) Value of Smoked Catfish Samples.

Duration (Week)	Samples	TRIMETHYLAMINE VALUES (mg N/100 g)				
		Control	2.5% Ginger	5% Ginger	7.5% Ginger	10% Ginger
	Fresh	15.50a	15.52a	15.41a	15.24a	15.27a
Day 0	Smoked	12.76a	9.67b	8.56c	8.45c	8.45c
Week 2	Smoked	10.57a	7.45b	6.24c	5.50d	5.43d
Week 4	Smoked	8.65a	6.43b	6.35b	5.12c	5.04c
Week 6	Smoked	8.22a	5.48b	5.37b	4.10c	4.02c
Week 8	Smoked	6.69a	3.43b	3.39b	3.28b	3.26b

Means in the same rows with different letters are significantly different ($p < 0.05$).

This result suggests that all the concentration of ginger inhibited the production of TMA from trimethylamine-oxide (TMAO) (Jay, 1987). However irrespective of treatment there was a decrease in TMA values of all samples after 7 weeks of storage at room temperature. This may be related to the high temperature and low relative humidity leading to the decrease in water activity, microbial activity and hence decrease in TMA values (Jay 19870). This result agrees with earlier results of (Negbenebor, *et.al.,* 1996) where 2.5% clove and ginger individually and in combination reduced the TMA values. Storage time seems to have more significant effects on the TMA values since at the eight weeks of storage there was no significant difference at P< 0.05 in TMA values of the treated samples.

3.2 Free Fatty Acids (FFA)

Following treatment the FFA values in the fresh fish ranged from 0.18-0.30% and was not significantly affected (P>0.05) by treatment (Table 3). However following smoke drying there was an increase in FFA values of all samples irrespective of treatment. However the control samples showed higher FFA values and it is significantly different (P<0.05) when compared with the treated samples. Results suggest that the various concentration of ginger used in this experiment inhibit FFA production. The FFA content in a product is an indication of the quality of the product (Clucas and Ward, 1996).

Table 3. The Effect of Different Concentration of Ginger on the Free Fatty Acid (FFA) of Smoked Catfish Samples.

Duration (Week)	Samples	FREE FATTY ACID				
		Control	2.5% Ginger	5% Ginger	7.5% Ginger	10% Ginger
	Fresh	0.30±0.05a	0.22±0.06 a	0.18±0.02a	0.21±0.03a	0.20±0.01a
Day 0	Smoked	4.30±0.03 a	3.66±0.02 b	3.60±0.03 b	3.51± 0.05b	3.50±0.03 b
Week 2	Smoked	4.28±0.03 a	3.64±0.01 b	3.59± 0.04b	3.52±0.12 b	3.38±0.05 b
Week 4	Smoked	4.24±0.07 a	3.51±0.03 b	3.60±0.07 b	3.32±0.03 c	3.25±0.01 c
Week 6	Smoked	4.68±0.04 a	3.57±0.06 b	3.46±0.04 b	3.12±0.02 c	3.05±0.03 c
Week 8	Smoked	3.82±0.06 a	2.34±0.04 b	2.39±0.01b	2.12±0.12 b	2.38±0.05 b

Means in the same rows with different letters are significantly different ($p < 0.05$).

3.3 Organoleptic Analysis

In the freshly smoked samples treated with 2.5% ginger was not significantly (P>0.05) different from the control in terms of taste, colour, and texture (Table 4). However the samples treated with 2.5% ginger was slightly more accepted than the control. In all the treated samples however, the 2.5% ginger is significantly rated higher (P<0.05) than the 5%, 7.5% and 10% respectively in the overall-acceptability. In the eight week, the control was already covered with moulds

Table 4. Organoleptic Attributes of Freshly Smoked and 8th Week Stored Catfish Treated with Different concentration of Ginger.

Treatment	Taste	Flavour	Texture	Appearance	Overall-acceptability
CONTROL	4.5a	4.4a	4.7a	4.5a	4.5a
FRESHLY SMOKED-2.5%	4.6a	4.5a	4.7a	4.6a	4.6a
5%	3.7b	3.8a	3.6b	3.7b	4.0b
7.5 %	3.2c	2.3b	3.0c	3.7b	2.4c
10 %	1.9d	1.9c	2.8c	3.6b	1.8d
CONTROL (8^{TH} WK)	**	**	**	**	**
8^{TH} WEEK OLD - 2.5%	4.2a	4.2a	3.9a	4.0a	4.1a
5%	4.3a	4.2a	4.0a	4.1a	4.3a
7.5%	3.7b	3.6b	3.5b	4.0a	3.0b
10%	1.8c	2.0c	3.8b	3.8a	2.3c

Means in a column with unlike letters differ significantly (P<0.05), **= Moldy, hence not tasted

4.0 CONCLUSION

The dipping of fish in a concentration of ginger before smoking has beneficial effects on the overall quality of the final products. This in a way will not only reduce the substantial losses associated with this type of product estimated at billions of naira but would also increase the rate of turn over as consumers would now find increased satisfaction with the processed fish as indicated by the sensory quality of the product. This would substantially improve fish protein intake in Nigeria and reduce protein malnutrition and its associated problems in the country.

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REFERENCES

[1] AOAC. Association of Official Analytical Chemists. Official Methods of Analysis 14th Edition, Washington D.C. 1984

[2] Adeniyi JP. Fish consumption in Nigeria. Implications for fishery development policies. J. West African Fisheries. 1987: 3(2):151-161.

[3] Clucks IJ, Ward AR. Post harvest Fisheries Development: A guide to handling, preservation, processing and quality. Chamita Maritime, Kent ME4 4TB, U.K. 1996.

[4] Collins CH, Lyne, PH. Microbiological Methods 3rd Ed. Butterworth and Co (Pub) Ltd, London. 1970.

[5] Duncan DB. Multiple range tests and multiple F test. Biometrics.1955: 11:1-44

[6] FAO. Simple methods of meat preservation. FAO paper No FAO, Rome, Italy. 1987.

[7] FAO. Fermented fish in Africa. A study on processing, marketing and consumption. FAO Fisheries Tech. paper 329, FAO, Rome. 1992.

[8] Harrigan WF, McCance ME. Laboratory Methods in Food and Dairy Microbiology. Academic Press, London. 1976.

[9] Jay JM. Modern Food Microbiology. 3rd Ed. EBS Publishers, New Delhi, India. 1987.

[10] Kent G. National fisheries policy of Thailand, FAO Fisheries circular No 777, FAO, Rome, Italy. 1984.

[11] Maurine MA, Pangram RM, Rosier ER. Principles of Sensory Evaluation of Foods. New York, Academic Press. 1965.

[12] Negbenebor C.A. Raw material supply for fish and animal processing Industries. In Proceedings of a workshop organized by the N/E Chapter of NIFST, Maiduguri, Nigeria. Pp12-24. Eds. I. Nkama and P. Sopade. 1990.

[13] Negbenebor CA, Kaduara SG, Igene JO, Chikwem J. Preliminary studies in the production of "Bunyi- youri" (A putrefied sun-dried fish product). Agrosearch, 1995: 1:25-33.

[14] Negbenebor CA, Adetunji IS. Igene JO. The effect of clove and ginger dip on quality of solar-tent dried (Clarias anguillaris). In FAO. Fisheries Report. 1996.

[15] Omojowo FS, Omojasola PF, Ihuahi JA. Microbial Quality of Citric Acid as Preservatives in Smoked Catfish (*Clarias gariepinus*). *In: Biological and Environmental Science Journal for the Tropics,* 2008 Vol. 5(3): 130-134.

[16] Omojowo FS, Omojasola PF, Idris GL, Ihuahi JA. Evaluation of Citric Acid and Potassium Sorbate as Preservatives on the Safety and Shelf-Life of Smoked Catfish. *In: Nature and Science Journal,* 2009a Vol. 7(11):1-8.

[17] Omojowo FS, Idris GL, Ihuahi JA. Comparative Assessment of potassium sorbate and Sodium metabisulphite on the safety and shelf life of Catfish. In: *Nature and Science Journal,* 2009b: Vol. 7(10):10-17.

[18] Pearson D. Chemical Analysis of Foods. 6th Ed. 1981, Pp 33-120. New-York, Church-hill Livingstone.

[19] Steel RGD, Torrie JH. Principles and procedures of Statistics. McGraw-Hill Book Co.Inc. 1980. New York.

[20] Vileg P, Body DR. Lipid content and fatty acid composition of some New-Zealand fish. NZJ Marine Fresh water Res. 1988: 22:151.

[21] Waterman JJ. The production of dried fish. FAO fish report, 1976. (F42), FAO, Rome.

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Diagnostic Role Of Resistin In Nonalchoholic Fatty Liver Disease

By

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Abstract: **Introduction:** Nonalcoholic fatty liver disease (NAFLD) is a major cause of liver-related morbidity and mortality. Insulin resistance is believed to be a key factor in the development of fatty liver. Moreover, insulin resistance states characterized by elevated expression and production of several cytokines; of particular adiponectin, leptin, resistin. Leptin and adiponectin have been implicated in the pathogenesis and progression of NAFLD but direct evidence of the role of resistin in NAFLD is lacking. **The aim of this study** was to determine the circulating resistin level in patients affected by NAFLD and to correlate resistin level with insulin sensitivity, liver function and histologic feature. **Subjects and methods**: This study included 100 subjects divided in to: Forty patients with NAFLD, forty obese person with BMI >30 with normal transaminases and normal liver ultrasound and twenty controls with BMI <20, for all subjects serum resistin was measured, Homeostasis model assessment (HOMA) was calculated and liver profile was assessed. Liver biopsy was done in NAFLD patients. **Results**: Serum resistin was higher in patients with NAFLD (16.2 ± 4) compared to obese and control groups (6.8 ± 4.1 and 3.4 ± 1.1) respectively ($p < 0.01$), serum resistin was higher in advanced cases of NAFLD compared to mild cases (19.2±3.6 vs. 13.5±2.7) respectively ($P < 0.01$). Moreover serum resistin was positively correlated to BMI, HOMA , highly sensitive CRP, AST and ALT. **Conclusion and recommendation**: Resistin has a role in pathogenesis of NAFLD, resistin level is a predictive of histology in NAFLD, so the use of serum resistin assay as a simple diagnostic biomarker for NAFLD is recommended. [Nature and Science 2010;8(4):64-68]. (ISSN: 1545-0740).

Key word: NAFLD, NASH, Obesity and Resistin.

Introduction: Nonalcoholic fatty liver disease (NAFLD) is increasingly recognized as a potential serious condition, which can progress to cirrhosis, liver failure and hepatocellular carcinoma and has a worldwide distribution.[1]The biological basis of variability in histological progression of NAFLD is unknown, consequently, it has become extremely important to understand the patho-physiology of NAFLD to develop sound therapeutic interventions. It is now recognized that non hepatic mechanisms are largely responsible for the development of insulin resistance, which causes hepatic steatosis.[2] Insulin resistance is believed to be a key factor in the development of fatty liver. Moreover, insulin resistance states characterized by elevated expression and production of several cytokines; of particular adiponectin, leptin, resistin[3] Resistin is a recently discovered signal molecule, which could help elucidation of the patho-physiology of the insulin resistance and its correlation with obesity.[4] Leptin and adiponectin have been implicated in the pathogenesis and progression of non-alcoholic steatohepatitis (NASH) and chronic hepatitis C (CHC), but little is known about the role of resistin in chronic liver diseases.[5] **The Aim of this work** was to determine the circulating resistin level in patients affected by NAFLD and to correlate resistin level with insulin sensitivity , liver function and histologic feature. **Subjects and methods**: 100 subjects recruited form the hepatology clinic of Ain Shams university hospital were enrolled in this study, they were divided into:

Group 1: Forty patients with NAFLD, diagnosis was based on chronic elevation of transaminases (>1.5 times the upper normal value for 3 months or longer), absence of hepatitis B and C virus markers, absence of autoantibodies indicative of autoimmune hepatitis , absent alcohol consumption and bright liver at ultrasound scanning, with body mass index (20-35 kg/m^2). In all patients, diagnosis was confirmed by liver biopsy .

Group 2: Forty obese persons with body mass index above 30 kg/m^2 with normal transaminases values and normal liver ultrasound.

Group 3: Twenty age and sex matched healthy subjects with body mass index 20-25 kg/m^2.

None of patients and control subjects were taking lipid-lowering medications, met-formin or thiazolidinediones. Written informed consent was obtained from all participants.

For all subjects the following was done:

1: *Full history taking***.** 2: *Clinical examination* **with special emphasis on calculation of body mass index,**

local abdominal examination.

3: Laboratory examination: (CBC, ESR, fasting and 2 h pp blood glucose, fasting insulin, renal function(S cr, BUN, Na, K) , liver profile(ALT, AST, GGT, ALP, bilirubin(total, direct), albumin, total proteins, PT, INR), lipid profile(total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol and triglycerides), high-sensitivity C-reactive protein (hs CRP). HCV Ab , HBs Ag, HBV C Ab, ANA, AMA, ASMA,LKM, ferretin level).

4: The level of serum resistin was measured by ELISA method.

5: Insulin resistance was estimated by homeostasis model assessment (HOMA) =Fasting insulin X Fasting glucose / 22.5.[6]

6. Abdominal Ultrasonography **using real time scanning device Toshiba, vision 200 (SSA, 320A) with convex probe 3.5-5 uHz, focusing on liver size, texture, visualization of intra-hepatic vessels and diaphragm, liver to kidney contrast ratio.**[7]

7:Percutaneous Liver biopsy was done only for NAFLD patients (group 1). It was performed under ultrasound guidance using 16-gauge needles. Specimens of at least 2.5 cm in length, including a minimum of 12 portal tracts. Thin serial sections (4 micrometers thick) from formalin-fixed, paraffin-embedded blocks of core liver biopsies were stained with hematoxylin & eosin then assessed for detection of fat globules to identify and quantify hepatic steatosis, inflammation, necrosis and fibrosis**.** A NAFLD activity score (NAS), which includes features of active injury, has been defined as the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3) and ballooning (0-2).

According to this scale, cases with scores ≥5 are diagnosed as NASH, and scores <3 are diagnosed as not NASH, 3-5 are diagnosed as borderine NASH . It has been clearly emphasized that the NAS is not intended to be used as a diagnostic tool, but rather to provide a uniform tool for assessing disease severity[8].

8: Statistical analysis: All the collected data were expressed as mean ± SD and analyzed by using SPSS version 13 using the following tests: student t, ANOVA, Perason correlation coefficient. P > 0.05 was considered non significant, P < 0.05 was considered significant P < 0.01 was considered highly significant.

Results: This study included 100 subjects they were divided into:

Group (1) forty patients with (NAFLD), they were 15 males and 25 females their mean age was 42± 13 with BMI 28.2 ±5.2 kg/m^2.

Group (2) forty obese patients, they were 13 males and 27 females their mean age was 39± 12 with BMI 32.7 ±1.4 kg/m^2.

Group (3) twenty healthy volunteers (controls), they were 6 males and 14 females their mean age was 40± 12 with BMI 25.2 ±2.6 kg/m^2.

NAFLD and obese patients had higher FBS, 2h PP, fasting insulin and HOMA compared to controls(P<0.01). NAFLD patients had higher serum resisrtin compared to obese and controls (16.2± 4vs.6.8± 4.1and 3.4 ±1.1) respectively p (<0.01) as shown in table 1.

Table(1): Comparison between the studied groups as regard blood glucose, insulin and resistin.

Prameter	Group1	Group2	Group3	1vs.2	1vs.3	2vs.3
FBS(mg/dl)	158±24	157±20	89 ±14	>0.05	**<0.01**	**<0.01**
2h pp(mg/dl)	255±58	250±680	127± 9	>0.05	**<0.01**	**<0.01**
Insuin(**µiu/ml**)	19.3 ±17	13.1± 6	12.1 ±4.1	>0.05	**<0.01**	**<0.01**
HOMA	135.2±123	104± 38	49.4 ±20.5	>0.05	**<0.01**	**<0.01**
Resistin **(ng/ml)**	16.2± 4	6.8± 4.1	3.4 ±1.1	**<0.01**	**<0.01**	>0.05

NAFLD patients were subsequently divided according to histological diagnosis using Kleiner et al., [8] scoring system in to:

Group 1a: Ten cases were classified as 'Not NASH' they were 4 males and 6 females with mean age 46±8.6.

Group 1b: Twelve cases were classified as 'Borderline NASH' they were 8 males and 4 females with mean age 51.3±9.6.

Group 1c: Eighteen cases were classified as 'NASH **'** they were 8 males and 10 females with mean age 32.7±11.4.

Group 1c (NASH) had higher BMI, Hs CRP as well as resistin compared to group1a'Not NASH' and group 1b'Borderline NASH' P < 0.01 as shown in table 2.

Table (2): Comparison between pathological grades of NAFLD among group one as regard different parameters.

	Group 1a(10)	Group 1b(12)	Group 1c(18)	Annova	P
BMI(kg/m^2)	21.8 ± 1.7	27.3± 2.5	33.6 ± 1.3	64.4	<0.01
ALT(U/L)	95 ± 11	96.3 ± 17.2	101.5 ±1 5.5	0.38	>0.05
AST(U/L)	71.1±12.5	76 ±7.8	82.2 ±15.4	1.3	>0.05
FBG(mg/dl)	150±20	153±27	173±19	1.06	>0.05
2hpp(mg/dl)	156.6±60	190±90	273±65	2.7	>0.05
Resistin (ng/ml)	13.5±2.7	14±2.2	19.2±3.6	7.8	<0.01
CRP (mg/dl)	2.3±2.2	3.9 ±1.5	5.9±1.7	6.5	<0.01

Resistin had positive correlation to blood glucose, insulin, HOMA , liver enzymes, LDL cholesterol, TG and Hs CRP as shown in table 3

Table3: Correlation of serum resistin to different parameters.

parameter	r	P	parameter	R	P
BMI	0.68	<0.05	T cholesterol	0.75	<0.01
FBG	0.66	<0.05	LDL	0.77	<0.01
2hpp	0.68	<0.05	TG	0.66	<0.01
Insulin	0.67	<0.05	AST	0.91	<0.01
HOMA	0.75	<0.05	ALT	0.91	<0.01
Hs CRP	0.73	<0.01			

Discussion:

This study essentially showed that NAFLD patients had higher serum resisrtin compared to obese and controls (16.2± 4 vs. 6.8± 4.1 and 3.4 ±1.1) respectively p (<0.01) and this increase was positively correlated with BMI, blood glucose and insulin resistance. The strong association between insulin resistance and NAFLD has been extensively demonstrated [9]. Available evidence suggests that insulin resistance affects hepatic fat accumulation by increasing release of free fatty acids from adipose tissue, increasing fatty acid and triglycerides synthesis in the liver, reducing fatty acid oxidation and reducing very low-density lipoprotein (VLDL) production. Binding of adiponectin to its receptors stimulates phosphorylation of PPARα activity and fatty acid oxidation in liver and reducing fatty acid synthesis through inhibition of acyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) expression and activity[10] , and this mechanism is inhibited by resistin, therefore increased resistin in NAFLD could result in increased fatty acid synthesis, accumulation of triglycerides, and reduced fatty acid oxidation via insulin resistance and inhibiting adiponectin action. **Al-Harithy and Al-Ghamdi** [11]found that serum resistin concentrations increased from lean (11.59 +/- 2.08) to OW/OB non-diabetic (16.29 +/- 2.29) to diabetic (19.42 +/- 3.60 ng/mL) (P<0.001). Furthermore, resistin correlated significantly and positively with insulin and HOMA in diabetic and non-diabetic subjects. **Baranova et al** [12]stated that serum resistin was higher in patients with insulin resistance than patients without insulin resistance and obese patients with insulin resistance have decreased serum adiponectin and increased serum resistin. **Ciba and Widhalm**[13] found an association between insulin resistance and NAFLD in obese children indicating that markers of insulin sensitivity could be useful screening parameters for NAFLD.

This study showed that advanced NAFLD was strongly associated with higher serum resistin, as Group 1c (NASH) had higher resistin compared to group1a'Not NASH' and group 1b'Borderline NASH' P < 0.01. The previous studies of the relationship between resistin and NAFLD were conflicting as **Aller et al**[14] confirmed that blood levels of resistin were higher in patients with a high grade of steatosis, on the other hand **Cho etal**[15] found that serum resistin levels were similar in Group I (normal liver), Group II (mild fatty liver)and Group III (moderate to severe fatty liver), while leptin levels increased with increasing degree of hepatic fat infiltration, moreover **Lee et al**[16] found that there were no significant differences in serum leptin and resistin levels between two normal and increased ALT groups, while serum adiponectin levels were lower in the increased ALT group than in the normal ALT group, furthermore **Tsochatzis et al**[5] **s**tated that there was no significant association between steatosis or necroinflammation and levels of adipokines, while the presence of moderate/severe fibrosis (stages 4-6) was associated lower resistin.

We found a positive correlation between serum resistin and AST,ALT, hs CRP(P<0.01). It is well known that inflammation is a key mechanism in the progression of fatty liver to hepatitis and cirrhosis. [17] Adipokines are believed to act through their effects on insulin

sensitivity. Insulin resistance and hyperinsulinemia are also associated with the inflammatory and fibrotic reaction that complicates advanced stages of the disease [18], but new lines evidence indicate an important action on stimulation/ inhibition of the inflamatory process. [19] **Mojiminiyi and Abdella**[20]stated that resistin may represent a link between obesity and insulin resistance via pro-inflammatory pathways. In NAFLD a self-perpetuating pathway between insulin resistance and inflammation may explain the necro-inflammation observed in the subset of patients with NASH. [21] in contradiction to our results **Pagano et al**[22] found no correlation between resistin and high-sensitivity C-reactive protein and positive correlation between resistin and histological inflammatory score. **Roberto et al**[23]confirmed the significant direct association between hs-CRP and resistin which might explain the inflammatory pathogenic role of resistin which aggravate liver histology at more severe stages in NAFLD.

Conclusion and recommendation: Resistin has a role in pathogenesis of NAFLD, resistin level is a predictive of histology in NAFLD, so it can be used as a simple diagnostic biomarker for NAFLD.

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References:

1: Farrell GC, Hall P, George J, McCullough AJ. Fatty Liver Disease: NASH and Related Disorders. Malden, MA: Blackwell, 2005; 23–37.
2: Charlton M, Angulo P, Chalasani N, Merriman R, Viker K, Charatcharoen witthaya P, et al.,. Low circulating levels of dehydroepiandrosterone in histologically advanced nonalcoholic fatty liver disease.Hepatology. 2008 Feb;47(2):484-92
3: Zou CC, Liang L, Hong F, Fu JF, Zhao ZY. Serum adiponectin, resistin levels and non-alcoholic fatty liver disease in obese children. Endocr J. 2005 Oct;52(5):519-24.
4: Stejskal D, Adamovská S, Bartek J, Juráková R, Prosková J. Resistin - concentrations in persons with type 2 diabetes mellitus and in individuals with acute inflammatory disease. : Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2003 Nov;147(1):63-9.
5:Tsochatzis E, Papatheodoridis GV, Hadziyannis E, Georgiou A, Kafiri G, Tiniakos DG, et al., . Serum adipokine levels in chronic liver diseases: Association of resistin levels with fibrosis severity. Scand J Gastroenterol. 2008 Apr 30:1-9.
6: Bonora, E.; Targher, G.; Alberiche, M. **Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: Studies in subjects with various degrees of glucose tolerance and insulin sensitivity. Diabetes Care. 2000; 23: 57-63.**
7: Hultcrantz, R. and Gabrielsson, N. Patients with persistant elevation of aminotransferase: Investigation with ultrasonography, radionucleatide imaging and liver biopsy. J. Intern. Med.1993;233: 524-31.
8: Poynard T, Ratziu V, Charlotte F, Messous D, Munteanu M, Imbert-Bismut F, et al., . Diagnostic value of biochemical markers (NashTest) for the prediction of non alcoholo steato hepatitis in patients with non-alcoholic fatty liver disease.BMC Gastroenterol. 2006 Nov 10;6:34.
9: Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R, et al., . Non-alcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology. 2003; 37 917.
10: Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, et al., . Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature. 2003; 423 762–769.
11: Al-Harithy RN and Al-Ghamdi S. Serum resistin, adiposity and insulin resistance in Saudi women with type 2 diabetes mellitus. Ann Saudi Med. 2005 Jul-Aug;25(4):283-7.
12: Baranova, Ancha; Gowder, Shobha; Schlauch, Karen; Elariny, Hazem; Collantes, Rochelle; Afendy, Arian; et al., .Gene Expression of Leptin, Resistin, and Adiponectin in the White Adipose Tissue of Obese Patients with Non-Alcoholic Fatty Liver Disease and Insulin Resistance .Obesity Surgery. 2006 September ;16(9):1118-1125.
13: Ciba I, Widhalm K. The association between non-alcoholic fatty liver disease and insulin resistance in 20 obese children and adolescents. Acta Paediatr. 2007 Jan;96(1):109-12.
14: Aller R, de Luis DA, Fernandez L, Calle F, Velayos B, Olcoz JL, et al., . Influence of insulin resistance and adipokines in the grade of steatosis of nonalcoholic fatty liver disease. Dig Dis Sci. 2008 Apr;53(4):1088-92.
15:Cho YK, Lee WY, Oh SY, Park JH, Kim HJ, Park DI, et al., .Factors affecting the serum levels of adipokines in Korean male patients with nonalcoholic fatty liver disease. Hepatogastroenterology. 2007 Jul-Aug;54(77):1512-6.
16: Lee YS, Cho YK, Pae JC, Oh SY, Kang MS, Park JH, et al.,.The relationship between serum adiponectin level and serum alanine aminotransferase elevation in Korean male with nonalcoholic fatty liver disease. Korean J Hepatol. 2006 Jun;12(2):221-9.
17: Diehl AM. Nonalcoholic steatosis and

steatohepatitis. Nonalcoholic fatty liver disease abnormalities in macrophage function and cytokines. American Journal of Physiology: Gastrointestinal and Liver Physiology. 2002; 282.
18: Hui JM, Sud A, Farrell GC, Bandara P, Byth K, Kench JG, et al.,. Insulin resistance is associated with chronic hepatitis C virus infection and fibrosis progression. Gastroenterology. 2003; 125 1695.
19: Shetty GK, Economides PA, Horton ES, Mantzoros CS, Veves A. Circulating adiponectin and resistin levels in relation to metabolic factors, inflammatory markers, and vascular reactivity in diabetic patients and subjects at risk for diabetes. Diabetes Care.2004;27:2450–2457.
20: Mojiminiyi OA and Abdella NA. Associations of resistin with inflammation and insulin resistance in patients with type 2 diabetes mellitus. Scand J Clin Lab Invest. 2007;67(2):215-25.
21: Machado, Mariana; Cortez-Pinto, Helena.Non-alcoholic fatty liver disease and insulin resistance. European Journal of Gastroenterology & Hepatology. 2005August; 17(8):823-826.
22: Pagano C, Soardo G, Pilon C, Milocco C, Basan L, Milan G, et a.,. Increased serum resistin in nonalcoholic fatty liver disease is related to liver disease severity and not to insulin resistance.J Clin Endocrinol Metab. 2006 Mar;91(3):1081-6.
23: Roberto Gambino, Simona Bo, Giovanni Musso, Barbara Uberti, Natalina Alemanno, Federica Ghione, et al., .Microsomal triglyceride transfer protein 493-T variant is associated with resistin levels and C-reactive protein. Clin Biochem. 2007 Aug 10; 17825806 .

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Assessing Environmental Flow Modeling For Water Resources Management: A Case of Sg. (River) Pelus, Malaysia

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Abstract: In Detailed Environmental Impact Assessment (DEIA), modeling of environmental flows is one of the main studies that need to be delivered in the final DEIA report. The model is important to the project proponent to engage suitable designs that can be suited to environmental needs, particularly on future water resources management. In this respect, Environmental Flow Assessment (EFA) is used to estimate the quantity and timing of flows to sustain the ecosystem values. The proposed of hydropower projects in Sg Pelus, Perak was studied aimed to evaluate existing river flow characteristics and to model EFA due to river diversion of Sg Pelus. Daily river flow (m^3/s) recorded at Sg Pelus (Station No. 6035) and Sg. Yum (Station No. 6044) gauging stations were used to design the flow duration curve. The low flow then calculated using the 7Q10 equation to estimate the lowest 7-day average flow that occurred on average once every 10 years. The results indicate that the average daily flows for both stations (6035 and 6044) are 5.080 m^3/s and 11.391 m^3/s, respectively. The flow duration curve shows that 50 percent of 4 m^3/s of discharge will be exceeded/ equaled in Station 6044 while 8.2 m^3/s of discharge will be exceeded or equaled in Station 6035. The requirement environmental flows for both parameters are 0.613 and 0.426 m^3/s for Environmental Flow Assessment, respectively. The results obtained in this model are important to managing the river at least in Class II after river diversion project. [Nature and Science 2010;8(4):69-76]. (ISSN: 1545-0740).

Keywords: Environmental Flow Assessment; Detailed Environmental Impact Assessment; Low flow; Flow duration curve.

1. Introduction

Recent developments of legislation in Malaysia tend to consider environmental flows in the context of environmental sustainability (Mohd Ekhwan et al. 2009). It is considered a basic principle in sustainable development and in the search for ways to reconcile multiple and competing water uses with environmental protection. One important tool for implementing this approach in the water allocation process is multi-criteria analysis, wherein an environmental flow assessment provides a way to quantify the environment criteria (Hafizan et al. 2008).

Practically, the concept of environmental flows was implemented for a very specific purpose, i.e. protecting the aquatic fauna downstream river diversion (Arthington et al. 1992). Since then several different applications and interpretations have evolved that extend the original meaning. In some recent cases, it is considered to be an instrument to achieve water quality targets - together with other measures. In Malaysia, environmental flows are not prescribed in the national legislation in general terms, as framework laws. Current norms consider environmental flows only in the form of a minimum in-stream flows to be present downstream of water diversions. Eventually, this approach is part of the Detailed Environmental Impact Assessment (DEIA), to be presented by the developers in their Water Protection Plans.

This case study describes a scheme to integrate Environmental Flow Assessments (EFA) with hydrologic modeling tools in the Sg. Pelus, Perak Malaysia. The purpose is mainly for hydropower generation. It shows how environmental objectives were incorporated in multi-criteria analysis to develop flow regulation policies, particularly the portion where the river flow will be diverted. Typically the main challenge in such circumstances is to define an environmental score that can be computed for different scenarios – one that is inaccessible to experimentation and measure. The approach described overcomes this problem by using existing low flow methodology, namely the 7Q10 equation to define EFA.

The main aim of this study was to study the flow characteristics of Sg Pelus in State of Perak, Malaysia and the development of environmental flows requirement as related to river diversion project. The information is important to stakeholder and project proponent to estimate how much waters can be diverted that also can fully supply at all times without deteriorating the water quality and quantity as a whole, particularly at the downstream sites of the respected project.

2. Conceptual Framework

In Malaysia, the total available electricity generating capacity was estimated at 19.3 GW in 2003, a jump of 23 % from 15.6 GW in 2002 due to the commissioning of several coal and gas based independent power plants in Perak, Perlis and Perai. The electricity generation in 2003 was 82,406 GWh, which represented an increase of 6 % from 77,501 GWh in 2002. The electricity generation in 2003, which was basically from thermal generation, contributed about 87 %, while hydroelectric only contributed 13 %. Out of 87 % thermal generation, 65 % was from gas turbine/ combined cycle block, 11 % was from coal-fired plant and 11 % was from gas/oil plant, which suggested that our electricity generation was highly dependant on natural gas (Hafez et al. 2009).

With increases in fuel (oil) prices, which was almost doubled in two years (2005 and 2006), hydropower is becoming increasingly appealing. Thus, hydropower is one of the alternatives to solve energy shortage in years to come. Despite the clean energy hydroelectric power plants can provide as an alternative of reducing dependence on non-renewable source, the government is constantly under criticisms for high cost of building dam, as well as environmental impacts of the dams (Mohd Ekhwan et al. 2009).

In certain activities which involved natural environment, particularly river diversion project, Environmental Impact Assessment is required by Department of Environment (DOE) to protect water source areas in headwater regions from degradation. A detailed Environmental Impact Assessment (DEIA) study must be carried out by the consultant at various perspectives, i.e. physical, biological, socio-economic including tourism, archeology and health to ensure that the impacts from the project are minimal.

One of the main criteria in DEIA, particularly in hydrological section is the need to EFA requirement. The idea is to address acceptable water quality for flow diversion, and at a same time to protect flora and fauna below the downstream river diversion. In a case of river diversion project, dry season become a subject matter where by water level normally at a minimum level. Therefore, the need to study minimum or low river flow characteristics is essential so that full hydropower electric can be supply at all times.

The most common low flow analyses for streams are twofold, namely minimum annual minimum flow and 7Q10 model analysis (Loneragan & Bunn, 1999; Rosenfeld et al. 2007). This study engages 7Q10 as this model is widely used throughout the world. In a case of Sg. Pelus, the 7Q10 was selected as a representative low streamflow value for regulatory and modeling purposes, particularly with respect to point-source pollution and concentration due to river flow diversion. Simply, the 7Q10 means "seven-day, consecutive low flow with a ten year return frequency; [or] the lowest stream flow for seven consecutive days that would be expected to occur once in ten years," (Mohd Ekhwan & Shukor 2006). According to the World Meteorological Organization, low flow is the "flow of water in a river during prolonged dry weather". Again, hydrologists use design flow statistics such as the 7Q10 or the lowest 7-day average flow that occurs on average once every 10 years to define low flow for the propose of setting permit discharge limits.

When the river is considered as unregulated natural river, the reliability of water availability is a function of the low flow characteristics (Petts 1984). The three main characteristics of low flow are:

- Duration - reflect the tolerance of the user to periods of water deficits.
- Magnitude - Low flow for specific duration will determine the amount of water that is available to the user (Pyrce 2004).
- Frequency of occurrence - The frequency of occurrence of low flow reflects the risk associated with the failure of water supply.

For this study, the 7Q10 flow was adopted as this method is the most commonly used single flow index (Table 1).

Table 1: Uses of the 7Q10 Flow

➲To protect/ regulate water quality (to prevent adverse biology/ecological impacts)
➲General indicator of prevalent drought conditions which normally cover large areas
➲Total maximum daily load to assess aquatic life protection
➲Minimum quantity of streamflow necessary to protect habitat during a drought situation
➲Considered as the wroth case scenario in water quality modelling
➲To compare the impacts of climate change and irrigation on low surface streamflows

3. Materials and Methods

The Sg. Pelus hydroelectric scheme is considered a mini-hydro utilizing Run-of-river type of hydroelectric power plant located within the Sg. Perak catchment. The scale of the project is considered relatively small, with minimal impact on already degraded natural environment of Sg. Pelus sub-catchment. The similar Sg. Perak catchment is currently exploited by hydroelectric power plants, such as Temengor, Bersia, Kenering and Chenderoh.

The Pelus river catchment is a sub-catchment of the Upper Perak River, which flows from its source

near the Thailand boarder, southwards through Perak State. On the east site of the Perak River, lies the Sg. Piah Basin, this is part of the Kenering sub-catchment. The Pelus catchment is similar in size and physiographic characteristics to the Piah catchments and lies directly to the south. The Sg. Pelus discharges into the Perak River about 10km downstream of Chenderoh. Total catchments size for Yum and Pelus are estimated at 135 km^2 to 170 km^2, respectively (Figure 1).

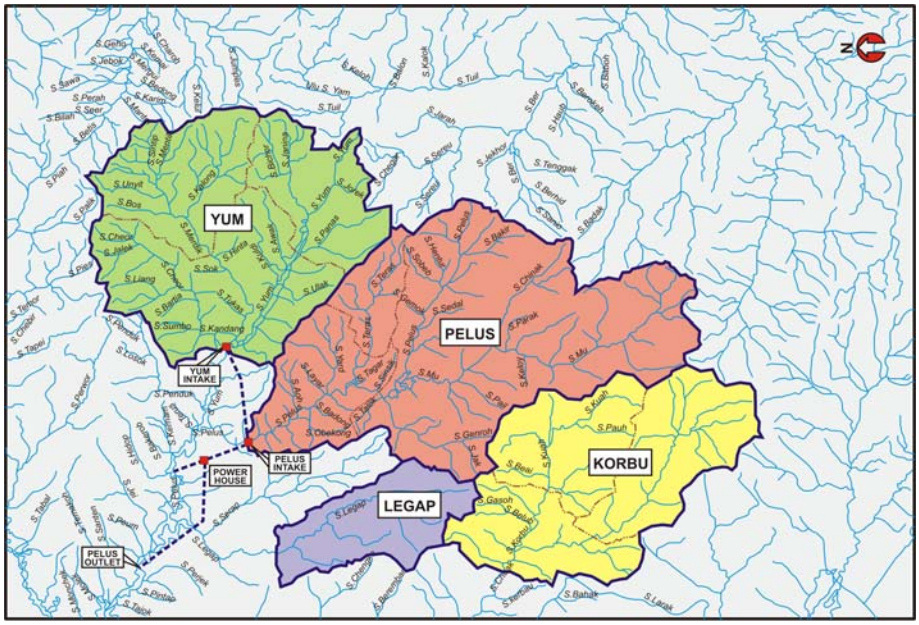

Figure 1: Pelus, Yum, Korbu and Legap Sub-Catchments

The bifurcation ratios as the ratio of the number of streams of one order to the number of streams of next highest order (n + 1). In this catchment, the average value of bifurcation ratio was 5.35. This value is within the threshold for the upper catchment as studied by Mohd Ekhwan & Shukor (2006) for Peninsular Malaysia where mean bifurcation ratio of most of the catchment in the Peninsular Malaysia tends to be approximately 5-7.

The rivers of this catchment are relatively short courses. Their gradients in the upper courses are steep. Some river reach can drop to more than 50 m creating gorgeous waterfall.

For this analysis, the stream flow was discussed at each single station. Stations 6044 (Sg. Yum at Kuala Yum) and 6035 (Sg Pelus below Kuala Yum) have a complete 13-year (Jan 1984-June 1997) and 14-year flow series, respectively (Jun 1985- October 1997). Meanwhile, the flow duration curve was developed by computing the percentage of time the various flow rates are equaled or exceeded and then plotting the discharge rates against the corresponding percentages of time.

Hydrological Procedure No. 12 (HPI2) 'Magnitude and Frequency of Low Flow in Peninsular Malaysia' describes a simple method to compute low flows. Like HP No.4, this procedure was developed based on regional frequency analysis. Four low flow regions (RC1; RC2; RC3 and RC4) were identified and using this procedure design low flows of return periods between 1, 10 and 25 years could be determined.

In the low flow frequency analysis, the total 7-day low flow for each year is identified. These total 7-days low flow value is then ranked starting with the lowest rank. Then, the percentage of ranking is computed for each rank. This is followed by plotting the log flow value against the respective percentage ranking on a probability paper. Information on Biological Oxygen Demand (BOD) and Total Suspended Sediment (TSS) were obtained using standard laboratory procedures.

4. Results and Discussions

The proposed scheme of Sg. Pelus hydropower project intends to abstract waters from Sg. Yum and Sg. Pelus which is then diverted to an underground power station (34.8 MW) at Kuala Legap (04^o 56" 47.8'E, 101^o 15" 45.4'N). The impact on water flow at the time the stream waters diverted into the tunnel is predicted – where the diversion will disrupt the flows, particularly the volume, velocity and water level especially stream section below the diversion intakes.

The channel platforms may also unstable in the early diversion period. Reducing flow can develop sediment deposition particularly in the inner bends of

the river. At the same time with decreased in water levels causing bank materials to be exposed and finally may lead to lateral erosion especially those in the step banks. These impacts however are temporary and localized and not considered causing any significant effects further downstream.

a. Sg. Yum - Daily Flow

Daily Q was constructed from the Station 6044. The station receives water from Sg Yum sub-catchment. Based on the figure, the maximum Q (m^3/s) recorded was 26.3, while the mean and minimum Q is 5.080 and 2.0, respectively (Figure 2).

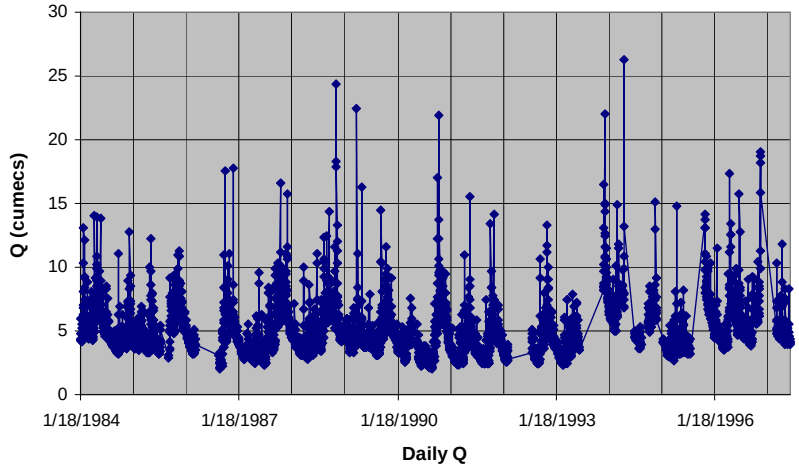

Figure 2: Daily Flow at Station 6044 (Sg. Yum at Kuala Yum)

b. Sg. Pelus- Daily flow

Daily flows recorded at Sg Pelus below Kuala Yum are expected to be higher compared to Sg Yum as this station received both discharges from Pelus and Yum catchments. Based on the flow data, the maximum daily flow was 66.7 m^3/s. The average over 12 years record is 11.391 m^3/s and the minimum flow is 0.6 m^3/s (Figure 3).

c. Flow Duration Curve

The flow duration curve is a plot that shows the percentage of time that flow in a stream is likely to equal or exceed some specified value of interest. For example, it can be used to show that the percentage of time river flow can be expected to exceed a design flow of some specified value (e.g., 5 m^3/s), to show the discharge of the stream, or to exceeded some percent of the time (e.g., 80% of the time).

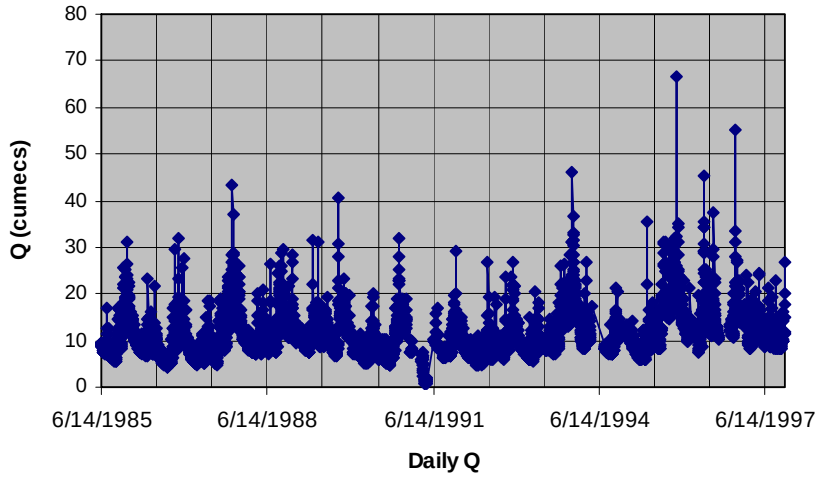

Figure 3: Daily flow at Station 6035 (Sg. Pelus below Kuala Yum)

The basic time unit used in preparing a flow-duration curve will greatly affect its appearance. For this study, mean daily discharges were used. The flow duration curve was developed by computing the percentage of time the various flow rates are equaled or exceeded and then plotting the discharge rates against the corresponding percentages of time.

Figures 4 and 5 show the daily flow duration curves calculated at Stations 6044 and 6035. It is estimated that for both stations, 50 percent of 4 m^3/s and 8.2 m^3/s of discharges will be exceeded or equaled. According to the figure to follow, minimum instream flow of approximate 2 m^3/s is likely to be available 100 % of the time for an average year. However, the demand of 5 m^3/s will only be available 25 % for Station 6044 and 80 % of the time. This implies that full supply will be available during a portion of the water year while a reduced supply will be available during other times of the year.

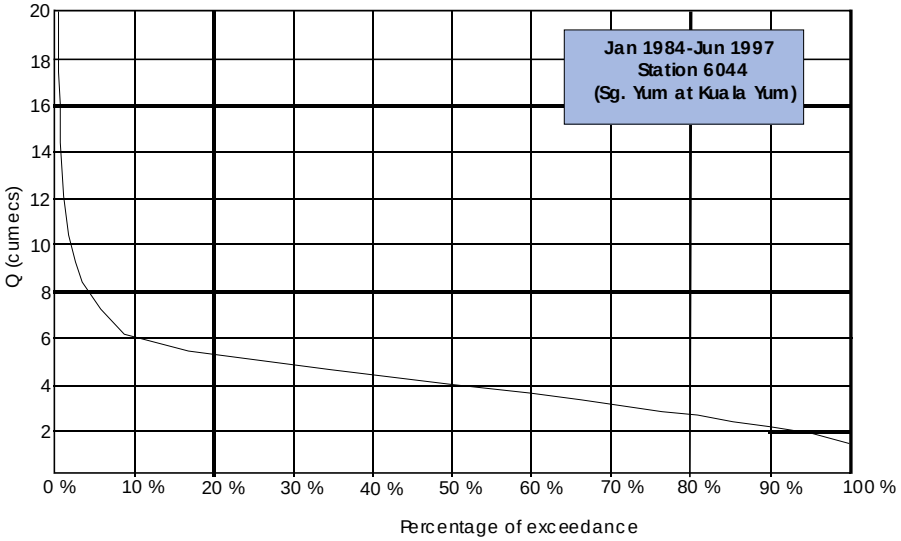

Figure 4: Flow Duration Curve for Sg Yum at Kuala Yum

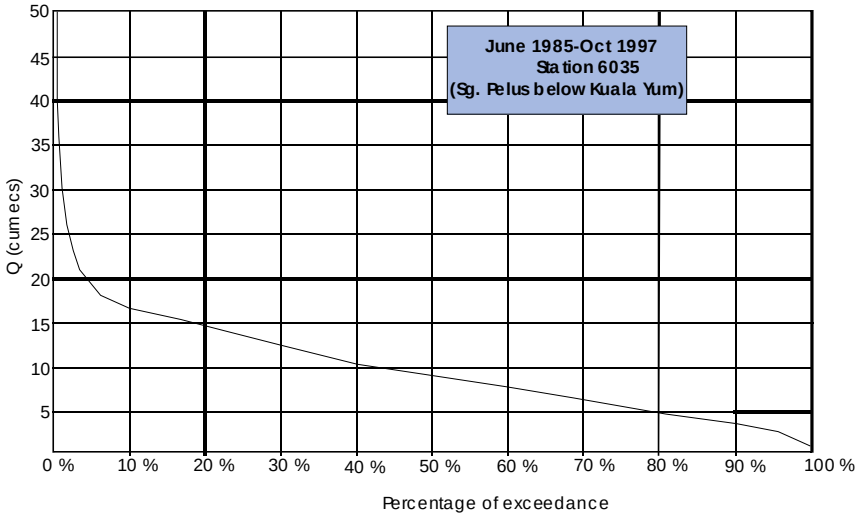

Figure 5: Flow Duration Curve at Sg Pelus below Kuala Yum

d) Low Flow

In the low flow frequency analysis, the total 7-day low flow for each year is identified. Line fitting is drawn to provide the representative 7-days low flow probability line as shown in Figure 6. The value was re-calculated from the mathematical model,

$$\hat{Y} = \acute{y} + S_{y\dot{z}} \text{ ..... [1]}$$

Where $\acute{y}$ is the population mean, S_y is standard deviation of the logarithms and $_{\dot{z}}$ is standard normal deviate. The estimated 7-day low flow for selected exceedence frequency (T) is tabulated in Table 2.

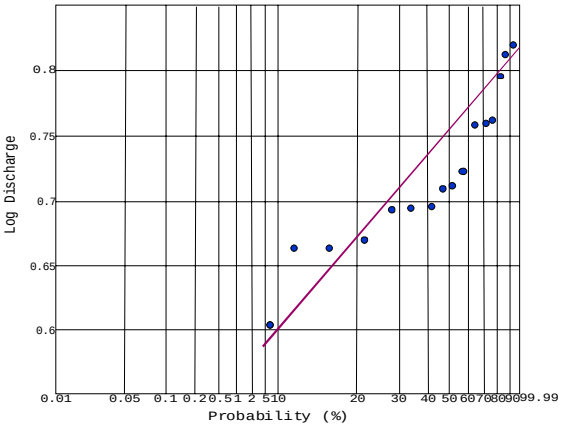

Figure 6: 7-Day Low Flow Frequency Curve

Table 2: 7-Day Low Flow Estimates for Sg. Pelus Catchment

T (Years)	*7-days low flow Q7, T(cumecs)*
01.5	2.413
02.33	1.602
05.0	1.175
10.0	**0.986**
20.0	0.890
50.0	0.801

e) Environmental Flow Assessment (EFA) For Water Resources Management

For Sg. Pelus river diversion project, the environmental group has adopted a suite of methods to determine environmental flows. These range from desktop studies in unstressed catchments to comprehensive studies of minimum flow requirements. The outputs from these assessments have been used to recommend Environmental Flow Assessment (EFA). EFA is a description of the flow regime required to maintain the ecosystem values, targeted by the assessment, at a low level of risk.

In this study, EFA is generally focused to those parts of the ecosystem and the specific times of the year that they are potentially at risk, particularly at the section where the stream will be diverted to the tunnel. For example, during the drought, the water use in a catchment may affect species that have particular requirements in these months (e.g. spawning, riparian germination and habitat availability) (Maidment 1993). At other times outside the months, water use may not have a great impact on the ecological processes in a river diversion section. It is therefore critical to set the environmental flows during the planning stage of the project to ensure that this value is adhered to during operations of the diversions.

For environmental flow requirement, the measured water quality values for BOD (biochemical indicator) and TSS (physical indicator) for various locations at Sg Pelus and it tributaries taken during the field works showed BOD concentration is between 1.8 mg/l (Sg Menlik, a tributary of Sg Yum and upstream of the proposed Yum Intake) to 3.1 mg/I (Sg Pelus at 500 m downstream of the proposed Pelus Outlet) while TSS concentration is between 8.0 mg/l (Sg Menlik) to 48.8 mg/l (Sg Pelus). The average values for both BOD and TSS parameters are 2.45 mg/l and 28.4 mg/l, respectively. This means that, both parameters are under the Class II. The required environmental flow for Sg Pelus is estimated. It is based on the average as represented by BOD and TSS against the 7-day low flows. The result is tabulated in Table 3.

To maintain at least Class II waters, the minimum environmental flows required for BOD and TSS are 0.279 m^3/s and 0.280 m^3/s, under 7-day low flow. Based on Table 2, the 7Q10 was calculated at 0.986 m^3/s. Both values shown are below the 7Q10, meaning that even during the dry season, the values are still can maintain at Class II as water volume is plenty enough to cater both parameters.

5. Conclusion

In conclusion, the work presented here should convey the need for reporting of low flow confidence limits, and the value of using these limits in the decision making process, particularly when it involves with river diversion works. In summary, the results obtained from this study can summarized as follows:

Table 3: Environmental Flow Assessment (EFA) Based on Mean Sampled Value of BOD and TSS under 7-Day Low Flow Conditions

	BOD	TSS
Mean (mg/L)	2.45	28.4
7-day Low Flow:		
m^3/s	0.986	0.986
L/s	986	986
Estimated Loading (mg/s)	2415.7	28002.4
Required Environmental Flow (m^3/s)	0.279	0.280

NOTE: The estimated loading was computed by multiplying mean BOD and TSS (mg/L) load with mean daily flow (L/s)/ 7-Day Low Flow.

- Total catchments size for Yum and Pelus are 135 km^2 and 170 km^2.
- Stations 6044 (Sg. Yum at Kuala Yum) and 6035 (Sg Pelus below Kuala Yum) are the gauged system used for the analyses.
- Mean daily flow for Sg. Yum is 5.080 m^3/s.
- Mean daily flow for Sg. Pelus is 11.391 m^3/s.
- 50 % of 4 m^3/s of discharge will be exceeded/ equaled in Station 6044
- 50 % of 8.2 m^3/s of discharge will be exceeded/ equaled in Station 6035
- BOD requirement for Environmental Flow Assessment (m^3/s) for Sg Pelus is 0.279 m^3/s.
- TSS requirement for Environmental Flow Assessment (m^3/s) for Sg Pelus is 0.280 m^3/s.

In conclusion, the work presented here should convey the need for reporting of low flow confidence limits, and the value of using these limits in the decision making process. Finally, the case study in Sg Pelus provides good exercise to identify acceptable limit threshold for the construction of the tunnel and at a same time maintaining the river water level for biotic and abiotic lives along the river system.

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Reference

Arthington, A.H., King, J.M., O'keefe, J.H., Bunn, S.E., Day, J.A., Pusey, B.J., Bludhorn, D.R. and Tharme, R., (1992). Development of an holistic approach for assessing environmental flow requirements of riverine ecosystems. In: Pigram, J.J. and Hooper, B.P. (Eds.), *Proceedings of an International Seminar and Workshop on Water Allocation for the Environment.* Centre for Water Policy Research, Armidale, 69-76.

Hafez Salleh, Azlan Shah Ali, Syahrul Nizam Kamaruzzaman, Loo Siaw Chuing. (2009). A case studies of intelligent buildings in Malaysia. *Malaysian Construction Research Journal.* Vol. 4,40-51.

Hafizan Juahir, Sharifuddin M. Zain, Mazlin B. Mokhtar, Mohd Ekhwan Toriman, Zaihan Jalaludin & Ijan Khushaida M. Jan. 2008. The Use of Chemometrics Analysis as a Cost-Effective Tool in Sustainable Utilisation of Water Resources in the Langat River Catchment, Malaysia. *American-Eurasian J. Agric. & Environ. Sci*. 4 (2): 258-265.

Hydrological Procedure No. 12 (HPI2) .Magnitude and Frequency of Low Flow in Peninsular Malaysia. Kuala Lumpur: Drainage Irrigation and Department Malaysia.

Loneragan, N.R. & Bunn, S.E., (1999). River flows and estuarine ecosystems: Implications for coastal fisheries from a review and a case study of the Logan River, southeast Queensland. *Australian Journal of*

Ecology, 24, 431-440.

Maidment, D. (1993).*Handbook of Hydrology*. McGraw Hill Publisher, United Kingdom.

Mohd Ekhwan Toriman & Shukor Md. Nor. (2006). An analysis of rainfall interception on the selected experimental plot of Pangkor Hill Reserved Forest. *Journal Wildlife and National Park.* December 2006.169-178.

Mohd Ekhwan Toriman, Mazlin Mokhtar, Muhamad Barzani Gasim, Sharifah Mastura Syed Abdullah, Osman Jaafar & Nor Azlina Abd Aziz. (2009). Water Resources Study and Modeling at North Kedah: A Case of Kubang Pasu and Padang Terap Water Supply Schemes. Research Journal of Earth Sciences 1 (2): 35-42.

Mohd Ekhwan Toriman, Muhamad Barzani Gasim & Hafizan Juahir. (2009). Application of Artificial Neural network in water level-discharge relationship of Sg Gumum-Tasik Chini Pahang. In Mushrifah Idris, Mohammad Shuhaimi Othman, Sahibin Abd Rahim, Khatijah Hussin, Nur Amelia Abas (eds.). *Sumber asli Tasik Chini.* Faculty Science and Technology Publishers. UKM. 89-105.

Petts, G. E. (1984). Impounded Rivers: Perspective for Ecological Management. Wiley, Chichester.

Pyrce, R. (2004). Hydrological Low Flow Indices and Their Uses, Watershed Science Centre, Trent University, Canada.

Rosenfeld J. S., Post J., Robins G. and Hatfield T. (2007). Hydraulic geometry as a physical template for the river continuum: application to optimal flows and longitudinal trends in salmonid habitat. Canadian Journal of Fisheries and Aquatic Sciences. 64, 755–767.

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Mutageneic and antimutagenic effects of some plant extracts *in Drosophilla melanogaster*

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Abstract: This study was designed to investigate the mutagenic potential of the anticancer drug vincristine and some plant extracts (fennel and parsley) on *Drosophilla melanogaster* using two test systems: the sex linked recessive lethal (SLRL) and the estimation of the activity of cholinesterase enzyme (ChE) in F1 and F2 bar eye females and F2 wild type males. A wild type strain Oregon-R (or-R) male flies of *D.melanogaster* were treated on a medium containing a concentration of only one of the three agents, followed by a combined treatment in an alternative way of fennel extract or parsley extract followed by vincristin, then vincristin followed by fennel extract or parsley extract and finally the three agents together. The results obtained, showed non significant increase in the percentage of the S.L.R.L in all stages of spermatogenesis in all treatments. Meanwhile, vincristine as a single treatment or combined with fennel or parsley extracts showed genotoxic effects in the three categories of the two generations of S.L.R.L: F1 females heterozygous F2 bar eye females and F2 wild type males on the genetic background of ChE in all treatments. [Nature and Science 2010;8(4):77-82]. (ISSN: 1545-0740].

Keywords: *Drosophilla melanogaster* - cholinesterase enzyme – vincristin – fennel – parsley.

1. Introduction

In the past, most of the studies on the genetic effects of anticancer drugs have been concentrated on cytogenetic damage (Clements etal., 1990). However, it is obviously important to learn more about the different types of mutagenic lesions induced by anticancer drugs. Marselos and Vainio(1991) reported that most of the cancer chemotherapeutic agents are mutagenic and carcinogenic. Vincristine (VCR) is a widely used anticancer drug in Arab countries.It contains the active substance vincristine sulphate.

Vincristine sulphate is a dimeric alkaloid found in the leaves of the plant *Catharanthus roseus,* Downing (2000). The natural vinca alkaloids and their synthetic derivatives are used as antineoplastices. These agents act by reacting with tubulin, altering the microtubule organization and dynamics disturbing the mitotic spindle and subsequently causing cell aneuploidy,Downing, (2000) and Ramirez et al. (2004) .The genotoxicity of VCR has been tested several times in vitro and in vivo in lower organisms. Various aspects of its effect have been reviewed on several occasions, Degraeve, (1978) & Kirsch-Volders,and Parrt (1996).

The available information on its genotoxicity is found to be contradictory to each other. Moreover in most of the earlier studies, either the doses tested were unusually high or the data generated were after chronic exposures to the chemical.Furthermore, Gonzalcz-Cid *et al*. (1999) found that VCR and vinorelbine (VRB) induced a significant increase in micronuclei (MN) frequencies in binucleated (BN) cells, as well as produced slowing of the cell cycle, causing a decrease in the percentage of BN cells in cultured human lymphocytes. Also, Tiburi *et al*. (2002) reported that vincristine (VCR), inblastine (VBL) and vinorelbine (VNR) induced genetic toxicity causing increments in the incidence of mutational events, as well as in somatic recombination in *Drosophila melanogaster*.

Now, the world is directed to depend on nature to decrease the side effects of the drugs. Herbal medicine is the oldest form of health care known to man kind. Herbs had been used by all cultures throughout history. It was an integral part of the development of modern civilization, the plant kingdom has provided an endless source of medicinal plants .Herb plants produce and contain a variety of chemical substances that act upon the body. Therefore, discovery and exploration of compounds possessing antimutagenic and anticarcinogenic properties are of great importance. Many substances with antimutagenic activity have been found by several investigators Soudamini et al., (1995) and Xie et al., (2006) . The present study was designed to detect the mutagenic effects of vincristine (VCR) anticancer drug and antimutagenic effects of some plant extracts in *Drosophila melanogaster* using two test systems, the sex liked recessive lethal mutations test (SLRL) and the estimation of the activity of Cholinesterase enzyme (ChE).

2. Materials and Methods

2.1-Materials:

2.1.1 Strains:

Two strains of *D.melanogaster* were used in the present study:

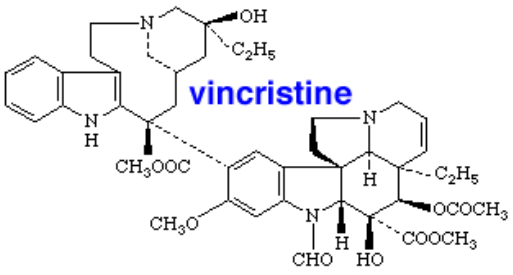
a- Muller-5 (M-5):
A marker strain of *D.melanogaster* used for the detection of Sex Linked Recessive Lethal mutations. Its X-chromosome carries a dominant marker bar eye (B) and a recessive mutant eye color, white apricot (W^a). It has also two inversions, the first is scute (Sc^{8r}) inversion and the second designated (in-s) is included in the first inversion.

b-Oregon- R (O-R):
This stock is a wild type strain that has always been used in *Drosophila* laboratories. It was obtained from the department of Genetics, Ain Shams University, Cairo, A.R.E. This strain was repeatedly tested to determine its spontaneous Sex-linked recessive lethal (S.L.R.L).

2.1.2.Chemicals:
a- Vincristine sulfate (Oncnvin):
Tablets product by Faulding Pharmaceuticals Pic/ Warwickshire CV31 3RW, United Kingdom.
(It is a dimeric alkaloid found in the leaves of the plant *Vinva rosea*.

b-Fennel (*Foeniculum vulgare Mill*)
The essential oil of the most important fennel variety (var. dulce) contains anethole (50 to 80%), limonene (5%), fenchone (5%), estragole (methyl-chavicol), safrole –pinene (0.5%), camphene, pinene, myrcene and p-cymene. **Parsley (*Petroselinum sativum)*** :
In this study, Parsley oil had been used for (CAP FARM) company no.22977/2002,Cairo, Egypt.

d- Kit for Cholinesterase estimation:
This kit was obtained from QUIMICA



CLINICA APLICADA for the estimation of the activities of the enzyme Cholinesterase (CHE).

2.2. Methods:

2.2.1.Two test systems were employed in this study:
a- Sex Linked recessive lethal (SLRL) assay for *Drosophila;* Mullar.(1972) and Brusick.(1980)
b-Estimation of enzyme Cholinesterase (ChE) activity in *Drosophila* using spectrophotometric analysis; Perparation of samples for Cholinesterase (ChE) activity estimation was carried as follow:
Sample prepared by homogenizing the whole body of 100 adults in 1.0 ml of refrigerated phosphate buffer (PH7.2)with glass homogenizer, centrifugation at 8.000 rpm for about 1 minute at 4Cºwas carried out . The particulated material was discarded, then 40 ul of the supernant was transferred in test tube. Use the kit of ChE as instruction .Measurment of transmission was done at 405 mu using spectronic spectrophotometer model.
2.2.2. Oregon-R of *D.melanogaster* males were treated as follows:
a. Single treatment of VCR with a concentration of 2ml/100 ml medium.
b. Single treatment of fennel with a concentration of 2ml/100 ml medium.
c. Single treatment of parsley with a concentration of 2ml/100 ml medium.
d. Combined treatments with VCR and parsley extract by arrangement of vinncristin followed by parsley extract then parsley extract followed by vinncristin and finally the two agents together.
e. Combined treatments with VCR and fennel extract by the arrangement of vinncristin then fennel extract, fennel extract followed by vinncristin and finally the two agents together.
3-SLRL have been estimated and three categories were analyzed for enzyme activity: F1, F2 females heterozygous and wild type males.

2.2.3. **Statistical Analysis:**
a-Significance of sex-linked recessive lethal results was detected by Kasten Baum and Bowman test Wurglar et al., (1975).
b- ANOVA test (SPSS program) was applied to determine significe of enzyme estimation.

3. Results

3.1 Induction of Sex-Linked Recessive Lethal:

a - The results obtained from the SLRL test after treatment with the one concentration of VCR (2ml/100ml of medium) are summarized in table (1). The frequencies for all broods were not significantly different from the control frequencies. Thus, it would be considered as conclusive results. Similarly, treated males with fennel and parsley extract as a single dose induced lethal mutation with a frequency of about 0.0 % in the first brood, and in the second brood about

0.12%, and about 0.40% in the third brood, with no lethality at the fourth brood. The frequencies for all broods were not significantly different from the control ones.

In addition, from table (1), it was noticed that data obtained showed that the single and combined treatments using sex linked recessive lethal mutations are inactive, producing a statistically insignificant increase in the frequency of total SLRL

3.2. Estimation activity of ChE enzyme:

This estimation was carried out in some insects of two generation of SLRL. VCR caused change in ChE activities in F1 females, F2 bar eye females and F2 wild type male due to its mutagenic potentiality. Statistical analysis indicated that the difference of F1 females, F2 females and F2 males with the control were significant (table2)

Table 1: Identification of sex linked recessive lethals occurring spontaneously and after different treatment with vincestine fennel and parsley plant extract in *D. melangaster*

Treatment	Sperms B1			Spermatides B2			Spermatocytes B3			Spermatogonia B4			Total		
	N.	L.	%	N.	L.	%	N.	L.	%	N.	L.	%	N.	L.	%
Control	906	1	0.11	913	1	0.10	88	2	0.22	815	1	0.12	3540	5	0.14
VCR 2%	1079	4	0.37	847	1	0.11	941	1	0.01	926	1	0.10	3793	7	0.18
Fennel plant Extract 2%	748	0	0.0	792	1	0.12	744	3	0.04	912	0	0.0	3232	4	0.12
Fennel 2% then VCR 2%	983	5	0.50	839	4	0.47	964	4	0.14	975	0	0.0	3761	13	0.34
VCR2% then Fennel 2%	852	0	0.0	847	1	0.11	986	3	0.03	915	2	0.21	3600	6	0.16
VCR2% and Feneel2% together	955	4	0.41	1018	3	0.29	820	1	0.12	1074	5	0.46	3867	13	0.33
Parsley 2%	632	4	0.6	402	2	0.4	344	3	0.8	268	-	0	1674	10	0.6
Parsley 2% then VCR2%	963	3	0.3	886	-	-	913	1	0.1	935	3	0.3	3697	5	0.3
VCR2% then Parsley 2%	968	1	0.1	762	6	0.7	853	-	-	941	1	0.1	3524	8	0.2
VCR2% and Parsley 2% together	910	3	0.3	955	3	0.3	1004	5	0.5	1030	2	0.2	3899	13	0.3

N.= Number of tested chromosomes, L.= Number of lethal mutations (SLRL), %= Frequency of SLRL

Table 2: Effect of vincerestine, fennel plant extract and parsley plant extract with different treatments on Cholinesterase (ChE) activity in the three categories of *D. melanogaster*

Category		ChE activity (units)*									
		Control	VCR	Fennel	Fennel then VCR	VCR then Feneel	VCR and Fennel	Parsley	Parsley then VCR	VCR then Parsley	VCR and Parsley
F1 ♀	B1	22827	14331**	28067	49419**	27148**	40551**	22945	39340**	75903**	51245**
	B2	24637	16518**	21338	90171**	65967**	46229**	25082	37722**	87720**	14672**
	B3	13767	13765**	12262	60206**	66868**	79104**	14226	41424*	64416*	36478*
	B4	30153	28650**	32390	46784**	68756**	62588**	31674	28486**	53925**	72876**
	Mean	22846	18314	23514	61645	57184.7	57118	23482	146972	281965	175273
F2 ♀	B1	37616	19866**	35153	38774**	47288**	47311**	41981	36743	70491	43818
	B2	26000	21002**	28889	33430**	77540**	76087**	25226	45900**	59707**	27049**
	B3	52753	19023**	55334	16048**	18342**	16874**	51016	14626**	22729**	25148
	B4	31509	26746**	38847	88948**	55004**	86773**	32656	16307**	14341**	23460**
	Mean	36969.5	21659.2	39556	44300	49543.5	56761.2	30447	39469**	14104	65711**
F2	B1	5305	31311**	15873	45176**	72481**	48606**	16235	16302	10883	11405

♂	B2	50227	43459**	46946	76124**	42499**	40377**	40588	29057	27720	35351
	B3	32363	21645**	37702	50637**	50499**	55493**	32957	66926**	25544**	74011**
	B4	34809	32910**	33537	49901**	65812**	42718**	33281	16055**	46627**	96634**
	Mean	33176	32312.2	33515	55459.5	57822.7	46798.5	30765	32090	27694	54350

* one unit of ChE activity is expressed one Ug of acetylcholine (substrat) reacting with ChE in on ml of 100 flies homogenate in one hour incubation at 37°C. * P 0.05 ** P 0.01e

4. Discussion

Results obtained from the SLRL test after treatment with the one concentration of VCR (2ml/100ml of medium) showed that the frequencies for all broods were not significantly different from the control frequencies. Thus, it would be considered as conclusive result.This result agreed with that obtained by Tood *et al*. (1983**)**, who found that the VCR produced many chromosomal effects but it is in the main, not mutagenic. Also, Clements *et al.* (1990) found that vincristine did not give positive results in the white-ivory somatic mutation test in *Drosophila.* However, positive results have been observed in somatic mutation and recombination test (SMART) of *Drosophilla melanogaster* (Tiburi,et al., 2002).

In addition,results showed that fennel and parsley extract had no mutagenic effect on *D.melanogaster* and that the frequencies for all broods were not significantly different from the control ones .These results agreed with that obtained by Zheng *et al*,(1992) who isolated five natural products compound from Umbelliferae, these compounds induced the detoxifying enzyme glutathione S-transferase (GST) in several mouse target tissues and the tumor was reduced from 68% to 11%. Also, the antioxidant activity of the fennel oils was evaluated as well as the antimicrobial activity, **(**Ruberto,2000**)**.Moreover, these results disagreed with the results obtained by Sanchez-Lamar, *et al*.(2002),who found that *Phyllantus orbicularis* plant extract induced micro nuclei and abnormal anaphase in Chinese hamster ovarian (CHO) cells. Moreover, the results of parsley extract agreed with that of Miller *et al*.(1983) who found that parsley and myristicin component didn't induce carcinogenic activities in the mouse and rat male livers.Also Nakashima,(1989), found that parsley can inhibit 88% of the mutagenicity in extracts from roasted beef Reults of applying the combined treatments (pre, co and post): fennel or parsley followed by vincristine; vincristine followed by fennel or parsley and finally the three components together showed non significant results .These results agreed with that of Shukla and Tanega (2005)who found that the pretreatment with garlic extract for 5 days prior to cyclophosphamide (CP) shows significant decrease in the chromosomal aberrations in Swiss albino mice. Moreover, it has been found that orange juice reduced the extent of DNA damage induced by Methyl methanesulfonate (MMS) in the pre and post-treatment by using comet assay in peripheral white blood cell, Franke et al., (2005).Also the pre- treatment with tomato and garlic significantly reduced the frequencies of N-methyl-N-nitro- nitrosoguanidine (MNNG) which induced bone marrow micronuclei in male swiss mice Kumaraguruparan et al., (2005).

Vincristine has been reported to be cytotoxic, namely as far as accumulation of mitotic figures, arrested of cells at metaphases with highly contracted chromosomes but failing of chromatid separation C-mitotic effects, inhibition of tubulin polymerization, disruption in the formation of microtubules and movement of chromosome, Degraeve (1978),Kirsch-Voldersand Parrt(1996) and Millerand Adler (1989).Thus simultaneous measurement of genotoxicity and cytotoxicity at different doses and exposure times may be an important consideration in the evaluation of genotoxicants. Also, the small numbers of biochemical and genetic investigations do not permit establishment of an exact mechanism of herbal therapies and antimutagenic action.

Further experiments are required to determine whether these substances are scavengers of genotoxic species or if their antimutagenic potential is demonstrated in more complicated ways, Xie, et al., (2006) and Andrew,(1997) Although the protective effects of coffee against somatic mutation and mitotic recombination induced by cyclophosphamide (CPH), mitomycin C (MMC) and urethane (URE) were evaluated in the wing spot test in *Drosophila melanogaster*, coffee showed significant dose-related inhibitory effects on the genotoxicity of MMC. The same protective effect was also observed with one concentration of coffee in combination with CPH (Abraham, and Graf, 1996).

Our results showed that VCR caused change in ChE activities in F1 females, F2 bar eye females and F2 wild type male due to its mutagenic potentiality. Statistical analysis indicated that the difference of F1

females, F2 females and F2 males with the control were significant.These results agreed with that of Kozik and Szczech,(1983) who observed that administration of therapeutic doses of vincristine to young rats brings about a drop of the neuronal AChE activity.

Meanwhile, niether the single treatment with fennel plant extract or parsley plant extract did not induce significant difference with the control of both generations in all broods.

The results showed nonsignificant increase in enzyme activity, which did not agree with the finding of Atta-ur-Rahman *et al* (2004) who mentioned that five steroidal alkaloids isolated from ethanolic extract of *Savcococca saligna* possessed cholinesterase inhibitory potential. Also Orhan *et al.*(2004)found that, the fumaria extracts displayed highly potent inhibition against both of the activity of AChE (Acetylcholinesterase) enzymes.

The combined treatments with fennel plant extract or parsley in different combination with vincrestine (pre, co and post treatments) showed a significant increase in enzyme activity for both generations in all broods.

5. Conclusion

vincrestine drug failed to increase the percentage of SLRL mutations and gave a non conclusive result. In contrast, it did record a significant difference when estimating the enzymatic activity of ChE which proved its ability to cause mutations. The treatment with both extracts of fennel and parsley plants didn't cause any significant increase in the SLRL mutations and no significant difference when estimating the enzymatic activity of ChE. Moreover, fennel and parsley extracts failed to induce any antimutagenic effect of vincrestine drug in *Drosophila melanogaster*.

References

1- Abdullaev, F.I., Riveron-Negrete, L., Caballero-Ortega, H., Manuel, H. J., Perez-Lotez., I., Pereda-Miranda, R and Espinosa-Aguirre, J.J.(2003). Use of *in vitro* assays to assess the potential antigenotoxic and cytotoxic effects of saffron (*Crocus Sativus L.*). *Toxicol In Vitro*. 17(5-6): 731-6.

2- Abraham, S.K and Graf, U.(1996). Protection by coffee against somatic genotoxicity in *Drosophila* : Role of bioactivation capacity.*Food and Chemical Toxicology.*, 34(1): 1-14.

3- Abrahams, S.K and Stooper, H.(2004). Anti-genotoxicity of coffee against N-methyl-N.nitro-N-nitrosoguanidine in mouse lymphoma cells. *Mutat Res*. 561(1-2):23-33.

4- Andrew, P. Odin.(1997). Vitamins as antimutagens: Advantages and some possible mechanisms of antimutagenic action. *Mutat Res*., 386:39-67.

5- Atta- ur- Rahman , M ., Feroz , F., Naeem , I., Zaheer- ul Hag , A., Nawaz , S.A., Khan, N and Khan , M. (2004) New Pregname-Type steroidal alkaloids From *Sarcococca salinga* and their cholinesterase inhibitory activity , *Steroids.* 2004 Oct-Nov;69(11-12):735- 41.

6- Brusick, D.(1980). Principle of genetic toxicology. Plenum Press, New York and London.

7- Clements, J., Hawe. D., Lowry, A and Phillips, M (1990). The effects of arrange of anti-cancer drugs in the white-ivory somatic mutation test in *Drosophila. Mutat Res*. 228: 171-176.

8- Degraeve, N. (1978) Genetic and related effects of Vincarosea alkaloids. *Mutant Res*., 55: 31-42.

9- Downing, K.H. (2000). Structural basis for the interaction of tubulin with proteins and drugs that affect microtubule dynamics, Ann. Re v. Cell Dev. *Biol.*, 16: 89-111.

10- Franke, SI., Pra, D., Erdtmann, B., Henriques, JA. and da Silva, J. (2005) Influence of orange juice over the genotoxicity induced by alkylating agents : an in vivo analysis . *Mutagenesis* ; 20(4) : 79-84.

11- Gonzalez-cid, M., Cuello, M. T and Larripa, I.(1999). Comparison of the aneugenic effect of vinorelbine and vincristine in cultured human lymphocytes. *Mutagenisis.*, 14(1):63-66.

12- Kirsch-Volders M, Parrt EM. (1996). Genetic toxicology of mitotic spindle inhibitors used as anticancer drugs. *Mutat Res*., 355:103-128.

13- Kozik, M. Band Szczech, J. (1983). Histoenzymatic and morphometic changes occurring in nerve cells of ggrus cinguli following treatment of gallng rats with vincristine. *Exp Pathol*., 24(4): 243-52.

14- Kumaraguruparan, R., Chandora Mohan, KV., Abraham, SK. and Nagini, S. (2005) Attenuation of N-methyl-N-nitro-nitroguanidine induced genotoxicity and oxidative damage stress by tomato and garlic combination . *Life Sci* ; 76(19) : 2247-2255.

15- Ludena,R.F.(1979). Interaction of lubuline with drugs and small molecules. In Roberts, Kand Hyams, J.S.(eds): "Microtubules New York: Academic Press,PP 94- 116.

16- Marselos, M and Vainio, H.(1991). Carcinogenic properties of pharmaceutic agents evaluated in the IARC monographs programme. *Carcinogenesis*., 12(10): 1751-1766.

17- Miller, B. M and Adler, I. D(1989). Suspect spindle poisons: analysis of C-mitotic effects in mouse bone marrow cells.*Mutagenesis*., 28: 215.

18- Miller, EC., Swanson, AB., Philips, DH., Fletcher, TL., Liem, A. and Miller, JA.(1983) Structure-activity studies of the carcinogenicities

in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Res*;43(3):1124-34.

19- Morales Ramirez,P., Vallarino-Kelly, T-and Cruz-Vallejo, V. (2004). Kinetics of micronucleated polychromatic erythrocyte (MN-PCE) induction in vivo by eneuploidogens. *Mutat Res*., 565: 79-87.

20- Muller, H.J.(1972). The problem of genetic modification Z. Induckt. Abstamn Verbelehre suppl.1,234-260. Cited after Ashuburner M. and E. novitisi., (1978)

21- Nakashima, K. (1989). Effects of vegetable juice on mutagenicity in extracts from roasted beef .*Jap.J.Nutr*; 47:229-303.

22- Orhan, I., B., Choudhary, M.I. and Khalid, A. (2004) Acetylcholinesterase an butyrylcholinesterase inhibitory activity of some Turkish medicinal plants *J Ethnopharmacol.* 91(1):57-60.

23- Panwar, M., Kumar. M., Samarth, R and Kumer, A (2005). Evaluation of chemopreventive action and antimutagenic effect of the standardized panax ginseng extract, EFLA400, in Swiss albinomice.(2005). *Phyother Res*.(1): 65-71.

24- Parejo, I., Viladomat, F., Bastida, J., Schmeda-Hirschmann, G., Burillo, J. And Codina, C.(2004). Bioguided isolation and identification of the non-volatile antioxidant compounds from fennel(Foeniculum Vulgare Mill.) Waste. *J Agric Food Chem*. 52(7): 1890-7.

25- Ruberto, G., Baratta, M.T., Deans, S. G and Dorman, H.J(2000). Antioxidant and antimicrobial activity of *Foeniculum Vulgare* and *Crithmum naritimum* essential oils. *Planta Med*., 66(8):687-93

26- Sanchez-Lamar A. Fuents, J., Fonseca, G., Capiro, N., Ferrer, M., Alonzo, A., Baluja, L., Cozzi, R., De Sakvia, R., Fiore, M and Liagostera, M.(2002). Assessment of the potential genotoxic risk of phyllantus orbicularis HBK aqueous extract using in vitro and in vivo assays. *Toxicol Lett*., 136(2): 87-96.

27- Satyanarayana,S., Sushruta, K., Sarma, G.S., Sirinivas, N and Subba Raju, G.V.(2004). Antioxidant activity of the aqueous extract of spicy food additives-evaluation and comparison with ascorbic acid in-vitro systems. *Herb Pharmacother*. 4(2): 1-10.

28- Shukla,Y. and Taneja,P.(2002)Antimutagenic effects of garlic extract on chromosomal aberrations.Cancer Lett;176(1):31-36.

29- Soudamini, K.K., Unnikrishnan, M.C., Sukumaran, K and Kuttan, R (1995). Mutagenicity and anti-mutagenicity of selected spices. *Indian J Physiol Pharmacol*. 39(4): 347-53.

30- Tiburi, M., Reguly, M.L., Schwartsmann, G., Cunha, K.S., Lehmann, M. and Rodrigues de Andrade, H.H (2002). Comparative genotoxic effect of vincristine , vinblostine and vinorelbine in somatic cells of *Drosophila melanogaster. Mutat Res*., 519: 141-149.

31- Todd, N., Clements, J., Zoeller, P and Phillips, M (1983). Absence of a mutagenic effect after feeding 4 anti-cancer drugs to *Drosophila melanogaster. Mutat Res*. 120: 121-125.

32- Winn, R.N., Kling, H and Norris, M.B (2005). Antimutagenicity of green tea polyphenols in the liver of transgenic medaka. Environ. Mol Mutagen.(2) 2005 Wiley-Liss, Inc.

33- Wurglar, F.E., Graf, U. And Berchtold, W.(1975) Statistical problems connected with sex-linked recessive lethal test in *Drosophila melanogaster.* Archiv fur genetick. 48:158-178.

34- Xie, J.T., Wang, C.Z., Wicks, S., Yin, J.J., Kong, J., Li, J., Li, Y.C. and Yuan, C.S.(2006). Genoderma lucidum extract inhibits proliferation of SW480 human colorectal cancer cells. *Exp Oncol*., 28(1): 25-29.

35- Zheng, G.Q., Kenney, P. M and Lam,L.K.(1992). Anethofuran, carvone, and limonene: Potential cancer chemopreventive agents from dill weed oil and caraway oil. *Planta Med*., 58(4):338-41.

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Evaluation of Proximate and Phytochemical Compositions of Fermented Raw and Fermented *Napoleona Imperialis* Seed and Their Feeding Values on Finisher Broilers

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Abstract: The high cost of feed in poultry enterprise is well established. This is blamed on limited availability of conventional feedstuff which is also in competition with man's dietary needs. This has necessitated the search for alternative protein sources such as *Napoleona imperialis* seed. Ripe *N. imperialis* seeds (NISs) were harvested in and around the Federal University of Technology, Owerri with the pods opened, the seeds extracted, and sun dried for 7 days. A portion of the sundried NIS was milled using hammer mill to produce the raw *N. imperialis* seed meal (NISM) while, the remaining portion was soaked in water for 4 days and sundried before milling to produce soaked NISM. Samples of raw and soaked NISMs were taken to the laboratory to determine its proximate and phytochemical compositions. Phytate, tannins, HCN, alkaloids, saponins and metabolisable energy value of the raw NISs were significantly ($p<0.05$) affected by the treatment. Birds on control diet performed significantly ($p<0.05$) better than those on 10% soaked NISM diet in terms of average daily feed intake and feed conversion ratio but similar ($p>0.05$) to those on 5% raw and 5% soaked NISMs. The average daily weight gain of birds on 5% raw and 10% soaked NISMs was significantly ($p<0.05$) lower than the control group. It is concluded that soaking for 4 days in water do not reduce the anti-nutritional content of *N. imperialis* seeds to a tolerable level for broilers. [Nature and Science 2010;8(4):83-88]. (ISSN: 1545-0740).

Keywords: novel seeds, proximate composition, phytochemistry, performance, broilers.

1. Introduction

The high cost of feed components as a variable in poultry enterprise is well established (Igboeli, 2000; Esonu, 2006). The high cost is blamed on limited availability of conventional feedstuff which are also competed for by man. This has necessitated the search for alternative feed ingredients. One of such alternative feed ingredient is *Napoleona imperialis* seed.

Napoleona imperialis (P. Beavr) belongs to the family *lecythidaceae* which is an evergreen non-timer plant that grows abundantly in bush fallows, secondary bushes and marginal lands in most of the tropical humid zone of West Africa (Koppel, 1990). People consume the juice from the pods and discard the seeds. The seeds appeared to have very low human food preference, little or no industrial use as at now.

Uchegbu *et al.* (2004) reported that raw *Napoleona imperialis* seed meal had 4.8% moisture, 11.7% crude protein, 4.9% ether extract, 3.6% crude fibre and 3.52% ash. The mineral content of *Napoleona imperialis* seed meal included 5.01g/kg calcium, 17.5g/kg potassium and 16.1 g/kg sodium (Ukpabi and Ukpabi, 2003). Iheukwumere and Okoli (2002) and Iheukwumere *et al.* (2002) fed the raw dried seeds to weaner rabbits and observed that at 15% inclusion, the seeds had no visible deleterious effects on growth rate, haematology and serum biochemical values. Uchegbu *et al.* (2004) recorded a good performance on finisher broilers fed 5% inclusion level of raw *Napoleona imperialis* seed meal, noting that beyond 5% level the performance was poor.

Napoleona imperialis seeds were reported by Uchegbu *et al.* (2004) to contain anti-nutritional factors and toxic elements such as saponin, tannin, flavonoid, phytate, alkaloid, cyanogenic glucosides and cardiac glucosides. In plants, saponin serves as anti-feedants and protects the plant against microbes and fungi attack. Saponins are often bitter to taste and so can reduce plant palatability. Radostits *et al.* (1997) reported that saponins could cause gastroenteritis, manifested by diarrhoea and dysentery. Westendarp (2005) reported negative effects of saponins on performance of farm animals. Oxalate decreases the availability of dietary essential minerals (calcium) at high concentration and causes death in animals due to its corrosive effects (Kumar and D'mello, 1991). Cyanogenic glucosides inhibit the energy giving oxygen linked respiratory activities in the cellular mitochondria (Lehnimger, 1975).

Numerous studies (Uchegbu *et al*., 2004; Ofoegbu, 2008; Osuagwu, 2008) have confirmed the presence of anti-nutritional factors in raw *Napoleona imperialis* seeds and the resulting negative performance of animals fed these seeds.

There is need to access the extent to which processing (soaking in water) will enhance the utilization of *Napoleona imperialis* seeds for monogastric animals, especially broilers. Therefore, the objective of the study was to evaluate the effect of four days fermentation on proximate and phytochemical compositions of raw *Napoleona imperialis* seed and as well as its feeding values in finisher broilers diets.

2. Materials and Methods

2.1 Experimental Site

This research was carried out in the Poultry Unit of the Teaching and Research Farm, Department of Animal Science and Technology, Federal University of Technology, Owerri, Imo state. It is situated in southeastern agro-ecological zone of Nigeria. The vegetation is typically rainforest with two seasons, the rainy and dry seasons. The period of rainy season is from the month of April to October, while the dry season runs through November to March. Imo state lies between latitude 4^{o} 4' and 6^{o}3' N and longitude 6^{o}15' and 8^{o}15' E.

2.2 Plant collection and authentication

The riped *Napoleona imperialis* seeds were harvested in and around the Federal University of Technology, Owerri. The scientific name was authenticated in the Department of Crop Science and Technology, Federal University of Technology, Owerri by Dr. I.I. Ibeawuchi.

2.3 Processing *Napoleona imperialis* seed into meal

Fresh matured *N. imperialis* pods opened, seeds removed (Plate 1) and sun dried for about 9 hours every day for 7 days. A portion of the sun dried *N. imperialis* seeds was milled using hammer mill to produce the raw *Napoleona imperialis* seed meal while, the remaining portion was soaked in water for four (4) days, sun dried and milled to produce soaked *N. imperialis* seed meal. Samples of raw and soaked *Napoleon imperialis* seed meals were taken to the laboratory for proximate and phytochemical analyses.

Plate 1. *Napoleona imperialis* seeds

2.4 Phytochemical and proximate analysis

Determination of tannin content of *Napoleon imperialis* seeds was done by the ferric chloride test as described by Harborne (1973). The presence of flavonoids in the test was determined by the acid alkaline test (Harborne, 1973). Saponin was gotten from emulsion test with aqueous extracts. Alkaloid was obtained by dispensing samples in Ethanol and Mayer's reagent. Phenol was determined according to the folic ciocciteon calorimetric method (A.O.A.C, 1990). HCN was gotten by the alkaline picrate colorimetric method. Phytate was determined by the spectrophotometer method of Oberlease (2003). The proximate composition of raw and soaked *Napoleon imperialis* seed meal was determined using the standard procedures of A.O.A.C. (1990).

2.5 Experimental design

Two hundred Anak finisher broilers were divided into four treatment groups of fifty birds each. Each treatment group was subdivided into five replicates of ten birds each. Four treatment diets 0% (control), 5% raw

($T_{5\%R}$), 5% soaked ($T_{5\%S}$) and 10% soaked ($T_{10\%S}$) NISMs were formulated (Table 1) and fed to finisher broilers in completely randomized design experiment for 35 days.

Table 1: The ingredient compositions of experimental diets fed to finisher broilers

Ingredient	% Diets (Napoleona imperialis seed meal)			
	$T_{0\%}$	$T_{5\%R}$	$T_{5\%S}$	$T_{10\%S}$
Maize	60	55	55	50
Napoleona imperialis seed meal	0	5.0	5.0	10.0
Calculated nutrient analysis				
Crude protein	20.4	20.7	20.7	20.8
Crude fibre	4.11	4.16	4.16	4.18
Ether extract	4.28	4.31	4.37	4.40
ME (Kcal/kg)	2950.99	2948.92	2947.83	2942.14

Each diet contained 20% soybean meal, 6% wheat offal, 3% palm kernel cake, 4% fishmeal, 3% blood meal, 2% bone meal, 1% oyster shell, 0.25% methionine, 0.25% lysine, 0.25% vitamin / mineral premix, 0.25% common salt. Vitamin/ premix provides the following per kg of feed: vitamin A, 10,000iu; vitamin D_3, 2000iu; vitamin E, 5iu; vitamin K, 2mg; riboflavin, 4.2mg; vitamin B_{12}, 0.01mg; panthothenic acid, 5mg; nicotinic acid, 20mg; folic acid, 0.5mg; Choline, 3mg; magnesium, 56mg; iron, 20mg; copper, 1.0mg; zinc, 5.0mg; cobalt, 1.25mg; iodine, 0.8mg; R - raw; S - soaked.

2.6 Data analysis

Statistical differences between treatment means were determined with the one way analysis of variance for completely randomized design (Steel and Torrie, 1980). Data on proximate composition and phytochemical composition were statistically analyzed using the t - test procedure of Snedecor and Cochran (1978). Where significant differences were detected between treatment means, mean separation was done using Duncan's New Multiple Range Test as outlined by Obi (1990).

3. Results

3.1 Phytochemical compositions: The phytochemicals compositions of raw and soaked *Napoleona imperialis* seeds are shown in table 2. The tannins, phytate, HCN, alkaloids and saponin values were significantly ($p<0.05$) reduced by soaking in water for 4 days. All other parameters measured including oxalate and autocyanin were similar ($p>0.05$) between the two treatments.

Table 2: The quantitative phytochemical composition of raw and soaked *Napoleona imperialis* seed

Parameters	Raw NIS	Soaked NIS
Oxalate	0.85	0.73
Tannins	1.35^a	0.84^b
Phytate	1.56^b	0.96^a
HCN (mg/kg)	18.74^a	9.52^b
Autocyanin	0.43	0.41
Alkaloid	0.56^b	0.38^a
Saponin	0.68^b	0.48^a

^{ab}Means within row with different superscripts are significantly ($p<0.05$) different. NIS - *Napoleona imperialis* seed.

3.2 Proximate compositions: The proximate compositions of raw and soaked *Napoleona imperialis* seeds are presented in table 3. The results showed that soaking *Napoleona imperialis* seeds in water for 4 days had no significant effect ($p>0.05$) on the crude protein, crude fibre, ether extract, ash, nitrogen free extract, moisture and dry matter. Soaking *Napoleona imperialis* seeds in water for four days significantly ($p<0.05$) lowered the metabolisable energy value.

Table 3: The proximate compositions of raw and soaked *Napoleona imperialis* seed

Parameters	Raw NISM	Soaked NISM
Moisture	11.26	12.81
Dry matter	88.74	87.19
Ash	3.82	3.31
Crude fibre	4.11	3.71
Ether extract	4.93	3.62

Crude protein	14.84	15.35
Nitrogen free extracts	61.04	61.2
ME (Kcal/kg)	2618.98[a]	2494.00[b]

[ab]Means within row with different superscripts are significantly ($p<0.05$) different; NISM - *Napoleona imperialis* seed meal.

3.3 Growth performance: The effects of raw and soaked *Napoleona imperialis* seed meals on performance of finisher broilers are shown in table 4. The average final body weights of the control birds were significantly ($p<0.05$) higher than the group fed 10% soaked *N. imperialis* seed meal diet. The average daily feed intakes of birds on control diet were not significantly ($p>0.05$) different from birds fed 5% raw and soaked NISM, but significantly ($p<0.05$) higher than those fed 10% soaked NISM diet. There was significant difference ($p<0.05$) in average daily weight gain of birds fed control diet relative to those on 10% soaked NISM diet. The mortality recorded in the study was numerically ($p>0.05$) higher and could be attributed to the *Napoleona imperialis* seed meal diet.

Table 4: The performance characteristics of finisher broilers fed raw and soaked *Napoleona imperialis* seed meal based diets

Parameter	***Napoleona imperialis* seed meal diets (%)**				**SEM**
	$T_{0\%}$	**$T_{5\%R}$**	**$T_{5\%S}$**	**$T_{10\%S}$**	
Avg. initial body weight (kg)	0.84	0.82	0.90	0.87	0.05
Avg. final body weight (kg)	1.24[a]	1.03[a]	1.19[a]	0.98[b]	0.07
Avg. daily weight gain (kg)	0.40[a]	0.21[ab]	0.29[ab]	0.11[b]	0.05
Avg. daily feed intake (g)	110.0[a]	100.0[a]	103.3a	70.0[b]	10.24
Feed conversion ratio (g feed / g gain)	4.94[b]	7.50[b]	8.50[b]	17.81[a]	2.25
Mortality (%)	3.30	4.80	10.60	14.30	

[a,b]Means within row with different superscripts are significantly ($p<0.05$) different. R - Raw, S - Soaked, SEM – Standard error mean.

4. Discussion

The reduction in phytate level of soaked *N. imperialis* seeds in the present study is in agreement with the earlier findings of Alonos *et al*. (1998) in *Faba* bean seeds soaked in water. The reduction in phytate content of raw *Napoleona imperialis* seeds during soaking could be attributed to leaching out along the concentration gradients (Kataria *et al*., 1989). The phytates (a poly phosphoric ester of inositol) are known to increase the requirement for minerals, especially phosphorus, which form insoluble complexes with phytic acid. The reduction in saponin content was in line with similar losses of saponin in fermented Baobab seeds (Umaru *et al.*, 2006). The 18.74 mg/kg HCN values found in raw *Napoleona imperialis* seed was much lower than those found in cowpea seeds (40 mg/kg), and was beyond the upper limit of 10 mg/kg HCN reported to be safe for human consumption (Oke *et al*., 1996; Makkar and Becker, 1997).

The crude protein content of the soaked *Napoleona imperialis* seeds was relatively higher when compared with the raw seeds. This agrees with the results of Yashim *et al.* (2009) that soaking improves the protein content of raw seeds. The reduction in ether extracts of *Napoleona imperialis* seeds soaked in water relative to raw *Napoleona imperialis* seeds was in contrast with the reports of Omafuvbe *et al*. (2004) on African locust bean seeds. The decrease in ash content of soaked *Napoleona imperialis* seeds was in agreement with the findings that ash decreased with soaking as reported on Delichous lablab beans by Osman (2007).

The decreases in final body weight of the birds fed 5% raw and 10% soaked NISMs could be attributed to the presence of anti-nutritional factors contained in raw *N. imperialis* seeds which prevent optimal utilization of nutrients in *N. imperialis* seed meal. The comparable result in final body weight of broilers fed control and 5% soaked NISM diets was an indication that soaking in water for 4 days might have reduced the anti-nutritional contents to a tolerable level. Higher level of anti-nutritional factors has been implicated in lowering nutrient availability and absorption in animals (Kumar and D'mello, 1991).

The significant difference in average daily weight gain of birds fed control diet relative to those on 10% soaked NISM diet. This reflects the inability of these birds to adequately handle and tolerate the anti-nutritional factors at this level of inclusion of soaked NISM. The results of the average daily weight gain of the birds on control diet were in agreement with the findings of Uchegbu *et al*. (2009) that birds fed control diet performed significantly better than those fed raw and soaked NISM diets. The reasons for the depressed performance in these birds could be associated with the anti-nutritional factors contained

in *Napoleona imperialis* seed meal (Uchegbu *et al*., 2009).

Feed conversion ratio (FCR) of the birds fed 10% soaked NISM was significantly ($p<0.05$) higher than the other three groups. The numerically lower FCR value recorded in control birds relative to those on the other 3 treatment groups was an indication that control diet was better utilized by these birds. The poorest result in FCR of birds on 10% soaked NISM diet was in support with the findings of Uchegbu *et al.* (2009) who reported the same result in finisher broilers fed 10% cooked NISM diet. The implication was that soaking in water for 4 days did not achieve a significant improvement in the reduction of the anti-nutritional content of *N. imperialis* seeds, and thus did not improve the utilization of *Napoloena imperialis* seed meal diets.

The negative effects of *N. imperialis* seed on feed intake have been reported by numerous researchers (Uchegbu *et al*., 2004; Westendarp, 2005; Radostits *et al*., 1997). It appeared that anti-nutritional factors (e.g. saponins) created a palatability problem (Ogbonna, 1983) which suppressed the intake of diet with 10% soaked *Napoleona imperialis* seed meal. The implication is that soaking slightly improved the utilization of NISM diet at 5% soaked relative when compared to those fed 5% raw NISM. In view of the fact that all the diets met the nutrient requirement for finisher broilers (Esonu, 2006) yet the birds assigned to these diets performed poorly support the report of Iyayi (2004) that a feed may contain adequate amount of nutrients in balanced proportions, yet these nutrients may not be available to the animals.

4. Conclusion

From the results obtained it can be concluded that inclusion of raw and soaked *Napoleona imperialis* seed meal in the diet of finisher broilers resulted to visible deleterious effect on performance. Although soaking *Napoleona imperialis* seeds in water for four days improved its utilization by broilers, but not to the extent of incorporating it up to 10%. Therefore more detail research is required to determine the appropriate methods of processing raw *Napoleona imperialis* seeds.

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References

[1] Igboeli G. Animal Production and Agriculture in the new millennium. Book of Proc. 25th Ann. Conference of Nigerian Society of Animal Production 2000; 1 - 3.

[2] Esonu BO. Animal nutrition and feeding: A functional approach. Rukzeal and Rukson Association Memory Press, Owerri, Nigeria 2006.

[3] Kopel CRS. The major significance of minor forest products in the humid forest zone. Food and Agricultural Organization, Rome 1990.

[4] Uchegbu MC, Okoli IC, Etuk EB, Anyanwu GA, Esonu BO, Udedibie ABI. Performance, carcass and organ characteristics of finisher broilers fed graded levels of raw *Napoleona imperialis* seed meal. Livestock Research for Rural Development 2004; 16 (6):24-30

[5] Ukpabi UH, Ukpabi UJ. (2003). Potential of seeds of *Napoleona imperialis* (p beauv) as a source of hemolytic saponin and feed ingredients. Livestock for rural development 2003; 15(12). http://www.Irrd.org/Irrd15/12/Ukpa 1512.

[6] Iheukwumere FC, Okoli IC. Preliminary studies on raw Napoleona imperialis as feed ingredients I: Performance and blood chemistry of weaner rabbits. Tropical Animal Production Investigation 2002; 5 (1): 113 – 119.

[7] Iheukwumere FC, Okoli IC, Okeudo NJ. Preliminary studies on raw Napoleona imperialis as feed ingredients II: Effect on performance, haematology and serum biochemistry, carcasses and organ weight of weaner rabbits. Tropical Animal Production Investigation 2002; 5 (1): 219 - 227.

[8] Radostits OM, Blood DC, Gray CC. Veterinary Medicine. Textbooks of the

diseases of cattle, sheep, goat, pigs and horses. 8th Edition. Saunders Publishers London 1997; 1580.

[9] Westendarp H. Saponin in nutrition of swine, poultry and ruminants. Deutsche Tierarztliche Wochenschrift 2005; 112: 65-70.

[10] Kumar R, D'mello JPF. Anti-nutritional factors in forage legumes. In: Tropical legumes in animal nutrition 1991.

[11] Lehnimger AL. Biochemistry. 2nd edition, Worth Publishers, New York 1975.

[12] Ofoegbu JC. Qualitative and quantitative evaluation of phytochemicals of broken, soaked and boiled *Napoleona imperialis* seeds, Project Report, Department of Animal Science and Technology, Federal University of Technology, Owerri 2008; 1-51.

[13] Osuagwu GC. Qualitative evaluation of anti-nutritional substances in raw, soaked and cooked *Napoleona imperialis* seed Project Report, Department of Animal Science and Technology, Federal University of Technology, Owerri 2008; 1-41.

[14] Harborne JB. Phytochemicals methods. A guide to modern techniques of plant analysis. London: Champman and Hall, New York 1973.

[15] AOAC. Official methods of analysis (15th edition.). Association of Official Analytical Chemist, Arlington, VA 1990.

[16] Oberlease D. Phytates. In: Toxicants occurring naturally in foods. Washington D.C. National Academic press 2003; 143-146.

[17] Steel RGB, Torrie JH. Principle and procedure of statistics. McGraw Hill, Books Co., New York1980.

[18] Snedecor GW, Cochran WG. Statistical Methods (6th edition). The Iowa state University press. Iowa - USA 1978.

[19] Obi IU. Statistical methods of detecting differences between treatment means, 2nd edition. Snaap press, Enugu, Nigeria 1990.

[20] Alonos R, Orue E, Marzo F. Effects of extrusion and conventional processing method of protein and anti-nutritional content in pea's seeds. Food Chemistry 1998; 63: 505-512.

[21] Kataria A, Chauhan BM, Punia D. Anti-nutrient and protein digestibility (in vitro) of Mung bean as affected by domestic processing and cooking. J. Food Chemistry 1989; 32: 9-17.

[22] Umaru HA, Adamu R, Dahiru D, Nadro MS. Levels of anti - nutritional factors in some wild edible fruits of Northern Nigeria. African Journal of Biotechnology 2006; 6(16): 1935-1938.

[23] Oke DB, Fetuga BL, Tewe OO. Effect of autoclaving on the anti-nutritional factors of cowpea varieties. Nigerian Journal of Animal Production 1996; 23: 33-38.

[24] Makkar HPS, Becker K. Nutrient and anti-nutrient factors in different morphological parts of *Oleifere* tree. Journal of Agricultural Science, Cambridge 1997; 128: 311-322.

[25] Yashim SM, Segun AJ, Abdu SB, Duru S, Adamu HY. Effects of duration of soaking of rattlebox seeds (*Crotalaria retusa L.*) on the level of some chemical composition and minerals. Proceeding of 34th Annual Conference of Nigerian Society of Animal Production 2009; 309-311.

[26] Omafuvbe BO, Falade OS, Osuntogun BA, Adewu SRA. Chemical and biochemical charges in Africa locust bean (*Parkia biglobosa*), and Melon (*Citrullus vulgaris*) seeds during fermentation to condiments 2004; 221-224.

[27] Osman MA. Effect of different processing methods on nutrient composition, anti-nutritional factors and in vitro digestibility of delichos Lablab Bean (*Lab lab purpureus* (L) sweet). Pakistan Journal of Nutrition 2007; 6: 299-303.

[29] Uchegbu MC, Ibekwe OA, Ogbuewu IP, Nwaodu CH, Obikaonu HO, Okoli IC. Feed intake and growth rate of finisher broilers fed diets containing raw and cooked Napoleona imperialis seed meal, Revista Cientifica UDO Agricola 2009. Accepted for publication.

[29] Ogbonna SC. Some chemical components of some woody species. M.Sc. Thesis, University of Ibadan 1983; 21-26

[30] Iyayi EA. Nigeria Farming Veterinary Services Food and Nutrition. Magazine 2004; 1 (6): 12-15.

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Plasmid Associated Anthracene Degradation by *Pseudomonas* sp. Isolated from Filling Station Site

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Abstract: Bacterial strains were isolated from oil contaminated soil of 5 different filling stations of Himachal Pradesh, India and screened for their anthracene degradation ability. Enriched media was used to isolate the anthracene degrading bacteria with 0.5% peptone and 0.1% w/v anthracene in basal salt mineral medium and during successive enrichment the peptone concentration was decreased to 0.25 g, 0.1 g and to 0.0 g. After one month of enrichment 5 strains were found to be potent anthracene degrader out of total 76 strains screened. These 5 strains were further subcultured for 10 days and on the basis of percent anthracene degradation strain E was found to degrade 74.8% anthracene supplemented in BSM medium at 0.1% as sole source of carbon and energy and identified as *Pseudomonas* sp. As evident by antibiotic sensitivity test, *Pseudomonas* sp. showed resistance against Cefadroxil and Ampicillin among tested 7 antibiotics. Acridine orange induced plasmid curing of isolate lead to complete loss of plasmid and anthracene degradation activity. The study suggests that the plasmid could have a role in anthracene degradation activity. [Nature and Science 2010;8(4):89-94]. (ISSN: 1545-0740).

Key words: anthracene, *Pseudomomas* sp., plasmid curing, acridine orange, marker antibiotic

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of compounds containing carbon and hydrogen, composed of two or more fused aromatic rings in linear, angular and cluster arrangements. They are lipophilic in nature and relatively insoluble in water (Hafez *et al.*, 2008 and Johnsen *et al.*, 2005). PAHs are ubiquitous pollutants and are generated from anthropogenic activities such as the burning of fossil fuels, the use of wood preservatives such as creosote and the generation of wastes from coal gasification plants (Ni Chadhain *et al.*, 2006). PAHs have been identified as hazardous chemicals by different State and Central Pollution Control Boards because of their toxic, carcinogenic and tetragenic effects on living body (Ruma *et al.*, 2007). Anthracene, together with other PAHs, is a persistent and toxic soil contaminant (Hyotylainen and Oikari, 1999; Lotufo, 1997). Pollution by PAHs is usually found on the sites of gas factories and wood preservation plants. Bioremediation is an economically and environmentally attractive solution for cleaning those sites (Kastner and Maho, 1996). Environmental anthracene contamination originates from a number of anthropogenic sources/practices such as manufacturing of dyes, production of synthetic fibers, plastics and pesticides, petroleum spills as a result of pipeline rupture and tanker failure. Its natural sources are coal and tar, and can be released by incomplete combustion of fuels (such as coal, oil, gas). Therefore it is a constituent of exhaust from automobile and charcoal grills.

Overall bioremediation is an attractive process due to its cost effectiveness and the benefit of pollutant mineralization to carbon dioxide and water (Trindade *et al.*, 2005). Degradation of anthracene, that is its conversion to both carbon dioxide and water (mineralization) or other organic substances (degradation products) which is not toxic is one of the inexpensive way of removing large concentrations of anthracene from soil and water. Transfer of genes responsible for biodegradation of hydrocarbons plays an important role in bioremediation of pollutants (Wilson and Jones, 1993). In this study we described our experiment designed to determine whether the anthracene degradation was plasmid associated or associated with chromosomal DNA.

2. Materials and methods

2.1 Collection of soil samples

For the isolation of anthracene degrading bacteria, soil samples were collected from 5 different Filling station sites of Himachal Pradesh, India. Samples were stored in sterilized polyethylene bags at 4°C for further use.

2.2 Enrichment and isolation of anthracene degrading microorganism

One gram of each soil sample was suspended in 100 ml Basal Salt Mineral (BSM) medium (g/l: K_2HPO_4, 0.38; $MgSO_4.7H_2O$, 0.2; NH_4Cl, 1.0; $FeCl_3$, 0.05; Distilled Water, 1000 ml and pH, 7.0) broth containing 1.0 g peptone and 0.1% w/v anthracene.

Flasks were incubated at 30°C on a rotary incubator shaker at 150 rpm. After 1 week of incubation, 5 ml of inoculum was transferred from each flask to the fresh 100 ml BSM broth containing 0.5 g peptone and 0.1% w/v anthracene. Further subculturing was done with 0.25 g, 0.1 g and 0.0 g peptone with constant concentration of anthracene. The anthracene degrading microorganisms from the flask containing 0.1% w/v anthracene and no peptone were isolated by spread and streak plate method and analyzed for their anthracene degradation activity.

2.3 Analytical techniques

For the determination of λ max of anthracene, a 10 ppm solution of anthracene in ethyl acetate was scanned from 190-600 nm on UV-VIS spectrophotometer. The λ max was used to determine the concentration of anthracene in ethyl acetate extracts. For preparation of standard curve of anthracene, a stock solution of 10 ppm in ethyl acetate was prepared and aliquots in the range of 0.2 to 1 ppm were separately read at λ max of anthracene.

2.4 Screening of anthracene degrading microorganisms

For screening of anthracene degrading microorganism, 30 ml BSM broth containing 0.1% w/v anthracene as sole carbon source was taken in different flasks and inoculated with 5% inoculums ($A_{600,}$ 0.70) of different enriched soil isolates. All the flasks were incubated for 10 days at 30°C on rotary shaker incubator at 150 rpm. During incubation the residual concentration of anthracene was monitored spectrophotometrically for 10 days by liquid-liquid extraction method as described by Manohar *et al.* (1999).

Further the anthracene degrading capability of most efficient anthracene degrading organism was monitored in 30 ml BSM broth containing 1.5% w/v anthracene/ethyl acetate and 5% inoculum (A_{600} 0.70). The degradation was monitored by sampling 2 ml from each reaction set for 240 h at an interval of 24 h. Identification of most efficient anthracene degrading microorganism was done on the basis of microscopic, morphological and biochemical characteristics.

2.5 Plasmid curing by acridine orange

BSM broth (18 ml) was taken in different flasks and it was inoculated with 1 ml culture inoculum (A_{600} 0.70) with different concentrations of acridine orange ranging from 10-100 µg/ml (Fujii *et al.*, 1997). The flasks were wrapped in black paper to prevent the photolysis of cells then incubated at 40°C for 7 days with gentle shaking at 100 rpm. After incubation acridine orange treated cultures were serially diluted up to 10^{-11} times in sterilized saline.

Seven different antibiotics were used to check the antibiotic sensitivity of most efficient anthracene degrading microorganism by agar cup method. The culture was spread plate on BSM agar medium and incubated for 30 min. The wells of 4 mm diameter were made with the help of a cork borer in the agar at equal distance. Seventy µl of each antibiotic was poured in these wells.

2.6 Preparation of master and replica plate

Master plate was prepared on nutrient agar plate. Eighty µl of different dilution preparations were spread on the plates and were allowed to incubate at 37°C for 24 h. Replica plates were prepared by transferring the exact imprint of master plate. For replica plate (Nutrient Agar plate with marker antibiotic, Cefadroxil) every single colony from master plate was picked with sterilized tooth prick tip and placed on the corresponding site on replica plate. All the replica plates were incubated at 37°C for 24 h and master plates were preserved at 4°C.

2.7 Plasmid isolation and agarose gel electrophoresis

The plasmid was isolated by alkali treatment methods described by Kado and Liu (1981) and was electrophoresed on 0.8 % agarose gel in presence of ethidium bromide (1 µg/ml). DNA bands were visualized under UV light under UV transilluminator and photographed.

3. Results

3.1 Screening for most efficient anthracene degrading microorganism

The λ max of anthracene in ethyl acetate was determined to be 254 nm (Figure 1). It was used to determine the concentration of anthracene in ethyl acetate extracts. Different soil samples from 5 filling station sites were analyzed for their anthracene degradation capability. After enrichment of 76 bacterial strains, 5 strains were found to be efficient anthracene degrader. Further the anthracene degrading capability was monitored for 10 days in BSM medium supplemented with anthracene as sole source of carbon. On the basis of anthracene degradation (%) ability strain E was found to be the most efficient anthracene degrader with maximum degradation rate of 70.6%. Strain B had shown the least anthracene degradation, 15.5% (Figure 2). Strain E was identified on the basis of cultural, microscopic, morphological and biochemical characteristics (Table 1). As determined by Bergey's manual of systematic bacteriology the strain E has been tentatively identified as *Pseudomonas* sp.

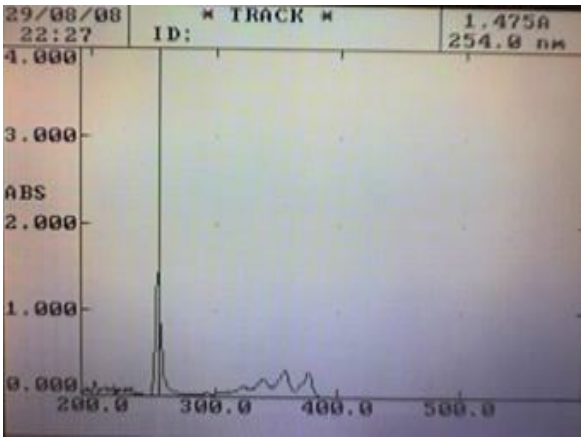

Figure 1. UV-Spectrum of anthracene in ethyl acetate. A single peak at 254 nm shows the max absorbance

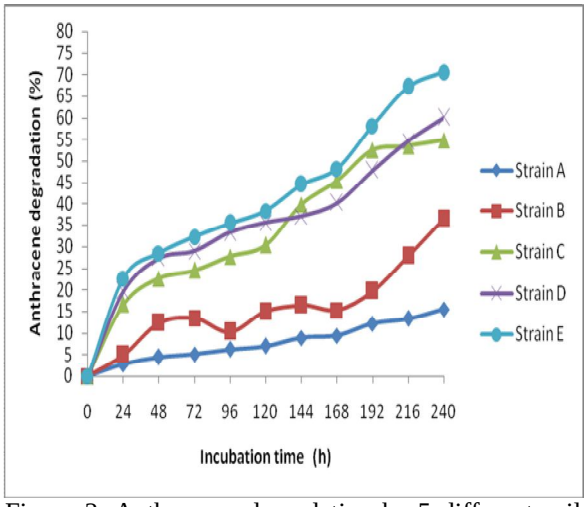

Figure 2. Anthracene degradation by 5 different soil isolates for 10 days of incubation period

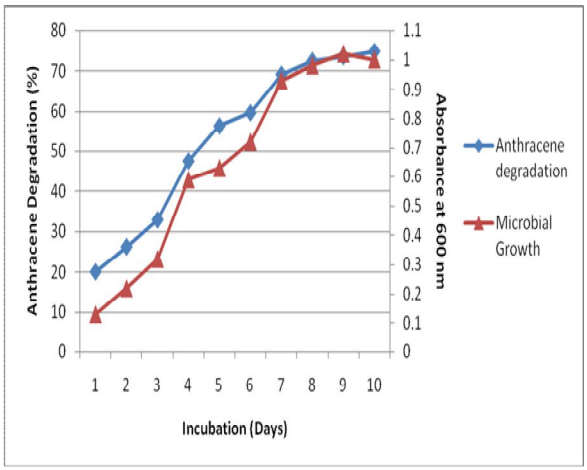

Figure 3. Growth (A_{600}) and anthracene degradation (%) by *Pseudomonas* sp.

3.2 Anthracene degradation study

The results of growth of the bacterium on anthracene and its utilization for different incubation periods were represented in Figure 3. Results revealed that there is an increase in cell growth with an increase in incubation period. The maximum growth of the bacterium was observed at 9th day of incubation (A_{600}, 1.02). The bacterium showed 74.8% utilization of anthracene at 10th day of incubation.

3.3 Plasmid curing by acridine orange

Pseudomonas sp. was tested for sensitivity and resistance against different antibiotics were used for sensitivity and resistance against *Pseudomonas* sp. Zone of inhibition was observed against oxytetracycline, azithromycin, erythromycin, cefixine and amoxyciline antibiotics while ampicillin and cefadroxil showed no zone of inhibition (Table 2, Figure 4). Replica plate showed the disappearance of colony when incubated in presence of acridine orange and marker antibiotic, cefadroxil (Figure 5). In contrast to this, the same colony was present on master plate which was untreated with acridine orange and incubated in presence of cefadroxil marker antibiotic (Figure 6). The curing of plasmid DNA was further supported by agarose gel electrophoresis of isolated plasmid DNA. A single band was observed for uncured culture while no band was visualized when the culture was treated with acridine orange at a concentration of 50 µg/ml (Figure 7).

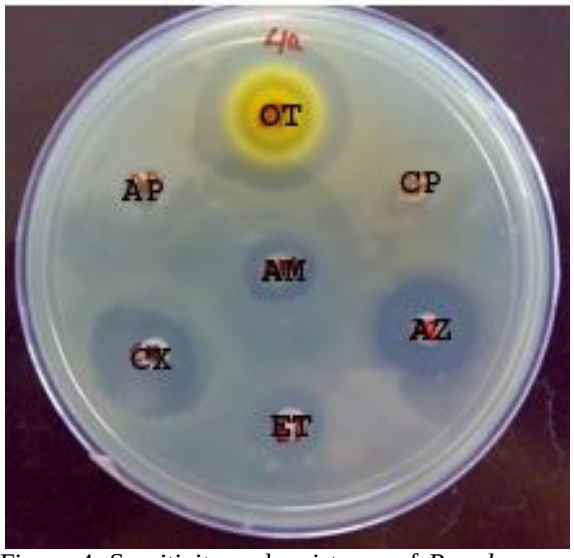

Figure 4. Sensitivity and resistance of *Pseudomonas* sp. against different antibiotics (OT- Oxytetracycline, CP- Cefadroxil, AZ- Azithromycin, ET- Erythromycin, CX- Cefixine, AP- Ampicillin, AM- Amoxicilline)

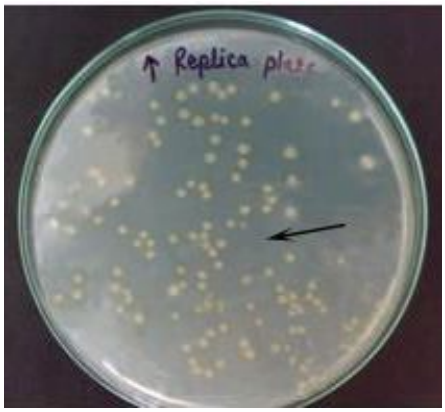

Figure 5. Replica plate showing the position of disappeared colony (arrow point) when incubated in presence of Cefadroxil, marker antibiotic.

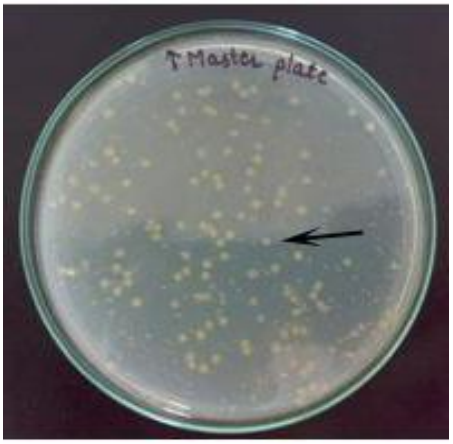

Figure 6. Master plate showing *Pseudomonas* sp. colonies on BSM agar plate when incubated in presence of Cefadroxil, marker antibiotic

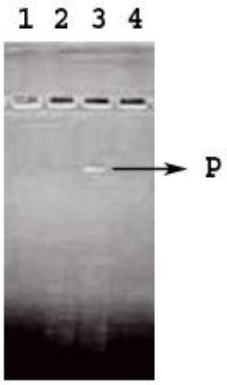

Figure 7. Gel Electrophoresis profile for DNA from *Pseudomonas* sp.. Partially purified Plasmid was separated on 0.8% Agarose. P, Plasmid DNA. Lane-2 and Lane 3 represent DNA profile of cured and uncured plasmid, respectively.

Table 1. Morphological and Biochemical characterization of *Pseudomonas* sp.

S.N.	Test	Results
	Morphological Characteristics	
1	Colony Character	Convex, Round, Creamish, Opaque, Greenish on white media
2	Simple Staining	+
3	Gram Staining	- , bacilli
4	Endospore	+, Green spores
5	Negative Staining	+
6	Motility	Motile
	Biochemical Characteristics	
7	Casein Hydrolysis	+
8	Urease	-
9	H_2S Production	-
10	Carbohydrate catabolism	-
11	Indole Test	-
12	Methyl-Red Test	-
13	Voges-Proskauer Test	-
14	Citrate Utilization	+
15	Catalase Test	+
16	Oxidase reaction	+
17	Nitrate reduction	+
18	Gelatin Liquefaction	+

Table 2. Zone of inhibition of different antibiotics against *Pseudomonas* sp.

S.N.	Antibiotics	Abbreviation	Zone of Inhibition (mm)
1	Oxytetracycline	OT	21
2	Cefadroxil	CP	0
3	Azithromycin	AZ	16
4	Erythomycin	ET	8
5	Cefixine	CX	17
6	Ampicillin	AP	0
7	Amoxicilline	AM	6

4. Discussion

Pseudomonas sp. strain E was isolated from filling station sites of Himachal Pradesh and cultured in BSM broth supplemented with 0.1% w/v anthracene as substrate. In contrast to other strains isolated in this study *Pseudomonas* sp. strain E degraded 74.8% in 10 days of incubation. The addition of anthracene to aqueous media was by dissolution in a carrier solvent, ethyl acetate (Moody

et al, 2001; Nadalig *et al*, 2002). Our results are in line with the results by Matthew *et al*. (2000), who have isolated *Pseudomonas aeruginosa, Alcaligenes eutrophus, Bacillus subtilis* and *Micrococcus luteus* from crude oil polluted soils using 0.1% w/v anthracene as the sole carbon and energy source resulted in a residual oil concentration of 22.2%, 33.3%, 39.3%, 44.0% and 91.7% respectively. Rodrigo *et al.* (2005) had also reported 71% of the anthracene degradation, added to the medium (250 mg L^{-1}) by *Pseudomonas sp.* isolated from a 14-year-old petrochemical sludge land farming site.

After screening, *Pseudomonas* sp. strain E was further cultured in BSM medium and during this period the maximum anthracene degradation was found to be 74.8% on 10^{th} day and maximum bacterial growth was measured on 9^{th} day. Similar studies have been reported for anthracene degradation & mineralization by *Pseudomonas, Sphingomonas, Nocardia, Beijerinckia, Rhodococcus* and *Mycobacterium* (Dean-Ross *et al*., 2000; Moody *et al.*, 2001). Another study has reported the complete degradation of added anthracene to autoclaved soil by *Burkholderia sp.* in 20 days (Somtrakoon *et al*., 2008). Manohar *et al*., (1999) had reported the complete anthracene degradation (2.8 mM) after 6 days of incubation and Eder *et al*., (2008) showed that after 48 days *Pseudomonas citronellolis* isolate 222A degraded 72% of anthracene.

We have tested several antibiotics for their resistance against *Pseudomonas* sp. strain E and observed that strain E was resistant against cefadroxil and ampicillin antibiotics. This suggests that strain E may posses the resistance gene for these two antibiotics and hence, these antibiotics were used as marker for the screening of plasmid cured bacterial colonies.

In the present study 50 µg/ml acridine orange concentration and 40°C temperatures was significantly effective for plasmid curing, suggesting that acridine orange can be used at sub-lethal temperature to cure the plasmid DNA. Curing agents such as acridine orange, if administered to bacterial populations in sub-lethal doses, can lead to the elimination of plasmid DNA without harming the bacterial chromosome and thus maintaining the ability to reproduce and generate offspring (Singleton and Sainsbury, 2001). Plasmid cured colony of strain E was not able to grow on BSM broth with anthracene as sole carbon source. It is assumed that this may be because of the removal/inactivation of gene(s) responsible for anthracene degradation from strain E. This indicates that gene(s) responsible for anthracene degradation might be associated with plasmid DNA that has been cured, thus not allowing the colony to grow in BSM broth. Moreover, the color of plasmid cured colony was changed to white from greenish (uncured). This study is in accordance with work carried out by Mesas *et al*. (2004) who had reported that the strains of *Oenococcus oeni*, RS2 (which carries the plasmids pRS2 and pRS3) were grown in the presence of different curing agents and at different temperatures. Sub lethal temperature together with acridine generated the cured strains, which lacking pRS3 plasmid and suggested that acridine orange is a better curing agent. Further the plasmid curing was confirmed by agarose gel electrophoresis of uncured and cured *Pseudomonas* sp. strain E. Gokhan and Serap (2005) also reported that catabolic pathways, which encode different aromatic hydrocarbon degradation routes, are frequently located on plasmids, although degradative genes can be located on either chromosome or plasmid.

The plasmid curing and agarose gel electrophoresis experiment suggest that anthracene degradation is plasmid associated. This is in accordance with the previous finding by Sanseverino (1993) who had proposed that NAH plasmid was involved in degradation of PAHs. Therefore, the possibility of the involvement of catabolic plasmid in the degradation of Anthracene by *Pseudomonas* sp. was investigated. In conclusion *Pseudomonas* sp. is an efficient anthracene degrading strain and could be use to develop an environmental friendly technology to overcome the problem of oil spills.

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References

[1] Hafez EE, Rashad M, Abd-Elsalam HE and EL-Hanafy AA. The Polyaromatic hydrocarbons as a serious environmental pollutants and the role of bioremediation to overcome this problem, Accepted for publication in Environment, Health and Nutrition-Global Issues. S.K. Basu and S. Datta Banik (Eds.), APH Publishing Corporation, New Delhi, India, 2008.

[2] Johnsen AR, Wick LY and Harms L. Principle of microbial PAH-degradation in soil. Environ. Pollut., 2005, 133: 71-84.
[3] Ni Chadhain SM, Norman RS, Pesce KV, Kukor JJ and Zylstra GJ. Microbial dioxygenase gene population shifts during polycyclic aromatic hydrocarbon biodegradation. Appl. Environ. Microbiol., 2006, 72: 4078-4087.
[4] Ruma R, Ray R, Chowdhury R, Bhattacharya P. Degradation of polyaromatic hydrocarbons by mixed culture isolated from oil contaminated soil—A bioprocess engineering study. Indian Journal of Biotechnology, 2007, 6: 107-113.
[5] Hyotylainen T and Oikari A. The toxicity and concentrations of PAHs in creosote contaminated lake sediment. Chemosphere, 1999, 38: 1135–1144.
[6] Lotufo GR. Toxicity of sediment-associated PAHs to an estuarine copepod: effects on survival, feeding, reproduction and behaviour. Mar. Environ. Res., 1997, 44: 149–166.
[7] Kastner M and Maho B. Microbial degradation of polycyclic aromatic hydrocarbons in soil affected by the organic matrix of compost. Appl. Microbiol. Biotechnol., 1996, 44: 668–675.
[8] Trindade PVO, Sobral LG, Rizzo ACL, Leite SGF and Soriano AU. Bioremediation of weathered and recently oil-contaminated soils from Brazil: a comparison study. Chemosphere, 2005, 58: 545-522.
[9] Wilson SC and Jones KC. Bioremediation of soil contaminated with polycyclic aromatic hydrocarbons. Environ. Pollut., 1993, 81: 229-249.
[10] Manohar S, Kim CK, Karegoudar TB. Degradation of Anthracene by a *Pseudomonas* strain, NGK1. Journal of Microbiology, 1999, 37(2): 73-79.
[11] Fujii T, Takeo M and Maeda Y. Plasmid-encoded genes sp.ecifying aniline oxidation from *Actinetobacter* sp. strain YAA. Microbiology, 1997, 143: 93-99.
[12] Kado CI and Liu ST. Rapid procedure for the detection and isolation of large and small plasmids. J. Bacteriol., 1981, 145(3): 1365-1373.
[13] Moody JD, Freeman JP, Doerge DR and Cerniglia CE. Degradation of Phenanthene and anthracene by cell susp.ensions of Mycobacterium sp. strain PYR-1. Appl. Environ. Microbiol., 2001, 67: 1476- 1483.
[14] Nadalig T, Raymond N, Ni'matuzahroh, Gilewicz M, Budzinski H and Bertrand JC. Degradation of phenanthrene, methylphenathrenes and dibenzothiophene by a *Sp.hingomonas* strain 2mpII. Appl. Microbiol. Biotechnol., 2000, 59: 79- 85.
[15] Matthew ONI and Amund DI. Degradation of Anthracene by Bacteria Isolated from Oil Polluted Tropical Soils. Z. Naturforsch., 2000, 55: 890-897.
[16] Rodrigo JSJ, Santos EC, Bento FM, Peralba MCR, Selbach PA, Sa ELS and Camargo FAO. Anthracene biodegradation by *Pseudomonas sp.*. isolated from a petrochemical sludge landfarming site. International Biodeterioration & Biodegradation, 2005, 56(3): 143-150.
[17] Dean-Ross D, Moody JD, Freeman JP, Doerge DR and Cerniglia CE. Metabolism of anthracene by a *Rhodococcus* sp. FEMS Microbiol. Lett., 2001, 204: 205-211.
[18] Somtrakoon K, Suanjit S, Pokethitiyook P, Kruatrachue M, Lee H and Upatham S. Enhanced biodegradation of anthracene in acidic soil by inoculated *Burkholderia sp.* VUN10013. Curr. Microbiol., 2008, 57: 102-106.
[19] Eder CS, Jacques RJS, Bento FM, Peralba M do C R, Selbach PA, Sá ELS and Camargo FAO. Anthracene biodegradation and surface activity by an iron-stimulated *Pseudomonas* sp. Bioresource Technology, 2008, 99(7): 2644-2649.
[20] Singleton P and Sainsbury D. Dictionary of Microbiology and Molecular Biology (3rd ed.). Chichester, UK: John Wiley & Sons, Ltd, 2001.
[21] Mesas JM, Rodriguez MC and Alegre MT. Plasmid curing of *Oenococcus oeni*. Plasmid, 2004, 51(1): 37-40.
[22] Gokhan C and Karagoz S. Isolation and characterization of phenanthrene-degrading bacteria from a petroleum refinery soil. Annals of Microbiology, 2005, 55(4): 255-259.
[23] Sanseverino J, Bruce M Applegate, Henry King JM and Sayler GS. Plasmid- mediated mineralization of naphthalene, phenanthrene and anthracene. Center for environmental technology and Dept. of microbiology, University of Tennessee, 1993.

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Anthelmintic comparative study of *Solanum lycocarpum* St. Hill extracts in mice naturally infected with *Aspiculuris tetraptera*.

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Abstract: This study intends to add new data on the helminthes parasites of laboratory mice. It has been investigated the antihelmintic activity of *Solanum lycocarpum* (*Solanaceae*) extracts against *Aspiculuris tetraptera* in mice naturally infected. The extracts were applied for oral saw (intragastric), into the volume of 0.04mL/g , with the employing of a dead and bend probe during three consecutive days. The fecal material, collected 24 hours after each application, performing a total of four fecal collection, have been softened previously, transferred about to sieve of network of 125 micrometers and tested under microscope stereoscope, with the objective of behave the identification and counting from the worms eliminated of the second to the fifth day of the experimental. Tukey-Kramer Multiple Comparisons Test was applied to compare the results. This approach intends to add new data on the helminthes parasites of laboratory mice. According to the analysis of the results it was observed that there were differences ($p<0.001$) in the %of elimination between 20%TM and 20% 20%UR (from 2.24 ± 3.33 to 2.92 ± 3.33), 20%TM and Nit (from 2.24 ± 3.33 to 64.0 ± 2.89), 20%TM and Meb (from 2.24 ± 3.33 to 100.0 ± 3.16), 20%UR and Nit (from 2.92 ± 3.16 to 64.0 ± 2.89) and ($p<0.01$) 20%UR and C (from 2.92 ± 3.16 to 1.56 ± 3.16). It was published that medicinal plants which were reported as useful in the treatment of diabetes the *S. lycocarpum* was the sixth most frequently mentioned. According to the results obtained in the present study, we can speculate that the anthelmintic effect of *Solanum lycocarpum* was noticed due to the concentration of steroidal alkaloid oligoglycosides and short-chain fatty acids. [Nature and Science 2010;8(4):95-100]. (ISSN: 1545-0740).

Key words: *Solanum lycocarpum* ; helminthes, mice; *Aspiculuris tetraptera* l;antihelmintic; medicinal plants

Introduction

The Brazilian flora is one the world richest sources of bioactive material due to its biodiversity. Several plants are currently used in Brazilian traditional medicine to treat diabetes. The starch obtained from the unripe fruits of *Solanum lycocarpum* St. Hill. (Solanaceae) has been widely used and commercialized as a hypoglycemic agent in Brazil. Recently studies carried out a chemical analysis of the starch and tried to correlate its supposed hypoglycemic activity with the polysaccharide content. However, these investigators did not conduct any experimental test to directly demonstrate the hypoglycemic effect attributed to the starch. As far as we know, no studies have evaluated the potential hypoglycemic effect of the starch of *S. lycocarpum* in experimental animals or the pattern of its use by a group of diabetic patients. *S. lycocarpum* is a plant which is shrubs ranging in height from 1.2 to 3 m. The fruit is yellow in color and resembles a medium sized tomato. Parts of the plant are poisonous if it gets in your system. When it is in bloom, it is medium blue. It blooms in the late winter, early spring, late fall, early winter, and mid winter. It is velvety or fuzzy. It needs water regularly. It is found in the Brazilian savannah but has been said to grow in San Antonio, Texas. *S. lycocarpum* is commonly used in Brazilian folk medicine. Solanaceae or lobeira is a plant used as a

hypoglycemic agent. A study reported that the extract reduces glycemia in alloxan induced diabetic rats. It was reported that the potential of *S.lycocarpum* as antioxidant was capable of reducing in 27% nitrate generation in diabetic animals. In literaturature has been demonstrated that *S.lycocarpum* is not ulcerogenic and restored haemoglobin and haematocrit to normal values in diabetic animals (Perez et al, 2006). Yoshikawa et al (2007) described that steroidal alkaloid oligoglycosides as solamargine, solasonine, and 12-hydroxysolasonine, inhibited the increase of rat serum glucose levels by suppressing the transfer of sucrose from the stomach to the small intestine.

It grows in wet, red clay. It needs water regularly. It doesn't need as much water in the winter because it needs full sunlight, and mild temperatures. They are edible by humans. This plant contains steroidal glycoalkaloids that can be transformed into an intermediate for steroidal drug production. In this way, it is very possible that these glycoalkaloids and its aglycone, once in the body by ingestion of *S. lycocarpum* fruits, may act by disrupting the endocrine system. Because its fruits may be consumed by pregnant animals in the fields, various studies determined the possible toxic effects of exposure to *S. lycocarpum* fruit from gestation. The unripe fruits contained 0.6% of solamargine and 0.9% of solasonine. It was related that *S. lycocarpum*, during gestation and the beginning of lactation reduces intrauterine growth. It is known that during adulthood, female offspring showed impaired sexual behavior and male offspring showed prominent degeneration of testis germinative cells, characterized by a reduced number of germ cells and vacuolation. It has been documented that the exposed offspring showed reduced hypothalamic norepinephrine (NOR), vanillylmandelic acid (VMA), 3-methoxy-4-hydrophenylglycol (MHPG) and homovanillic acid (HVA) levels, and reduced striatum NOR, HVA, VMA, MHPG, dopamine (DA), dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindolacetic acid (5-HIAA) levels. It is suggest that the fruit may act as an estrogen, with a long-term effect, impairing the receptive lordosis behavior of female offspring and promoting testis abnormalities in male offspring at adulthood. It appears to disrupt brain organization since important central monoamine level alterations were also related (Schwarz et al, 2005).

It was described by Vieira et al (2003) the anti-inflammatory effects of the crude ethanol extract and its alkaloid fraction from *S. lycocarpum* fruits. Due to the referred study the alkaloid fraction induced a dose-dependent reduction in ear edema formation and leukocyte migration, suggesting that *S. lycocarpum* fruits may contain steroidal alkaloids accounting for the anti-inflammatory effect of the crude ethanol extract.

Maruo et al (2003) demonstrated the embryotoxic effects of *S. lycocarpum* fruit ingestion during preimplantation and during organogenesis in rats. In this study few differences were observed in food and water consumption without biological importance. It was observed that the placental weight in the group that received the plant during the organogenesis period was decreased. An increase in sternebra abnormalities was observed in animals treated with the plant during organogenesis. Olfactory bulb hemorrhage was increased in the group that received the plant during preimplantation when compared to the control group. These results indicate that consumption of *S. lycocarpum* at 3% in diet during pregnancy cause slight toxicological effects. Chang et al (2002) evaluating the toxic effects of lobeira during the fetus genesis period, related that no clinical signs of maternal toxicity were observed. The placenta weights of the treated rats were lower than those of the control. Lungs and kidneys of the fetuses treated with lobeira were also significantly reduced, suggesting a fetus toxic effect of this plant. Rodents, as mice and rats are the most common laboratory animals used in research and testing. They are seldom investigated for autochthonous ecto- and endoparasites prior their utilization in the experiments. Pinworms commonly infecting laboratory rodents include mainly the mice pinworms *Syphacia obvelata* and *Aspiculuris tetraptera*, and in rats *Syphacia muris* (Matysiak et al, 2006). Fecal specimens obtained from rats and mice in general are infected with one or more helminth species. *Syphacia muris* and *Syphacia obvelata* are more frequently in rats, and *Aspiculuris tetraptera*, *S. obvelata* , in mice (Senlik et al, 2005).

Farina et al (2010) verified that serum glucose, water and food intake, urine excretion, and urine sodium concentration were reduced in S. lycocarpum flour-treated diabetic rats (TDRs), compared with diabetic control rats (DCRs). In addition, TDRs did not show signs of kidney hypertrophy, unlike those in the DCR group. They had suggested that the use of S. lycocarpum flour can be an effective support in diabetes mellitus treatment.

Some plant extracts are efficient due to their anthelmintic activity. It was related that ethanolic and aqueous extracts obtained from nine plant species from seven families selected depending on their use in Turkish folk medicine, including *Citrillus lanatus* (Thunb.) *Matsum.* (seed), *Jasminum fruticans L.* (branches), *Juniperus drupacea Labill.* (fruits), *Juniperus nana L.* (fruit and leaves), *Juniperus oxcycedrus L* (fruit and leaves), *Mentha longifolia L.* (herba), *Pinus nigra ssp. pallasiana* (Lamb.) *Richt.* (fruits), *Plantago lanceolata L.* (leaves), and *Zea mays L.* (seed) were evaluated for their in vivo anthelmintic

activity. Among the plant extracts studied, both ethanolic and aqueous extracts of *Jasminum fruticans, Mentha longifolia and Pinus nigra ssp. pallasiana*, the aqueous extracts of *Zea mays*, the ethanolic extracts of *Citrillus lanatus*, *Juniperus drupacea* (fruit), *Juniperus oxcycedrus* and *Plantago lanceolata* displayed significant anthelmintic activity against pinworms, *Syphacia obvelata* and *Aspiculuris tetraptera*, in mice. Rest of the extracts from plants did not show any remarkable anthelmintic activity (Kozan et al, 2006).

Some plant extract may act differently due to its action against the parasite. In a study the anthelmintic activity of the extracts obtained from *Luxemburgia octandra* was evaluated naturally infected mice with *Aspiculuris tetraptera*. The leaves extracts were given to the animals during three days. The ethanolic and ethyl acetate extracts did not present the nematicide effect against *A. tetraptera* (Silva et al, 2005). In the present study we evaluated the anthelmintic activity of *Solanum lycocarpum* extracts in a concentration of 20% in mice naturally infected *Aspiculuris tetraptera.*

Material and Method

Vegetal extracts: Dried leaves of units of had been used in the anthelmintic tests *Solanum lycocarpum* collected in the City of Três Marias, State of Minas Gerais and in the City of Seropédica, State of Rio de Janeiro. The botanical identification was carried through in the Department of Botany of the Rural Federal University of Rio de Janeiro, having been the exsiccates deposited under numbers RBR 28010 and RBR 14071. For the execution of the tests, the extracts had been gotten by infusion (tea), submitted to the filtration in nylon and the express concentrations in g/100 ml (p/v).

Animals and anthelmintic tests: For anthelmintc test have been used lots of albinos mice, male and females weighted in media of 25g and naturally infecting for *Aspiculuris tetraptera*, originated from Oswaldo Cruz Foundation – FIOCRUZ and held into the Institute of Biology from Rural Federal University from Rio de Janeiro. The animals have been held into bird cages individual of polypropylene (30x 20 x 13cm), it has at the bottom road of screen stark and stiff (network of 7x 7mm) upon a sheet of absorbent paper with the aim to facilitate the collection diary of excrement (Steward,1955, Amorim et al., 1987 e Amorim e Borba, 1990).

The extracts were applied for oral saw (intragastric), into the volume of 0.04mL/g , with the employing of a dead and bend probe during three consecutive days. The excrement, collected 24 hours after each application, performing a total of four fecal collection, have been softened previously, transferred about to tames of network of 125 micrometers (µm) and evaluated under microscope stereoscope, with the objective of behave the identification from the worm eliminated of the second to the fifth day of the experimental. Into the fifth and last days from the tests, the mice have been sacrificing for inhalation of vapors of ether ethyl, examining in the colon the number of the *A. tetraptera* remnants (Amorin et al., 1999). On the tests have been used the extracts of *Solanum lycocarpum* (leaves dried from Três Marias in the concentration of 10% and 20%) and (leaves dried from UFRRJ in the concentration of 10% and 20%). Additional lots of mice have been used with standard, they receiving doses of 20mg/kg/day of mebendazol and 100mg/kg/day of nitroscanato and they were submitted to the identical assessment anthelmintic description about to the animals treated with the plant extracts. A batch control, without a treatment served about to appraise the elimination spontaneous from the helminthes studied. The outcome antinematode also was denominated in terms percentile average of roundworm eliminated, considering the number of roundworm eliminated in the faecal material in relation to the total number. Statistical analysis were performed and Tukey-Kramer Multiple Comparisons Test was applied to compare the results.

Results

According to the analysis of the results it was observed that there were no differences ($p>0.05$) in the % of elimination between TM and UR (from 5.64 ± 3.33 to 3.15 ± 3.16), UR and C (from 3.15 ± 3.16 to 1.56 ± 3.16) and an extremely significant difference between TM and C (from 5.64 ± 3.16 to 1.56 ± 3.16) (Table 1).

According to the analysis of the results it was observed that there were differences ($p<0.001$) in the % of elimination between 20%TM and 20% 20%UR (from 2.24 ± 3.33 to 2.92 ± 3.33), 20%TM and Nit (from 2.24 ± 3.33 to 64.0 ± 2.89), 20%TM and Meb (from 2.24 ± 3.33 to 100.0 ± 3.16), 20%UR and Nit (from 2.92 ± 3.16 to 64.0 ± 2.89) and ($p<0.01$) 20%UR and C (from 2.92 ± 3.16 to 1.56 ± 3.16).

Table 1. Antihelmintic activity of the extracts obtained of *Solanum lycocarpum* in the elimination of *Aspiculuris tetraptera* in mice naturally infected.

Used Parts	Administration form	Number of animals	Number of Helminthes		Elimination (%)
			Fecal Exam	Necropsy	
Leaves Dried from Três Marias (TM)	10%	10	61	1082	5.64 ± 3.33
Leaves Dried from UFRRJ (UR)	10%	12	54	1717	3.15 ± 3.16
Nitroscanato (NIT)		12	499	282	64.0 ± 0.00
Mebendazol (MEB)		10	324	0.0	100 ± 0.00
Control (C)		10	45	2836	1.56 ± 3.16

The extracts were applied for oral saw (intragastric), into the volume of 0.04mL/g , with the employing of a dead and bend probe during three consecutive days. The excrements, collected 24 hours after each application, performing a total of four fecal collection, have been softened previously, transferred about to tames of network of 125µm and evaluated under microscope stereoscope, with the objective of behave the identification of the worm eliminated of the second to the fifth day of the experimental. Tukey-Kramer Multiple Comparisons Test was applied to compare the results.

Table2. Antihelmintic activity of the extracts obtained of *Solanum lycocarpum* in the elimination of *Aspiculuris tetraptera* in mice naturally infected.

Used Parts	Administration form	Number of animals	Number of Helminthes		Elimination (%)
			Fecal Exam	Necropsy	
Leaves Dried from Três Três Marias (TM)	20%	07	09	393	2.24
Leaves Dried from UFRRJ (UR)	20%	10	22	729	2.92
Nitroscanato (NIT)		12	499	282	64.0
Mebendazol (MEB)		10	324	0.0	100
Control (C)		10	45	2836	1.56

The extracts were applied for oral saw (intragastric), into the volume of 0.04mL/g , with the employing of a dead and bend probe during three consecutive days. The excrements, collected 24 hours after each application, performing a total of four fecal collection, have been softened previously, transferred about to tames of network of 125µm and evaluated under microscope stereoscope, with the objective of behave the identification of the worm eliminated of the second to the fifth day of the experimental. Tukey-Kramer Multiple Comparisons Test was applied to compare the results.

Discussion

Although the objective of the present study was not to carry out a toxicological investigation of *S. lycocarpum* starch, we observed that the animals treated with the starch did not differ from those treated with the vehicle in terms of body weight changes during the experimental period. Many studies carried out on experimental animals have shown that steroidal alkaloids are generally toxic. Baker et al, (1989) have shown that Syrian hamsters orally treated with ground material obtained from Solanaceae species developed gastric and intestinal mucosal lesions. In addition, treatment of mice with steroidal alkaloids isolated from plants of this family also induced alterations of liver weight, arrhythmic beating in neonatal heart cells and neural-tube defects (Schwarz et al, 2005). Animal models have been exhaustively investigated regarding aspects related to their suitability for the development of experimental protocols under laboratory conditions. Nevertheless, in most of the adopted procedures, the prior detection of their ecto and endo parasites are generally overlooked related to the really effects of natural extracts in their biological cycle.

In the Brazilian cerate, a preparation obtained from the fruits of *Solanum lycocarpum* St.-Hill. (Solanaceae), popularly known as 'fruta-de-lobo' (wolf-fruit), have been widely employed for diabetes management, obesity and to decrease cholesterol levels. The medicinal preparation consists of the green fruits

which are ground in aqueous solution and filtered. The white 'gum' deposited is decanted and slowly dried providing a powder which is commercialized in capsules with the name of 'polvilho-de-lobeira'. Through phytochemical analysis of this phytomedicine and the fruit of *S. lycocarpum* were found polysaccharides as the main component. Some polysaccharides slow gastric emptying and act on the endocrinous system affecting the liberation of gastrointestinal hormones, lowering blood glucose levels. According to Schwarz et al (2005) it is well known that this plant contain steroidal glycoalkaloids that can be transformed into an intermediate for steroidal drugs production, like oral contraceptives. In this way, it is very possible that these glycoalkaloids and its aglycone, once in the body by ingestion of *S. lycocarpum* fruits, may act disrupting to the endocrine system as well as it may probably affect the reproductive system of helminthes. The hypocholesterolemic activity could be due to the increased fecal bile acid excretion as well as to the action of the short-chain fatty acids, coming from fermentation, on the synthesis of delta-aminolevulinate and by the increase of the cholesterol 7-alpha-hydroxylase and 3-hydroxy-3-methylglutaryl CoA reductase synthesis (Dall and Lino, 2000).

Soares-Motta et al (2009) had observed in their study that the serum biochemical parameters showed triglyceride reductions in treated animals of both sexes with the refereed extract in the concentration of 10%; in females, an increase in albumin and alanine aminotransferase levels and a reduction in total protein levels were noted. The study therefore demonstrate sex-related differences in *S. lycocarpum* toxicity. This study would suggest that this extract could induce a depression in the hepatic metabolism which could actually lead to lower nutrient availability level inetstinal biologically providing an environment suitable for growth of parasites.

Due to the effect related it may be possible that these fatty acids could act as an anthelmintic, although in he present study there was not observed differences between TM and UR extracts related to % of elimination in comparison one to another, although in comparison to the control group was evident a significative difference due to the UR group. Related to the obtained results due to the action of the UR extract it may be explained by their concentration as well as originated region which may explain the effect due to the biochemistry compounds in the equivalents proportions in spite of different conditions as soil composition, light and water availability.

The effect of UR extract may be support by possible modifications in ribosomal DNA spacer region suggesting that it could result in genetic and geographical variability as well as different bioactivity which may not be effective depend on the concentration of the extract (Arruda et al, 2003).

We can speculate that the other effect would be related to the low concentration of steroidal alkaloid oligoglycosides which in a optimal concentration may suppress the transfer of sucrose from the stomach to the small intestine which could diminish the support of glucose to helminthes together with its antioxidant effect which is capable of reducing the nitrate generation which can be used in the protein synthesis as well as the possible inflamatory effect induced by the extract in the gastric and intestinal mucosal which could interfere in local homeostasis which is essentially to the develop of helminthes.

Conclusion

The results of the present study point to the need for a careful evaluation of the phytotherapic product in researching even when it may be widely used by the population. Based on the results we can suggested that the anthelmintic effect of *Solanum lycocarpum*, TM and UR extracts, is related to the possible concentration of steroidal alkaloid oligoglycosides as well as the short-chain fatty acids presents in the extract. The similar action of the extracts may be explained by adaptation mechanisms related to the genetic and geographical variability. In additional, one can speculate that the concentration of biologically active compounds may interfere with the manifestation of biological effects related to the extract.

References

1. J Ethnopharmacol. 2006, 19: 442-444. Effect of *Solanum lycocarpum* St. Hill on various haematological parameters in diabetic rats. Perez AC, Franca V, Daldegan VM Jr, Duarte ID.
2. J Nat Prod. 2007, 70:210-214. Structures of steroidal alkaloid oligoglycosides, robeneosides A and B, and antidiabetogenic constituents from the Brazilian medicinal plant *Solanum lycocarpum*. Yoshikawa M, Nakamura S, Ozaki K, Kumahara A, Morikawa T, Matsuda H.
3. Neurotoxicol Teratol. 2005, 27: 861-870. Rats exposed to *Solanum lycocarpum* fruit in utero and during lactation: neurochemical, behavioral and histopathological effects. Schwarz A, Soares MR, Florio JC, Bernardi MM, Spinosa HS.

4. Appl Physiol Nutr Metab. 2010, 35(1):40-4. Glycemic and urinary volume responses in diabetic mellitus rats treated with *Solanum lycocarpum*. Farina F, Piassi FG, Moysés MR, Bazzolli DM, Bissoli Nde S.
5. Phytother Res. 2003, 17: 892-896. Anti-inflammatory effect of *Solanum lycocarpum* fruits. Vieira G Jr, Ferreira PM, Matos LG, Ferreira EC, Rodovalho W, Ferri PH, Ferreira HD, Costa EA.
6. Exp Toxicol Pathol.,2009, Aug 10. [Epub ahead of print] Toxicological evaluation of 10% *Solanum lycocarpum* St. Hill fruit consumption in the diet of growing rats: Hematological, biochemical and histopathological effects. Soares-Mota MR, Schwarz A, Bernardi MM, Maiorka PC, Spinosa HD.
7. Neurotoxicol Teratol. 2003, 25: 627-631. Embryotoxic effects of *Solanum lycocarpum* St. Hill fruits consumption during preimplantation and organogenesis in rats. Maruo VM, Soares MR, Bernardi MM, Spinosa HS.
8. J Ethnopharmacol. 2002, 81: 265-269. Fetal toxicity of *Solanum lycocarpum* (Solanaceae) in rats. Chang CV, Felicio AC, Reis JE, Guerra Mde O, Peters VM.
9. Wiad Parazytol. 2006, 52: 99-102. Helminth parasites of laboratory mice and rats. Perec-Matysiak A, Okulewicz A, Hildebrand J, Zalesny G.
10. J Ethnopharmacol. 2006, 108: 211-216. Epub 2006 May 16. Evaluation of some plants used in Turkish folk medicine against parasitic infections for their in vivo anthelmintic activity. Kozan E, Kupeli E, Yesilada E.
11. Rev Bras Parasitol Vet. 2005, 14: 106-118. Evaluation of the anthelmintic activity of extracts from Luxemburgia octandra St. Hill. in mice naturally infected with *Aspiculuris tetraptera* and *Vampirolepis nana*. Alves CC, Borba HR, Carvalho MG, Bomfim TC.
12. Turkiye Parazitol Derg. 2005, 29:123-125. Helminths detected in some laboratory animals by fecal examinations. Senlik B, Diker AI, Kucukyildiz F.
13. Rev Bras Farmácia. 1990, 71: 85-87. Ação anti-helmíntica III. Efeito de extratos aquosos de *Punica granatum* L. (romã) na eliminação de *Vampirolepis nana* e de oxiurídeos em camundongos. Amorin, A. & Borba, H.R.
14. J Ethnopharmacol. 1999, 64: 255-258. Anthelmintic activity of látex of *Fícus* species. Amorim, A., Borba, H.R., Carauta, J.P.P., Lopes, D. & Kaplan, M.A.C.
15. Rev Bras de Farmácia. 1987, 68: 64-70. Ação anti-helmíntica de plantas. Amorim, A., Borba, H.R. & Silva, W.J.
16. Toxicon, 1989, 27: 1331-1337. Pathology in hamsters administered Solanum plant species that contain steroidal alkaloids. Baker DC, Keeler RF & Gaffield W.
17. Parasitology, 1955, 45: 231-241. Anthelmintic studies: A controlled critical entero-nemacidal test. Steward, J. S
18. J Ethnopharmacol. 2000, 71: 337-341. The use of complex polysaccharides in the management of metabolic diseases: the case of *Solanum lycocarpum* fruits. Dall'Agnol R, Lino von Poser G.
19. J Ethnopharmacol. 2000, 73: 283-287. Evaluation of the toxicity of *Solanum lycocarpum* in the reproductive system of male mice and rats.de Cassia da SeSa R, Vireque AA, Reis JE, Guerra MO.
20. Mycol Res. 2003, 107: 25-37. Nuclear and mitochondrial rDNA variability in Crinipellis perniciosa from different geographic origins and hosts. de Arruda MC, Ferreira MA, Miller RN, Resende ML, Felipe MS.
21. Pharmacol Biochem Behav. 2005, 81: 928-934. Impaired female sexual behavior of rat offspring exposed to Solanum lycocarpum unripe fruits during gestation and lactation: lack of hormonal and fertility alterations. Schwarz A, Felippe EC, Bernardi MM, Spinosa HS.
22. J of Ethnopharmacol. 2000, 71: 337-341. The use of complex polysaccharides in the management of metabolic diseases: the case of Solanum lycocarpum fruits. Dall'Agnol R, Von Poser, GL.

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A Review on the Future of Ecotourism in the Valley of Flowers National Park: A Case Study of Garhwal Himalaya, India

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Abstract: This paper reports the future of Ecotourism in the Valley of Flowers national park in Garhwal Himalaya, Uttarakhand, India. The valley has an unusually rich flora of over 600 species with many rarities. Animals found are nationally rare or endangered. 13 species of mammals are recorded for the Park and its vicinity although only 9 species have been sighted directly. Other factors that are contributing to ecotourism are beautiful landscapes, peaks, lakes and tarns etc. But now-a-days the problem of Solid waste is increasing at an alarming rate because of the heavy influx of tourists and improper management practices. This paper reviews the various ecotourism resources of the area and their future prospects. [Nature and Science. 2010;8(4):101-106]. (ISSN: 1545-0740).

Keywords: Fauna Flora, Glaciers, Tarns.

1. Introduction

Ecotourism has been developed following the environmental movement which appeared at the beginning of the seventies. The growing interest of people for environment and trips oriented towards fresh air, in addition to the growing dissatisfaction towards mass tourism, highlighted to the tourism industry a need for ecotourism. Besides, the understanding and the agreement with the principles of nature preservation and durability for a growing portion of the population took part in the evolution of the term "**ecotourism**".

Ecotourism is often considered as a form of tourism with "a strong motivation". There is no universal definition for ecotourism. It is usually considered as a "tourism favorable to the environment", which is, on a practical level, variously interpreted according to the country.

In the absence of a clear and recognized definition, the definition for the International Society for Eco-Tourism (1991) is: "... a responsible tourism in natural environment which preserves it and participates to the well-being of local populations".

According to the World Conservation Union (1996), it can be defined as "... the visit of natural environments remained relatively intact... with a low negative impact... including a socio-economical implication for the local populations which is at the same time active and beneficial".

Characteristics of Ecotourism

Although it is difficult to define **ecotourism**, it presents several characteristics:

- the destination is generally a natural environment which is not polluted;
- its attractions are its flora and its wildlife, and more generally it bio-diversity;
- ecotourism must support the local economy and the specificity of the place;
- it must contribute to the preservation of the environment, and more generally, promote the preservation of nature;
- eco-tourist stays often include an educational aspect.

In the last twenty years India has opened its door to the international tourists and is now fostering tourism largely to gain an increase in the foreign earnings to help its economy. Majority of the tourists are involved with nature tourism as India has lot of potential for this form of tourism. Garhwal Himalaya presents an example where tourism reached some of the most sensitive ecosystem of the high Himalayan region unprepared and unguarded. The region offers dramatic mountain scenery to be imagined anywhere on the earth that mountaineers would never be tired of singing its praise (Fukuda, 1971). The result is obviously, for more eco-negatives and few eco-positives (Kaur, 1977).

Valley of Flowers (VOF) National Park nearby protects one of the most beautiful mountain wildernesses of the Western Himalayas, celebrated for its meadows of endemic alpine flowers where more than 600 Himalayan species grow in an area of less than 2,500 hectares. It is also the habitat of the endangered snow leopard, Asiatic black bear, and brown bear, Himalayan musk deer and bharal. Together, the parks preserve a transition zone between the eastern and western Himalayan flora, the Zanskar mountains and the Great Himalayas, long praised in Hindu mythology and for over a century by botanists and mountaineers (UNEP Report). Marked by difficult geographic terrain and hard accessibility the region has long enjoyed self-sufficiency, which was supplemented by beneficial pilgrim economy (Pauw, 1986). The biological significance of VOF lies in its exquisite floral and faunal biodiversity with myriads of alluring flowers.

2. The Study Area

The VOF National Park (87.50 sq. km.; lat 30° 41` – 30° 48` N and long 79° 33` – 79° 46` E) is located in Chamoli Garhwal, about 595 km northeast of Delhi(capital of India) in the state of Uttarakhand. Its altitude ranges from 3,200 m asl to 6,675m asl. (Figure 1). The VOF has a highly heterogeneous landscape, ranging from low lying flat and gentle slopes to steep slopes, unstable glacial moraines, stream banks, forest meadow edges and snow bound areas. Such a geomorphological heterogeneity has resulted in a rich diversity of flowering plants, which attracts a number of botanists and tourists across the world.

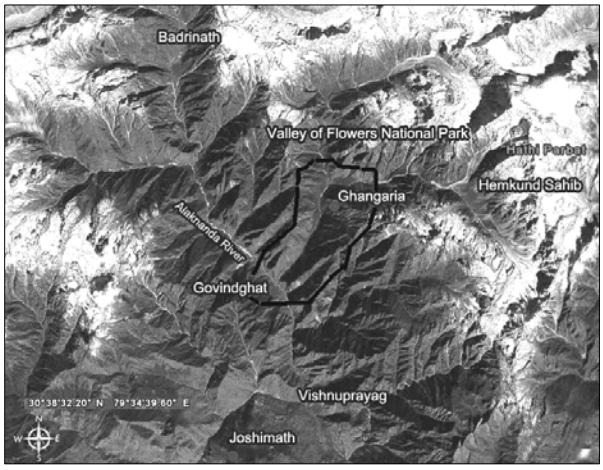

Figure 1. Study site

Climate

Climate is of particular significance to tourism. In fact, tourist market of hills depends on climate. The month of May and June attracts maximum number of tourists and pilgrims to the region whereas the number starts to decline in the month of July. This increase and decrease in the number of tourists cab be attributed to weather and climatic changes in the region. The area under investigation is varied in climate. Conditions are generally dry with low annual precipitation, but there is heavy monsoon rainfall from late June to early September. Prevailing mist and low cloud during the monsoon keeps the soil moist, hence the vegetation is lusher than is usual in the drier inner Himalayan valleys. From mid April to June temperatures are moderate to cool (19°C maximum). The Valley of Flowers also has the microclimate of an enclosed inner Himalayan valley, and is shielded from the full impact of the southwest summer monsoon by the Greater Himalaya range to its south. There is often dense fog and rain especially during the late summer monsoon. Both Basin and Valley are usually snow-bound for six to seven months between late October and late March, the snow accumulating deeper and at lower altitudes on the shadowed southern than on the northern side of the valleys (Lavkumar, 1979; Lamba, 1987). Attitudinally the area can be divided into the following climatic zones viz,

- Warm up to 1300m asl
- Temperate 1300-2100m asl
- Cold 2100-3030m asl
- Glacial above 3400m asl.

3. Potential Ecotourism Resources of the Area

Flora

The Valley of Flowers National Park can be divided into three broad eco-climatic zones viz. sub alpine (2800-3500 m asl.). Lower alpine (3500-3700 m asl.) and higher alpine (3700m asl.). The valley has an unusually rich flora of over 600 species with many rarities. These comprise 25% of the vascular plants found in the Chamoli district though the valley is only 1.3% of its area. The habitats include valley bottom, river bed, small forests, meadows, eroded, scrubby and stable slopes, moraine, plateau, bogs, stone desert and caves. The lower surrounding hills in the buffer zone are thickly forested. The Forest Research Institute in 1992 recorded 600 species of angiosperms and 30 pteridophytes in the valley and

surroundings, discovering 58 new records for the valley of which 4 were new for Himalayan Uttar Pradesh. Of these plants, 5 out of 6 species globally threatened are not found in Nanda Devi National Park or elsewhere in Uttaranchal: *Aconitum falconeri, A. balfouri,* Himalayan maple *Acer caesium,* the blue Himalayan poppy *Mecanopsis aculeate* and *Saussurea atkinsoni* (Green & Peard, 2005). 31 species are classified as nationally rare. The dominant family is the Asteraceae with 62 species. 45 medicinal plants are used by local villagers and several species, such as *Saussurea obvallata* (Brahmakamal) are collected as religious offerings to Nanda Devi and other deities. The site is designated a Centre of Plant Diversity. Characteristic of the sub-alpine zone are high altitude forests which help to retain moisture and snow and support a large number of floral and faunal communities. It is dominated by the uncommon Himalayan maple *Acer caesium,* west Himalayan fir *Abies pindrow,* Himalayan white birch *Betula utilis*, and *Rhododendron campanulatum* with Himalayan yew *Taxus wallichiana, Syringa emodi* and *Sorbus lanata*. Some of the common herbs are *Arisaema jacquemontii, Boschniakia himalaica, Corydalis cashmeriana, Polemonium caerulium, Polygonum polystachyum* (a rampant tall weed), *Impatiens sulcata, Geranium wallichianum, Helinia elliptica, Galium aparine, Morina longifolia, Inula grandiflora, Nomochoris oxypetala, Anemone rivularis, Pedicularis pectinata, P. bicornuta, Primula denticulate and Trillidium govanianum*. In trampled areas where past livestock congregated, Himalayan knotweed *Polygonum polystachium* is a rampant weed.

The Valley's lower alpine zone has greater moisture and deeper soil. A large number of herbaceous communities grow in great profusion and it supports the greatest diversity of alpine plants. Characteristic of the zone are dwarf shrubs, cushion herbs, grasses and sedges. Common and singleseed junipers *Juniperus communis* and *J. squamata, Rhododendron anthopogon, Salix spp., Lonicera myrtillus, Cotoneaster microphyllus,* and *Rubus ellipticus* are the major shrub species in this zone. The herbaceous flora gives a spectacular multicoloured array of flowers during the growing season. Their growth cycle is very short, and they give way to other communities later in the season. The dominant herbs of this zone are *Potentilla atrosanguinea, Geranium wallichianum, Fritillaria roylei, Impatiens sulcata, Polygonum polystachyum, Angelica archangelica, Selinum vaginatum.* The common grasses of the zone are *Danthonia cachemyriana, Calamogrostis emodensis, Agrostis pilosula and Trisetum spicatum*; the main sedge species are *Kobresia roylei and Carex nubigena*.
The higher alpine zone is an area of pioneer species dispersed among moraines, boulders, and rocky slopes, dominated by scattered and stunted herbs with delicate flowers, mosses and lichens. On northern aspects and in sheltered areas are extensive shrubby patches of *Rhododendron lepidotum, Cassiope fastigiata and Juniperus communis*. The zone's dominant species are *Kobresia royleana, Trachydium roylei and Danthonia cachemyriana*. There are also several colourful herbs like *Saussurea simpsoniana, Potentilla argyrophylla, Geum elatum, Senecio spp., Bistorta affinis, Bergenia stracheyi* and the flagship species blue Himalayan poppy *Mecanopsis aculeate* (UNEP World Conservation Monitoring center 2005).

Fauna

Animals found are nationally rare or endangered. 13 species of mammals are recorded for the Park and its vicinity although only 9 species have been sighted directly: common langur *Presbytes entellus*, flying squirrel *Petaurista petaurista*, Himalayan black bear *Selenarctos thibetanus* (VU), red fox *Vulpes vulpes*, Himalayan weasel *Mustela sibirica*, and Himalayan yellow marten *Martes flavigula*, goral *Naemorhedus goral*, Himalayan musk deer *Moschus chrysogaste*, Indian mouse deer *Moschiola meminna*, Himalayan thar *Hemitragus jemlahicus* (VU) and serow *Capricornis sumatrensis* (VU). The tahr is common, the serow, goral, musk deer and bharal, blue sheep are rare. The common leopard *Panthera pardus* is reported from lower parts of the valley closer to the villages. Local people have also reported evidence of Himalayan brown bear *Ursus arctos* and bharal or blue sheep *Pseudois nayaur*. A recent faunal survey in October 2004 has established the presence of snow leopard *Uncia uncia* (EN) in the National Park.

The area is within the West Himalayan Endemic Bird Area but there have been no surveys specific to the Valley. 114 species were seen in 1993 in Nanda Devi Park. Species frequently seen in the valley include *lammergeier Gypaetus barbatus*, *Himalayan griffon Gyps himalayensis*, yellow billed and red billed choughs *Pyrrhocorax graculus and P. pyrrhocorax, koklass pheasant Pucrasia macrolopha,* the nationally listed monal pheasant *Lophophorus impejanus*, found in rhododendron thickets, scaly-bellied woodpecker *Picus squamatus*, greater yellow

naped woodpecker *P. flavinucha*, great barbet *Megalaima virens*, blue throated barbet *M. asiatica*, snow pigeon *Columba leuconota* and spotted dove *Streptopelia chinensis*. The area is relatively poor in reptiles: most often seen are the high altitude lizard *Agama tuberculata*, Himalayan ground skink *Leiolopisma himalayana* and Himalayan pit viper *Gloydius himalayanus*. Along with the flowers are wild bees and many species of butterfly which need to be more researched. A few of the more evident species are lime butterfly *Papilio demoleus demoleus*, common yellow swallowtail *Papilio machaon*, common mormon *Papilio polytes romulus, spangle Papilio protenor protenor* and common blue apollo *Parnassius hardwickei*. (UNEP World Conservation Monitoring center 2005).

Cultural Heritage

The Valley of Flowers 07 kms south of the park entrance, at Ghangrea, a track leads off to the Hemkund Sahib shrine sacred to Sikhs, and the Hindu temple to Lord Lakshman, (brother of Lord Rama), beside Lake Lokpal. About 400,000-500,000 pilgrims visit them every year. (UNEP World Conservation Monitoring center 2005).

Faith Scapes (Pilgrimage)

The faith scapes of the region are discussed below:

- Panch Badri
- Shri Badrinath
- Yogadhyan Badri
- Briddha badri
- Adi Badri

Social attractions/Interests

- Mana, etc

Landscape and Peaks

An attractive landscape is an asset on which mountain tourism depends. It can be stated that mountains are the foundations of the tourism industry. The entire study area is mountainous. The rugged landmass is thoroughly and artistically punctuated with natures superlatives. Mountains any where in the world, with their pronounced scuklpturing carry greater aesthetic appeal than low reliefed land forms. Here landscapes are most enchanting. The actual attraction is the towering snow covered peaks that makes the scenery challenging and beautiful to adventurers. The chains of the high mountain peaks are divided into different mountain groups by the mighty rivers of Garhwal. The high peak of Bunderpunch (6302m asl), Kalanag (6387m asl) lie between the tons and Bhagirathi rivers, Matri (6721 m asl), Chirbas (6525m asl), Trimukh Parvat (6422m asl) lie between Jadh ganga and the Bhagirathi rivers.

Another cluster of peaks lie between Bhagirathi and Saraswati river. Famous among the peaks are Chaukhamba situated on the west of Badribath temple, Neelkanth (6600 m asl) and the Sameru Parvat (6350m asl).

Glaciers

There are numerous glaciers in the area. Valleys between 2000m asl and 3000m asl show the glacial feature wherever knot blot out by fluvial action. Some of the glaciers, which need mention at this point, are:

- Doonagiri Glacier
- Tiprabamak Glacier
- Satopanth, Bhagirathi-Khark Glacier.

Water systems

It consists of rivers, Streams, Tarnns and Torrents. The rivers are running deep into the gorges from where it cannot be utilizerd for the purpose of irrigation. The main rivers are:

- Alaknanda
- Saraswati
- Dhauli Ganga

Lakes and Tarns

Upper Garhwal Himalaya is famous for its tranquil tarns, which are found around 3000m asl. The landslide and heaps of debris, partly blocking the rivers or streams forms most of these tarns, though some are fed by the underground sources. Plugging of valley by moraines deposited by ancient glaciers forms most glacial lakes. Mostly, they are sweet water lakes but few are brine water too. Unfortunately, some of the lakes have dried due to improper management practices like Ghona Lake in Chamoli district is the latest example of such happening. This lake came into existence near Ghona village on 06th august 1893 with a huge landslide-blocking river Birahi Ganga. In 1930 forest department released some trout fishes and soon it became angler's paradise. But the cloudburst of 22nd

July 1970 made the lake to overflow and break the wall of one side, resulting in devastating flood. Suddenly on 26th July 1970 the lake disappeared.

Some of the Tals of the area are:

- Hemkund Lokpal
- Satopanth
- Vasundhara fall
- Deotal
- Roopkund
- Vednikund.

4. Conservation value

The Valley is one of the two core zones of the Nanda Devi Biosphere Reserve which protects one of the most spectacular mountain wildernesses of the western Himalayas, among which the Paspawati valley is celebrated for its flowers. More than 500 species grow there in an area of less than 2,500 hectares. It is also the habitat of the endangered snow leopard and Himalayan musk deer. The whole area lies within a Conservation International-designated Conservation Hotspot, in a WWF Global 200 Eco-region, is in a WWF/IUCN Centre of Plant Diversity and in one of the world's Endemic Bird Areas. It is also a UNESCO Biosphere Reserve.

5. Need for Conservation

The Park is a natural laboratory for the conservation and study of the western Himalayan flora. In 2002-03 in cooperation with the villagers' Eco-Development Committee and Forest Committee of Bhyundar the Forestry Department oversaw the clearing of 50 tons of litter and removed 120 temporary stalls from the pilgrim trail from Govindhar to Hekmund. The Committee is also spreading awareness of the need to suppress the rampant Himalayan knotweed. Management is done within the 2003-2013 plans for Nanda Devi Biosphere Reserve which is implemented annually in consultation with local, district and state bodies but does not manage the parks directly.

Management constraints

The main management issues are control of invasive knotweed within the Valley, and, on the way to it, tourist and pilgrim litter. Some 1,000 ha of meadow are infested with the tall fast growing Himalayan knotweed which controls erosion but crowds out and smothers the subalpine flora. Its increase where livestock used to congregate is related to the prohibition of grazing. While livestock overgraze and over-enrich the soil, they may enhance floral diversity by limiting the growth of taller more vigorous plants. Its eradication and regular monitoring is expected to be a major expense (Srivastava, 1999). The litter piles up by the tonne from the thousands of tourists that visit the shrines: 300,000 plastic bottles a year and 5-600 kg of human and mule dung per day. The local people have now combined to clear this. A past threat to the forests surrounding the pilgrim route was the destruction of trees for firewood but this is now forbidden. There is no pollution and little danger from avalanches except on the approach road from Govindghat. There is, nevertheless, a constant threat from local poachers, especially to the snow leopard, and to ungulates when they come down to the valleys in winter; also from local indifference to wildlife conservation. This is aggravated by lack of adequate funding for the training needed for high altitude monitoring.

6. Conclusion

Valley of Flowers is known for its pristine beauty. Tourism has emerged in form of industry today for generating revenue. The number of tourists visiting Valley is increasing every season. Ecotourism in VOF should be promoted in a manner that it should not destroy the beauty of natural ecosystem. Community participation has a key role to play in spreading awareness among the local villagers, tourists, etc for having a sustainable form of ecotourism.

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7. References

1. Fukada, K., 1971. *The Great Himalayas*. In Shirakawa Yoshikazu (Ed.) Himalayas. Harry N. Abrahams. Inc., New York.
2. Kaur, J., 1977. The Valley Of Flowers: Exploratory Survey of Bhyundar Valley

along the Alaknanda for Recreation Resource Planning. *Tourism Recreation Resource*, Vol II, NO. 2.
3. Lamba, B 1987. Status survey report of fauna. Nanda Devi National Park. *Records of the zoological survey of India Occasional Paper* No. 103.50pp
4. Lavkumar, K. 1977. Report on the preliminary survey of the Nanda Devi basin. *WWF-India*, Bombay. 27pp.
5. Pauw, E.K., 1986. *Report on the Tenth Settlement of Garhwal District*, Govt. Press, Allahabad.
6. Shiva Garg 2006. An Assessment of Solid Waste management through public participation in the Valley of Flowers National park, Uttarakhand. Submitted to Gurukul Kagri University for partial fulfillment of M.Sc degree
7. Srivastava, S. 1999. Management plan for the valley of flowers national park. *Wildlife Preservation Organization*, Uttar Pradesh.
8. UNEP-World Conservation Monitoring Center 2005. Under World Heritage sites Nanda Devi and valley of flowers national Parks, Uttaranchal India.

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Diabetogenic Effect of Pregnancy in Sprague-Dawley (SPD) Rats: Potential use as Experimental Model of Human Gestational Diabetes

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Abstract: The effect of pregnancy on the pattern of oral glucose tolerance was investigated using Sprague-Dawley (SPD) rats. Adult virgin, timed-pregnant and non-pregnant rats were subjected to brief ether anaesthesia after 18-hour overnight fasting period to allow for oro-gastric administration of glucose load at 3.0g/kg body weight (b. wt.) as 30% solution. Glucose concentration determined from the tail blood shows that the starting glucose concentration of the pregnant rats was 6.9±0.4 mmol/l, a significantly higher ($P<0.05$) value than 5.8 mmol/l, the starting blood glucose concentration of the non-pregnant animals (Controls). The peak blood glucose level attained at the 60th minute was significantly higher ($p<0.05$) in the pregnant rats (13.5±0.3 mmol/l) as compared to that of the non-pregnant rats (8.5±0.3 mmol/l). After 120minutes, the blood glucose level of the non-pregnant rats dropped to a near starting level while the corresponding value in the pregnant rats remained comparatively higher ($P<0.05$). Assessment of the rate of appearance and disappearance of glucose in the blood and the determination of glucose response and glucose tolerance indexes (GRI and GTI) respectively showed that pregnancy caused poor glucose utilization in the rats. The results of this short-term study suggest that pregnancy is largely diabetogenic in Sprague-Dawley (SPD) rats. The diabetogenic effect of pregnancy did not necessitate administration of any other diabetogenic agent such as streptozotocin or fructose. Thus, pregnancy induced diabetes in this strain of rats may have potential value as model of gestational diabetes in human. [Nature and Science 2010; 8(4):107-111]. (ISSN: 1545-0740).

Keywords: Gestational diabetes; glucose response index; glucose tolerance index; insulin resistance

1.0 Introduction

Diabetes is a life-threatening disorder characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. (Odom et al, 2004). The vast majority of diabetes fall into two broad categories namely type 1 and type 2 diabetes. Type 1 diabetes is caused by an absolute deficiency of insulin secretion due to pancreatic β-cell destruction. The other major category, type 2 diabetes, is more prevalent especially in developing countries where there are gradual changes to western life styles. Type 2 diabetes forms a spectrum of pathophysiological conditions ranging from predominantly an insulin resistance state with relative insulin deficiency to predominantly an insulin secretory defect combined with insulin resistance (Alberti and Zimmet, 1998).

In many developing countries like Nigeria, these two categories of diabetes are well known, and considerable attention is being focused on their prevention and proper management. Another important class of diabetes that has not been given comparable level of attention particularly in developing countries is gestational diabetes(Alberti and Zimmet, 1998). It is characterized by carbohydrate intolerance of variable severity with onset or first recognition during pregnancy. In many developing countries, several fetal and neonatal deaths are associated with diabetes-related pregnancy. Babies born of diabetic pregnant mothers are macrosomic (large), and unhealthy. In the United States and other developed countries, the frequency of gestational diabetes is increasing. The long-term implications for developing countries like Nigeria are important. In view of the increasing prevalence of gestational diabetes and the associated risk of maternal and neonatal morbidity and mortality, gestational diabetes remains a significant challenge and increasing attention should be focused on the problem.

Experimental animal models of gestational diabetes are of immense value in this respect. It is generally known that the use of human subjects in biomedical research sometimes has obvious limitations which include ethical and other considerations. Development of experimental animal

models remains an important way of enhancing understanding the pathophysiological mechanisms of conditions that affect human. Considering gestational diabetes and its associated problems, experimental findings from animal models would enhance the development of preventive and management strategies with a view to improving diabetic pregnancy outcomes. Previous studies relating to animal models of gestational diabetes involved administration of streptozotocin (Lopez-Soldado and Herrera, 2003) or fructose (Olatunji-Bello and Nwachukwu, 2000) before or during pregnancy. However the adequacy of these models in reproducing human gestational diabetes has been questioned (Caluwaerts et al., 2003) partly due to the fact that these agents (STZ and fructose) are themselves diabetogenic (Rakieten et al., 1963; Zavaroni et al., 1980); thus, the diabetogenic effect of pregnancy is complicated by the effect of these agents. Thus, induction of diabetes in animals by pregnancy without administering other diabetogenic agents may represent more appropriate animal models of human gestational diabetes in human. The aim of this study is to assess susceptibility of Sprague-Dawley (SPD) rats to pregnancy-induced diabetes with a view to evaluating the use of this strain as experimental models of gestational diabetes.

2.0 Materials and Methods

2.1 Experimental Animals

Sprague-Dawley albino rats of both sexes weighing 160-180g were obtained from the Laboratory Animal Unit of the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, and transferred to the Animal Room of the Biological Garden, University of Lagos, where they are kept in plastic cages with mesh grid floors for acclimatization and mass breeding in the new environment. The cages were thoroughly cleaned and the animals examined on a daily basis. Clean tap water and rat feed were made available *ad libitum*. The temperature of the animal room was 30±3°C with 12h:12h light darkness cycle. On this regime, the animals remained uniformly healthy and active. Rats that became pregnant were separated into solid floor maternity cages. Fine sterilized wood shavings were provided in the cages as bedding and nesting materials. Immediately after the offspring were weaned (i.e. before attaining sexual maturity), they were transferred into new cages where they were kept separately as females or males only to avoid mating before induction of pregnancy. This was to ensure that the animals were still virgin males and females before induction of pregnancy. In this study, strict adherence of experimental procedures to ethics in animal experimentation was ensured.

2.2 Induction of Pregnancy

After 90 days of life, 20 virgin rats (10 females:10 males) obtained from mass breeding above were housed in 10 mating groups of monogamous pairs (1 females: 1 male per cage). At this period of life, the animals would have attained sexual maturity, and the virgina would have opened. To check for successful mating, the virgina was examined every morning, and virgina smears were obtained to see if sperm cells were present. In addition, the virgina and the floor of the cages were observed to check for the presence of cornified plug. The presence of sperm cells in the virgina smear or the availability of cornified plug in the virgina or on the floor of the cage indicated successful mating, and this was regarded as Day-1 of gestation. Such females were separated into maternity cages to constitute pregnant rats for oral glucose tolerance test (OGTT) which was carried out on D-17 of gestation.

2.3 Oral Glucose Tolerance

Since successful mating does not imply successful pregnancy, the criterion for inclusion in oral glucose tolerance test (OGTT) among the rats placed n the Pregnant Group was successful pregnancy. Female rats (n=18) consisting of 8 pregnant rats (treatment) and10 non-pregnant rats (control) were used for OGTT on Day-17 as described in previous reports (Odeigah et al., 1994). Essentially, the animals were fasted for 18 hours before the test, and a glucose load of 3.0/kg body weight (b. wt.) was delivered into the stomach through the buccal cavity as 30% solution by oro-gastric intubation under light ether anaesthesia. Blood glucose was obtained from cut tail tips for the determination of blood glucose concentration (glucose-oxidase method) using an automated digital blood glucose analyzer, glucometer (Accu-Chek Advantage, Roche, USA), just before oral glucose loading (0 minute) and at 30, 60, and 120 minutes of OGTT to obtain the glucose tolerance curve of each rat.

2.4 Analysis of Data

2.4.1 Statistical Analysis

All data were input into the computer for statistical analysis using a software package – GraphPad Prism Version 5.00. The results are expressed as mean±SEM. Statistical difference between means was determined by Student's t-test,

and P<0.05 was considered significant while P<0.01 was taken to be highly significant.

2.4.2 Rate of Appearance and Disappearance of Glucose from the Blood

The mean rate of appearance of glucose in the blood was obtained by determining the positive slope of the glucose tolerance curve from the starting (0-minute) blood glucose level to the peak level according to the formular below:

Rate of appearance/disappearance of glucose (mmol/min) = ΔG/ΔT (Note: ΔG=change in blood glucose concentration in mmol. while ΔT=time in minutes (mins.).

The rate of disappearance of glucose was similarly calculated using the negative slope of the curve from the peak level to the final glucose level.

2.4.3 Glucose Response Index (GRI) and Glucose Tolerance Index (GTI)

Glucose utilization was assessed by Glucose response index (GRI). The GRI for each rat was estimated as the incremental area under its glucose tolerance curve. It is calculated by summation of the areas of the trapezoids defined by individual points on the curve (Lebovitz and Feinglos, 1983).This area which is above the fasting blood glucose level during the two-hour oral glucose tolerance determination is inversely related to glucose tolerance index (GTI) which gives a direct assessment of glucose utilization in each animal. Thus, GTI was estimated as the reciprocal of GRI.

3. Results

Figure 1 depicts the plasma glucose profiles of the pregnant and the non-pregnant rats as revealed by their glucose tolerance curves. It could be observed that the starting blood glucose concentration of the pregnant rats was 6.9±0.4, a significantly higher (P<0.05) value than 5.8 mmol/l, the starting blood glucose concentration of the non-pregnant animals. After 60 minutes of OGTT, the blood glucose concentration reached a peak level in both groups. However, the peak plasma glucose concentration in the pregnant rats (13.5±0.3 mmol/l) was significantly higher (P<0.05) than that of the non-pregnant rats (8.5±0.3 mmol/l). This blood glucose rise resulted in a rate of appearance of glucose of 0.11mmol/min. and 0.05 mmol/min respectively in the pregnant and the non-pregnant rats respectively (Table 1).The rate of disappearance of glucose after attaining the peak concentration was, however, higher in the non-pregnant rats. After 120minutes, the blood glucose level of the non-pregnant rats dropped to 6.2±0.3, a value which was near the starting level; however, the corresponding in the pregnant rats remained significantly high (P<0.05). Determination of glucose tolerance index (GTI) as revealed by Figure 2 showed that pregnancy caused a very significantly higher GTI of 483.3.0±35.0 mmol.min/l (P<0.01) when compared to the non-pregnant state (GTI=175.5±25.0 mmol.min/l).

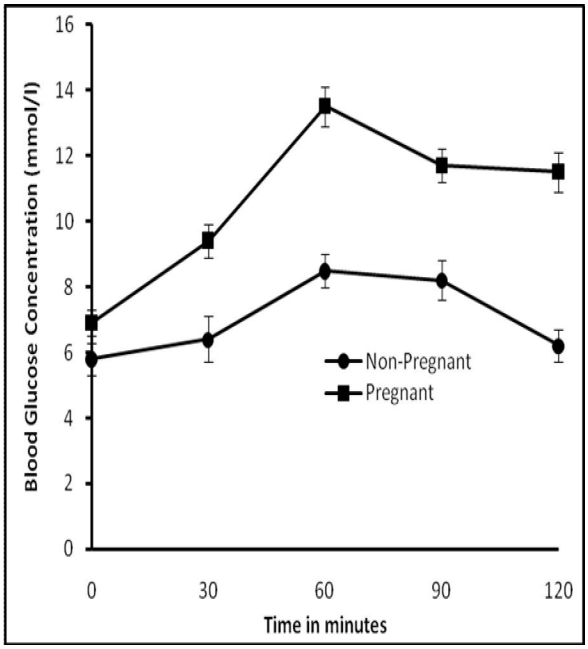

Figure 1. Increased Glycaemic Response in Pregnant Rats as Compared to non-Pregnant Rats.

Table 1. Rate of Appearance and Disappearance of Glucose (mmol/min.) in the Blood of Pregnant and Non-Pregnant Rats

Animal Groups	Rate of Appearance (mmol/min.)	Rate of Disappearance (mmol/min.)
Non-Pregnant	0.05	0.05
Pregnant	0.11	0.03

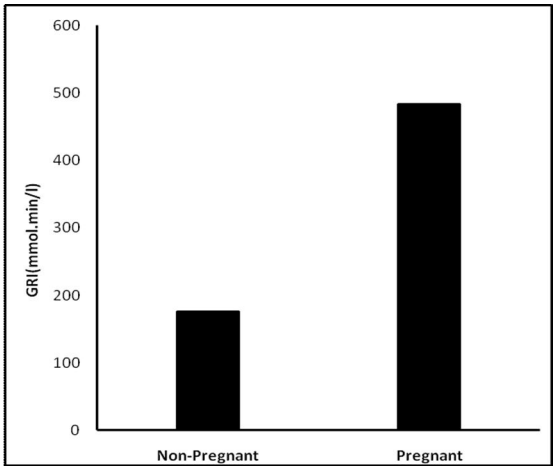

Figure 2. Higher Glucose response Index (GRI) in the pregnant Rats as Compared to that of Non-Pregnant Rats

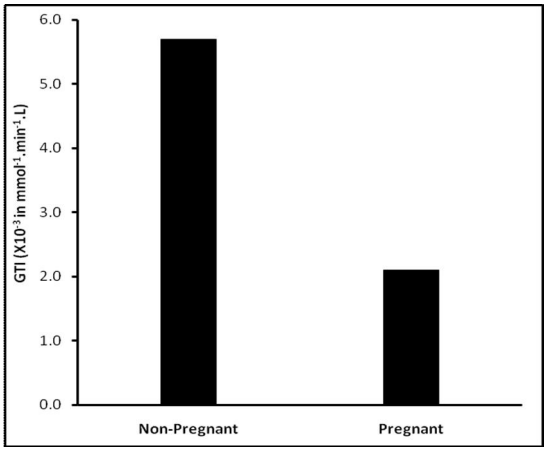

Figure 3. Higher Glucose Tolerance in Pregnant Rats than the Non-Pregnant Rats as Assessed by Glucose Tolerance Index

4.0 Discussion

In the present study, oral glucose tolerance test or OGTT was performed on the 17th day of gestation in the rats, a period that is equivalent to the third trimester of pregnancy in human. Although the cause of pregnancy induced diabetes is not fully known, there are some theories explaining the etiology of the condition. According to a popular theory, some hormones (oestrogen, cortisol and human placental lactogen) produced during pregnancy are responsible for insulin resistance observed in gestational diabetes. Normally, the pancreas produces additional insulin to overcome insulin resistance, but when insulin production is not enough to overcome the effect of these hormones, gestational diabetes results. Xiang et al. (1999) suggested that the degree of insulin resistance increases with gestational period and that insulin resistance plays a major role in the development of diabetes mellitus during pregnancy. Subsequent studies in rats by Olatunji-Bello and Nwachukwu (2000) agreed with the findings of Xiang et al. (1999). Thus, on Day-17 of pregnancy in rats, insulin resistance or, possibly, diabetes associated with pregnancy would have fully developed.

The elevated blood glucose concentration at every time-point of OGTT in the pregnant rats indicated the presence of impaired glucose tolerance. It was therefore not surprising that glucose tolerance index or GTI was significantly higher in the pregnant rats than in those that were not pregnant. Several reports have indicated that in late gestation, women with gestational diabetes have increased fasting insulin concentrations and less suppression of hepatic glucose production during insulin infusion, thereby indicating decreased hepatic insulin sensitivity in women with gestational diabetes compared with a weight-matched control group (Catalano et al., 1993). It is not clear whether impaired glucose tolerance due to pregnancy was associated with hyperinsulinemia in SPD rats. Direct determination of insulin concentration concurrently with glucose measurement would be elucidating.

The underlying pathophysiology of pregnancy-induced diabetes is associated with decreased maternal insulin resistance, a situation whereby a defined concentration of insulin is unable to effect a predictable biological response of nutrient metabolism at the target tissue (Catalano et al., 2003). This agrees with the observation of Xiang et al. (1999) that significant alteration of glucose metabolism occurs in women who develop gestational diabetes. The increased rate of appearance of glucose in the blood of pregnant rats and its slow rate of disappearance as observed in this study was an indication of poor glucose metabolic state resulting from insulin resistance during pregnancy in the rats. Decreased maternal insulin sensitivity in women with gestational diabetes may increase nutrient availability to the foetus, possibly accounting for an increased risk of foetal overgrowth and adiposity. This explains why babies of diabetic pregnant women are macrosomic (Catalano et al., 2003).

Considering the significantly elevated starting glucose level and the general blood glucose profile during OGTT in the rats used in this study, it may be suggested that pregnancy is largely diabetogenic particularly in SPD rats. Unlike other studies where

the diabetogenic effect of pregnancy is complicated by other agents like streptozotocin (STZ) and fructose (Lopez-Soldado and Herrera, 2003; Olatunji-Bello and Nwachukwu, 2000) that are themselves diabetogenic (Rakieten et al., 1963; Zavaroni et al., 1980), the glucose tolerance pattern observed in the present study was due to pregnancy alone. This does not rule out the presence of underlying genetic factors predisposing the animals to pregnancy diabetes. Similar observation in human had suggested that the metabolic stress of pregnancy may unmask a genetic susceptibility that causes alterations in glucose metabolism leading to gestational diabetes. If this observation is confirmed by other workers, one may suggest the presence of genetic factors in the susceptibility of SPD rats to pregnancy-induced diabetes.

In order to characterize the genetic component more accurately, selective breeding is in progress in our laboratory to create two strains of rats having different susceptibility to gestational diabetes. Other genetic and molecular studies are also in progress to determine the heritability of this trait and possible association of molecular markers such as RFLPs which may lead to easy identification of people at risk.

Conclusion

Pregnancy is largely diabetogenic in SPD albino rats, and they may serve as models for human gestational diabetes.

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References

1. Odom DT, Zizlsperger N, Gordon B, Bell GW, Rinaldi NJ, Murray HK, Volkert, TL, Schreiber J, Rolfe PA, Gifford, DK, Ernest F, Bell GI, Young RA (2004). Control of pancreas and liver gene expression by HNF transcription factors. Science 2004: 303:1378-1381.
2. Alberti KGMM, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1:Diagnosis and classification of diabetes mellitus. Provisional Report on WHO Consultation. Diabetic Medicine 1998; 15: 539-553.
3. Lopez-soldado I Herrera E. different diabetogenic response to moderate doses of streptozoticin in pregnant rats, and its long-term consequences in the offspring. Exp. Diabesity Res 2003;4:107-118
4. Olatunji Bello II nwachukwu D. glucose tolerance during pregnancy in fructose-fed rats. J. of Med. & Medical sci. 2000 ; 2 (1): 65-67
5. Caluwaerts S, Holenmans K, Van bree R, Verhaeghe J, Van Assche FA. Is low-dose strptozotocin in rats an adequate model for gestational diabetes mellitus? J. Soc. Gynecol. Investig. 2003; 10: 216-221
6. Rakieteen N, Rakieten ML, Nadkarni MV. Studies on the diabetogenic action of streptozotocin. Cancer Chemother. Rep. 1963; 29: 91-98
7. Zavaroni I, sander S, scott S, reaven GM. Effect of fructose feeding on insulin secretion and insulin action in the rat. Metabolism 1980;29: 970-973
8. Odeigah PGC, Taiwo IA, Onokpite, GO. Effects of salt and chilli pepper on oral glucose tolerance in normal and alloxan-diabetic rats. International Diabetes Digest 1994; 5: 70-73.
9. Lebovitz HE, Feinglos MN. Mechanism of Action of second generation sulfonylurea, glipizide. The American Journal of Medicine 1983 Syposium: 46-54.
10. Xiang AH, peters RH, trigo E, kjos SL, leeWP, bucham.TA. (1999) multiple metabolism defects during late pregnancy in women at high risk of type 2 diabetes. Diabetes 1999; 48: 848-854.
11. Catalano PM, Kirwan JP, Mouzon SH, King J. Gestational diabetes and insulin resistance: Role in short- and long-term implications for mother and foetus. J. Nutr. 2003; 113:1674-1683.

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Studies on growth, nutritional and microbiological status of citrus seedlings infested with root-rot disease

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Abstract: This research aims to evaluate the suppressive effects of compost fortified with Trichoderma harzianum and Top.Zn formulations on citrus root-rot and plant growth. Pathogenicity test proved that isolate no.1 of Fusarium solani and Macrophomina phaseolina were the most frequently causing infection of all orange plants with 87.5 and 93.75% disease severity respectively. Soil infested with F. solani or M. phaseolinea decreased plant growth and N, P and K contents in the orange leaf tissues compared to the control. Meanwhile, application of Top.Zn compound alone raised up N, P and K contents (%) in leaves of orange and mandarin survived in soil infested with F. solani and M. phaseolina. Use of compost with T. harzianum and Top.Zn simultaneously with a pathogen inoculation caused a significant increase in plant growth, chlorophyll a and b, macronutrients (N, P and K) content, micronutrient (Fe, Zn, Mn and Cu) contents orange and mandarin seedlings. The total fungal and bacterial counts in the orange and mandarin rhizosphere were increased progressively as the plant grew up reaching their maximum at the last count which was taken after 90 days (seedlings were 1-year old). In soil infested with F. solani and M. phaseolina, treatment with compost fortified with T. harzianum increased the total fungal count 3.34 and 28.98 times, respectively in orange and 2.60 and 21.99 times, respectively in mandarin compared with non treated control. In soil infested with F. solani and M. phaseolina, the treatment with compost fortified with T. harzianum in combination with Top.Zn decreased the average number of total bacterial counts in the rhizosphere of orange 85.04 and 78.92% respectively and 59.32 and 92.74 % respectively in the rhizosphere of mandarin. [Nature and Science. 2010;8(4):112-121]. (ISSN: 1545-0740).

Key words: Citrus root rot, rhizosphere, compost, Trichoderma harzianum, Top.Zn formulation.

1. Introduction

Citrus root-rot caused by *Fusarium solani* (Mart) Snyd & Hans and *Macrophomina phaseolina* was reported to attack citrus varieties (Kore and Mane 1992; Rensburg, *et al.* 2001; Kung'u *et al.* 2002; El-Mohamedy and Ahmed 2009). Although root-rot is under control due to (Verma *et al.* 1999) the pressure against the use of fungicides due to high coast, fear of resistance and potential hazards to the environment have resulted in a shift towards biological control using compost fortified with antagonistic microorganisms could be the way to control root-rot citrus disease.

Compost applied to the soil improves its quality by altering the chemical and physical properties, increase organic matter content, water holding capacity, overall diversity of microbes, provide macro-and micronutrients for plant growth and suppress diseases which indirectly contribute to plant growth enhancement (Scheuerell and Mahaffe 2004; Sylvia 2004; Healther *et al* 2006).

Certain microorganisms present in the compost such as *Trichoderma* spp. are known to stimulate plant growth (Ozbay and Newman 2004; Sylvia, 2004). These microbes benefit for the plant through different mechanisms action, including the production of secondary metabolities, antibiotics and hormone like substances (Ozbay and Newman 2004; Harman *et al.* 1996). The production of siderophores, antagonistic to soil borne root pathogens (Dubeikovsky *et al.* 1993; Siddiqui *et al.* 2008) has been also reported.

The bio-efficiency of compost therefore, could also be further enhanced by fortifying it with plant nutrients or biocontrol inoculants such as *Trichoderma* spp. *Trichoderma harzianum* alone or in combination with compost has been documented as the most common and effective biocontrol agent for disease control in various host-pathogen systems (Elad 2000; Ibrahim 2005; Siddiqui *et al.* 2008).

Therefore, this study was carried out to determine the efficiency of compost fortified with *T. harzianum* as an alternative to chemical fungicide and Top.Znformulation on morpho-physical growth and occurrence of root rot disease of orange and mandarin citrus seedlings. The effect of different treatments on rhizosphere soil microflora was also studied.

2. Material and Methods:

Pathogens isolation and identification

Since 2006, thirty diseased citrus seedlings (one-two years old) grown under greenhouse conditions in Behera and El-Giza Governorates were sent to the laboratory of National Research Centre (NRC), Egypt, to identify the organisms causing root-rot disease. Selected root segments of diseased plants were washed by running water for one hours and surface disinfected by immersion in a 0.5% sodium hypochlorite solution for 2 min, rinsed with sterilized water, dried under a laminar airflow hood, and cut into pieces (3-5 mm). The root pieces were placed on plates containing potato dextrose agar (PDA) for 3-7 days at 28°C±2. Fungal isolates were purified using hyphal tip and single spore culture techniques (Booth 1971).

Identification of pure culture was carried out according to Gilman (1957), Booth (1971), Nelson *et al* (1983), Barnett and Hunter (1986).

Pathogenicity tests

Autoclaved oat (100g oat / 70 cm^3 water) was infected with plugs of a 10 days-old pure culture of each isolated fungi and incubated for 2-3 weeks. Sterilized oat grains without fungi were used as control. Soil was infested with the rate of 10% (w/w), watered every two days for one week before planting. One transplant (1 year old) of citrus orange seedlings (cv. valincia) was transplanted in each pot. Four pots were used for each isolate as replicates. Four months after planting disease symptoms were registered daily on shoot system as follows: 0 = healthy, 25% = yellowish, 50% = plant wilted, 75% = whole plant wilted and 100% = plant dead showed severe wilt. When foliar symptoms appeared, rotted root segments were surface disinfected as described before and placed on PDA plates for reisolation of the pathogen.

Compost (farmyard manure)

One year old farmyard manure produced by NRC, Giza, Egypt was used and analyzed. The chemical properties were determined using the method described by A.O.A.C. (1990). The chemical analyses are summarized in table 1.

Table 1. Chemical characteristics of the compost (farmyard manure)

pH	EC(dS/m)	C%	N%	C/N ratio	Total P%	Total K%	OM%
6.90	4.30	17.60	1.18	14.92	0.41	1.07	26.38

A representative soil sample taken from the used soil was analyzed and listed in table 2.

Table 2. Some physical and chemical properties of the soil used in the experiment.

Particle size distribution [%]			Texture soil	EC dS/m	PH	Available nutrients [mg/]						
Sand	Silt	Clay				N	P	K	Fe	Mn	Zn	Cu
41.18	30.95	27.87	Clay loam	2.3	7.8	30	10	286	5.8	4.7	0.9	0.31

Compost fortified with biocontrol agent

The highly antagonistic isolate of *T. harzianum* obtained from previous work by Haggag and Saber (2000) exhibited a broad-range of antibiosis towards many plant pathogens was grown on PDA broth medium in 500 ml capacity conical flask for 28°C±2. Conidia spores and mycelial growth were harvested to obtained fungal suspension of 3x 10^8cfu/ml. Farmyard manure compost was inoculated with 10 ml/ 100g on oven dry basis of *T. harzianum* spore suspension and inoculated in plastic containers in the dark at room temperature (25-28°C). **Top. Zn formulation**. Top.Zn (8 hydroxy quinoline sulphate) was kindly obtained from soil and water use Dept. NRC, Egypt.

Greenhouse experiments

During two consecutive seasons (2008-2009) a pot experiment was carried out to evaluate the role of compost fortified with *T. harzianum* and Top.Zn at rate of kg/600L against *F. solani* and *M. phaseolina* the causal agents of root-rot disease of orange cv. valincia and mandarin cv. Egyptian balady citrus plants. Each pots containing 5kg soil somewhat wet (about 60% water holding capacity) were inoculated with a propagules of *F. solani* and *M. phaseolina* at a rate of 50g/pot, 3days before cultivation to ensure the distribution of the inocula. Four orange and mandarin seedlings one year old were used as a replicates for each treatment. Untreated seedlings were used as a control. *F. solani*

and *M. phaseolina* prepared as mentioned in the pathogenicity tests were used.

On the other hand, bioagent was used at the rate of 50g/pot by drenching soil with compost individual and in combined treatments to soil before transplanting citrus seedlings.

Treatments were categories for each citrus varieties into the following:

1-Un-infested soil (control).
2-Infested with *F. solani*
3-*M. phaseolina*
4-*T. harzianum*
5-*F. solani* + *T. harzianum*
6-*M. phaseolina* + *T. harzianum*
7-*F. solani* + Top.Zn
8-*M. phaseolina* + Top.Zn
9-*F. solani* + *T. harzianum* + Top.Zn
10-*M. phaseolina* + *T. harzianum* + Top.Zn

Plant growth analysis On the late of September, a leaf sample consisted of 50 leaves was taken from the mid shoots transplant according to Jones *et al*. (1991). Plant height (cm), branch length (cm), stem thickness at 5 cm above the crown was measured by gauge (mm) and leaf area using Li- 3100 area meter were recorded. Number of mature leaves was counted on each seedling.

Chemical characteristics

a- Chlorophyll (a and b)

Chlorophyll a and chlorophyll b content were determined using spectrophotometer at a wave length, 647 and 664 nm proposed by Coombs *et al.* (1987).

b- Macro and Micronutrients analysis

To analyze macro and micronutrients in citrus, samples were taken from each treatment, then dried at 70°C, and it was grounded using stainless steel equipments. From each sample 0.2 g was digested using 5 cm^3 of the mixture of sulfuric (H_2SO_4) and perchloric ($HCLO_4$) acids (1:1) as described by Peterburgski (1968). Total nitrogen was determined by micro-Kjeldahl method and phosphorus was determined calorimetrically at wavelength 680 nm using spectrophotometer (Spekol) as well as potassium was determined by using Gallen Kamp flame photometer. Micronutrients, i.e., Zn, Fe, Mn and Cu were measured using atomic absorption spectrophotometer Perkin Elmer model 5000 (Cottenie *et al.* 1982).

Microbiological analysis

The total bacterial and fungal counts in the rhizosphere of the two citrus varieties were obtained from each treatment after 30, 60 and 90 days. The method adopted by Louw and Webely (1959) for studying the microorganisms of rhizosphere soil region was used. A portion of root system was taken with great care to obtain soil very closed to root-system as much as possible and transferred to a wide mouth reagent bottle of known weight containing 90 cm^3 distilled water under aseptic conditions. The plate count technique according to Allen (1961) was followed for total count of bacteria and fungi. Soil extract agar medium (Buent and Rovira 1955) and dilutions of $1/10^5$ to $1/10^7$ were used for bacteria and incubated at 35°C±2 for 48 hrs. Martin medium (Allen 1961) was used for fungi at dilutions of $1/10^3$ to $1/10^5$. Plates were incubated at 28°C±2 for 5-7 days.

Statistical analysis

Data of the present study were statistically analyzed and the differences between the means of the treatments were considered significantly when they were more than least significant differences (LSD) at the confidence level of 5% as outlined by Duncan (1955).

3. Result Analysis

Root-rot pathogens of citrus seedlings

Isolation from rotted roots seedlings revealed the association of one or more of the following six fungal species, i.e., *Alternaria tenuis*, *Chaetomium globosum*, *Fusarium solani*, *Macrophomina phaseolina*, and *Rhizoctonia solani***.** *F. solani* and *M. phaseolina* in particular were more frequent than any of the other fungi. These fungi were previously reported to be associated with root-rot of citrus in other countries (Kore and Mane 1992, Rensburg *et al*. 2001, Kung'u *et al.* 2002). In Egypt, El-Mohamedy and Ahmed (2009) reported that dry root-rot disease of mandarin caused by *F. solani* attack most citrus varieties.

Upon testing the pathogenicity of these isolated fungi, isolates of *Fusarium* and *Macrophomina* were found more or less able to attack orange citrus seedlings (Table 3). Orange seedlings were highly vulnerable to attack by the all three isolates of *F. solani* and the two isolates of *M. phaseolina*. The isolates no. 1 of *F. solani* and *M. phaseolina* caused 100% plant infection causing 87.5 and 93.75% disease severity on plant growth of orange and mandarin, respectively. *F. solani* and *M. phaseolina* have been known to be the main organisms causing root-rot of citrus (Strauss and Labuschagen 1999, Catara and Polizza 1999).

Table 3. Disease incidence on one year seedlings of citrus (orange cv.) artificially inoculated with isolates representing fungal species associated with root-rot disease and their severity on plant

Tested fungal Isolates	Isolate No.	Plants infected [%]	Severity on plant growth [%]
Alternaria tenuis		0	-
Chaetomium globosum		0	-
Fusarium solani	1	100	87.5
Fusarium solani	2	50	25.0
Fusarium solani	3	75	25.0
Macrophominea phaseolinea	1	100	93.75
M. phaseolinea	2	50	25.0
Rhizoctonia solani	1	25	6.25
R. solani	2	50	12.50
R. solani	3	50	31.25
LS D (0.05)		18.77	

Effect of compost fortified with *T. harzianum* and TopZn

1. plant growth parameters

It could be noticed from data presented in Table (4) that orange or mandarin seedlings which survived in infested soil with *F. solani* and *M. phaseolina* never attained the normal growth in height or other growth parameters. The stem height of orange seedlings survived in *F. solani* and *M. phaseolina* infested soil reduced more 3.66 and 11.69% respectively compared to the control. A similar trend was observed with the other growth parameters.

Data also showed that different growth parameters of orange and mandarin seedlings were significantly increased over those of seedlings growing in control pots (untreated) and also seedlings growing in soil infested with the root-rot pathogens (*F. solani* and *M. phaseolina).* The increment in seedlings height, branch length, number of branches and leaves, stem diameter and leaf surface area were increased significantly in seedlings treated with compost fortified with *T. harzianum* alone or together with *F. solani* and *M. phaseolina.* Possible explanations of this phenomenon include: control of pathogens leading to stronger growth and nutrient uptake (Ousley *et al.* 1993), solubilization of insoluble micro nutrients in soil (Altomare *et al.* 1999), and production of growth hormones (Windham *et al.* 1986).

In addition treatment of orange and mandarin seedlings infested with *F. solani* and *M. phaseolina* with Top.Zn alone or in combination with compost fortified with *T. harzianum* exhibited a highly significant effect. The stem height of orange and mandarin seedlings treated with compost fortified with *T. harzianum* alone was increased from 45.67 to 64.67 cm (41.60% increase) and from 39.67 to 58.67 cm (47.89% increase) respectively. The same trends were also observed with the other growth characters. Data also showed that orange plants survived in soil infested with *F. solani* and *M. phaseolina*, used fertilized compost fortified with *T. harzianum* + Top.Zn increased the number of leaves orange from 15.00 to 26.67 (77.8%) and from 13.00 to 26.33 (102.54%) respectively for both pathogens, while on mandarin plants the number of leaves was increased 83.36% and 111.08 % respectively for both pathogens.

Chlorophyll a and b contents recorded in citrus leaves infected with *F. solani* and *M. phaseolina* were lower than in the uninfected control. In addition treatment of orange and mandarin seedlings infested with *F. solani* and *M. phaseolinea* with Top.Zn alone or in combination with compost fortified with *T. harzianum* exhibited a highly significant effect. Since, chlorophyll a in the leaves of orange seedlings treated with Top. Zn and compost fortified with *T. harzianum* alone was increased from 73.67 to 89.38 (21.32% increase) and from 73.67 to 87.44 (18.69% increase) in soil infested with *F. solani* and *M. phaseolina* respectively. As reported by several investigators that compost made of agricultural and industrial wastes have been widely used as soil amendment and induced suppression of soil borne pathogen through biological mechanisms (De Couster and Hoitink 1999; Muhammed and Amusa 2003; Rivera *et al*. 2004)**.**

Table 4. Effect of compost fortified with *T. harzianm* and Top.Zn on the growth parameters of two citrus seedlings infected with *F. solani* and *M. phaseolina*

Growth parameters / Treatments	Untreated	Soil infested with								
		T. harz	*F. solani*				*M. phaseolina*			
			Infes.	*T. harz.*	Top.Zn	Top.Zn.+ *T. harz*	Infes.	*T. harz.*	Top.Zn	Top.Zn + *T. harz*
Orange										
Stem length [cm]	* 45.67g	64.67 a	44.00 h	57.00 d	52.33ef	62.33 b	40.33 f	53.33e	51.00f	60.33 c
Branch length [cm]	6.27g	10.17 a	5.67h	8.60d	70.17f	9.60b	5.40h	8.13e	6.50g	9.10c
No. of branches	2.00bc	4.00a	1.00c	2.00b c	1000bc	3.00a b	1.00c	2.00bc	2.67abc	3.00a b
No. of leaves	18.33f	28.67 a	15.00 g	24.33 c	2.67e	26.67 b	13.00 h	22.33d	20.00a	26.33 b
Main stem diam. [cm]	0.73d	1.17a	0.54g h	0.71d e	0.63ef	0.92b	0.51h	0.65ef	0.61fg	0.83c
Branch diam. [cm]	0.60c	0.80a	0.53d	0.64c	0.62e	0.74b	0.52d	0.63c	0.61e	0.65c
Leave area [cm]	28.74g	40.06 a	27.12 h	33.42 d	29.59f	37.80 b	26.40 h	31.39e	29.11g	35.41 c
Chlorophyl a	73.67h	91.03 a	70.28 f	58.34 d	78.42f	89.38 b	68.47 f	80.38e	75.47g	87.44 c
Chlorophyl b	27.40f	33.32 a	26.17 g	28.34 d	27.45ef	30.35 b	25.33 h	28.23d e	27.57de f	29.25 c
Mandarin										
Stem length [cm]	39.67g	58.67 a	38.00 h	51.00 d	46.33ef	56.33 b	34.33 f	47.33e	45.00f	53.67 c
Branch length [cm]	6.10e	9.13a	5.33f	8.20c	6.17e	8.60b	5.17f	7.10d	5.50f	8.10c
No. of branches	1067cd	3.00a	1.00d	1.00d	1.67cd	2.67a b	2.00b c	1.00d	2.33abc	2.67a b
No. of leaves	17.33f	27.67 a	14.00 g	23.33 c	19.67e	25.67 b	12.00 h	21.33d	19.00e	25.33 b
Main stem diam. [cm]	0.63d	1.1a	0.44f	0.61d	0.53e	0.82b	0.41f	0.55e	0.51e	0.73c
Branch diam. [cm]	0.50c	0.70a	0.43d	0.54c	0.52e	0.64b	0.42d	0.53c	0.51e	0.55c
Leave area [cm]	27.74g	39.06 a	26.12 h	32.42 d	28.95f	36.00 b	25.40 h	30.39e	28.11g	34.41 c
Chlorophyl a	63.67h	90.03 a	60.28 e	75.34 d	68.42e	79.38 b	60.47 f	79.38b	65.47g	77.44 c
Chlorophyl b	26.40e	31.31 a	25.17 f	27.33 d	26.42e	29.35 b	24.33 g	27.23d	26.54e	28.25 c

*Means values followed by the same letter within the treatments are not significantly different ($p < 0.05$) according to the Duncan's multiple range tests

2. Nutritional status

Data in Table 5 show the effect of soil treatment with compost fortified with *T. harzianum* and Top.Zn on leaf of micro- and macronutrients of orange and mandarin seedlings. Compost fortified

with *T. harzianum* and Top.Zn applications had a significant effect on nutrient contents (%) in orange and mandarin citrus leaves (*p< 0.05).* Soil infested with *F. solani or M. phaseolina* decreased N, P and K content in the orange leaves compared to the control. Opposite trend was observed in mandarin leaves. Data also indicate that soil infested with compost fortified with *T. harzianum* alone improved N content in orange leaves while, P and K contents were decreased compared with untreated control. On the contrast, the same treatment improved the macronutrients, i. e., N, P and K in the leaves of mandarin.

Application of Top.Zn compound alone raised up the macronutrients in leaves of orange and mandarin survived in infested soil with *F. solani* and *M. phaseolina*. As, the percentage of N, P and K in orange leaves infested with *F. solani* were 3.35, 0.236 and 1.88%, respectively compared with 1.66, 0.214 and 1.72% respectively in the control. The same trends were also observed in leaves of orange and mandarin when soil was treated with Top.Zn + compost fortified with *T. harzianum*

Concerning micronutrients in the leaves, results cleared that soil infested with the two root-rot pathogens reduced Fe, Zn, Mn and Cu in orange leaves , while in mandarin leaves only Fe content was decreased comparing to the control. Application of Top.Zn alone or in combination with compost fortiied with T. *harzianum* also raised up the micronutrients percentage in leaves of orange and mandarin survived in soil infested with *F. solani* and *M. phaseolina* respectively. Possible explanations of this phenomenon include: control of minor pathogens leading to stronger growth and nutrients uptake (Ousley *et al.* 1993) solubilization of insoluble minor nutrients in soil (Altomare *et al.,* 1999) and production of growth hormones (Windham *et al.* 1986). *Trichoderma harzianum* may enhance plant growth by increasing the solubility of zinc, copper, iron and manganese ions, all plant nutrients with low solubility (Yedidia *et al.* 2001). Altomare *et al.* 1999 reported that *T. harzianum* increases plant nitrogen efficiency and also solubilize phosphate and micronutrients that could be made available to provide plant growth. They also concluded that the improvement of plant nutritional level might be directly related to a general beneficial growth effect of the root system following *T. harzianum* inoculation. The results of present study was in line of earlier studies which indicated that *T. harzianum* had a positive effect on citrus transplant growth.

Table 5. Effect of compost fortified with *T. harzianm* and Top.Zn on the nutritional status of two citrus seedlings infected with *F. solani* and *M. phaseolina*

Nutritional status / Treatments	Untreated	Soil infested with								
		T. harz	*F. solani*				*M. phaseolina*			
			Infes.	*T. harz.*	Top.Zn	T. harz	Infes.	*T. harz.*	Top.Zn	Top.Zn + T. *harz*
Orange										
Macronutrients [%]										
N	* 1.77e	2.30d	1.77e	1.70g	3.35b	3.47a	1.73f	1.46h	2.50c	3.46a
P	0.214 b	0.215b	0.176c	0.158 e	0.236a	0.238 a	0.164 d	0.112 f	0.215b	0.238 a
K	1.72a b	1.78ab	1.52ab	1.78a b	1.88ab	2.32a	1.40b	1.03b	1.78ab	2.00a b
Micronutrients [µ/ g^{-1}]										
Fe	60.0e	73.6bcd	70.0cd	69.3d	75.0b	88.0a	69.7c d	73.0b cd	74.3bc	75.3a b
Zn	41.7a b	43.0ab	39.0b	41.0a b	44.0ab	45.3a	39.0b	39.0b	43.3ab	45.0a
Mn	27.7c	39.0bc	27.3c	36.3c	41.0ab	43.0a	27.0e	37.8c	40.3ab	41.0a b
Cu	5.7f	7.3b	6.9c	6.1e	7.5ab	7.6ab	6.7d	7.0c	6.7d	7.6a

Mandarin										
Macronutrients [%]										
N	1.54g	2.13d	1.93e	1.96e	2.43c	2.91a	1.81f	1.94e	2.09d	2.69b
P	1.00e	0.175b	0.164bc	0.139 d	0.213a	0.215 a	0.160 c	0.165 bc	0.206a	0.213 a
K	1.32b	2.32a	1.40b	1.52a b	1.88ab	2.00a b	1.33b	1.72a b	1.78ab	1.78a b
Micronutrients [µ/ g^{-1}]										
Fe	69.0c	87.70a	63.0d	70.7b c	73.0bc	72.0b c	69.7b c	70.0b c	72.7bc	73.3b
Zn	35.7a b	45.3a	37.0cd	41.1a bc	39.0bc d	43.0a b	39.0b cd	40.7a bc	41.7abc	43.7a b
Mn	28.0d	39.0c	36.7c	37.3c	40.7ab	42.3a	37.6c	38.7b c	40.7ab	41.3a b
Cu	5.8c	7.1ab	6.9ab	6.6b	7.3ab	7.7a	6.8ab	7.0ab	7.2ab	7.4ab

*Means values followed by the same letter within the treatments are not significantly different ($P < 0.05$) according to the Duncan's multiple range tests.

3. Biological activity populations

Tables 6 and 7 show the effect of compost fortified with *T. harzianum* and Top.Zn on the microflora of the two citrus seedlings. It is clear that the total fungal and total bacterial counts in the rhizosphere of both orange and mandarin were increased progressively as the plant grew up reaching their maximum at the last count which was taken after 90 days. This increase may be attributed to the increase around and richness of root exudates and sloughs. Rhizosphere microorganisms utilize compounds and materials released from the roots and provide microorganisms with nutrients, consequently, the rhizosphere supports large active microbial populations (Akhtar and Siddiqui 2008)

Table 6. Effect of compost fortified with *T. harzianm* and Top.Zn on the total fungal counts of two citrus seedlings infected with *F. solani* and *M. phaseolina.*
(cfu x10^3/g soil)

Nutritional status / Treatments	Untreated	Soil infested with								
		T. harz	*F. solani*				*M. phaseolina*			
			Infes.	*T. harz.*	Top.Zn	Top.Zn + *T. harz.*	Infes.	*T. harz.*	Top.Zn	Top.Zn + *T. harz.*
Orange										
After 30 days	3.0	182.6	21.9	34.7	12.4	11.1	28.5	82.6	9.2	6.4
After 60 days	14.2	1641.9	28.7	81.9	7.3	104.4	19.1	181.0	23.8	27.9
After 90 days	1670. 2	3348.7	138.0	417.2	81.9	89.9	2560.7	4540.0	144.9	117.0
Mean	56.2	1724.4	62.9	177.9	33.9	68.5	869.4	1628.7	59.3	50.4
Mandarin										
After 30 days	0.6	47.3	7.8	73.7	0.8	26.9	1.1	67.8	2.3	33.0
After 60 days	23.9	80.9	34.4	48.1	9.1	27.8	32.0	425.3	13.9	38.4
After 90 days	120.5	406.5	160.8	254.8	39.1	82.8	261.1	2693.9	85.42	43.0
Mean	48.3	178.2	67.7	125.6	16.3	45.8	98.1	1062.3	33.9	38.1

a-Total fungal count

Inoculation of *T. harzianum* to soil alone increased the total fungal community in the rhizosphere of orange from 56.2 to 1724.4 cfu/g (30.7

times) and from 40.3 to 178.2 cfu/g (4.42 times) in mandarin. Fungal population in the rhizosphere of infested soil with *F. solani* or *M. phaseolina* was stimulated than that of the comparable healthy ones. As, inoculation of orange and mandarin citrus plant with *F. solani* resulted in an 11.92% and 40.16% increase in fungal count, respectively. The same trend was also observed when soil inoculated with *M. phaseolina.* Such infection would lead to the damage of the root and hence resulting in increased activity of saprophytic microorganisms.

Treatment with compost fortified with *T. harzianum* to infested soil with *F. solani* and *M. Phaseolina* increased the fungal population density by 3.34 and 28.98 folds respectively in orange and by 2.60 and 21.99 times respectively in mandarin compared with non treated control. As reported by Hoitink *et al.* (1991) that compost treatment cause a slow release of nutrients which supports beneficial activity of microflora.

Data in Table 6 also revealed that soil treated with Top.Zn alon decreased the total fungal count in the rhizosphere of orange from 56.2 to 33.9 cfu/g (39.18% decrease) and from 48.3 to 16.3 (66.25% decrease) in the rhizosphere of mandarin. It is proved that Top.Zn may have been fungicide effect toward fungi in soil. The same trend was also observed when Top.Zn was treated in combination with *T. harzianum.* Similar inhibitory effects of fungicides were recorded by Domsch (1960) ; Sahab *et al.* (1985).

b-Total bacterial count

It is clear from Table 7 that treatment of orange and mandarin seedlings with compost fortified with *T. harzianum* alone decreased the total bacterial counts from 488.1 to 58.9 cfu/g (87.93 % decrease) and from 549.6 to 124.5 cfu/g (77.35 % decrease) respectively.

Table 7. Effect of compost fortified with *T. harzianm* and Top.Zn on the total bacteria of two citrus seedlings infected with *F. solani* and *M. phaseolina* .
(cfu x10^5/g soil)

Nutritional status / Treatments	Untreated	Soil infested with								
		T. harz	*F. solani*				*M. phaseolinea*			
			Infes.	*T. harz.*	Top.Zn	Top.Zn + *T. harz*	Infes.	*T. harz.*	Top.Zn	Top.Zn + *T. harz*
Orange										
After 30 days	8.0	43.9	73.56	90.4	73.7	93.7	18.2	138.6	35.14	31.6
After 60 days	17.9	31.9	166.1	61.9	44.7	101.4	32.1	37.9	68.1	55.9
After 90 days	1438.3	2.5	1003.4	2031.6	3039.8	23.9	2732.9	178.6	2207.2	22.13
Mean	488.1	26.1	635.0	727.9	1052.7	73.0	927.7	118.4	770.1	102.9
Mandarin										
After 30 days	37.6	11.7	32.7	15.0	14.7	11.2	13.8	29.9	36.9	13.3
After 60 days	37.6	26.3	21.8	13.6	28.5	17.7	23.7	32.2	199.6	15.9
After 90 days	1573.6	335.4	773.8	583.9	332.0	641.8	386.8	474.1	458.3	150.5
Mean	549.6	124.5	276.1	204.2	125.1	223.6	141.3	178.7	231.6	59.9

In this respect many investigators noted that compost treatment highly reduced population density of some fungi and bacteria. In addition, rhizobacteria which produce antibiotic that suppress deleterious microbes (El-Mohammedy and Ahmed 2009 ; Ziedan, 2000). On the other hand, the average number of bacteria was much higher in the rhizosphere of orange soil infested with *F. solani* and *M. phaseolina* than in non-infested soil (control). While opposite trend was observed in the rhizosphere of mandarin, as the average number of total bacterial count were decreased from 549.6 to 226.1 cfu/g (49.76% decrease) and from 549.6 to 141.3 cfu/g (74.29% decrease) in soil infested with *F. solani* and *M. phaseolina,* respectively.

In infested soil with *F. solani* and *M. phaseolina* , the treatment with Top.Zn and compost fortified with *T. harzianum* decreased the average

number of total bacterial counts in the rhizosphere of orange by 85.04 and 78.92%, respectively and by 59.32 and 92.74 %, respectively in the rhizosphere of mandarin.

The obtained results throughout the present work highlighted the efficiency of compost as appropriate carriers for *T. harzianum* and Top.Zn used as biological control of citrus root-rot disease.

REFERENCES

1. Akhtar M. S., Siddiqui Z. A. 2008. Biocontrol of root-rot disease complex of chickpea by *Glomus intraradices, Rhizobium* sp. and *Pseudomonas straita*. Crop protection 27: 410-417.
2. Allen O. N. 1961. Experiments in soil bacteriology. Burgess Pub. Co. USA. 147 pp.
3. Altomare C., Norvell W. A., Bjorkman T., Harman G. E. 1999. Solubilization of phosphates and micronutrients by the plant growth promoting and biocontrol fungus *T. harzianum* Rifai,1295-22. Applied Environ. 65: 2926-2933.
4. A.O.A.C. 1990. Official Methods of Analysis Association of Official Analytical Chemists. 15th Ed. Inc. Wash. D.C.
5. Barnett H.L., Hunter B. B. 1996. Illustrated genera of imperfect fungi. Burgess Pub. Co. Minnesota, 241 pp.
6. Booth C. 1971. The genus *Fusarium.* Commonwealth Mycological, Institute, Kew, Surrey, England, 273 pp.
7. Buent J. S., Rovira A. O. 1955**.** Microbiological studies of some subantarctic soils. J. Soil Sci. 6: 119.
8. Catara A., Polizzi G. 1999. Dry root rot of citrus symptoms, causing and susceptibility of rootstock. Rivisto di fruticolture 6: 38-41.
9. Coombs P. O., Hall S. P., Schlock, J. M. O. 1987. Techniques in bioproductivity and photosynthesis. Pergamon Press, Oxford, 159.
10. Cottenie A., Verloo M., Velghe G., Comerlynk R. 1982. Chemical analysis of plant and soil. Ghent, Belgium, Laboratory of Analytical and Agro-chemistry State University.
11. De Couster T. J. H., Hoitink A. J. 1999. Using compost to control plant diseases. Bio Cycle Magazine, June: 61.
12. Domsch K. H. 1960. The efficiencies of soil fungicides. IV. Spectrum changes in the soil flora. Z. Pjkrankh. 67: 129.
13. Dubeikovsky A. N., Mordukhova E. A., Kochetkov V. V., Polikarpova F. Y., Boronin A. M. 1993. Growth promotion of blackcurrant softwood cuttings by recombinant strain *Pseudomonas fluorescens* BSP53a synthesizing an increased amount of indol-3-acetic acid. Soil Biology and Biochemistry 25: 1277-1281.
14. Duncan D. B. 1955. Multiple range and multiple F Test. Biometrics 11: 1-42.
15. Elad Y. 2000. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. Crop Protection 19: 709-714.
16. El-Mohamedy R. S., Ahmed M. A. 2009. Effect of biofertilizers and humic acid on control of dry root-rot disease and improvement yield quality of mandarin. Research J. Agric. And Biol. Sci. 5: 127-139.
17. Gilman C. J. 1957. A manual of soil fungi. 2nd ed. Iowa State College Press, 45o pp.
18. Haggag W. M., Saber M.S. 2000. Evaluation of three composts as multi-strain carriers for biofertilizer and biocontrol agents against *Fusarium* wilt of some legume plant. Arab J. Biotech. 13: 133-144.
19. Harman G. E., Latorre B., Agosin E., Martin R.S., Riegel R. S., Nielson P. A., Tronsmo A., Pearson R. C. 1996. Biological control and integrated control of Botrytis Bunch rot of grape using *Trichoderma* spp. Biological control 7: 259-266.
20. Healther M. D., Alexandra G. S., Richard P.D. 2006. Compost and manure mediated impacts on soilborne pathogens and soil quality. Soil Science Society of American J. 70: 347-358.
21. Hoitink H. A. J., Inbar Y., Boehm M. J. 1991. Status of compost-amended potting mixes naturally suppressive to soilborne diseases of floricultural crops. Plant Disease 75: 869-873.
22. Ibrahim M. D. 2005. Delivery system of *Trichoderma* inoculants for control of *Rhizoctonia* disease in *Brassica rapa*. Ph.D. Thesis, UPM, Malysia.
23. Jones Jr. J. B., Wolf B., Mills H. A. 1991. Plant analysis Handbook. Micro-Macro Publishing. Inc., Georgia, USA. Chapter 7. pp.45-88.
24. Kore S. S., Mane A. V. 1992. A dry root disease of Kagilime (*Citrus aurantifolia*) seedlings caused by *Fusarium solani*. Hournk Maha Aric. India 17: 276-278.
25. Kung'u J. N., Sief A.A., Gatumbi R. W. 2002. *Macrophomina* root rot of citrus in Kenya, E. Afr. Agric. For. J. 68: 1
26. Louw H. A., Weblely D. W. 1959. The bacteriology of root region of the oat plant grown under controlled pot culture conditions. J. Appl. Bacteriol. 22: 216
27. Muhammed S., Amusa N. A. 2003. *In-vitro* inhibition of growth of some seedling blight inducing pathogens by compost inhabiting microbes. African J. of Biotechnology 2: 161-

164.
28. Nelson P. E., Toussoum T. A., Marasas W.F.O 1993. *Fusarium* spp. An illustrated manual for identification. Philadelphia(PA): Pennsylvania Univ. P. 189.
29. Ousley M. A., Lynch J. M., Whips J. M. 1993. Effect of *Trichoderma* on plant growth ;a balance between inhibition and growth promotion. Microbial. Ecology 26: 277-285.
30. Ozbay U., Newman, S. 2004. Effect of *Trichoderma harzianum* strains to colonize tomato roots and improve transplant growth. Pakistan J. Biol. Sci. 7: 253-257.
31. Peterburgski A.V. 1968. Handbook of Agronomic Chemistry. Kolop Publishing House, Moscow, Russia.
32. Rensburg J. C., Labuschagne N., Nemic S. 2001. Occurrence of *Fusarium*-produced naphthazarins in citrus trees sensitivity of relation to citrus blight. Plant Pathology 50: 258-265.
33. Rivera M. C., Wright E. R., Lopez M. V., Garda D., Barraque M. Y. 2004. Promotion of growth and control of damping – off (*R. solani*) of greenhouse tomatoes amended with vermicompost. Phyton 04: 229-235.
34. Sahab A. F., Osman A. R., Soleman N. K., Mikhail M. S. 1985. Studies on root-rot of lupin in Egypt and its control. Egypt. J. Phytopath. 17(1): 23-35.
35. Scheuerell S. J., Mahaffe W.F. 2004. Compost tea as a container medium drench for suppressing seedling damping-off caused by *P. ultimum*. Phytopathology 94: 1156-1163.
36. Siddiqui Y., Meon S., Ismail R. Rahmani M., Ali A. 2008. Bio-efficiency of compost extracts on the wet rot incidence , morphological and physiological growth of okra (*Abelmoschus esculentus*) Sci. Horticulturae 117: 9-14.
37. Strauss J., Labuschagen N. 1999. Pathogenicity of *Fusarium solani* isolates on citrus roots and evaluation of different inoculum types. Toage Paste Plant Watenskap 9: 48-52.
38. Sylvia E. W. 2004. The effect of compost extract on the yield of strawberries and severity of *Botrytis cinerea*. J. of Sustainable Agriculture 25: 1-8
39. Verma K. S., Nartey S., Singh N. 1999. Occurrence and control of dry root rot of citrus seedlings. Plant Disease Research 14: 31-34.
40. Windham M. T., Elad Y., Baker R. 1986. A mechanism for increased plant growth induced by *Trichoderma* spp. Phytopathol. 76: 518-521.
41. Ziedan H. E. 2000. Soil treatment biofertilizers for controlling peanut rot and pod rot disease in Nobaria province. Egypt J. Phytopathol. 28: 17-26.
42. Yedidia I., Srivastva A. K., Kapulnik Y., Chet I. 2001. Effect of *T. harzianum* on microelement concentrations and increased growth of cucumber plants. Plant and Soil 235: 235-242.

2/1/2010

Scanning Electron Microcopy Studies on Mango Malformation

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Abstract: Mango malformation disease (MMD) is an economically important disease of *Mangifera indica* globally. *Fusarium subglutinans* has been associated with mango floral and vegetative malformation although confusion still remains regarding the etiology of the disease. In order to determine the *Fusarium subglutinans* penetration site, artificial inoculation of mango seedlings variety Alfonso were conducted. When soil was infested with *F. subglutinans*, the malformation was detected in the buds, three months post inoculation. Symptoms of the disease include loss of the apical dominance and swelling of vegetative buds, proliferation of leaves and flowers, phyllody and hypertrophy of panicle axes. Using scanning electron microscope (SEM), symptoms of vegetative and floral malformation appeared where mycelium of *Fusarium subglutinans* were present in the tissue at high concentrations compared to that of the untreated controls. Studies also revealed the presence of, pin-sized to large holes, disorganised cells and fungal mycelial infection at the base of the malformed buds during bud-inception stages. Moreover, *Fusarium* isolate colonized seedling root systems and became systemic, spreading to above-ground plant tissues include apical and lateral buds. *Fusarium subglutinans* proved to be the dominant fungus. [Nature and Science. 2010;8(4):122-127]. (ISSN: 1545-0740).

Key words: Egypt, *F. subglutinans,* Mango Malformation, *Mangifera indica*.

1-Introduction

Mango (*Mangifera indica* L.) is universally considered one of the most important fruit crop in tropical and subtropical areas of the world. Mango is attacked by various animate and inanimate diseases, which causes 50–80% loss in yield and was described as the abnormal inflorescence (Singh 2006). Malformation is the most notorious malady amongst the animate problems affecting both vegetative and floral parts of mango. Apical or axillary buds turn into deformed and compact structures. In affected panicles, primary and secondary axes are shortened which result in fruit abortion or no fruit setting (Ploetz., 2003). Malformation is noticed on seedlings and saplings organs. Malformation is the most threatening disease causing colossal losses every year (Iqbal *et al.*, 2006). Mango malformation disease (MMD), was first recorded in India in 1891 (Campbell and Marlatt, 1986). Symptoms of the disease include loss of the apical dominance and swelling of vegetative buds, proliferation of leaves and flowers, phyllody and hypertrophy of panicle axes. The vegetative deformation may also affect immature trees and nursery stock, which can lead to the spread of infected plants. More important, however, is the affect of malformation on fruit set: fruit in affected panicles either do not set or abort. Primary and secondary axes on affected panicles are shortened, thickened and greatly branched (Kumar *et al.*, 1993). Mango malformation has been intriguing scientists as to its cause and control for more than 100 years. The earliest hypothesis that mites caused the disorder did not last long as acaricides failed to control the problem (Yadav, 1999). The disease has been associated with physiologic disorders and hormonal imbalances (Tapan *et al.*, 2006) and attacks of an eriophyid mite, *Aceria* (*Eriophyes*) *mangifera* (Doreste, 1984). Similarly, nutrient deficiency or toxicity were discounted (Shah *et al*., 2009). However, *Fusarium subglutinans* [*Gibberella fujikuroi* var. *subglutinans*] appears to have a significant role in malformation. However, the latest citations confirm that a fungus *Fusarium subglutinans* is the cause of mango malformation (Ploetz and Gregory, 1993 and Britz *et al.*, 2002) as the causal agent of malformation. In 2002, a new species, *F. mangiferae*, was established based on nuclear and mitochondrial DNA sequences; it included strains of *F. subglutinans* from Egypt, Oman, Florida, Israel, Malaysia, and South Africa, some of which had been shown to cause MMD by artificial inoculation (Ploetz *et al.*, 2002 , Freeman, *et al.*, 2004 and Kvas *et al*., 2008). Currently, the disease has spread where mangos are grown and causes the most severe damage in Egypt (Ploetz *et al.*, 2002). The scanning electron microscope (SEM) has been shown by Scholefield (1982) and many others to be an excellent tool for presenting detail of plant structure with great depth of focus. Thus whole flowers and floral parts can be photographically presented in more detail than has been possible with light microscopy. The

present paper was further extended include scanning electron microscopy to examine the role and behavior of *F. subglutinans* in incidence of mango malformation under greenhouse.

2-Materials and Methods

2.1.Cultures

Fusarium subglutinans was evaluated for their pathogenic potential of mango seedlings cv El fonse sown in pots -30 cm diam- containing a 50:50 (v/v) mixture of vermiculite and perlite. Seedling of mango was sown into soil inoculated with 10^5 colony forming units | g of soil of pathogenic fungi. Four replications of six seedlings each were evaluated. Sterilized water was used as a control. Transplanted seedlings were monitored for development of wilt and/or foliar malformation. When malformed were developed (after 120 days), ten roots, stem and malformed pieces, each approximately 5 mm in length, from each seedling, were surface sterilized in a 10% bleach solution (0.525% aqueous sodium hypochlorite; 1 part standard household bleach in 10 parts water), rinsed in sterile, distilled water, and placed on a selective agar medium for *Fusarium* spp. (Komada 1975). Plates were incubated under diurnal cycles of cool, fluorescent light at about 24 ^{0}C for 7-10 days. Emerging fungi were compared with inoculated isolates to determine whether they were the same morphological species.

2.2. SEM procedure.

Apical, lateral buds , root and stem cross section of malformed seedlings were collected and fixed in glutaraldehyde. The samples were dehydrated using a graded ethanol series and critical point dried in CO_2. The pressure was decreased very slowly to prevent tissue damage. Samples were examined by Scanning Electron Microscope using an accelerating voltage of 6 kV and a spot size of 125 nm. With some specimens, up to 30 min observation was possible. All images were computer processed.

3. Results and Discussion

The present studies were aimed to determine the fungus, *Fusarium subglutinans* associated with malformed tissues of inoculated mango seedlings growing under greenhouse conditions. *In vitro*, studies with light and SEM microscopes showed fungal mycelial infection at the base of the fully swollen malformed buds (Fig. 1 A, B and C). As the fungus could not be identified with scanning electron micrographs, healthy and mal formed tissues were cultured *in vitro*. When soil was infested with the fungus, 12 weeks post inoculation, strong colonization of micro and macroconidia of *F. subglutinans* was observed on the malformed lateral and apical vegetative buds (Fig. 2). Also, symptoms of floral malformation appeared where mycelium of *Fusarium subglutinans* were present in the tissue at high concentrations (Fig. 3). White cottony mycelial growth of *Fusarium subglutinans* formed on the fourth day from malformed tissues only. No such mycelial growth was observed in healthy buds. SEM studies also revealed the presence of damage in malformed tissue caused by the fungus, pin-sized to large holes, disorganised cells and fungal mycelial infection at the base of the malformed buds during bud-inception stages. At the same time, morphological and microscopical examination , using SEM in inoculated seedlings with *F. subglutinans* revealed the presence of fungal mycelial and micro and macroconidia infection in the stem and root vessels (Fig. 4). Percent colonization of *Fusarium* was significantly higher in either stem or root sections. In this study we have shown that *F. subglutinans* proved to be the dominant fungus infecting majority of the tissues. Little is know about the epidemiology of the disease, dissemination of conidia, location of infection sites, modes of infection and colonization of plant tissue. This data indicated that the primarily infection via root, completely colonized the seedling root systems and became systemic, spreading to apical plant tissues (apical buds). Apart from competition for nutrients, the fungus may release secondary metabolites, which could create further hormonal imbalance and inhibit the normal growth of the meristematic tissue of the buds (Tapan, *et al.*, 2006). The second infection for long distance dispersal of the pathogen is hypothesized to be via infected nursery stock or by the mango bud mite.

Among other possibilities this may suggest that the fungus, which is closer to vascular channels of the mother plant, competes for the nutrients by acting as a more powerful sink than the buds of the malformed inflorescence and could be a reason for the low uptake of assimilates by the malformed buds as observed in tracer studies (Freeman, *et al*., 2004) . The fungus *Fusarium monilifome* var. *subglutinans* was isolated from malformed parts of mango and its pathogenicity was also proved by demonstrating identical etiology for vegetative and floral malformation, but foliar applications with different fungicides failed in checking malformation (Chakrabarti and Ghosal, 1985) probably due to the fungi is systemic. The results of these studies will be helpful for future statistics, management, forecasting and experimental designing.

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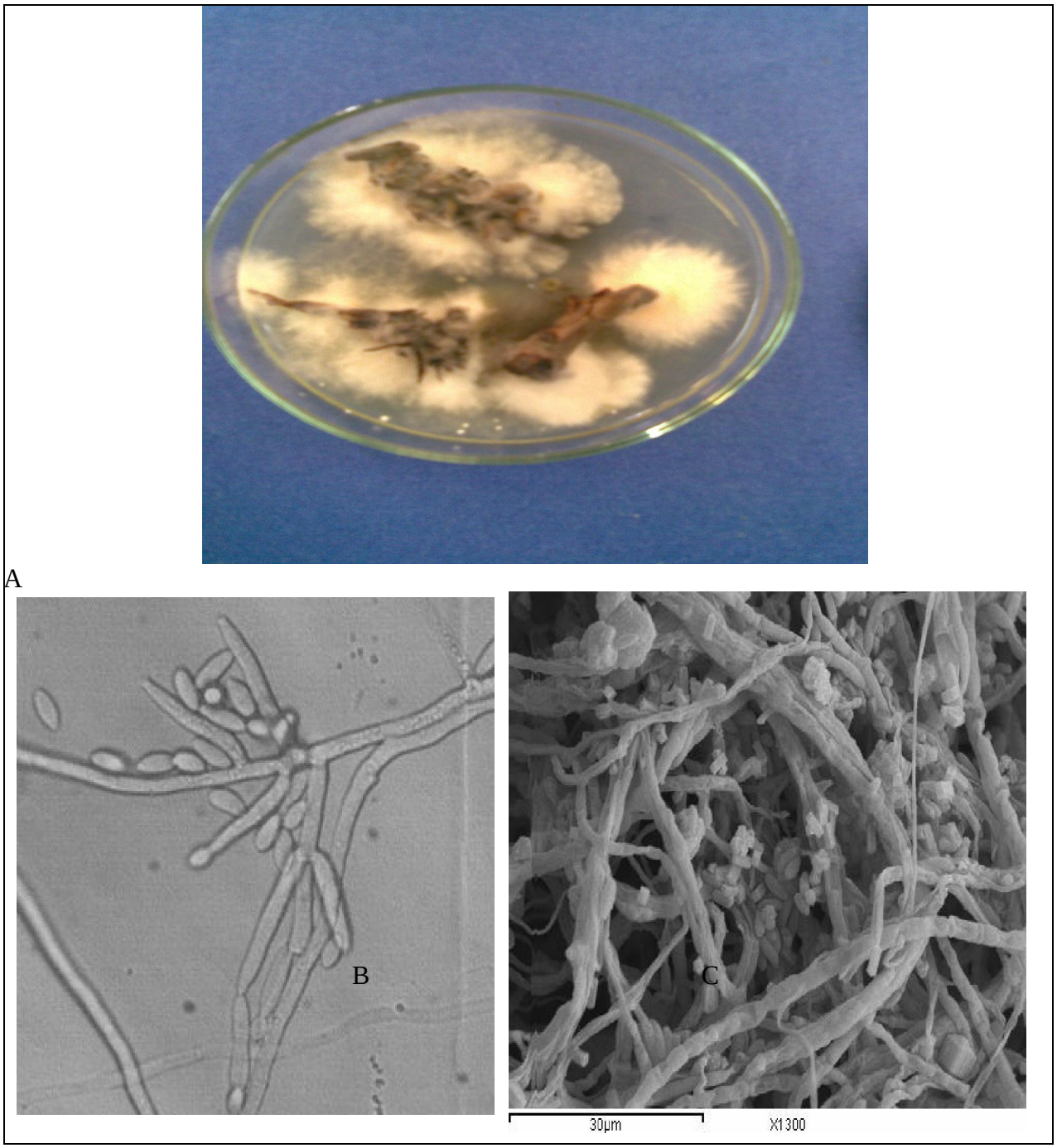
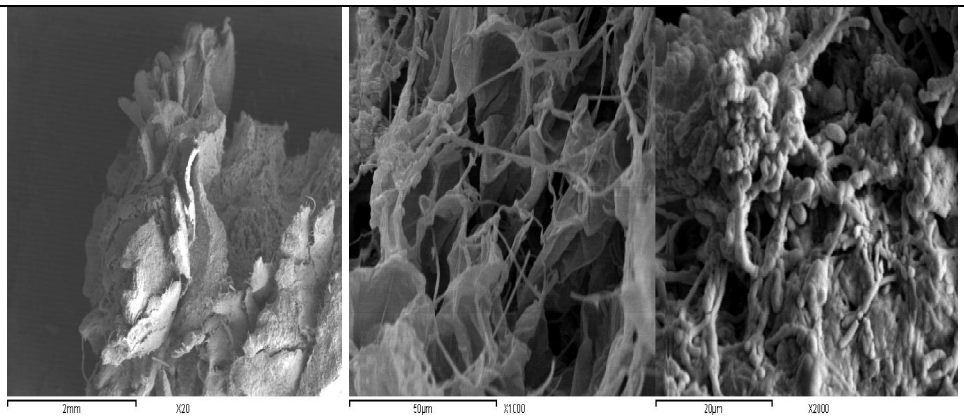
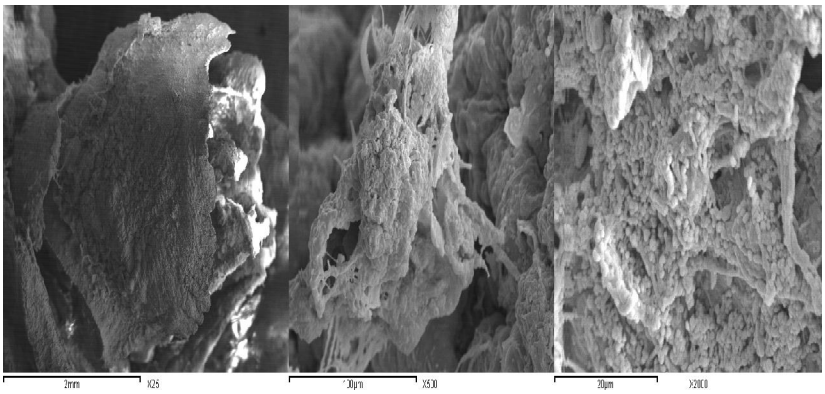


Figure 1. A) Isolation of *Fusarium subglutinans* from buds and stem
B) *In vitro,* Light microscopy of *Fusarium subglutinans*
C) *In vitro,* SEM of *Fusarium subglutinans*

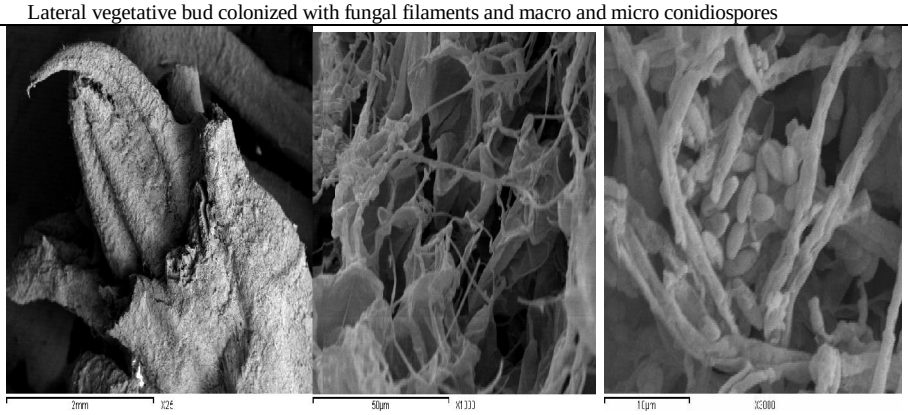


A

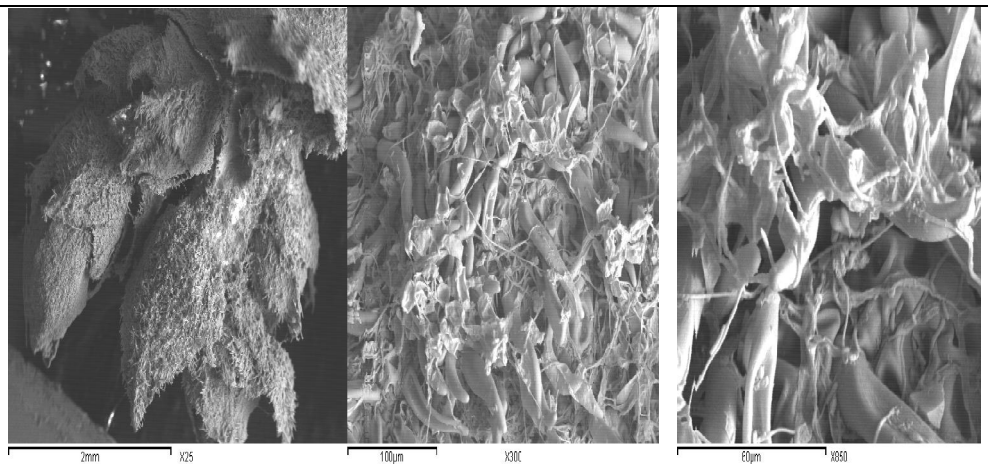


B

Figure 2 . Scanning Electron Microscope of mango tissue seedlings cv.Al-Fonso inoculated with *F. subglutinans*
A) Apical vegetative bud colonized with fungal filaments and macro and micro conidiospores
B) Lateral vegetative bud colonized with fungal filaments and macro and micro conidiospores



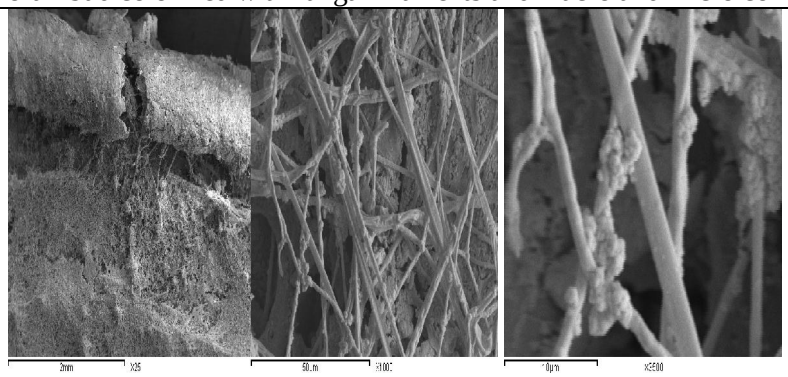
A



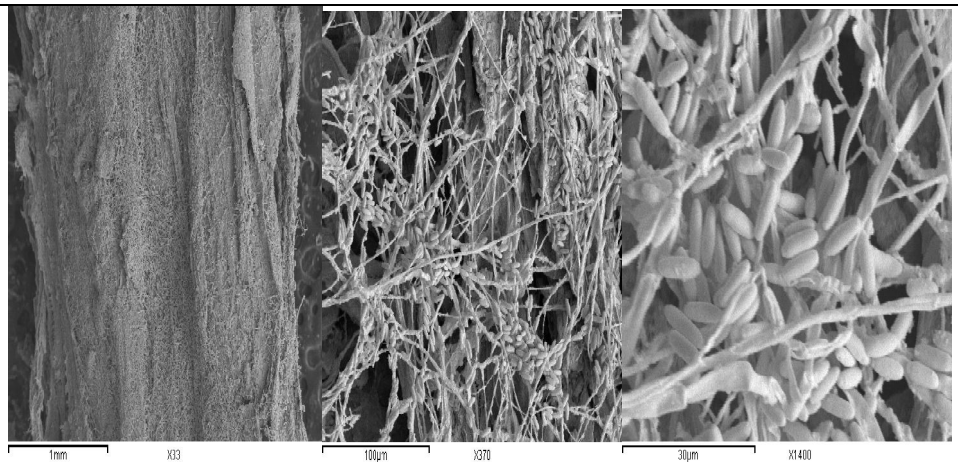
B

Figure 3 . Scanning Electron Microscope of mango tissue seedlings cv.Al-Fonso inoculated wit *F. subglutinans*

A) Apical floral bud colonized with fungal filaments and macro and micro conidiospores
B)Lateral floral bud colonized with fungal filaments and macro and micro conidiospores



A



B

Figure 4 .Scanning Electron Microscope of mango tissue seedlings cv.Al-Fonso inoculated wit *F. subglutinans*

A) Stem vessels colonized with fungal filaments and macro and microconidiospores
B)Tip root colonized with fungal filaments and macro and micro conidiospores

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References

[1] Britz H, Steenkamp ET, Coutinho, TA, Wingfield BD, Marasas WFO , Wingfield, MJ . Two new species of *Fusarium* section Liseola associated with mango malformation. Mycologia, 2002; 94(4): 722-730.
[2] Campbell, CW , Marlatt R .Current status of mango malformation disease in Florida. Proc. Inter Am. Soc. Trop. Hortic. 1986; 30: 223–6
[3] Chakrabarti DK , Ghosal, S . Effect of *Fusarium monilifoomze var. subglutinans* infection on mangiferin production in the twigs of Man&era in&a L. J. Phytopathol. 1985; 113, 47-50.
[4] Doreste SE. Información sobre el eriófido del mango, *Eriophyes mangiferae* (Sayed), en Venezuela. Rev. Facultad Agrociencia (Universidad Central de Venezuela) 1984; 13:91-100.
[5]Freeman S, Klein-Gueta D, Korolev N , Sztejnberg A. Epidemiology and survival of *Fusarium mangiferae*, the causal agent of mango malformation disease, Acta Hort. 2004; 645, 487-491.
[6] Kumar J, Singh U S, Beniwal SPS , Mango malformation: One hundred years of research. Annu. Rev. Phytopathology 1993; l.31:217-232.
[7]Kvas M, Steenkamp E, Al-Adway A, Deadman M, Al-Jahwari A, Marasas W, Wingfield B, Ploetz R , Wingfielf M .*Fusarium mangiferae* associated with mango malformation in the Sultanate of Oman. European J. Plant Pathology 2008; 121(2): 195-199.
[8] Iqbal Z, Ahmad A, Saleem A. Role of *Fusarium mangiferae* in causation of mango malformation disease. J.. of Research Sc.., 2006; 17(1), 09-14
[9] Ploetz R C. Diseases of mango. Pages 327-363 in: Diseases of Tropical Fruit Crops. R. C. Ploetz, ed. CABI Publishing, Oxford, UK. 2003.
[10] Ploetz R, Zheng QI, Vázquez Á, Sattar MAA . Current status and impact of mango malformation in Egypt A *Fusarium* sp. different from *Fusarium oxysporum* and *F. mangiferae* is associated with mango malformation in Michoacán, Mexico**.** International Journal of Pest Management. 2002; 48:(4) 279-285.
[11] Scholefield P. Scanning electron microscope study of flowers of avocado, litchi, macadamia and mango. *Scientia Horticulturae*, 1982; 16 :263-272 263
[12] Shah 1, Iqbal Z, Ahma K, Khan Z, Shaheen M, Arshad M, Ahmad S, Sher M, Valeem, E. The extent of micro minerals in healthy and malformed organs of mango. Pak. J. Bot., 412009 ; (6): 2817-2820, 2009.
[13] Singh VK . Physiological and biochemical changes with special reference to mangiferin and oxidative enzymes level in malformation resistant and susceptible cultivars of mango (*Mangifera indica* L.). Scientia Horticulturae 2006; 108 : 43–48.
[14] Tapan N, Anitha G, Gurdeep B S, Alok S , Pant R . Mango (*Mangifera indica* L.) malformation : Role of stress ethylene and cyanide. Physiology and Molecular Biology of Plants. 2006; 12 (2): 163-165.
[15] Usha K, Goswami AM, Sharma HC, Singh B and Pande PC. Scanning electron microscopic studies on floral malformation in mango. Scientia Horticulturae 1997; 71 : 127-1
[16] Yadav V K .Mango malformation - a review. J. Appl. Hort. 1999; 1(1)70-78.

2/3/2010

Epidemiology and the Association of the *Fusarium* Species with the Mango Malformation Disease in Egypt

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Abstract: Mango malformation disease (MMD) is an economically important disease of *Mangifera indica* globally. This disease is caused by a complex of fungal pathogens, of which various *Fusarium* spp. dominate. This study was conducted to assess the epidemiology and its pathogenesis of mango malformation disease in Egypt. In three main Governorates of mango production, El Giza, Esamalîa and El-Bohera, disease incidence reached up to 80%. Maximum infection of traditional cultivars was observed in Hindi Sennara, Alfonso, Timour and Zebda. Exotic Tomy , Keet and Kent cultivars appeared to be moderate infection . Nine additional taxa have been isolated, i.e., *F. subglutinans, F. oxysporum, F. sterilihyphosum, F. proliferatum, F. culmorum, F. nygamai, F. pseudonygamai, F. nelsonii* and *F. verticilioides* from Egypt. *Fusarium subglutinans* proved to have the high frequency in all mango cultivars in tested area, while, *F. oxysporum, F. sterilihyphosum, F. proliferatum* frequently were less. To date, Koch's postulates have been applied with *Fusarium* for their pathogenic potential on mango cultivars seedlings under greenhouse conditions. Apparently, not all isolates of this *Fusarium* species are equally virulent on mango seedlings. *Fusarium subglutinans* proved to be the dominant fungus in all varieties. At the same time, *F. oxysporum , F. sterilihyphosum, F. proliferatum*, displayed also moderate virulence. Moreover, isolates colonized seedling root systems and became systemic, spreading to above-ground plant tissues include apical and lateral buds. *Fusarium subglutinans* proved to be the dominant fungus. Complex Strains of *F. subglutinans, F. oxysporum, F. sterilihyphosum* and *F. proliferatum* induced typical malformation symptoms on mango seedlings and trees in Egypt [Nature and Science. 2010;8(4):128-135]. (ISSN: 1545-0740).

Key words: Egypt, *F. subglutinans, F.oxysporum , Fusarium sterilihyphosum* and *F. proliferatu*, Mango Malformation, *Mangifera indica*.

1. Introduction

Mango (*Mangifera indica* L.) is universally considered one of the most important fruit crop in tropical and subtropical areas of the world. Major producers include India, Pakistan, Brazil, Australia, South Africa, Egypt, and USA (Ploetz *et al*., 2002). Egypt produce 232,000 tone of mangos annually and export moderate amounts (1500 tones) to 20 countries in the near East and Europe . Mango suffers from several diseases at all stages of its life (Ploetz., 2003). Malformation is the most threatening disease causing colossal losses every year (Iqbal *et al.*, 2006). Mango malformation disease (MMD), was first recorded in India in 1891. It is found elsewhere in Asia (Israel, Malaysia, and Pakistan), Africa (Egypt, South Africa, Sudan, Swaziland, and Uganda), and the Americas (Brazil, El-Salvador, Mexico, the United States, and Venezuela) (Marasas *et al.*, 2006). The disease is endemic as a tree once infected never recovered, mango malformation can be classified into vegetative and floral malformation. (Zheng and Ploetz 2002). Three main symptoms of this phenomenon were recorded in Egypt i.e., malformed and stunted growth of seedlings in the nursery stage, vegetative growth malformation and inflorescence malformation in the bearing trees. The earliest hypothesis that mites caused the disorder did not last long as acaricides failed to control the problem (Yadav, 1999). Symptoms of the disease include loss of the apical dominance and swelling of vegetative buds, proliferation of leaves and flowers, phyllody and hypertrophy of panicle axes. The vegetative deformation may also affect immature trees and nursery stock, which can lead to the spread of infected plants. More important, however, is the affect of malformation on fruit set: fruit in affected panicles either do not set or abort. Primary and secondary axes on affected panicles are shortened, thickened and greatly branched (Kumar *et al*., 1993). The disease has been associated with physiologic disorders and hormonal imbalances (Singh *et al.*, 1991 and Tapan *et al.*, 2006) and attacks of an eriophyid mite, *Aceria*

(*Eriophyes*) *mangifera* (Doreste, 1984). However, *Fusarium subglutinans* [*Gibberella fujikuroi var. subglutinans*] appears to have a significant role in malformation. Koch's postulates have only been completed for *Fusarium subglutinans* and *F. oxysporum* (Covarrubias, 1989) as the causal agents of malformation. Yet some controversy remains regarding species identification and the inoculation methods used . In 2002, a new species, *F. mangiferae*, was established based on nuclear and mitochondrial DNA sequences; it included strains of *F. subglutinans* from Egypt, Oman, Florida, Israel, Malaysia, and South Africa, some of which had been shown to cause MMD by artificial inoculation (Ploetz *et al.*, 2002 and Kvas *et al*., 2008). Three or more additional taxa have been associated with MMD: *F. sterilihyphosum* from Brazil and South Africa, and *Fusarium* sp. nov. and *F. proliferatum* (teleomorph: *Gibberella intermedia*) from Malaysia (Marasas, *et al*., 2006 and Alvarado *et al*., 2006). Currently, the disease has spread where mangos are grown and causes the most severe damage in Egypt (Ploetz *et al.*, 2002 and Haggag, Wafaa and Abd Wahab, 2009).

The present study had two objectives: (i) to determine the frequency of different fungi associated with malformed tissues and establish the cause of mango malformation in Egypt , and (ii) to evaluate pathogenic potential of selected *Fusarium* isolates in mango malformed diseased plants. Tests were conducted under controlled conditions on seedlings in a greenhouse.

2.Materials and Methods

2.1. Disease survey.

A disease survey in El-Behera, El-Giza and Esamali Governorates was performed on complete differentiation of healthy and malformed plants during the vegetative and flowering growth cycle (December to July) of 2008 season. Six traditional varieties (Hindi Sennara, Alfonso, Timour , Zebda, Awais and Dabsha) and three exotic verities (Keet, Kent and Tomy) were kept under the study. Each location contributed five panicles along with 6-8 cm shoot portion representing one of each variety. From each of the 5 districts, 10 samples of every cultivar were collected. Tissue pieces 5 mm long, were surface sterilized in 1% NaOCl solution for 2 minutes and placed onto Potato Dextrose agar (PDA) medium in 9 cm diameter Petri plates. The plates were kept in a incubator at 25^0C under fluorescent illumination to give a 12 hour photoperiod to ensure maximum macroconidial production. After 6-7 days of incubation, the isolated fungi were identified on the basis of morphological characters (Summerell *et al*. 2003).

Disease assessment.

Three branches at the four cardinal points were labeled per tree canopy. During each assessment, the total number of healthy and diseased shoots (vegetative and floral) were counted on each branch and averaged over the three branches per tree. The disease progress was determined as the accumulated proportion of diseased shoots per tree (*Yic*) corrected for host growth. At each time, (*i), Yic* was calculated as: $Yic = Yi/N$, in which, *Yi* is the accumulated number of diseased shoots at time *I* and *N* is the total number of weekly in the vegetative and floral stage (January to May) of mango growth .

2.2.Disease progress.

The experiment was conducted during 2008 growing cycles in a 6-year-old commercial orchard of the mango cultivars in the Noubaria station, Behera Govarnorate . The soil was -sandy, lightly compacted one . Trees averaged 3m height, with a mean trunk diameter of 0.5 m and a spacing of 5 m between tree rows. The trees had an average of 25 floral and vegetative deformations at the beginning of the experiment. A randomized block design was used. The experimental unit was a tree, and the same trees were used.

2.3.Pathogenicity.

Isolates of *Fusarium* species were tested for their ability to cause malformation by inculcation of mango healthy plants. Two sets of pathogenicity experiments were done in 2008 and 2009; the first set ; mango seedling cv. Seddek (two years old) was inoculated with 10^5 colony forming units of *Fusarium* spp either as apical buds injection or as inoculated soil . The second set seedlings of mango cvs. Timor, Seddek, Awes, Zebdia, El fonse, Fagar Kelan, Handi Besenra, Keet, Kant and Tomy were sown into soil inoculated with 10^5 colony forming units | g of soil inoculated with pathogenic fungi. For each experiment and tested isolate, four replications of six seedlings each were evaluated. Sterilized water was used as a control. Transplanted seedlings were monitored for development of malformation. At the end of the experiment (120 days), all surviving seedlings were examined for apical disease symptoms. Data were recorded on symptoms manifestation as diseases incidence and severity (from 1-4 scale) . Post inoculation colonization by *Fusarium i*solates in inoculated plants was determined by reisolation. Root , stem and malformed pieces, each approximately 5 mm in length, from each seedling, were surface sterilized in a 10% bleach solution (0.525% aqueous sodium hypochlorite; 1 part standard household bleach in 10 parts water), rinsed in sterile, distilled water, and placed on a selective agar medium for *Fusarium* spp. (Komada 1975). Plates were incubated under diurnal

cycles of cool, fluorescent light at about 24 ^{0}C fo2r 7-10 days. Emerging fungi were compared with inoculated isolates to determine whether they were the same morphological species.

Statistical analysis.

The obtained data were statistically computed using the software SPSS for Windows (release 9.0.0, Dec. 18, 1998, standard version, SPSS Inc.). All treatments in the previous experiments consisted of three or four replicates.

3. Results and Discussion

3.1. Disease survey.

A disease survey in El-Behera, Giza and Ismailia Governorates was performed during the growing period as a preliminary study. Mean percentages of the disease incidence in cultivars were calculated on vegetative and blossom clusters (Table 1). Many isolates of *Fusarium* were obtained during routine isolations from seedlings, large trees exhibiting malformation disease symptoms. Data indicated that nine additional taxa were associated with MMD inculde *F. subglutinans, F. oxysporum, F. sterilihyphosum, F. proliferatum, F. culmorum, F. nygamai, F. pseudonygamai*, *F.nelsonii* and *F. verticilioides*. *F. subglutinans* proved high frequancy from all cultivars and location , ranging from 94.7to 84.5%. *F. oxysporium , F. sterilihyphosum* and *F. proliferatum* solates, exhibited moderate frequency, ranging from 11.5 to 1.56. The other isolates displayed very low in frequency from some cultivars , ranging from 0.23 to 0.76 %. Maximum infection of traditional cultivars was observed in Timour, Zebda, Awais and Alfonso. Exotic Tomy , Keet and Kent cultivars appeared to be the moderate infection varieties to all *Fusarium* spp. Mango malformation disease (MMD) is a serious disease in many areas where this important crop is grown. This disease causes abnormal development of vegetative shoots and inflorescences (Kumar *et al* 1993 and Yadav, 1999). Floral malformation is the most prominent symptom and is characterized by abnormal, thick and fleshy panicles (Kumar *et al* 1993). Affected panicles bear no fruit, resulting in significant economic losses (Kumar *et al* 1993 and Ploetz, 2003). Mango malformation disease was first described in India in 1891 (Marasas *et al.*, 2006), and has since been shown to occur multiple location in Asia, Africa, and the Americas. Despite this fact, relatively little is known about the disease. The recent discovery that several *Fusarium* spp. are associated with MMD is intriguing. At least four taxa of *Fusarium* have been associated with MMD worldwide, including *Fusarium subglutinans* (previously *F. mangiferae*) in many growing regions, *Fusarium sterilihyphosum* in Brazil and South Africa, and *Fusarium* sp. nov. and *F. proliferatum* (teleomorph: *Gibberella intermedia*) from Malaysia. To data, only *F. subglutinans* has been reported in Egypt (Ploetz *et al.,* 2002). The origins of the associated *Fusarium* species are unknown. Thus, the present study were to determine the frequency of different fungi in malformed tissues and establish the cause of mango malformation in Egypt and determine of fungi associated with malformed tissues of mango on different traditional and exotic cultivars. Complete resistance has not been observed in anyone variety . Nine unique *Fusarium* spp. were isolated, identified and named *F. Subglutinans, F. oxysporium, F. sterilihyphosum, F. proliferatum, F. culmorum, F. nygamai, F. pseudonygamai*, *F. nelsonii* and *F. verticilioides*. We have routinely isolated high levels of *F. Subglutinans* from malformed disease mango trees . This species is most often isolated from the interior of stems, branches, and vegetative and blossom of trees displaying malformation symptoms. The infection frequency (within tissue infection) of *F. subglutinans* confirms its role in causation of malformation symptoms. However, *Fusarium subglutinans* appears to have a significant role in malformation. Symptoms of vegetative and floral malformation appeared where mycelium of *Fusarium* species were present in the tissue at high concentrations. Since, *F. oxysporum , Fusarium sterilihyphosum* and *F. proliferatum* solates, exhibited moderate frequency from most of cultivars and location. *Fusarium subglutinans* was commonly isolated from mango trees displaying malformation symptoms (Ploetz and Gregory, 1993, Freeman *et al.*, 2004, Iqbal *et al*., 2006, Marasas *et al.*, 2006). Reports that *F. oxysporum* Schlecht emend. Snyder & Hansen causes MMD indicate that a new, chlamydospore-producing taxon is involved (Marasas, *et al*., 2006). (Marasas, *et al*., 2006). *Fusarium sterilihyphosum*, on the other hand, has been isolated from malformed mango tissue in South Africa, meanwhile, *F. proliferatum* are associated with MMD in Malaysia (Marasas, *et al*., 2006 and Alvarado *et al*., 2006). Other numbers of *Fusarium* associated with either healthy or diseased plants are likely saprophytic; a much smaller number are capable of eliciting disease.

3.2. Disease progress.

Data in Fig. (1 and 2) indicated that symptoms of malformation were initially observed on January on vegetative stage and continues on blossom clusters of March and maximum symptoms was appeared in March and decline in May. Maximum disease severity was observed in the selected orchards. All tested cultivars were susceptible to infection with malformation expressed as disease progress. Traditional cultivars i.e. Al-Fonso, Timour, Awais and Zebda were the most susceptible cultivars, as they gave

the highest percentage of infection. Meanwhile, Dabsha and Dabsha were the moderate resistance. Data also show that in all exotic cultivars as Tomy , Keet and Kent appeared to be the least infection . In general, the highest disease incidence was found during the warm season February to April. Seasonal variations in the occurrence and severity of problem correlate with ambient temperature at flowering (Majumdar and Sinha, 1972). In Egypt panicles appearing on spring shoots are most severely affected (Shawky *et al.*, 1980). In Florida the heaviest infection occurs under unusually wet conditions (Campbell and Marlatt, 1986). The severity of the disease varies from variety to variety and tree to tree in the same variety.

3.3. Pathogenicty

Eight fungi viz. *F. subglutinans, F. solani, F. oxyspoum, F. sterilihyphosum, F. proliferatum, F. moniliforme, F.avena* and *F.chlamydspore* were tested using susceptible Seddek cultivar as apical injection or inoculated soil (Table 2). Data pertaining to artificial inoculations revealed that effort to produce disease by spores injection or soil inoculation. Soil inoculation was successful method. Four *Fusarium subglutinans* proved to be the dominant fungus with 100% sample's infection in inoculated soil. Fungi *F. oxysporum, F. sterilihyphosum* and *F.proliferatum* showed moderate infection in induced typical malformation symptoms in inoculated mango seedling and were re-isolated . Other *Fusarium* spp. give grown and root rots symptoms.

Four fungi viz. *F. subglutinans, F.oxysporum, F. sterilihyphosum* and *F.proliferatum* were elected for the other test study using eleven cultivars (Table 3). Significant differences (P=0. 05) were found among different isolates for the infection. *Fusarium subglutinans* proved to be the dominant fungus with 100% sample's infection in the seven local cultivars include Al-Fonso, Hindi Sennara, Seddek, Timour, Dabcha, Zebda, Ewais and Fagrkelan. Exotic keet, Tomy and kent appeared to be the moderate infection varieties giving 92.0 , 96.0 and 93.7% tissue infection, respectively. Other fungi like *F. oxysporum* and *F. proliferatum* showed moderate infection level. One tested *F. sterilihyphosum* isolate was weakly virulent.

Data in Table 3 indicated that *F. subglutinans, F.oxysporum, F. sterilihyphosum* and *F. proliferatum* were found to be associated with malformed parts as well as colonized root stem. Maximum recovery (100%) was exhibited by *F. subglutinans* in all local cultivars include Al-Fonso, Hindi Sennara, Seddek, Timour, Dabcha, Zebda, Ewais and Fagrkelan. Moderate recovary was recorded in exotic keet, Tomy and kent .Other fungi like *F.oxysporum and F. sterilihyphosum* showed moderate recovery level from either root or apical buds. *F. proliferatum* showed the least one. On healthy mango seedlings, a small conical apical bud gradually attaining its normal shape .

We planed to re-test these isolates in subsequent greenhouse inoculation trials to confirm their pathogenic behavior (Table 4). *Fusarium* isolates obtained from diseased mango trees, varied widely in their virulence on inoculated mango seedlings under greenhouse conditions. Isolate of *F. subglutinans* was pathogenic and the other tested isolate was moderately virulent on inoculated seedlings, that caused malformation on all cultivars.

In this study we have shown that at least four distinct *Fusarium* spp. are associated with mango malformation symptoms. *F. subglutinans* proved to be the dominant fungus infecting majority of the tissues. *Fusarium mangiferae* has been isolated from mango malformation symptoms in various geographical areas, such as South Africa, Florida, Egypt, India, Israel and Malaysia (Ploetz *et al*., 2002 and Kvas *et al*., 2008). The infection frequency and disease incidence of other fungi remained much less. Complex Strains of *F. subglutinans, F. oxysporum, F. sterilihyphosum* and *F. proliferatum* induced typical malformation symptoms on mango seedlings and trees in Egypt . Also, this results of this study, together with those of Steenkamp *et al* (2000), have shown that mango malformation in South Africa is associated with two distinct species, *F. subglutinans var mangiferae* and *F. sterilihyphosum*. The pathogenic interaction with floral buds resulted in high incidences of malformation which started early in the floral season, extended up to February and re-established in July. The results of these studies will be helpful for future statistics, management, forecasting and experimental designing.

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Table 1. Fungi associated with malformed parts of mango in Egypt.

Cultivars	El-Bohera		Giza		Ismalia	
	Fungi isolated	(%) Infection frequency	Fungi isolated	(%) Infection frequency	Fungi isolated	(%) Infection frequency
Hindi Sennara	*F. subglutinans* *F. oxysporum* *F. sterilihyphosum* *F .proliferatum* *F. culmorum* *F. nygamai* *F .pseudonygamai*	84.7 6.80 3.52 2.43 0.34 0.46 0.25	*F. subglutinans* *F. oxysporum* *F. sterilihyphosum* *F.proliferatum* *F. culmorum* *F.nygamai* *F.pseudonygamai*	88.5 5.43 3.72 3.23 0.34 0.36 0.36	*F. subglutinans* *F.oxysporum* *F. sterilihyphosum* *F.proliferatum* *F. culmorum*	88.4 4.06 3.33 2.35 0.37
El-Founso	*F. subglutinans* *F. sterilihyphosum* *F. proliferatum* *F.oxysporum* *F.nygamai* *F.pseudonygamai*	89.4 4.42 3.34 2.54 0.30 0.54	ND*		*F. subglutinans* *F.oxysporum* *F.proliferatum* *F. sterilihyphosum*	90.0 4.24 3.23 2.54
Sadeka	*F. subglutinans* *F.proliferatum* *F.oxysporum* *F.nygamai* *F.nelsonii*	88.5 7.76 3.43 0.32 0.54	*F. subglutinans* *F.oxysporum* *F.proliferatum* *F.nygamai*	*87.5* *8.87* *3.34* *0.98*	*F. subglutinans* *F.oxysporum* *F.nygamai* *F.ndsonii*	87.7 6.87 0.65 0.13
Timour	*F. subglutinans* *F.oxysporum* *F.nygamai* *F. sterilihyphosum* *F.proliferatum* *F. culmorum*	91.7 2.43 0.67 1.56 1.56 0.45	*F. subglutinans* *F.oxysporum* *F.nygamai* *F. sterilihyphosum* *F.nelsonii*	*93.6* *2.65* *0.76* *1.65* *0.76*	*F. subglutinans* *F.oxysporum* *F.nygamai* *F. culmorum* *F. proliferatum* *F. sterilihyphosum* *F.ndsonii*	93.6 2.54 0.34 0.65 2.65 1.87 0.23
Dabcha	*F. subglutinans* *F. proliferatum* *F.nygamai* *F.acuminatum*	*94.8* *4.76* *0.54* *0.27*	*F. subglutinans* *F. proliferatum* *F.nygamai* *F.oxsporum*	*92.6* *4.87* *3.53* *0.76*	*F. subglutinans* *F.nygamai*	*95.8* *0.65*
Zebda	*F. subglutinans* *F.oxysporum* *F. proliferatum* *F. sterilihyphosum*	*91.8* *3.54* *2.43* *2.43*	*F. subglutinans* *F.oxysporium* *F. proliferatum* *F. sterilihyphosum*	*92.4* *4.34* *2.42* *2.56*	*F. subglutinans* *F.oxysporium* *F. proliferatum* *F. sterilihyphosum* *F.verticilioides*	*91.3* *2.43* *2.54* *2.54* *0.24*
Ewais	*F. subglutinans* *F. sterilihyphosum* *F.oxysporum* *F.proliferatum* *F.nygamai*	*91.5* *3.66* *3.33* *2.66* *0.75*	*F. subglutinans* *F.pseudonygamai* *F. sterilihyphosum* *F.nygamai* *F.nelsonii*	*94.4* *2.34* *3.54* *0.54* *0.43*	*F. subglutinans* *F.oxysporum* *F. sterilihyphosum* *F.proliferatum* *F.verticilioides*	*91.3* *4.32* *3.43* *3.12* *0.32*
Fagrkelan	*F. subglutinans* *F.oxysporium* *F.nygamai*	*93.7* *5.65* *0.46*	*F. subglutinans* *F.oxysporum* *F.nygamai*	*94.7* *5.76* *0.48*	*F. subglutinans* *F.oxysporium* *F.nygamai*	*94.6* *4.76* *0.45*
Keet	*F. subglutinans* *F. sterilihyphosum* *F. proliferatum* *F.nygamai*	*88.5* *5.0* *5.5* *0.54*	*ND*		*F. subglutinans* *F. sterilihyphosum* *F. proliferatum* *F.nygamai*	*89.1* *6.9* *5.5* *0.23*
Tomy	*F. subglutinans* *F. proliferatum* *F.nelsonii*	*84.5* *10.5* *0.32*	*ND*		*F. subglutinans* *F.proliferatum* *F.ndsonii*	*88.5* *11.5* *0.24*

*ND: Not detected

Figure 1**.** Mango malformation on blossom clusters.

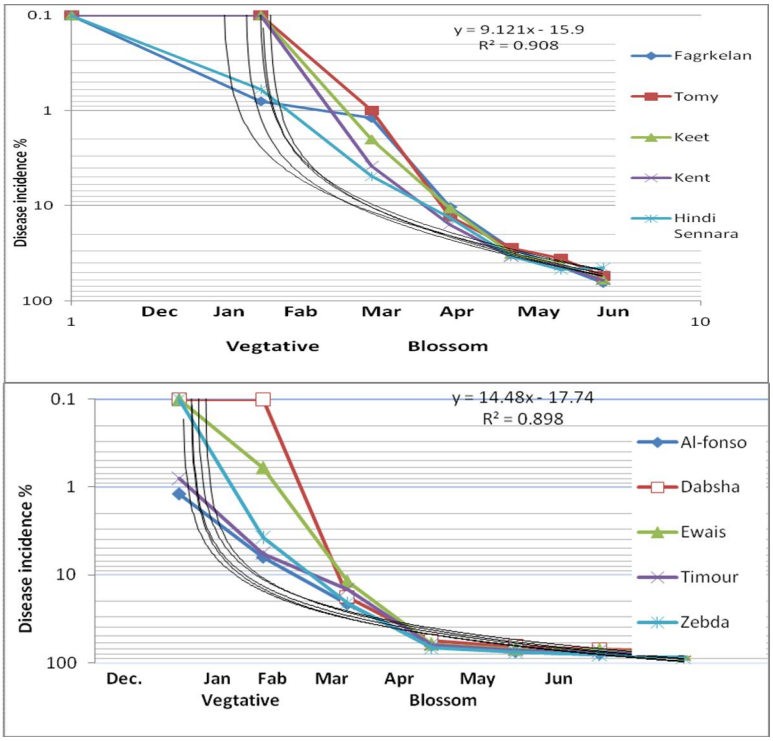

Figure 2. Disease progress curves of malformation in vegetative and floral shoots of mango in El Bohera Governorate

Table 2. Comparative virulence of selected *Fusarium* isolates on inoculated mango cv. Seddek seedlings

Treatment	Infested soil		Injection buds	
	Disease incidence %	Disease severity	Disease incidence %	Disease severity
F. subglutinans	100.0	4.0	75.0	3.3
F. solani	0.0	0.0	0.0	0.0
F. oxyspoum	50.0	1.3	25.0	0.3
F. sterilihyphosum	50.0	2.3	25.0	2.0
F. proliferatum	50.0	1.6	25.0	1.3
F.moniliforme	0.0	0.0	0.0	0.0
F.avena	0.0	0.0	0.0	0.0
F.chlamydspore	0.0	0.0	0.0	0.0
LSD	25.0	0.5.	12.0	0.5

Table 3. Comparative virulence of selected *Fusarium* isolates on inoculated mango cultivars seedlings

Cultivars	% Infection				Mean
	F. subglutinans	*F. oxyspoum*	*F. sterilihyphosum*	*F. proliferatum*	
Hindi Sennara	100.0	4.56	1.76	3.76	27.5
Seddek	100.0	9.58	3.87	8.56	30.5
Timour	100.0	11.4	5.87	9.54	31.7
Dabcha	100.0	8.54	6.87	6.34	30.4
Zebda	100.0	13.7	8.56	11.7	33.4
Ewais	100.0	10. 5	7.26	9.65	31.1
Fagrkelan	100.0	8.08	5.26	7.98	30.3
Al Fonso	100.0	13.5	9.65	9.45	34.6
Keet	90.0	2.61	1.34	1.65	23.9
Tomy	96.0	0.94	0.00	0.00	24.2
Kent	93.7	0.75	0.00	0.00	23.6
Mean	97.9	7.96	4.77	5.91	
LSD	2.43	2.95	2.54	2.76	

Table 4. Percent recovery of *Fusarium sp.* from inoculated mango cultivars.

Cultivars	*F. subglutinans*		*F.oxysporum*		*F. sterilihyphosum*		*F. proliferatum*	
	Root colonized	Apical colonized	Root colonized	Apical colonized	Root colonized	Apical colonized	Root colonized	Apical colonized
Hindi Sennara	100.0	100.0	2.36	3.65	2.06	1.26	3.53	2.13
Seddek	100.0	100.0	5.27	6.76	5.26	4.27	4.08	3.00
Timour	100.0	100.0	6.07	5.43	8.24	5.00	5.04	2.04
Dabcha	100.0	100.0	8.07	6.76	4.04	6.17	4.24	2.24
Zebda	100.0	100.0	6.96	5.43	7.17	4.66	3.17	2.11
Ewais	100.0	100.0	6.46	8.45	7.55	3.26	3.25	2.25
Fagrkelan	100.0	100.0	4.74	6.87	4.58	3.64	1.08	0.58
Al Fonso	100.0	100.0	7.54	6.98	6.98	5.34	3.87	2.43
Keet	66.6	80.0	1.04	1.70	0.75	0.95	0.90	0.34
Tomy	63.0	76.0	1.95	1.45	0.50	0.45	0.41	0.64
Kent	83.7	80.0	1.75	1.65	0.84	0.95	0.50	0.45
LSD	3.67	4.23	0.89	0.95	0.92	0.78	0.75	0.45

References

[1] Alvarado R G, Fernández-Pavía SP, Ploetz RC. *Fusarium* sp. characterization causing mango malformation in Michoacan Mexico. Phytopathology 2006; 96, S99.

[2] Campbell CW, Marlatt R. Current status of mango malformation disease in Florida. Proc. Inter Am. Soc. Trop. Hortic., (1986;30: 223–6

[3] Covarrubias C C Pruebas de patogenicidad de *Fusarium* sp. como agente causal de la escoba de bruja del mango *Mangifera indica* L. 1989; Page 36 in: XVI Congr. Nal. Fitopatol. Montecillos, México.

[3] Doreste S E . Información sobre el eriófido del mango, *Eriophyes mangiferae* (Sayed), en Venezuela. Rev. Facultad Agrociencia(Universidad Central de Venezuela) 1984; 13:91-100.

[4] Freeman S, Klein-Gueta D, Korolev N, Sztejnberg A. Epidemiology and survival of *Fusarium mangiferae*, the causal agent of mango malformation disease, Acta Hort. 2004;45, 487-491.

[5] Haggag Wafaa M, Abd Wahab, I M . First Report of *Fusarium sterilihyphosum and F. proliferatum*-Induced Malformation Disease of Mango in Egypt. *J. of Plant Pathol*. 2009; 91(1), 232.

[6] Komada H . Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Review of Plant Protection Research (Japan) 1975; 8:114-125.

[7] Kumar J, Singh U S, Beniwal S PS . Mango malformation: One hundred years of research. Annu. Rev. Phytopathology 1993; .31:217-232.

[8] Kvas M, Steenkamp E, Al-Adway A, Deadman M, Al-Jahwari A, Marasas W, Wingfield B, Ploetz R, Wingfielf, M . *Fusarium mangiferae* associated with mango malformation in the Sultanate of Oman. European J. Plant Pathology . 2008; 121(2): 195-199.

[9] Iqbal Z, Ahmad AD , Saleem A .Role of *Fusarium mangiferae* in causation of mango malformation disease. Journal of Research Science 2006; 17(1), 09-14

[10] Majumdar PK , Sinha GC . Seasonal Variation in the incidence of malformation in *Mangifera Indica* L. Acta Hort 1972; 24: 221–23

[11] Marasas WFO, Ploetz RC, Wingfield MJ, Wingfield, BD, Steenkamp ET. Mango malformation disease and the associated *Fusarium* species. Phytopathology2006; 96, 667-672.

[12] Ploetz R C . Diseases of mango. Pages 327-363 in: Diseases of Tropical Fruit Crops. R. C. Ploetz, ed. CABI ; Publishing, Oxford, UK.

[13] Ploetz RC, Gregory N. Mango malformation in Florida: Distribution of *Fusarium subglutinans* in affected trees, and relationships among strains within and among different orchards. Acta Hort. 1993; 34, 388-394.

[14] Ploetz R, Zheng QI, Vázquez Á, Sattar MAA. Current status and impact of mango malformation in Egypt A *Fusarium* sp. different from *Fusarium oxysporum* and *F. mangiferae* is associated with mango malformation in Michoacán, Mexico. International Journal of Pest Management. 2002; 48:(4) 279-285.

[15] Shawky I, Zidan Z, Tomi AEl, Dahshan DI. Flowering malformation in relation to vegetative growth of Taimour mangoes. Egyptian J. Hort. 1980; 5: 123–32

[16] Singh Z, Dhillon BS, Arora CL. Nutrient levels in malformed and healthy tissues of mango (*Mangifera indica*L.). Plant Soil 1991; 133:9-15.

[17] Steenkamp ET, Britz H, Coutinho TA, Wingfield BD, Marasas WFO , Wingfield M. Molecular characterization of *Fusarium subglutinans* associated with mango malformation. Mol Plant Pathol 2000; 1:187–193.

[18] Summerell BA, Salleh B, Leslie JF . A Utilitarian Approach to *Fusarium* Identification. Plant Diseases. 2003; 87 (2): 117-128.

[19] Tapan N, Anitha, G, Gurdeep BSN, Alok S, Pant R . Mango (*Mangifera indica* L.) malformation : Role of stress ethylene and cyanide. Physiology and Molecular Biology of Plants. 2006; 12 (2): 163-165.

[20] Yadav V K. Mango malformation - a review. Journal Appl. Hort. 1999;1(1)70-78

[21] Zheng Q, Ploetz RC. Genetic diversity in the mango malformation pathogen and development of a PCR assay. Plant Pathology 2002; 51, 208-216.

3/1/2010

Table 1. Fungi associated with malformed parts of mango in Egypt.

Cultivars	El-Bohera		Giza		Ismalia	
	Fungi isolated	(%) Infection frequency	Fungi isolated	(%) Infection frequency	Fungi isolated	(%) Infection frequency
Hindi Sennara	*F. subglutinans* *F. oxysporum* *F. sterilihyphosum* *F .proliferatum* *F. culmorum* *F. nygamai* *F .pseudonygamai*	84.7 6.80 3.52 2.43 0.34 0.46 0.25	*F. subglutinans* *F. oxysporum* *F. sterilihyphosum* *F.proliferatum* *F. culmorum* *F.nygamai* *F.pseudonygamai*	88.5 5.43 3.72 3.23 0.34 0.36 0.36	*F. subglutinans* *F.oxysporum* *F. sterilihyphosum* *F.proliferatum* *F. culmorum*	88.4 4.06 3.33 2.35 0.37
El-Founso	*F. subglutinans* *F. sterilihyphosum* *F. proliferatum* *F.oxysporum* *F.nygamai* *F.pseudonygamai*	89.4 4.42 3.34 2.54 0.30 0.54	ND*		*F. subglutinans* *F.oxysporum* *F.proliferatum* *F. sterilihyphosum*	90.0 4.24 3.23 2.54
Sadeka	*F. subglutinans* *F.proliferatum* *F.oxysporum* *F.nygamai* *F.nelsonii*	88.5 7.76 3.43 0.32 0.54	*F. subglutinans* *F.oxysporum* *F.proliferatum* *F.nygamai*	*87.5* *8.87* *3.34* *0.98*	*F. subglutinans* *F.oxysporum* *F.nygamai* *F.ndsonii*	87.7 6.87 0.65 0.13
Timour	*F. subglutinans* *F.oxysporum* *F.nygamai* *F. sterilihyphosum* *F.proliferatum* *F. culmorum*	91.7 2.43 0.67 1.56 1.56 0.45	*F. subglutinans* *F.oxysporum* *F.nygamai* *F. sterilihyphosum* *F.nelsonii*	*93.6* *2.65* *0.76* *1.65* *0.76*	*F. subglutinans* *F.oxysporum* *F.nygamai* *F. culmorum* *F. proliferatum* *F. sterilihyphosum* *F.ndsonii*	93.6 2.54 0.34 0.65 2.65 1.87 0.23
Dabcha	*F. subglutinans* *F. proliferatum* *F.nygamai* *F.acuminatum*	*94.8* *4.76* *0.54* *0.27*	*F. subglutinans* *F. proliferatum* *F.nygamai* *F.oxsporum*	*92.6* *4.87* *3.53* *0.76*	*F. subglutinans* *F.nygamai*	*95.8* *0.65*
Zebda	*F. subglutinans* *F.oxysporum* *F. proliferatum* *F. sterilihyphosum*	*91.8* *3.54* *2.43* *2.43*	*F. subglutinans* *F.oxysporium* *F. proliferatum* *F. sterilihyphosum*	*92.4* *4.34* *2.42* *2.56*	*F. subglutinans* *F.oxysporium* *F. proliferatum* *F. sterilihyphosum* *F.verticilioides*	*91.3* *2.43* *2.54* *2.54* *0.24*
Ewais	*F. subglutinans* *F. sterilihyphosum* *F.oxysporum* *F.proliferatum* *F.nygamai*	*91.5* *3.66* *3.33* *2.66* *0.75*	*F. subglutinans* *F.pseudonygamai* *F. sterilihyphosum* *F.nygamai* *F.nelsonii*	*94.4* *2.34* *3.54* *0.54* *0.43*	*F. subglutinans* *F.oxysporum* *F. sterilihyphosum* *F.proliferatum* *F.verticilioides*	*91.3* *4.32* *3.43* *3.12* *0.32*
Fagrkelan	*F. subglutinans* *F.oxysporium* *F.nygamai*	*93.7* *5.65* *0.46*	*F. subglutinans* *F.oxysporum* *F.nygamai*	*94.7* *5.76* *0.48*	*F. subglutinans* *F.oxysporium* *F.nygamai*	*94.6* *4.76* *0.45*
Keet	*F. subglutinans* *F. sterilihyphosum* *F. proliferatum* *F.nygamai*	*88.5* *5.0* *5.5* *0.54*	*ND*		*F. subglutinans* *F. sterilihyphosum* *F. proliferatum* *F.nygamai*	*89.1* *6.9* *5.5* *0.23*
Tomy	*F. subglutinans* *F. proliferatum* *F.nelsonii*	*84.5* *10.5* *0.32*	*ND*		*F. subglutinans* *F.proliferatum* *F.ndsonii*	*88.5* *11.5* *0.24*

*ND: Not detected

Figure 1**.** Mango malformation on blossom clusters.

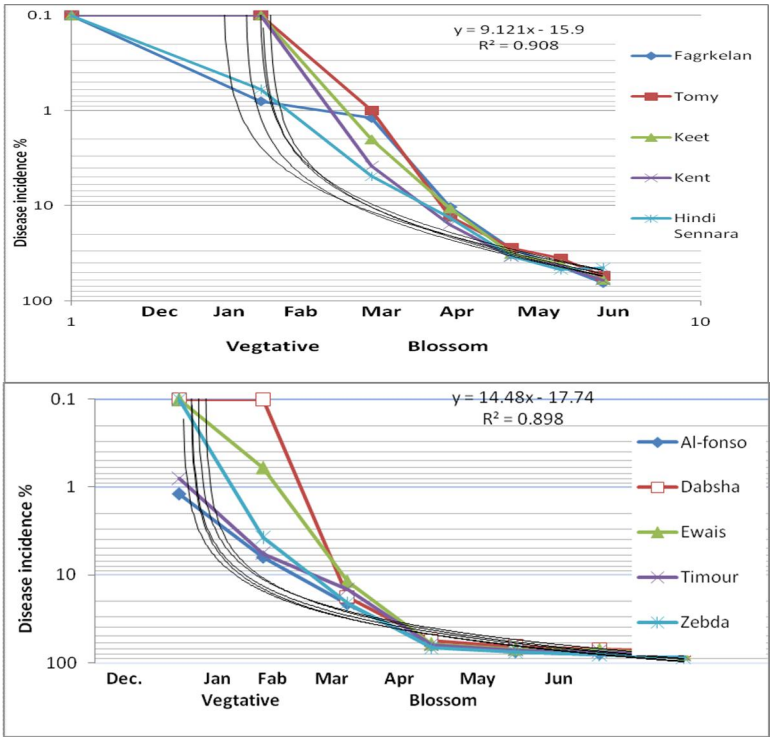

Figure 2. Disease progress curves of malformation in vegetative and floral shoots of mango in El Bohera Governorate

Table 2. Comparative virulence of selected *Fusarium* isolates on inoculated mango cv. Seddek seedlings

Treatment	Infested soil		Injection buds	
	Disease incidence %	Disease severity	Disease incidence %	Disease severity
F. subglutinans	100.0	4.0	75.0	3.3
F. solani	0.0	0.0	0.0	0.0
F. oxyspoum	50.0	1.3	25.0	0.3
F. sterilihyphosum	50.0	2.3	25.0	2.0
F. proliferatum	50.0	1.6	25.0	1.3
F.moniliforme	0.0	0.0	0.0	0.0
F.avena	0.0	0.0	0.0	0.0
F.chlamydspore	0.0	0.0	0.0	0.0
LSD	25.0	0.5.	12.0	0.5

Table 3. Comparative virulence of selected *Fusarium* isolates on inoculated mango cultivars seedlings

Cultivars	% Infection				Mean
	F. subglutinans	*F. oxyspoum*	*F. sterilihyphosum*	*F. proliferatum*	
Hindi Sennara	100.0	4.56	1.76	3.76	27.5
Seddek	100.0	9.58	3.87	8.56	30.5
Timour	100.0	11.4	5.87	9.54	31.7
Dabcha	100.0	8.54	6.87	6.34	30.4
Zebda	100.0	13.7	8.56	11.7	33.4
Ewais	100.0	10. 5	7.26	9.65	31.1
Fagrkelan	100.0	8.08	5.26	7.98	30.3
Al Fonso	100.0	13.5	9.65	9.45	34.6
Keet	90.0	2.61	1.34	1.65	23.9
Tomy	96.0	0.94	0.00	0.00	24.2
Kent	93.7	0.75	0.00	0.00	23.6
Mean	97.9	7.96	4.77	5.91	
LSD	2.43	2.95	2.54	2.76	

Table 4. Percent recovery of *Fusarium sp.* from inoculated mango cultivars.

Cultivars	*F. subglutinans*		*F.oxysporum*		*F. sterilihyphosum*		*F. proliferatum*	
	Root colonized	Apical colonized	Root colonized	Apical colonized	Root colonized	Apical colonized	Root colonized	Apical colonized
Hindi Sennara	100.0	100.0	2.36	3.65	2.06	1.26	3.53	2.13
Seddek	100.0	100.0	5.27	6.76	5.26	4.27	4.08	3.00
Timour	100.0	100.0	6.07	5.43	8.24	5.00	5.04	2.04
Dabcha	100.0	100.0	8.07	6.76	4.04	6.17	4.24	2.24
Zebda	100.0	100.0	6.96	5.43	7.17	4.66	3.17	2.11
Ewais	100.0	100.0	6.46	8.45	7.55	3.26	3.25	2.25
Fagrkelan	100.0	100.0	4.74	6.87	4.58	3.64	1.08	0.58
Al Fonso	100.0	100.0	7.54	6.98	6.98	5.34	3.87	2.43
Keet	66.6	80.0	1.04	1.70	0.75	0.95	0.90	0.34
Tomy	63.0	76.0	1.95	1.45	0.50	0.45	0.41	0.64
Kent	83.7	80.0	1.75	1.65	0.84	0.95	0.50	0.45
LSD	3.67	4.23	0.89	0.95	0.92	0.78	0.75	0.45

References

[1] Alvarado R G, Fernández-Pavía SP, Ploetz RC. *Fusarium* sp. characterization causing mango malformation in Michoacan Mexico. Phytopathology 2006; 96, S99.

[2] Campbell CW, Marlatt R. Current status of mango malformation disease in Florida. Proc. Inter Am. Soc. Trop. Hortic., (1986;30: 223–6

[3] Covarrubias C C Pruebas de patogenicidad de *Fusarium* sp. como agente causal de la escoba de bruja del mango *Mangifera indica* L. 1989; Page 36 in: XVI Congr. Nal. Fitopatol. Montecillos, México.

[3] Doreste S E . Información sobre el eriófido del mango, *Eriophyes mangiferae* (Sayed), en Venezuela. Rev. Facultad Agrociencia(Universidad Central de Venezuela) 1984; 13:91-100.

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[5] Haggag Wafaa M, Abd Wahab, I M . First Report of *Fusarium sterilihyphosum and F. proliferatum*-Induced Malformation Disease of Mango in Egypt. *J. of Plant Pathol*. 2009; 91(1), 232.

[6] Komada H . Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Review of Plant Protection Research (Japan) 1975; 8:114-125.

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[8] Kvas M, Steenkamp E, Al-Adway A, Deadman M, Al-Jahwari A, Marasas W, Wingfield B, Ploetz R, Wingfielf, M . *Fusarium mangiferae* associated with mango malformation in the Sultanate of Oman. European J. Plant Pathology . 2008; 121(2): 195-199.

[9] Iqbal Z, Ahmad AD , Saleem A .Role of *Fusarium mangiferae* in causation of mango malformation disease. Journal of Research Science 2006; 17(1), 09-14

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[14] Ploetz R, Zheng QI, Vázquez Á, Sattar MAA. Current status and impact of mango malformation in Egypt A *Fusarium* sp. different from *Fusarium oxysporum* and *F. mangiferae* is associated with mango malformation in Michoacán, Mexico. International Journal of Pest Management. 2002; 48:(4) 279-285.

[15] Shawky I, Zidan Z, Tomi AEl, Dahshan DI. Flowering malformation in relation to vegetative growth of Taimour mangoes. Egyptian J. Hort. 1980; 5: 123–32

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[17] Steenkamp ET, Britz H, Coutinho TA, Wingfield BD, Marasas WFO , Wingfield M. Molecular characterization of *Fusarium subglutinans* associated with mango malformation. Mol Plant Pathol 2000; 1:187–193.

[18] Summerell BA, Salleh B, Leslie JF . A Utilitarian Approach to *Fusarium* Identification. Plant Diseases. 2003; 87 (2): 117-128.

[19] Tapan N, Anitha, G, Gurdeep BSN, Alok S, Pant R . Mango (*Mangifera indica* L.) malformation : Role of stress ethylene and cyanide. Physiology and Molecular Biology of Plants. 2006; 12 (2): 163-165.

[20] Yadav V K. Mango malformation - a review. Journal Appl. Hort. 1999;1(1)70-78

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Table 1. Fungi associated with malformed parts of mango in Egypt.

Cultivars	El-Bohera		Giza		Ismalia	
	Fungi isolated	(%) Infection frequency	Fungi isolated	(%) Infection frequency	Fungi isolated	(%) Infection frequency
Hindi Sennara	*F. subglutinans* *F. oxysporum* *F. sterilihyphosum* *F .proliferatum* *F. culmorum* *F. nygamai* *F .pseudonygamai*	84.7 6.80 3.52 2.43 0.34 0.46 0.25	*F. subglutinans* *F. oxysporum* *F. sterilihyphosum* *F.proliferatum* *F. culmorum* *F.nygamai* *F.pseudonygamai*	88.5 5.43 3.72 3.23 0.34 0.36 0.36	*F. subglutinans* *F.oxysporum* *F. sterilihyphosum* *F.proliferatum* *F. culmorum*	88.4 4.06 3.33 2.35 0.37
El-Founso	*F. subglutinans* *F. sterilihyphosum* *F. proliferatum* *F.oxysporum* *F.nygamai* *F.pseudonygamai*	89.4 4.42 3.34 2.54 0.30 0.54	ND*		*F. subglutinans* *F.oxysporum* *F.proliferatum* *F. sterilihyphosum*	90.0 4.24 3.23 2.54
Sadeka	*F. subglutinans* *F.proliferatum* *F.oxysporum* *F.nygamai* *F.nelsonii*	88.5 7.76 3.43 0.32 0.54	*F. subglutinans* *F.oxysporum* *F.proliferatum* *F.nygamai*	*87.5* *8.87* *3.34* *0.98*	*F. subglutinans* *F.oxysporum* *F.nygamai* *F.ndsonii*	87.7 6.87 0.65 0.13
Timour	*F. subglutinans* *F.oxysporum* *F.nygamai* *F. sterilihyphosum* *F.proliferatum* *F. culmorum*	91.7 2.43 0.67 1.56 1.56 0.45	*F. subglutinans* *F.oxysporum* *F.nygamai* *F. sterilihyphosum* *F.nelsonii*	*93.6* *2.65* *0.76* *1.65* *0.76*	*F. subglutinans* *F.oxysporum* *F.nygamai* *F. culmorum* *F. proliferatum* *F. sterilihyphosum* *F.ndsonii*	93.6 2.54 0.34 0.65 2.65 1.87 0.23
Dabcha	*F. subglutinans* *F. proliferatum* *F.nygamai* *F.acuminatum*	*94.8* *4.76* *0.54* *0.27*	*F. subglutinans* *F. proliferatum* *F.nygamai* *F.oxsporum*	*92.6* *4.87* *3.53* *0.76*	*F. subglutinans* *F.nygamai*	*95.8* *0.65*
Zebda	*F. subglutinans* *F.oxysporum* *F. proliferatum* *F. sterilihyphosum*	*91.8* *3.54* *2.43* *2.43*	*F. subglutinans* *F.oxysporium* *F. proliferatum* *F. sterilihyphosum*	*92.4* *4.34* *2.42* *2.56*	*F. subglutinans* *F.oxysporium* *F. proliferatum* *F. sterilihyphosum* *F.verticilioides*	*91.3* *2.43* *2.54* *2.54* *0.24*
Ewais	*F. subglutinans* *F. sterilihyphosum* *F.oxysporum* *F.proliferatum* *F.nygamai*	*91.5* *3.66* *3.33* *2.66* *0.75*	*F. subglutinans* *F.pseudonygamai* *F. sterilihyphosum* *F.nygamai* *F.nelsonii*	*94.4* *2.34* *3.54* *0.54* *0.43*	*F. subglutinans* *F.oxysporum* *F. sterilihyphosum* *F.proliferatum* *F.verticilioides*	*91.3* *4.32* *3.43* *3.12* *0.32*
Fagrkelan	*F. subglutinans* *F.oxysporium* *F.nygamai*	*93.7* *5.65* *0.46*	*F. subglutinans* *F.oxysporum* *F.nygamai*	*94.7* *5.76* *0.48*	*F. subglutinans* *F.oxysporium* *F.nygamai*	*94.6* *4.76* *0.45*
Keet	*F. subglutinans* *F. sterilihyphosum* *F. proliferatum* *F.nygamai*	*88.5* *5.0* *5.5* *0.54*	*ND*		*F. subglutinans* *F. sterilihyphosum* *F. proliferatum* *F.nygamai*	*89.1* *6.9* *5.5* *0.23*
Tomy	*F. subglutinans* *F. proliferatum* *F.nelsonii*	*84.5* *10.5* *0.32*	*ND*		*F. subglutinans* *F.proliferatum* *F.ndsonii*	*88.5* *11.5* *0.24*

*ND: Not detected

Figure 1**.** Mango malformation on blossom clusters.

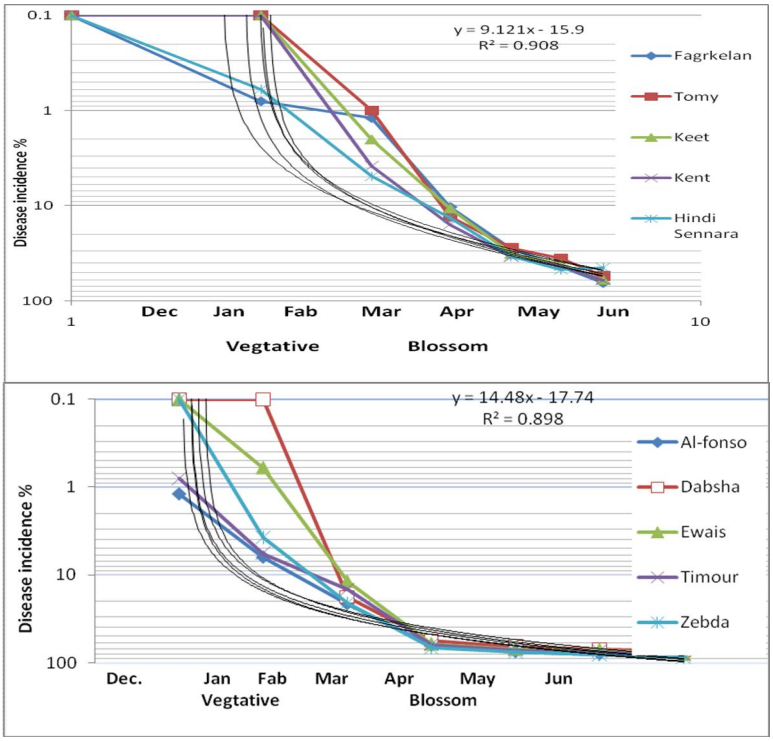

Figure 2. Disease progress curves of malformation in vegetative and floral shoots of mango in El Bohera Governorate

Table 2. Comparative virulence of selected *Fusarium* isolates on inoculated mango cv. Seddek seedlings

Treatment	Infested soil		Injection buds	
	Disease incidence %	Disease severity	Disease incidence %	Disease severity
F. subglutinans	100.0	4.0	75.0	3.3
F. solani	0.0	0.0	0.0	0.0
F. oxyspoum	50.0	1.3	25.0	0.3
F. sterilihyphosum	50.0	2.3	25.0	2.0
F. proliferatum	50.0	1.6	25.0	1.3
F.moniliforme	0.0	0.0	0.0	0.0
F.avena	0.0	0.0	0.0	0.0
F.chlamydspore	0.0	0.0	0.0	0.0
LSD	25.0	0.5.	12.0	0.5

Table 3. Comparative virulence of selected *Fusarium* isolates on inoculated mango cultivars seedlings

Cultivars	% Infection				Mean
	F. subglutinans	*F. oxyspoum*	*F. sterilihyphosum*	*F. proliferatum*	
Hindi Sennara	100.0	4.56	1.76	3.76	27.5
Seddek	100.0	9.58	3.87	8.56	30.5
Timour	100.0	11.4	5.87	9.54	31.7
Dabcha	100.0	8.54	6.87	6.34	30.4
Zebda	100.0	13.7	8.56	11.7	33.4
Ewais	100.0	10. 5	7.26	9.65	31.1
Fagrkelan	100.0	8.08	5.26	7.98	30.3
Al Fonso	100.0	13.5	9.65	9.45	34.6
Keet	90.0	2.61	1.34	1.65	23.9
Tomy	96.0	0.94	0.00	0.00	24.2
Kent	93.7	0.75	0.00	0.00	23.6
Mean	97.9	7.96	4.77	5.91	
LSD	2.43	2.95	2.54	2.76	

Table 4. Percent recovery of *Fusarium sp.* from inoculated mango cultivars.

Cultivars	*F. subglutinans*		*F.oxysporum*		*F. sterilihyphosum*		*F. proliferatum*	
	Root colonized	Apical colonized	Root colonized	Apical colonized	Root colonized	Apical colonized	Root colonized	Apical colonized
Hindi Sennara	100.0	100.0	2.36	3.65	2.06	1.26	3.53	2.13
Seddek	100.0	100.0	5.27	6.76	5.26	4.27	4.08	3.00
Timour	100.0	100.0	6.07	5.43	8.24	5.00	5.04	2.04
Dabcha	100.0	100.0	8.07	6.76	4.04	6.17	4.24	2.24
Zebda	100.0	100.0	6.96	5.43	7.17	4.66	3.17	2.11
Ewais	100.0	100.0	6.46	8.45	7.55	3.26	3.25	2.25
Fagrkelan	100.0	100.0	4.74	6.87	4.58	3.64	1.08	0.58
Al Fonso	100.0	100.0	7.54	6.98	6.98	5.34	3.87	2.43
Keet	66.6	80.0	1.04	1.70	0.75	0.95	0.90	0.34
Tomy	63.0	76.0	1.95	1.45	0.50	0.45	0.41	0.64
Kent	83.7	80.0	1.75	1.65	0.84	0.95	0.50	0.45
LSD	3.67	4.23	0.89	0.95	0.92	0.78	0.75	0.45

References

[1] Alvarado R G, Fernández-Pavía SP, Ploetz RC. *Fusarium* sp. characterization causing mango malformation in Michoacan Mexico. Phytopathology 2006; 96, S99.

[2] Campbell CW, Marlatt R. Current status of mango malformation disease in Florida. Proc. Inter Am. Soc. Trop. Hortic., (1986;30: 223–6

[3] Covarrubias C C Pruebas de patogenicidad de *Fusarium* sp. como agente causal de la escoba de bruja del mango *Mangifera indica* L. 1989; Page 36 in: XVI Congr. Nal. Fitopatol. Montecillos, México.

[3] Doreste S E . Información sobre el eriófido del mango, *Eriophyes mangiferae* (Sayed), en Venezuela. Rev. Facultad Agrociencia(Universidad Central de Venezuela) 1984; 13:91-100.

[4] Freeman S, Klein-Gueta D, Korolev N, Sztejnberg A. Epidemiology and survival of *Fusarium mangiferae*, the causal agent of mango malformation disease, Acta Hort. 2004;45, 487-491.

[5] Haggag Wafaa M, Abd Wahab, I M . First Report of *Fusarium sterilihyphosum and F. proliferatum*-Induced Malformation Disease of Mango in Egypt. *J. of Plant Pathol*. 2009; 91(1), 232.

[6] Komada H . Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Review of Plant Protection Research (Japan) 1975; 8:114-125.

[7] Kumar J, Singh U S, Beniwal S PS . Mango malformation: One hundred years of research. Annu. Rev. Phytopathology 1993; .31:217-232.

[8] Kvas M, Steenkamp E, Al-Adway A, Deadman M, Al-Jahwari A, Marasas W, Wingfield B, Ploetz R, Wingfielf, M . *Fusarium mangiferae* associated with mango malformation in the Sultanate of Oman. European J. Plant Pathology . 2008; 121(2): 195-199.

[9] Iqbal Z, Ahmad AD , Saleem A .Role of *Fusarium mangiferae* in causation of mango malformation disease. Journal of Research Science 2006; 17(1), 09-14

[10] Majumdar PK , Sinha GC . Seasonal Variation in the incidence of malformation in *Mangifera Indica* L. Acta Hort 1972; 24: 221–23

[11] Marasas WFO, Ploetz RC, Wingfield MJ, Wingfield, BD, Steenkamp ET. Mango malformation disease and the associated *Fusarium* species. Phytopathology2006; 96, 667-672.

[12] Ploetz R C . Diseases of mango. Pages 327-363 in: Diseases of Tropical Fruit Crops. R. C. Ploetz, ed. CABI ; Publishing, Oxford, UK.

[13] Ploetz RC, Gregory N. Mango malformation in Florida: Distribution of *Fusarium subglutinans* in affected trees, and relationships among strains within and among different orchards. Acta Hort. 1993; 34, 388-394.

[14] Ploetz R, Zheng QI, Vázquez Á, Sattar MAA. Current status and impact of mango malformation in Egypt A *Fusarium* sp. different from *Fusarium oxysporum* and *F. mangiferae* is associated with mango malformation in Michoacán, Mexico. International Journal of Pest Management. 2002; 48:(4) 279-285.

[15] Shawky I, Zidan Z, Tomi AEl, Dahshan DI. Flowering malformation in relation to vegetative growth of Taimour mangoes. Egyptian J. Hort. 1980; 5: 123–32

[16] Singh Z, Dhillon BS, Arora CL. Nutrient levels in malformed and healthy tissues of mango (*Mangifera indica*L.). Plant Soil 1991; 133:9-15.

[17] Steenkamp ET, Britz H, Coutinho TA, Wingfield BD, Marasas WFO , Wingfield M. Molecular characterization of *Fusarium subglutinans* associated with mango malformation. Mol Plant Pathol 2000; 1:187–193.

[18] Summerell BA, Salleh B, Leslie JF . A Utilitarian Approach to *Fusarium* Identification. Plant Diseases. 2003; 87 (2): 117-128.

[19] Tapan N, Anitha, G, Gurdeep BSN, Alok S, Pant R . Mango (*Mangifera indica* L.) malformation : Role of stress ethylene and cyanide. Physiology and Molecular Biology of Plants. 2006; 12 (2): 163-165.

[20] Yadav V K. Mango malformation - a review. Journal Appl. Hort. 1999;1(1)70-78

[21] Zheng Q, Ploetz RC. Genetic diversity in the mango malformation pathogen and development of a PCR assay. Plant Pathology 2002; 51, 208-216.

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Biochemical evaluation of the effect of *Rhazya stricta aqueous* leaves extract in liver and kidney functions in Rats

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Abstract: Rhazya *stricta* (*R. stricta*) is an important medicinal species used in indigenous medicinal herbal drugs to cure various diseases in South Asia and Middle East Countries. Over 100 alkaloids have been isolated, from *R. stricta* leaves, stems, roots and legumes and mixtures of aerial parts. The aim of this study was evaluatation of the beneficial effects of oral administration of extracts of the *R. stricta* leaves on serum lipid profile concentrations, the activity of liver enzymes and the kidney functions, using doses comparable to those applied by humans in the folkloric medicine. To achieve this goal, fifty five male Wistar rats were divided into four groups as follows: group 1 (control, n= 10) received a daily single oral dose of 0.5 ml of distilled water, groups 2, 3 and 4 (each of 15), each animal received a daily single oral dose of 0.5 ml of distilled water containing 0.1 gm/ml (group 2), 0.125 gm/ml (group 3) and 0.150 gm/ml (group 4) of the *Rhazya* leaf aqueous extract, for 18 weeks. Blood samples were collected, after an overnight fast, 1, 2, 4, 8, 12 and 18 weeks post-treatment. The aqueous extract of the *R. stricta* leaves significantly decreased concentrations of TGs, LDL-c, cholesterol, uric acid and creatinin, but increased concentration of HDL-c. It triggered all these activities without affecting liver enzyme activities or kidney functions. These findings may have a positive impact on the cardiovascular patients and may provide a new therapeutic strategy to reduce hypertriglyceridemia. [Nature and Science. 2010;8(4):136-142]. (ISSN: 1545-0740).

Key words: *Rhazya stricta*; lipid profile; liver enzymes; aqueous extracts; uric acid

1. Introduction

Rhazya stricta Decne (*R. stricta*), locally known as Harmal, is a member of family Apocynaceae. It is widely distributed throughout Western Asia from Yemen to Arabia, to the North West Province of India and abundantly found in various regions of Pakistan. The plant is a glabrous erect shrub with smooth central stem and dense erect branches, Western (1989) . It is widely used in traditional medicine as a reputed tonic and curative for rheumatic pain, sore throat, syphilis, diabetes, helminthiasis, inflammatory conditions, fever and other diseases , Ageel et al., (1987); Ali,et al., (1995) and Ali,et al., (1998) The leaves of the plant contain alkaloids with β-carboline nucleus (akuammidine, rhazinilam and tetrahydrosecamine), Bashir et al;., (1994). The *R. stricta* leaves have been shown to contain flavonoids, glycosides, triterpenes, tannins, volatile bases and probably other substances, Ahmed et al;., (1983) and AL-Yahya et al;., (1990). Extensive studies on the phytochemistry, Baherji et al;., (1970); Rahman et al;., (1988); Bashir et al; (1994) and Wasfi et al; (1994), antimicrobial activity Bashir et al; (1994), central nervous system depression, Ali et al; (1995) and general pharmacology and toxicity of the plant[13] have been reported. Its leaf extracts were found to cause sedation, analgesia, decreased motor activity, antidepressant-like activities, complex effects on brain endogenous monoamine oxidase activity and centrally-mediated hypotension in mice and rats, Ali, et al., (1995); Ali, et al., (2000); Tanira et al., (2000); and et al., (2000). Recently, Baeshin's team run a series of elegant experiments proving that the aqueous extract *Rhazya stricta* leaves had mutagenic activities on *wide* range of cell types including *S. cervisae*, Baeshin et al., (2005) *Aspergillus terreus*, Baeshin et al., (2008). *Allium cepa* root tip meristem, Baeshin et al., (2009) and the primary culture of human lymphoctes Baeshin et al., (submitted).

To our best knowledge, there is no documented report elucidating the effects of this plant on serum HDL-c concentrations. In light of the ample use of this plant, and as part of our ongoing research in exploring possible curable effects of some indigenous medicinal herbs in KSA, we decided to investigate the effect, if any, of the *R. stricta* leaf extract on lipid profile concentrations, especially HDL-c and HDL.

2-Effects of the *R. stricta* leaf aqueous extract after two weeks of treatment

The data presented in Table 2 shows that, after two weeks of treatment, the *Rhazya* extract consistently recapitulated its dose-dependent decreasing mode of action on TGs, specially in context of group 4, since it turned down concentrations of TGs to as nearly as 60% of their concentration in the control group. It also consistently increased, in a dose-dependent manner,

concentrations of HDL-c and LDL.

However, it did not significantly affect concentrations of cholesterol in all treated groups. Although a highly significantly (P<0.01) increase in activity of AST was observed in group 4, no effects was noticed for ALT or ALP. For effects of the extract on creatinine, uric acid and urea, its best effects were clearly seen in the context of group 4, where it significantly decreased concentrations of uric acid and urea; however, it did not bring effects on creatinine level. Nonetheless, it did increased concentration of creatinine in group 2.

Materials and Methods

1-Materials:

1.1-Animals

Fifty five locally bred adult male Wistar rats, initially weighing 150-200 gm, were obtained from King Fahad Medical Research Center (KFMRC), King Abdul-Aziz University, Jeddah, KSA. They were housed in groups of five animals at a temperature of 22°C under a 12 h dark-light cycle. They were fed *ad libitum* a standard pellet diet (Grain Soils and Flourmills Organization Jeddah, KSA) and given distilled drinking water.

1.2-Plant material and extract preparation

The plant was collected from a nearby area of Jeddah, KSA in May 2005. Leaves were shade-dried and ground to a fine powder with a blender. The resulting powder was stored at 4°C. Aqueous solutions were freshly prepared daily from this powder and used in all tests. Three different concentrations of the powdered leaves were prepared (0.1 gm/ml, 0.125 gm/ml and 0.150 gm/ml) by macerating 4 gm, 5 gm and 6 gm, respectively, in 40 ml distilled water for 12 h at room temperature, with occasional shaking. The extract was then filtered. The filtrate was giving directly to the rats; the aqueous extract was always administrated orally in a volume of 0.5 ml of the prepared dose.

2-Material and Methods:

2.1-Experimental design

Animals were divided into four groups and acclimatized for 4 days prior to experimentation. Group 1 (control, n= 10), each animal was given, by oral gavage, 0.5 ml distilled water. Groups 2, 3 and 4 (each of 15), each animal was given, by oral gavage, a daily single dose of 0.5 ml distilled water containing 0.1 gm/ml (group 2), 0.125 gm/ml (group 3) and of 0.150 gm/ml (group 4) of the extract, for 18 weeks.

Blood samples were collected, after an overnight fast, on weeks 1, 2, 4, 8, 12 and 18 post-treatment. At the time of the collection, information including body weights, food and water intakes as well as any abnormal physical behavior was recorded for each animal. Collected blood samples were centrifuged at 3000 rpm for 10 minutes and sera were stored immediately at - 80°C until time of analysis.

2.2 Biochemical assays

Sera were used for measuring concentrations of total cholesterol, high density lipoprotein (HDL-c), low density lipoprotein (LDL-c) and triglycerides (TG), for assaying activities of aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP), and for determining concentrations of uric acid, urea, and creatinine. The last task was achieved by commercial kit-based enzymatic colorimetry methods (Dade Behring, USA) using an automated chemistry analyzer (Dimension R Clinical Chemistry System). These measurements were carried out in the biochemistry lab, King Abdul Aziz University Hospital (KAUH), Jeddah, KSA.

2.3 Statistical evaluation

Statistical analysis was performed using SPSS 10 for Windows. The data were expressed as means ± standard deviation. Comparison of variables between groups was performed using one-way analysis of variance (ANOVA). The least significance difference test (LSD) was employed to compare means for pairs of groups. A difference was considered to be statistically significant when *P*-value ≤ 0.05.

3. Result Analysis

1-Effects of the *R. stricta* leaf aqueous extract after one week of treatment

The results are summarized in Table 1. Statistical analysis indicated that oral administration of the aqueous extract the *R. stricta* leaves significantly (*P*<0.05) decreased concentration of TGs in group 4, but it did not bring significant effect in group 2 or 3. On the other hand, it exerted a dose-dependent increasing mode on concentrations of HDL-c in all treated groups, whereas the lower dose of the extract, 0.1 gm/ml (group 2), significantly (*P*<0.05) increased HDL-c concentration, the higher doses of the extract, 0.125 gm/ml (group 3) and of 0.150 gm/ml (group 4), highly significantly (*P*<0.01) increased concentrations of HDL-c. Meanwhile, we did not observe significant (*P*>0.05) difference between all treated and control groups, regarding alterations in the concentration of LDL-c or cholesterol, neither did we notice change in activity of serum AST, ALT or ALP. In addition, serum creatinine concentration was lower in group 2 than its concentration in group 1 (control) and, finally, concentrations of uric acid and urea were significantly lower in group 4 than in control group.

3-Effects of the *R. stricta* leaf aqueous extract after four weeks of treatment

As shown in Table 3, TG concentrations were consistently decreased (P<0.05-0.01) in all treated groups compared to the control group. The extract, however, did not alter concentrations of HDL-c in all treated group, but it consistently decreased concentrations of LDL in all treated groups. Furthermore, neither did the extract alter concentrations of cholesterol, nor did it affect activity of AST, ALT, or ALP in all treated groups. Finally, the extract decreased concentration of creatinine in the context of group 4, but it did not alter concentration of uric acid or urea in any groups.

3-Effects of *Rhazya stricta* after eight weeks of administration of treatment

After eight weeks of treatment the extract highly significantly (P<0.01) augmented serum levels of TGs, especially in groups 2 and 3. It also boosted level of HDL-c in group 2; on the other hand, levels of LDL and cholesterol dwindled. In addition, the extract neither did affect activity of AST, ALT, nor ALP. The extract did not either alter concentrations of creatinine, but it significantly decreased and increased concentrations of uric acid and urea, respectively.

4-Effects of the *R. stricta* leaf aqueous extract after twelve weeks of treatment

Effects of the *Rhazya* extract after twelve weeks are displayed in Table 5; pair-wise comparison of TG levels in all treated and control groups show that the extract has neutralizing effect on TG levels. On the other hand, it significantly raised level of HDL-c in group 4. The extract did not affect either concentration of LDL or cholesterol; neither did it alter activity of AST, ALT nor ALP. The extract did, however, decrease activities of creatinine group 3), uric acid (groups 3 and 4) and urea (groups 2 and 3).

Effects of the *R. stricta* leaf aqueous extract after eighteen weeks of treatment

When we monitored effects of the *Rhazya* extract after eighteen weeks of treatment (Table. 6), we found that all treated and control animals have a more or less comparable levels for lipid profile, TGs, HDL-c, LDL and cholesterol. The same observation was noticed too for activities of the liver enzymes, AST, ALT and ALP, whereas the extract has no ability to alter their activities. Additionally, effects of the extract on concentrations of creatinine and urea were insignificant, but it significantly kept its decreasing effect on uric acid in groups 2 and 3.

Table1: Lipid profile concentrations, Liver enzyme concentrations and kidney function test results among the groups after one week of treatment with *Rhazya stricta*

Variable n	Group 1 Control 10	Group 2 (0.1gm/ml) 15	Group 3 (0.125gm/ml) 15	Group 4 (0.150gm/ml) 15	*P*-value
Triglycerides(mmol/l)	1.02±0.27	0.82±0.25	1.02±0.25	0.72±0.30	$<0.05^{(c)}$
HDL (mmol/l)	0.43±0.06	0.50±0.07	0.51± 0.08	0.512± 0.07	$<0.05^{(a)}$ $<0.01^{(b,c)}$
LDL (mmol/l)	0.89±.245	0.94±0.219	0.84±0.173	0.88±0.23	NS
Cholesterol (mmol/l)	1.79±0.21	1.72±0.43	1.81±0.24	1.70±0.22	NS
AST (U/L)	85.1±12.06	72.07±14.59	71.80±24.69	72.27±30.49	NS
ALT (U/L)	53.8±7.67	51.8±10.04	54.80±8.43	58.53±11.81	NS
ALP (U/L)	198.6±19.8	172.87±36.0	205.8±60.02	221.47±38.99	NS
Creatinine (umol/l)	31.89±4.48	28±4.62	30.40±4.39	32.20±4.78	$<0.05^{(a)}$
Uric acid (U/L)	61.70±25.62	49±15.80	70.66±23.69	39.40±19.45	$<0.05^{(c)}$
Urea (umol/l)	7.52±0.95	6.82±0.83	6.87±0.86	6.32±1.03	$<0.01^{(c)}$

Values were represented as the mean ± SD; the data were statistically analyzed using ANOVA followed by LSD test. (a: control vs. group 2, b: control vs. group 3, c: control vs. group 4).
P- value ≤ 0.05 was used as a criterion of significance.
P- value ≤ 0.01 was used as a criterion of highly significance.
NS: Not significant

Table2: Lipid profile concentrations, Liver enzymes concentrations and kidney function test results among the groups after two weeks of treatment with *Rhazya stricta*

Variable N	Group 1 Control 10	Group 2 (0.1gm/ml) 15	Group 3 (0.125gm/ml) 15	Group 4 (0.150gm/ml) 15	*P*-value
Triglycerides(mmol/l)	1.0± 0.23	0.86± 0.21	0.93± 0.20	0.61± 0.20	$<0.001^{(c)}$

HDL (mmol/l)	0.49± 0.07	0.52± 0.08	0.54± 0.08	0.56± 0.07	<0.05[c]
LDL (mmol/l)	0.83± 0.28	0.88± 0.16	0.86± 0.15	1.03± 0.11	<0.05[c]
Cholesterol (mmol/l)	1.78± 0.26	1.79± 0.21	1.83± 0.19	1.87± 0.22	NS
AST (U/L)	86.6± 22.65	86.8± 13.97	98.13± 17.88	108.83± 15.76	<0.01[c]
ALT (U/L)	61.5± 12.71	62.2± 9.78	56.6± 17.5	66.31± 7.94	NS
ALP (U/L)	177.8± 19.53	160.4± 18.29	186.46± 17.79	172.9± 28.78	NS
Creatinine (umol/l)	33.6± 3.06	35.8± 3.73	37.86± 5.01	32.76± 5.13	<0.05[b]
Uric acid (U/L)	79.4± 26.60	79.4± 18.48	68.5± 23.17	61.61± 16.41	<0.05[c]
Urea (umol/l)	7.72± 1.05	7.59± 0.99	7.09± 0.83	6.11± 0.87	<0.001[c]

Values were represented as the mean ± SD; the data were statistically analyzed using ANOVA followed by LSD test. (a: control vs. group 2, b: control vs. group 3, c: control vs. group 4).
P- value ≤ 0.05 was used as a criterion of significance.
P- value ≤ 0.01 was used as a criterion of highly significance.
NS: Not significant

Table3: Lipid profile concentrations, Liver enzymes concentrations and kidney function test results among the groups after four weeks of treatment with *Rhazya stricta*

Variable **n**	**Group 1 Control** **10**	**Group 2 (0.1gm/ml)** **15**	**Group 3 (0.125gm/ml)** **15**	**Group 4 (0.150gm/ml)** **15**	***P*-value**
Triglycerides (mmol/l)	1.16± 0.49	0.89± 0.14	0.81± 0.17	0.86± 0.34	<0.05[a,c] <0.01[b]
HDL (mmol/l)	0.50± 0.07	0.49± 0.06	0.53± 0.08	0.52± 0.07	NS
LDL (mmol/l)	0.28± 0.06	0.23± 0.044	0.24± 0.038	0.24± 0.036	<0.05[a,b,c]
Cholesterol (mmol/l)	1.96± 0.23	1.7± 0.46	1.79± 0.19	1.78± 0.19	NS
AST (U/L)	74± 44.25	90.13± 24.03	95.08± 18.49	83.77± 15.02	NS
ALT (U/L)	62.3± 7.65	62.73± 8.6	63.57± 7.9	59.57± 4.69	NS
ALP (U/L)	189.4± 53.83	179.2± 34.85	194± 20.46	181.92± 29.02	NS
Creatinine (umol/l)	36± 6.48	34.2± 6.39	38.29± 4.64	30.85± 6.04	<0.05[c]
Uric acid (U/L)	63.6± 30.02	51.73± 15.63	92.5± 44.65	60.75± 32.64	NS
Urea (umol/l)	7.47± 1.18	6.89± 1.12	7.19± 0.92	6.96± 1.09	<0.05[c]

NS: Not significant
Values were represented as the mean ± SD; the data were statistically analyzed using ANOVA followed by LSD test. (a: control vs. group 2, b: control vs. group 3, c: control vs. group 4).
P- value ≤ 0.05 was used as a criterion of significance.
P- value ≤ 0.01 was used as a criterion of highly significance.
NS: Not significant

Table 4: Lipid profile concentrations, Liver enzymes concentrations and kidney function test results among the groups after eight weeks of treatment with *Rhazya stricta*

Variable **n**	**Group 1 Control** **10**	**Group 2 (0.1gm/ml)** **15**	**Group 3 (0.125gm/ml)** **15**	**Group 4 (0.150gm/ml)** **15**	***P*-value**
Triglycerides (mmol/l)	0.39± 0.07	0.46± 0.08	0.46± 0.06	0.40± 0.05	<0.01[a,b]
HDL (mmol/l)	0.49± 0.05	0.57± 0.05	0.52± 0.07	0.48± 0.06	<0.01[a]
LDL (mmol/l)	0.25± 0.02	0.24± 0.06	0.21± 0.02	0.19± 0.03	<0.05[b] <0.001[c]
Cholesterol (mmol/l)	1.94± 0.4	2.1± 0.25	1.72± 0.15	1.71± 0.23	<0.05[b,c]
AST (U/L)	127.4± 24.57	120.79± 23.16	147.5± 44.06	127.8± 19.54	NS
ALT (U/L)	55.8± 8.01	58.79± 9.7	62.71± 8.2	57.73± 7.14	NS

ALP (U/L)	140.3± 23.86	140.71± 24.56	150.21± 18.91	131.6± 20	NS
Creatinine (umol/l)	41.1± 5.69	38.79± 4.88	43.57± 4.79	39.07± 6.97	NS
Uric acid (U/L)	88. 7± 34.27	64.71± 16.79	76.86± 21.89	69.4± 18.26	$<0.05^{(a,c)}$
Urea (umol/l)	5.26± 0.66	5.61± 0.71	6.03± 0.59	5.69± 0.82	$<0.05^{(b)}$

Values were represented as the mean ± SD; the data were statistically analyzed using ANOVA followed by LSD test. (a: control vs. group 2, b: control vs. group 3, c: control vs. group 4).
P- value ≤ 0.05 was used as a criterion of significance.
P- value ≤ 0.01 was used as a criterion of highly significance.
NS: Not significant

Table 5: Lipid profile concentrations, Liver enzymes concentrations and kidney function test results among the groups after twelve weeks of treatment with *Rhazya stricta*

Variable **n**	**Group 1 Control** **10**	**Group 2 (0.1gm/ml)** **15**	**Group 3 (0.125gm/ml)** **15**	**Group 4 (0.150gm/ml)** **15**	***P*-value**
Triglycerides(mmol/l)	0.40± 0.07	0.45± 0.09	0.46± 0.07	0.44± 0.06	NS
HDL (mmol/l)	0.42± 0.04	0.48± 0.06	0.48± 0.06	0.50± 0.06	$<0.05^{(a,b)}$ $<0.01^{(c)}$
LDL (mmol/l)	1.1± 0.22	1.03± 0.17	0.97± 0.14	1.12± 0.12	NS
Cholesterol (mmol/l)	1.73± 0.21	1.72± 0.18	1.68± 0.21	1.82± 0.18	NS
AST (U/L)	127.1± 31.22	127.3± 33.37	121.77± 24.19	123.5± 24.18	NS
ALT (U/L)	61.3± 6.3	63.42± 12.22	59.64± 13.31	64.28± 7.64	NS
ALP (U/L)	127.7± 20.83	121.17± 12.15	133.43± 17.1	128± 21.89	NS
Creatinine (umol/l)	49.4± 9.05	45.83± 3.1	42.14± 3.21	51.86± 5.20	$<0.01^{(b)}$
Uric acid (U/L)	77.9± 17.69	72.58± 16.08	60.86± 16.03	60.79± 14.96	$<0.05^{(b,c)}$
Urea (umol/l)	6.47± 0.81	5.63± 0.73	6.21± 0.66	5.72± 0.83	$<0.05^{(a,c)}$

Values were represented as the mean ± SD; the data were statistically analyzed using ANOVA followed by LSD test. (a: control vs. group 2, b: control vs. group 3, c: control vs. group 4).
P- value ≤ 0.05 was used as a criterion of significance.
P- value ≤ 0.01 was used as a criterion of highly significance.
NS: Not significant

Table6: Lipid profile concentrations, Liver enzymes concentrations and kidney function test results among the groups after eighteen weeks of treatment with *Rhazya stricta*

Variable **n**	**Group 1 Control** **10**	**Group 2 (0.1gm/ml)** **15**	**Group 3 (0.125gm/ml)** **15**	**Group 4 (0.150gm/ml)** **15**	***P*-value**
Triglycerides (mmol/l)	0.52± 0.06	0.49± 0.19	0.52± 0.06	0.49± 0.08	NS
HDL (mmol/l)	0.45± 0.06	0.48± 0.07	0.48± 0.07	0.49± 0.05	NS
LDL (mmol/l)	1.13± 0.19	1.18± 0.23	1.11± 0.16	1.12± 0.13	NS
Cholesterol (mmol/l)	1.77± 0.18	1.75± 0.55	1.82± 0.21	1.86± 0.14	NS
AST (U/L)	142.3± 57.34	125.69± 32.15	118.43± 22.60	142.07± 54.16	NS
ALT (U/L)	69.9± 17.58	65± 14.66	63.64± 10.95	73.57± 21.76	NS
ALP (U/L)	123.3± 25.05	115.15± 20.85	115.29± 11.72	122.23± 20.94	NS
Creatinine (umol/l)	41.56± 7.97	45.83± 6.45	44.93± 5.06	39.86± 4.11	NS
Uric acid (U/L)	66.60± 23.54	54.92± 8.50	45.86± 6.2	54.36± 13.42	$<0.01^{(b,c)}$
Urea (umol/l)	6.17± 2.12	6.69± 1.95	7.44± 0.69	6.85± 0.74	NS

Values were represented as the mean ± SD; the data were statistically analyzed using ANOVA followed by LSD test. (a: control vs. group 2, b: control vs. group 3, c: control vs. group 4).
P- value ≤ 0.05 was used as a criterion of significance.
P- value ≤ 0.01 was used as a criterion of highly significance.
NS: Not significant.

4. Conclusions

Rhazya stricta is commonly used in folk medicine of the Arabian Peninsula for the treatment of many diseases. Therefore, we decided to elucidate the effect, if there any, of the *R. stricta* on blood lipid indices. Our findings indicated that the aqueous extract of the *R. stricta* leaves significantly decreased concentrations of TGs, LDL-c, cholesterol, uric acid and creatinin, but increased concentration of HDL-c. It triggered all these activities without affecting liver enzyme activities or kidney functions. In many studies, relatively large doses of the plant extract were used to determine the pharmacological and toxicological actions, Tanira *et al*.,(1996) and Adam,*et al*.,(2002) . Therefore, it was necessary to study the biochemical effects of this plant using low doses, in other words, doses comparable to those applied in folk medicine. The present study confirmed the low toxic potential of the plant extract. Our study showed that the daily oral administration of single doses of the extract of plant leaves (0.1, 0.125 and 0.150 gm/ml) for 18 weeks, did not produce significant changes in the activity of serum AST, ALT and ALP, neither did the extract produced significant rise in the concentrations of uric acid, urea nor creatinine. Therefore, kidney functions have not been affected by such treatment. Paradoxically, another study reported emergence of hepatonephrotoxicity for chicken grown on 100 g/kg *Rhazya stricta* diet after 4 and 7 weeks of treatment, Al-Homidan *et al.*,(2002).One possible explanation for emergence of this toxicity is the dosage effect, where applying as high as 100 g/kg *Rhazya stricta* diet elicited toxicity consequences. Our work demonstrates for the first time that the aqueous extracts of *Rhazya stricta* significantly reduced serum TG, LDL-c, cholesterol, uric acid and creatinine levels and increased HDL-c concentrations. These results indicated that the aqueous extracts of *Rhazya stricta* are effective on improving blood lipids status without bringing about a significant hepato- or nephro-toxicity. The mechanisms by which aqueous extracts of the plant leaves reduced serum lipids are unclear. Other point, our data seemed to contradict a study of report Adam *et al*.,(2002) who found that cholesterol levels were elevated in sheep fed on 0.25 g/kg/day *Rhazya stricta* leaves for 42 days. This is could be due to the relatively high dose of the plant extract. Another study reported that chronic treatments of rats or mice with *Rhazya stricta* lyophilized extract at oral doses of 0.5, 1, or 2 g/kg for 28 days produced no significant effect on any of the hematologic or biochemical indices measured Tanira et al., (1996). This suggests that the method of extraction is affecting the plant leaves. Most researches have used lyophilized extract containing about 18.3% of the original material and aqueous solutions were prepared from this lyophilized product Rasheed et al.,(1994) and Ali et al.,(1999). It has been reported previously that the leaves of the plant contain volatile bases and probably other substances, Ahmed et al.,(1983)and AL-Yahya et al.,(1990), which might alter ratio/properties of the other constituents of the extract prepared from the lyophilized leaves using a freeze drier. Confirming to this notion is that freeze-drying may diminish some medicinal plant actions, Abascal et al.,(2005).Therefore, researchers and practitioners should carefully consider how the use of freeze-dried material may affect pharmacological and clinical study results.

In conclusion, the marked hypotriglyceridemic and hypocholesterolemic effect of this plant may have therapeutic implications on patients with hypertriglyceridemia and hypercholesterolemia. However, more work is needed , with the world growing interest in complementary and alternative medicinal investigations, to explore possible mechanisms of action of *Rhazya stricta* leaves in human cardiovascular disorders, using the same method of extraction that has been used by humans in the folk medicine. Furthermore, the mutagenicity of the leaf extract demonstrated by

Baeshin's *et al(2005),and submitted).*, should be taken in consideration , as how to be avoided during therapeutic implications.

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References

1. Abascal K, Ganora L, Yarnell E. (2005). The effect of freeze-drying and its implications for botanical medicine. *Phytother Res*., 19(8): 655-660.
2. Adam SE, Al-Yahya MA, Al-Farhan AH. (2002). Toxicity of Nerium oleander and *Rhazya stricta* in
3. Najdi sheep: hematologic and clinicopathologic alterations. *Am J Chin Med*., 30(2-3): 255-262.
4. Ahmad Y, Fatima K, Le-Quesne PW. (1983). Further alkaloidal constituents of the leaves of *Rhazya stricta*. *Phytochem*, 27: 1017-1019.
5. Ageel AM, Mossa JS, Al-Yahya MA, Tariq M, Al-Said MS. (1987) Plants Used in Saudi Folk Medicine. King Abdul Aziz City for Science and Technology. Riyadh (KSA): King Saud University Press; AL-Yahya MA, AL-Meshal IA, Mossa JS, AL-Badr AA, Tariq M. (1990). Saudi Plants: A

Phytochemical and Biochemical Approach. King Abdul Aziz City for Science and Technology. Riyadh (KSA): King Saud University Press.
6. Ali BH, Bashir AK, Banna NR, Tanira MO. (1995). Central nervous system activity of *Rhazya stricta* Dence in mice. *Clin Exp Pharmacol Physiol*, 22: 248-253.
7. Ali BH, Tanira MO, Bashir AK, Al-Qarawi AA. (2000). Effect of *Rhazya stricta* Decne on monoamine oxidase and cholinesterase activity and brain biogenic amine levels in rats. *J Pharm Pharmacol*, 52(10):1297-1300.
8. Ali BH, Al- Qarawi H, Mousa HM, Bashir AK, *et al*. (2000). Concentration of amino acids in brains of mice treated with the traditional medicinal plant *Rhazya stricta* Dence. *Indian J Pharmacol*., 32: 253-254.
9. Ali BH, Bashir AK, Tanira MOM. (1999). The effect of *Rhazya stricta* Decne, a traditional medicinal plant, on spontaneous and drug-induced alterations in activity of rats. *Pharmacol Biochem Behav*., 64(3):455-460.
10. Al-Homidan, A, Al-Qarawi AA, Al-Waily SA, Adam SE. (2002). Response of broiler chicks to dietary *Rhazya stricta* and Nigella sativa. *Br Poult Sci*., 43(2):291-296.
11. Ali BH, Bashir AK, Tanira MO, Banna S. (1995). Some central nervous system actions of *Rhazya stricta* in mice. *Clin Exp Pharmacol Physiol*., 20: 496- 502.
12. Ali BH, Bashir AK, Tanira MO. (1998). The effect of *Rhazya stricta*, a traditional medicinal plant, on the forced swimming test in rats. *Pharmac Biochem Behav*, 59: 547-50.
13. Bashir AK, Abdalla AA, Wasfi IA, Hassan ES, Amiri MH, Crabb TA. (1994). Phytochemical and antimicrobial studies on the leaves of *Rhazya stricta* growing in United Arab Emirates. *Fitotrepia*, LXV: 84-85.
14. Baherji A, Majumden L, Chaterjee A. (1970). Occurrence of geissoschizine and other minor bioenergetically related alkaloids in *Rhazya stricta Phytochemistry*, 9: 1491.
15. Bashir AK, Abdalla AA, Hassan ES, Wasfi IA, Amiri MH, Crabb TA. (1994). Alkaloids with antimicrobial activity from the roots of *Rhazya stricta* Dence growing in United Arab Emirates. *Arab Gulf J Sci Res*., 12: 119- 131.
16. Baeshin, N. A., Twaty, N. and Al-Hebshi, A. (2005). Evaluation the genotoxicity of *Rhazia stricta* leaves extract by the *Saccharomyces cerevisiae* auxotrophic mutant test. *Egyp. J. Nat. Toxi*., 2: 87-100.
17. Baeshin, N. A; Qari, S. H.; Sabir, J. S. M. and ALhejin, A. M. (2008) Biochemical and Molecular Evaluation of Genetic Effects of *Rhazya stricta* (Decne) Leafs Extract on *Aspergillus terreus.* Saudi Journal of Biological Sciences, 15 (1) 25-33.
18. Baeshin, N. A; Sabir, J. S. And Qari, S. H. (2009). Cytogenetic and molecular evaluation of genetic effects of leaf extract of *Rhazya stricta* (Decne) on *Allium cepa* root tip meristem. *Egypt. J. Cytol*., 38: 73-83.
19. Baeshen N. A; , Sabir, J. S.; Abo-Aba, S. E and Qari, S. H. Evaluation of Cytogenetic and Molecular Effects of Aqueous Extract of *Rhazia stricta* Leaves on Human Lymphocytes *in vitro*. (submitted).
20. Rahman AU, Fatima T, Khanum S. (1988). β Hydroxyvincadiffuromine, an alkaloids from *Rhazya stricta*. *Phytochemistry*, 27: 3721- 3723.
21. Rasheed RA, Bashir AK, Ali BH, Padmanabhan R. (1997). Effect of *Rhazya stricta* on the developing fetus. *Reprod Toxicol*., 11(2-3): 191- 199.
22. Tanira MO, Ali BH, Bashir AK, Chandranath I. (1996)Some pharmacologic and toxicologic studies on *Rhazya stricta* Decne in rats, mice and rabbits. *Gen Pharmacol*., 27(7): 1261-1267.
23. Tanira MO, Ali BH, Bashir AK, Dhanasekaran S, Tibirica E, Alves L. (2000). Mechanism of of the hyptotensive action of *Rhazya stricta* leaf extract in rats. *Pharmac Res*., 41(3): 369- 378.
24. Wasfi IA, Bashir AK, Amiri MH, Abdalla AA. (1994). The effect of *Rhazya stricta* on glucose homeostasis in normal and streptozotocin diabetic rats. *J Ethnopharmacol*., 43(2): 141- 147.
25. Western AR.(1989) The flora of the United Arab Emirates: An introduction. United Arab Emirates University. Dubai (UAE): Al Bayan Commercial Printing Press;

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Some biochemical Studies on Friesian Suffering from Subclinical Mastitis

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Abstract: The present study was conducted to investigate the effect of subclinical mastitis on clinicopathological changes in Mastitic friesian. A total of 400 individual milk samples from clinically normal udder quarters of 100 diary friesians were examined microbiologically as well as by using California mastitis test (C.M.T.) for detection of subclinical mastitis and designing rapid diagnostic tests for other infection. Blood samples were analysed for hemogram, cortisol, alanine aminotransferase, asparate aminotransferase, total protein, inorganic phosphorous and calcium. Also L.DH in milk was detected. The results indicated that there is a significant elevation of cortisol, Sgot, p.cv, L.DH activity in milk while a notable decrease in total protein, serum calcium and Hemogram. was observed. However; Serum phosphorous level did not exhibit obvious changes**.** [Nature and Science. 2010;8(4):143-146]. (ISSN: 1545-0740).

Key words: Microbiology of mastitis, Pathology of mastitis, Enzymes in mastitis, changes in blood

1. Introduction

Mastitis is the most frequent, disease responsible for early culling of milking animals, this culling sometimes takes place before the animal reaches the age of maximum production. The colonization of mamary gland by pathogenic microorganisms results in series events which lead to major alteration in the composition of milk, and on the disease set in inflamatory reactions takes place for several days (Elsagheer *et al,* 1992). Preacute coliform mastitis is of great economic importance in the dairy industry since the infection with coliform organisms and the following production of endotoxin leads to high mortality. Akira (1989)reported that when diagnosis of preacute coliform mastitis is given by clinical signs and hematological hematobiochemical findings only misdiagnosis on the prognosis is common. Thus the presence of endotoxin in blood plasma should be checked for the precise diagnosis of bovine preacute coliform mastitis. This is characterized by severe quarter Inflammation (Schalm *et al,* 2006). Stress in the form of muscular exertion causes alterations in the different blood constituents (Agarwal *et al,* 1984; Bhasrekar *et al,* 1984 and Cabona *et al,* 1990). This work was intitiated so as to investigate clinopathological changes among infected friesian. The aim of the present work was also to study the bacteriological incidence of subclinical mastitis among friesian and to find the relationship between clinicopathological changes and mastitis in subclinical mastitic friesian.

2. Material and Methods

For conducting this work 400 milk samples were aseptically collected for clinically normal quarters of friesian selected from Mounofia governorate. Samples were examined using the following tests:

(A) California mastitis test C.M.T. according to the procedure described by American Public Health.

(B) Microbiological examinations which include cultivation of milk sediment. The milk sediment obtained by centrifugation of 10 ml of the samples for 20m at 3000 rpm was seeded on a plates of nutrient agar, blood agars, Edward medium, Mackonkey's agar and subarouds dextrose agar.

Examination of incubated milk

Loopfuls from the incubated samples over night at 37^{o} were streaked on the same forementioned media and inoculated plates were incubated at 37° for 48hr except sabarouds agar plates which were incubated at 25° and checked daily for the growth of fungi for 3 weeks. Suspected colonies appearing on different

media were examined microscopically and ^identification was carried out according to Ajello *et al.* (1966) and El-Sagheer *et al.* (1992).

Blood samples from friesian were collected by jugular venepuncture in test tubes with or without EDTA.

Serum was harvested by centrifugation at 3000 rpm. Calcium, Inorganic phosphorous, total protein Sgot, Sgpt were determined in serum using kits from Diamond Diagnostica company, Egypt and measured by spectrophotometer in the UV range (240nm). Cortisol was assayed by R.I assay technique using kits from Diagnostic Products Coporation, Los Angles USA, according to method of Kowalaski (1976). A complete blood picture was manually performed as outlined by Jain (1986).

LDH assay

L.D.H in milk was measured by specials kits according to methods of Kachmar and Moss (1976).

The samples were processed by centrifugation to remove fat and pellet and intermediate layers obtained were further centrifuged at 30,000 Xg for 15 min essentially according to the method of Bagin *et al.* (1977). The supernatent obtained was filtered through filter paper and used as the enzyme source.

LD.H activity was assayed spectrophotometrically at 340nm by special kits according to Kachmar and Moss (1976).

3. Result

The results obtained from Table 1 revealed that out of 100 lactating friesian 14% were infected with *E.coli* 6% infected with S.*agalactia* 3.5% S. *aureus &* 1.5% *Pseudomonas aeruginosa.*

From the Table 1 it was obvious that C.M.T. reaction is positive for friesian with preacute coliform mastitis. Further prognostic diagnosis have commonly been done based on clinical symptoms and hematological & biochemical examinations.

ABLE 1. Bacteriological examination of infected friesian.

100 lactating friesian	**14% infected with E. coli**	6% **S. Agalactia**	**3.5% Pseudomonas Aeruginosa**	**1.5% S. Aureus**

The Hemogram showed a significant decrease in R.B.Cs, P.C.V. and hemoglobin while there was a significant increase in E-SR & W.B.C.S ($p<0.01$) (Table 2).

Cortisol levels

The present investigation (Table 3) indicated that mastitic cows had significant elevation of serum Cortisol levels as compared with non mastitic cows ($p<0.01$).

Liver junctions

Enzymatic activity

Sgot values were significantly ($p < 0.01$) higher in mastitic cows while Sgpt values revealed no obvious changes as compared with control group.

Calcuim and Inorganic phosphorous

Slight non significant decrease were recorded in calcium level while inorganic phosphorous revealed no obvious changes.

Total protein

T.P. values were significantly lower in mastitic cows ($p < 0.01$). ***L.DH in milk***

L. DH level in milk were significantly higher in mastitic cows ($p < 0.01$).

4. Discussion

Subclinical mastitis is of great economic importance *ixy* the dairy industry since the infection with coliform organism leads to high mortality. When diagnosis is given by clinical signs only Ederhart (2007) suggested further investigations including hematological and biochemical investigations for confirmation. It is clear that incidence of subclinical mastitis among examined dairy cows is relatively high with reduction in milk yield which causes a heavy economic losses. The colonization of mamary glands by pathogenic microorganisms results in a series of events which lead to major alterations of milk compositions secreted from cells. Therefore C.M.T is a suitable measure for use on large scales monitoring programs. Elsagheer *et al.* (1992) suggest that application of C.M.T. leads to early detection of subclinical infected quarters and aids in the selection

of dairy animals for either segregation or therapy for less than costs of the disease including the large losses in milk production for cows with preacute coliform mastitis. The level of LDH seems to increase in mastitic milk, (Kerumori *et al*, 1989).

Table 2. Effect of mastitis on Hemogram of infected cows.

E. coli	Hemoglobin g/dl	P.C.V%	R.B.C.S 10^6/wd	W.B.C.D 10^3/wd	E.SR mrn/2hrs
Control	9.10 ±0.23	31 ± 0.32	8.10 ±0.18	10.35 ±0.64	1.03 ±0.092
Infected	8.60 ±1.94*	27.5 ±0.62	6.33 ±0.90	12.27 ±0.053*	1.83 ± 0.045**
Streptococcous agalactiae					
Control	9.9 ±0.20	32 ±0.67	9.3 ±0.74	10.00 ±0.26	1.00 ±0.35
Infected	7.80 ±0.58**	31 ±0.070*	8.8±0.80*	13.00 ±0.63*	1.72 ±0.072** '
S. oureus					
control	9.45 ±0.05	34 ±0.69	9.00 ±0.07	10.57 ±0.71	1.23 ±0.021
Infected	7.94 ± 0,08**	28.00 ± 0.23**	8.40.09*	14.00 ±0.53**	2.2 ± 0.052**
Pseudomonas Aeruginosa					
Control	9.3 ± 0.13	32 ±0.72	9.00 ±0.74	10.00 ±1.83	1.00 ±0.64
Infected	7.00 ± 0.69	30 ± 0.93*	8.03 ±0.33*	12.00 ±0.77*	2.0 ±0.0.82**

** $p < 0.01$ * $p < 0.05$

Table 3. Effects of subclinical mastitis on Biochemical changes and Cortisol hormone level of infected cows.

E. coli	Total protein	L.DH activity U/ml	Cortisol mg/dl	Sgot U/L	Sgpt U/L	Calcium mg/dl	phosphorous mg/dl
control	7.95 ±0.73	57.3 ±2.23	0.93 ±0.32	75.3 ±0.64	13.23 ±0.37	8.93 ±0.74	6.94 ±0.78
Infected	6.33 ±0.27	10.14 ±0.62**	l.34±0.23	16.3 ±68**	L5.00 ±0.26*	7.10 ±1.09*	6.74 ±0.53
Streptococous agalactia							
control	7.84 ±0.14	67.00 ±0.40	0.70 ±0.13	80 ±0.62	12.00 ±0.074	8.00 ±0.52	7.00 ±0.35
Infected	6.10 ±0.37*	127 ±17**	1.83±0.29**	1.94 ±54**	14.00 ±0.19**	7.33 ±0.34**	6.89 ±0.92
S. aureus							
Control	7.90 ±1.23	50 ±0.27	0.90 ±0.54	94 ±0.40	13.00 ±1.23	8.73 ±0.51	7.5 ±0.68
Infected	6.59 ±0.22*	134±16**	1.91±0.82**.	158±l3**	14.3 ±1.73*	7.10±0.14**	6.8±0.88
pseudomonas aeruginosa							
control	7.97 ±0.62	73 ±0.48	0.83 ±0.34	0.94 ±1.20	14.00 ±0.73	8.51 ± 0.27	7.00 ±0.23
Infected	6.83±0.33	178±50**	1.93 ±0.33**	139 ±1.00*	1.48 ±1.70*	7.85 ± 0.34*	7.79 ±0.40

**** p< 0.01 * p < 0.05**

Prognostic diagnosis have commonly been done on clinical symptoms and the hemotological and biochemical examinations blood. Biochemical analysis of mastitic animals may help in diagnosis of subchemical abnormalities and become a helpful means for practice under field conditions (Rose, 1987). The present results in mastitic cows fell in the range given by Jain (1986) and Koneko (1989).

As shown in Table 1 the significant changes in hemogram and other biochemical values are due to infection with mastitis. The highly significant increases detected in Sgot values & Cortisol are in line with the results of Sloss & Dufty (1980), Symons *et al.* (1974). Agarowal *et al.* (1984) however attributed these changes to stressful conditions. In the present study we have shown that L.D.H activities were enhanced in mastitic milk. The enhancement can be at least partly explained by the participation of leucocytes which have L. DH activity at the 1,000 U/mg protien level in mastitic milk (Kasumori *et al*., 1989). Protien concentration as well as somatic cell

count and L. DH is increased when compared to normal milk.

In the present investigation we have also measured L. D.H of 4 species of bacteria which were isolated from the mastitic milks used in the present study .and the activity was detected in the extracts of *E. coli, S. aureus, S. agolacaltiae & Pseudomonas aeroginosa.* The enzyme activities were much higher in case of the infected udders as compared to the control.

We could not determine the pattern of mastitic udders because the udders contained large number of leucocytes by washing small pieces of udder tissue with mechanical shaking.

Conclusively in mastitic animals the application of C.M.T. leads to early detection of subclinically infected quarters and aids in the selection of dairy animals for either segregation or therapy. Also we conclude that mastitis causes anemia in cows detected by dercrease of hemoglobin, R.B.C.S, and P.C.V. L.D.H activity in milk increases, as well as Cortisol, Sgot and calcium in serum.

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References

1. Agarwal, S.P., Agarwal ,V.K., and Dwaraknoth, P.K. (1984) Effect of graded work load on circulating levels of Cortisol and thyroid hormones incros bred bullocks. Nat. Anim. phsiol. Research work conf. Thiland A.P. 4.
2. Akira, A. (1989) Detection endotoxin in affected milk from cows with coliform mastitis, Japanese J. Vet Science 41 (4) 845.
3. Ajella, L., George, L.K., Kaplow and Houfman (1966) Laboratory Manual for Medical Mycology, M.S. Dept of Health Education and Walfor Georgia
4. American Public Health Association APH (1978) "Standard Methods for Examination of Dairy Products" 14th ed., Amer public health Ass. Washington.
5. Bagin, E., Zin Advidor, J., Rivetz, B., Gordin, S. and Saran (1977) Distribution of lactate dehydrogenase isoenzymes in normal and inflammed udders and milk, Res. Vet Sci. 22, 198.
6. Bhasrekar, M.R., Parage, M.S., Jashi, B.M., and Mangurkar, B.R. (1984) Draftability of crossbed bullocks in comparison to local bullock, Nat Animal Physiology Research Work Conf .Thialand A. P8.
7. Cabona, E.M., Adriano, E.A. and Encarnacion, R.O. (1990) Hematologic observations on swamp buffallow harvested to increased on pulling, lods Buffallow Bull. 9, 27.
8. Ederhart, R.J. (2007). Am. Vet. Med. Assoc. 206, 1973.
9. Elsagheer, M.A., Ahmed, Liala Ali and Hegazi, A.G. (1992) Proc. Inter. Meet work Animal, 134.
10. Join, W.C.(1986) "Schalm's Vet Hematology" 4th ed lee and Febiger Philedelphia U.S.A.
11. Kerumori, K., Kanae, M. and Mario, K. (1989) Contribution of leucocytes to the origin of lactate dehydrogenase by isoenzymes in milk of bovine, Mastitis Japanese Journal of Vet. Science, 530.
12. Koneka, J.J. (1989) Clinical Biochemistry of Domestic Animals 4th ed Academic press In New York U.S.A.
13. Kowalaski, A. and Paul, W. (1976) Determination of serum Cortisol, Clin Chemistry (5) 1152p.
14. Rose, R.J. (1987) Poor performance syndrome investigation and diagnostic technologies in current therapy in equine medicine, (Ed) W.E Robinoson, Philadelphia W.B. Saunders.
15. Schalam, O.W., Caroll, E.J. and Jain, W.C. (2006) Bovine Mastitis Febriger philadephia.
16. Sloss, V., and Dufty, J.H. (1980) Hand Book of Bovine Obstetrics. Williams and Wilkins Blathimore, London.
17. Sndecor, C.W., and Cochran, W.B. (1976) "Statistical Methods". 6th ed Iowa State University Press Iowa U.S.A.
18. Symons, D.A. and Wright, L.J. (1974) Changes in bovine mamary gland permeability after intramamary exotoxin infusion, J. Comp. Pathology, 84, 9.
19. Verma, R. (1988) Studies on clinical and subclinical bovine mastitis, Indian Journal of Comparative Microbiology Immology and Infectious Disease. 9 (1) 28.

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Bioaccumulation and histopathological alterations of the heavy metals in *Oreochromis niloticus* fish

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Abstract: Copper, lead, cadmium and mercury concentrations were recorded in water and tissues of *Oreochromis niloticus* from Egyptian fish farms in 2007-2009. Histopathological alterations in fish tissues were also studied. Bioconcentration factors of copper, lead, mercury and cadmium in liver and muscle tissue were (3.93 & 3.87), (8.10 & 7.60), (0.79 & 50.0) & (38.25 & 30.25), respectively. Mercury was the most bioaccumulated and biomagnified metal in the muscles, while Cu was the least. The concentration of cadmium, lead and copper were highest in liver and lowest in kidney tissue, while mercury (Hg) concentrations were highest in muscles, lowest in kidney tissue. Several histopathological changes were noted in muscles, liver, gills, kidney and intestine tissue attributable to heavy metals exposure. [Nature and Science. 2010;8(4):147-156]. (ISSN: 1545-0740).

Key words: Bioconcentration, copper, lead, cadmium, mercury, Tilapia, Pollution, histopathology.

1. Introduction

Metal contamination of aquatic ecosystems has long been recognized as a serious pollution problem. When fish are exposed to elevated levels of metals in a polluted aquatic ecosystem, they tend to take these metals up from their direct environment (Seymore 1994). Heavy metal contamination may have devastating effects on the ecological balance of the recipient environment and a diversity of aquatic organisms (Farombi *et al*. 2007).

Transport of metals in fish occurs through the blood where the ions are usually bound to proteins. The metals are brought into contact with the organs and tissues of the fish and consequently accumulated to a different extent in different organs and tissues of the fish. Most heavy metals released into the environment find their way into the aquatic environment as a result of direct input, atmospheric deposition and erosion due to rainwater, therefore aquatic animals may be exposed to elevated levels of heavy metals due to their wide use for anthropogenic purposes (Kalay and Canli, 2000). Heavy metals are non-biodegradable and once they enter the environment, bioconcentration occurs in the fish tissue in the case of aquatic environment, by means of metabolic and biosorption processes (Wicklund-Glynn 1991).

Heavy metals such as cadmium, lead, cupper and more specifically mercury are potentially harmful to most organisms even in very low concentrations and have been reported as hazardous environmental

Pollutants able to accumulate along the aquatic food chain with severe risk for animal and human health.

Toxic heavy metal contamination mostly occurred in aquaculture farms and frequently occurs in groundwater, rivers, estuaries, wetland and coastal areas. Of particular concern are the highly toxic non-nutrient elements such as mercury (Hg), lead (Pb), and cadmium (Cd).

The presence of pollutants have been associated with decreased fertility and other reproductive abnormalities in birds, fish, shellfish and mammals and also altered immune function. Heavy metals like mercury and cadmium are known to accumulate in marine organisms and cause rapid genetic changes (Nimmo *et al.* 1978, Nevo *et al.* 1986).

The toxicity of these elements is due to their ability to cause, oxidative damage to living tissues. Damage includes enhanced lipid peroxidation, DNA damage, enzyme inactivation and the oxidation of protein sulfydryl groups (Taiz and Zeiger 1998). Toxic heavy metal can cause dermatological diseases, skin cancer and internal cancers (liver, kidney, lung and bladder), cardiovascular disease, diabetes, and anaemia, as well as reproductive, developmental, immunological and neurological affects in the human body. Metal contamination sources are typically derived from natural sources: mining, industrial waste discharges, sewage effluent, harbor activities and agrochemicals etc.

It is also possible that environmental toxicants may increase the susceptibility of aquatic animals to various diseases by interfering with the normal functioning of their immune, reproductive and developmental processes (Couch and John, 1978).

Prolonged exposure to water pollutants even in very low concentrations have been reported to induce morphological, histological and biochemical alterations in the tissues which may critically influence fish quality.

According to EPA guidelines, "the BCF

(Bioconcentration Factors) is defined as the ratio of chemical concentration in the organism to that in the surrounding water. Bioconcentration occurs through uptake and retention of a substance from water only, through gill membranes or other external body surfaces. In the context of setting exposure criteria it is generally understood that the terms "BCF" and "steady-state BCF"' are synonymous. A steady-state condition occurs when the organism is exposed for a sufficient length of time that the ratio does not change.

The present study was carried out to investigate the bioaccumulation of heavy metals (lead, copper, cadmium and mercury) in the tissues of *Oreochromis niloticus* and to determine the histopathological changes caused by the residues of these metals in their organs.

2. Material and Methods

SAMPLING

The water samples were obtained from different farms derived their water supply from some River Nile ramifications. Forty eight water samples and one hundred adult freshwater tilapia (*Oreochromis niloticus*) ranged between 100- 150 g in weight were collected from 12 Tilapia farms located in 6 Governorates (Kafer Al-Sheikh, Ismailia, Kaliobea, Damiatta, Al-Fayum and Behera) during 2007-2009. At laboratory, the fish samples were washed with deionized water and wrapped separately in acid washed polyethylene bag and stored frozen at -20°C until analysis was carried out.

PROCEDURES:

PREPARATION AND ANALYSIS OF WATER SAMPLES:

The analysis of water samples was carried out according to A.P.H.A. (1992). The water samples were preserved by the addition of one ml of concentrated nitric acid per liter until the time of analysis. The water samples were filtered through 0.45µl membrane filter. The required volume (100 ml) of the filtrate was collected to measure lead, cadmium, mercury and copper levels in water samples by using Air/Acetylene Flame Atomic Absorption Spectrophotometer (UNICAM 696 AA Spectrometer). Flameless Atomic Absorption Spectrophotometer equipped with (MHS) mercury hydride system "Cold Vapour Technique" was used for determination of mercury levels in examined water samples.

PREPARATION AND ANALYSIS OF FISH SAMPLES:

Procedure (A): Each sample was represented by one gram of tissues dissected from the gills, liver, kidney and muscles, then placed in a clean screw-capped tube and digested according to the method described by Finerty *et al*. (1990). The obtained solutions were then analyzed by using Air/ Acetylene Flame Atomic Absorption Spectrophotometer (UNICAM 696 AA Spectrometer) for determination of copper (Cu), lead (Pb), cadmium (Cd) and mercury (Hg) levels in examined samples.

Procedure (B): The measurement of the mercury concentration in examined fish samples was carried out at minimal temperature for all fish samples where 0.5 gram macerated fish tissues was digested according to the technique described by Diaz-Ravina *et al*. (1994). About 5 ml stannous chloride solution were added to the obtained solutions to reduce mercury to elemental form and then analyzed by using Flameless Atomic Absorption Spectrophotometer equipped with "MHS" mercury hydride system "Cold Vapour Technique".

HISTOPATHOLOGICAL EXAMINATION

Tissue specimens from fresh Nile Tilapia were taken (gills, muscles, livers, intestine and kidney) and fixed in 15 % buffered neutral formalin. They were processed to obtain five micron thick paraffin sections then stained with Hematoxylin and Eosin (Bancroft *et al.*, 1996) and examined under light microscope.

STATISTICAL ANALYSIS

Data were analyzed using Analysis of Variance (ANOVA) and means were separated by Duncan at a probability level of < 0.05 (SAS Institute 2000).

3. Result

Results are shown in Table 1 (Heavy metal concentrations in water of Nile Tilapia farms) and Table 2 (Concentration of heavy metals in fresh Nile tilapia tissues). Figure A-1-12 **(**The histopathological alterations in Tilapia tissues) , Figure (B)-2(Mean concentrations of Cu, Pb, Cd and Hg in water of fish farms in different Governorates and the permissible limits according to WHO,1984) and Figure (C)-3 (Mean residual accumulations of Cu, Pb, Cd and Hg in tissues of *Oreochromis niloticus* and the permissible limits according to WHO,1984).

Table 1, showed that the mean concentration of copper in water of Tilapia farms was 0.65 ± 0.01 ppm, while Table 2 showed the mean concentrations of copper in gills, liver, kidney and muscles of Tilapia (were 4.8 ± 0.05 , 2.56 ± 0.21, 1.52 ± 0.06 and 2.54 ± 0.05 ppm, respectively). The BCF of copper in liver and muscles was 3.93 and 3.87, respectively. The mean concentration of lead in water of Tilapia cultures was 0.20 ± 0.07 ppm, whiles the mean concentrations of lead in gills liver, kidney and muscles, were 0.483 ± 0.05, 1.523 ± 0.02, 0.155 ± 0.02 and 1.521± 0.02 ppm,

respectively. The BCF of lead in liver and muscles was 8.10 and 7.60 ppm, respectively. The mean concentration of cadmium in water of Tilapia farms was 0.04 ± 0.009 ppm, while the mean concentrations of cadmium in gills liver, kidney and muscles were 0.891 ± 0.05 , 1.523 ± 0.02, 0.212 ± 0.02 and 1.21 ± 0.05 ppm, respectively. The BCF of cadmium in liver and muscles was 38.25 and 30.25 ppm, respectively.

The mean concentration of mercury in water of Tilapia farms was 0.07 ± 0.009 ppm, while the mean concentrations of mercury in gills liver, kidney and muscles were 0.04 ± 0.002, 0.055 ± 0.003, 0.020 ± 0.005 and 3.50 ± 0.22 ppm, respectively. The BCF of mercury in liver and muscles was 0.79 and 50 ppm, respectively. The histopathology of different Tilapia tissues revealed that there are several histopathological changes in different Tilapia organs (muscles, liver, gills, kidney and intestine) as shown in Figure (A1).

Gills showed mild congestion and edema of the primary lamellae (Figure A1-8). Severe edema, hyperplasia, fusion and focal desquamation of the epithelial lining of the secondary lamellae as seen in Figure (A1)-9. The gill arch, especially at the bases of the gill filaments, showed numerous mononuclear leukocytic infiltration, edema and congestion. The apex of gill filaments showed congestion, hyper activation of the mucous and chloride cells with epithelial vacuolation of the secondary lamellae.

Liver showed degeneration of the hepatocytes and intravascular haemolysis in blood vessels as shown in Figure (A1 -2), congestion of central vein, hemorrhages (Figure A1-3), nuclear pyknosis in the majority of hepatic cells (FigureA1-4) and the metal-binding proteins were accumulated in the nuclei of hepatocytes 42% of the examined adult freshwater tilapia *(Oreochromis niloticus)* were showed histopathological alterations.

Kidney The kidney is composed normally of numerous renal corpuscles with well developed glomeruli and a system of tubules. The proximal segment is covered by tall columnar epithelial cells with basal nuclei and brush border located along the cell apices. The distal segment was lined with large, relatively clear columnar epithelial cells with central nuclei and the brush border was reduced or not present. The glomerulus is larger in diameter than the distal segment, containing columnar epithelial cells with basal nuclei and no brush border (Figure A1-10). In our study, the kidney showed hydropic swelling of tubules, sometimes with pyknotic nuclei and many necrotic areas as well as swollen proximal epithelial cells with necrotic nuclei as noticed in Figure A1- 11.

Muscular tissues Several histopathological alterations were seen in the muscles of Tilapia which included degeneration in muscle bundles with aggregations of inflammatory cells between them and focal areas of necrosis. Also, atrophy and edema of muscle bundles as well as splitting of muscle fibers were seen as in Figure A1-6.

The pathological findings in the intestine included atrophy in the muscularis, degenerative and necrotic changes in the intestinal mucosa and submucosa with necrotized cells aggregated in the intestinal lumen, edema and atrophy in the submucosa as shown in Figure A1-12.

Table 1. Heavy metal concentrations in water of Nile Tilapia farms.

	In water samples (mg/L)			Occurren-ce %
Metal	Min.	Max.	Mean ± SE	
Copper	0.044	0.887	0.65 ± 0.01	35%
Lead	0.04	0.29	0.20± 0.07	82%
Cadmium	0.001	0.082	0.04± 0.009	72%
Mercury	0.01	0.11	0.07 ± 0.009	12%

Table 2. Concentration of heavy metals (ppm) in fresh Nile Tilapia tissues.

Metal / Tissue	Copper	Lead	Cadmium	Mercury
Gills				
Mean	4.8±0.05	0.483±0.05	0.891±0.05	0.04±0.002
Min	1.32	0.02	0.11	0.002
Max	6.22	1.21	1.82	0.24
Liver				
Mean	2.56 ± 0.21	1.523 ±0.02	1.523 ± 0.02	0.055 ± 0.003
Min	1.22	0.01	0.20	0.001
Max	3.55	3.20	2.43	0.72
Kidney				
Mean	1.52±0.06	0.155±0.02	0.212±0.02	0.02±0.05
Min	0.21	0.11	0.09	0.002
Max	2.42	2.02	0.89	0.12
Muscles				
Mean	2.54±0.05	1.52±0.02	1.21±0.05	3.50±0.33
Min	0.21	0.892	0.55	1.32
Max	2.8	1.00	1.780	5.240

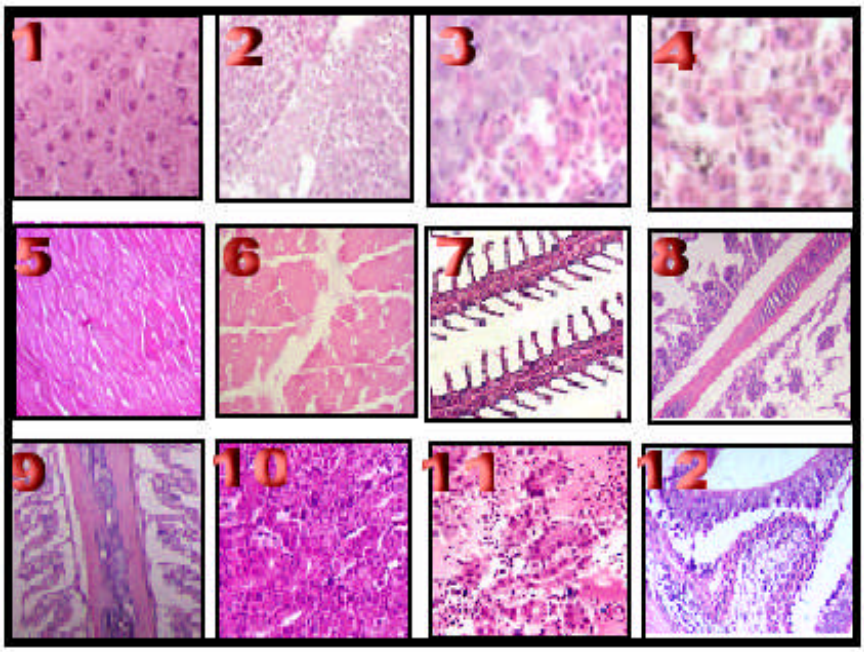

Figure A-1 (1-12).1: *Liver of Tilapia nilotica fish showing the normal structure (X400).* ***2:*** *Liver of Tilapia nilotica fish showed degeneration of the hepatocytes and intravascular haemolysis in blood vessels.* ***3:*** *Liver of Tilapia nilotica fish showing haemorrhage (X400).* ***4:*** *The liver showed congestion and central vein, nuclear pyknosis in the majority of hepatic cells. (X400).* ***5:*** *Muscle bundles of Tilapia nilotica fish showing the normal structure (X400).* ***6:*** *Degeneration in muscle bundles with focal area of necrosis (X400).* ***7:*** *Gills: Gills of Tilapia nilotica fish showing the normal structure (X100).* ***8:*** *Degenerative and necrotic changes in the epithelium of gill filaments and secondary lamellae (X400).* ***9:*** *Edema in secondary lamellae and gill filaments (X400).* ***10:*** *Kidney showing the normal structure (X400).* ***11:*** *Severe degenerative and necrotic changes in the renal tubules with focal areas of necrosis(X400) and aggregations of inflammatory cells.* ***12:*** *Degeneration, haemorrhage in the submucosa and aggregations of inflammatory cells in the mucosa and submucosa (X400).*

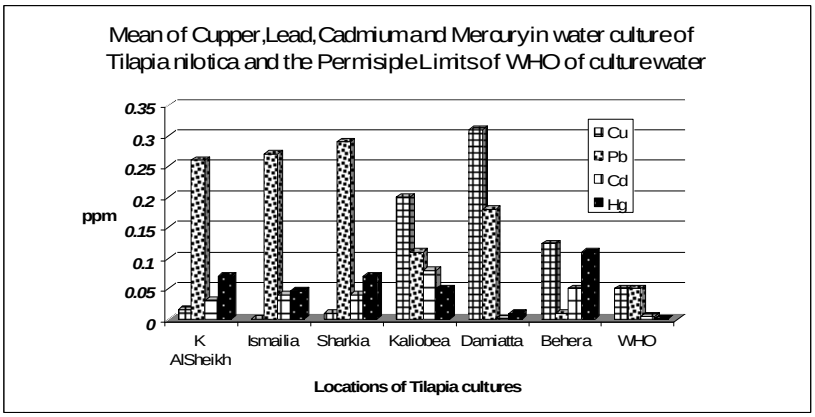

Figure (B)-2: Mean concentrations of Cu, Pb, Cd and Hg in water of fish farms in different Governorates and the permissible limits according to WHO (1984).

Table 3. Permissible limits of various heavy metals

Metal	Permissible	Country and reference
Copper	1.00 ppm 20.0 ppm 20.0 µg/g	WHO (1984) South Africa (Foodstuffs, cosmetics and disinfectants Act. No. 54 of 1972) Spain: Boletin Official del Estado (1991)
Lead	0.05 ppm 0.1 mg/kg 0.5 ppm 5.0 µg/g	WHO (1984) Egypt "E.O.S.Q.C. (1993) FAO/WHO (1992) Spain: Boletin Official del Estado (1991)
Cadmium	0.005 ppm 0.05 ppm 0.1 mg/kg 1.0 pg/g	WHO (1984) FAO/WHO (1992) Egypt "E.O.S.Q.C. (1993) Spain: Boletin Official del Estado (1991)
Mercury	0.001 ppm 0.5 mg/kg 0.5 ppm 1.0 µg/g	WHO (1984) Egypt "E.O.S.Q.C. (1993) FAO/WHO (1992) Spain: Boletin Official del Estado (1991), Schuhmacher and Domingo (1996)

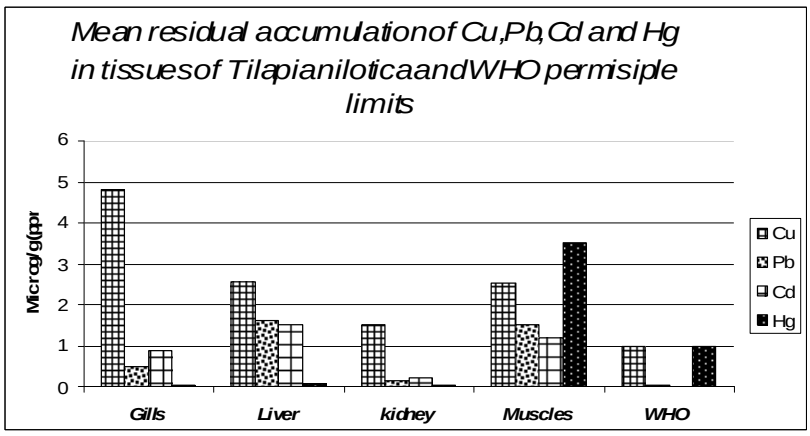

Figure (C)-3: Mean residual accumulations of Cu, Pb, Cd and Hg in tissues of *Oreochromis niloticus* and the permissible limits according toWHO (1984).

4. Discussion

Mean copper concentration in water of Tilapia farms was 0.65 ± 0.01 ppm and the maximum permissible limits recommended by WHO (1984) is 0.05 ppm, while in flesh was 2.54 ± 0.05 ppm. The recorded results of copper concentrations in fish were lower than the permissible limits intended by Foodstuffs, Cosmetics and Disinfectants (1972) [20.0 ppm] and Boletin Official del Estado (1991) in Spain [20.0 µg g–1] and Schumacher and Domingo (1996). The BCF were; 3.93 and 3.87ppm in liver and muscles, respectively.

It is shown from Table.1 that the lead concentration in Tilapia tissues was exceed the permissible limit recommended by E.Q.S.Q.C. (1993). This result was nearly higher than those reported by Seddek *et al*. (1996) and Marouf and Dawoud (2006), they recorded levels ranged from 0.42 to 0.74 ppm. This result was much higher than those recorded by Suppin *et al.*, (2005) and Celik and Oehlenschlager (2007), they recorded levels varied from 0.04 ppm to 76.1 ppb.

High levels of lead may be attributed to presence of industrial and agricultural discharges, motor boat traffics and also from mine and smelting operations.

Lead is non-essential element and higher concentrations can occur in aquatic organisms close to anthropogenic sources. It is toxic even at low concentrations and has no known function in biochemical processes (Burden *et al*., 1998). It is known to inhibit active transport mechanisms, involving ATP, to depress cellular oxidation reduction reactions and to inhibit protein synthesis (Waldorn and Stofen 1974). Lead was found to inhibit the impulse conductivity by inhibiting the activities of monoamine oxidase and acetylcholine esterase to cause pathological changes in tissue and organs (Rubio *et al*., 1991) and to impair the embryonic and larval development of fish species (Dave and Xiu, 1991).

Mean cadmium concentration in water of *Tilapia* farms was 0.04 ± 0.009 ppm and the maximum permissible limits recommended by WHO (1984) is 0.005 ppm, while in flesh was 1.21 ± 0.05 ppm. The recorded results of cadmium concentrations in fish were higher than the permissible limits intended by Boletin Official del Estado (1991) in Spain [l.0 µg g^{-1}], FAO/WHO (1992) [0.05 ppm] and Egyptian Organization for Standardization and Quality Control "E.O.S.Q.C".[0.1 mg kg^{-1}]. The BCF were; 38.25 and 30.25ppm in liver and muscles, respectively. This result agree with that obtained by Daoud (1999) who reported that the cadmium concentrations in water and fish were higher than the maximum permissible limits recommended by WHO (1984). The presence of cadmium in fish in Egypt was detected by Seddek (1996) with mean levels of 0.62 ppm in Oreochromis fish and 0.39 ppm in *Bagrus Byad* fish. Our result was nearly parallel to those reported by Celik and Oehlenschlager (2007) who recorded Cd concentration with levels varied from 0.1 to 0.8 ppm. Cadmium is highly toxic non-essential heavy metal and it does not have a role in biological processes in living organisms. Thus even in low concentration, cadmium could be harmful to living organisms (Burden *et al*., 1998). The value of cadmium accumulation in liver of Tilapia was (1.523 ± 0.02) µg g^{-1} dry weight. High accumulation of cadmium in liver may be due to its strong binding with cystine residues of metallothionein.

The high levels of Cd may be attributed to industrial and mining operations as well as the phosphate fertilizer which is considered the main source of Cd in the environment (Dimari *et al.* 2008).

Mean mercury concentration in water of Tilapia cultures was 0.07 ± 0.009 ppm and the maximum permissible limits recommended by WHO (1984) is 0.001 ppm, while in flesh was 3.50 ± 0.22ppm. The recorded results of mercury concentrations in Tilapia tissues were higher than the permissible limits intended by Boletin Official del Estado (1991) in Spain [1.0 µg g^{-1}], FAO/WHO (1992) [0.5 p.p.m] and Egyptian Organization for Standardization and Quality Control (E.O.S.Q.C) (1993) [0.5 mg kg^{-1}]. The BCF were; 0.79 and 50.0 in liver and muscles respectively. These findings coincide with those reported by Daoud *et al*. (1999) and Tantawy (1997).

Conama (2005) recommend a maximum concentration of 0.0002 mg Hg l^{-1} in water supplies used for rearing fish species destined for human consumption in Brazil. This value is very similar to those recommended by Malaysia National Water Quality Standards (Doe-Um, 1986). Meanwhile, the most notorious mercury compounds in the environment are monomethyl and dimethyl salt of mercury which are soluble. They are produced from inorganic mercury in sediment by anaerobic bacteria through the action of methyl-cobalamine and intermediate in the synthesis of methane and get into natural water (Manahan, 1989). The average (88.9%) of total mercury in fish musculature was in the form of methyl mercury (Bishop and Neary, 1974) which is lipid soluble and easily absorbed and distributed through biological system.

This element is one of the most toxic metals, which are introduced into the natural environment by human interferences (Buhl, 1997). Some papers have reported situations where high mercury levels were detected in water, mainly nearby gold extraction locations (Maurice-Bourgoin *et al*. 2000; Dolbec *et al*. 2001) and industrial zones (Kime 1998, Sunderland and Chmura 2000). According to Allen (1994), the exposure of *Orechromis aureus* to 0.5 mg Hg l^{-1} caused

a raise in the number of leukocyte and erythrocyte within 24 hours. Gill and Pant (1985) also reported hematological anomalies in *Barbus conchonius* exposed to 0.18 mg Hg l^{-1} in acute test.

It can be noticed that the highest bioaccumulation were observed in the organs mainly implicated in metals metabolism. The concentration of cadmium (Cd), lead and copper in tissues was high in the following order; liver> muscles > gills > kidney, while mercury (Hg) concentrations were high in the muscles > liver > gills > kidney. Oladimeji and Offem (1989) noticed that the gills of *Oreochromis niloticus* consistently accumulated higher amount of lead as lead nitrate.

BCF obtained for Pb, Cu, Cd and Hg in the muscles of Tilapia were all greater than 1.00ppm which indicated that the metals were highly bioaccumulated and biomagnified (according to Falusi and Olanipekun 2007). Mercury was the most bioaccumulated and biomagnified of all metals studied in the muscles of the *O. niloticus*, while Cu was the least one.

From the results of this study, the concentrations of different metals investigated in the tissues of Tilapia (gills, liver, kidney and muscle) except copper exceed the acceptable levels proposed for human consumption (USEPA 1995).

The histopathological alterations attributed to the prolonged exposure to heavy metals resulted in respiratory, osmoregulatory and circulatory impairment. These findings were demonstrated by Fernandes *et al*., (2008). Moreover, Alvarado *et al.* (2006) reported that, the dramatic increase of chloride cells in the gills that produces epithelial thickening of the filament epithelium enhances migration of chloride cells up to the edge of the secondary lamellae and provokes the hypertrophy and fusion of secondary lamellae. These could be considered as unspecific biomarker responses of heavy metals exposure and disturbed health of fish.

Gills showed edema of the primary lamellae; severe edema, hyperplasia, fusion and focal desquamation of the epithelial lining of the secondary lamellae were observed. According to Mallatt (1985), the edema of the gill epithelium is one of the main structural changes caused by the exposure to heavy metals. Our results confirm this lesion of heavy metals exposure. These alterations have been reported for other species exposed to heavy metals particularly Cd (Gardner and Yevich 1970; Karlsson-Norrgren *et al.* 1985; Pratap and Wendelaar Bonga 1993; Thophon *et al.* 2003) and sometimes referred as a first sign of pathology (Thophon *et al.* 2003). Cellular proliferation in the gill epithelium is also observed in fish exposed to different pollutants as described by Gardner and Yevich 1970 and Thophon *et al*. 2003. Lifting, swelling, and hyperplasia of the gill epithelium could serve as a defense function, as these alterations increase the distance across which waterborne irritants must diffuse to reach the bloodstream. Lamellar fusion could be protective once it reduces the amount of vulnerable gill surface area (Mallatt 1985). However, branchial responses that serve to slow entry of toxicants have the undesirable side effect of impairing gas exchange. This was described by Benson *et al.*, (1987) who observed a fall in respiratory function of *Notemigonus crysoleucas* exposed to Cd.

The liver showed degeneration of the hepatocytes, congestion of central vein and nuclear pyknosis in the majority of hepatic cells. These findings were apparent as the liver considered the organ of detoxification, excretion and binding proteins such as metallothionein (MTs). The metal-binding proteins were present in the nuclei of hepatocytes suggested that the increase in the cell damages (De Smet and Blust 2001). Similar results were observed by Van Dyk (2003) and Mela *et al.* (2007). Liver of fish is sensitive to environmental contaminants because many contaminants tend to accumulate in the liver and exposing it to a much higher levels than in the environment, or in other organs (Heath 1995).

Pandey *et al*., (1994) described the alterations in liver and intestine of *Liza parsia* exposed to Hg Cl2 (0.2 mg Hg l^{-1}) for 15 days. Similarly, Oliveira Ribeiro *et al.* (2002) reported serious injuries in gills and olfactory epithelium of *Salvelinus alpinus* exposed to 0.15 mg Hg l^{-1}.

Similar alterations in muscles and kidney of Tilapia were observed in several species of fish exposed to heavy metals and these alterations were described by Oliveira Ribeiro *et al*. (2002), Jiraungkoorskul *et al.* (2003), Thophon *et al*. (2003) and Gupta and Srivastava (2006).

The result indicates that the heavy metal contamination definitely affects the aquatic life of the fresh water fish. Hence, a scientific method of detoxification is essential to improve the health of these economic fish in any stressed environmental conditions. However, the high concentrations of the analyzed metals in the whole body tissues investigated could be due to the storage role played by these tissues.

Fish contaminated by heavy metals suffers pathological alterations, with consequent inhibition of metabolic processes, hematological changes, and decline in fertility and survival.

It can be conclusively deduced from this study that fish has the tendency to bioaccumulate heavy metals in a polluted environment. Since virtually all metals investigated were found in higher concentration, so government should intact laws that will ensure that industries make use of standard waste treatment plants for the treatment of their wastes before they are being discharged into water bodies.

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References

1. Allen P. 1994.Changes in the haematological profile of the cichlid *Oreochromis aureaus* (Steindachner) during acute inorganic mercury intoxication. *Comparative Biochemistry and Physiology* 108 C : 117-121.
2. Alvarado NE, Quesada K, Hylland I, Marigómez, L, Soto M. 2006. Quantitative changes in metallothionein expression in target cell-types in the gills of turbot (*Scophthalmus maximus*) exposed to Cd, Cu, Zn and after a depuration treatment. Aquatatic Toxicology 77: 64-77.
3. American Public Health Association (APHA) (1992). Standard methods for the examination of water and wastewater. 18th ed. American Public Health Association, Washington, DC.
4. Bancroft D, Stevens A, Turner R. 1996. Theory and practice of histological techniques. Fourth edn., Churchill Livingstone, Edinburgh, London, Melbourne.
5. Benson WH, Baer KN, Stackhouse RA, Watson CF 1987. Influence of cadmium exposure on selected hematological parameters in freshwater teleost, *Notemigonus crysoleucas*. Ecotoxicology and Environmental Safety 13: 92–96.
6. Bishop JN , Neary BP. 1974. The form of mercury in fresh water fish. Proc. Int. Conf. Transp. Persist. Chem. Aquatic Ecocyst, Ottawa, III-25-III-29.
7. Boletin Oficial Del Estado (1991). Separates del Boletin Oficial del Estado Espanol No. 195. Gaceta de Madrid, P: 27154. In: Schuhmacher, M. and Domingo, J. L. (1996).
8. Buhl KJ. 1997. Relative sensitivity of three endangered fishes, Colorado Squaw fish, Bonytail, and Razorback Sucker, to selected metal pollutants. *Ecotoxicology and Environmental Safety* 37: 186-192.
9. Burden VM, Sandheinrich CA, Caldwell A. 1998. Effects of lead on the growth and alpha amino levulinic acid dehydrates activity of juvenile rainbow trout, *Oncorhynchus mykiss*. Environmental Pollution 101: 285-289.
10. Celik U, Oehlenschlager D. 2007. High contents of Cd, Pb, Zn and Cu in popular fishery products sold in Turkish Supermarkets. Food Control 18: 258-261.
11. Conama 2005 Resolução no 357, de 17 de março de 2005. Brasília: Ministério do Meio Ambiente. Conselho Nacional do Meio Ambiente. Publicação D.O.U. 18/03/2005. Disponível em: http://www.mma.gov.br/port/conama/res/res05/ res35705.pdf Acesso em: 18/abril/2006.
12. Couch C, John A. 1978. Diseases, parasites, and toxic responses of commercial penaeid shrimps of the Gulf of Mexico and South Atlantic soasts of North America. EPA-600/J-78-072. U.S. Fish and Wildlife Service. Fishery Bulletin 76: 1-44. (ERL, GB 283).
13. Daoud JR, Amin AM, Abd El-Khalek MM. 1999. Residual analysis of some heavy metals in water and *Oreochromis niloticus* fish from polluted areas. Veterinary Medical Journal 47: 351-365.
14. Dave G, Xiu R. 1991. Toxicity of mercury, copper, nickel, lead and cobalt to embryos and larval of Zebra fish *Brachydanio rerio*. Archive of Environmental Contamination and Toxicology 21: 126-134.
15. De Castro E, Ferreira H, Cunha M, Bulcao C, Sarmento C, De Oliveira I , Fregoneze JB. 1996. Effect of central acute administration of cadmium on drinking behavior. Pharmacology Biochemistry and Behaviour 53: 687-693.
16. De Smet H, Blust R. 2001. Stress responses and changes in protein metabolism in carp (*Cyprinus carpio L.*) during cadmium exposure. Ecotoxicology and Environmental Safety 48: 255-62.
17. Diaz-Ravina M, Baath E, Frostegard A. 1994. Multiple heavy metal tolerance of soil bacterial communities and its measurement by a thymidine incorporation technique. Applied Environmental Microbiology 60: 2238–2247.
18. Dimari GA, Abdulrahman JC, Garba ST. 2008. Metals concentrations in tissues of *Tilapia galli, Clarias lazera* and Osteoglossidae caught from Alau Dam, Maiduguri, Borno State, Nigeria. American Journal of Environmental Sciences 4: 373-379.
19. Doe-um M. 1986. Water quality criteria and standards for Malaysia. Executive Summary. Final Report. Department of Environment, Ministry of Science, Technology and Environment, Malaysia/ Consultant group of water quality, Institute of Advanced Studies, Univ. of Malaya, Kuala Lumpur, Malaysia, 1, V-Xll.
20. Dolbec J, Mergler D, Larribe F, Roulet M, Lebel J, Lucotte M. 2001. Sequential analysis of mercury levels in relation to fish diet of an

Amazonian population, Brazil. Science of the Total Environment 271: 87-97.
21. Egyptian Organization for Standardization and Quality Control (EOSQC). 1993. Maximum residue limits for heavy metals in food. Ministry of Industry. No. 2360/1993. PP. 5.
22. Falusi BA, Olanipekun EO. 2007. Bioconcentrations factors of heavy metals in tropical crab (*Carcinus* sp.) from River Aponwe, Ado-Ekiti, Nigeria. Journal of Applied Science and Environmental Management 11: 51-45.
23. FAO/WHO (1992) Food Monitoring and Assessment Programme, WHO, Geneva 5, UNEP, Nairobi. 52. Report of the Third Meeting of the GEMS/Food
24. Farombi EO, Adelowo OA, Ajimoko YR. 2007. Biomarkers of oxidative stress and heavy metal levels as indicator of environmental pollution in African Catfish (*Clarias gariepinus*) from Nigeria Ogun River. International Journal of Environmental Research and Public Health 4: 158-165.
25. Fernandes C, Fernandes AF, Ferreira M, Salgado M. 2008. Oxidative stress response in gill and liver of *Liza saliens*, from the Esmoriz-Paramos coastal lagoon, Portugal. Archives of Environmental Contamination and Toxicology 55: 691-700.
26. Finerty MW, Madden JD, Feagly SE, Grodner RM. 1990. Effect of environs and seasonality on metal residues in tissues of wild and pond-raised Crayfish in southern Louisiana. Archives of Environmental Contamination and Toxicology 19: 49-55.
27. Foodstuffs,Cosmetics,Disinfectants (1972) Regulation No. R 2064. Marine Food Government Gazette. Government Printer, Pretoria. Act. No. 54 of 1972.
28. Gardner GR, Yevich PP. 1970. Histological and hematological responses of an estuarine teleost to cadmium.Canadian Journal of Fish Research 27:2185-2196.
29. Gill TS, Pant JC. 1985. Mercury-induced blood anomalies in the freshwater teleost (*Anguilla rostrata*. Water, Air and Soil Pollution: 165-171.
30. Gupta P, Srivastava N. 2006. Effects of sublethal concentrations of zinc on histological changes and bioaccumulation of zinc by kidney of fish *Channa punctatus* (Bloch). Journal of Environmental Biology 27:211-215.
31. Heath AC. 1995. Water pollution and fish physiology. 2nd edn., Lewis Publishers, Boca Raton. pp. 125-140.
32. Jiraungkoorskul W, Uptham ES, Kruatrachue M, Sahaphong S, Vichasri-Grams S, Pokethitiyook P. 2003. Biochemical and histopathological effects of glyphosate herbicide on Nile tilapia (*Oreochromis niloticus*).Environmental Toxicology 18:260-267.
33. Kalay M, Canli M. 2000. Elimination of essential (Cu and Zn) and non essential (Cd and Pb) metals from tissues of a fresh water fish, *Tilapia zillii*.Tropical Journal of Zoology 24:429-436.
34. Karlsson-Norrgren L, Runn P, Haux C, Förlin L. 1985. Cadmium-induced changes in gill morphology of zebra fish *Brachydanio rerio* (Hamilton-Buchanan) and rainbow trout *Salmo gairdneri* Richardson. Journal of Fish Biology 27: 81–95.
35. Kime J. 1998. Endocrine Disruption in Fish. Kluwer, Dordrecht
36. Manahan SE. 1989. Toxicological Chemistry; a guide to toxic substances in chemistry. pp. 256-257. Brooks/Cole Publishing Co, C.A.
37. Mallatt J. 1985. Fish gill structural changes induced by toxicants and other irritants: a statistical review. Canadian Journal of Aquatic Science 42:630-648.
38. Marouf HA, Dawoud AS. 2006. Evaluation of heavy metals content in freshwater Crayfish in Damietta. Journal of Veterinary Medical Association,Egypt 66:217-225.
39. Mela MR, Ventura F, Carvalho DF, Pelletier CE, Ribeiro CA. 2007. Effects of dietary methylmercury on liver and kidney histology in the neotropical fish *Hoplias malabaricus*. Ecotoxicology and Environmental Safety 68: 426 - 35.
40. Maurice-Bourgoin L, Quiroga I, Chincheros J, Courau P. 2000. Mercury distribution in waters of the upper Madeira Rivers and mercury exposure in Riparian Amazonian populations. Science of the Total Environment 260: 73-86.
41. Nevo E, Noy R, Lavie B, Beiles A, Muchtar S. 1986. Genetic diversity and resistance to marine pollution. Biological Journal of the Linnean Society 29: 139-144.
42. Nimmo DR, Rigby RA, Bahner LH, Sheppard JM. 1978. The acute and chronic effects of cadmium on the estuarine mysid *Mysidopsis bahia*. Bulletin of Environmental Contamination and Toxicology 19: 80–85.
43. Oladimeji AA, Offem BO. 1989. Toxicity of lead to *Clarias lazera*, *O. niloticus*, *Chironomus tantans* and *Benacus* sp. Water, Air and Soil Pollution 44: 191-201.
44. Oliveira-Ribeiro CA, Belger L, Pelletiter E, Rouleau C. 2002. Histopathological evidence of inorganic mercury and methylmercury toxicity in the arctic charr (*Salvelinus alpinus*).

Environmental Research 90: 217-225.
45. Pandey AK, Mohamed MP, George KC. 1994. Histopathological alterations in liver and intestine of *Liza parsia* (Hamilton-Buchanan) in response to mercury toxicity. Journal of Advanced Zoology 15: 18-24.
46. Pratap HB, Wendelaar Bonga SE. 1993. Effect of ambient and dietary cadmium on pavement cells, chloride cells, and sodium, potassium-ATPase activity in the gills of the freshwater teleost *Oreochromis mossambicus* at normal and high calcium levels in the ambient water. Aquatic Toxicology 26: 133–150.
47. Rubio R, Tineo P, Torreblance A, Del-Romo J, Mayans JD. 1991. Histological and electron microscopical observations on the effects of lead on gills and midget gland of *Procamarus clarkii*. Toxicology and Environmental Chemistry 31: 347-352.
48. SAS Institute. 2000. SAS User's Guide: statistics, SAS Institute, Cary, NC.
49. Schumacher M, Domingo JL. 1996. Concentrations of selected elements in oysters (*Crassostrea angulata*) from the Spanish coast. Bulletin of Environmetal Contamination and Toxicology 56: 106-113.
50. Seddek AS, Salem DA, El-Sawi NM, Zaky ZM. 1996. Cadmium, lead, nickel, copper, manganese and flourine levels in River Nile fish. Assiut Veterinary Medical Journal,Egypt 35: 95-102.
51. Seymore T. 1994. Bioaccumulation of metals in *Barbus marequensis* from the Olifants River, Kruger National Park, and lethal levels of Mn to juvenile *Oreochromis mossambicus*. MSc thesis, Rand Afrikaans University, South Africa.
52. Sunderland EM, Chmura GL. 2000. An inventory of historical mercury emissions in maritime Canada: implications for present and future contamination. Science of the Total Environment 256: 39-57.
53. Suppin D, Zahlbruckner R, Krapfenbauer CC, Hassan I, Hauser C, Smulders F. 2005. Mercury, lead and cadmium content of fresh and canned fish collected from Austrian retail operations. Ernahrung Nutrition 29: 456-460.
54. Taiz L, Zeiger E. 1998. Plant defenses: surface protectants and secondary metabolites. In: Taiz L and Zeiger E (eds.). Plant physiology. Sinauer Associates, Massachusetts. pp. 347-377.
55. Tantawy EA. 1997. Safety and quality of fishes infested with parasites. PhD thesis (Meat Hygiene), Faculty of Veterinary Medicine, Cairo University, Egypt.
56. Thophon S, Kruatrachue M, Upatham ES, Pokethitiyook P, Sahaphong S, Jaritkhuan, S. 2003. Histopathological alterations of white seabass, *Lates calcarifer*, in acute and subchronic cadmium exposure. Environmental Pollution 121: 307–320.
57. U.S. Environmental Protection Agency (USEPA). 1995. National Water Quality Inventory: 1994 Report to Congress. EPA 841-R-95-005. U.S. Environmental Protection Agency, Office of Water, Washington, DC.
58. Van Dyk JC. 2003. Histological changes in the liver of *Oreochromis mossambicus* (Cichlidae) after exposure to cadmium and zinc. MSc thesis, Rand Afrikaans University, South Africa.
59. Waldorn HA, Stofen S. 1974. Sub-clinical lead poisoning. Academic Press, New York, pp: 1-224.
60. World Health Organisation (WHO). 1984. Guidelines for drinking water quality. WHO, Geneva, No.111.
61. Wicklund-Glynn A. 1991. Cd and Zn kinetics fish: studies on water-borne Cd and Zn turnover and intracellular distribution in minnows, *Phoxinus phoxinus*. Pharmacology and Toxicology 69: 485-491.

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Bioaccumulation of cadmium in the fresh water prawn *Macrobrachium rosenbergii*

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Abstract: The effects of Cd on mortality, resistance and bioaccumulation in giant freshwater prawn *Macrobrachium rosenbergii* in Egypt were studied. Survival of prawns exposed to cadmium doses over 60 μgL^{-1} were significantly lower than of those exposed to lower doses. After 96 hours prawns exposed to >40 $\mu g\ L^{-1}$ of cadmium had a greater reduction in total haemocyte count and phagocytic activity than those exposed to lower concentrations. Bioaccumulation of Cd in the gills, hepatopancreas and muscleswas variable. Cadmium accumulated in gills and hepatopancreas, but muscles had a moderately significant Cd level increase. *Macrobrachium rosenbergii* manifested histopathological alterations in gills, hepatopancreas and muscles when exposed to different concentrations of cadmium. [Nature and Science 2010; 8(4):157-168]. (ISSN: 1545-0740).

Keywords: toxicity, survival, haemocyte count.

1. Introduction

Heavy metals are considered a major source of environmental pollution. Cadmium (Cd) which is one of these pollutants has taken considerable attention for its great different toxic effects on living individuals. Metal contamination sources are typically derived from different sources: mining, industrial waste discharges, sewage effluent, harbor activities and agrochemicals. Cadmium is unique among the other metals because of its toxicity at a very low dosage and long biologic half life (30 years in human) and its low rate of excretion from the body (Jones and Cherian 1990).

Heavy metals like cadmium are known to accumulate in marine organisms, and cause rapid genetic changes (Nimmo *et al.* 1978; Nevo *et al.* 1986). It is also possible that environmental toxicants may increase the susceptibility of aquatic animals to various diseases by interfering with the normal functioning of their immune, reproductive and developmental processes (Couch 1978; Brock 1997). In decapods crustaceans, 3 types of circulating hemocytes are recognized: hyaline, semi-granular and large granular cells (Tsing et al. 1989). They are involved in cellular immune responses that include phagocytosis and constitute the primary method of eliminating microorganisms or foreign particles (Bayne, 1990). In addition to phagocytosis, hemocytes are involved in coagulation and in the production of melanin via the prophenoloxidase system (Johansson and Söderhäll 1989 and Söderhäll *et al*. 1996). Enzymes for the prophenoloxidase system are contained in the granular hemocytes, released as proenzymes upon stimulation by microbial cell components such as 1 ,3-glucan or lipopolysaccharide from fungal cell walls, and activated by a serine protease (Söderhäll 1983, Smith *et al.* 1984, Söderhäll et al. 1996). The activity of phagocytosis has been reported for many crustaceans (Söderhäll *et al.* 1996) including the brown shrimp *Penaeus californiensis* (Hernández-López et al. 1996), the tiger shrimp *P. monodon* (Sritunyalucksana *et al.* 1999) and *Macrobrachium rosenbergii* (Cheng et al., 2002). Several physico-chemical parameters and environmental contaminants have been reported to affect the immune response in crustaceans and these have been reviewed by Le Moullac and Haffner (2000). Environmental toxicants have been reported to cause a reduction in hemocyte count in the common shrimp *Crangon crangon* (Smith and Johnston 1992)

Pollution of aquatic environments with heavy metals has seriously increased worldwide attention and under certain environmental conditions, fish may concentrate large amounts of some metals from the water in their tissues. Heavy metals such as cadmium, is potentially harmful to most organisms even in very low concentrations and have been reported as hazardous environmental pollutants able to accumulate along the aquatic food chain with severe risk for animal and human health. Bioconcentration is the increase in concentration of a chemical in an organism resulting from tissue absorption levels exceeding the rate of metabolism and excretion. Neurotoxicity on the CNS appears in a variety of neurochemical and behavioral changes due to cadmium exposure (Desi *et al.* 1998). Toxic heavy metal can cause dermatological diseases, skin cancer and internal cancers (liver, kidney, lung and bladder), cardiovascular disease, diabetes, and anaemia, as well as reproductive, developmental, immunological and neurological effects in the human body. Cd can enter into the brain

parenchyma and neurons causing neurological alterations in humans (Rose *et al*. 1992) and animal models (Lukawski et al. 2005). Cd is listed by the U.S. Environmental Protection Agency as one of 126 priority pollutants. Acute-Cd exposure results in pulmonary edema and respiratory tract irritation, whereas chronic exposure to Cd often leads to renal dysfunction, anemia, osteoporosis, and bone fractures (Friberg *et al.* 1986 and Goering et al. 1995). Cadmium is carcinogenic for a number of tissues (Waalkes 2000) and is classified by IARC (1993) as a human carcinogen. In laboratory animals, acute Cd poisoning produces primarily hepatic and testicular injury, whereas chronic exposure results in renal damage, anemia, and immuno- and osteotoxicity (Goering *et al.* 1995, Klaassen *et al.* 1999). It has been suggested that the mechanism of Cd toxicity involves the production of reactive oxygen species and free radicals (Manca *et al*. 1994, Stohs *etal.* 2001).

The aim of this study was to investigate the effect of Cd toxicity on mortalities and resistance in giant freshwater prawn *(Macrobrachium rosenbergii)* and also to investigate the bioaccumulation of cadmium residues in their tissues.

2- Materials and Methods

Experimental design

Freshwater was adjusted with the desired parameters according to New (1995) as followed (temperature of 20-28 °C, pH 7-7.8, dissolved oxygen 5-8 mg/L, salinity 2 ppt, hardness 100-150 ppm Ca(CO)3, total ammonia less than10 ppm, nitrate 20 ppm and nitrite 1ppm).

Stock cadmium solution: 100 mg $CdCl_2$ metal dissolve in a solution composed of 20 mL water plus 5mL concentrated HCL and make up to 1000 mL with water (1.00 mL = 100 µg Cd). Ten different concentrations of Cd were then prepared from the stock solutions (10, 20, 30, 40, 50, 60, 70, 80, 90,100 µg L^{-1}).

Macrobrachium rosenbergii were obtained from commercial farms in Alexandria and Al-Kalubia, Egypt, and acclimated in the laboratory for two days before experimentation.

The toxicity tests were conducted according to the standard procedures of FAO (1985). Ten concentrations of Cd ranged between 10 until 100 µg and a control were set up. Ten shrimps of the same size (ranged from 13.2 to 16.5 g with mean of 15.32 ±0.15g) were separately transferred from the holding tanks into the control and experimental tanks. The whole set was aerated continuously, while the test solution in each tank was changed with requisite fresh solution every 24 hrs to maintain the definite concentration of Cd for 96 hrs. Observations for mortality were made twice (10.0 am and 6.0 pm) daily.

Analysis: The 96 hrs $_{LC50}$ values were calculated using probit analysis according to Finney (1971).

Cell counts

Hemolymph (100 µL) was sampled individually at the beginning of each test and at 96 hrs post exposure to Cd. It was withdrawn from the ventral sinus of each prawn into a 1 ml sterile syringe (25 gages) containing 0.9 ml anticoagulant solution (trisodium citrate 0.114 M, sodium chloride 0.1 M, pH 7.45, osmolality 490 mOsm kg–1). A drop of the anticoagulant-hemolymph mixture was placed on a hemocytometer to measure THC using an inverted-phase contrast microscope.

Culture of *Lactococcus garvieae*

The bacterial strain *L. garvieae* isolated from diseased *Macrobrachium rosenbergii* after artificial infection was used in this study. The bacterium was cultured on tryptic soy agar (TSA) for 24 h at 28 °C before being transferred to 10 mL of tryptic soy broth (TSB) for 24 h at 28 °C as a stock culture. The stock cultures were then centrifuged at 7155 x g for 15 min at l4 °C. The supernatant fluid was removed and the sediment was resuspended in saline solution (0.85 NaCL) and adjusted at 10^{10} cfu mL^{-1} as stock bacterial suspensions for testing.

Phagocytic activity of *M. rosenbergii* to *L. garvieae*

After 72 hrs of Cd exposure in each treatment, prawns were injected in the cephalothorax with 20 µl of the bacteria suspension (10^{10} cfu ml^{-1} in 0.85% NaCI) resulting in 2 x 10_8 cfu prawn l^{-1}. After injection, the prawns were held in their respective solutions for 3 h (s). Hemolymph (200 µl) was collected from the ventral sinus and mixed with 200 µl of sterile anticoagulant containing sodium citrate (0.8 g), EDTA (0.34 g), Tween 80 (10 µl) and distilled water (100 ml with pH of 7.45).

Phagocytic activity was measured using the method described by Weeks-Perkins et al., (1995) where 200 µL *of* diluted hemolymph sample was mixed with 0.2 ml of 0.1% paraformaldehyde for 30 min at 4 °C to fix the hemocytes. They were then centrifuged at 800x g at 4 °C, washed and resuspended in 0.4 ml of sterile phosphate buffer solution. The suspension (50 µL) was spread onto a slide glass and air-dried and stained with Diff-Quick stain. About 200 hemocytes were counted using light microscope and the phagocytic rate was estimated as follows:

PR = [(phagocytic hemocytes) / (total hemocytes)] x 100.

Preparation and analysis of tissue samples

Procedure A: Each sample was represented by one gram of tissues dissected from the gills, hepatopancreas, and muscles, then placed in a clean screw-capped tube and digested according to the method described by Finerty *et al*. (1990).

Procedure B: The obtained solutions were then

analyzed by using Air/Acetylene Flame Atomic Absorption Spectrophotometer (UNICAM 696 AA Spectrometer) for determination of cadmium levels in examined samples.

Histopathological examination:

Tissue specimens from *Macrobrachium rosenbergii* were taken (gills, hepatopancreas and muscles) and fixed in 15 % buffered neutral formalin. They were processed to obtain five micron thick paraffin sections then stained with Hematoxylin and Eosin, (H&E) according to Bancroft *et al.*, (1996) and examined under light microscope.

Statistical analysis:

Data were analyzed using Analysis of Variance (ANOVA) and means were separated by the Duncan posthoc test at a probability level of < 0.05 (SAS, 2000).

3. Results:

After 96 h(s), mean (±SD) survival of prawns in control tanks (0 Cd) was 94±2.20% and significantly higher *(P* < 0.05) than that of prawns in all other treatments (Table 1). At 96 h(s), survival of prawns exposed to 10-50 µg/L^{-1} concentrations of cadmium were significantly greater *(P* < 0.05) than for prawns exposed to higher concentrations (60 µg/L^{-1} or greater) (P < 0.05). Survival of prawns exposed to 60, 70, 80, 90 and 100 µg/L^{-1} of cadmium was significantly lower (P < 0.05), with means *of(±SD)* 57 ± 0.70%, 50 ± 0.70%, 50 ±.70%, 40 ± 0.20% and 40 ± 0.21%, respectively as shown in Table 2. The regression analysis of prawn survival (%)was highly significant (P < 0.001; r2 = 0.964).

Table 1 and Figures B and C show that at 96 hours, prawns exposed to 40, 50, 60, 70, 80 and 90 µg/L^{-1} concentrations of cadmium were significantly had greater reduction in THC and Phagocytic activity than for prawns exposed to lower concentrations (10 – 30 µg/L^{-1}), *(P* < 0.05).

The LC50 of Cd on *M. rosenbergii*

The 96-h(s) $_{LC50}$ for cadmium-exposure in *M. rosenbergii* was calculated to be 74 µg/L^{-1}.

Bioaccumulation of Cd in different tissues of *M. rosenbergii*

The highest bioaccumulation of cadmium was observed in the organs mainly implicated in metal intoxication. Cadmium (Cd) in tissues was high in the gills > hepatopancreas> muscles. Cadmium accumulations were increased in gills, hepatopancreas and muscles, *with the increasing exposure of concentrations* respectively.

Gills: The rate of accumulation of cadmium was maximum in gills of exposed prawn .The rate of accumulation increased along with the increasing of cadmium concentration reaching up to 1.1± 0.025 µg gm^{-1} after 96 h(s) exposure for Cd at 100 µg/L^{-1} as shown in Table 2.

Hepatopancreas: Cadmium could not be traced in the hepatopancreas of control test as well as at very low concentration 10 µg/L^{-1}, even though the quantity of accumulated cadmium was less in the case of hepatopancreas when compared to gills.

Muscles: The rate of accumulation of cadmium in muscles increased along with exposure concentrations. The mean rate of accumulation at 100 µg/L^{-1} was 0.065± 0.008 µg gm^{-1}. The rate of accumulation was less as compared with other tissues, Table 2.

Histopathological alterations in different tissues of *M. rosenbergii*

Results of the present study revealed that *Macrobrachium rosenbergii* manifested histopathological changes in gills, hepatopancreas and muscles.

Gills showed mild congestion, swelling and edema at low doses of Cd intoxication. Severe edema, hyperplasia, at highest doses of intoxication was observed as shown in Figure 3.

Muscular tissues

Figure 5 shows the normal structures of the muscles. Several histopathological alterations were seen in the muscles *Macrobrachium rosenbergii.* The pathological findings included degeneration in muscles with infiltration and aggregations of hemocytes between them and focal areas of necrosis. Also, atrophy of muscle bundles, edema, hyaline degeneration and splitting of muscle fibers were seen.

Table 1: Effect of cadmium on survival, total hemocyte count (THC) and phagocytic % of freshwater prawns, *Macrobrachium rosenbergii,* exposed to cadmium at different concentrations for 96 hours post-treatment. Values are means± SD (n =4 prawns in each case).

Cd[1] Con.	Survival %	Immune response	
		THC[2]	Phagocytic%
0	94±2.20	196±70	90±7.70
10	86±1.70*	195±16	90±.8.70
20	86±1.60*	199±12*	84±7.00
30	70±1.67*	170±9.0*	70±7.00*
40	63±0.87*	170±8.0 *	62±7.00*
50	60±0.30*	145±11*	50±2.70*
60	57±0.70*	138±9.0*	40±0.70*
70	50±0.70 *	136±8.0*	40±0.70*
80	50±.70*	130±12*	40±3.00*
90	40±0.20*	130±8.0*	40±0.00*
100	40±0.21*	120±0.0*	30 ±3.00*

[1]: [1]: Cd^{2+} µgL^{-1} , [2]:x $10^5 ml^{-1}$, *Significant($P < 0.05$).

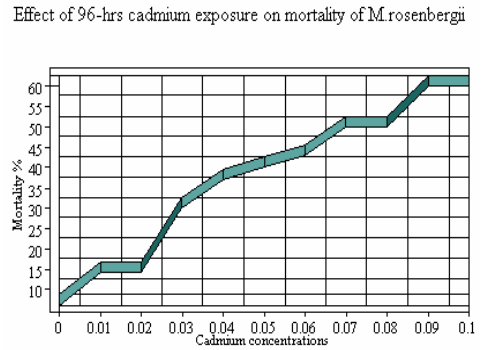
Table 2. The residual analysis of cadmium in freshwater prawns, *Macrobrachium rosenbergii,* exposed to cadmium at different concentrations for 96 hours post-treatment. Values are means± SD.

Conc. of Cd[1]	Bioaccumulation in tissues[2]		
	Gills	Hepatopancreas	Muscles
0	-	-	-
10	-	-	-
20	0.05 ±0.008	0.02±0.006	0.005±0.001
30	0.05 ±0.009	0.025±0.008	0.01±0.001
40	0.06 ±0.018	0.03±0.012	0.02±0.003
50	0.065 ±0.021	0.04±0.009	0.02±0.005
60	0.08± 0.022	0.06±0.011	0.03±0.01
70	0.90±0.011	0.065±0.011	0.05±0.009
80	1 ±0.011	0.08 ±0.012	0.055±0.011
90	1.1±0.02	2± 0.01	0.06 ±0.022
100	1.1±0.025	2.2±0.02	0.065±0.008

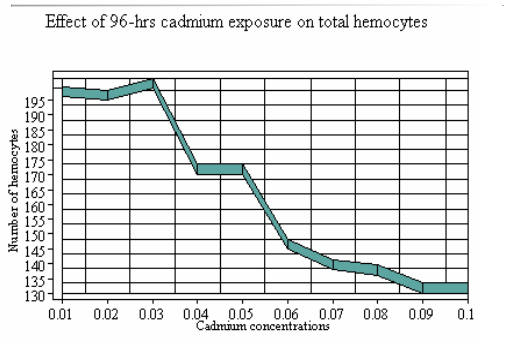
Cd^{2+} µg gm^{-1}. = mg kg^{-1} = ppm. :[2] [1]: [1]: Cd^{2+} µg L^{-1},

Table 3 .The permissible limits of Cd

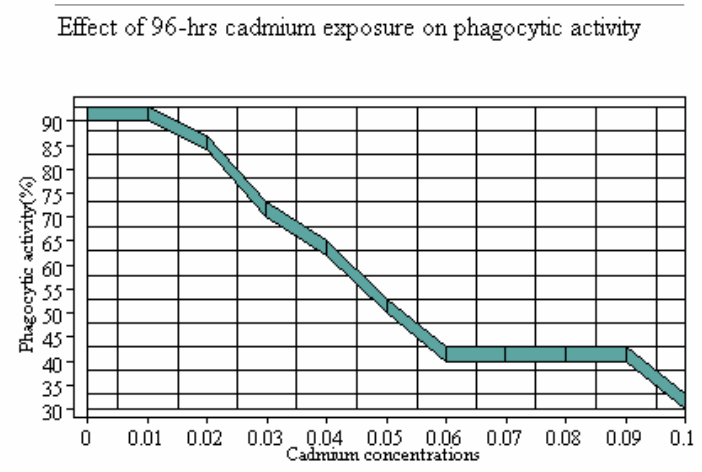
Metal	Permissible	Country and references
Cadmium	0.005 ppm	WHO (1984)
	0.05 ppm	FAO/WHO (1992)
	0.1 ppm	Egypt, E.O.S.Q.C. (1993)
	1.0 pg/g^{-1}	Spain: Boletin Official del Estado (1991)



A-Regression Equation(y) = a + bx=33.09+58.30x
Slope (b) = (NΣXY - (ΣX) (ΣY)) / (NΣX^2 - $(\Sigma X)^2$)
Intercept (a) = (ΣY - b (ΣX)) / N



B-Regression Equation(y) = a + bx=157.18+1999.48x
Slope (b) = (NΣXY - (ΣX) (ΣY)) / (NΣX^2 -



C-Regression Equation(y) = a + bx=57.82+640.52x Slope (b) = (N ΣXY - (ΣX) (ΣY)) / (NΣX2 - (ΣX)2)
Intercept (a) = (ΣY - b (ΣX)) / N.Correlation coefficient =-0.96

Figure1 (A, B, C) The relationship of mortality and immune response (total hemocyte count and phagocytic activity) to different concentrations of Cd^{2+}

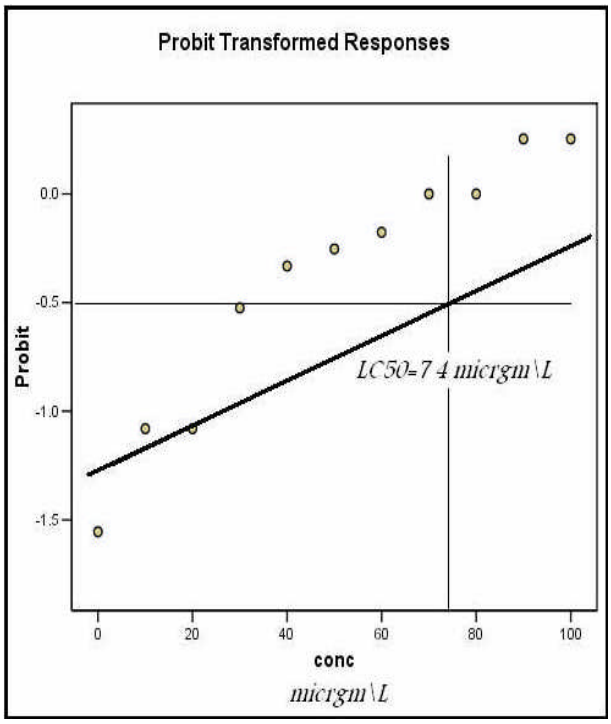


Figure2: LC50 of cadmium on *M. rosenbergii* for 96-h exposure using the resulting regression equation, in *M. rosenbergii*, the 96-hours $_{LC50}$ for cadmium was calculated to be 7 4 µg L^{-1}, cadmium.

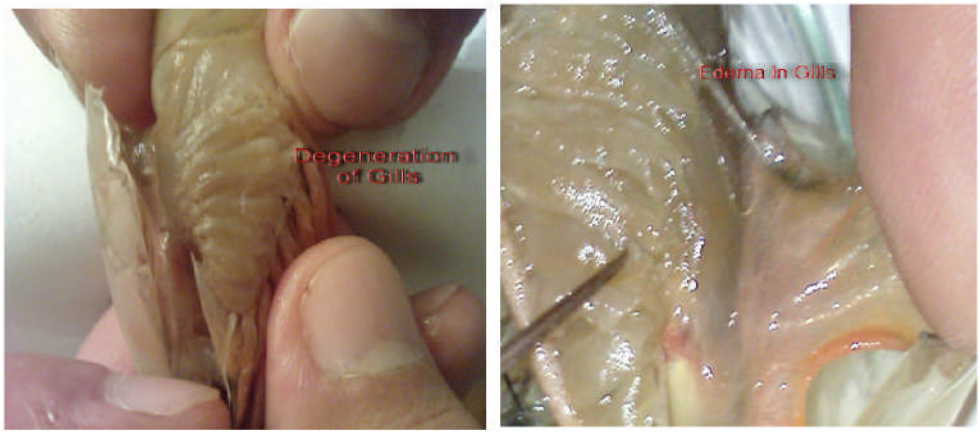


Figure 3: Gills showed congestion, swelling, edema and hyperplasia, at highest doses of intoxication

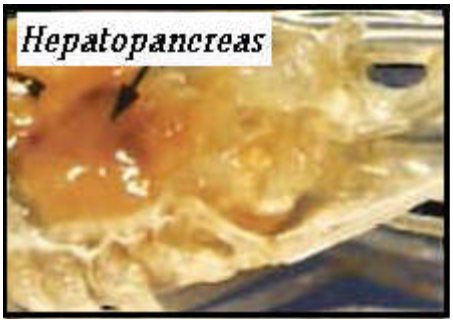
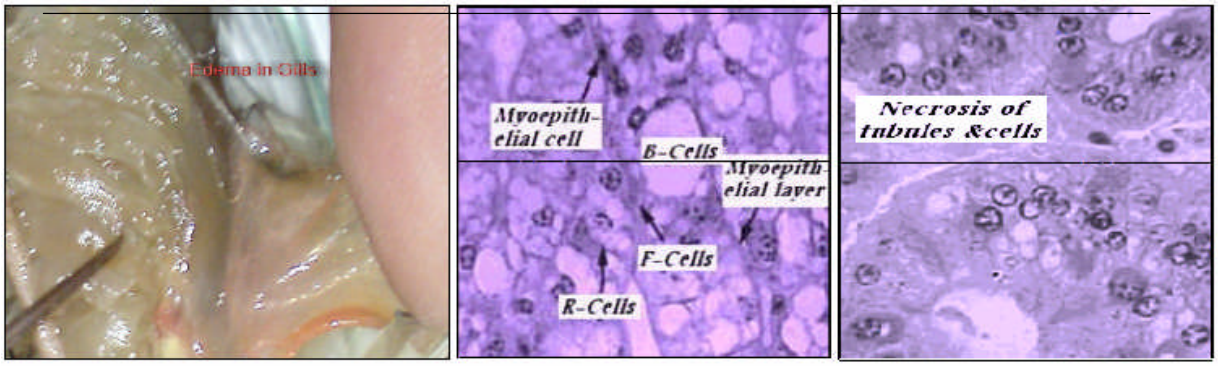


Figure 4: Hepatopancreas showed degeneration of the hepatocytes and haemolysis (the findings were apparent as the hepatospleen consider the organ of detoxification, excretion and binding proteins such as metallothionein).



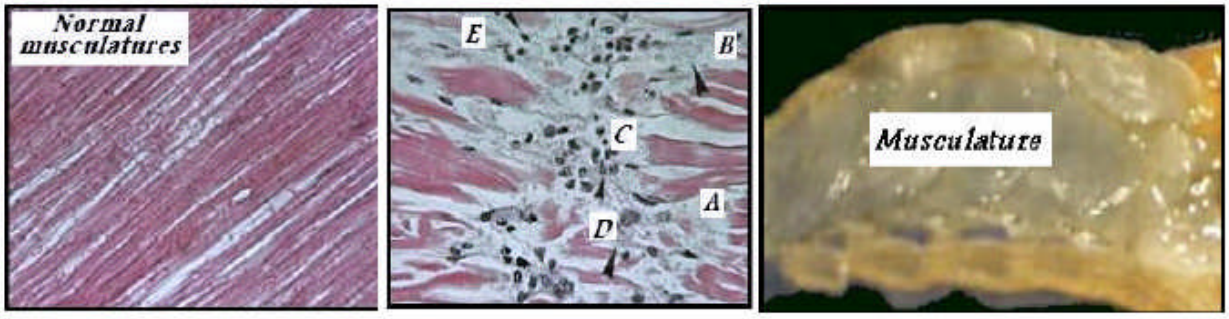


Figure 5: Muscular tissues showed pathological alterations; included degeneration in muscles with infiltration and aggregations of hemocytes between them and focal areas of necrosis. Also, atrophy of muscle bundles, edema, hyaline degeneration and splitting of muscle fibers. A; splitting of muscle fibers, B: hyalin e degeneration, C: infiltration of hemocytes, D: focal areas of necrosis, E: atrophy of muscles bundles and edema.

4. Discussion:

After 96 hrs, survival of prawns exposed to 10-50 µg L^{-1} concentrations of cadmium were significantly greater ($P < 0.05$) than prawns exposed to higher concentrations (60 µg/L^{-1}or greater)

Cheng (1979) tested Hg, Cu, Cd and Zn in *Penaeus monodon* and found that Hg was the most toxic of all metals, followed by Cu, Cd and Zn and he added that Cd toxicity was the most rapid one.

Kuo *et al* (1984) suspected that Cd and Cu were the cause of mortalities in hatchery farms in Taiwan in 1980-1981, with the heavy metals coming from the waste water discharged by nearby industries.

Prawns exposed to 40, 50, 60, 70, 80 , 90 and 100 µgL^{-1} concentrations of cadmium were significantly had greater reduction in THC and Phagocytic activity than for prawns exposed to lower concentrations (10 – 30 µg/L^{-1}), ($P < 0.05$).

Several scientists have investigated the effects of environmental parameters on crustacean defense mechanisms. Dean and Vernberg (1966) reported that temperature affects hemolymph clotting time, hemocyte counts and serum protein concentration in the hermit crab *Uca pugilator*. Truscott and White (1990) found tide-associated rhythms in the total hemocyte count for freshly captured shore crab *Carcinus maenas*, with peak count occurring at high tide. Increased hemocyte numbers provide an enhanced immune capability during periods of high activity. Hauton *et al.*, (1995) reported a significant negative correlation between phenoloxidase activity and tidal height in *C. maenas*, and this indicated cyclical changes in immunocompetence. An increased prevalence in the shell disease of marine decapods crustaceans has been reported to result from polluted environments, also suggesting a decrease in immunocompetence (Gopalan and Young ;1975, Young and Pearce; 1975).

Carolina (2009) studied the effect of Manganese on the immune system of marine invertebrates and found that Mn severely suppresses the number of circulating hemocytes in *Nephrops norvegicus* by inducing apoptosis. However, Mn increased the number of circulating hemocytes in *Asterias rubens* and at the same time affected their ability to phagocyte. The sensibility of exposed gills to bacterial infection has been previously described in other shrimps exposed to cadmium (Couch 1977, Darmono 1990). Their presence has also been observed in gills of *P. japonicus* (Souheil 1995) and of the crayfish *Astacus leptodactylus* (Maesteracci and Vey 1989) infected by fungi.

A significant reduction in phagocytosis of *Bacillus cereus* was observed in the shore crab *Carcinus maenas* following 14 day exposure to 500 µg/L^{-1} Cd (Truscott and White 1990).

The 96-hour LC_{50} for cadmium in *M. rosenbergii* was calculated to be 7 4 µg/$L^{-1.}$ However, Fafioye and Ogunsanwo (2007) found that the lethal concentration (LC_{50}) for 96 hrs exposure to cadmium for *M. rosenbergii* post larvae was 3.23 m/L .The 96 h(s) LC_{50} values of 2.88, 3.02 and 3.11 mg/ L of Cd reported to be toxic to *P. monodon* (Diaz 1995), *P. pencillatus* and *P. indicus* (Chinni and Yallapragda 2000), respectively.

The highest bioaccumulation of cadmium was observed in the organs mainly implicated in metal intoxication. Cadmium (Cd) in tissues was high in the gills > hepatopancreas> muscles.
The highest Cd concentration in gills might be related to the important quantity of this metal in the hemolymph and or the necrosed tissues, or these organs might constitute the entry sites of the metal and act as a transient store for accumulated Cd (Martin and Rainbow 1998). The relatively higher Cd concentration in the hepatopancreas could originate from a progressive transfer of Cd from gills to the hepatopancreas could originate from a progressive transfer of Cd from gills to the hemolymph (Bjerregaard 1990), and/or from a process of differentiation of hepatopancreatic epithelium as observed by AliKhan (1989) in the Isopod *Porcellio spinicornis* leading to transfer of the metal into the intestinal lumen and from this site to the exterior as observed by Brown (1982) in Cray fish. However, the higher Cd concentration in hepatopancreas suggested that this organ plays a role in metal storage and or in detoxification process by a metal binding component (White and Rainbow 1986)

cadmium accumulation in muscles of *M. rosenbrgii* was ranged from 0.005-0.065 (ppm) and the maximum permissible limits recommended by WHO, (1984) is 0.005 ppm. The recorded results of cadmium concentrations in muscles of*M. rosenbrgii* were higher than the permissible limits intended by Boletin Official del Estado (1994) in Spain [l.0 pg/g^{-1}] and FAO/WHO (1992)[0.05 p.p.m] but lower than Egyptian Organization for Standardization and Quality Control (E.O.S.Q.C) (1993) [0.1 mg kg^{-1}].

Cadmium is highly toxic non essential heavy metal and it does not have a role in biological processes in living organisms. Thus even in low concentration, cadmium could be harmful to living organisms (Burden *et al.*, 1998). High accumulation of cadmium in liver may be due to its strong binding with cystine residues of metallothionein (Klaassen *et al*. 1999).

Agricultural activities are likely to add important amounts of Cd to the natural levels. Fertilizers are important sources of Cd based agrochemicals which are widely used in intensive agriculture (Alloway 1990).

The histopathological alterations in different tissues of *M. rosenbergii*

Gills showed mild congestion, swelling and edema at low doses of Cd intoxication. Severe edema, hyperplasia, at highest doses of intoxication. Similar effects such as necrosis, cell proliferation, epithelial lifting and dilated lamellae were observed in gills of

fish exposed to metals, including cadmium as observed by Malia (1985). Since high Cd concentrations result in serious damage to the gills, the metal may consequently inhibit the physiological functions of these organs. Since the gills of the shrimp are probably involved in gas exchange, we suppose that these alterations resulting in disruption of respirations (Thurberg 1973).

The effects of Cd on fish gill morphology have been studied in some species (Gardner and Yevich 1970; Karlsson-Norrgren *et al.* 1985; Pratap and Wendelaar Bonga 1993and Thophon *et al.* 2003).

Hepatopancreas showed degeneration of the hepatocytes and haemolysis (Fig.4). These findings were apparent as the hepatospleen consider the organ of detoxification, excretion and binding proteins such as metallothionein (MTs). The metal-binding proteins were present in the nuclei of hepatocytes suggested that the increase in the cell damages (De Smet, Blust 2001). Similar results were observed by Van Dyk (2003) and Mela *et al.* (2007).

Frías-Espericueta et al., (2008) studied the effect of three concentrations of Cu (3.512, 1.756 and 0.877 mg\ l- 1) on the juvenile *Litopenaeus vannamei* and he found that there were severe time- and dose-dependent structural damages, such as necrosis, loss of regular structure and infiltration of hemocytes in the gill tissues, as well as atrophy, necrosis and irregular tubular structure in the hepatopancreas.

4. Conclusions:

This study reveals an important precaution for prawn cultivation. Knowledge of the toxicity of cadmium will be helpful to water quality management in fish farms with specialty to prawn cultures; they affect the immune response and cause a reduction in hemocyte count in *Macrobrachium rosenbergii*. Caution should be exercised against water source contamination and exposure to fertilizer and industrial pollution. For this reason, the assessment of risk and the safe levels of toxic substances added to any natural environment through human or natural sources, should not neglect the effects on biological systems caused by the interaction of minute amounts of toxicants.

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References:

Alikhan MA. 1989. Magnesium and manganese regulation during moult-cycle in *Porcellio spinicornis* Say (Porcel- lionidae, Isopoda), Bulletin of Environmental Contamination & Toxicol. 42; 699-706.

Alloway BJ. 1990. Heavy metals in soils, 339 pp. Blackie and Son, New York.

Bancroft JD, Stevens A, Turner DR. 1996. Theory and Practice of Histological Techniques. 4th Ed. New York, Churchill, Livingstone.

Bayne CJ. 1990. Phagocytosis and non-self recognition in invertebrates. Phagocytosis appears to be an ancient line of defense. Bioscience 40: 723–73 1.

Bjerregaard P .1990. Influence of physiological condition on cadmium transport from hemolymph to hepatopancreas in Carcinus maenas. Marine Biol. 106, 199-209.

Boletin Oficial Del Estado. 1991. Separates del Boletin Oficial del Estado Espanol No. 195. Gaceta de Madrid, P: 27154. In: Schuhmacher M, Batista J, Domingo JL, Corbella J. Mercury concentrations in autopsy tissues from inhabitants of Tarragona Province, Spain. Trace Elem Elect 1996; 13: 75-79.

Brock JA. 1997. Special topic review: taura syndrome, a disease important to shrimp farms in the Americas. World Journal of Microbiology and Biotechnology 13: 415–418.

Brown BE .1982. The form and function of metal containing 'granules' in invertebrate tissues. Biology Rev. 57: 621 -667.

Burden VM, Sandheinrich MB, Caldwell CA .1998. Effects of lead on the growth and alpha amino levulinic acid dehydrates activity of juvenile rainbow trout, Oncorhynchus mykiss. Environmental Poll. 101: 285-289.

Carolina O. 2009. Immunotoxicology in marine invertebrates: effects of Mn on the immune responses. M.D. thesis, School of Life Sciences, Heriot-Waff University, Edinburgh, UK.

Cheng W, Liu C, Chen J. 2002. Effect of nitrite on interaction between the giant freshwater prawn Macrobrachium rosenbergii and its pathogen Lactococcus garvieae. Disease of Aquatic Org.Vol. 50: 189–197.

Cheng HC. 1979. Acute toxicity of heavy metals to some marine prawns. China Fish. 316: 3-10. (In Chinese, with English abstract.)

Chinni S, Yallapragda PR. 2000. Toxicity of copper, cadmium, zinc and lead to Penaeus indicus postlarvae: Effects of individual metals. J. of Environmental Biol.21: 255-258.

Couch JA. 1977. Ultrastructural study of lesions in gills of a marine shrimp exposed to cadmium. J. Invertebrate Pathol. 29: 267-288.

Couch JA. 1978. Diseases, parasites, and toxic responses of commercial penaeid shrimps of the Gulf of Mexico and South Atlantic coasts of North America. Fish. Bull. 76:1 -43.

Darmono D, Denton G, Campbel R. 1990. The pathology of cadmium and nickel toxicity in the banana shrimp (Penaeus merguiensis de Man), Asian Fish. Sci. 3: 287-297.

Dean JM, Vernberg FJ. 1966. Hypothermia and the blood of crabs. Comparative Biochemistry and Physiol.l7 B: 19-22.

Desi I, Nagymajtenyi L, Schuiz H. 1998. Behavioural and neurotoxicological changes caused by cadmium treatment of rats during development. J. Applied Toxicol. 18: 63-70.

DeSmet H, Blust R. 2001. Stress responses and changes in protein metabolism in carp (Cyprinus carpio) during cadmium exposure. Ecotoxic. and Environmental Safety 48: 255-62.

Diaz, V.R. 1995. Preliminary results of acute toxicity tests for mercury and cadmium on Milkfish (Chanos chanos Forsskal) juveniles. In: Watson, D., K.S. Ong and G. Vigers (eds.). ASEAN Criteria and Monitoring: Advances in Marine Environmental Management and Human Health Protection. Proceedings of the ASEAN-Canada Midterm Technical Review Conference on Marine Science (24-28 October 1994), Singapore. EVS Environment Consultants, Vancouver, and National Science and Technology Board, Singapore.

Egyptian Organization for Standardization and Quality Control (E.O.S.Q.C.). 1993. Maximum residue limits for heavy metals in food. Ministry of Industry No. 2360/1 993. PP. 5. Cairo, Egypt.

Fafioye OO, Ogunsanwo BM. 2007. The comparative toxicities of cd, cu and pb to M. rosenbergii and Penaeus mondon post larvae. African J. of Agricultural Research. 2: 31-35.

FAO. 1985. Manual of methods in aquatic environment research. Part 4. Bases for selecting biological tests to evaluate marine pollution: FAO fish tech paper 164.

FAO/WHO 1992. Food Monitoring and Assessment Programme, WHO, Geneva 5, UNEP, Nairobi. 52. Report of the Third Meeting of the GEMS/Food

Finerty MW, Madden JD, Feagly SE, Grodner RM. 1990. Effect of environs and seasonality on metal residues in tissues of wild and pond raised Cray fish in Southern Louisiana. Arch Environ. Contamination & Toxicology, 19: 49-55.

Finney DJ. 1971. Probit Analysis. Third edition, Cambridge University Press, London, pp. 13-28.

Frías-Espericueta MG, Abad-Rosales S, Nevárez-Velázquez AC, Osuna-López I, Páez-Osuna F, Lozano-Olvera R, Voltolina D. 2008. Histological effects of a combination of heavy metals on Pacific white shrimp Litopenaeus vannamei juveniles. Aquatic Toxicology 89: 152-157.

Frías-Espericueta1 MG, Osuna-López I., Voltolina, D, Beltrán-Velarde MA, Izaguirre-Fierro G, López-López G, Maria D, Muy-Rangel, Rubio-Carrasco W. 2009. The contents of Cd, Cu, Pb and Zn of the white shrimp, *Litopenaeus vannamei* (Boone, 1931) of six coastal lagoons of Sinaloa, NW Mexico. Revista de Biología Marina y Oceanografía 44: 197-201.

Friberg L, Elinder CG, Kjellstrom T, Nordberg, GF. 1986. Cadmium and health: a radiological and epidemiological appraisal. CRC Press, Boca Raton, FL Gao, S., Zou D. 1995. Acute toxicity of Cd, Zn and Mn to larvae of. *Penaeus pencillatus*. Bulletin of Marine Science. 14: 83-86.

Gardner GR, Yevich PP. 1970. Histological and hematological responses of an estuarine teleost to cadmium. Canadian Journal of Fish Research .27: 2185–2196.

Gopalan UK, Young JS. 1975. Incidence of shell disease in shrimp in the New York Bight. Bulletin of Marine Pollut. 6: 149-153.

Goering PL, Waalkes MP, Klaassen CD. 1995.

Toxicology of cadmium. In: Goyer, RA, Cherian MC (eds). Toxicology of metals: biochemical aspects. Handbook of experimental pharmacology. Springer-Verlag, New York. pp. 189-213.

Hauton C, Hawkins LE, Williams JA. 1995. Circatidal rhythmicity in the activity of phenoloxidase enzyme in the common shore crab (Carcinus maenas). Comparative Biochemistry and Physiol. 111 B: 374-352.

Hernández-López J, Gollas-Galván T, Vargas-Albores F. 1996. Activation of the prophenoloxidase system of the brown shrimp (Penaeus californiensis Holmes). Comparative Biochemistry and Physiol. C 113:61–66.

International Agency for Research on Cancer (IARC). 1993. Cancer monographs on the evaluation of the carcinogenic risks to humans, Vol. 58. IARC Scientific Publications, Lyon.

Johansson MW, Söderhäll K. 1989. Cellular immunity in crustaceans and the propo system. Parasitology Today. 5: 171–176.

Jones MM, Cherian C. 1990. Cadmium, a unique metal. Toxicology J., 62: 1-25

Karlsson-Norrgren L, Runn P, Haux C, Forlin L. 1985. Cadmium-induced changes in gill morphology of zebra fish (Brachydanio rerio) (Hamilton-Buchanan) and rainbow trout (Salmo gairdneri) Richardson. Journal of Fish Biol. 27: 81–95.

Klaassen CD, Liu j, Choudhuri S. 1999. Metallothionein: an intracellular protein to protect against cadmium toxicity. Annual Review of Pharmacology and Toxicol.39: 267-294.

Kuo GH, Lin YS, Chen HC, Lo CF. 1984. Diseases and mortalities of cultured marine fish and shellfish in Taiwan. In: Liao IC, Hirano R. (eds.). Proceedings of ROC-Japan symposium on mariculture; 14-15 December 1981, Taipei, Taiwan. Pingtung, Taiwan: Tungkang Marine Laboratory: 173-192. (TML Conference Proceedings, No. 1).

Le Moullac G, Haffner P. 2000. Environmental factors affecting immune response in Crustacea. Aquaculture 191: 121–131.

Lukawski K, nieradko B, Sieklucka- Dziuba M. 2005. Effects of cadmium on memory processes in mice exposed to transient cerebral oligemia. Neurotoxicology & Teratol. 27: 575 -84.

Maestracci V, Vey A. 1989. Fungal infections of gills in crayfish: histological, cytological and physiopathological aspects of the disease. In: Goedlin P. (ed.). Fresh water crayfish. 7: 187-194.

Malia J. 1985. Fish gill structural changes induced by toxicants and other irritants: a statistical review, Canadian Journal of Fish Aquatic Sci.42: 630-648.

Manca D, Ricard, AC, Van Tra H, Chevalier G. 1994. Relation between lipid peroxidation and inflammation in the pulmonary toxicity of cadmium. Archives of Toxicol. 65: 364-369.

Martin DJ, Rainbow PS. 1998. The kinetics of zinc and cadmium in the hemolymph of the shore crab *Caivinus maenas*,Aquatic Toxicol. 40: 203-231.

Mela MR, Ventura F, Carvalho DF, Pelletier CE, Ribeiro CA. 2007. Effects of dietary methylmercury on liver and kidney histology in the neotropical fish *Hoplias malabaricus*. Ecotoxicology and Environmental Safety 68: 426 - 35.

Nevo E, Noy R, Lavie B, Beiles A, Muchtar S. 1986. Genetic diversity and resistance to marine pollution. *Biological Journal of the Linnean Society* 29: 139-144.

New MB. 1995. Status of freshwater prawn farming: a review. Aquatic Res. 26: l-54.

Nimmo DR, Rigby RA, Bahner LH, Sheppard JM. 1978. The acute and chronic effects of cadmium on the estuarine mysid, *Mysidopsis bahia*. Bulletin of environmental Contamination & Toxicol.19: 80–85.

Pratap HB, Wendelaar Bonga SE. 1993. Effect of ambient and dietary cadmium on pavement cells, chloride cells, and sodium, potassium-ATPase activity in the gills of the freshwater teleost *Oreochromis mossambicus* at normal and high calcium levels in the ambient water. Aquatic Toxicol. 26: 133–150.

Roesijadi G, Robinson WE. 1994. Metal regulation in aquatic animals: mechanisms of uptake, accumulation, and release. In: Malins DC, Ostrander GK (eds). Aquatic toxicology: molecular, biochemical and cellular perspectives; pp. 387-420. Lewis Publishers, Boca Raton.

Rose CS, Heywood PG, Costanzo RM. 1992. Olfactory impairment after chronic occupational cadmium exposure. Journal of Occupational Med.34: 600 -5.

SAS Institute. 2000. SAS User's Guide: statistics, SAS Institute, Cary, NC.

Smith VJ, Johnston PA. 1992. Differential haemotoxic effect of PCB congeners in the common shrimp, *Crangon crangon*. Comparative Biochemistry and Physiol. C 101: 64 1–649.

Smith VJ, Söderhäll K, Hamilton M. 1984. 1,3-glucan induced cellular defense reaction in the shore crab, *Carcinus maenas*. Comparative Biochemistry and Physiol. A 77: 636–639.

Söderhäll K. 1983. 1,3-glucan enhancement of protease activity in crayfish *hemocyte lysate*. Comparative Biochemistry and Physiol. B 74: 221–224.

Söderhäll K, Cerenius L, Johansson MW. 1996. The prophenoloxidase activating system in invertebrates. In: Söderhäll K, Iwanaga SGR, Vasta GR (eds). New directions in invertebrate immunology. SOS Publications, Fair Haven NJ, p 229-253.

Souheil H. 1995. Pathogénicité et action des champignons dugenre *Fusarium* Link sur la capacité osmorégulatrice etles tissus osmorégulateurs de *Penaeus japonicus* (Bate). Thèse dr., Universite Montpellier-II, 164 pp.

Stohs SJ, Bagchi D, Hassoun E, Bagchi M. 2001. Oxidative mechanisms in the toxicity of chromium and cadmium ions. Journal of Environmental Pathology, Toxicol and Oncol. 20: 77-88.

Sritunyalucksana K, Sithisarn P, Withyachumnarnkul B, Flegel TW. 1999. Activation of prophenoloxidase, agglutinin and antibacterial activity in hemolymph of the black tiger prawn, *Penaeus monodon*, by immunostimulants. Fish and Shellfish Immunol. 19:2 1–3 0.

Thophon S, Kruatrachue M, Upatham ES, Pokethitiyook P, Sahaphong S, Jaritkhuan G. 2003. Histopathological alterations of white seabass, *Lates calcarifer*, in acute and sub chronic cadmium exposure. Environmental Pollut. 121: 307–320.

Thurberg FP, Dawson M, Collier R. 1983. Effects of copper and cadmium on osmoregulation and oxygen consumption in two species of estuarine crab. Marine Biol 23: 171-175.

Truscott R, White KN. 1990. The influence of metal and temperature stress on the immune system of crabs. Functional Ecol l4: 455–461.

Tsing A, Arcier JM, Brèhèlin M. 1989. Hemocytes of penaeids and palaemonid shrimps: morphology, cytochemistry and hemograms. Journal of Invertebrate Pathol53: 64–77.

Waalkes M. 2000. Cadmium carcinogenesis in review. Journal of Inorganic Biochemi.79: 241-244.

WHO.1984. Guidelines for drinking water quality., Geneva No.111. Volume 1. Recommendations.

Weeks-Perkins BA, Chansue N, Wong-Verelle D. 1995. Assay of immune function in shrimp phagocytes: techniques used as indicators of pesticides exposure. In: Stolen JS, Fletcher TC, Smith SA, Zelikoff JT, Kaattari SL, Anderson RS, Söderhäll K, Weeks-Perkins BA (eds). Techniques in fish immunology, Vol 4. SOS Publications, Fair Haven, NJ, p 223–231.

White SL, Rainbow PS. 1986. Accumulation of cadmium by *Palaemon elegans* (Crustacea: Decapoda). Marine Ecology Progress Ser.32: 17-25.

Yang ZB, Zhao YL, Li N, Yang J. 2007. Effect of waterborne copper on the microstructure of gill and hepatopancreas in *Eriocheir sinensis* and its induction of metallothionein synthesis. Archives of Environmental Contamination and Toxicology 52: 222-228.

Young JS, Pearce JB. 1975. Shell disease in crabs and lobsters from New York Bight. Bulletin of Marine Pollut. 6: 101-105.

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