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	73A-11 and C-K-2 were identified as desirable for seed production in all the three plant population environments. Genotypes 69B-88Z, Domu and 73A-97 were identified as desirable genotypes for cultivation in 133,333 plants ha^{-1} environment, C-K-2 in 166,667 plants ha^{-1} environment and 93A-97, 73A-11, 69B-88Z and C-K-2 in 266,667 plants ha^{-1} environment to obtain seed of high and stable germination and emergence. These genotypes were superior in seed quality and therefore deserve a place in commercial seed production and future seed improvement strategies. [Nature and Science 2010;8(2):7-14]. (ISSN: 1545-0740). **Keywords**: environment, interaction, plant population, seed quality	
3	**Turritopsis nutricula** Hongbao Ma, Yan Yang Brookdale University Hospital and Medical Center, Brooklyn, New York 11212, USA, hongbao@gmail.com **Abstract:** *Turritopsis nutricula* is a hydrozoan that can revert to the sexually immature (polyp stage) after becoming sexually mature. It is the only known metazoan capable of reverting completely to a sexually immature, colonial stage after having reached sexual maturity as a solitary stage. It does this through the cell development process of transdifferentiation. This cycle can repeat indefinitely tha offers it biologically immortal. It is not clear if stem cells are involved in this immortality or not. Upto now, there is little academic report in the Turristopsis nutricula studies. To study the mechanism of the biological immortality of Turritopsis nutricula possibly supplies the way finding the biological immortality for human. [Nature and Science 2010;8(2):15-20]. (ISSN: 1545-0740). **Keywords:** immortal; immortality; sexual maturity; stem cell; transdifferentiation; *Turritopsis nutricula*	Full Text
4	**Oral Vaccination of Nile Tilapia (*Orechromis niloticus*) Against Motile Aeromonas Septicaemia** *Noor El Deen Ahmed Ismail[(1)], Nagwa Sad. Atta I[(2] and Abd E Aziz ,Mohamed .Ahmed[(3)]* *[1)] Dept. of Hydrobiology , National Research Centre(, N.R.C).* *[(2)] Dept. of Microbiology, N.R.C.* *[3)] Dept. of Fish Diseases, Fac.of Vet Med. Cairo.* dr_ahmednoor2002@yahoo.com **Abstract:** The present study was planned for preparation of formalin inactivated wet-packed whole cells Aeromonas *hydrophila* bacterin for oral vaccination. The humeral antibody response of vaccinated Nile tilapia *(Orechromis niloticus (O. niloticus)* was determined by micro-agglutination test. Moreover efficacy of the prepared bacterin against infection with *Aeromonas hydrophila* was detection and calculated as a relative level of protection. Nile tilapia *(O. niloticus)* immunized orally with formalin-inactivated *Aeromonas hydrophila* .wet-packed while cells had low level of antibody titer reached 2 and 3 by log2 at first and fourth week post-immunization respectively while Nile tilapia *(O. niloticus)* fed on minced meat without vaccine had antibody titer reached 1 by log 2 throughout the experimental period . The relative level of protection among Nile tilapia *(O. niloticus)* immunized orally were 86.8. [Nature and Science 2010;8(2):21-26]. (ISSN: 1545-0740). **Keywords:** *Aeromonas hydrophila* - bacterin -vaccination- humeral antibody- Nile tilapia	Full Text
5	**Forest community structure and composition along an elevational gradient of Parshuram Kund area in Lohit District of Arunachal Pradesh, India** C.S. Rana and Sumeet Gairola Department of Botany, HNB Garhwal University, Srinagar Garhwal- 246 174, Uttarakhand, India drcsir@gmail.com, sumeetgairola@gmail.com **Abstract:** The present study was conducted in natural Himalayan forests of Parsuram Kund area in Lohit district of Arunachal Pradesh to understand the effect of altitudinal variation on structure and composition of the vegetation. Three altitudinal zones viz., upper zone (U) = 550-850 m asl, middle zone (M) = 500-700 m asl and lower zone (L) = 350-450 m asl were selected for the study. Tree Species richness (SR) was recorded to be highest (26) on the middle altitude followed by lower (21) and upper altitude (13). Species diversity (richness) and dominance (Simpson Concentration of dominance index) were found to be inversely related to each other. Shrub and herb species richness were also recorded to be higher on middle altitude followed by lower and upper altitudes. [Nature and Science 2010;8(2):27-35]. (ISSN: 1545-0740).	Full Text

	Key words: Phytosociology, species richness, diversity indices, altitude	
6	**Effects of some Artificial diets on the Growth Performance, Survival Rate and Biomass of the fry of climbing perch, *Anabas testudineus* (Bloch, 1792)** Md. Jobaer Alam, Md. Ghulam Mustafa, Md. Mominul Islam Department of Fisheries, University of Dhaka, Dhaka-1000, Bangladesh. Email: jobaerviu@gmail.com, mghulam@univdhaka.edu **Abstract:** An experiment on culture of climbing perch (*Anabas testudineus*) in cemented tanks using different protein level diets was conducted to find the appropriate feeding diets and their effects on the growth, survival and biomass of 15-days old fry of *Anabas testudineus* in intensive culture . The experiment was carried out for duration of 60 days with 4 treatments in 8 cemented tanks each of size 12×6×1.5 feet. The initial length and weight were 14.5 ± 0.4 mm and 0.95 ± 0.05 g respectively. The feeds were applied twice a day at the rate of 10 %(initially) to 5% (later on) of the body weight of the fry/day. The results showed that the growth of fry varied significantly ($P<0.05$) with different diets. The highest growth, survival rate and biomass were found in the trial where fishes were fed on Sabinco feed containing 50.92% protein (on dry matter basis), followed by Feed-3 containing 30% protein. The poorest growth rate was shown by Feed- 1 (prepared by rice bran, wheat bran, fish meal and soybean meal) containing 20% protein. There was no significant difference in survival rates among the fry fed with Sabinco and prepared diets. The experiment suggests that Feed-2 (Sabinco Feed) can be recommended for the intensive culture of climbing perch. [Nature and Science. 2010;8(2):36-42]. (ISSN: 1545-0740). **Key words:** Climbing perch, Artificial diets, Survival rate, Growth Performances and Biomass	Full Text
7	**Diminution Of Aflatoxicosis In Tilipia Zilli Fish By Dietary Supplementation With Fix In Toxin And Nigella Sativa Oil** *Mona S. Zaki[(1)] ; Olfat M. Fawzi[(2)]; Suzan Omar[(2)]; medhat khafagy[(3)]; mostafa fawzy[(1)];* Isiz Awad [(2)] [1]Department of Aquaculture, Vet. Division National Research Centre, Giza, Egypt. [2]Department of Biochemistry, National Research Centre, Giza, Egypt. [3]National Cancer Institute, Cairo University, Egypt. *dr_mona_zaki@yahoo.co.uk* **Abstract:** Mycotoxins are toxic metabolites of fungal origin, they are produced by certain strains of the fungi *Aspergillus flavus* and *Aspergillus parasiticus.* Under favorable conditions of temperature and humidity, these fungi grow on certain foods and feeds, resulting in the production of aflatoxins, which can enter into the human food chain directly through foods of plant origin (cereal grains), indirectly through foods of animal origin (kidney, liver, milk, eggs); however their continuous intake even in microdoses can result in their accumulation. Aflatoxins are hepatotoxic, hepatocarcinogenic and immunotoxic and cause growth retardation in animals and exposed human populations.Fix in Toxin is a kind of pentonite (clay) consists of (sodium calcium aluminosilicate), a non toxic agent and absorbent for a wide variety of toxic agents. It acts as an enterosorbant that rapidly binds aflatoxins in the gastrointestinal tract resulting in decreased aflatoxin uptake and bioavailability. *Nigella sativa* is a spicy potent belonging to ranunculacea seeds oil showed antibacterial, fungicidal effects.This study was conducted to evaluate the ability of Fix in Toxin 0.2 % and *Nigella sativa* oil 1% to diminish the clinical signs of aflatoxicosis in *Tilapia Zilli* fish, and based on this evidence, it's hypothesized that clay based entersorption of Aflatoxin may be a useful strategy for prevention of Aflatoxicosis in human population.60 *Tilapia Zilli* fish were divided into three groups, 20 fish for each group: Group 1 served as control and will be fed on commercial fish diet. Group 2 were be supplied by Aflatoxin contaminated ration with corn 80 ug toxin /kg ration. Group 3 were be supplied by aflatoxin contaminated ration with corn 80 ug toxin/kg ration and treated with 0.2 % Fix in Toxin and 1 % Nigella sativa oil injected daily I/P. Analysis of hematological parameters, clinical chemistry revealed significant differences between the control groups and the aflatoxicotic groups. administration of Fix in Toxin 0.2% and *Nigella sativa oil* injection 1% of body weight reduced the aflatoxicosis in liver and kidney by improving all liver and kidney enzymes.The dietary HSCAS clay remedy is novel, inexpensive and easily disseminated and proves its efficacy in diminishing the clinical signs of aflatoxicosis in fish, where it acts as an alfatoxin enterosorbant that tightly and selectively binds the poison in the gastrointestinal tract of the fish, decreasing their bioavailability and associated toxicities. In addition the *Nigella sativa oil* has a synergistic effect with Fix in Toxin in diminishing aflatoxicosis in fish. These findings support	Full Text

	their use for dietary intervention studies in human populations at high risks for aflatoxicosis, specially in Egypt, where studies have shown that concurrent infection with the hepatitis B virus (HBV) during aflatoxin exposure increases the risk of hepatocellular carcinoma (HCC). [Nature and Science 2010;8(2):43-49]. (ISSN: 1545-0740). **Key words**: Aflatoxicosis, *Tilapia Zilli* fish, Fix in Toxin effect, *Nigella sativa oil* effect, Hematological parameters, Clinical chemistry dynamic simulation; model; composting; domestic solid waste	
8	**Integrated Application of Poultry Manure and NPK Fertilizer on Performance of Tomato in Derived Savannah Transition Zone of Southwest Nigeria** [1]Ayeni L.S, .[2]Omole T.O, [2]Adeleye, .E.O. and [3]Ojeniyi, S.O. [1]University of Agriculture, Department of Soil Science and Land Mgt, PMB 2240, Abeokuta, Nigeria [2]Adeyemi College of Education, Department of Agricultural Science, Ondo, Nigeria. [3] Federal University of technology Department of Crop Pest And Soil Management Akure, Nigeria [1]Corresponding author: leye_sam@yahoo.com **Abstract:** Field experiments were conducted in two locations at Owo in early and late crop seasons (2007) to compare the effects of poultry manure at 0, 10, 20, 30, 40 t ha^{-1} and 300 kg ha^{-1} NPK 15:15:15 fertilizer on nutrient uptake and yield of tomato. The sites were located within the forest savannah transition zone of southwest Nigeria. The experiments were replicated three times in randomized complete block design. The test soil was slightly acidic, low in OM, N and P. Application of poultry manure and 300kg ha^{-1} NPK fertilizer significantly ($P<0.05$) increased plant N, P and K. Poultry manure at 20, 30 and 40 t ha^{-1} and NPK 15:15:15 fertilizer significantly ($P<0.05$) increased plant leaf, area height, number of leaves, branches fruits and fruit yield. Application of 10 t ha^{-1} poultry manure gave similar values of plant N, P and K and yield components compared with 300 kg ha^{-1} NPK fertilizer. The cumulative yield for the two seasons at 0, 10, 20, 30, 40 t ha^{-1} and 300kg/ha NPK were 9.6, 12.0, 18.1, 19.3, 14.4 and 13.5 t ha^{-1} respectively. [Nature and Science. 2010;8(2):50-54]. (ISSN: 1545-0740). **Key words**: early crop, late crop, nutrient concentration, yield	Full Text
9	**Antigenotoxic Efficacy of Some Vitamins against the Mutagenicity Induced by Ifosfamide in Mice** Souria M. Donya , Fawzia A. Aly, Mona A. M. Abo-Zeid Genetics and Cytology Department, Genetic Engineering and Biotechnology Division, National Research Center, El-Behooth St. 31, Dokki 12622, Cairo, Egypt. monaabozeid@yahoo.com **Abstract:** Ifosfamide (Holoxan, IFO) is an oxazaphosphorine alkylating agent with a broad spectrum of antineoplastic activity. IFO can damage DNA during any phase of the cell cycle and therefore, is not phase-specific. Thus the objective of this investigation is to measure the potential cytotoxicity of IFO alone and in combination with vitamins (FA, VB12 and VC) The genotoxic potential of IFO was evaluated *in vivo* using different mutagenic end points. Male Swiss mice were injected with different doses of IFO intraperitoneally to investigate the genotoxicity in somatic and germ cells. The doses were 8, 16 and 24mg IFO/kg body wt. as single doses, and 8mg IFO/kg body wt. as a repeated dose for three consecutive days. Samples were collected after 24h, 7 and 14 days after treatments. IFO induced chromosomal aberrations (in somatic and germ cells), SCEs and sperm shape abnormalities, which were highly significant in a dose dependent manner 24h after treatments. Chromosomal aberrations were declined with increasing the time of recovery. However, the tetraploid cells in mouse bone marrow were increased. IFO increased the percentage of DNA fragmentation in mouse spleen cells as measured by diphenylamine (DPA) assay, and confirmed by agarose gel-electrophoresis. Oral administration of folic acid (10 mg/kg body wt.), vitamin B12 (0.3 mg/kg) and vitamin C (50 mg/kg body wt.) declined the chromosomal aberrations in somatic and germ cells 24h after concurrent treatment with IFO. The used doses of vitamins reduced the percentage of DNA fragmentation induced by 24mg IFO/kg body wt. with DPA assay. **In conclusion**, the study indicates that the anticancer drug IFO is a mutagenic agent in mouse somatic and germ cells. Vitamins (FA, VB12 and VC) play a beneficial role against the mutagenicity of this drug. [Nature and Science 2010;8(2):55-66]. (ISSN: 1545-0740). **Keywords:** IFO, FA, VB12, VC, cytogenetic parameters, DNA damage	Full Text
10	**Use of InfoWork RS in modeling the impact of urbanisation on sediment yield in Cameron Highlands, Malaysia**	Full Text

	Mohd Ekhwan Toriman[1], Othman A. Karim[2], Mazlin Mokhtar[3], Muhammad Barzani Gazim[4], Md. Pauzi Abdullah[4] 1. School of Social, Development & Environmental Studies, Universiti Kebangsaan Malaysia. 2. Department of Civil Engineering, National University of Malaysia. 3. Institute of Environment and Development (LESTARI) Universiti Kebangsaan Malaysia 4. School of Environmental and Natural Resource Sciences, National University of Malaysia. ikhwan@ukm.my **Abstract:** Hydrodynamic model and sediment transport model were investigated in the Sg Telom and Sg Bertam, Cameron Highlands as a result of rapid urbanization and agriculture activities over the past 30 years. This article, from the point of view of the river catchment as a whole system, presents an integrated approach by combining the hydraulic and hydrology simulations with numerical model of sediment transport and change in river bed level before and after the Ringlet reservoir. To accomplish this purpose, InfoWork RS, a well developed numerical model for sediment transport and river bed variations were used. The application shows that it can properly simulate change of river bed variation over 10 months simulation period. [Nature and Science 2010;8(2):67-73]. (ISSN: 1545-0740). **Key words**: dynamic simulation; model; composting; urbanization; sediment	
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, Faculty of Social Sciences and Humanities, 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(2):74-81]. (ISSN: 1545-0740). **Keywords:** Environmental Flow Assessment; Detailed Environmental Impact Assessment; Low flow; Flow duration curve	Full Text
12	**Growth inhibitory effect on microorganisms by a D-galactose-binding lectin purified from the sea hare (*Aplysia kurodai*) eggs: An *in vitro* study** Sarkar M. A. Kawsar[1*], Sarkar M. A. Mamun[2], Md Shafiqur Rahman[3], Hidetaro Yasumitsu[1], Yasuhiro Ozeki[1*] 5. Department of Genome System Science, Graduate School of Nano Biosciences, Yokohama City University, 22-2 Seto, Kanazawa-Ku, Yokohama 236-0027, Japan 6. Department of Botany, Faculty of Science, University of Chittagong, Chittagong-4331, Bangladesh 7. Department of Microbiology, Faculty of Science, University of Chittagong, Chittagong-4331, Bangladesh kawsoral@yahoo.com; ozeki@yokohama-cu.ac.jp **Abstract:** A D-galactose specific lectin purified from the eggs of sea hare, *Aplysia kurodai* (AKL) by lactosyl-agarose affinity chromatography has been evaluated for screening of antimicrobial activities. AKL was disulfide	Full Text

	bonded dimeric lectin consisted of two 32 kDa polypeptides. This lectin has significant hemagglutinting activity against trypsinized rabbit and human erythrocytes and it was inhibited by galactose and galacturonic acid. AKL has been screened for *in vitro* both antibacterial activity against eleven human pathogenic bacteria and antifungal activity against six phytopathogenic fungi. Antimicrobial evaluation of standard antibiotics, ampicillin and nystatin were used as comparative study. AKL significantly inhibited the growth of gram-positive bacteria. *Staphylococcus aureus* (12 mm) and *Bacillus megaterium* (11 mm) were exhibited the highest zone of inhibition by the addition of the lectin (250 µg/disc). However, AKL did not inhibit the growth of gram-negative bacteria as *Escherichia coli*. On the other hand, AKL (100 µg/mL) has also inhibited the mycelial growth of *Curvularia lunata* (21.53%). These antimicrobial activities by the lectin will provide an effective defense ability of the sea hare eggs against invading microbes. [Nature and Science 2010;8(2):82-89]. (ISSN: 1545-0740). **Key words**: *Aplysia kurodai*, lectin, organisms, mycelial growth, SDS-popyacrylamide gel electrophoresis	
13	**Urban land use classification and functional zoning of Ulaanbaatar city, Mongolia** Gantulga Gombodorj and Chinbat Badamdorj Faculty of Earth Sciences, National University of Mongolia, Ikh surguulliin gudamj 2 -NUM building 6, Ulaanbaatar 210646, Mongolia. gantulga100@yahoo.com lis@num.edu.mn **Abstract:** As Mongolia moves to a market economy and begins to encourage land ownership and the creation of private land and housing markets, it will need to revise its approach to the spatial planning and control of land uses and development by individuals and businesses. Designed for a regime of exclusive state ownership of land, the existing "target use" restrictions for individual parcels as shown in the detailed Master Plans hinders the exercise of market-driven choices by individuals and businesses wishing to put land and infrastructure to their most productive uses. [Nature and Science 2010;8(2):90-97]. (ISSN: 1545-0740). **Keywords:** Urban land use classification, land use zoning and regulation, functional zones	Full Text
14	**Studies On Susceptibility Of Methicillin –Resistent Staphylococcus aureus To Some Nigerian Honey** (1)Yenda, E. N. *(2)De, N. (2)Lynn, M and (2)Aliyu, T B (1) Health Services Management Board, P.M.B. 1082, Jalingo, Taraba State, Nigeria, e-mail: ebeny@justice.com (2) Department of Microbiology, Federal university of Technology, Yola, e-mail: nanditamicrobio@yahoo.com * To whom all correspondence should be addressed **Abstract:** This study was aimed at determining the susceptibility of methicillin-resistant S. aureus (MRSA) isolates to some Nigerian honey. Sixty isolates of S. aureus were obtained from patients attending State Hospital, Jimeta Yola, Adamawa State. Twenty out of the sixty isolates were MRSA which were assessed for susceptibility or resistance to three (one processed and two crude) local honey samples in different concentrations and two commonly used antibiotics namely ciprofloxacin and ofloxacin using disk diffusion assay. All the twenty MRSA were susceptible to undiluted Sardauna plateau honey and its different concentrations of 50%, 25% and 13% (with growth inhibition zone ranging from 13 to 33 mm) but 25% of the isolates were resistant at concentrations of 6%. Against the MRSA isolates, undiluted Hong honey recorded 85% antibacterial activity, followed by 65%, 55%, and 5% respectively for its lower dilutions of 50%, 25% and 13% (with growth inhibition zone 12 or less than 12 mm). The undiluted Abuja honey sample recorded 85% antibacterial activity, followed by 35% and 15% respectively for its lower dilutions of 50% and 25%. Eighty five percent (85%) of the isolates were resistant to ofloxacin and 80% of the isolates were resistant to cipfrofloxacin (growth inhibition zone 20 mm or less for ciprofloxacin and 15 mm or less for ofloxacin, respectively). Values of the minimum inhibitory concentration and the minimum bactericidal concentration of S.P. honey were in the range of 0.4%-0.5% and 0.8 - 1% respectively whereas the values for H. honey and A. honey were in the range of 0.9-0.1% and 1.9-2.0% and 3.5-4.0% respectively. [Nature and Science 2010;8(2):98-108]. (ISSN: 1545-0740). **Keywords:** MRSA, honey, methicillin, MIC, MBC	Full Text
15	**The relationship between serum adiponectin and steatosis in patients with chronic hepatitis C genotype-4** Esmat Ashour, PhD, Nervana Samy, MD, Magda Sayed, PhD and Azza Imam*, MD. Biochemistry Department -National Research Center- Cairo * Internal Medicine Department - Faculty of medicine - Ain Shams University. nervana91@hotmail.com	Full Text

	Abstract: The mechanisms underlying steatosis during hepatitis C virus (HCV) infection are complex and multifactorial. The aim of our study was to assess whether host metabolic factors influence the degree of hepatic steatosis and fibrosis in patients infected with hepatitis C virus genotype 4 by investigating the role of adiponectin, leptin and insulin resistance. **Methods:** Adiponectin and leptin levels, HCV genotypes, HCV-RNA, IR (HOMA-IR), body mass index and liver steatosis and fibrosis were assessed in 74 chronic patients with HCV genotype 4. **Results:** Chronic HCV patients with steatosis showed lower serum adiponectin levels and higher levels of leptin, HOMA, alanine aminotransferase, γ glutamiltransferase and fibrosis scores. Low adiponectin levels were independently associated with grades of steatosis and HOMA-IR. Adiponectin levels showed significant inverse correlation between adiponectin and steatosis grade, BMI, HOMA and fibrosis stage. The multivariate analysis of factors showed that steatosis was significantly associated with low adiponectin concentration while, leptin, Insulin, HOMA, ALT, γ-GT and cholesterol were positively associated with steatosis. **Conclusion:** This study stated that Egyptian patients with HCV genotype-4 suffering from steatosis had lower adiponectin level that is inversely correlated with insulin resistance. These data support a role for adiponectin in protection against liver injury and that hypoadiponectinemia may contribute to hepatic steatosis progression. Further molecular and genetic studies with larger numbers of patients are required to confirm these results. [Nature and Science 2010;8(2):109-120]. (ISSN: 1545-0740). **Key words**: Adiponectin, steatosis, hepatitis C, leptin	
16	**Comparative studies on the impact of Humic acid and formalin on ectoparasitic infestation in Nile tilapia *Oreochromis niloticus*** *Noor El- Deen, A.E.* [1]*, Mona M.Ismaiel*[2]*, Mohamed A. E*[3]*. and Omima A.A.El-Ghany*[3] 1. Hydroiology Dept. National Research Center Dokki, Egypt 2. Fish diseases and management Dept .Fac.of Vet .Med. Seuz Canal Univ. 3. Fish disease Dept. Animal Health Research institute Dokki Egypt. dr_ahmednoor2002@yahoo.com **Abstract:** Naturally infested *Oreochromis niloticus* (*O. niloticus*) were collected and examined for external parasites (*Trichodina* and *Cichlidogyrus*). The aim of present study was to investigate the efficacy and difference in treatment of formalin as a chemical and Humic acid as a natural product in treatment of ectoparasitic infestation of *O.niloticus*. The prevalence of isolated parasites showed high infestation rate of *Trichodina* (100%) and *Cichlidogyrus* (65%). The main clinical sings of infested fish were slimy dark skin with signs of asphyxia, rapid opericular movement, aggregation of fish on the water surface with gulping the atmospheric air. Flashing of fish, detached scales with frayed fins with presence of hemorrhagic lesions on the skin, fins, gills with congested gills, sluggish movement, finally off food and loss of escape reflex. Hematological parameters of infested fish showed significant decrease in red blood cell counts, hemoglobin percentage and packed cell volume. Total WBCs showed non significant difference. Serum total protein and cholesterol were significantly decreased, while, AST, ALT, alkaline phosphatase, urea sodium and potassium were significantly increased. Chemical treatment using formalin 20 ppm for 24h. As a long bath and natural treatment using Humic acid 3 ppm for 24h. As along bath showed complete eradication of *Trichodina* and *Cichlidogyrus*. [Nature and Science 2010;8(2):121-125]. (ISSN: 1545-0740). **Keywords:** *Oreochromis niloticus- Trichodina – Cichlidogyrus*- Humic acid and formalin	Full Text

Optimization of Asparaginase Production by *Pseudomonas aeruginosa* Using Experimental Methods

R. Manikandan[1], CN Pratheeba[2], Pankaj Sah[3] and Stuti Sah [4]

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Abstract: Evaluation of fermentation process parameter interactions for the production of l-asparaginase by *Pseudomonas aeruginosa*. Box-Behnken design of experimentation was adopted to optimize nutritional sources, physiological (incubation period) and microbial (inoculum level).The experimental results and software predicted enzyme production values were comparable. Incubation period, inoculum level and nutritional source (soybean) were major influential parameters at their individual level. Interaction data of the selected fermentation parameters could be classified as least and most significant at individual and interactive levels. All selected factors showed impact on l-asparaginase enzyme production by this isolated microbial strain either at the individual or interactive level. Incubation temperature, inoculums concentration, and nutritional source (soybean) had impact at individual level. Significant improvement in enzyme production by this microbial isolate was noted under optimized environment. [Nature and Science. 2010;8(2):1-6]. (ISSN: 1545-0740).

Key words: box-Behnken; *pseudomonas aeruginosa*; L- asparaginase; response surface

1. Introduction

L-Asparaginase has received increased awareness in current years for its ant carcinogenic potential. Cancer cells distinguish themselves from normal cells in diminished expression of l-asparagine (Swain *et al.* 1993; Manna *et al.* 1995). Hence, they are not capable of producing l-asparagine, and mainly depend on the l-asparagine from the circulating plasma pools (Swain *et al.* 1993).l-Asparaginase (l-asparagine amidohydrolase EC 3.5.1.1) catalyses the conversion of l-asparagine to l-aspartate and ammonium, and this catalytic reaction is essentially permanent under physiological conditions. If l-asparaginase is given to cancer patients then there will be nonstop reduction of l-asparagine. This extradinary behavior of cancerous cells was broken by scientific community (Story *et al.* 1993; Swain *et al.* 1993). Asparaginase is used for treating acute lymphoblastic leukemia, lymphosarcoma. This therapy brought a major breakthrough in modern oncology. With the development of its new functions, a great demand for l-asparaginase is expected in the coming years. The biochemical and enzyme kinetic properties vary with the microbial source. However, *Erwinia* asparaginase had a shorter half life than E. coli (Asselin et al. 1993); suggesting the need to discover new l-asparaginases that are serologically different, but have similar beneficial effects. This requires selection of soil samples from various sources for isolation of possible microbes, which have the ability to produce the most wanted enzyme.

Experimental designs nowadays have been regarded as one of the most favorable techniques in covering a large area of practical statistics and obtain unambiguous results with the least expense. Response surface method (RSM) designs help to quantify the relationships between one or more measured responses and the vital input factors. The most popular response surface methodologies are Central Composite, Box-Behnken designs.

Box-Behnken design is an efficient and creative three-level composite design for fitting second-order response surfaces. It is an independent quadratic design. The methodology is based on the construction of balance designs which are rotatable and enable each factor level to be tested several times. Each factor or

independent variable can be placed at one of three equally spaced values (coded as –1, 0, and +1). In this design the treatment combinations are at the midpoints of edges of the cubical design region and at the center. Box-Behnken designs provide excellent predictability within the spherical design space and require fewer experiments compared to the full factorial designs or central composite designs. The number of required experiments for Box-Behnken design can be calculated according to $N = k^2 + k + c_p$, where k is the factor number and c_p is the replicate number of the central point.

In the present investigation**,** we study about optimization of asparaginase production by *Pseudomona aeruginosa using* design of experiments by Box-Benhken Design.

2. Materials and methods:

2.1 Maintenance and cultivation of Microorganism

The strain *Pseudomonas aeruginosa* was obtained from NCIM, Pune, India. The strain was subcultured in nutrient broth. The broth was incubated in the shaker with 175 rpm and at 37°C overnight. Sterile plates containing nutrient agar of specified composition were streak plated with the overnight cultures. In 100 ml nutrient broth, the cultures are grown overnight. The culture on the broth was used as the source for the entire experiment. Cultivation was achieved by solid-state fermentation (SSF) as previously reported by Ramesh and Lonsane (1987).2.24 g of soyabean is moistened with 5 ml of phosphate buffer containing culture. The plates are incubated for 48 hrs & are checked for enzyme activity.

2.2 Estimation of L-asparaginase activity

Reaction mixture consisting of 0.5 ml of 0.08 mol/l of 1 l-asparagine, 1ml of 0.05mol/l borate buffer (pH 7.5) and 0.5 ml of enzyme solution was incubated for 10 min at 37 ^{0}C. The reaction was stopped by the addition of 0.2 ml of 15% trichloroacetic acid solution. The liberated ammonia was coupled with 1 ml Nessler's reagent & OD is measured at 500 nm, and was quantitatively determined using standard curve.

2.3 Optimization of the process parameters

Process optimization was carried out by conducting 17 experiments to identify the best combinations of the parameters which involved in the production biomass to obtain high yield of crude extract. The parameters, soybean (10, 12.5,15 gms), inoculums (300, 450, 600 µl) and incubation (48, 72, 60 h) were selected. 17 different cultures were obtained by varying the three parameters. The concentration of the enzyme was measured using standard plot. The data obtained from 17 experiments, were used to find out the optimum point of the process parameters by using Box-Behnken Design in Response surface methodology. All the data were treated with the aid of Design Expert from Stat-Ease.

3. Results and Discussion

3.1 Analysis of variance

Based on design of experiment, 17 combination were developed (Table 1) and processed to obtain asparaginase as mentioned in this paper. The data obtained from the experiments were used to the analysis of variance (Table 2 and 3). The Model F-value of 6.366E+007 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, AC, BC, A^2, B^2, C^2, A^2B, A^2C, $AB^2$are significant model.

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Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Analysis of process variables by response surface plots

The optimum values of the selected variables were obtained by solving their regression equation and analyzing response surface contour plots. Response Surface plots as a function of two factor at a time maintaining all other factors at a fixed level (zero for instance) are more helpful in understanding both the main and interaction effects of the two factors. The plots can be easily obtained by calculating the data from the model. The values were taken by one factor, where the second varies with constant of a given Y -values. The yield values of the different concentrations of the variable can also be predicted from respective response surface plots. Figure 1 to 6 shows the relative effect of the two variables with protein concentration level. The coordinates of the central point within the highest

contour levels in each of these figures corresponded to the optimum concentrations of the respective components.

Figure 1 and 2 show their contour and response surface plot obtained as a function of incubation period vs. medium with asparaginase concentration, while all other variables are maintained at zero level (coded units). Figure 3 and 4 show their contour and response surface plot obtained as a function of volume of inoculum vs. medium with soybean concentration, while all other variables are maintained at zero level (coded units).Figure 5 and 6 show their contour and response surface plot obtained as a function of Incubation period vs. medium with asparaginase concentration, while all other variables are maintained at zero level (coded units).

Final equation in terms of terms of coded factors:

Asparaginase (mg/ml) = 5.56 + (0.4985 * A) - (0.544 * B) - (0.2155 * C) - (0.25825 * A * B) - (0.537 * A * C) - (0.092 * B * C) + (0.069375 * A^2) - (0.11963 * B^2) + (0.526125 * C^2) + (0.76725 * A^2* B) + (0.8625 * A^2* C) - (0.94925 * A * B^2)

Optimum values

The protein production was predominantly influenced by the amount of soybean, incubation period and inoculum. The contour plots show the region of the desirability for the production of protein content. The point prediction from the analysis of variable for response surface cubic model for the production of protein concentration (5.566 mg/ml) is 12.5 ml of medium, 450 μl of inoculum, and 60 h of incubation.

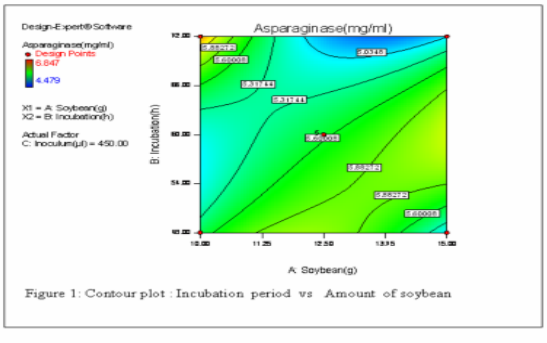

Figure 1: Contour plot : Incubation period vs Amount of soybean

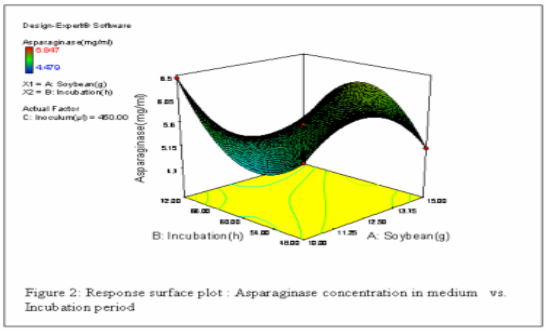

Figure 2: Response surface plot : Asparaginase concentration in medium vs. Incubation period

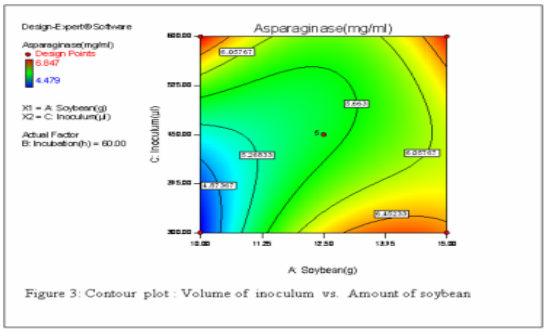

Figure 3: Contour plot : Volume of inoculum vs. Amount of soybean

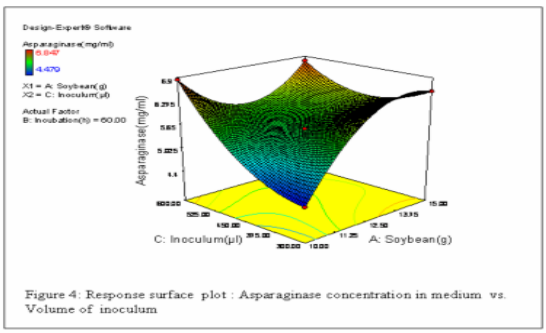

Figure 4: Response surface plot : Asparaginase concentration in medium vs. Volume of inoculum

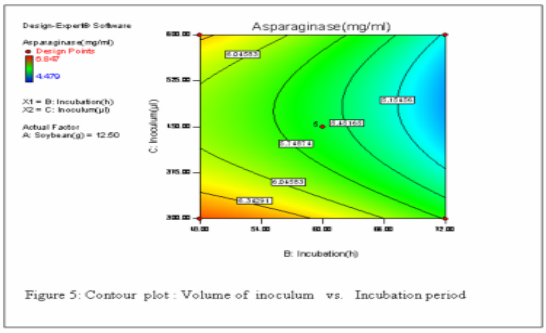

Figure 5: Contour plot : Volume of inoculum vs. Incubation period

Table 1. Combination of process variables

Run	A: Soybean (g)	B: Incubation (h)	C: Inoculum (µl)	Asparaginase (mg/ml)
1	10	72	450	6.448
2	10	48	450	5.485
3	15	60	600	6.770
4	10	60	600	6.847
5	12.5	60	450	5.566
6	12.5	60	450	5.566
7	12.5	60	450	5.566
8	12.5	48	600	6.393
9	15	72	450	5.030
10	15	60	300	6.550
11	12.5	72	300	5.736
12	12.5	48	300	6.640
13	10	60	300	4.479
14	12.5	60	450	5.566
15	12.5	60	450	5.566
16	12.5	72	600	5.121
17	15	48	450	5.100

Table 2. ANOVA for Response Surface Cubic Model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob >F	
Model	7.741125	12	0.645094	6.366E+007	<0.0001	significant
A-Soybean(g)	0.994009	1	0.994009	6.366E+007	<0.0001	
B-Incubation(h)	1.183744	1	1.183744	6.366E+007	<0.0001	
C-Inoculum(µl)	0.185761	1	0.185761	6.366E+007	<0.0001	
AB	0.266772	1	0.266772	6.366E+007	<0.0001	
AC	1.153476	1	1.153476	6.366E+007	<0.0001	
BC	0.033856	1	0.033856	6.366E+007	<0.0001	
A^2	0.020265	1	0.020265	6.366E+007	<0.0001	
B^2	0.060253	1	0.060253	6.366E+007	<0.0001	
C^2	1.165505	1	1.165505	6.366E+007	<0.0001	
A^2B	1.177345	1	1.177345	6.366E+007	<0.0001	
A^2C	1.487813	1	1.487813	6.366E+007	<0.0001	
AB^2	1.802151	1	1.802151	6.366E+007	<0.0001	
AC^2	0	0				
B^2C	0	0				
BC^2	0	0				
A^3	0	0				
B^3	0	0				
C^3	0	0				
Pure Error	0	4	0			
Cor Total	7.741125	16				

Table 3. Regression Analysis

Std. Dev.	0	R-Squared	1
Mean	5.78	Adj R-Squared	1
C.V. %	0	Pred R-Squared	N/A
PRESS	N/A	Adeq Precision	0

Table 4. Coefficient value of the factor

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	5.566	1				
A-Soybean(g)	0.4985	1				2
B-Incubation(h)	-0.544	1				2
C- inoculum(µl)	-0.2155	1				2
AB	-0.25825	1				1
AC	-0.537	1				1
BC	-0.092	1				1
A^2	0.069375	1				1.005882
B^2	-0.11963	1				1.005882
C^2	0.526125	1				1.005882
ABC ALIASED Intercept						
A^2B	0.76725	1				2
A^2C	0.8625	1				2
AB^2	-0.94925	1				2

Table 5. Predicted value from Box - Behnken design

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding	
A	Soybean(g)	12.5	10	15	0	Actual	
B	Incubation(h)	60	48	72	0	Actual	
C	Inoculum (µl)	450	300	600	0	Actual	
Response	Prediction	SE Mean	95% CI low	95% CI high	SE Pred	95% PI low	95% PI high
Asparaginase (mg/ml)	5.566	0	5.566	5.566	0	5.566	5.566

PI - Prediction interval
CI - Confidence interval
SE Mean – Standard error of the mean.
SE Pred – Standard error of prediction

3. Conclusion

In this work the process parameters the amount of soybean, incubation time and inoculum were selected and optimized to produce asparaginase. Design Expert from Stat-Ease was used to develop design of experiment. Box Behnken design in Response surface method was used to optimize the process condition. Thus it has been concluded that the point prediction from the analysis of variable for response surface cubic model for the production of protein concentration (5.566 mg/ml) is 12.5 ml of medium, 450 µl of inoculum, and 60 h of incubation.

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Stability Analysis of Seed Germination and Field Emergence Performance of Tropical Rain-fed Sesame Genotypes

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Abstract: The work was carried out to determine the stability of two seed quality traits (seed germination and field emergence) in 14 sesame genotypes that were grown in three plant population environments in Abeokuta, southwest Nigeria in each of two seasons. Seeds harvested from each environment were tested for these quality traits. Data obtained were subjected to analysis of variance of Finlay-Wilkinson regressions and stability analysis. Each genotype was defined by three stability parameters: (1) mean seed germination and field emergence over all environments, (2) the linear regression (b values) of genotype mean seed germination and field emergence in each environment, (3) the mean square deviation from the regression for each genotype (S^2d value). The genotypes varied considerably in the two seed quality traits and genotype x environment (GxE) interactions were significant. Regression coefficients ranged from 0.19 to 1.70 for seed germination and 0.14 to 3.01 for field emergence. Genotype 530-6-1 with a regression coefficient close to unit (b=1.03), smaller S^2d value and a relatively high seed germination of 79% had general adaptability and somehow averagely stable. The highest field emerging genotypes proved less stable and selection solely for high emergence could result in discarding many genotypes that were relatively better adapted to environmental changes. Genotypes 530-6-1, 73A-11 and C-K-2 were identified as desirable for seed production in all the three plant population environments. Genotypes 69B-88Z, Domu and 73A-97 were identified as desirable genotypes for cultivation in 133,333 plants ha^{-1} environment, C-K-2 in 166,667 plants ha^{-1} environment and 93A-97, 73A-11, 69B-88Z and C-K-2 in 266,667 plants ha^{-1} environment to obtain seed of high and stable germination and emergence. These genotypes were superior in seed quality and therefore deserve a place in commercial seed production and future seed improvement strategies. [Nature and Science 2010;8(2):7-14]. (ISSN: 1545-0740).

Keywords: environment, interaction, plant population, seed quality

1. Introduction

Seed quality is defined as a standard of excellence in certain characters or attributes that will determine the performance of the seed when sown or stored (Hampton, 2002). It relates to the characteristics of seeds which result in the high field performance and eventually high seed/grain yield. Seed germination and field emergence have been identified as good indicators of seed quality in different crops.

Most of the quality characteristics are polygenically inherited, and will therefore be influenced by the environment to a large extent (Labuschangne *et al*., 2002). Studies have shown that seed quality can be largely influenced by a wide range of environmental factors during seed production, harvesting, processing, storage and treatments such as seed priming (Tektrony *et al*., 1980; McDonald, 2000; Adebisi and Ojo, 2001; Tesnier, 2002; Adebisi and Ajala, 2007). Those factors of the production environment which dictate the quality of seeds produced include temperature, available moisture during seed development and maturation, incidence of diseases and pests in the field and at storage, management practices, harvest and post-harvest seed handling (Tekrony *et al*., 1980; Adeyemo *et al*., 1998; Adebisi and Ojo, 2001).

Different attempts have been made to solve the problems created by genotype x environment interactions (Hanson *et al*., 1956; Comstock and Moll, 1963). Most of the estimates, however, only provide information on their existence and magnitude, but give no measurements of the individual genotype. Selection of stable genotype that performs consistently across environments can reduce the magnitude of these interactions. Besides, stability of sesame performance is of special importance under rain-fed conditions in developing countries where environmental conditions varied considerably and the technologies of modifying the environments are far from adequate (Adebisi, 2004). Interest has been focused on the regression analysis, an approach originally proposed by Yates and Cochram (1938) and later modified by Finlay and Wilkinson (1963) and Eberhart and Russel (1966). Regression analysis has been widely used in comparing and measuring genotypic performances of common bean (Beaver *et al.*, 1985), Soybean (Ojo, 2002 and Ojo *et al*., 2002), cashew (Adebola and

Esan, 2002), navy bean (Gebeyehu and Assefa, 2003) and sesame (Adebisi, 2004)

Most of the sesame (*Sesamum indicum* L.) genotypes grown in the South-west Nigeria were selected based only on their desirable seed weight or yield per hectare with little or no reference to stability of seed quality performance. This has resulted in poor yield and quality of seed obtainable. Although sesame is grown in diverse plant population environments in Nigeria, there is currently no information on the seed quality stability and response of different tropical sesame genotypes under these environments. There is the need to identify outstanding genotypes with stable, desirable and superior seed quality for the farmers.

A genotype is stable if, at a given location or plant population it exhibits very little fluctuation in seed quality from year to year. An ideal sesame selection (genotype) is therefore one that combines high seed quality and stable performance in most of the ecological environments where it is cultivated. Therefore, the present work was conducted to determine the stability of seed germination and field emergence performance in some tropical rain-fed sesame genotypes grown in south-west Nigeria under three plant population environments and identify genotypes that performed well under such environments.

2. Materials and Methods

Fourteen sesame genotypes sourced from the National Cereals Research Institute, Badeggi, Niger State, Nigeria were evaluated in trials conducted at the Teaching and Research Farm of the University of Agriculture, Abeokuta (7^o15'N, 3^o25'E). Seeds of the 14 sesame genotypes were grown under three plant populations during the rainy seasons of 2001 and 2002. The treatments formed experimental environments as follows: Environment 1 = 50 cm x 15 cm (133,333 plants ha^{-1}), Environment 2 = 60 cm by 10cm (166,667 plants ha^{-1} and Environment 3 = 75 cm x 5 cm (266,667 plants ha^{-1}). The plant populations and seasons, therefore, constituted six environments.

The experimental fields were well-drained sandy-loamy soil with a pH range of 6.81 to 7.80, nitrogen status between 0.07% and 0.14%, organic matter between 1.42% and 2.86% and carbon status between 0.82% and 1.66 %. The average rainfall for the two seasons ranged from 500 mm annum $^{-1}$ in 2001 to about 800 mm $annum^{-1}$ in 2002. At each plant population and in each season, the 14 entries were arranged in randomized complete blocks with three replications. Sowing was done by hand in four-row-plots of 3 m long and spaced 50 cm x 15 cm, 60 cm x 10 cm and 75 cm x 5 cm. Seeds were mixed with sand and hand drilled while seedlings were thinned at 3 weeks after sowing to about 15 cm, 10 cm and 5 cm between plants. Following thinning, a post emergence fertilizer application of NPK 15:15:15 was applied by drilling at the rate of 60kgN, 30kg P_20_5 and 50kg K_20 ha^{-1}. Weeding was carried out twice before and after fertilizer application.

Seeds harvested from each of the environments were evaluated in the seed laboratory for seed germination and field emergence thus:

Seed germination: The test was performed according to ISTA (1995). Three 100-seed replicates of each genotype were germinated in 11cm diameter petri dishes inside a moistened paper towels with 5ml of distilled water. The petri dishes were arranged inside an incubator at 30^0C temperature in a completely randomized design. After seven days of germination, the proportion of germinated seed (visibly emerged normal radicle) was expressed as normal germination percentage.

Field emergence: Four sub samples of 50 seeds for each genotype under each environment were hand-sown in furrows of 2.0m, 0.30m apart and 0.05m deep in the field. Soil medium was kept sufficiently wet for emergence. The number of emerged seedlings was counted at 14 days after sowing and expressed as percentage of seed sown.

2.1. Data Analysis

Data generated were firstly transformed using angular transformation (arcsine). and then subjected to analysis of variance of Finlay-Wilkinson regressions using GENSTAT (2001) 10.0 statistical package.

Stability parameters for each genotype were determined using the regression procedure of Eberhart and Russel (1966). Each genotype was defined by three values: (1) mean seed germination and field emergence over all environments, (2) the linear regression (b values) of genotype mean seed germination and field emergence in each environment, (3) the mean square deviation from the regression for each genotype (S^2d value). Significance of regression co-efficient (b-values) was tested by the student's t-test (Steel *et al*., 1997). For the regression analysis of variance, the residuals from the combined analysis of variance were used as a polled error to test the significance of the S^2d values (Osman, 1991). A significant F-value would indicate that S^2d was significantly different from zero. Co-efficients of determination (r^2 values) were computed from individual linear regression analysis (Pinthus, 1973).

Stimulation of current experiment by varying the number of plant density was used to determine the most efficient plant density for sesame seed quality testing under rain fed tropical conditions.

3. Results

Results of analysis of variance of Finlay-Wilkinson regressions for seed germination and field emergence are presented in Table 1. There were high significant mean squares for environment and genotype x environment interaction for seed germination and field emergence. Genotype effects were highly significant for seed germination and field emergence.

Stability parameters of seed germination of 14 sesame genotypes evaluated in six environments are presented in Table 2. Regression co-efficients ranged from 0.19 (for genotype 73A-97) to 1.70 (for genotype Type A). Six genotypes (Goza, Type-A, E8, Domu, C-K-2 and 530-3) had regression co-efficients greater than 1.0. One of these genotypes (C-K-2) had higher seed germination than the mean of all the genotypes. However, seven genotypes (73A-97, Pbtil No1, 69B-88Z, 73A-94, 73A-11, 93A-97 and Yandev 55) had regression co-efficients less than 1.0. Genotype 530-6-1 had regression co-efficient close to unit (b = 1.03).

Results in Table 3 show the stability parameters of field emergence of 14 sesame genotypes evaluated across six plant population environments. Regression co-efficients for field emergence trait ranged from 0.14 (for Pbtil No1) to 3.01 (for 73A-94). Eight genotypes (93A-97, 93A-11, Type-A, 530-6-1, 73A-94, Domu, 73A-97 and 530-3) had regression co-efficients higher than 1.0. Four of these genotypes (73A-11, 530-6-1, 73A-94 and 73A-97) had higher field emergence than the mean of all the genotypes. Regression co-efficients of Goza, 69B-88Z, Yandev 55, E8, C-K-2 and Pbtil No1 were less than 1.0 with field emergence below the mean of all the genotypes except for Yandev 55, 69B-88Z and C-K-2 which had higher mean than mean of all the genotypes.

As shown in Table 4, seed germination of the sesame genotypes showed significant differences in each of the three plant population environments. Genotypes 69B-88Z (78%), 530-6-1 (77%) and Domu (77%) as well as 73A-97 (76%) had higher seed germination at 133,333 plants ha^{-1}. Similarly, C-K-2 (80%), 73A-11 (78%), 93A-97 (78%), 530-6-1 (77%) and 73A-94 (77%) recorded remarkably higher seed germination at 166,667 plant ha^{-1} while 73A-97, Yandev 55, C-K-2, 73A-11 and 530-6-1 with seed germination above 80% were among genotypes with significant higher seed germination at 266,667 plant ha^{-1}.

In Table 5, 73A-97, 73A-94, Yandev 55, 73A-11, 69B-88Z and 530-6-1 were among genotypes that had significant greater field emergence at 133,333 plants ha^{-1} while Pbtill No1 (85%) followed by C-K-2 (75%) and E8 (71%) recorded significant higher emergence at 166,667 plants ha^{-1}. At 266,667 plants ha^{-1}, 73A-97, 5306-1, C-K-2and 93A-97 and 73A-11had significant higher emergence of 73, 71, 70, 69 and 69%, respectively.

Table 1: Analysis of variance of Finlay-Wilkinson regressions for seed germination and field emergence over 14 sesame genotypes in six environments.

Source of variation	DF	Mean Square Values	
		Seed germination	Field emergence
Replication	12	6.69	34.37
Genotype (Gen.)	13	195.61**	267.12**
Environment (Env) (Linear)	5	1069.90**	266.86**
Gen.xEnv.(Linear)		154.68**	147.49**
Pooled Error	156	11.33	18.08

** Significant at 0.01 level of probability ns = not significant

Table 2. Mean seed germination and estimates of stability parameters in 14 sesame genotypes evaluated over six environments

Genotype	[+] Mean seed germination (%)	R^2	FWb	S^2d	T
Yandev 55	77[a]	0.22	0.69[ns]	0.64[ns]	1.07
93A-97	76[a]	0.23	0.57[ns]	0.52[ns]	1.09
Goza	68[d]	0.44	1.47[ns]	0.83[ns]	1.78
Type-A	70[cd]	0.68	1.70*	0.59[ns]	2.88
73A-11	77[a]	0.56	0.79[ns]	0.35[ns]	2.25
530-6-1	79[a]	0.82	1.03**	0.24[ns]	4.27
73A-94	73[bc]	0.53	0.84[ns]	0.40[ns]	0.17
69B-88Z	76[ab]	0.60	0.98[ns]	0.40[ns]	2.43
E8	71[c]	0.91	2.31**	0.37[ns]	6.22
Domu	72[c]	0.41	1.58**	0.94[ns]	1.68
73A-97	78[a]	0.21	0.19[ns]	0.52[ns]	0.36
C-K-2	77[a]	0.38	1.12[ns]	0.71[ns]	1.56
530-3	72[c]	0.53	1.67[ns]	0.08[ns]	2.11
Pbtil No1	71[cd]	0.07	0.21[ns]	0.38[ns]	0.56
Mean	74		1.00		

Mean values within a column with a letter superscript in common are not significantly different at $P < 0.05$
*, ** FWb value significantly different at 5% and 1% levels of probability respectively
FWb: Finlay-Wilkinson regression co-efficient,
R^2 = coefficient of determination
S^2d = Mean square deviation from the regression
t = 't' test value
[+] Mean standard germination after angular transformation

Table 3. Mean field emergence and estimates of stability parameters in 14 sesame genotypes evaluated over six plant population environments

Genotype	[+] Mean Field emergence (%)	R^2	FWb	S^2d	T
Yandev 55	67[ab]	0.11	0.90[ns]	1.27[ns]	0.71
93A-97	62[bc]	0.38	2.09[ns]	1.33[ns]	1.58
Goza	58[c]	0.01	0.21[ns]	0.94[ns]	0.22
Type-A	59[bc]	0.36	2.17[ns]	1.44[ns]	1.50
73A-11	68[ab]	0.89	1.40**	0.23[ns]	5.98
530-6-1	66[b]	0.68	2.29*	0.79[ns]	2.91
73A-94	66[b]	0.73	3.01*	0.93[ns]	3.25
69B-88Z	66[b]	0.01	0.17[ns]	0.90[ns]	0.18
E8	63[bc]	0.01	0.39[ns]	1.78[ns]	0.22
Domu	64[bc]	0.78	2.29**	0.60[ns]	3.77
73A-97	69[a]	0.72	2.37*	0.74[ns]	3.21
C-K-2	71[a]	0.02	0.35*	1.32[ns]	0.26
530-3	63[bc]	0.66	2.80*	1.02[ns]	2.76
Pbtil No1	61[c]	0.00	0.14[ns]	1.34[ns]	0.10
Mean	65		1.00		

Mean values within a column with a letter superscript in common are not significantly different at $P < 0.05$
*, ** FWb value significantly different at 5% and 1% levels of probability respectively
FWb: Finlay-Wilkinson regression co-efficient,
R^2 = coefficient of determination
S^2d = Mean square deviation from the regression
t = 't' test value
[+] Mean field emergence after angular transformation

Table 4. Performance of seed germination under three plant population environments over two cropping seasons.

Genotype	Seed germination (%) 133,333 plants ha-1	166,667 plants ha-1	266,667 plants ha-1
Yandev 55	72	74	84
93A-97	72	78	78
Goza	70	71	54
Type A	70	75	85
73 A-11	73	78	80
530-6-1	77	77	82
73A-94	69	77	74
69B-88Z	78	73	75
E8	71	73	68
Domu	77	75	65
73A-97	76	76	84
C-K-Z	74	80	80
530-3	70	76	71
Pbtil No1	71	70	74
Mean	73	75	75
Lsd(0.05)	5.19	5.52	5.45

Data presented according to method of Choo *et al.* (1984) of determination of stability of performance

Table 5: Performance of field emergence under three plant population environments over two cropping seasons.

Genotype	Field emergence (%) 133,333 plants ha-1	166,667 plants ha-1	266,667 plants ha-1
Yandev 55	69	68	64
93A-97	61	55	69
Goza	60	61	56
Type A	51	63	63
73 A-11	68	68	69
530-6-1	67	67	71
73A-94	71	61	65
69B-88Z	68	62	68
E8	53	71	54
Domu	65	63	63
73A-97	71	63	73
C-K-Z	66	75	70
530-3	59	62	66
Pbtil No1	60	85	65
Mean	64	66	65
Lsd(0.05)	4.41	5.02	5.19

Data presented according to method of Choo *et al.* (1984) of determination of stability of performance

4. Discussion

The results of joint regression analysis revealed that the GXE (linear) effect due to environment showed significant differences between regression co-efficients pertaining to the regression of genotype seed germination and field emergence on environmental seed germination and field emergence. The result revealed differences among slopes of regression lines and the regression model was adequate in explaining stability of the 14 sesame genotypes in respect of their seed quality (seed germination and field emergence). These observations are in agreement with that reported by Adebisi and Ajala (2006) for sesame seed yield in south- west Nigeria.

In this study, the coefficients of determination (R^2) ranged from 0..07 to 0.91 Since the environmental sum of squares contributed to the

regression sum of squares, Moll *et al*., 1978 and Osman (1991) showed serious concern in the interpretation of R^2 values. Osman (1991) reported that linear regressions accounted for 76-99% of the variation in sesame seed yield. Similarly, Adebisi and Ajala (2006) observed that linear regression accounted for 0.65-1.25 of the variation in seed yield of Nigerian sesame genotypes. In this study, linear regressions contributed as much as between 07 and 91% of the variation in seed germination and between 01 and 89% in field emergence. The significant differences in b values suggested that all the 14 sesame genotypes responded differently to the different plant population environments. Variability in environments was an important factor and largely determined the usefulness of b values (Pfahler and Linskens, 1979).

The stability result of seed germination indicated that Goza, Type-A, 530-6-1, E8, Domu, C-K-2 and 530-3 had regression coefficients greater than 1.0, they were, therefore, sensitive to environmental changes in respect of seed germination. However, one of these genotypes (C-K-2) with higher seed germination than the overall genotype mean suggests that it could be recommended for cultivation under productive environments for higher seed germination. Genotypes 73A-97, Pbtil No1, 69B-88Z, 73A-94, 73A-11, 73A-97 and Yandev 55 had regression co-efficients less than 1.0. These genotypes were relatively better adapted to poor environment and were insensitive to environmental changes in respect of seed germination. Such genotypes could be recommended only for cultivation in unfavourable conditions. Also genotype 530-6-1 with regression co-efficient close to unit (b = 1.03) had general adaptability and somehow averagely stable.

For field emergence performance, genotypes 73A-11, Type-A, 530-6-1, 73A-94, Domu, 73A-97 and 530-3 had regression co-efficients above 1.0, and they were therefore sensitive to environmental changes for field emergence. Four of these genotypes (73A-11, 530-6-1, 73A-94 and 73A-97) recorded higher field emergence than the genotype mean, and hence, could be recommended for production under productive environments. Conversely, field emergence of six genotypes (Goza, 69B-88Z, Yander 55, E8, C-K-2 and Pbtil No1) had regression co-efficient values less than 1.0, with mean emergence of either below or above genotype mean, hence, they were relatively better adapted to environmental changes and could be suggested for cultivation in unfavourable conditions, without any adverse effect on field emergence.

According to Eberhart and Russel (1966), a genotype considered as stable should meet criteria of high mean performance, with b equal to unity and S^2d approaching zero. Using these criteria, seed germination of genotype 530-6-1 with regression coefficients of 1.03, S^2d approaching zero and with relatively high seed germination of 78.50% could be considered widely adapted and stable. It has the ability to express its germination potential when produced in a range of environmental conditions. The highest field emerging genotypes proved less stable and selection solely for high emergence could result in discarding many genotypes that were relatively better adapted to environmental changes.

In a similar vein, Choo *et al.* (1984) described a desirable genotype as one with high mean, at least average performance, in all environments and an undesirable genotype as having either a low mean performance or below-average performance in some environments. Following Choo *et al.* (1984) criteria and defining high mean seed germination as at least 5% above the grand mean (Table 4), only 530-6-1 showed itself to be desirable in each of the plant population environments. However, for field emergence (Table 5), the performance at individual plant population environment indicated that 73A-11 and C-K-2 maintained above average emergence in each of the three plant population environments evaluated.

The method of Choo *et al*. (1984) coupled with the regression analysis have jointly pointed out genotypes 530-6-1, and 73A-11 and C-K-2 as desirable genotypes that will give good germination and field emergence, respectively over an array of environments encountered in the south-west of Nigeria and similar ecologies. Moreover, when applied to individual plant population environment, the method of Choo *et al*. (1984) pointed out 69B-88Z, Domu and 73A-97 as being most suitable for seed production in 133,333 plants ha^{-1} environment and genotypes 73A-11 and C-K-2 in 166,667 plants ha^{-1} environment. However, genotypes 93A-97, 73A-11, 73A-97, 69B-88Z and C-K-2 would be appropriate in 266,667 plants ha^{-1} environment to obtain stable and high seed germination and emergence.

5. Conclusion

The investigation of stability of sesame genotypes clearly showed that most of the test genotypes were sensitive to production environments. Hence, their wider adaptability, stability and general performance to the fluctuating growing conditions within and across plant population environments were considerably lowered. The stability analysis provides meaningful information regarding stability and consistency of seed quality performance of sesame genotypes across different environments. These genotypes can be obtained from the University of Agriculture, Abeokuta, Nigeria and National

Cereal Research Institute (NCRI), Badeggi, Nigeria. The identified genotypes may be used as parents in future sesame crop improvement programmes. Sesame seed must be tested for germination and vigour in different environments to determine the favourable conditions for sesame seed production, as discussed by Heydecker (1972); Dickson (1980); Odiemah (1991) and Adebisi (2004).

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Turritopsis nutricula

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Abstract: *Turritopsis nutricula* is a hydrozoan that can revert to the sexually immature (polyp stage) after becoming sexually mature. It is the only known metazoan capable of reverting completely to a sexually immature, colonial stage after having reached sexual maturity as a solitary stage. It does this through the cell development process of transdifferentiation. This cycle can repeat indefinitely tha offers it biologically immortal. It is not clear if stem cells are involved in this immortality or not. Upto now, there is little academic report in the Turristopsis nutricula studies. To study the mechanism of the biological immortality of Turritopsis nutricula possibly supplies the way finding the biological immortality for human. [Nature and Science 2010;8(2):15-20]. (ISSN: 1545-0740).

Keywords: immortal; immortality; sexual maturity; stem cell; transdifferentiation; *Turritopsis nutricula*

1. Introduction

Turritopsis nutricula is a hydrozoan that can revert to the sexually immature (polyp stage) after becoming sexually mature. It is the only known metazoan capable of reverting completely to a sexually immature, colonial stage after having reached sexual maturity as a solitary stage. It does this through the cell development process of transdifferentiation. This cycle can repeat indefinitely that offers it biologically immortal. To study the reason of the biological immortality of *Turritopsis nutricula* possibly supplies the way finding the biological immortality for human.

Turritopsis nutricula is a species of jellyfish with a very unusual quality: it is biologically immortal. Also known as the immortal jellyfish, this fascinating animal, in theory, has the ability to sustain life indefinitely, so long as its nerve center remains intact.

Typically, jellyfish die after reproducing, but the immortal jellyfish is capable of returning to a polyp after producing offspring. This essentially means that this type of jellyfish is able to return itself to a much younger state. As a result of reversing its life cycle, the immortal jellyfish can evade death. If the jellyfish continues to reverse its life cycle following reproduction, it can live on for an indefinite period. In laboratory tests, the species reverted back to the immature polyp stage 100% of the time.

Turritopsis nutricula is capable of rejuvenating itself due to a process called transdifferentiation. Transdifferentiation occurs when a non-stem cell turns itself into another type of cell. But, it is not clear if stem cells are involved in this immortality or not. As my opinion, the transdifferentiation in *Turritopsis nutricula* has related mechanism to stem cell when the life cycle reverted. It is important to reveal the relationship of this *Turritopsis nutricula* transdifferentiation and stem cell. Transdifferentiation is rare, and when it does occur, it most commonly occurs in parts of the organsism, like in the eye of the salamander. However, the immortal jellyfish has incorporated transdifferentiation into its lifecycle. In the process, all of the old cells are regenerated. At the end of the cycle, the immortal jellyfish is a young polyp, ready to start life anew (Wendy, 2009).

While colonial animals can have their immortality, solitary animal individuals are to die. Hydrozoan cnidarians usually have a complex life cycle, wherein a colonial stage leads to the sexually mature, solitary, adult stage. Eggs and sperms from solitary, sexual, adult medusa (jellyfish) develop into an embryo and planula larva, and they then form the colonial polyp stage. Medusae are formed asexually from polyps. These medusae have a limited lifespan and die shortly after releasing their gametes.

The hydrozoan *Turritopsis nutricula* is diferent, which is biological immortality. The solitary medusa of this species can revert to its polyp stage after becoming sexually mature (Bavestrello et al., 1992; Piraino et al., 1996). In the laboratory, 100% of these medusae regularly undergo this change. The cells that accomplish the building of a new stolon are probably those of the exumbrella. However, it is not known whether the sensory cells, myoepithelial cells, and cnidocytes are derived from the exumbrella or the endodermal component.

In the past, because of men's desire to be forever young, inspired by legends, epics, myths, stories of the gods and goddesses, they engaged in the quest for the so-called 'fountain of youth' but nobody was able to find one. Based on scientific studies, some organisms or creatures are considered or thought to be immortal.

As the nature will, to live eternally is an extracting dream in all the human history. Stem cell is the original of life and all cells come from stem cells (Hongbao Ma 2005a). Germline stem cell is the cell in the earliest of the cell stage. It is possible to inject the germline stem cell into adult human body to get the eternal life. This article is to try to describe the stem cell and to explore

the possibility of the eternal life with the stem cell strategy (Ma Hongbao 2007).

2. Description

Diameter *of Turritopsis nutricula* is about 5 mm. It has an equally high and bell-shaped figure. The walls are uniformly thin. The bright red, big stomach has a cruciform shape in its cross section. Young specimens have only 8 tentacles along the edge, while adult specimens have 80-90 tentacles.

Turritopsis nutricula is the first case in which a metazoan is capable of reverting completely to a sexually immature, colonial stage after having reached sexual maturity as a solitary stage (Gilbert, 2006).

Jellyfish usually die after propagating but Turritopsis *nutricula* reverts to a sexually immature stage after reaching adulthood and is capable of rejuvenating itself. The jellyfish and its reversal of the ageing process is now the focus of research by marine biologists and geneticists. It is thought to achieve the feat through the cell development process of transdifferentiation, in which cells transform from one type to another.

The switching of cell roles is usually seen only when parts of an organ regenerate. However, it appears to occur normally in the Turritopsis life cycle (Lech Mintowt-Czyz, 2009).

Turritopsis nutricula, a type of jellyfish, is gaining notoriety for its uncanny and unprecedented capacity to de-evolve instead of dying. These jellyfish are the first evidenced metazoan, or multi-celled creature, to demonstrate the ability to revert back to a colonial stage after reaching sexual maturity. After sexually reproducing, most animals inevitably die. Turritopsis nutricula, however, undergo a transformation in which they return to a stage of sexual immaturity after reproducing, only to mature and reproduce again, then return to sexual immaturity, and so on. What does this mean? Turritopsis nutricula do not die, by nature, and are believed to have an indefinite potential lifespan.

Turritopsis nutricula are about 5 mm in diameter in sexually mature stage. They have 8-24 tentacles when they are young and up to 90 tentacles as mature adults. Shaped like a bell, their external walls are transparent and their stomachs are large and have a distinctive red color.

Turritopsis nutricula rejuvenate from sexually mature to colonial through two processes: cell transformation and cell transdifferentiation. Transdifferentiation is one cell transform into a completely different type of cell. By transdifferentiating, these cells are able to change their entire make-up, much like the much-publicized stem cells. After sexually reproducing, the jellyfish reabsorbs all of its external parts and turns into a cyst, which looks like an ameba-esque blob. The cyst then attaches to the ground and grows into a stalk-shaped polyp colony. These polyps begin a new cycle, where they form into mature jellyfish - all genetically identical. This specimen of *Turritopsis nutricula* vibrantly shows its majestic being, with its deep red stomach clearly showing.

2.1 *Turritopsis nutricula*

Classification
Kingdom – Animalia
Phylum – Cnidaria
Class – Hydrozoa
Order – Hydroida
Family – Clavidae
Genus – Turritopsis
Species – T. nutricula

Turritopsis nutricula is a Hydrozoan, which is a class of Cnidarians including the Portuguese Man-of-War, Hydra, and freshwater jellyfish. Hydrozoans are, for the most part, colonial animals. *Turritopsis nutricula* is a colonial organism as a polyp, though it is an independent, pelagic creature. The organism has a diameter of 5 millimeters, or one fifth of an inch. Although a young specimen would only have 8 tentacles, an adult would have between 80 and 90 tentacles. It has an obvious red stomach in the center of the main part of its body. Once eggs are fertilized, they develop in the stomach and in the screen formed by the cave in the planula, or free-swimming, ciliated form of some Cnidarian species. The eggs must then be deposited on the seabed in colonies of the larval stage of polyps. The jellyfish will then hatch in 2 days and will become sexually mature after just 2 weeks, which it then can revert back to a polyp in the process of transdifferentiation. T. Nutricula can be found worldwide.

2.2 Hydra

Classification:
Kingdom – Animalia
Phylum – Cnidaria
Class – Hydrozoa
Subclass – Leptolinae
Order – Anthomedusae
Suborder – Capitata
Family – Hydridae
Genus – Hydra

3. Distribution, range and related species

Turritopsis nutricula are originally from the Caribbean but have spread all over the world. *Turritopsis nutricula* are found in temperate to tropical regions in all of the world's oceans. It is believed to be spreading across the world as ships are discharging ballast water in ports. Since the species is immortal, the

number of individuals is spiking. "We are looking at a worldwide silent invasion" said Smithsonian Tropical Marine Institute scientist Dr. Maria Miglietta.

3.1 Plants

- Great Basin Bristlecone Spine – 4,862 years
- Fitzroya Cupressoides – 3,622 years
- Fortingall Yew – 2,000 – 5,000 years (not verified)
- Sacred Fig – 2,293 years, if the planting date of 288 B.C. is correct

3.2 Animals

- Antarctic Sponge – 1,550 years (approx.)
- Icelandic Cyprine – 405 years
- Koi Fish – 215 years
- Bowhead Whales – 210 years (unconfirmed)

3.3 *Turritopsis nutricula*

One of the most bizarre organisms that ever existed is the *Turritopsis nutricula*. It is a hydrozoan with a life cycle in which it reverts to the polyp stage after becoming sexually mature. It is said that it is the only known case of a metazoan that is capable of reverting completely to a sexually immature, colonial stage after having reached sexual maturity as a solitary stage. This cycle can repeat indefinitely, rendering it biologically immortal until its nerve center is removed from the rest of the body. This hydrozoan can be found in tropical and temperate regions and a diameter of about 5 mm.

3.4 Hydra

Hydra is a kind of cnidarians that is claimed to be immortal. It is simple fresh-water animal that possesses radial symmetry. A Hydra is a predatory animal that can be found in most unpolluted freshwater ponds, lakes and streams in the temperate and tropical regions. It can be captured by gently sweeping a collecting net through weedy areas. A Hydra undergoes aging very slowly and has the ability to regenerate.

3.5 Hydra oligactis

The Brown Hydra or scientifically known as Hydra oligactis can be found in the northern temperate zone. It is a common organism found in still waters from early spring to late autumn. A Brown Hydra is commonly found attached to the stems of water plants, the undersides of leaves, submerged twigs and on the surface of stones.

3.6 Hydra viridissima

The Green Hydra or formally called Hydra viridissima can be found in both temperate and tropical fresh waters. The characteristic green color comes from cells of the unicellular alga Chlorella within the cells of the gastrodermis. It also retracts to a small green blob when disturbed.

3.7 Hydra Viridis

The Hydra viridis, like the Hydra oligactis, can be found widely dispersed in the northern temperate zone and is also a common organisms found in still waters from early spring to late autumn. The characteristic green color comes from cells of the unicellular alga Chlorella within the cells of the gastrodermis. Like also the Hydra oligactis, this species when disturbed retracts to a small green blob which is easily overlooked.

3.8 Hydra vulgaris

Hydra vulgaris is a freshwater hydroid that has 4-12 tentacles that protrudes from just outside of its mouth. It is about 12 mm in length and feed by extending their tentacles and waiting for food to touch the tentacles, they than bring the food to their mouth, inject, and digest the organism. Anything that cannot be digested is egested. Ingestion and ejection occur through the mouth. This peculiar organism can reproduce by sexual reproduction, budding and indirectly through regeneration.

3.9 Actinobacteria

A certain Rachel Sussman has traveled the world to take photographs of actinobacteria - the oldest living things in the world. These bacteria are from Siberia and thought to be around 400,000 years old. Actinobacteria are a group of Gram-positive bacteria with high G+C ratio and can be terrestrial or aquatic.

3.10 Bacteria

In the Earth, bacteria exist everywhere even in the hottest and coldest places. As colony, bacteria are thought to be immortal. They reproduce through a very unusual way called cell division. A parent bacterium will split itself into two identical daughter cells and then these daughter cells will split themselves in half. This process repeats, thus making the bacterium colony essentially immortal. But, essentially to say, the life cycle of bacteria is not really immortal. Individually, the daughter bacteria are not the parent bacteria, as the daughters/sons of human are not heir parents.

3.11 Bristlecone Pine

A Bristlecone Pine's oldest known living specimen is over 4,800 years old. This species of tree is thought to reach an age far greater than that of any other single living organism known and is speculated to be potentially immortal (Nobert Soloria Bermosa, 2009).

4. Life cycle

The fertilized eggs develop in the stomach and in the screen formed by the cave in the jellyfish planula. The eggs are then planted on the seabed in polyp colonies. The jellyfish hatches after two days. The jellyfish becomes sexually mature after a few weeks (the exact duration depends on the ocean temperature; at 20°C it is 25-30 days and at 22°C it is 18-22 days). It is the only known animal that is capable of reverting to its juvenile polyp state. Theoretically, this cycle can repeat indefinitely, rendering it potentially immortal. Found in warm tropical waters Turritopsis nutricula is believed to be spreading across the world as ships' ballast water is discharged in ports. Though solitary, they are predatory creatures and mature asexually from a polyp stage. While most members of the jellyfish family usually die after propagating, the *Turritopsis nutricula* has developed the unique ability to return to a polyp state (Qossay Takroori, 2009).

5. Biological immortality

Jellyfish usually die after propagating; however, the *Turritopsis nutricula* has developed the ability to return to a polyp state. This is done through a cell change in the external screen, exumbrella. In it's life cycle, the medusa is transformed into a stolon and the polyps into a hydroid colony. The umbrella turns inside out; middle section and tentacles are reabsorbed before the polyp spawns. Stolons form two days before the polyps differentiate. The ability to reverse the life cycle is probably unique in the animal kingdom, and allows the jellyfish to bypass death, rendering the *Turritopsis nutricula* biologically immortal. Laboratory tests showed that 100% of specimens reverted to the polyp stage (Wikipedia, 2009). It can do this because it can alter the differentiated state of a cell, transforming it into another cell type, called transdifferentiation, and it is usually seen only when parts of an organ regenerate. However, it appears to occur normally in the Turritopsis nutricula life cycle. In this transdifferentiation process, the medusa is transformed into the stolons and polyps of a hydroid colony. First, the umbrella everts and the tentacles and mesoglea are resorbed. The everted medusa attach to the substrate by the end that had been at the opposite end of the umbrella, and spawning occurs shortly thereafter. The cnidarian then secretes a perisarc and stolons. Two days after the stolons are first seen, polyps differentiate. These polyps feed on zooplankton and soon are budding off new medusae.

6. The Secrets of Immortality

Turritopsis nutricula can achieve immortality by reverting back to a polyp, which is its larval stage, after become sexually mature. This cycle is called transdifferentiation, and theoretically, can be repeated indefinitely. It is the only known case of a sexually mature metazoan, which is basically an animal, changing back into a colonial, immature state. On the other hand, Hydrae become immortal by other means. These animals do not undergo senescence, or the process of aging. They have a regenerative ability that can dilute poisons by going through the process of mitosis or cell division.

7. Application of *Turritopsis nutricula* researches in immortal life

The application of a study of the *Turritopsis nutricula* could be boundless, as stem-cell research appears at the forefront of many medical studies on organ reproduction, cancer treatments, and brain injury treatments to name a few. By using the cells of the jellyfish, which transdifferentiate, scientists can continue to research solutions for these problems without mucking about in the moral dilemmas that come with researching embryonic stem cells. The jellyfish's cells are also similar in make to cancer cells, which are able to affect the order and process of genetic systems. By studying these cells, scientists may be able to gain insight in the never-ending search for a cure for cancer. These jellyfish, rumored to be plotting world-domination, are in fact spreading in droves. What some scientists now refer to as a "widespread invasion" could affect the structure and functionality of the oceanic ecosystem. It is believed that they spread when the jellyfish stow away in the ballast tanks of large ships and are carried from place to place. This is a major pathway for the global spread of "invasive" species. Native to the Caribbean, these jellyfish are now being found in waters surrounding Italy and Spain, Japan, Panama, and even Florida. It's anyone's guess where they end up next (Jenny Riegel, 2009).

All the life in the Earth, human, animals, plants and everything grows would be dying finally? Meet *Turritopsis nutricula*, the only immortal jellyfish on planet earth. Its only 5 mm long and spreading around the world really fast because they don't die.

Research were done on the phylum of Cnidaria, which includes, in their common names, jellyfish, coral, sea pens, hydras, and animals of the like. This phylum contains some of the most primitive, diverse, and beautiful animals on this planet. It also has some of the strangest animals this planet has to hold. I will be presenting these eccentric animals and hope you will view them with awe and interest. These animals are not "immortal", but "biologically immortal", meaning they can still die from accidents and being killed or destroyed. No animal is perfectly immortal. Some people say that the prospect of a possibly immortal creature disproves the Qur'an, a holy Islamic text that states that no living thing is immortal. Anyways, I have made several editions to this, hoping to reduce the

reading difficulty of the content to the level of the common viewer. If you see any errors within this article, please tell me and I shall edit it accordingly. Other than these biologically immortal animals, long various multi-cellular eukaryotes (have cells with nucleus) have been known to live for a very time, here with their longest known lifespan:

8. Discussions

Everything in earthly existence, including human life in all of its facets, is involved in a process of ongoing change. Hence, permanence seems unattainable, and thereby especially desirable. The wish for immortality thus becomes one of the most important original reasons for the appearance of religions, and the motives of many scientific research fields can also be traced to this motive (Edmondson 2005). Life is a physical and chemical process. From ontology aspect, the world is timeless and the life exists forever as any other body in the nature. The nature of life is that life is a process of negative entropy, evolution, autopoiesis (auto-organizing), adaptation, emergence and living hierarchy. Up to now, there is no scientific evidence to show that life body and non-life body obey the same natural laws. But, all the researches are made by the methods of biology, biochemistry and molecular biology, etc. I t is very possible that the life and non-life are essential different in the biophysics, i.e. the quantum level. In the future, it is possible to make artificial life by either biological method or electronic technique (Hongbao Ma 2005b).

Immortal jellyfish, like most other species of jellyfish are either male or female. They do not have a specialized reproduction system. The male releases his sperms into the column of water. They come in contact with eggs that are present in the stomach of the female jellyfish. During the embryonic stage, they are either settled onto the mouth or the oral arms of the female. After they have passed this stage, they transform into free swimming planulae and separate themselves from the body of their mother. They float along the surface of the water for a few days and then settle on a hard, stationary object like the surface of a rock.

At this stage, they become transformed into polyps. These polyps become stationary as well. They continue to feed on microscopic plankton and zooplankton at this stage. This polyp then begins to grow multiple identical polyps until it becomes a colony. This colony of multiple polyps is also stationary and attached to the hard surface at its base. All the polyps are connected with minute feeding tubes and they receive equal nourishment from their microscopic diet. The colony of polyp can remain in this stage for years at a time. When the condition is right, this colony of polyp begins to grow horizontal grooves. The groove at the top is the fastest to mature, and will soon free itself and become a free swimming jellyfish.

This process of reproduction is common to most species of jellyfish. What is unique about the immortal jellyfish is that after reproducing sexually, they are able to return to their polyp stage. When most fish die after their sexual maturity, immortal jellyfish get transformed back into a polyp and restart the process of asexual reproduction. In this way, the jellyfish is able to convert itself back into a polyp, start a polyp colony again and give birth to a number of new jellyfish.

How does the immortal jellyfish accomplish this feat? It is through cell development process of transdifferentiation. What this means is that it can alter the differentiated state of the cell and transform it into a new cell. In this process of transdifferentiation, the medusa of the immortal jellyfish is transformed into the polyps of a new polyp colony. First, the umbrella reverts itself and then the tentacles and mesoglea get resorbed. The reverted medusa then attaches itself to the substrate by the end that had been at the opposite end of the umbrella and starts giving rise to new polyps to form the new colony. Theoretically, this process can go on infinitely, effectively rendering the jellyfish immortal.

The immortal jellyfish is one of the most unique animals not just within the species of jellyfish, but within the entire history of the animal kingdom. It has actually managed to accomplish the one feat that has been yearned by many and accomplished by none.

The genus Hydra includes 17 species. Hydras also show radial symmetry like T. Nutricula. Hydras are simple, predatory Hydrozoans that feed on minute aquatic invertebrates, such as copepods. They are sessile animals that retract into a ball if startled. They can move their location by either detaching from their substrate and floating away in the current or by sticking to the substrate with its mouth and tentacles, detaching its foot, and reattaching its foot in a new location. Repeating this, a Hydra can move a couple of inches a day. If it loses a body part, or is even totally obliterated into separate cells, it can still regenerate all of the missing body parts into a fully functional organism.

This regenerative ability, which can regenerate any body part or tissue, is known as morphallaxis, a key function of the Hydra that helps to enable it biologically immortal. When there is a large bounty of food available, or a small amount of potential mates, the Hydra may undergo asexual budding. Hydrae exemplify asexual budding, in which an identical copy of the parent is replicated in an organism that will eventually separate from the parent. Asexual budding is a great survival tool for a case in which a mate cannot be found. If conditions are bleak, then Hydrae may undergo sexual reproduction. The body wall will produce testes or a rudimentary ovary (Kaio Ken, 2009).

Immortality has been a subject of fascination to humanity since at least the beginning of history and has been a major point of focus of religion, as well as the subject of speculation, fantasy, and debate.

Upto now, there is little academic report in the *Turristopsis nutricula* studies. To study the reason of the biological immortality of *Turritopsis nutricula* possibly supplies the way finding the biological immortality for human.

Related to human activities, there are two aspects of the world: One is the observed world (epistemology) and the other is the existed world (ontology). From the epistemology angle, time and space are relative (observed) (Ma 2003). From the ontology angle, time and space are absolute (existed) and the universe is a timeless world, which means that all the past, the present and the future exist eternally. All the life is a kind of existence in the universe, and from this aspect the life exist eternally.

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Oral Vaccination of Nile Tilapia (*Orechromis niloticus*) Against Motile Aeromonas Septicaemia

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Abstract: The present study was planned for preparation of formalin inactivated wet-packed whole cells Aeromonas *hydrophila* bacterin for oral vaccination. The humeral antibody response of vaccinated Nile tilapia *(Orechromis niloticus (O. niloticus)* was determined by micro-agglutination test. Moreover efficacy of the prepared bacterin against infection with *Aeromonas hydrophila* was detection and calculated as a relative level of protection. Nile tilapia *(O. niloticus)* immunized orally with formalin-inactivated *Aeromonas hydrophila* .wet-packed while cells had low level of antibody titer reached 2 and 3 by log2 at first and fourth week post-immunization respectively while Nile tilapia *(O. niloticus)* fed on minced meat without vaccine had antibody titer reached 1 by log 2 throughout the experimental period . The relative level of protection among Nile tilapia *(O. niloticus)* immunized orally were 86.8. [Nature and Science 2010;8(2):21-26]. (ISSN: 1545-0740).

Keywords: *Aeromonas hydrophila* - bacterin -vaccination- humeral antibody- Nile tilapia.

1. Introduction

Recently many countries practice fish culture very successfully not only as food industry but also as major source of income. Bacterial diseases among cultured fish either primarily or secondarily are considered to be a major cause of fish mortalities Grisez,,L. and Ollevier,F.(1995). *Aeromonas hydrophila* is known to be one of the most important bacteria associated with diseases in marine and freshwater fishes. The diseases caused by *Aeromonas hydrophila* ranged from acute rapidly fatal septicemia to latent infections and has been referred as hemorrhagic septicaemia or *Aeromonas septicemia.* At present, most of the cultured fish diseases are treated with drugs such as antibiotics, sulfonamides, nitrofurans and others. The chemotherapeutic measures are effective, particularly when used as early as possible, and have wide spectrum of pathogen control. However, several difficulties are often encountered by chemotherapy as, the cost of drugs is expensive, the resistant strains of pathogens are easily induced in water and the drug residues may deposit in fish body may introduce potential hazard to public health and to the environment by the emergence of drug resistant microorganisms and antibiotic residues and retain in water system as toxicants or pollutants Sugita et al (1991). In order to avoid the side effects of chemotherapy, the control measure by immunization of fish with vaccines gains the effort and rapid development. Many experimental and practical approaches to stimulate the immune response of fish were reported (Badran 1984, Abdel-Kader 1994 and Aly *et. al*., 2000**)**. Such immune response could be detected either by the presence of specific antibodies in the blood or by protection against infection. In the past, the presence of antibodies in the blood is well revealed when the immune system is stimulated by the injection of the antigen, but not when given orally or by immersion. After which, the trials were attempted to increase the production of antibodies and prolonging their presence in the blood by emulsifying the antigen in adjuvant Krantz, *et. al* (1963) who used mineral oil emulsion, Collins, *et. al* (1976) Fruend's incomplete adjuvant (FIA) and Badran (1990**)** FIA and Fruend's complete adjuvant (FCA). Moreover, several techniques have been successfully used for fish vaccination. Such techniques included injection immersion and oral routes.

The present study was planned for: Preparation of formalin inactivated wet-packed whole cells for oral vaccination. Determination of the humeral antibodies by microagglutination test in parent fish and fingerlings. Examination of the efficacy of the prepared bacterines by infection of the tested fish with *Aeromonas hydrophila* and calculation the relative level of protection (R.L.P).

2-Material and methods

2-1-Fish: - A total of (210) live apparently healthy. Nile tilapia (*(O. niloticus*) divided as follow(Forty adult fish for biological test, Forty adult fish for innocuty test, Eighty adult fish for oral vaccination, Twenty-five male of body weight from 120:130 gm, Twenty five female of body weight from 100:120 gm.

2-2-Ponds:-Twenty-three cement ponds in a private hatchery fish in Kafr El Sheikh Governorate with dimensions of 3x 8x 2 meter and Twelve glasses aquaria with dimension of 70 x 53 x 53 cm. in Labe of Hydrobiology Dept. NRC, were used for the biological and innocuty test Cement ponds and glasses aquaria were supplied with dechlorinated water with a temperature(25:28 C^{o}**).**

2-3-Diet: diet with 35% and 25% protein for feeding of fingerlings and adult Nile tilapia respectively. Food in ratio of 3% of fish body weight per day was considered to be the optimal maintenance amount required for adult fish and 5% for fingerlings according Noor El Deen (2OO7) .

2-4-Bacterial strain used:-Aeromonas hydrophila were isolated from liver or ascetic fluid of diseased fishes on Brain heart infusion broth followed agar with 0.5, 1, 2 and 4% NaCl Chen and Levin (1975).

2-5-Biological test (Virulence test): To detect the level of virulence of the obtained *Aeromonas hydrophila* strain, laboratory test were conducted using Nile tilapia (*O. niloticus*) as the fish of choice. According to Wakabayashi, et. al (1981) the Bacterial solution for fish inoculation was prepared by suspending 20 hs culture from brain heart infusion agar of the obtained strain in sterile physiological saline solution to give a concentration of 5 mg bacterial cells by wet weight/ml which was estimated to be between 1.8 / 10^6 and 1.6 / 10^9 C.F.U/ml .

2-6-Vaccine preparation for oral vaccination: Wet-packed, whole cell bacterin was prepared according to Rohovec, et. al (1981) as: Ten ml of brain heart infusion broth were inoculated with *Aeromonas hydrophila* . After 12 hs. Incubation at 25°C, 2 ml of this broth culture were used to inoculate one liter broth culture which was in turn incubated for 12 hs at 25°C. The prepared one liter was used as an inoculum for 15 liters of the broth medium and subjected to an incubation period of 10 : 12 hs at 25°C. Finally, 250 ml of 20% dextrose solution was added and the culture was incubated for an additional 12 hs. The bacterial cells were killed by addition of formalin to give a final concentration of 0.3 % over night. The cells were harvested by centrifugation and stored at 20 °C.

2-7- Innocuity test: This test was performed according to Anderson , et. al (1970) by inoculation of susceptible fish (*O. niloticus*) intraperitonealy with the prepared bacterin to insure that there is no infection or disease will be occur from living bacteria .

2-8-Sterility test: This test was done as described by Aly (1981) by cultivation of the prepared bacterins on brain heart infusion agar to insure that there's no growth of *Aeromonas hydrophila* or other organisms may be occurred.

2-9-Preparation of stained antigen used in antibody titration. Preparation of *Aeromonas hydrophila* antigen for antibody titration was established. The formalin inactivated bacterial cells by wet-weight was diluted with equal volume of sterile physiological saline solution. One drop of Loffler's alkaline methylene blue, prepared as described by Cruickshank (1985) was added to each 10 ml of the diluted antigen

2-10-Biological test "virulence test":-Forty Nile tilapia with (110 ±10) g body weight were divided into two groups each contains Twenty fish. Fish of the first group were injected intramuscularly through the back with 0.2 ml of the bacterial suspension (5 mg bacterial cell by wet weight /ml) /100g body weight Badran (1987). The fish of the second group (control group) injected with 0.2ml of sterile physiological saline solution. The tested fish were placed under observation for 2 weeks. The strain of *Aeromonas hydrophila* can be classified into 3 categories of virulence:-High virulence: - All tested fish were dead in a week. Moderate virulence: - Not all tested fish were dead in a week Virulence: - No fish were dead without show any clinical signs.

2-11- Safety test (Innocuity test):-The safty test was performed according to Anderson et. al (1970) by inoculation of the susceptible Nile tilapia (*O. niloticus*) intraperitonealy (I.P) with washed bacterin cells from the prepared vaccine. Two groups of Nile tilapia corresponding to the vaccinated and control, each contained 20 fish with (100 ±10 g) body weight were used. Nile tilapia (*O. niloticus*) of the first group were injected intraperitonealy with 0.1 mg bacterin cells) / fish. The fish of the second group (Control) were injected intraperitonealy with sterile physiological saline solution . The fish of both groups were placed under investigation during 15 days after injection. After that, fish were tested for re-isolation of injected organism on brain heart infusion broth that incubated at $25C^{0}$ for 24 hs.

2-12-Sterility test : - An inoculum from the bacterin was cultivated on brain heart infusion agar and incubated at 25°C for 24 hs. The cultures were examined for positive bacterial growth.

2-13- Vaccination method:- Eighty fish of *O.niloticus* (Forty male and Forty female)were used with separation of male from female and placed under observation in 2 cement ponds for 2 weeks for acclimatization and insuring the freedom of fish from diseases. Forty male and Forty female) were placed in 10 groups each contain 4 male and 4 female with attention that 9 groups of vaccinated were fed on diet

contain wet-packed whole cell bacterin at level of 5 mg/g of diet Fryer, et. al (1976), while the other Eight fish (Four male and Four female) fed diet without bacterin in one group as a control. Food containing vaccine was given at ratio of 3 % of the fish body weight per day for 8 days. The blood collected and sera separation was performed from Thirty-six fish (Eighteen males and Eighteen females from vaccinated fish) and two male and two female from control. Antibody titration of the collected serum was evaluated by microagglutination (MA) test. The other Thirty-six fishes were placed in 6 groups each contain 3 male+3 female with attention that 5 groups of vaccinated and one group nonvaccinated fish to give chance for normal breeding. Fingerlings from each group collected and 50 of each group squeezed and body fluid collected for microa-gglutination and biochemical analysis while other 50 fingerling from each group used for challenge test.

2-14- Challenge test:-The 6 groups used for breeding gave fingerlings after different periods post vaccination: (1- first group gave fingerlings after 12 day p.v, Second group gave fingerlings after 18 day p.v ,third group gave fingerlings after 20 day p.v , Forth group gave fingerlings after 25 day p.v ,fifth group didn't gave fingerlings , 6th group (control group) gave fingerlings after 14 days. Six groups of Nile tilapia (*O. niloticus*) breeder fish and six groups of fingerlings each contain 50 fingerlings corresponding to each group of breeder fish were used .one group of 50 fingerling from nonvaccinated fish not exposed to challenge used as negative control. Breeder fish subjected to challenge one month post vaccination, while fingerling subjected to challenge at age of one month. The organism for challenge was cultured in brain heart infusion broth at 25 ± 1°C for 24hs. The cultured broth was diluted with sterile saline solution to give a final concentration of 1.0 g bacterial cell by wet weight/L Badran (1993).Before immersing the experimental fish in the diluted broth culture for 10 minutes. The fish were pre-immersed in 1.5% Nacl solution for 5 minutes. Then immersed in the prepared broth culture, the challenged fish were placed under observation for 2 weeks and the dead fishes were used for *Aeromonas hydrophila* resonation. The relative level of protection (RLP) in each challenge was determined according to Newman and Majinarish (1982). using the equation

$$(\text{RLP}) = 1 - \frac{\text{Present immunized mortality}}{\text{Present control mortality}} \times 100$$

3-Result:-

3-1-The results of biological test of *Aeromonas hydrophila* among Nile tilapia were documented in Table (1). The results explained that, sixteen fish died in the second day post-infection. Then Eight fish were died in the third day post-infection, then three fish died in the fourth day post-infection and finally, four fish was died in the fifth day post-infection. No fish of the control group were died during the experiment.

Table (1) : The result of biological test of *Aeromonas hydrophila* among Nile tilapia (Orechromis niloticus)

* Group 1			**Group 2			Days
No. of fish	%	% of Total mortality	No. of fish	%	% of Total mortality	
0	0	0	0	0	0	1st day
16	40	40	0	0	0	2nd day
8	20	60	0	0	0	3rd day
12	30	90	0	0	0	4th day
4	10	100	0	0	0	5th day
-	-	100	0	0	0	6th day
-	-	100	0	0	0	7th day

* The fish of group 1 were injected I/M with 0.2 ml of the bacterial suspension/ 100 g fish body weight.

** The fish of group 2 were injected I/M with 0.2 ml of sterile physiological saline solution/ 100 g fish body weight.

3- 2-Tests performed to insure safty and sterility of the bacterin:-The injected fish showed no signs of *Aeromonas hydrophila* infection and there were no postmortem changes. The cultures of resolution showed no microbial growth of *Aeromonas hydrophila*.

4-3-The cultivated plates showed neither *Aeromonas hydrophila* nor other bacterial growth after 24 hs. of incubation at 25 °C . This result indicated that the prepared vaccine was sterile and safe to be used in the vaccination process.

3-4- The results revealed that, food supplied to fish in ratio of 2 and 2.5% of their body weight per day were not sufficient for maintenance while the food supplied in ratio of 3.5% of fish body weight was more than the fish requirement.

4-5-The results of immune response of Nile tilapia (*O. niloticus*) vaccinated orally with *Aeromonas hydrophila* wet-packed whole cells bacterin in comparison with those fed on untreated food were slight increase in the immune response of vaccinated fish where the antibody titers were 2 at 1st and 2nd week post-vaccination and 3 at 3rd and 4th week post vaccination. On the other side the antibody titer in the control group was 1 by log2 throughout the experiment. While, microa-gglutination and biochemical analysis increase in vaccinated fish than nonvaccinated (Table,2).

Table (2) :Results of the micro-agglutination titer, double immunodiffusion test, and total protein, g/dl, in Fingerlings samples at 4th week.

Fingerlings from control fish	**Fingerlings from oral vaccinated fish**	**Sample Test**
12	150	Micro -agglutination titer
-	+	Double immunodiffusion test
2	3	Total protein (g/dl)

3-6- The results of challenge were documented in Table (3). The results explained that, the fish vaccinated by oral methods were protected against challenge with *Aeromonas hydrophila* where the RLP were 86.8 .

Table (3): Comparison between the relative levels of protection (RLP) afforded by the different route of Aeromonas hydrophila vaccines of O. niloticus.

Route of vaccine	Results*	Percent of survivals	RLP
injection	4/50	92	91.2
immersion	5/50	70	86.8
oral	5/50	90	86.8
control	0/50	100	100

4-Discussion

Throughout this work the biological properties of *Aeromonas hydrophila* strain were tested for determining its virulence to Nile tilapia *(O. niloticus)* After which preparation of *Aeromonas hydrophila* bacterin (formalin inactivated bacterial cells) was performed.

The bacterin was tested for safety and sterility before immunization of Nile tilapia *(O. niloticus)* was done by the oral method of vaccination. Beside, the determination of humoral antibody titers and RIP of vaccinated and non-vaccinated fish. Several trials for vaccine preparation were performed on organisms other than *Aeromonas hydrophila* . The results of these trials were successful in the production of several vaccines for immunization against fish pathogens, such as *Aeromonas hydrophila* (Azad, et. al., 1999) and *Aeromonas salmonicida* (Cipriano, 1983) and some of them had been commercialized. The present trial also explained the ability of Nile tilapia *(O. niloticus),* to produce high level of specific antibody titer after Indicated that the bacterin prepared from *Aeromonas hydrophila* after treatment with formalin was antigenic in nature being able to against the inoculated antigen.

The results obtained from this investigation recorded that Nile tilapia *(O. niloticus),* collected for the present study had a light natural antibodies specific to *Aeromonas hydrophila* which detected by microagglutination test and calculated by log2. natural antibody titers were 1 throughout the experiment. These results agree with those reported by(Badran, 1994) who recorded low level of natural antibodies (1 by log2) against *Aeromonas hydrophila* in *O. niloticus*. The author explained that, the natural antibody against *Aeromonas hydrophila* produced as a result of contact of the normal fish with *Aeromonas hydrophila* present in the fish environment. The level of natural antibody against *Aeromonas hydrophila* was about the half of natural antibody against *Aeromonas hydrophila* Nile tilapia (*O. niloticus*) Badran, 1990 (1991 B). The high level of natural antibody against A. hydrophila was produced as a result of continuous contact of Nile tilapia (O. niloticus) with *Aeromonas hydrophila* was normally present in the fresh water and normally inhabit the intestinal tract of fish . Concerning to oral vaccination, the result revealed that, Nile tilapia *(O. niloticus)* vaccinated by wet –packed whole cells bacterin by oral route at ratio of 5mg bacterial cells/gm minced meat for 15 days produced low level of humeral antibody (2 at first and second week and 3 at 4th and 4th week post vaccination) not greatly different from those of control fish . Unfortunately, there are no available literature dealt with oral vaccination with *Aeromonas hydrophila* bacterin. On the other hand, the result of the present study nearly agree with those recorded by many authors on organisms other than P. *fluorescens* (Rohovec, et.al., 1975; Fryer,et. al., 1976; Rodegers and Austin, 1981; Badran, 1991 B, and Azad et. al (1999). Regarding to the relative level of protection (R.L.P) Amend (1981) Suggested that the RLP of over 60% provided acceptable protection. Our result revealed that the RLP of Nile telapia *(O. niloticus)* vaccinated by injection, immersion, and oral rout were 91.2, 86.8 and 86.8 % respectively. These results explained that, there is no great difference between the R.L.P of fish vaccinated by different methods inspite of the humeral antibody titers resulted from immersion and oral vaccination were low when compared with those of injection vaccination. The protection against infection of fish vaccinated by immersion and oral routes was related to agglutinins secreted in the muscus of body surface, gills; and intestinal mucosa (Kawai et. al., 1981; Badran, 1991 B, 1995 A, 1995 B and Sabry. N.M.(2008). The secreted agglutinins inhibit the organism to move freely and grow on the surface of the body and the mucus with trapped organism are removed leaving the skin clean and intact Badran (1991 B).

Indeed, the protocol of oral vaccination is very attractive since it's suitable for mass administration to

fish of all size, imposes on stress on the fish because handling is not required and therefore dose not interfere with routine husbanding practices. Moreover, oral vaccination is the only method studies concerning the success of laboratory and field application of oral vaccines, according to the R.L.P against vibriosis (Rohovec et. al., 1975 Kawai and Kusuda, 1985 Fryer et. al., 1987 and Kusuda et. al., 1987), furunculosis (Austin and Rodgers , 1981., Rodgers and Austin, 1985 and Fryer, 1987) and motile Aeromonas hydrophila (Badran, 1991 A and 1991 B and Aly et. al., 2000)

Conclusion : The bacterin prepared from *Aeromonas hydrophila* had antigenic in nature where it's able to stimulate the immune system of immunized Nile tilapia *(O. niloticus).* Nile tilapia *(O. niloticus)* vaccinated by oral routes had low level of humeral antibody titers not greatly different from those of control . The protection of vaccinated fish against infection not dependent only on the humeral antibody responds where immersed and orally vaccinated fish, which had low level of humeral antibody titer, were protected against infection at the same level of fish vaccinated by injection route.

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Forest community structure and composition along an elevational gradient of Parshuram Kund area in Lohit District of Arunachal Pradesh, India.

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Abstract: The present study was conducted in natural Himalayan forests of Parsuram Kund area in Lohit district of Arunachal Pradesh to understand the effect of altitudinal variation on structure and composition of the vegetation. Three altitudinal zones viz., upper zone (U) = 550-850 m asl, middle zone (M) = 500-700 m asl and lower zone (L) = 350-450 m asl were selected for the study. Tree Species richness (SR) was recorded to be highest (26) on the middle altitude followed by lower (21) and upper altitude (13). Species diversity (richness) and dominance (Simpson Concentration of dominance index) were found to be inversely related to each other. Shrub and herb species richness were also recorded to be higher on middle altitude followed by lower and upper altitudes. [Nature and Science 2010;8(2):27-35]. (ISSN: 1545-0740).

Key words: Phytosociology, species richness, diversity indices, altitude.

1. Introduction

The Indian Himalayan region occupies a special place in the mountain ecosystems of the world. These geodynamically young mountains are not only important from the stand point of climate and as a provider of life, giving water to a large part of the Indian subcontinent, but they also harbor a rich variety of flora, fauna, human communities and cultural diversity (Singh, 2006). Understanding of forest structure is a pre-requisite to describe various ecological processes and also to model the functioning and dynamics of forests (Elourard et al., 1997). Species diversity has functional consequences, because the number and kinds of species present in any area determine the organismal traits, which influence ecosystem processes. The components of species diversity that determine the expression of traits include the number of species present (species richness), their relative abundance (species evenness), presence of the particular species (species composition), the interactions among species (non-additive effects), and the temporal and spatial variation in these properties. In addition to its effects on current functioning of ecosystems, species diversity influences the resilience and resistance of ecosystems to environmental changes (Chapin et al., 2000).

The altitude and aspect play a key role in determining the temperature regime and atmospheric pressure of any site. Within one altitude the cofactors like topography, aspect, inclination of slope and soil type affect the forest composition (Shank and Noorie, 1950). Ellu and Obua (2005) have suggested that different altitudes and slopes influence the species richness and dispersion behavior of tree species. Moreover, Kharkwal et al. (2005) have pointed out that altitude and climatic variables like temperature and rainfall are the determinants of species richness. The distribution and species richness pattern of different species are largely regulated by altitude and physiographic factors (Sharma et al., 2009a, 2009b). The micro-environment of different aspects of hill slopes is influenced by the intensity and duration of available sunlight (Yadav and Gupta, 2006). This type of ecological knowledge is fundamental for conservation and sustainable utilization, and may provide important information for the policy makers for drafting management plans of fragile mountain ecosystems. Under the backdrop of the aforesaid facts, the present study was undertaken in natural Himalayan forests of Parsuram Kund area of Lohit district in Arunachal Pradesh to understand the effect of altitude on the structure and composition of the vegetation of natural forests.

2. Material and methods

The study was conducted in the natural Himalayan forest of Parsuram Kund area of Lohit district in Arunachal Pradesh. The Lohit district is one

of the districts of the state of Arunachal Pradesh in India. The Lohit District is located at the north-eastern edge of the state. The district stretches from 97° 24' E longitude to 95° 15' E longitude and 29° 22' N latitude to 27° 33' N latitude. The main ethnic groups that inhabit the place are Tai Khamti, Mishmi and Singpho. This river originates from the eastern part of Tibet and becomes a part of India at a place called Kibithoo. The district headquarters are located at Tezu. According to Indian census of 2001, it has a total area of 11402 Km^2 and a population of 143,478. The district is named after one of the important river of the state, Lohit River, from the Sanskrit Louhitya, reddish- or rust-coloured, and consists of the river valley and hills/mountains to the North and South. The area is highly inaccessible, and it is only in 2004 that a permanent bridge has been made operational across the Lohit at the holy site of Parashuram Kund, giving round-the-year connection to Tezu.

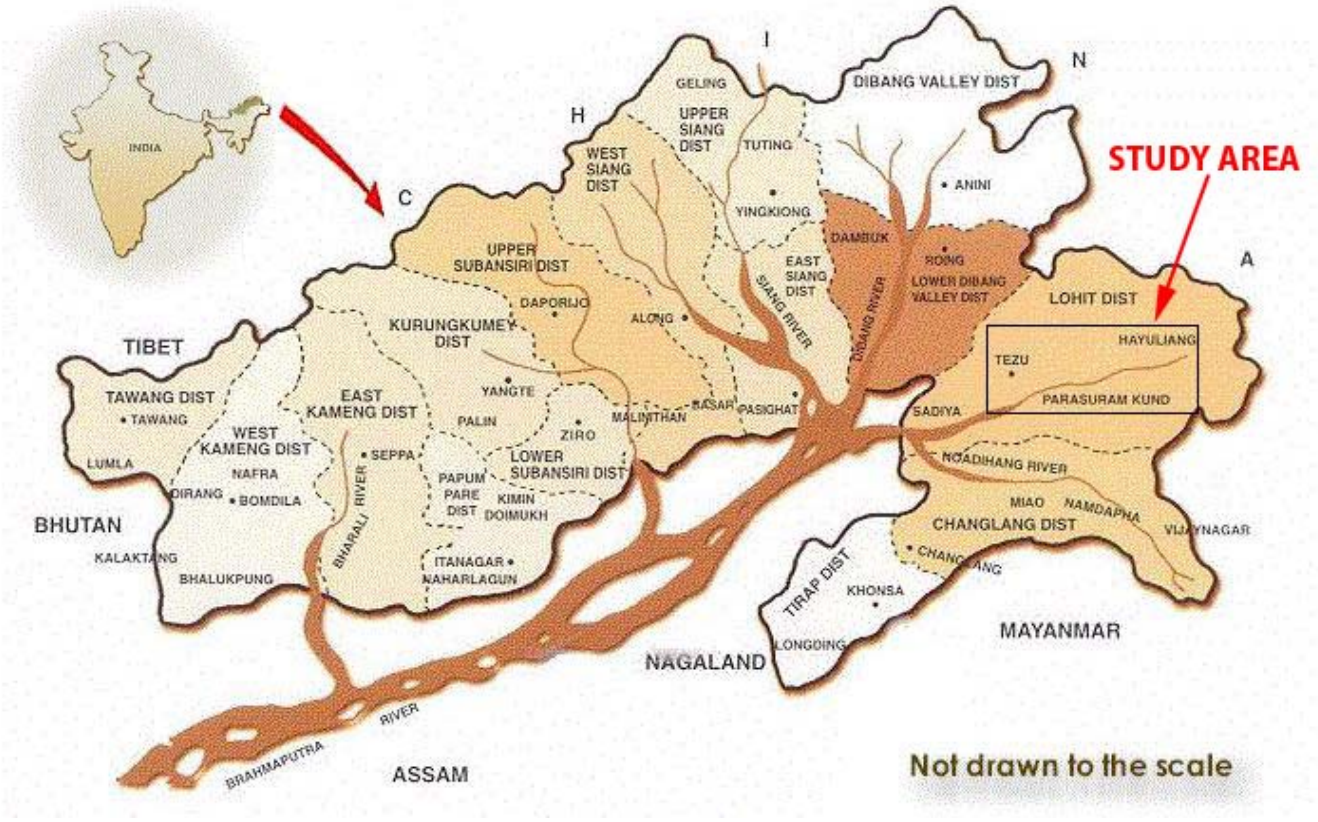

Figure 1: Map of the study area.

The climate of this place is mainly of two types. The high-altitude places are slightly cold, while the low-altitude places as well as the valleys are quite humid and hot. The period between the later part of November to the early part of March is winter time. The rainy season is experienced by the place between June and October. The climate is hot and highly humid in the lower elevations and in the Valleys and mildly cold in the higher elevations. The winter prevails during the months from late November to early March. The period from March to May is the pre-monsoon season. It is followed by monsoon from June to October. After the reconnaissance survey three altitudinal zones viz., upper zone (U) = 550-850 m asl, middle zone (M) = 500-700 m asl and lower zone (L) = 350-450 m asl were identified to study the effect of altitudinal variation on structure and composition of the vegetation.

The composition of the forest along the altitudinal gradient was analysed by using nested quadrat method or centre point quadrat method for trees, shrubs and herbs species as per Kent and Coker (1992). Three vegetation layers, (i.e., trees, shrubs and

herbs) were analyzed for species richness, density and diversity. A total of 60 plots (twenty plots in each forest type) measuring 10m × 10m each were sampled. Trees (≥10cm dbh) were analyzed by 10m × 10m sized quadrats, whereas shrubs by 5m × 5m sized quadrats. Further, quadrats of 1m × 1m size were randomly laid out with in each 10m × 10m sized quadrat at each site, to study plants in the herbaceous layer. Circumference at breast height (cbh= 1.37m) was taken for the determination of tree basal area and was calculated as πr^2, where r is the radius. Total basal area is the sum of basal area of all species present in the forest. Species Richness was simply taken as a count of number of species present in that forest type. Basal area (m^2/ha) was used to determine the relative dominance of a tree species. The diversity ($\overline{H}$) was determined by using Shannon-Wiener information index (Shannon and Weaver, 1963) as: $\overline{H} = -\sum n_i /n \log_2 n_i /n$; where, n_i was the density of a species and n was the sum of total density of all species in that forest type. The Simpson's concentration of dominance (Simpson, 1949) was measured as: $Cd= \sum Pi^2$, where, $\sum Pi = \sum n_i /n$, where, ni and n are same as in Shannon-Wiener diversity index. Simpson's diversity index (Simpson, 1949) was calculated as: D = 1-Cd, where, D = Simpson's diversity and Cd = Simpson's concentration of dominance.

3. Results

Results of forest community structure and composition are given in tables 1 to 4.

Trees: At upper altitude *Albizia lucida* was the dominant tree species with highest density (240 ind/ha) and TBC (8.19 m^2/ha). At middle altitude *Artocarpus chaplasha* had highest density (120 ind/ha) and TBC was highest in *Terminalia myriocarpa* (28.12 m^2/ha). At lower altitude *Albizia lucida* had highest density (120 ind/ha) and *Dubanga grandiflora* had highest TBC (9.62 m^2/ha). Tree Species richness (SR) was recorded to be highest at middle altitude (26) followed by lower (21) and upper (13) altitudes. Highest tree density was recorded at middle zone (860 ind/ha) followed by upper (600 ind/ha) and lower (550 ind/ha) altitudinal zone. Cd was found to be highest (0.2078) on upper altitude followed by lower (0.1094) and middle (0.0719) altitude. Value $\overline{H}$ was found to be highest (4.171) at middle altitude followed by lower (3.740) and upper (2.893) altitude.

Table 1: Phytosociology and diversity of tree strata.

Species	Density (ind/ha)			Basal Area (m^2/ha)			Cd			Shannon-Wiener ($\overline{H}$)		
	U	M	L	U	M	L	U	M	L	U	M	L
Actinodaphne obovata	-	20	-	-	0.07	-	-	0.0005	-	-	0.126	-
Ailanthus excelsa	-	10	-	-	2.58	-	-	0.0001	-	-	0.075	-
Alangium begoniaefolia	50	20	-	0.78	0.09	-	0.0069	0.0005	-	0.299	0.126	-
Albizia lucida	240	-	120	8.19	-	7.21	0.1600	0.0000	0.0476	0.529	-	0.479
Albizia sp.	-	80	-	-	3.03		-	0.0087	-	-	0.319	-
Artocarpus chaplasha	-	120	20	-	1.15	0.01	-	0.0195	0.0013	-	0.396	0.174
Bombax ceiba	-	-	10	-	-	8.60	-	0.0000	0.0003	-	-	0.105
Brassiopsis glomerulata	10	10	-	0.1	0.05	-	0.0003	0.0001	-	0.098	0.075	-
Callicarpa arborea	-	-	10	-	-	1.08	-	-	0.0003	-	-	0.105
Cinnamomum sp.	-	-	10	-	-	0.01	-	-	0.0003	-	-	0.105
Cyanometra polyandra	-	-	10	-	-	4.03	-	-	0.0003	-	-	0.105
Dalbergia sisso	80	20	50	6.8	3.2	5.60	0.0178	0.0005	0.0083	0.387	0.126	0.314
Dalbergia sp.	10	-	-	0.39	-	-	0.0003	-	-	0.098	-	-
Dendrocalamus hamiltonii	-	80	-	-	0.56	-	-	0.0087	-	-	0.319	-
Dilenia indica	-	-	10	-	-	3.20	-	0.0000	0.0003	-	-	0.105
Duabanga grandiflora	70	20	100	3.45	1.94	9.62	0.0136	0.0005	0.0331	0.362	0.126	0.447
Dysoxylon hamiltonii	-	20	-	-	1.26	-	-	0.0005	-	-	0.126	-
Engelhardtia spicata	-	-	50	-	-	6.80	-	-	0.0083	-	-	0.314
Ficus cunia	-	10	-	-	0.16	-	-	0.0001	-	-	0.075	-
Ficus roxburghii	-	10	-	-	0.39	-	-	0.0001	-	-	0.075	-

Ficus semecordata	-	40	10	-	15.81	3.56	-	0.0022	0.0003	-	0.206	0.105
Garuga gamblei	-	-	20	-	-	9.60	-	-	0.0013	-	0.126	0.174
Gynocardia odorata	10	20	10	0.29	1.3	2.20	0.0003	0.0005	0.0003	0.098	-	0.105
Knema angustifolia	-	10	-	-	0.07	-	-	0.0001	-	-	0.075	-
Kydia calycina	-	10	20	-	0.96	4.38	-	0.0001	0.0013	-	0.075	0.174
Laportea sp.	-	-	10	-	-	2.13	-	-	0.0003	-	-	0.105
Leea sp.	-	10	-	-	0.15	-	-	0.0001	-	-	0.075	-
Macaranga denticulata	20	90	20	0.9	3.39	2.00	0.0011	0.0110	0.0013	0.164	0.341	0.174
Macropanax dispermus	-	10	10	-	0.07	0.72	-	0.0001	0.0003	-	0.075	0.105
Mallotus tetracoccus	20	-	-	0.3	-	-	0.0011	-	-	0.164	-	-
Ostodes paniculata	-	20	-	-	0.26	-	-	0.0005	-	-	0.126	-
Pandanas odoratissima	30	40	-	0.19	0.39	-	0.0025	0.0022	-	0.216	0.206	-
Pterospermum acerifolium	20	60	10	1.36	8.84	2.30	0.0011	0.0049	0.0003	0.164	0.268	0.105
Sapindus rarak	-	-	30	-	-	4.50	-	-	0.0030	-	-	0.229
Sarcosperma griffithii	-	30	-	-	0.51	-	-	0.0012	-	-	0.169	-
Saurauria nepalensis	-	10	-	-	0.05	-	-	0.0001	-	-	0.075	-
Stercularia villosa	30	-	-	0.31	-	-	0.0025	-	-	0.216	-	-
Talauma hodgsonii	-	-	10	-	-	0.78	-	-	0.0003	-	-	0.105
Terminalia myriocarpa	10	80	-	1.15	25.12	-	0.0003	0.0087	-	0.098	0.319	-
Toona ciliata	-	10	-	-	3.51	-	-	0.0001	-	-	0.075	-
Trema orientalis	-	-	10	-	-	0.01	-	-	0.0003	-	-	0.105
	600	860	550	19.61	74.91	78.32	0.2078	0.0719	0.1094	2.893	4.171	3.740

Shrubs: At upper altitude *Musa nagensium* was the dominant shrub species with highest density (680 ind/ha) and TBC (16.24 m^2/ha). At middle altitude also Musa nagensium was the dominant shrub species with highest density (560 ind/ha) and TBC (4.24 m^2/ha). At lower altitude *Piper peepuloides* was the dominant shrub species with highest density (540 ind/ha) and highest TBC was recorded for *Grewia disperma* (1.45 m^2/ha). Shrub Species richness (SR) decreased from upper altitude to lower altitude with highest SR at upper (12) altitude followed by middle (10) and lower (10) altitude. Highest (3180 ind/ha) density was recorded at upper altitude followed by lower (2640 ind/ha) and middle (2380 ind/ha) altitudinal zone, where as highest TBC (17.45 m^2/ha) was recorded at upper altitude followed by middle (9.31 m^2/ha) and lower (1.93 m^2/ha) altitudes. Cd was found to be highest (0.143) on middle altitude followed by upper (0.120) and lower (0.118) altitude, whereas $\bar{H}$ was found to be highest (3.27) at upper altitude followed by lower (3.21) and middle (2.99) altitude.

Table 2: Phytosociology and diversity of shrub strata.

Species	**Density (ind/ha)**			**Basal Area (m^2/ha)**			**Cd**			**Shannon-Wiener ($\bar{H}$)**		
	U	**M**	**L**	**U**	**M**	**L**	**U**	**M**	**L**	**U**	**M**	**L**
Acacia pinnata	40	280	-	0.03	0.02	-	0.0002	0.0138	-	0.079	0.363	-
Artemisia nelagirca	160	-	200	0.01	-	0.02	0.0025	-	0.0057	0.217	-	0.282
Bambusa pallida	-	60	-	-	2.17	-	-	0.0006	-	-	0.134	-
Boehmeria longifolia	360	-	220	0.04	-	0.02	0.0128	-	0.0069	0.356	-	0.299
Boehmeria macrophylla	240	-	-	0.4	-	-	0.0057	-	-	0.281	-	-
Calamus leptospadix	440	-	-	0.38	-	-	0.0191	-	-	0.395	-	-
C. floribundus	120	-	340	0.02	-	0.09	0.0014	-	0.0166	0.178	-	0.381
Debregeasia longifolia	160	240	120	0.19	0.62	0.12	0.0025	0.0102	0.0021	0.217	0.334	0.203
Dendrocalamous gigneus	-	40	-	-	0.35	-	-	0.0003	-	-	0.099	-
Ficus urophylla	-	100	-	-	0.22	-	-	0.0018	-	-	0.192	-

Girardinia diversifolia	180	-	280	0.02	-	0.06	0.0032	-	0.0112	0.234	-	0.343
Grewia disperma	-	-	240	-	-	1.45	-	-	0.0083	-	-	0.314
Jasminium sp.	300	-	-	0.03	-	-	0.0089	-	-	0.321	-	-
Mesea indica	420	-	-	0.05	-	-	0.0174	-	-	0.386	-	-
Murraya paniculata	-	120	-	-	0.17	-	-	0.0025	-	-	0.217	-
Musa nagensium	680	560	-	16.24	4.24	-	0.0457	0.0554	-	0.476	0.491	-
Piper peepuloides	-	-	540	-	-	0.12	-	-	0.0418	-	-	0.468
P. griffithii	-	340	240	-	1.44	0.01	-	0.0204	0.0083	-	0.401	0.314
Rubus ellipticus	-	400	-	-	0.06	-	-	0.0282	-	-	0.432	-
Sida rhombifolia	-	-	160	-	-	0.01	-	-	0.0037	-	-	0.245
Trevesia palmata	80	-	-	0.04	-	-	0.0006	-	-	0.134	-	-
Triumfetta bartramia	-	240	-	-	0.02	-	-	0.0102	-	-	0.334	-
Zanthoxylum nepalensis	-	-	300	-	-	0.03	-	-	0.0129	-	-	0.356
	3180	2380	2640	17.45	9.31	1.93	0.1202	0.1434	0.1175	3.274	2.997	3.206

Monsoon Herbs: At upper altitude *Imperata cylindrica* was the dominant herb species with highest density (3300 ind/ha) followed by *Equisetum* sp. (2100 ind/ha) and *Saccharum spontaneum* (1400 ind/ha). At lower altitude *Bidens pilosa* was the dominant herb species with highest density (4000 ind/ha) followed by *Elatostemma* sp. (1800 ind/ha) and *Imperata cylindrical* (1600 ind/ha). At middle altitude *Elatostemma* sp. was the dominant herb species with highest density (1900 ind/ha) followed by *Pilea* sp. (1800 ind/ha) and *Phyrinum pubinerve* (1600 ind/ha). Herb Species richness (SR) was found to be higher at middle altitude (29) followed by upper and lower altitude with 21 species each. Highest (14200 ind/ha) density was recorded at upper altitude followed by middle (13100 ind/ha) and lower (13800 ind/ha) altitudinal zone. Cd was found to be highest (0.133) on lower altitude followed by upper (0.105) and lower (0.077) altitude, whereas $\bar{H}$ was found to be highest (4.21) at middle altitude followed by upper (3.78) and lower (3.55) altitude.

Table 3: Phytosociology and diversity of herb strata in monsoon and post monsoon seasons.

Species	**Density (ind/ha)**			**Cd**			**Shannon-Wiener ($\bar{H}$)**		
Monsoon Season Herbs	**U**	**M**	**L**	**U**	**M**	**L**	**U**	**M**	**L**
Begonia sp.	400	100	100	0.0008	0.0001	0.0001	0.145	0.054	0.051
Bidens pilosa	-	-	4000	-	-	0.0840	-	-	0.518
Colocossia sp.	-	300	-	-	0.0005	-	-	0.125	-
Commelina bengalensis	-	200	-	-	0.0002	-	-	0.092	-
Cyanotis vaga	-	200	400	-	0.0002	0.0008	-	0.092	0.148
Cyperus sp.	-	200	200	-	0.0002	0.0002	-	0.092	0.089
Elatostemma sp.	1100	1900	1800	0.0060	0.0210	0.0170	0.286	0.404	0.383
Equisetum sp.	2100	400	-	0.0219	0.0009	-	0.408	0.154	-
Eupatorium adenophorum	-	-	1000	-	-	0.0053	-	-	0.274
Eupatorium odoratum	200	200	-	0.0002	0.0002	-	0.087	0.092	-
Forrestica sp.	-	200	-	-	0.0002	-	-	0.092	-
Imperata cylindrica	3300	500	1600	0.0540	0.0015	0.0134	0.489	0.180	0.360
Lygodium flexuosum	300	100	-	0.0004	0.0001	-	0.118	0.054	-
Mastersia sp.	-	200	-	-	0.0002	-	-	0.092	-
Mikania micrantha	600	300	100	0.0018	0.0005	0.0001	0.193	0.125	0.051
Molinera cucurboides	100	200	200	-	0.0002	0.0002	0.050	0.092	0.089
Nephrolepis cordifolia	400	200	300	0.0008	0.0002	0.0005	0.145	0.092	0.120
Ophiopogon intermedius	200	300	300	0.0002	0.0005	0.0005	0.087	0.125	0.120
Paederia foetida	500	100	500	0.0012	0.0001	0.0013	0.170	0.054	0.173

Paspalam sp.	600	300	300	0.0018	0.0005	0.0005	0.193	0.125	0.120
Photos scandens	700	200	200	0.0024	0.0002	0.0002	0.214	0.092	0.089
Phyrnium pubinerve	500	1600	-	0.0012	0.0149	-	0.170	0.370	-
Pilea sp.	-	1800	-	-	0.0189	-	-	0.393	-
Polygonum capitatum	-	-	200	-	-	0.0002	-	-	0.089
Polypodium sp.	400	300	200	0.0008	0.0005	0.0002	0.145	0.125	0.089
Pteris sp.	300	700	300	0.0004	0.0029	0.0005	0.118	0.226	0.120
Saccharum spontaneum	1400	1200	1000	0.0097	0.0084	0.0053	0.329	0.316	0.274
Senecio cappa	200	200	-	0.0002	0.0002	-	0.087	0.092	-
Sonchus oleraceus	300	200	200	0.0004	0.0002	0.0002	0.118	0.092	0.089
Thladiantha calcarata	300	100	-	0.0004	0.0001	-	0.118	0.054	-
Thysanolaena maxima	300	200	200	0.0004	0.0002	0.0002	0.118	0.092	0.089
Urtica dioica	-	700	700	-	0.0029	0.0026	-	0.226	0.218
	14200	13100	13800	0.1053	0.0770	0.1332	3.784	4.212	3.552
Post-monsoon Season Herbs	**U**	**M**	**L**	**U**	**M**	**L**	**U**	**M**	**L**
Begonia sp.	100	100	200	0.0001	0.0001	0.0002	0.052	0.055	0.089
Bidens pilosa	-	-	3800	-	-	0.0781	-	-	0.514
Colocossia sp.	-	100	-	-	0.0001	-	-	0.055	-
Commelina bengalensis	-	200	-	-	0.0002	-	-	0.094	-
Cyanotis cappa	200	-	-	0.0002	-	-	0.089	-	-
Cyanotis vaga	-	200	300	-	0.0002	0.0005	-	0.094	0.121
Elatostemma sp.	1200	2100	1600	0.0078	0.0269	0.0138	0.309	0.428	0.363
Equisetum sp.	2200	700	-	0.0262	0.0030	-	0.425	0.229	-
Eupatorium adenophorum	-	-	800	-	-	0.0035	-	-	0.240
Eupatorium odoratum	400	200	-	0.0009	0.0002	-	0.150	0.094	-
Forrestica sp.	-	200	-	-	0.0002	-	-	0.094	-
Imperata cylindrica	2900	700	1400	0.0455	0.0030	0.0106	0.475	0.229	0.338
Lygodium flexuosum.	200	100	-	0.0002	0.0001	-	0.089	0.055	-
Mastersia sp.	-	200	-	-	0.0002	-	-	0.094	-
Mikania micrantha	500	300	300	0.0014	0.0005	0.0005	0.175	0.127	0.121
Molinera cucurboides	-	100	200	-	0.0001	0.0002	-	0.055	0.089
Nephrolepis cordifolia	400	200	200	0.0009	0.0002	0.0002	0.150	0.094	0.089
Ophiopogon intermedius	300	300	500	0.0005	0.0005	0.0014	0.121	0.127	0.175
Paederia foetida	400	200	600	0.0009	0.0002	0.0019	0.150	0.094	0.199
Paspalam sp.	800	400	200	0.0035	0.0010	0.0002	0.240	0.156	0.089
Photos scandens	500	200	800	0.0014	0.0002	0.0035	0.175	0.094	0.240
Phyrnium pubinerve	800	1000	-	0.0035	0.0061	-	0.240	0.287	-
Pilea sp.	-	1900	-	-	0.0220	-	-	0.408	-
Pogonetum sp.	-	200	100	-	0.0002	0.0001	-	0.094	0.052
Polygonum capitatum	-	-	100	-	-	0.0001	-	-	0.052
Polypodium sp.	200	0	100	0.0002	-	0.0001	0.089	-	0.052
Pteris sp.	500	800	400	0.0014	0.0039	0.0005	0.175	0.250	0.121
Saccharum spontaneum	1000	1000	1000	0.0054	0.0061	0.0054	0.277	0.287	0.277
Senecio cappa	300	100	-	0.0005	0.0001	-	0.121	0.055	-
Sonchus oleraceus	100	300	200	0.0001	0.0005	0.0002	0.052	0.127	0.089
Thladiantha calcarata	200	100	-	0.0002	0.0001	-	0.089	0.055	-
Thysanolaena maxima	400	200	300	0.0009	0.0002	0.0005	0.150	0.094	0.121
Urtica dioica	-	700	600	-	0.0030	0.0019	-	0.229	0.199
	13600	12800	13700	0.1012	0.0795	0.1233	3.795	4.150	3.634

Post-monsoon Herbs: At upper altitude *Imperata cylindrica* was the dominant herb species with highest density (2900 ind/ha) followed by *Equisetum* sp. (2200 ind/ha) and *Saccharum spontaneum* (1000 ind/ha). At middle altitude *Elatostemma* sp. was the dominant herb species with highest density (2100 ind/ha) followed by Pilea sp. (1900 ind/ha). At lower altitude *Bidens pilosa* was the dominant herb species with highest density (3800 ind/ha) followed by *Elatostemma* sp. (1600 ind/ha) and *Saccharum spontaneum* (1000 ind/ha).

Table 4: Total diversity indices of different forest strata at different altitudes.

Variable	Site	Trees	Shrubs	Herbs	
				Monsoon	Post-monsoon
Density (Ind/ha)	Upper	600	3180	14200	13600
	Middle	860	2380	13100	12800
	Lower	550	2640	13800	13700
Cd	Upper	0.208	0.12	0.105	0.101
	Middle	0.072	0.1434	0.077	0.079
	Lower	0.109	0.118	0.133	0.123
$\overline{H}$	Upper	2.89	3.27	3.78	3.79
	Middle	4.17	2.99	4.21	4.15
	Lower	3.74	3.21	3.55	3.63
SR	Upper	13	12	21	21
	Middle	26	10	29	28
	Lower	21	10	21	21

4. Discussion

The diversity of trees is fundamental to total forest biodiversity, because trees provide resources and habitat for almost all other forest species (Huang et al., 2003). At large scales, species diversity generally was found related to climate and productivity (Rahbek, 2005). Franklin et al. (1989) proposed that long-term productivity of natural forest ecosystems with high tree species diversity may be greater than that of forests with low diversity as a result of increased ecosystem resilience to disturbance. Slobodkin and Sanders (1969) opined that species richness of any community is a function of severity, variability and predictability of the environment in which it develops. Therefore, diversity tends to increase as the environment becomes more favourable and more predictable (Putman, 1994). Tree species diversity varied greatly from place to place mainly due to variation in biogeography, habitat and disturbance (Sagar et al., 2003), which have also been considered as the important factors for structuring the forest communities (Burslem and Whitmore, 1999). Srivastava et al. (2005) reported that the community characters differ among aspect, slope and altitude even in the same vegetation type. In our study we found that tree diversity decreased from lower altitude to higher altitude which means in our study area the environment at lower altitude was favourable for increasing tree diversity as compared to higher altitude.

Post-monsoon Herb Species richness (SR) was found to higher on middle altitude (28) followed by lower and upper altitude both with 21 species each. Highest (13700 ind/ha) density was recorded at lower altitude followed by upper (13600 ind/ha) and middle (12800 ind/ha) altitudinal zone. Cd was found to be highest (0.123) on lower altitude followed by upper (0.101) and middle (0.079) altitude, whereas $\overline{H}$ was found to be highest (4.15) at middle altitude followed by upper (3.79) and lower (3.63) altitude.

In many other studies, the mean $\overline{H}$ values for the other forests of temperate Himalaya varied from 0.4 to 2.8 (Singh et al., 1994), 0.08 to 1.29 (Shivnath et al., 1993) and 1.55 to 1.97 (Mishra et al., 2000), whereas in our study values reported by us are bit higher. Whittaker (1965) and Risser and Rice (1971) have reported the range of values of Cd for certain temperate vegetation from 0.19 to 0.99. The values of concentration of dominance (Cd) of the present study were more or less similar to the earlier reported values for temperate forests. Mean Cd values of 0.31 to 0.42 (Mishra et al., 2000) and 0.07 to 0.25 (Shivnath et al., 1993) were reported earlier from other parts of Indian Himalaya. The higher value of Cd in the forest growing on upper altitude was due to lower species richness. According to Baduni and Sharma (1997) the Cd or Simpson's index was strongly affected by the IVI of the first three relatively important species in a community. Species diversity (richness) and dominance (Simpson index) are inversely related to each other (Zobel et al., 1976). The Himalayan region is bestowed with a variety of natural resources which have been exploited by mankind since time immemorial. The link between forest management and the well-being of communities in forested areas has traditionally been defined by forest sector employment opportunities (Sharma and Gairola, 2007). The reported values of diversity and density in these forests can be utilized for management purposes and in the future for technological advancement,

economic prosperity and providing employment opportunity to the local people.

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Effects of some Artificial diets on the Growth Performance, Survival Rate and Biomass of the fry of climbing perch, *Anabas testudineus* (Bloch, 1792)

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Abstract: An experiment on culture of climbing perch (*Anabas testudineus*) in cemented tanks using different protein level diets was conducted to find the appropriate feeding diets and their effects on the growth, survival and biomass of 15-days old fry of *Anabas testudineus* in intensive culture . The experiment was carried out for duration of 60 days with 4 treatments in 8 cemented tanks each of size 12×6×1.5 feet. The initial length and weight were 14.5 ± 0.4 mm and 0.95 ± 0.05 g respectively. The feeds were applied twice a day at the rate of 10 %(initially) to 5% (later on) of the body weight of the fry/day. The results showed that the growth of fry varied significantly ($P<0.05$) with different diets. The highest growth, survival rate and biomass were found in the trial where fishes were fed on Sabinco feed containing 50.92% protein (on dry matter basis), followed by Feed-3 containing 30% protein. The poorest growth rate was shown by Feed- 1 (prepared by rice bran, wheat bran, fish meal and soybean meal) containing 20% protein. There was no significant difference in survival rates among the fry fed with Sabinco and prepared diets. The experiment suggests that Feed-2 (Sabinco Feed) can be recommended for the intensive culture of climbing perch. [Nature and Science. 2010;8(2):36-42]. (ISSN: 1545-0740).

Key words: Climbing perch, Artificial diets, Survival rate, Growth Performances and Biomass

1. Introduction

Fish is the major protein sources in the diet of the Bangladeshi people. Fish contributes about 60% of the available protein in the diet. It indicates the importance of fish in contributing to the level of nutrition of the people of Bangladesh (DOF, 1998; FAO, 1992). In spite of having large fisheries resources, Bangladesh is facing an acute malnutrition problem due to the shortage of animal protein supply in the diet.

Among various production inputs, the choice of fast growing species with desirable aquaculture traits is a pre-requisite for augmenting fish production in culture-based fisheries. Natural food based culture of major carp is still in practice in Bangladesh but carp culture could not be widely practiced in the shallow and seasonal ponds. In this regard koi fish (*A. testudineus)* is an excellent fish for growing in the shallow and seasonal ponds in a country like Bangladesh (Hussain *et al*. 1989, Gupta 1992, Kohinoor *et al*. 1993, Akhteruzzaman *et al*. 1993, Gupta *et al*. 1994) because Bangladesh enjoys very suitable climatic and ecological conditions for culture of warm-water species.

MAEP (1995) has shown that pond size affects the production of major carps. Small ponds below 0.1 acre size may not be profitable for conventional carp polyculture. Mustafa and Brown (1984) reported that growth rate in small ponds was rapid than in large impounded ponds. They commented that small ponds were more productive and easily manageable in our country. Edible fish production per unit area, *A. testudineus* is more productive than most farm fishes at the same level of intensification.

The labyrinth fishes, Anabantoidie, derived their name for having a labyrinth like accessory - breathing organ on either side of the head. Two widely known Asian members of the groups are climbing perch (*A. testudineus)* and gourami *(Ospharonemus).*Climbing perch, *A. testudineus* is a fresh water fish indigenous to South and Southeast Asia. It can thrive well in low dissolved oxygen (DO) waters and it can migrate between ponds. Wild climbing perch (*A. testudineus)* is a popular fish in Asia with larger fish (over 60 g) fetching a high market price.

Due to its air breathing ability and tolerance of adverse environmental conditions this fish turns out to be a very good candidate of fish culture. It is found in fresh and brackish waters mostly in ponds, swamps and lakes of Bangladesh, India and Southeast Asian countries. It is commonly called as koi fish in Bangladesh.

Larvae and young fry of *A. testudineus* fed on phytoplankton and zooplankton, larvae fry and adults feed on crustaceans, worms, moluscs and insect, algae, soft higher plants and organic debris (Potongkam,1972). For fish,

the optimum amount of protein in formulated feeds is important because either low or high levels of protein may lead to poor growth. As well, excess protein in fish diet may be wasteful and cause the diets to be unnecessarily expensive.

Therefore, attempts were taken to investigate the requirement of optimum protein level in formulated diets for *A. testudineus* fish. The objectives of the experiment were- a) to explore a suitable artificial feed for *A. testudineus* fry to obtain its maximum survival and growth b) to develop culture techniques through determination of effective feeding rate and locally available suitable feeds in on-station and on-farm condition and c) to estimate the proximate chemical composition of feed ingredients to be used for the formulation and development of quality fish feeds.

2. Materials and methods

The experiment was conducted in 8 cemented tanks of equal size (12×6×1.5 feet) for a period of 60 days at Tongi Fish Seed Multiplication Farm, Gazipur. Four different feeds were used in this experiment to observe their effects on growth, survival and biomass of *A. testudineus.*

2.1 Fry source

The fry of *A. testudineus* used in this experiment were obtained from a private hatchery named Reliance Aqua Farms situated at Bailor, Trishal, Mymensingh. Fry's were carried to the study area under well oxygenated condition.

2.2 Experimental design

Eight cemented tanks of 12×6×1.5 feet each were used in this experiment. The tanks were divided into four treatments namely treatment T_1, T_2, T_3 and T_4 each having two replications. Four different feeds namely Feed I, Feed II, Feed III and Feed IV were applied to the treatment T_1, T_2, T_3 and T_4 respectively. 15 days old 100 fry of *A. testudineus* of almost equal sizes were stocked in each tank.

2.3 Preparation of the tanks and release of fry

Cemented tanks with 2-4 m^3 water volume was used for nursing the fry. The tank was filled with well filtered water approximately one week before stocking. Water outlet of tanks was covered with proper mesh size net. The mesh size was changed (increased) during fish rearing to facilitate washing out the faeces and waste foods. The proper mesh size was 1.0-1.5 mm at the beginning of rearing. After the preparation of the tank the collected fingerlings were gently acclimatized with the tank water and released carefully.

2.4 Management of the tanks & fry

For maintenance of good health and growth of fry, frequently cleaning of tanks and feeding of fry are necessary. All the tanks were rubbed daily for removing bacteria accumulated on inner surface of rearing tanks. Faeces, waste particles of food and dead bodies of fish were siphoned at regular interval. From the 5^{th} or 6^{th} day of rearing antibiotic treatment was used. The most efficient antibiotic is terramycin. Four-six tablets of terramycin (2-3 g) produced for veterinary treatment can control the outbreak of bacterial diseases. Moreover, when there was deterioration in water quality, 10-20% water was replaced.

2.5 Collection of feed ingredients and Feed preparation

Sabinco (formulated feed; Feed II), Fish meal, Soybean meal, rice bran, wheat bran and vitamin pre-mix were collected from local fish and poultry feed traders of Gazipur town. Poultry viscera were collected from local grocery market. Rodovit GSS of Rhone Poolenc was used as vitamin pre-mix. Depending on the chemical composition and caloric values of the ingredients different diets were prepared. Physical properties like size, flavour and odor, texture, color; density (sinking rate), dehydration capacity and water stability were considered. Diets in the form of meal, paste and cakes were tried; however at the later stages soft pellets were prepared.

Table 1: Composition of the test feeds (%)

Ingredients	Feed - I	Feed II (Sabinco)	Feed - III	Feed - IV
Fish meal	18.0	40	30.0	22.0
Soybean meal	8.0	25	15.0	11.0
Wheat bran	30.0	15	21.0	20.0
Rice bran	43.0	19	31.0	44.0
Poultry viscera	-	-	2.0	2.0
Vitamin & Mineral mixture	1.0	1.0	1.0	1.0

The selected ingredients as mentioned above were used for the preparation of three different isocaloric fish feeds, denoted by Feed I, III and IV according to the treatment groups, by mixing the ingredients in such a manner so as to give crude protein values of 20, 30 and 24.7 % respectively. The amounts of ingredients needed to prepare 1 kg of feed were calculated from their proximate chemical composition and adjusted in such a manner that all the feeds contain nearly the same amount of energy per kg of feed. The feed were made into bite size pellets by adding starch solution or liquid from boiled rice and dried in an oven at 40° C for 2 days; or extracted as pellets from a pelleting machine and stored, sealed and frozen until used. These pellets were spread out on polythene paper and allowed to sundry for 4-6 hrs. Then the dried feed was stored in an air tight plastic bag.

2.6 Proximate analysis of the test feed

Proximate composition of the test of feeds were determined following the standard methods given by Association of official Analytical Chemists (AOAC, 1980) in the Nutrition Laboratory of Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh. The proximate composition of different feeds is shown in Table-2.

Table 2: Proximate analysis (% dry matter basis) of test feed

Parameters	Feed - I	Feed – II	Feed - III	Feed - IV
Dry matter	86.70	88.90	87.78	89.62
Crude protein	20.60	50.92	30.10	24.7
Crude lipid	11.61	8.35	10.40	12.01
Ash	12.30	16.36	15.30	13.80
Moisture	13.30	11.10	12.22	10.38
NFE*	31.80	24.36	9.9	20.3
Energy (Kcal/100g)	419.25	580.6	472	470. 5

- Nitrogen Free Extract calculated as: 100 - %(Moisture Protein + Lipid + Ash + Crude fibre).

2.7 Methods of feeding

After acclimatization of the released fish in the tank the *A. testudineus* fry were fed with Sabinco feed and three other prepared supplemental feeds at the rate of 10% (initially) and 5% (later on) of total body weight of stocked fry twice daily up to satiation at 09:00 AM and 5.00 PM. In the early stage the feed was diluted with small amount of water and then it was applied in the tank and later on it was spread directly. The fry were considered to be satiated when they stopped feed up taking or searching for food. After half an hour of feed supply the uneaten food particles and faeces were removed by siphoning.

2.8 Sampling & health monitoring

Sampling was done at 7 days interval and random samples of 20 *A. testudineus* fry were caught by glass nylon hapa from all the treatments. The fry were checked once a week and inspected for signs of malnutrition or disease. Dead fishes were immediately removed from the tank. Inefficient feeding practices can place the fish under stress which can result in parasitic infections. However, during this stage, sibling cannibalism was probably the greatest cause of mortality.

Total length and weight were recorded using a graph paper attached Petri dish and a Metler AJ 100 digital balance respectively. After careful measurement the fry were released in the respective place.

2.9 Evaluation of the water quality parameter

Water quality parameters such as temperature, pH and dissolved oxygen were monitored every 2 days of interval during the experimental period. Temperature was recorded using a Celsius Thermometer; dissolved oxygen and pH were measured directly by a portable digital DO meter (WPA OX 20) and a portable digital pH meter (WPA CD 70).

3. Results

Detailed result of the study on the growth performance, survival rate, biomass, water quality parameter and all other aspects reared in the eight cemented tanks fed on four different diet as recorded in during the period of study are presented below-

3.1 Growth

The initial average length of fry of *A. testudineus* increased from 14 mm to 40 mm, 15 mm to 66 mm, 14.5 mm to 56 mm and 14.2 mm to 51 mm for T_1, T_2, T_3 and T_4 treatments respectively after the experimental period. The initial average weight of *A. testudineus* fry increased from 0.9 g to 3.5 g, 1.0 g to 7.3 g, 0.95 g to 5.82 g and 0.92 g to 5.4 g for the treatments T_1, T_2, T_3 and T_4 respectively. The highest and lowest average final length was found for T_2 and T_1 treatment respectively. Again the highest and lowest average final weight was also found in the same treatments respectively.

3.2 Survival rate

The highest survival rate was found in treatment T_2. . The survival rates were recorded 74, 85, 81 & 79% in the treatments T_1, T_2, T_3 and T_4 respectively**.** However, these differences were not significant (P>0.05) among treatments of each experiment.

3.3 Biomass

After 60 days of study period, the highest biomass (total weight of fish) was found in treatment T_2 (620.5 g) and lowest biomass was found in treatment T_1 (259.74 g).

3.4 Water quality parameter

The physico-chemical parameters of water in different cemented tanks under different treatments during the trial are shown in Table-7. Temperature pH and DO of water in different cemented tanks under different treatments ranged from 28.8 to 29.8 ^{0}C, 7.6 to 8.1 and 5. 5 to 6.2 mg/l.

The differences in results among replications of a treatment were invariably small. Consequently, all values for a treatment were determined by combining the results of replications and averaging them.

Table 3: Growth of the fry of *A. testudineus* in cemented tanks during the trail period of 60 days (replications averaged).

Feed (Treatments)	Sampling day									
	1		15		30		45		60	
	Length (mm)	Weight (gm)	Length (mm)	Weight (gm)	Length (mm)	Weight (gm)	Length (mm)	Weight (gm)	Length (mm)	Weight (gm)
T_1	14	0.9	18	1.35	24	1.92	34	2.8	40	3.51
T_2	15	1.0	24	1.9	35	3.52	53	5.89	66	7.3
T_3	14.5	0.95	21	1.75	30	2.8	45	4.73	56	5.82
T_4	14.2	0.92	20	1.65	28	2.58	42	4.44	51	5.4

Table 4: Growth parameters of *A. testudenius* fed on different feeds after the trail period of 60 days.

Parameters	**T_1**	**T_2**	**T_3**	**T_4**
Initial length (mm)	14	15	14.5	14.2
Final length (mm)	40	66	56	51
Initial weight (g)	0.9	1.0	0.95	0.92
Final weight (g)	3.5	7.3	5.82	5.4
Condition factor	0.83	0.83	0.78	0.71
FCR	4.63	2.88	3.50	3.92
Specific growth rate (% day/fish)	0.98	1.43	1.30	1.26

Means in the same row with different superscripts are significantly different (P<0.05).

Table 5: Total number of fish and survival rate during the trail period of 60 days (replications combined). where "No." = number of fish and "S.R." = survival rate (%).

Feed (Treatments)	Sampling day									
	1		15		30		45		60	
	No.	S.R.	No.	S.R.	No.	S.R.	No.	S.R.	No.	S.R.
T_1	100	100	92	92	84	84	78	78	74	74
T_2	100	100	95	95	91	91	87	87	85	85
T_3	100	100	91	91	87	87	83	83	81	81
T_4	100	100	89	89	85	85	81	81	79	79

Table 6: Biomass of the fry of *A. testudineus* during the trail period of 60 days in grams (replications averaged).

Feed (Treatments)	Sampling day				
	1	15	30	45	60
T_1	90	124.2	161.28	218.4	259.74
T_2	100	180.5	320.32	512.43	620.5
T_3	95	159.25	243.6	392.59	471.42
T_4	92	146.85	219.3	359.64	426.6

Table 7: Water quality parameters during the trail period of 60 days.

Parameters	**T_1**	**T_2**	**T_3**	**T_4**
Average temp. (^{0}C)	29.6	28.8	29.2	29.8
Average pH	7.9	8.1	7.6	7.8
Average DO (mg/l)	5.7	6.2	5.9	5.5

3.5 Statistical Result

ANOVA was done with a view to finding out any significant difference among the SGR%, Feed efficiency and Survival (%) of *A. testudineus* fry on Sabinco Feed and three types of formulated Feed in four pairs of experimental tanks with replications. The F value from the result of SGR (%), Feed efficiency and Survival had been found to be 5.63, 310.94 and 2.8. SGR (%) and Feed efficiency are significant at 5% level but Survival is non-significant. Duncan's New Multiple Range Test is done with a view to finding out which fish feed will be the best in bringing growth and increasing weight, length etc. with proportional amount of nutrients.

4. Discussion

Availability of quality feed or ingredients of feed are the vital factors that affect commercial fish culture. In the present study Feed II was Sabinco Feed and Feed I, Feed III and IV were prepared from locally available ingredients. Among the feeds, Feed II contained highest protein percentage (dry matter basis) followed by Feed III, IV and I.

Better growth, survival and FCR were observed in fish fed with Feed II. Feed III produced second highest growth and survival. Growth and production in fish culture are generally dependent on the daily feed consumption, qualities of feed and feeding frequency (Mookerjee and Mazumdar, 1946). According to Chakraborty *et al.* (1995) the growth of carp (*Cyprinus carpio)* increases with protein levels, and there was an approximately linear increase of growth with feeding level for any given diet.

At the end of the present experiment on rearing fish in concrete tanks, the best growth was observed in treatment II with fish fed dietary that contained 50.92% protein. Results showed that fish fed on Sabinco feed grew with the weight gain of 0.11 g/day and SGR of 1.43%/day that were significantly higher ($p<0.05$) compared to other treatments. The significant lower growth was observed in fish fed Feed-I where the growth rate and SGR were 0.04 g/day and 0.98%/day, respectively. There was no significant difference

($p<0.05$) between treatment III and IV. The mean weight and length of fish in treatment II were the highest because of high protein content in the test diets.

In the present experiment maximum average length and weight were 66 mm and 7.3 g respectively obtained from the treatment fed with Sabinco feed (containing 50.92% protein). The experimented result is in agreement with the report of Sangrattanakhul, C. (1989) in which he estimated required dietary protein level for climbing perch ranged from 35-45%. Doolgindachabaporn (1994) also recommended that the feed containing 38.6% protein as the best feed formula in term of growth and survival for *Anabas* fry. Mookerjee, H.K. and S.R. Mazumdar, 1946, tested the performance of different diets containing 30, 34.7, 39.5, 44.1 and 48.9% protein in dry weight basis and reported that 39.5% protein is optimum in diet for commercial rearing of *Anabas testudineus*. In this experiment, the highest growth and survival were found with the Sabinco Feed containing 50.92% protein. In a trial conducted by Ray and Patra (1989) they indicated that climbing perch can achieve a rate of growth from 0.5-0.9 g/day when culture in earthen pond. The daily weight gain was observed in fish fed Feed-II that was 0.11 g/day. The experimented result is in agreement with the report of Sangrattanakhul (1989) reported that the ADG of *A. testudincus* fish ranging from (0.10-0.12). The above finding has more or less similarities with us.

It is evident from the results of SGR values of *A. testudineus* fish fed on Sabinco Feed and other formulated feeds with the increase of age the value of SGR decreases. From this point of view the formulated feed II gives best result in comparison with the other three feed. This finding resembles the Medawars (1945) fifth law "the specific growth declines more and more slowly as the organism increases in age". Minot (1908) was the first person to recognize that for most animals the specific growth rate is highest early in life and that it typically decreases with increasing age, becoming zero in some animals. The SGR% value of koi fish in our experiment also shows the same trend.

FCR were higher in the diets with the lowest protein content. In this experiment fish fed Feed I had the highest FCR that is 4.63. Fish fed Sabinco feed performed the lowest (2.88), and no significant differences were found in treatment III and treatment IV that was 3.50 and 3.92 respectively at ($p<0.05$) level. Doolgindachabaporn, (1994) found that the FCR value of *A. testudineus* ranges from 1.8-3.0. The above findings had similarities with us. Potongkam (1972) reported that FCR of climbing perch fed on trash fish and pellet were 2.07 and 1.89, respectively. In this study, feed conversion ratio was higher at higher feeding level due to feed losses increased with feeding level. Moreover, increase of feed conversion ratio at feeding rates might result from the in-completed digestion of feed (Rao, 1971).

During nursing period, most water parameters in cemented tanks were in suitable ranges for fish growth. Dissolve oxygen ranged from 5.5 to 6.2 mg/l whereas temperature ranged from 28 to 29.8˚C. Although there were little fluctuation in the parameters of water temperature and dissolved oxygen concentration from morning to afternoon in four treatments of experiment the ranges of these values were still suitable for the growth of climbing perch fry (Khan *et al.*, 1996).

5. Conclusion

The investigation reported the effect of some artificial feeds on growth, survival and biomass of the fry of *A. testudineus*. Optimum level of protein for the fish growths was also determined. Sabinco and three formulated fish feeds were used in the feeding and rearing trial of the fish. Different bio parameters, such as condition factor, survival rate, feed conversion ratio (FCR), specific growth rate (SGR) etc. were used to see the growth performance and feed utilization during the study period. Data were analyzed statistically using ANOVA & DMRT (Duncan's New Multiple Range Test). Probabilities of 0.05 were considered statistically significant. Based on results of this experiment, it can be concluded that the culture of climbing perch in tanks using Sabinco and Feed III feed can be applied in order to improve the income of farmers in our country. The feed containing 50.92% protein resulted in the best growth, survival, FCR and yield of climbing perch, which can be recommended for the culture of climbing perch.

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Diminution Of Aflatoxicosis In Tilipia Zilli Fish By Dietary Supplementation With Fix In Toxin And Nigella Sativa Oil

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Abstract: Mycotoxins are toxic metabolites of fungal origin, they are produced by certain strains of the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Under favorable conditions of temperature and humidity, these fungi grow on certain foods and feeds, resulting in the production of aflatoxins, which can enter into the human food chain directly through foods of plant origin (cereal grains), indirectly through foods of animal origin (kidney, liver, milk, eggs); however their continuous intake even in microdoses can result in their accumulation. Aflatoxins are hepatotoxic, hepatocarcinogenic and immunotoxic and cause growth retardation in animals and exposed human populations.Fix in Toxin is a kind of pentonite (clay) consists of (sodium calcium aluminosilicate), a non toxic agent and absorbent for a wide variety of toxic agents. It acts as an enterosorbant that rapidly binds aflatoxins in the gastrointestinal tract resulting in decreased aflatoxin uptake and bioavailability. *Nigella sativa* is a spicy potent belonging to ranunculacea seeds oil showed antibacterial, fungicidal effects.This study was conducted to evaluate the ability of Fix in Toxin 0.2 % and *Nigella sativa* oil 1% to diminish the clinical signs of aflatoxicosis in *Tilapia Zilli* fish, and based on this evidence, it's hypothesized that clay based entersorption of Aflatoxin may be a useful strategy for prevention of Aflatoxicosis in human population.60 *Tilapia Zilli* fish were divided into three groups, 20 fish for each group: Group 1 served as control and will be fed on commercial fish diet. Group 2 were be supplied by Aflatoxin contaminated ration with corn 80 ug toxin /kg ration. Group 3 were be supplied by aflatoxin contaminated ration with corn 80 ug toxin/kg ration and treated with 0.2 % Fix in Toxin and 1 % Nigella sativa oil injected daily I/P. Analysis of hematological parameters, clinical chemistry revealed significant differences between the control groups and the aflatoxicotic groups. administration of Fix in Toxin 0.2% and *Nigella sativa oil* injection 1% of body weight reduced the aflatoxicosis in liver and kidney by improving all liver and kidney enzymes.The dietary HSCAS clay remedy is novel, inexpensive and easily disseminated and proves its efficacy in diminishing the clinical signs of aflatoxicosis in fish, where it acts as an alfatoxin enterosorbant that tightly and selectively binds the poison in the gastrointestinal tract of the fish, decreasing their bioavailability and associated toxicities. In addition the *Nigella sativa oil* has a synergistic effect with Fix in Toxin in diminishing aflatoxicosis in fish. These findings support their use for dietary intervention studies in human populations at high risks for aflatoxicosis, specially in Egypt, where studies have shown that concurrent infection with the hepatitis B virus (HBV) during aflatoxin exposure increases the risk of hepatocellular carcinoma (HCC). [Nature and Science 2010;8(2):43-49]. (ISSN: 1545-0740).

Key words: Aflatoxicosis, *Tilapia Zilli* fish, Fix in Toxin effect, *Nigella sativa oil* effect, Hematological parameters, Clinical chemistry dynamic simulation; model; composting; domestic solid waste

1. Introduction

When certain types of fungus grow on food, they produce minute amounts of toxins called *mycotoxins*. Most fungi-produced mycotoxins are harmless, and even helpful. For example, the antibiotic penicillin came from a fungus, and it is a mycotoxin [Magan N 2005] [1].

The aflatoxins are a group of structurally related toxic compounds produced by certain strains of the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Under favorable conditions of temperature and humidity, these fungi grow on certain foods and feeds, resulting in the production resulting in the production of aflatoxins, which can enter into the human food chain directly through foods of plant origin (cereal grains), indirectly through foods of animal origin (kidney, liver, milk, eggs) Rojas-Duran T 2006 [2].

The most pronounced contamination has been encountered in tree nuts, peanuts, and other oilseeds, including corn and cottonseed. The major aflatoxins of concern are designated B1, B2, G1, and G2. These toxins are usually found together in various foods and feeds in various proportions [Takatori K 2006] [3]; however, aflatoxin B1 is usually predominant and is the most toxic. Aflatoxin M a major metabolic product of aflatoxin B1 in animals and is usually excreted in

the milk and urine of dairy cattle and other mammalian species that have consumed aflatoxin-contaminated food [Martins HM 2007] [4]. These poisons are completely heat stable, so neither cooking nor freezing destroys the toxin. They remain on the food indefinitely. Aflatoxins grow on grains and legumes mostly during storage, so the grains and legumes must be stored correctly to limit this problem [Kabak S 2006] [5]. Aflatoxins produce acute necrosis, cirrhosis, and carcinoma of the liver in a number of animal species; no animal species is resistant to the acute toxic effects of aflatoxins; hence it is logical to assume that humans may be similarly affected. Aflatoxin B1 is a very potent carcinogen in many species, including nonhuman primates, birds, fish, and rodents. In each species, the liver is the primary target organ of acute injury [Gong Y 2004], [Egal S 2005], [Wagacha JM 2008] [6],[7] and [8].*Nigella sativa* is a spicy potent belonging to ranunculacea seeds oil showed antibacterial fungicidal effects (Akguil, 1989) [9]. Nigella sativa inhibited chemical carcinogenesis, some investigators reported that its antioxidants effect inhibited chemical carcinogenesis. Ascorbic acid and Nigella sativa could reduce aflatoxin induced liver cancer (Newperne *et al.*1999) [10].Fix in toxin is a kind of pentonite (clay) consists of (sodium calcium aluminosilicate) a non toxic agent and absorbent for a wide variety of toxic agents (El-Bouhy *et al.*, 1993) [11]. It acts as an enterosorbant that rapidly and preferentially binds aflatoxins in the gastrointestinal tract resulting in decreased aflatoxin uptake and bioavailability [Phillips TD 2002] [12].

Aim of the present work:

This study was conducted to evaluate the ability of Fix in toxin 0.2 % and Nigella sativa oil 1% to diminish the clinical signs of Aflatoxicosis in *Tilapia Zilli*, and based on this evidence, it's hypothesized that clay based entersorption of Aflatoxin may be a useful strategy for prevention of Aflatoxicosis in human population.

Material and Methods:

Experimental Design: 60 *Tilapia Zilli* fish (50-100g each) were obtained from Abbassa and were acclimatized to laboratory conditions. They were kept in glass aquaria supplied with dechlorinated tap water at a rate of one liter for each cm of fish body.

The 60 *Tilapia Zilli* fish were divided into three groups, 20 fish for each group :

- Group 1 served as control and will be fed on commercial fish diet.
- Group 2 were supplied by Aflatoxin contaminated ration with corn 80 ug toxin /kg ration.
- Group 3 were supplied by Aflatoxin contaminated ration with corn 80 ug toxin/kg ration and treated with 0.2 % Fix in Toxin and 1 % Nigella sativa oil injected daily I/P.

The fish were fed by hand twice daily and feed consumption in all groups was recorded daily, also mortality and body weight due to Aflatoxin were recorded.

Samples Analysis: serum was collected 3 times at 3 months interval and sera were frozen at -20^{o}C.

Biochemical and Hormonal studies: The activities of aspartic aminotransferase (AST) and alanine aminotransferase (ALT) as well as cholesterol, urea and creatinine levels were determined according to the method of Varley *et al.*, () [13] by using commercial kits (Bio Merieus, France), total lipids were estimated according to the method of Siesta (1981) [14]. Total serum protein was estimated according to Drupt () [15].

Haematological studies: Blood hemoglobin was assessed and hematocrit value was carried out by using microhematocrit capillary tubes centrifuged at 2000 P.M. for 5 min according to the method of Drabkin (1964) [16].

Statistical analysis: Data are collected, summarized then tabulated for st Statistical analysis according to the method of Gad and Weil (1986) [17].

Results:

Table 1 showed that Aflatoxicosis produce a significant decrease in body weight if compared with the control group.

Table 2, 3, 4 showed that there is a significant decrease in PCV and Hemoglobin ($P < 0.01$). There is a significant decrease in mean of total protein and a significant increase in AST, ALT. There is a significant increase in urea, creatinine, total lipid, cholesterol and alkaline phosphatase ($P < 0.01$).

Post treatment with Fix in Toxin 0.2% and Nigella sativa oil injection 1% for 3 months. All this parameters return to normal level gradually as shown in Tables 1, 2, 3 and 4 if compared with control group.

TABLE (1): Effect of Aflatoxin on body weight of fish during the course of the experiment.

Groups	1 month	2 months	3 months
Group 1	57 ± 0.21p	68 ± 0.16*	101 ± 0.72*
Group 2	51 ± 0.10	61 ± 0.2*	74 ± 0.13*
Group 3	54.5 ± 0.06	64 ± 0.73*	84 ± 0.64*

(*P <0.01)TABLE

TABLE (2): Effect of Aflatoxin after one month on biochemical and hematological parameters in fish and after treatment with Fix in Toxin 0.2 % + *Nigella sativa* **1 %**

Parameters	Group 1 Control N =20	Group 2 Aflatoxin N =20	Group 3 Aflatoxin + Fix in Toxin 0.2 % *Nigella sativa* 1% N=20
Total protein mg/L	5.5 ± 0.18	3.13 ± 0.73**	4.4 ± 0.71
AST U/L	81 ± 0.24	112 ± 0.05**	103 ± 0.04
ALTU/L	17 ± 0.68	27 ± 0.73**	24 ± 0.75
Urea mg/dl	2.88 ± 0.28	4.4 ± 0.63**	5.2 ± 0.28*
Creatinine mg/dl	0.72 ± 5.5	0.98 ± 0.63**	0.88 ± 0.35
Total lipids cholesterol mg/dl	98 ± 0.98	143 ± 0.24**	104 ± 0.28*
Cholesterol mg/l	188.0.78	210 ± 2.3**	198 ± 0.34*
Alkaline phosphatase mg/dl	18.9 ± 0.38	28.7 ± 0.34**	22 ± 0.14
Hemoglobin mg/dl	8.3 ± 0.24	5.4 ± 0.75**	7.1 ± 1.61
P.C.V%	39.1 ± 64	34.1 ± 0.04*	34.1 ± 0.08

****p < 0.01**

TABLE (3): Effect of Aflatoxin after two months on biochemical and hematological parameters in fish and after treatment with Fix in Toxin 0.2 % + *Nigella sativa* **1 %**

Parameters	Group 1 Control N =20	Group 2 Aflatoxin N =20	Group 3 Aflatoxin + Fix in Toxin 0.2 % *Nigella sativa* 1% N=20
AST U/L	84 ± 1.27	121 ± 2.3**	94.6 ± 0.09
ALT U/L	18 ± 0.72	31 ± 0.89**	19 ± 0.16
Urea mg/dl	2.8 ± 0.74	5.1 ± 913**	3.1 ± 0.21
Creatinine mg/dl	0.83 ± 0.26	1.3 ± 0.51**	0.83 ± 0.27
Total protein mg/l	5.7 ± 0.22	3.1 ± 0.14**	4.7 ± 0.27
Total lipids mg/dl	98 ± 0.14	184 ± 13**	99 ± 0.77
Cholesterol mg/dl	186 ± 0.64	239 ± 3.6**	189 ± 2.3
Alkaline phosphates U/L	18.8 ± 0.18	33.9 ± 0.28**	201 ± 0.13
Hemoglobin %	8.6 ± 0.29	4.8 ± 0.73**	7.1 ± 114
P.C.V%	42 ± 0.71	28 ± 0.03**	37 ± 0.28

(P <0.01)**

TABLE (4): Effect of Aflatoxin after three months on biochemical and hematological parameters in fish and after treatment with Fix in Toxin 0. 2 % + *Nigella sativa* **1 %**

Parameters	Group 1 Control N =20	Group 2 Aflatoxin N =20	Group 3 Aflatoxin + Fix in Toxin 0.2 % *Nigella sativa* 1% N=20
Total protein mg/l	5.7 ± 0.23	3.1 ± 0.44**	5.5 ± 0.76
AST U/L	81 ± 0.18	133 ± 6.3**	82 ± 0.28
ALT U/L	19.1 ± 0.23	25 ± 0.38**	19.3 ± 0.08
Urea mg/dl	2.77 ± 0.23	5.1 ± 0.19**	2.78 ± 0.36
Creatinine mg/dl	0.79 ± 0.47	1.5 ± 0.53**	0.82 ± 0.33
Total lipids mg/dl	96 ± 0.74	189 ± 1.4**	95 ± 0.83
Cholesterol mg/dl	184 ± 0.95	254 ± 2.4**	183 ± 0.74
Alkaline phosphatase U/L	18.6 ± 0.28	35.2 ± 0.92**	18.3 ± 0.33
Hemoglobin %	8.5 ± 0.43	4.7 ± 0.72**	8.6 ± 0.38
P.C.V%	38 ± 0.22	26 ± 0.16**	39 ± 034

(P <0.01)**

Discussion:

Aflatoxicosis is poisoning that results from ingestion of aflatoxins in contaminated food, so humans are exposed to aflatoxins by consuming foods contaminated with products of fungal growth. Such exposure is difficult to avoid because fungal growth in foods is not easy to prevent [Bennett JE 2005] [18]. Aflatoxins produce acute necrosis, cirrhosis, and carcinoma of the liver and also impair immunity which ultimately led to increased susceptibility to disease in a number of animal species; no animal species is resistant to the acute toxic effects of aflatoxins [Pier AC 1987, Pier AC 1999, Wildi Dis J 2004] [19, 20, 21]; hence it is logical to assume that humans may be similarly affected.

Evidence of acute aflatoxicosis in humans has been reported from many parts of the world [Williams JH 2004, Kovacs M 2004, Gong Y 2004, Wagacha JM 2008] [22, 23,6, 8] and there is a positive association between dietary aflatoxins and hepatocellular carcinoma (HCC). especially aflatoxin B1, is potent carcinogens in some animals, In 1988, the IARC placed aflatoxin B1 on the list of human carcinogens.

Studies have shown that concurrent infection with the Hepatitis B virus (HBV) during aflatoxin exposure increases the risk of hepatocellular carcinoma (HCC). As HBV interferes with the ability of hepatocytes to metabolize aflatoxins, This effect is synergistic with the resulting damage far greater than just the sum of aflatoxin or HBV individually (Williams, 2004) [22].

The biochemical results detected in Table 2, 3, 4, showed significant increase in AST, ALT, while there was significant decrease in total protein level in group 2. These findings agreed with those found by by Jassar and Balwant (1993), Rasmassen *et al*. (1986), Sisk *et al*. (1988), due to liver injury induced by Aflatoxicosis [24, 25, 26]. The elevation of ALP activity comes in consisitence with that mentioned by Jassar and Balwant (1993), Svobodava *et al*. (1999) in chicken due to degenerative changes in the liver causing leakage of enzymes into serum and cause the highest concentration of alkaline phosphatase [24, 27].

The biochemical results, detected in table 2, 3, 4 showed significant increase in urea and creatinine, which are indicative of abnormal kidney functions group 2, Similar finding were reported by Newperne (1999) [12]. These changes due to necrosis of kidneys reported by Jindal and Mahipal (1994) Mansfeld (1989), Pier (1987) [28, 29, 19]. The lipid metabolism was altered during Aflatoxicosis as judged by increase of total lipid content. In the present experiment, there is a highly elevation of total lipid and cholesterol in serum which agree with Sippel, *et al*. (1983), Sisk *et al*. (1988) [30, 31].

It is obvious that administration of Fix in Toxin 0.2% and Nigella sativa oil injection 1% of body weight reduced the Aflatoxicosis in liver and kidney, group 3. These findings agreed with those found by Harvey RB 1991, Phillips TD 1999, 2002, 2008, Wang JS 2005, Afriyie Gyawu E 2005, 2008 in relation to the dieatry HSCAS clay [32, 33, 12, 34, 35, 36, 37]. In addition the *Nigella sativa oil* has a synergistic effect with Fix in Toxin in diminishing aflatoxicosis in fish due to its antibacterial fungicidal antioxidants effects [El-Bouhy *et al*. (1993)] [11]

The present study showed a significant decrease in PCV, HB concentration in the affected fish that was proportionally correlated with the severity of aflatoxicosis. This result is in accordance with Robert (1989), El-Bouhy *et al*. (1993) [38,11]. They found similar results in broilers chinckens common carp. Fish and this indicates that the toxin causes a deleterious effect on the hemopoeitic system.

In conclusion, based on the present research, the dietary HSCAS clay remedy is novel, inexpensive and easily disseminated and proves its efficacy in diminishing the clinical signs of aflatoxicosis in fish, where it acts as an alfatoxin enterosorbant that tightly and selectively binds the poison in the gastrointestinal tract of the fish, decreasing their bioavailability and associated toxicities. In addition that, *Nigella sativa oil* has a synergistic effect with Fix in Toxin in diminishing this aflatoxicosis in fish These findings support their use for dietary intervention studies in human populations at high risks for aflatoxicosis, specially in Egypt, where studies have shown that concurrent infection with the hepatitis B virus (HBV) during aflatoxin exposure increases the risk of hepatocellular carcinoma (HCC).

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Integrated Application of Poultry Manure and NPK Fertilizer on Performance of Tomato in Derived Savannah Transition Zone of Southwest Nigeria

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Abstract: Field experiments were conducted in two locations at Owo in early and late crop seasons (2007) to compare the effects of poultry manure at 0, 10, 20, 30, 40 t ha^{-1} and 300 kg ha^{-1} NPK 15:15:15 fertilizer on nutrient uptake and yield of tomato. The sites were located within the forest savannah transition zone of southwest Nigeria. The experiments were replicated three times in randomized complete block design. The test soil was slightly acidic, low in OM, N and P. Application of poultry manure and 300kg ha^{-1} NPK fertilizer significantly ($P<0.05$) increased plant N, P and K. Poultry manure at 20, 30 and 40 t ha^{-1} and NPK 15:15:15 fertilizer significantly ($P<0.05$) increased plant leaf, area height, number of leaves, branches fruits and fruit yield. Application of 10 t ha^{-1} poultry manure gave similar values of plant N, P and K and yield components compared with 300 kg ha^{-1} NPK fertilizer. The cumulative yield for the two seasons at 0, 10, 20, 30, 40 t ha^{-1} and 300kg/ha NPK were 9.6, 12.0, 18.1, 19.3, 14.4 and 13.5 t ha^{-1} respectively. [Nature and Science. 2010;8(2):50-54]. (ISSN: 1545-0740).

Key words: early crop, late crop, nutrient concentration, yield

1. INTRODUCTION

Adequate nutrients supply is essential for optimum production of tomato. Due to high cost and scarcity of mineral fertilizer, most farmers cannot afford the use of chemical fertilizer. This necessitates research into organic wastes that are cheap, readily available and environmentally friendly that can be used as fertilizer.

Recently, there has been boost in poultry production in urban centers in Nigeria which leads to piling of poultry wastes. This constitutes environmental pollution and health hazard to the inhabitants, whereas studies carried out in Nigeria and elsewhere confirm poultry manure as effective nutrient sources for increasing yield and nutrient status of crops such as maize , amaranths, sorghum and pepper (Adeniyan and Ojeniyi 2005, Akanni and Ojeniyi, 2005) and it also improves soil fertility and physical properties. However, research information is scarce on response of tomato, nutrient composition and yield to application of poultry manure in the forest savanna transition zone of Nigeria. Hence, the objective of this work was to determine the optimum rate of poultry manure and its effect on nutrient uptake and yield of tomato relative to the recommended level of NPK 15:15:15 fertilizer in the rain forest-savanna transition zone.

MATERIAL AND METHODS

Field Experiment

Experiments were conducted at Owo (Ehinogbe area and Ondo state Ministry of Agriculture, Zonal office, Owo) on soils that were slightly acidic, skeletal, clay kaolinitic and Oxic tropuldalf Owo lies within the forest savannah transitional zone of southwest Nigeria in the latitude5^0 12^1 N and longitude 5^0 35^1E.

In April 2007 (early cropping season) field trials was sited each at two sites. The poultry manure (PM) treatments were 0, 10, 20, 30 and 40 t ha^{-1} while NPK15:15:15 fertilizer was 300kg/ ha (recommended). The treatments were arranged using randomized complete block design with three replications. Heaps were spaced 1m apart in a plot of 3m x 3m to give a plant population of 10,000 plants / ha. Seeds of local variety of tomato were sowed in the nursery one month before the seedlings were transplanted to the field. Poultry manure was applied at heaping, two weeks before transplanting while NPK fertilizer was applied one week after transplanting. The trials were repeated at both sites in September 2007 (late season) after the harvest of early season crop.

Soil and poultry manure Analysis

Surface (0-20 cm) soil samples were collected before the conduct of the experiment at the two sites bulked, air – dried and 2mm sieved (for routine analysis) (11TA. 1979). Total N was determined by kjedahl method, p was extracted by

bray -1- p. exchangeable. Cations were extracted using normal ammonium acetate. The available K was determined by flame photometer while Ca and Mg were determined by atomic absorption spectrophotometer, Soil pH (1:2 soil-water) was determined by pH meter. Organic matter (OM) was determined by dichromate oxidation method. Soil texture was determined by the hydrometer method.

Poultry manure used for the conduct of the experiment was air –dried and milled. Samples were dry ashed at 500^0C for 6 hrs in furnace and extracted with nitric – perchloric acid mixtures (AOAC, 1990). N was determined by Kjedahl method, P by colorimeter, K by flame photometer and Ca and Mg were determined on AAS.

Growth and yield data

Five plants were randomly selected per plot. The number of leaves, branches and tap root length at harvest were determined. Ripe fruits from the randomly selected plants were harvested on plot basis, counted and weighed. Fruit yield was extrapolated to t ha^{-1}.

Plant Analysis

At 50% flowering, leaf samples were selected from 5 plants / plot. The leaf samples were washed with distilled water and oven dried at 65^0C to constant weight and ground. The N, P, K, Ca and Mg were determined as done in poultry manure analysis.

Statistical Analysis

The mean agronomic parameters for each season were subjected to analysis of variance and mean data were compared using least significance difference at 5% level of probability.

Results

Table 1 shows data on the pre treatment soil analysis of the experimental site and the poultry manure used for (Table 1)the experiment. The soils were slightly acidic, deficient in OM, N, and available P (Aune and Lal, 1997). The manure used contained N, Ca, Mg, P and K. The nutrient composition of the poultry manure suggests that it would serve to increase the soil fertility and hence improve the performance of the crop.

Table 1: Initial soil analysis and nutrient composition of poultry

Parameters	**Soil Value**	**Poultry (%)**
pH (H_20)	5.73	-
Organic matter %	1.81	-
Total N%	0.15	0.56
Available P mg kg^{-1}	11.3	1.38
Exchangeable K (c mol k g^{-1})	0.55	1.28
Exchangeable Ca ‘	1.70	0.68
Exchangeable Mg ‘	1.50	2.09
Sand %	68	-
Silt %	14	-
Clay %	18	-
Textural class sandy loam		-

Tables 2 and 3 show the effects of poultry manure rates and NPK 15: 15:15 fertilizer on nutrient concentration in tomato plants in early and late season respectively. Relative to control, poultry manure applications and NPK 15:15:15 fertilizer significantly ($P<0.05$) increased plant N and P in both seasons. In early season, 20 t ha^{-1} poultry manure significantly ($P< 0.05$) increased plant K while 30 t ha^{-1} poultry manure increased K in late season compared with control. Manure significantly increased ($P<0.05$) plant N, P, Ca, and Mg in both seasons. The 30 t ha^{-1} manure gave highest leaf nutrients content in both seasons with exception of K in early season.

Table 2: Effect of poultry manure rates and NPK 15:15:15 fertilizer on nutrient concentration in early (**E**) season tomato plant (%)

Treatment t ha^{-1}	**N**	**P**	**K**	**Ca**	**Mg**
Poultry manure			**%**		
0	1.54	0.29	1.70	0.25	0.40
10	1.68	0.32	1.88	0.43	0.77
20	3.20	0.40	2.08	0.50	0.96
30	5.80	0.48	1.81	0.60	0.99
40	5.89	0.41	1.76	0.49	0.96
NPK 300	1.70	0.35	1.80	0.25	0.38
LSD 0.05	0.08	0.05	0.21	0.14	0.36

Table 3: Effect poultry manure and NPK fertilizer on nutrient concentration in late (**L**) season tomato plant (%).

Treatment t ha^{-1} N	**P**	**K**	**Ca**	**Mg**
poultry manure		%		
0	1.35	0.20	1.50	0.20	0.36
10	1.60	0.30	1.78	0.40	0.69
20.	3.80	0.45	1.80	0.52	0.85
30.	4.90	0.50	2.50	0.65	0.96
40.	5.10	0.30	1.60	0.40	0.60
NPK 300	1.68	0.30	1.62	0.20	0.35
LSD 0.05	1.2	0.07	0.19	0.21	0.39

Table 4 shows the effect of poultry manure (pm) and NPK 15:15:15 fertilizer on plant growth parameters of tomato in the two cropping seasons and Table 5 contains data on fruit yield. Relative to control, poultry manure and NPK 15: 15:15 fertilizer significantly ($P < 0.05$) increased number of fruits; number of leaves, branches and fruit weight, but root length was not increased significantly. The 30 t ha^{-1} Pm gave highest values of the growth and yield parameters. Compared with 300kg ha ^{1} of NPK fertilizer, the 20, 30 and 40 t ha^{-1} had higher values of the measured parameters. Therefore, 20 and 30 t ha^{-1} PM significantly ($P<0.05$) increased number of leaves, branches and fruit yield in both seasons. The percentage increase in fruit yield (weight) in early season was in the decreasing order of 30 t ha^{-1} PM > 20 t ha^{-1} PM > 40 t ha^{-1} PM > 300 Kg / ha NPK fertilizer > 10 t ha^{-1} PM. The late season followed the same trend. The cumulative yield (t ha^{-1}) for the two seasons were: 9.6 t ha^{-1} for control, 12.0 t ha^{-1} for 10 t ha^{-1} PM, 18.1 t ha^{-1} for 20 t ha^{-1} PM, 19.3 t ha^{-1} for 30 t ha^{-1} PM, 14.4 t ha^{-1} for 40 t ha^{-1} PM and 13. 5 t ha^{-1} for NPK 15:15:15 fertilizer.

Table 4: Effect of poultry manure and NPK fertilizer on the growth of early (E) and late (L) season tomato (Trials 1 & 2)

Treatment t ha^{-1}	**No of Branches**		**No of Leaves**		**Tap root length (cm)**	
PM	E	L	E	L	E	L
0	3.1	5.1	29	21	19.9	20.0
10	6.2	8.9	44	31	22.5	21.6
20	10.7	14.6	80	48	25.7	26.2
30.	12.1	17.9	93	64	28.7	29.8
40	8.5	10.2	59	44	24.6	23.9
NPK 300	8.3	11.0	48.0	36	23.0	23.10
LSD 0.05	1.20	1.46	12.0	6.50	NS	NS

Table 5: Effect of poultry manure on yield of early (E) and late (L) season tomato

Treat PM t ha^{-1}	No of fruits/plant E	L	fruit weight /plant (g) E	L	cumulative fruit weight t ha^{-1}	increase in fruit yield %
0	9	11	4.5	5.1	9.6	-
10	14	15	5.9	6.1	12	25
20	14	21	9.0	9.1	18.1	88.5
30	15	20	9.4	9.9	19.3	90.6
40	14	17	7.1	7.3	14.4	50
NPK 300	13	17	6.6	6.9	13.5	41
LSD (0.05)	0.3	0.7	NS	NS		

3. Discussion

The significant increases in yield components of tomato due to poultry manure and NPK fertilizer confirm the deficiency of OM, N and P in the soil and poultry manure as effective source of plant nutrients. Therefore, addition of poultry manure and mineral fertilizer made more nutrients available to tomato plants.

This is in line with the work of Adenawoola and Adejoro (2005) that, poultry manure increased growth and yield of *Corchorus olitorus.* Plant height, number of leaves, leaf area, number of fruits and tomato yield as well as N, P and K were increased with the increase in the level of poultry manure up to 30 t ha^{-1}. The soil treated with 30 t ha^{-1} poultry manure gave highest plant K with corresponding increase in yields. The yield and growth parameters were found to decrease at 40 t ha^{-1} compare to 30 t ha^{-1} poultry manure indicating nutrient imbalance at the highest rate of application. The better performance of 30 t ha^{-1} poultry manure might be as a result of higher nutrient uptake especially N, P and K. It was indicated in the result that 40 t ha^{-1} PM reduced plant P, K, Ca and Mg compared to 20 t ha^{-1} of poultry manure. The least plant N, P and K contents recorded for tomato without poultry manure agrees with the observation that poultry manure supplied N, P and K (Ayeni, 2008, Ayeni *et al*., 2008). 20, 30 and 40 t ha^{-1} poultry manure performed better than 300 kg ha^{-1} NPK 15:15:15 fertilizers. This work shows that increase in poultry manure up to 30 t ha^{-1} maximizes yield than 20 t ha^{-1} of poultry manure earlier recommended by Akanni and Ojeniyi. (2007) as, optimum level for the production of tomato in the rain forest zone of southwest Nigeria.

4. Conclusion

Poultry manure significantly enhanced growth, yield and macronutrients content of tomato in savanna – forest transition zone of southwest Nigeria. The manure at 20 and 30 t ha^{-1} increased nutrient status and yield of tomato compared with 300 kg ha^{-1} N.P.K 15:15:15 fertilizer. The poultry manure at 30t ha^{-1} maximizes yield and N, P, K and Ca content of tomato plan.

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Antigenotoxic Efficacy of Some Vitamins against the Mutagenicity Induced by Ifosfamide in Mice

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Abstract: Ifosfamide (Holoxan, IFO) is an oxazaphosphorine alkylating agent with a broad spectrum of antineoplastic activity. IFO can damage DNA during any phase of the cell cycle and therefore, is not phase-specific. Thus the objective of this investigation is to measure the potential cytotoxicity of IFO alone and in combination with vitamins (FA, VB12 and VC) The genotoxic potential of IFO was evaluated *in vivo* using different mutagenic end points. Male Swiss mice were injected with different doses of IFO intraperitoneally to investigate the genotoxicity in somatic and germ cells. The doses were 8, 16 and 24mg IFO/kg body wt. as single doses, and 8mg IFO/kg body wt. as a repeated dose for three consecutive days. Samples were collected after 24h, 7 and 14 days after treatments. IFO induced chromosomal aberrations (in somatic and germ cells), SCEs and sperm shape abnormalities, which were highly significant in a dose dependent manner 24h after treatments. Chromosomal aberrations were declined with increasing the time of recovery. However, the tetraploid cells in mouse bone marrow were increased. IFO increased the percentage of DNA fragmentation in mouse spleen cells as measured by diphenylamine (DPA) assay, and confirmed by agarose gel-electrophoresis. Oral administration of folic acid (10 mg/kg body wt.), vitamin B12 (0.3 mg/kg) and vitamin C (50 mg/kg body wt.) declined the chromosomal aberrations in somatic and germ cells 24h after concurrent treatment with IFO. The used doses of vitamins reduced the percentage of DNA fragmentation induced by 24mg IFO/kg body wt. with DPA assay. **In conclusion**, the study indicates that the anticancer drug IFO is a mutagenic agent in mouse somatic and germ cells. Vitamins (FA, VB12 and VC) play a beneficial role against the mutagenicity of this drug. [Nature and Science 2010;8(2):55-66]. (ISSN: 1545-0740).

Key words: IFO, FA, VB12, VC, cytogenetic parameters, DNA damage.

1. Introduction

Ifosfamide (IFO) is an oxazaphosphorine alkylating agent with a broad spectrum of antineoplastic activity. It is used alone or in combination regimens for the treatment of a variety of haematological malignancies such lymphomas and multiple myeloma, and solid tumors including sarcoma, ovarian, testicular, cervical, breast, lung cancer and bone tumors (Dechant et al., 1991; Zhang et al., 2005; Goto et al., 2007) and is also used as systemic anticancer therapy in gynecological cancer patients with renal dysfunction (Li et al., 2007). IFO destroys tumor cells through apoptosis initiated by DNA damage, modulation of cell cycle and other antiproliferative effects. IFO is used concurrently with the uroprotective mesna to avoid hemorrhagic cystitis (Siu and Moore, 1998).

According to many authors who studied the action of vitamins *in vivo, the* treatment protocols that yield the best result in terms of reduction of chromosomal damage were those in which vitamins (A, B12, C, E, and FA) were administered as pre-treatment or in simultaneous treatment with the clastogenic agent (Ghaskadbi, 1992; Aly et al., 2002; Costa and Nepomuceno, 2006).

The present study was undertaken to assess the cytogenetic effect of IFO at different doses. The protective roles of FA, VB12, and VC on the induced chromosomal aberrations were also studies.

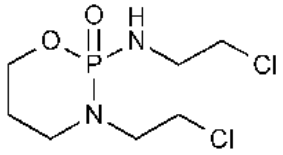

Figure 1: Ifosfamide

2. Materials and Methods

2.1. Tested subastances:

Ifosfamide (Figure 1) was purchased from Holoxan, Baxter Frankfurt am Main, Germany. Based on Paget and Barnes (1964) evaluation of drug activities, it was used as reference to convert the human therapeutic dose to mice therapeutic dose.
Folic acid (FA): Nile Co. for Pharm. and Chem. Ind. Cairo, Egypt. Vitamin B12 (B12): Amriya Pharm. Ind., Alexandria, Egypt. Vitamin C (VC): S.D.Fine-Chem.Ltd., Mumbai, India.

Table 1: Number and mean percentage of different types of chromosomal aberrations induced in mouse bone marrow cells 24h., 7 and 14 days after treatment with different doses of Ifosfamide.

Dose	Duration of treatments	No. and (%) of cells with different types of structural aberrations														(%) of cells with different types of numerical aberrations				Total Chromosomal Aberrations			
		Gap		Br or F.		Del.		C. F.		C. A.		G+ Br. and/or F.		Br.+ F.		41 Ch.		Tetrap.		Including Gaps		Excluding Gaps	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
I. Control	24h.	10	2.00	5	1.00	0	0.00	1	0.20	0	0.00	0	0.00	0	0.00	0	0.00	1	0.20	17	3.40	7	1.40
	7 Days	10	2.00	4	0.80	0	0.00	1	0.20	1	0.20	0	0.00	0	0.00	0	0.00	1	0.20	17	3.40	7	1.40
	14 Days	7	1.40	4	0.80	0	0.00	0	0.00	1	0.20	1	0.20	1	0.20	0	0.00	2	0.40	16	3.20	9	1.80
II. IFO Single Dose 8mg/kg	24h.	69	13.8	37	7.40	0	0.00	1	0.20	2	0.40	2	0.40	33	6.60	0	0.00	7	1.40	151***	30.2	82***	16.4
	7 Days	48	9.60	33	6.60	0	0.00	2	0.40	0	0.00	9	1.80	1	0.20	0	0.00	13	2.60	106***	21.2	58***	11.6
	14 Days	42	8.40	21	4.20	0	0.00	2	0.40	3	0.60	4	0.80	2	0.40	0	0.00	18	3.60	92***	18.4	50***	10.0
16mg/kg	24h.	75	15.0	49	9.80	0	0.00	2	0.40	3	0.60	10	2.00	82	16.40	1	0.20	10	2.00	232***	46.4	157***	31.4
	7 Days	65	13.0	50	10.0	0	0.00	0	0.00	12	2.40	5	1.00	13	2.60	0	0.00	14	2.80	159***	31.8	94***	18.8
	14 Days	47	9.40	30	6.00	0	0.00	0	0.00	0	0.00	9	1.80	6	1.20	0	0.00	20	4.00	112	22.4	65	13.0
24mg/kg	24h.	91	18.2	35	7.00	3	0.60	1	0.20	10	2.00	9	1.80	130	26.0	0	0.00	13	2.60	292***	58.4	201***	40.2
	7 Days	67	13.4	58	11.6	2	0.40	1	0.20	1	0.20	11	2.20	17	3.40	0	0.00	16	3.20	173***	34.6	106***	21.2
	14 Days	66	13.2	40	8.00	1	0.20	0	0.00	3	0.60	11	2.20	6	1.20	1	0.20	20	4.00	148	29.6	82	16.4
Repeated Dose 8mg/kgX3days	24h.	71	14.2	64	12.8	2	0.40	5	1.00	1	0.20	4	0.80	33	6.60	0	0.00	12	2.40	192***	38.4	121***	24.2
	7 Days	65	13.0	43	8.60	0	0.00	1	0.20	0	0.00	5	1.00	7	1.40	0	0.00	13	2.60	134***	26.8	69***	13.8
	14 Days	40	8.00	26	5.20	0	0.00	2	0.40	3	0.60	6	1.20	2	0.40	0	0.00	20	4.00	99	19.8	59	11.8

The total number of scored cells is 500 (5 animals/ group); ***p<0.001 G.: Gaps, Br.: Breaks, F.: Fragments, Del.: Deletions, C. F.: Centric Fusions, C. A.: Centromeric Attenuations, Tetrap.: Tetraploidy.

2.2. Cytogenetic studies:

2.2.1. Animals:

Laboratory-bred strain Swiss albino male mice of 8-10 weeks old with an average weight of 27.5±2.5g obtained from the National Research Center, Cairo, Egypt, were used. Animals were housed in groups (5 animals/ group) and maintained under standard food and water *ad libitium.*

2.2.2. Doses and Treatments:

IFO doses were i.p. administered at 8, 16, 24 mg/kg b.wt. as a single doses and repeated treatment for three consecutive days were conducted using the lowest dose 8 mg IFO /kg b. wt. Oral treatment of vitamins at 10 mg FA/kg b. wt., 0.3 mg VB12 /kg b.wt. and 50 VC /kg b. wt. were taken concurrently with 24 mg IFO/ kg. The anticancer drug IFO and vitamins were dissolved in distilled water.

Control groups of animals received distilled water and others received vitamins alone were collected concurrently with the treated groups.

2.2.2.1. Chromosomal Aberrations in Somatic and Germ Cells:

Samples were harvested after 24 h, 7 and 14 days of treatments with the different doses of IFO. The groups of animals received the concurrent administration of 24mg IFO/kg b. wt. with each of the vitamins separately were sacrificed after 24h.

2.2.2.2. Sister Chromatid Exchange (SCEs):

Samples were harvested 24h after treatment with the different doses of IFO.

2.2.2.3-Sperm-Shaped Abnormalities:

Mice were treated once i.p. with each of the three doses of IFO. Samples were collected after 35 days from the treatments

2.3. Cytogenetic Paramters:

- For **chromosomal aberrations** in somatic and germ cells, bone-marrow metaphases were prepared according to Yosida and Amano (1965). The diakinase –metaphase I cells collected from the spermatocytes were made following the air-drying technique of Evans *etal.* (1964).Slides were stained with 7 % Giemsa stain in phosphate buffer (pH6.8). 100 well spread metaphases per animal were analyzed for chromosomal aberrations. The types of aberrations in bone-marrow cells included gaps breaks, deletions, fragments, centric fusions, centromeric attenuations. The types of aberrations in spermatocytes were XY univalents, autosomal univalents, fragments and breaks.

- For **sister-chromatid exchanges,** the method described by Allen (1982**)** was adopted with some modifications. Bone - marrow cells were fixed and stained with fluorescence plus Giemsa method of Perry and Wolff (1974). The frequency of SCE's was recorded for each animal in at least 30 metaphases.

- For **sperm- shape abnormalities,** the epididymides were excised and, minced in isotonic sodium citrate solution (2.2%). Smears were prepared and sperms were stained with Eosin Y (Wyrobek and Bruce, 1978). At least 1000 sperm per animal (5000/group) were assessed for morphological abnormalities of the sperm shape.

Table 2: Frequency of sister chromatid exchanges in mouse bone marrow cells induced by different doses of Ifosfamide.

Dose	No. of Scored Cells	Total No. of SCE's	Mean ± SE
I. Control	**150**	**583**	**3.89±0.058**
II-IFO Single Dose			***
8mg/kg	**161**	**2183**	**13.56 +0.31**
			***
16mg/kg	**160**	**3842**	**24.01+0.81**
			***
24mg/kg	**156**	**4030**	**25.83+0.56**
Repeated Dose			***
8mg/kgX3day	**159**	**2638**	**16.59+0.54**

***P<0.001

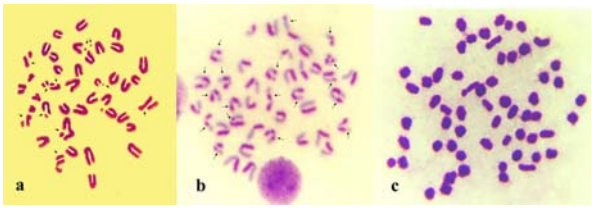

Figure 2: Metaphases with (a) chromosomal aberrations: breaks and fragments, (b) sister chromatid exchanges in bone-marrow cells and (c) triploid diaknesis- metaphase I cell in mouse spermatocyte after i.p. treatment with IFO.

2.4. DNA Fragmentation Assay:

The groups of animals treated with different doses8, 16 and 24 of IFO were collected 24h after treatments and repeated dose 8mg IFO/kg b.wt. for three days. The other groups of animals received concurrently 24mgIFO/kg b.wt. with each of the vitamins doses and were sacrificed 24h after treatment.

The method of DNA fragmentation assay was carried out according to Perandones *et al.* (1993). Mouse spleen was mechanically dissociated in hypotonic lysis buffer. The cell lysate was centrifuged

Table 3: Number and mean percentage of different types of diakinase metaphase I cells with chromosomal aberrations induced in mouse spermatocytes 24h., 7 and 14 days after treatment with different doses of Ifosfamide.

Dose	Duration of treatments	No. and % of cells with different types of chromosomal aberrations										Total Chromosomal Aberrations	
		XY un.		Auto. un.		XY+ Auto. un.		Br. or F.		3n			
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
I. Control	24h.	8	1.60	3	0.60	0	0.00	0	0.00	0	0.00	11	2.20
	7 Days	7	1.40	4	0.80	0	0.00	1	0.20	0	0.00	12	2.40
	14 Days	6	1.20	3	0.60	0	0.00	0	0.00	0	0.00	9	1.80
II. IFO.Single Dose 8mg/kg	24h.	16	3.20	12	2.40	3	0.60	3	0.60	1	0.20	*** 35	7.00
	7 Days	9	1.80	9	1.80	1	0.20	3	0.60	0	0.00	n.s. 22	4.40
	14 Days	14	2.80	1	0.20	1	0.20	1	0.20	0	0.00	n.s. 17	3.40
16mg/kg	24h.	29	5.80	22	4.40	7	1.40	7	1.40	0	0.00	*** 65	13.0
	7 Days	31	6.20	6	1.20	0	0.00	7	1.40	1	0.20	*** 45	9.00
	14 Days	15	3.00	6	1.20	3	0.60	3	0.60	1	0.20	** 28	5.60
24mg/kg	24h.	27	5.40	17	3.40	14	2.80	9	1.80	0	0.00	*** 67	13.40
	7 Days	24	4.80	15	3.00	2	0.40	8	1.60	0	0.00	*** 49	9.80
	14 Days	21	4.20	8	1.60	3	0.60	4	0.80	1	0.20	*** 37	7.40
Repeated Dose 8mg/kgX3days	24h.	15	3.00	23	4.60	6	1.20	2	0.40	0	0.00	*** 46	9.20
	7 Days	16	3.20	12	2.40	2	0.40	5	1.00	0	0.00	*** 35	7.00
	14 Days	15	3.00	6	1.20	1	0.20	3	0.60	0	0.00	** 25	5.00

The total number of scored cells is 500 (5 animals/ group); XY un.: XY univalents, Auto. un.: Autosomal univalents, XY+ Auto. un.: XY univalents plus Autosomal univalents, Br.: Breaks, F.: Fragments. ** $p<0.01$; *** $p<0.001$; n.s. = not significant.

Table 4: Number and mean percentage of different types of sperm shape abnormalities in mouse sperms induced by different doses of Ifosfamide.

Dose	No. of Scored sperms	Abnormal sperms		(%) of Different types of abnormal sperms							
		No.	Mean (%) ± SE	Amor.	Triang	W. Hook	Ban. Shape	Big Head	Small Head	Forked Head	Coiled Tail
I. Control	5099	99	1.94±0.23	1.02	0.33	0.24	0.04	0.04	0.04	0.00	0.23
II. IFO Single Dose											
8mg/kg	5146	146	2.84+0.19 *	1.52	0.82	0.25	0.09	0.00	0.02	0.02	0.12
16mg/kg	5331	331	6.21+0.09 ***	1.99	3.08	0.49	0.09	0.06	0.04	0.02	0.46
24mg/kg	5430	430	7.92+0.21 ***	2.71	3.52	0.76	0.07	0.04	0.07	0.11	0.64
Repeated Dose 8mg/kgX3 day	5212	212	4.07+0.22 ***	1.78	1.06	0.49	0.13	0.00	0.04	0.02	0.55

* p<0.05; *** p<0.001; Amor.: Amorphous, Triang.: Triangular, W. Hook : Without Hook, Ban. Shape: Banana Shape

at 13.000 xg for 15 min. then, the supernatant containing small DNA fragments was separated immediately and half the supernatant was used for gel-electrophoresis. The other half, as well as the pellet containing large pieces of DNA were used for the colorimetric determination by Diphenylamine (DPA) assay.

2.5. Statistical Analysis:

The significance of the results from the control data was calculated using ($2X^2$ contingency table) for chromomosal aberrations in somatic and germ cells and t- test for SCE's ,sperm- shape abnormalities and DNA fragmentation assays.

3. Results

3.1. Cytogenetic effect of IFO

3.1.1. Effect of IFO on somatic cells:

a- Chromosomal aberrations in bone marrow cells:

Table (1) illustrates a detailed study of single and repeated treatments with IFO for 24h, 7 and 14 days on the induction of chromosomal aberrations in mouse bone marrow. The percentage of induced aberrations was increased by increasing the dose of IFO. It was found to be statistically highly significant (p<0.001) after excluding gaps. The percentage of chromosomal aberrations decreased with increasing the time of recovery (Figure 2a).

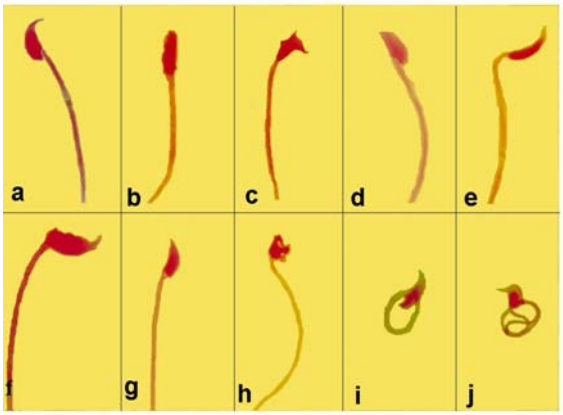

Figure 3: Types of sperm-shape abnormalities found in normal and IFO treated mice (a) normal sperm with a definite head by a marked hook and tail, (b and c) amorphous head, (d) without hook, (e) banana –shape, (f) big head, (g) small head, (h) triangular, (i and j) coiled tail.

b- Sister chromatid exchanges (SCE's):

All the tested doses induced a statistically significant increase in the frequency of SCE's (p <0.001) over that of the control (Table 2; Figure 2b).

3.1.2. Effect of IFO on germ cells:

a- Chromosomal aberrations in diakinase metaphase I (spermatocytes):

Aberration rates of control animals showed no

Table 5: Number and mean percentage of chromosomal aberrations in mouse bone marrow cells induced by different doses of Ifosfamide plus different doses of vitamins FA, VB12, and VC.

Treatment and Doses	No. of cells with different types of structural aberrations							No. of cells with numerical aberration		Total Chromosomal Aberrations				Inhibition (%) Of Aberrant Cells Excluding Gaps
	G.	Br. or F.	Del.	C.F.	C.A.	G+Br. and/or F.	Br.+ F.	41 Ch.	Tetrap.	Including Gaps No.	%	Excluding Gaps No.	%	
I. Control	10	5	0	1	0	0	0	0	1	17	3.40	7	1.40	-
FA (10mg/kg)	11	9	0	0	0	2	0	0	3	25	5.00	14	2.80	-
VB12 (0.3mg/kg)	12	8	0	0	0	1	0	0	2	23	4.60	11	2.20	-
VC (50mg/kg)	10	8	0	2	0	0	0	0	4	24	4.80	14	2.80	-
II. Treatment and Protection for Single Dose														
IFO (24mg/kg)	91	35	3	1	10	9	130	0	13	*** 292	58.4	*** 201	40.2	-
IFO+ FA (24 +10)	36	25	0	1	5	9	80	0	9	••• 165	33.0	••• 129	25.8	35.82
IFO+ VB12(24+0.3)	36	29	0	0	0	7	95	1	13	••• 181	36.2	••• 145	29.0	27.86
IFO+ VC(24+50)	32	22	1	0	3	2	101	0	17	••• 178	35.6	••• 146	29.2	27.36
III. Treatment and Protection for Repeated Dose														
IFO(8mg/kgX3days)	71	64	2	5	1	4	33	0	12	*** 192	38.4	*** 121	24.2	-
IFO+ FA(8+10)	38	24	0	0	4	3	15	0	11	••• 95	19.0	••• 57	11.4	52.89
IFO+ VB12(8+0.3)	35	30	0	0	1	2	16	0	16	••• 100	20.0	••• 65	13.0	46.28
IFO+ VC(8+50)	40	37	1	0	5	3	28	1	13	••• 128	25.6	• 88	17.6	27.27

The total number of scored cells is 500 (5 animals/ group); *** $p<0.001$: Significance compared to Control.

•$p<0.05$; •••$p<0.001$: Significance compared to treatment with IFO; G.: Gaps, Br.: Breaks, F.: Fragments, Del.: Deletions, C. F.: Centric Fusions, C. A.: Centromeric Attenuations, 41 Ch.: 41 Chromosomes, Tetrap.: Tetraploidy, IFO: Ifosfamide, FA: Folic acid, VB12: Vitamin B12, VC: Vitamin C.

Table 6: Number and mean percentage of diakinase metaphase I cells with chromosomal aberrations in mouse spermatocytes induced by different doses of Ifosfamide plus different doses of vit. FA, VB12, VC.

Treatment and Doses	Normal Cells	XY un.	Auto. un.	XY+ Auto. un.	Br. or Frag.	3n	Total Aberr.ations No.	%	Inhibition (%) of Aberrant Cells
I. Control	489	8	3	0	0	0	11	2.20	-
FA (10mg/kg)	487	7	6	0	0	0	13	2.60	-
VB12 (0.3mg/kg)	488	8	1	0	3	0	12	2.40	-
VC (50mg/kg)	490	8	2	0	0	0	10	2.00	-
II. Treatment and Protection for Single Dose									
IFO (24mg/kg)	433	27	17	14	9	0	67***	13.4	-
IFO+ FA (24 +10mg/kg)	472	15	8	2	3	0	28•••	5.60	58.21
IFO+VB12(24 +0.3mg/kg)	474	13	8	2	3	0	26•••	5.20	61.19
IFO+ VC(24 +50mg/kg)	471	17	8	2	3	0	30	6.00	55.22
III. Treatment and Protection for Repeated Dose									
IFO(8mg/kgX3days)	454	15	23	6	2	0	46***	9.20	-
IFO+ FA (8 +10mg/kg)	478	11	6	2	2	1	22••	4.40	52.17
IFO+ VB12 (8 +0.3mg/kg)	477	11	8	0	3	1	23•••	4.60	50.00
IFO+ VC (8 +50mg/kg)	480	14	5	1	0	0	20	4.00	56.52

The total number of scored cells is 500 (5 animals/ group); *** p<0.001: Significance compared to Control.
•• p<0.01 ••• p<0.001: Significance compared to treatment with IFO; Br.: Breaks, F.: Fragments, IFO: Ifosfamide FA: Folic acid, VB12: Vitamin B12, VC: Vitamin C.

variation. The significant effect of IFO on the induction of chromosome aberrations in spermatocytes was observed after single and repeated treatment. The percentage of chromosomal aberrations was dose dependent and decreased as the time after treatment increased (Table 3; Figure 2c).

b- Sperm-shape abnormalities:

IFO induced a dose dependent and statistically significant increase in the percentage of sperm shape abnormalities. Table (4) shows the different types of observed abnormalities (Figure 3).

3.1.3. The protective effect of vitamins:

Tables (5, 6) demonstrated the protective effect of FA, VB12& VC on the induction of the chromosomal aberrations in somatic and germ cells after concurrent treatment with IFO. The results showed that FA, VB12 & VC exerted a significant reduction in the percentage of chromosome aberration induced by 24 mg IFO/kg b. wt. as a single dose and 8mgIFO/kg b. wt. for 3 consecutive days as a repeated dose.

3.2. DNA fragmentation assay:

3.2.1. Effect of IFO:

a. DPA assay:

Mean percentage of DNA fragmentation in mouse spleen cells was markedly increased ($p<0.001$) after treatment with the single and repeated doses of IFO (Table 7).

b. Agarose gel-electrophoresis:

DNA fragmentation assessed by agarose gel-electrophoresis was increased in a dose dependent manner with the increasing of IFO (Figure 4)

3.2.2. Protective effect of vitamins:

Table (7) illustrates the mean percentage of DNA fragmentation induced in mouse spleen cells after i.p. treatment with 24mg IFO /kg b. wt. and oral concurrent treatment with 10, 0.3 and 50mg /kg b. wt. FA, VB12 and VC, respectively. The percentage of DNA fragmentation decreased to 12.68%, 10.64% and 11.15% after treatment with FA, VB12 and VC, respectively, compared with 21.77% for IFO alone. Figure (4) shows the DNA fragmentation assessed by agarose gel- electrophoresis, which was decreased after treatment with vitamins compared to that with 24mgIFO/ kg b.wt.

4. Discussion

Ifosfamide, as all other alkylating agents, destroy tumor cells through apoptosis initiated by DNA damage, modulation of cell cycle and other anti-proliferative effects. Thus it can damage DNA during any phase of cell cycle, and therefore, it is not phase specific. The main mechanism is inhibition of DNA replication, as the interlinked strands cannot separate. (Zhang *et al.*, 2005).

Table 7: Mean percentage of DNA fragmentation induced in mouse spleen cells after treatment with different doses of IFO alone and in combination with FA, VB12 or VC using DPA assay.

Dose (mg/kg)	DNA Fragmentation Mean (%)±SE	DNA Fragmentation Inhibition (%)
I. Control	**2.95±0.621**	**-**
FA (10mg)	**2.56±0.869**	**-**
VB12 (0.3mg)	**3.17±0.787**	**-**
VC (50mg)	**2.87±0.716**	**-**
II. IFO		
Single dose	***	
8	**20.73+1.441** ***	**-**
16	**21.03+1.511** ***	**-**
24	**21.77+1.076** ***	**-**
Repeated dose		
8X3days	**21.25+0.898**	**-**
III. IFO+Vitamins	•••	
IFO+FA (24+10)	**12.68±1.123** •••	**41.74**
IFO+VB12 (24+0.3)	**10.64±1.116** •••	**51.12**
IFO+VC (24+50)	**11.15±0.944**	**48.76**

(5 animals/ group);
*** $p<0.001$: Significance compared to Control.
••• $p<0.001$: Significance compared to treatment with IFO.

IFO induced highly significant percentage of structural chromosomal aberrations in mouse bone marrow cells and diakinesis metaphase I cells (spermatocytes) which increased with dose increasing. Adler and El-Tarras, (1990) demonstrated that cisplatine, an alkylating anticancer drug, induced chromosomal aberrations in primary spermatocytes and spermatogonial stem cells of male mice at 5, 7.5 and 10 mg/kg b.wt. Alvarez-Gonzalez *et al.,* (2001) found that IFO induced micronuclei in muose bone marrow cells. Also this was reported when injected i.p. tumor bearing mice at 90 mg/kg for 1-3 days led to loss of IP+/-19q (Leurand *et al.,* 2004). Although the structural aberrations in mouse cells decreased with increasing the time of recovery, the numerical aberrations increased and reached their maximum after 14 days of recovery. The positive correlation between tetraploid cells and long duration of treatment may lead to induction of secondry carcinoma. This hypothesis supported with the study of Kubota *et al*., (1997) who found that induction of secondary carcinoma appeared in patient treated with therapeutic regimens containing daunorubicin and

cyclophosphamide. Also, they observed a third malignancy caused by alkylating agents (therapeutic regimens) containing IFO.

Separation of chromosomes forming XY and autosomal univalents was the most common type of aberrations in mouse spermatocytes in the present study. Concerning numerical aberrations, it is worth to mention that a very low frequency of triploid diakinasis metaphase I cells were observed after treatment with different doses of IFO. This phenomenon may be attributed to the effect of the anticancer on the spindle apparatus (Temtamy *et al.*, 1982; Hemavathy and Krishnamurthy, 1988). Such results agree with Amer *et al.,* (2002) who observed triploid spermatocytes in mice treated with 1mg Mitomycine C /kg b.wt.

IFO at single and repeated doses induced a highly significant and a dose dependent increase in SCE's frequencies in mouse bone marrow cells. The mean values of SCE's/cell were higher than three folds of the control indicating that IFO is a strong inducer of SCE. Induction of SCE's was observed in cultured V79 chinese hamster cells after treatment with IFO and cyclophosphamide (Sirianni and Huang 1980). Aly *et al*. (2003) demonstrated that both cisplatin and gemcitabine separately induced SCE's in mouse bone marrow cells in a dose dependent manner.

Sperm-head abnormalities are usually taken as a characteristic criterion and as an applied test for monitoring the mutagenic potential for many chemicals (Brusick, 1980). Tail deformities were reported to reduce fertility in human and animals (Topham.1983).

The mean percentage of sperm shape abnormalities were dose dependent with IFO. The maximum percentage was 7.92+0.21($p<0.001$)24h after treatment with 24mg IFO/kg b. wt., such results coincide with the results obtained by cisplatin (Giri *et al.,* 1998), Mitomycin C 1mg/kg b.wt (Farghaly and Ibrahim, 2003) and cyclophosphamide at 20, 60 mg/kg b. wt. (El-Nahas et al., 1989; Kumar et al., 2004; Hassan et al., 2006) which induced highly significant sperm shape abnormalities in mice.

Apoptosis is a form of programmed cell death was shown to play a key role in normal development and oncogenesis. Its hall mark biochemical feature of endonuclease activation, was giving rise to internucleosomal DNA fragmentation (Perandones *et al.,* 1993). The present study indicated the apoptotic changes induced by IFO in mouse spleen cells (*in vivo*) revealed a significant increase in the percentage of DNA fragmentation with (DPA) assay and was confirmed by agarose gel electrophoresis. The observed increase in DNA fragmentation might be due to the induction of DNA strand breaks by this compound.

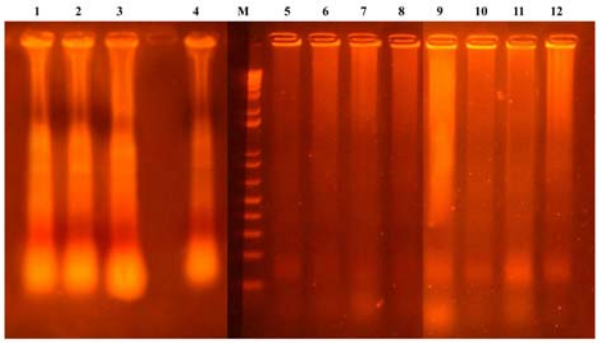

Figure (4): Effect of IFO on DNA fragmentation. Lanes 1-3: DNA of mice treated with 8, 16 and 24mg IFO/kg b wt. respectively. Lane 4: treatment with repeated dose of IFO. Lane M: 1K base DNA ladder. Lane 5: control .Lanes 6-8: DNA of mice administered FA, VB12 or VC respectively. Lane9: DNA of mice treated with 24 mg IFO/kg b. wt. Lanes10-12: DNA of mice treated concurrently with IFO plus FA, VB12 or VC respectively (FA, 10mg/kg b. wt., VB12 0.3mg/kg b. wt., VC 50mg/kg b. wt.).

Many studies demonstrated that IFO have the potential to induce DNA fragmentation and apoptosis in various tissues *in vivo* and *in vitro* .Latz *et al.*, (1997) demonstrated that 1ug/ml IFO for 2h induced DNA fragmentation in different cell lines *in vitro* such as V79 Chinese hamster, caski- (squamous ca.), widr-(colon ca.) and MRI-221 (melanoma) cells. Hartley *et al.,* (1999) observed the presence of DNA cross linking in the lymphocytes of patients treated with IFO at 3.09/m^2 /day by continuous intravenous infusion over 3-5 days or as a 3h infusion daily for 3 days. Ypsilantis *et al*.,(2004) demonstrated that IFO induced enterocyte apoptosis and DNA fragmentation in the rabbit small and large intestine in a dose and intestinal site- dependent manner and it had a dose related apoptotic, but steady anti-mitotic effect on intestinal crypt cells, which led to mucosal atrophy in the small intestine of the rabbit.

The inhibition of DNA synthesis, specially the cellular DNA may be induced by cross links between the anticancer drugs and the DNA molecules. IFO generates bifunctional alkylating nitrogen mustards which are converted to chemically reactive carbonium ions at neutral PH and react with the 7-nitrogen atom of purine bases in DNA, especially when they are flanked by adjacent guanines.The second arm in phosphoramide mustard can react with a second guanine moiety in an opposite DNA strand or in the same strand to form cross links. The O^6 atom of guanine may also be a target for oxazaphosphorines (Zhang et al., 2005)

In a trial to minimize the genotoxicity effect of IFO. FA, VB12 and VC were administered simultaneously with single and repeat doses. The

results showed that the maximum effect of FA appeared after repeated treatment in mouse bone marrow cells and spermatocytes. The possible mechanism of FA action is connected with thymidylate synthetase activity and through DNA synthesis (Glover, 1982) and with modifying cellular nucleotide pools (Kunz, 1988). Also, FA is involved in both methyl metabolism and in DNA synthesis and repair (Duthie and Hawdon, 1998).

Donya and Aly (2003) found that FA caused a highly significant inhibition in the percentage of aberrant metaphases induced in mice somatic and germ cells after treatment with methotrexate (anticancer drug).

VB12 is required for the synthesis of methionine and S-adenosyl methionine, the common methyl donor required for the maintenance of DNA conformation (Zingg and Jones, 1997). It is essential for one-carbon metabolism and cell division thus its synthesis is very complex and restricted to microorganisms (Afman *et al.,* 2001; Ulleland *et al.,* 2002).

The percentage of inhibition reached 61.12% and 50.00% in mouse spermatocytes after single and repeated treatments with IFO plus VB12. The present results agreed with the studies carried out by Joksic *et al.* (2006), who demonstrated that VB12 reduced the incidence of micronuclei induced by ribavirin, a synthetic purine nucleoside analogue with a broad spectrum of antiviral activity, in phytohemaglutinine-stimulated human lymphocytes.

Oral administration of VC to mice treated with IFO (concurrent administration) minimized the percentage of chromosomal aberration induced in somatic and germ cells after both single and repeated treatments. VC is a powerful reducing agent (antioxidant) and plays a part in intracellular oxidation/reduction system, and binding oxidants (free radicals) produced endogenously. Besides,VC can promote the removal of oxidative DNA damage from the DNA and/or nucleotide pool,through the upregulation of repair enzymes ,perhaps induced by the vitamin's proxidative properties(Cooke *et al.,* 1998).

Ghaskadbi *et al.,* 1992 and Vijayalaxmi and Venu 1999 reported that the modifying effect of ascorbic acid at low doses on cyclophosphamide induced micronuclei in mice. Also, Giri *et al*., (1998) found that the frequency of all mutagenic parameters in Swiss albino mice treated with VC plus cisplatin were significantly less than those treated with cisplatin alone suggesting a protective role of ascorbic acid against cisplatin.

The concurrent administration of IFO at 8mg/kg b. wt. with vitamins FA, VB12, and VC reduced the percentage of DNA fragmentation in mouse spleen cells as measured by DPA and agarose gel electrophorasis. Simultaneous treatment of human peripheral blood mononuclear cells (*in vitro*) with cisplatin and melatonin (free radical scavenger and general antioxidant pineal hormone) decreased cisplatin induction of DNA fragmentation from 45% to 28%.(Hassan *et al.,* 1999).VC diminished the extent of DNA damage evoked by selenium-cisplatin conjugate but had no effect on the kinetics of DNA repair in human lymphocytes (Blasiak and Kowalik, 2001).But post treatment of VC for mice treated with cyclophosphamide did not affect DNA damage level using comet assay in peripheral white blood cells (Franke et al ., 2005).

The present study indicates that the anticancer drug IFO is a mutagenic agent in mouse somatic and germ cells. Vitamins (FA, VB12 and VC) play a beneficial role against the mutagenicity of this drug.

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Use of InfoWork RS in modeling the impact of urbanisation on sediment yield in Cameron Highlands, Malaysia

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Abstract: Hydrodynamic model and sediment transport model were investigated in the Sg Telom and Sg Bertam, Cameron Highlands as a result of rapid urbanization and agriculture activities over the past 30 years. This article, from the point of view of the river catchment as a whole system, presents an integrated approach by combining the hydraulic and hydrology simulations with numerical model of sediment transport and change in river bed level before and after the Ringlet reservoir. To accomplish this purpose, InfoWork RS, a well developed numerical model for sediment transport and river bed variations were used. The application shows that it can properly simulate change of river bed variation over 10 months simulation period.
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Key words: dynamic simulation; model; composting; urbanization; sediment

1. Introduction

Flow and sediment transport are important in relation to several hydrology-hydraulic and engineering topics, e.g. erosion around structures, backfilling of dredging channels and river morphological change (Wilson, 1981; Cooper, et al. 1987; Woolhiser, 1990). It closely related to soil erosion or soil loss although the process mechanism is not the same as sediment yield- A unit which is defined as the total sediment outflow from a catchment, and normally expressed in absolute terms t ha^{-1} $year^{-1}$. Eroded soil may be redeposited a few meters from where it was dislodged, whereas sediment yield from a basin is that portion of the eroded soil which leaves the basin. In the context of river basin sediment models, the principal objective is to link the on-site rates of erosion and soil loss within the basin to the outlet sediment yield. In is clear from field studies that the dominant response mechanisms behind the link, along with the sediment yield itself, was the process of sediment being transported from the source to the outlet. This includes the sediment transport modes (wash load, bed load or suspended load), sediment properties (size and shape of grain), bedforms (ripple, dunes and antidunes), bed roughness, *ks* and effective shear stress, τb. Combination of these factors with other physical aspects such as basin area scale and hydro-meteorological conditions have been shown to influence the sediment transport of many catchment areas as studied by Bruijnzeel, 1990, Wan Ruslan, 1996, Mohd Ekhwan, 1997; 2002; Mohd Ekhwan & Noorazuan, 2003. Reports of the impact of urbanization on sediment yield have been reported by many researchers in Malaysia and elsewhere (Wan Ruslan 1997; Douglas, 1967; Mohd Ekhwan, 2005). The reports are particularly important not only for project formulation but also in land use planning. Most of the previous studies agreed that rapid urbanization such as land clearance for agriculture activities and industralisation have accelerated it impact on sediment yield as studied by Wan Ruslan (1997) in Sg Relau, Penang (30 times greater of sediment output), Krishnaswamy, et al (2001) in Costa Rica (15 times), Mozzherin (1994) in Russia (21 times) and Walling & Gregory (1970) in Devon, England (10 times). In this respects, knowledge of sediment behaviors including its yield potential can allow more accurate land use decisions to be made.

Over the last 30 years, an advance in computer modeling provides large opportunity to study the sediment behavior particularly in relation to hydrologic (i.e SHETRAN, HEC-6, MIKE SHE) or by combination of hydrology and hydraulic models (i.e. InfoWork RS, SWMM, SED2D, XP-STORM, BASINS). The use of such computer modeling can simulate event-based or continuous periods, much larger river or basins and more importantly was its capability to integrate with other spatial modeling such as Geographical Information System (GIS) data interface. The proposed of the article is to discuss both hydrodynamics and sediment transport mechanism which later will be transformed into the sediment yield in Cameron Highland Pahang, specifically the process

of bed level changed before and after the Ringlet reservoir. The objective is to model the sediment transport characteristics of Sg Telom and Sg Bertam as a result of rapid urbanization (established or on-going property development such as housing, shop lots, hotels, etc) and agricultural activities which generate most of the eroded soils and sediments to Sg Telom.

2. Study Area and Sedimentation Issue

Cameron Highlands is a district located in the state of Pahang (Figure 1). With a total area of approximately 71,218 hectares (175,978 acres), it occupies around 2 per cent of the state area and is bordered by Kelantan on the north, by Perak on the west and the Pahang's District of Raub on the south and east. The area experienced daily temperatures which fluctuated between 27.4^{0}C in February/April, and 13.5^{0}C in January. Precipitation is generally common throughout the year with most of it recorded during the Northeast and Southwest monsoons. Record obtained from the Malaysian Meteorological Services (MMS) shows that the Cameron Highlands receives an average rainfall of 2,800mm with the western foothills area receiving higher precipitation compared to the higher mountainous area. Topographically, Cameron Highlands is located on the main mountain range of Banjaran Titiwangsa with elevations ranging from 100 metres above m.s.l. at the eastern part, to 2031 metres above m.s.l. at the western part of the area, and with the highest peak being Gunung Brinchang (2,031m).

Cameron Highlands is drained by three main river systems namely Sg. Telom, Sg. Bertam and Sg. Lemoi which drain the northern, middle and southern parts of the district, respectively. These rivers flow eastwardly and joining up with Sg. Pahang. Sg Telom and Sg Bertam, in particular play a vital role to Cameron Highlands as freshwater supply sources, irrigation water sources for the agricultural activities, and as sources for hydroelectricity generation and recreational activities.

For decades, Cameron Highlands rapid land development and human activities like agriculture, urbanisation, infrastructure development, deforestation and etc. have contributed to severe upland soil erosions. These activities have led to tremendous pressure to the existing river system and water courses. Forest coverage in Cameron Highlands has reduced quite tremendously in the last 5 years. From the current land use, almost 2,000 hectares of forest have been converted to agricultural lands within the catchments of Upper Telom and Upper Bertam. This is a reduction to 51% from the total land area in the two catchments compared to 62% in 1997. For years during heavy rainfall rivers in Cameron Highlands have to imbue the high rate of eroded sediments coming from these sources. Agricultural activities generate most of the eroded soils in Cameron Highlands. Activities like market gardening, floriculture, mixed agriculture, tea and orchard constitute more than 11,000 hectares of active land that produces silts to the water courses. Agricultural activities almost 36% of the total land use in Upper Telom and Upper Bertam catchments while constitute of more than 16% of the total land area in Cameron Highlands.

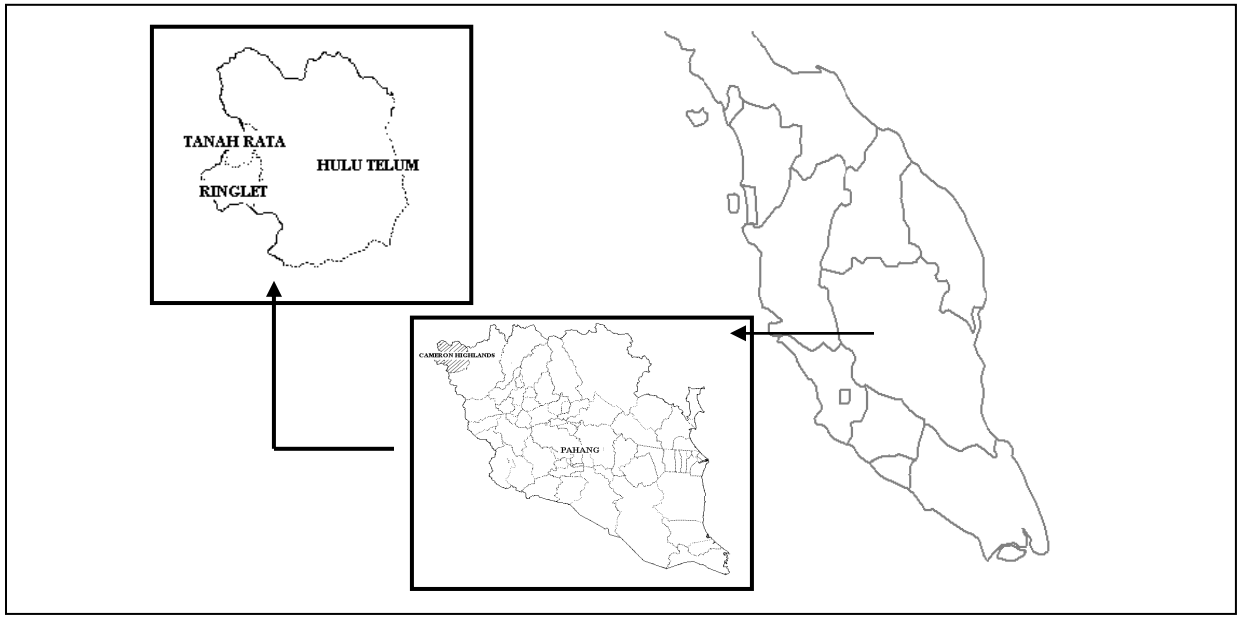

Figure 1. Location of Cameron Highlands

The land developments (housing, shop lots, hotels and etc.) within the urban areas and others constructions activities were identified as the second largest sediment contributor to the existing river in Cameron Highland. Table 1 shows the ranking of activities which significantly generated the sediment loading to the existing river systems in Cameron Highland (Tew, 2003). Next is followed by infrastructure like road construction, water supply pipeline contracts and etc. These activities have reduced the once ample forest reserve in the Cameron Highlands areas. Out of the three river systems, Sg Telom and Sg Bertam are the one receives intensively developed for agriculture and urbanization. The sediments within this catchment will directly enter the Ringlet Reservoir. Figure 2 indicates that the sediment accumulated inside the reservoir started to increase slightly between 1966 and 1967, decrease between 1968 and 1969, increase again between 1970 and 1975, decrease again between 1976 and 1981 and increase substantially 1982 onwards.

Table 1: The lists of activities, which identified as the major sediment contributor to the existing rivers in Cameron Highland

Activities	Estimated Soil Loss	
	Tons/yr	Rank
Agriculture	218,150	1
Mixed Residential	14,260	2
Road	10,642	3
Private Bungalows	10,117	4
Water Body/River	8,887	5
Govt. Institution/Quarters	7,945	6
Apartment/Hotel	5,070	7
Forest	2,573	8
Commercial	1,597	9
Golf/Recreational	1,381	10
Orang Asli Settlement	941	11

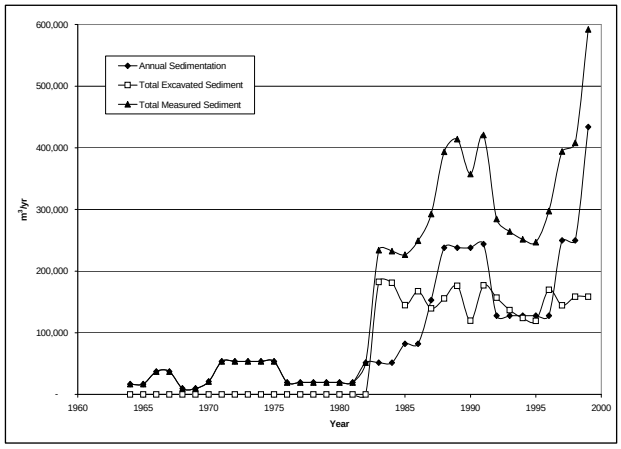

Figure 2: Ringlet reservoir sedimentation

3. Model Development

During the hydrographic survey, it was found that Sg Telom and Sg Bertam are one of the major contributions of sediment yield to Ringlet dam. Therefore, for sediment transport modeling, this study only engaged both rivers. In this study, InfoWork RS, a one-dimensional hydrodynamic simulation program developed by the Wellingford, UK was utilised to model sediment transport in Sg Telom and Sg Bertam. The software simulates one-dimensional channel flow by solving the fully dynamic de Saint-Venant equations, which define the conservations of mass and momentum. The computational grids are created with alternating *Q* (discharge) and *h* (water level) points. The *h* points are created at the location where cross sectional data are available, and Q points are generated automatically in between the *h* points. The InfoWork RS provides an option where bed resistance (Manning's *n*) can be calculated as a function of hydraulic parameters such as water depth, hydraulic radius, and flow velocity. To initiate the process of modeling all the identified streams of the study area, the following information and data is compiled and analysed:

- Details of the main catchments of Sg. Telom and Sg Bertam (including data from hydrographic survey and riverbed sediment- these samples were dried, weighed and then run through sieves of various sizes.).
- Physical characteristics of the rivers, such as cross-sectional levels and dimensions, longitudinal bed profiles and slopes.
- Meteorological and hydrological data, such as rainfall, for Cameron Highlands and surrounding areas.

A detailed chart for modeling process is shown in Figure 3.

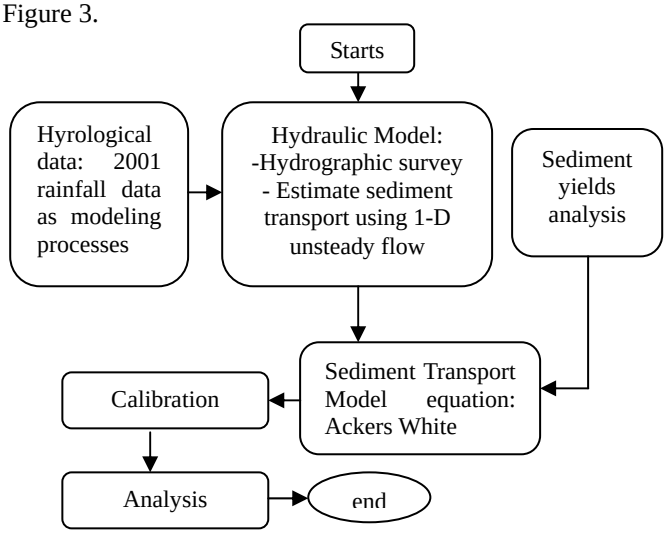

Figure 3: Modeling flow chart

4. Results and Discussion

There are numbers of research findings show that, the impact of a particular land use activity on the increase of sediment yields. These studies confirmed that the sediment loads of many rivers may have increased by an order of magnitude or more as a result of cultural land use changes within the watershed. Table 2 shows the rate of sediments entering the river for each catchment. Based on the current output, Upper Telom catchment was found to have very high degree of erosion estimated at almost 140 tonne/ha/yr. Due to the fact that almost all the sediments produced in this catchment flow into Ringlet Reservoir, Upper Telom only contributed as much as 450,000 m^3 of silts into Ringlet in 2003. Due to this sediment reduction, Sungai Telom has received almost 380,000 m^3 of sediments in the same year.

For the case of Sungai Bertam, the existence of Ringlet Reservoir has stopped the sediments from flowing downstream. The production of sediments from Plau'ur (very small catchment flowing into Sungai Nenggiri, Kelantan), Upper Telom and Upper Bertam catchments have reached almost 600,000 m^3. The remaining sediment flow into Sungai Bertam generated from Middle and Lower Bertam catchments have been estimated to be 280,000 m^3.

Table 2: Rate of sediment entering river system

Sub-catchment	Catchment area (km^2)	Soil loss (m^3/yr)	Soil loss (m^3/km^2/yr)	Estimated sediment rate (m^3/km^2/yr)
Upper Telom	100.33	529, 712	5, 279.70	3, 827.78
Lower Telom	194.23	616, 094	3, 171.98	2, 299.69
Upper Bertam	78.58	304, 847	3, 879.45	2, 812.60
Moddle Bertam	101	328, 130	3, 248.81	2, 355.39
Lower Bertam	94.33	58, 626	621.50	450.59

The riverbed analysis indicates that the materials in the upstream of the Sg Bertam are gravels of granite or metasediment and few sand and silt. Meanwhile, the riverbed materials of the Sg Telom and its tributaries are mainly boulders of granite and minor metasedimentary rocks, sand and silt. The size of the boulders varies from few centimeters to few ten centimeters. The results of particle size distribution for both river systems are illustrated in Table 3.

To provide suitable sediment concentration profile in the model, an assumption was made that low flow has to be generated from the ground water (base flow), which could transport less sediment. Some trials were made and sediment concentration in Table 4 below was used in the calibration process. Depending on the amount of flow, sediment concentration rises as the flow capacity rises.

Table 3: Median particle sizes of sampled river bed material

River	Reach	Slope	Sampling Location	d_{50} (mm)	average d_{50} (mm)
Sg. Telom	T1	> 10%	14	2.64	2.64
	T2	7 - 10%	15	1.06	1.06
	T3	4 - 7%	28	2.64	2.64
	T4	2 - 4%	27	1.50	1.50
	T5	< 2%	25	1.41	1.41
Sg. Bertam	B1	> 10%	34	0.52	0.48
			41	0.44	
	B2	< 2%	37	1.54	1.65
			39	1.77	

Table 4: Sediment concentration vs flow

Flow (m3/s)	Sediment concentration mg/l
1	10
2	20
3	50
4	100
5	200
6	500
7	1500
8	3000
10	6000
15	10000
25	16000
35	22000
60	35000

The simulation process was carried out for a period of 10 month. Figures 4 and 5 below show the result of the bed level changed at upstream and downstream sites of the Ringlet reservoir while the subsequent figures (Figures 6-9) illustrate bed level and flow in different scenarios.

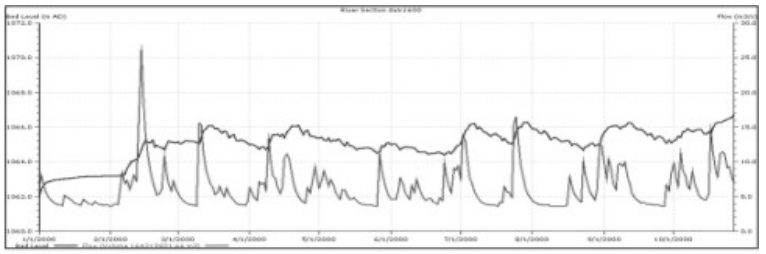

Figure 4: Bed level change at upstream of the reservoir

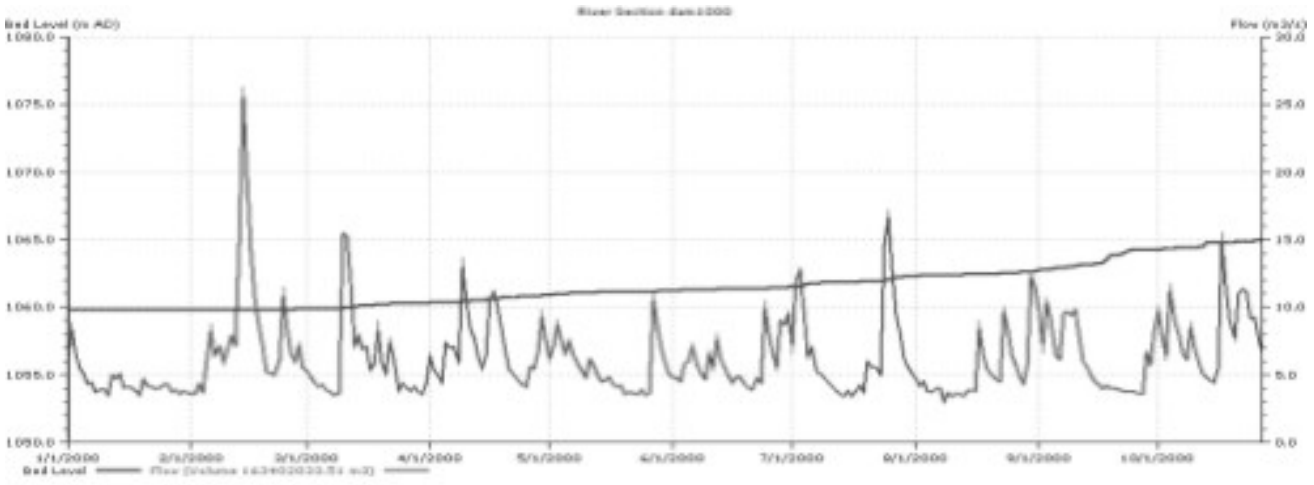

Figure 5: Bed level change further downstream of the reservoir

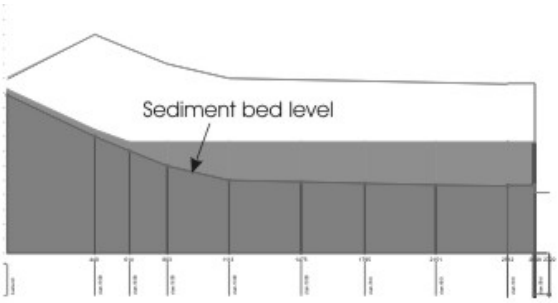

Figure 7: Simulation start

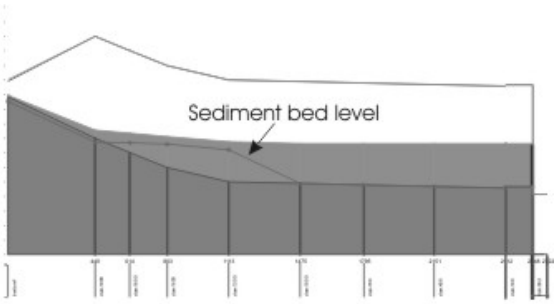

Figure 8: After 2 month

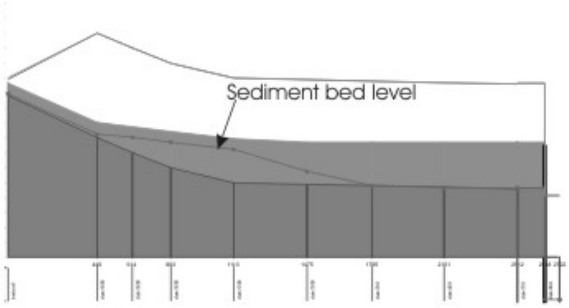

Figure 9: After 7 months

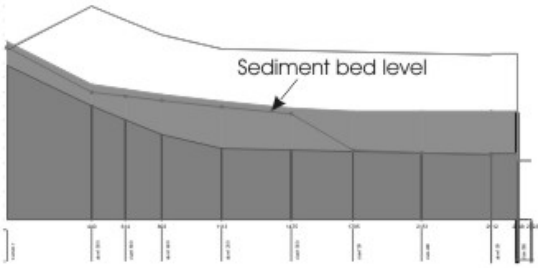

Figure 10: After 10 months

Total volume of sediment accumulated in the dam is equivalent to 530000 m^3/year. This value is about 70% from the estimation obtained using the USLE model, which was 800,000 m^3/yr (Adroit, 2006). The reasons for these discrepancies may be deduced to the particles size utilized in the model itself. The particle size distribution obtained from sample 11 (The utilized bed material was taken from sample 11, which originates near the dam site) fell short from indicating the profile of silts and clay i.e. sediments size less than 0.0625mm. With the current sediment profile (sample 11), the transport model would under estimated the amount of sediments accumulated inside the reservoir. In reality, with smaller silt and clay inserted into the model, the contribution of accumulated sediments inside the reservoir would increase substantially.

With these results, the calibration process for the current exercise should be considered as successful. In the future exercise, further analysis can be carried out for other scenarios especially within the sub-catchments.

5. Conclusion

Rapid developments such as agriculture, urbanisation, infrastructure development, and deforestation in Cameron Highlands have contributed to severe upland soil erosions and sedimentation in the river. These activities have led to tremendous pressure to the existing river system and water courses. For years, during heavy rainfall, rivers in Cameron Highlands have to accommodate the high rate of eroded sediments coming from these sources. Poor sediment control has resulted in the filling up of the Ringlet Reservoir just after 30 years of it's commissioning in the 1960's. This is very much less than the expected design life span of the storage capacity of such structure, which is normally designed to accommodate for sedimentation between 50 to 100 years. In fact, the Ringlet Reservoir was designed with a targeted life-span of 80 years. In environmental point of view, the agricultural activities and construction activities within the Cameron Highlands Rivers basin are known to be significantly impacting on the natural environment as a consequence of increasing runoff peak, accelerated sedimentation, the removal of vegetation filters and increased pollutant loading of nutrients into waterways. The major settlement areas and rapid land development within that areas, such as at Blue Valley, Kg Raja, Brinchang, Tanah Rata, Ringlet and Bertam Valley has also contributed significantly to the sediment loading into the existing rivers system. This has led to severe localised flood as a result of shallow bed level of the major rivers.

Acknowledgements

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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(2):74-81]. (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).

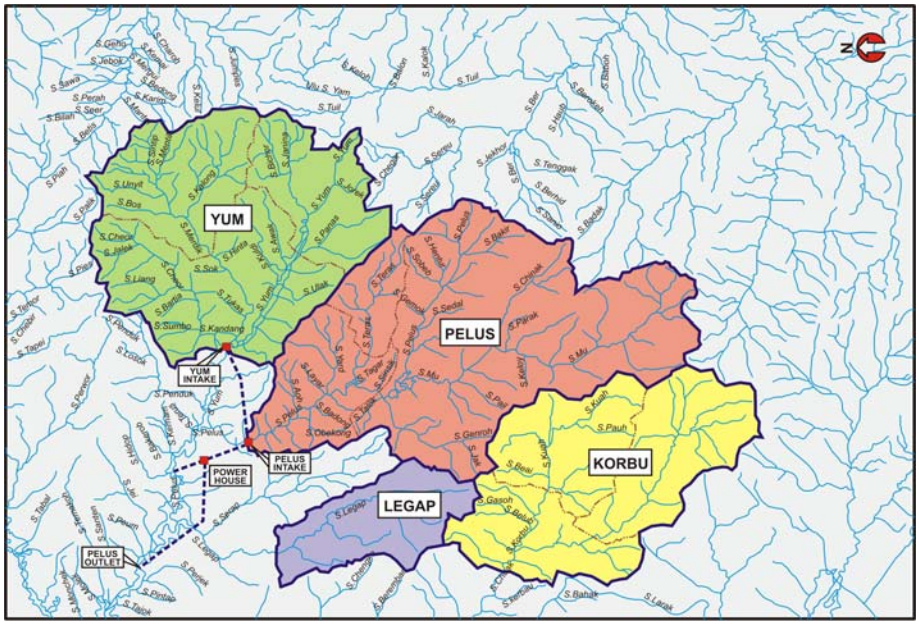

Figure 1: Pelus, Yum, Korbu and Legap Sub-Catchments

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).

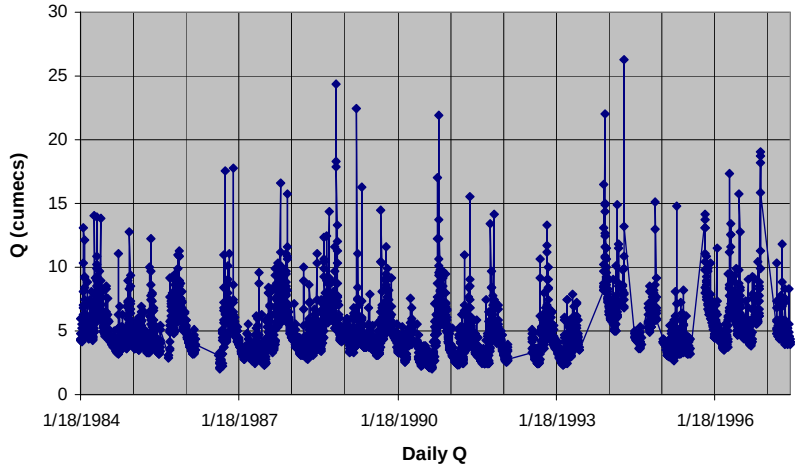

Figure 2: Daily Flow at Station 6044 (Sg. Yum at Kuala Yum)

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).

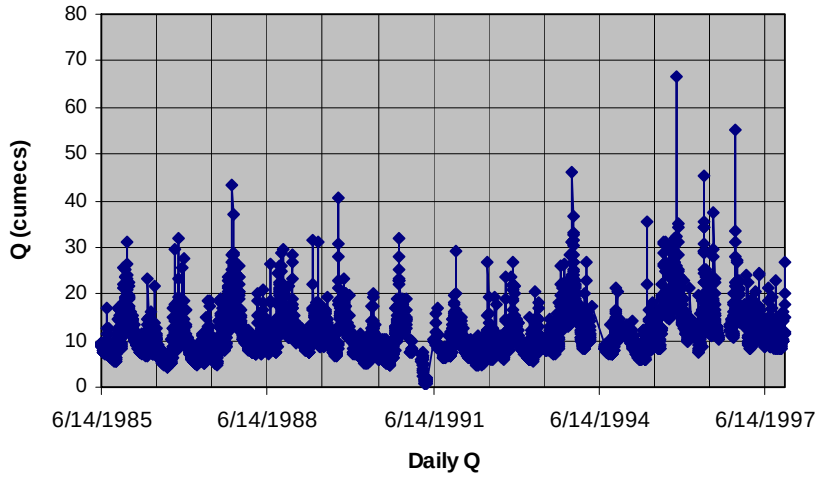

Figure 3: Daily flow at Station 6035 (Sg. Pelus below Kuala Yum)

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.

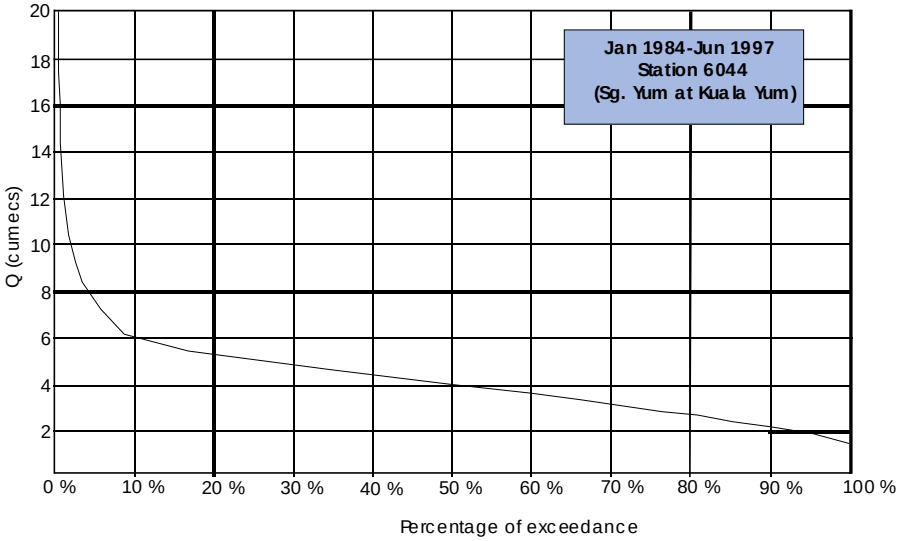

Figure 4: Flow Duration Curve for Sg Yum at Kuala Yum

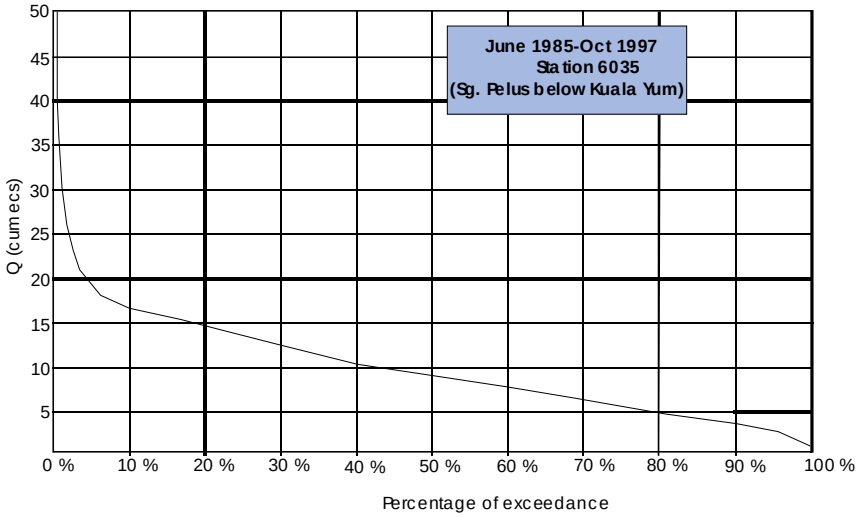

Figure 5: Flow Duration Curve at Sg Pelus below Kuala Yum

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.

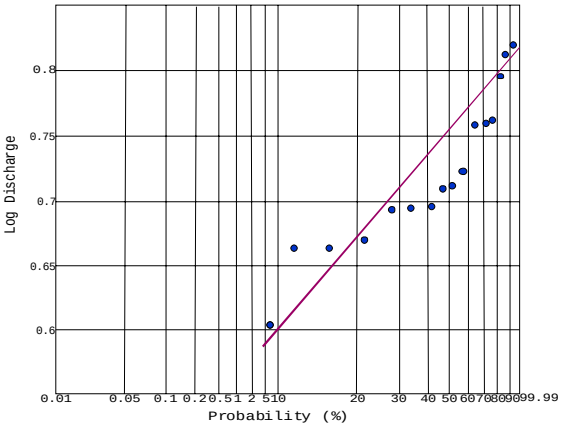

Figure 6: 7-Day Low Flow Frequency Curve

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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Growth inhibitory effects on microorganisms by a D-galactose-binding lectin purified from the sea hare (*Aplysia kurodai*) eggs: An *in vitro* study

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Abstract: A D-galactose specific lectin purified from the eggs of sea hare, *Aplysia kurodai* (AKL) by lactosyl-agarose affinity chromatography has been evaluated for screening of antimicrobial activities. AKL was disulfide bonded dimeric lectin consisted of two 32 kDa polypeptides. This lectin has significant hemagglutinting activity against trypsinized rabbit and human erythrocytes and it was inhibited by galactose and galacturonic acid. AKL has been screened for *in vitro* both antibacterial activity against eleven human pathogenic bacteria and antifungal activity against six phytopathogenic fungi. Antimicrobial evaluation of standard antibiotics, ampicillin and nystatin were used as comparative study. AKL significantly inhibited the growth of gram-positive bacteria. *Staphylococcus aureus* (12 mm) and *Bacillus megaterium* (11 mm) were exhibited the highest zone of inhibition by the addition of the lectin (250 µg/disc). However, AKL did not inhibit the growth of gram-negative bacteria as *Escherichia coli*. On the other hand, AKL (100 µg/mL) has also inhibited the mycelial growth of *Curvularia lunata* (21.53%). These antimicrobial activities by the lectin will provide an effective defense ability of the sea hare eggs against invading microbes. [Nature and Science 2010;8(2):82-89]. (ISSN: 1545-0740).

Key words: *Aplysia kurodai*, lectin, organisms, mycelial growth, SDS-popyacrylamide gel electrophoresis

1. Introduction

Lectins are multivalent carbohydrate-binding proteins that are widely distributed in various organisms from microorganisms to higher vertebrates. The chemical properties of animal lectins, such as the sugar specificity, divalent ion requirement and structure of carbohydrate binding domains, provide the classification of these lectins into several families. By virtue of their sugar-binding property, they are useful candidates for detection of cell-surface carbohydrates (Yu et al., 2001), biomedical applications (Pryme et al., 2002), and purification of glycoconjugates (Yamamoto et al., 1984). Physiologically, animal lectins have been postulated to perform important roles in various endogenous biological processes including self defense, cell-cell recognition, sugar transportation, development, biomineralization, immunity as antibacterials and others (Dong et al., 2004; Kamiya et al., 2002; Kilpatrick, 2002, Suzuki et al., 2003, Iijima et al., 2003). Many animal lectins with various carbohydrate-binding specificities have been discovered from eggs or reproductive organs (Ozeki et al., 1991; Hosono et al., 1999), the carbohydrate recognition mechanisms are seemed to be important for the biological events as fertilization or their early development. In phylum Mollusca, some lectins with anitibacterial, opsonizing, and cytotoxic activities were found from their organs (Banerjee et al., 2004; Melo et al., 2000).

Sea hares of the species *Aplysia kurodai* belong to the subclass Opisthobranchia of the mollusca. They lay yellow eggs in gelatinous strings in their spawning season (May-June). Sea hare species have attracted the interest of many workers investigating the chemical compounds secreted by the purple gland or present in different tissues, possibly involved in the defense of these invertebrates. Thus, some sea hare species have been shown to contain low molecular mass substances with antimicrobial (Ichida & Higa, 1986) and antitumor activities (Usami et al., 2008). From the species, a 70 kDa hexameric galacturonic acid-biniding lectin consisting of 13 kDa subunits has been isolated from eggs using galacturonic acid-conjugated Sepharose gel (Kamiya & Shimizu, 1981) and demonstrated potent agglutinins in extracts of *A. kurodai* egg masses which could agglutinate mammalian erythrocytes and marine bacteria. Two 28 kDa and 26 kDa D-galactose binding

lectins with cell attachment potency against human sarcoma cells were purified from the mantle (Ozeki, 1998). Very recently, two β-1,3-glucanases were purified from the digestive fluid (Kumagai & Ojima, 2010) of *A. kurodai*. *Aplysia* gonad lectin (AGL) has been purified a galactophylic lectin from gonad of *Aplysia depilans* (Gilboa-Garber et al., 1985) as first, appearing antibacterial activity and cytotoxity for carcinoma cells (Zipris et al., 1986). However, *Aplysia juliana* showed the antibacterial and antineoplastic activity against gram-positive bacteria (Kamiya et al., 1989).

Especially, eggs of sea hare are kept in tide pool until hatch, they need to prevent enemies biochemically. Sometime, lectins and toxins are closely related as shown as a galactose-binding lectin, RCA 120 presents together with harmful toxin Ricin in the beans and many lectins present in snake venom. As seen in bullfrog eggs, a sialic acid-binding lectin (SBL) has activity as ribonuclease and apoptotic activity against mouse lymphoma cells P388 (Nitta et al., 1994). Sea hare eggs may possess antibiotic factors, as the egg masses appear to be free of bacteria. Egg mass of sea hare exhibited the antibacterial activity and suggested that antibacterial factors were produced in the albumen gland, such that each egg was coated with antibacterially active albumen before passing down the oviduct (Kamiya et al., 1984). Aplysianin-A with 320 kDa glycoprotein has been purified from the albumen gland of a sea hare, *Aplysia kurodai* and it showed 50% inhibition growth of *Bacillus subtilis* gram-positive bacteria (Kamiya et al., 1986). Also plant pathogens, like animal ones, use protein-carbohydrate interactions in their strategy for host recognition, attachment and invasion (Kostlanova et al., 2005).

We previously evaluated the affect of the lectin from *Aplysia kurodai* eggs for cell proliferation of lymphoma cells and determined the kinetics against the glycoprotein recognized by the lectin using surface plasmon resonance (Kawsar et al., 2009). This study aimed to evaluate the antibacterial and antifungal activity of the AKL purified from the sea hare *Aplysia kurodai* eggs against some human and phytopathogens.

2. Materials and Methods

2.1. Reagents

Lactosyl-agarose was purchased from Seikagaku Kogyo Co. Ltd., Japan. A standard protein marker mixture (Daiichi-III) for SDS-PAGE was purchased from Daiichi Pure Chem. Co. Ltd., Japan. Bicinchoninic acid (BCA) kit was purchased from Pierce Co. Ltd., USA. Agar, dextrose, peptone, beef extract were purchased from Merck Ltd., India and BDH Ltd., Bangladesh.

2.2. Animals

Sea hare *Aplysia kurodai* and its eggs were collected in the tidal zone at the Zushi coast, Kanagawa prefecture, Japan from spring to summer season. Eggs and animals were stored at -80^0C or used after collection according to the situation.

2.3. Purification of *A. kurodai* lectin (AKL)

A galactose-binding lectin was purified from the sea hare *Aplysia kurodai* (AKL) as previously reported (Kawsar *et al.,* 2009). In brief, two hundred grams freezed sea hare, *A. kurodai* eggs as yellow string noodles was crushed into particles in a mortor, then mixed it with 10 volumes (w/v) of Tris-buffered saline (TBS) (10 mM Tris(hydroxymethyl)aminomethane-HCl, pH 7.4, containing 150 mM NaCl) containing 10 mM of a protease inhibitor mixture. The homogenates were centrifuged at 14,720 *g* in 500-ml centrifuge bottles for 1 h at 4°C with a Suprema 21 centrifuge equipped with an NA-18HS rotor. The supernatant was centrifuged again at 27,500 *g* for 1 h at 4°C for two times and was applied to a lactosyl-agarose affinity column that was fitted with a Sephadex G-75 pre-column. After application of the extracts, the column was washed extensively with TBS. The lectin was eluted with 50 mM lactose in TBS and each 1 mL of elution was collected in tubes with a fraction collector. Each chromatography step during washing and elution was monitored using a UV monitor by the measurement of the absorbance at 280 nm. The eluted fractions as identified by UV spectrophotometer at 280 nm were combined, and dialyzed against 1,000 times volumes of TBS to remove free from sugar.

2.4. Hemagglutinating activity

Hemagglutinating activity was performed using 1% (w/v) trypsinized and 0.25% glutaraldehyde-fixed rabbit and human erythrocytes as described previously (Matsui, 1984). Erythrocytes were suspended at a concentration of 1% (w/v) in TBS. In the general assay, 20 μL each of TBS, TBS containing 1% Triton X-100, and erythrocytes were added to 20 μL of the two times-serially-diluted lectin with TBS in 96 well

V-shape titer plates for 1 h. The hemagglutination activity of the lectin was expressed as the titer defined as the reciprocal of the highest dilution giving positive hemagglutination. To determine the sugar binding specificity of the lectin, 20 µL of each of the sugar (200 mM) and the glycoprotein (5 mg/mL) was serially diluted with TBS and added to lectin with the titer of 16, 1% Triton X-100, and erythrocytes in 96 well V-shape titer plates for 1 h incubation. The minimum inhibitory sugar concentration against the lectin was expressed as negative activity.

2.5. SDS-polyacrylamide gel electrophoresis and molecular mass

The molecular mass of the polypeptide was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Purified lectin was mixed with an equal amount of sample buffer (20 mM Tris-HCl, pH 6.8; 0.2% SDS, and 20% glycerol) and then heated at 70^0C for 15 min. Aliquots of 30 µL were applied to the well of a mini-slab gel (gel size: 80 mm × 100 mm with 1 mm thickness; 12% and 5% polyacrylamide were used in separation and upper gels, respectively, constant current at 30 mA for 1 h) according to a previous report (Laemmli, 1970). The following polypeptides were used as molecular mass markers; phosphorylase b (M_r 94 kDa), bovine serum albumin (M_r 66 kDa), ovalbumin (M_r 42 kDa), carbonic anhydrase (M_r 30 kDa), trypsin inhibitor (M_r 20 kDa), and lysozyme (M_r 14 kDa). After SDS-PAGE, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue (CBB) R-250 in 40% (v/v) and 10% acetic acid (v/v) followed by discoloration by excessive staining with 40% methanol and 10% acetic acid.

2.6. Protein determination and sugar content

Protein concentrations were determined using BCA protein assay kit (Smith et al*.,* 1985 and Wiechelman et al., 1988) with bovine serum albumin as the standard by measuring absorbance at 562 nm with spectrophotometer ND-1000 (Nano Drop Tech. Inc., USA). The carbohydrate content of the purified lectin was determined by the phenol-sulfuric acid method (Dubios et al*.,* 1956), using D-glucose as the standard by measuring absorbance at 340 nm.

2.7. Strains

The bacterial and fungal strains used in this study were obtained from the Microbiology Laboratory, Department of Microbiology, University of Chittagong, Bangladesh. Gram-positive bacterial strains were *Bacillus subtilis* BTCC 17, *Bacillus cereus* BTCC 19, *Bacillus megaterium* BTCC 18 and *Staphylococcus aureus* ATCC 6538 and Gram-negative bacterial strains were *Salmonella typhi* AE 14612, *Salmonella paratyphi* AE 146313, *Shigella dysenteriae* AE 14396, *Shigella sonnei* CRL (ICDDR,B), *Escherichia coli* ATCC 25922, *Vibrio cholerae* (CRL (ICDDR,B) and *Pseudomonas sp.* CRL (ICDDR,B). The fungal pathogens were *Alternaria alternata* (Fr.) Kedissler, *Botryodiplodia theobromae* Pat., *Curvularia lunata* (Wakker) Boedijin, *Colletotrichum corcori* Ikata (Yoshida), *Fusarium equiseti* (Corda) Sacc., and *Macrophomina phaseolina* (Tassi) Goid.

2.8. Medium and culture

Standard NA (Nutrient Agar) medium was used for growing bacterial strains throughout the work. A 20 g of agar powder, 5 g of peptone, 3 g of beef extract and 0.5 g of NaCl was added slowly to 1000 mL water and the solution was mixed thoroughly with a glass rod. After 10 minutes of boiling, the medium was transferred into 500 mL conical flask and flask was closed with a cotton plug. The medium was autoclaved for 15 minutes at 121^0C and 15 psi and ready to use bacterial culture. Older cultures were transferred to freshly prepared NA slants separately for each species via sterilized bacterial loop. In such a way, four test tubes were freshly prepared for each bacterial pathogen. These test tubes of inoculated slants were incubated at $(35\pm2)^0$C in incubator for 18-24 hours and each culture was used throughout for antibacterial screening studies. For preservation of the stock culture, one set of culture slants were kept in polythene bag, properly tied and preserved at 10^0C.

2.9. Antibacterial assay

The *in vitro* sensitivity of the bacteria to the test purified lectin was done by disc diffusion method (Bauer *et al*.1996). In this method sterilized paper discs of 4 mm in diameter and petridishes of 150 mm in diameter were used throughout the experiment. The autoclaved Mueller-Hinton agar medium, cooled to 45^0C, was poured into sterilized petridishes to a depth of 3 to 4 mm and after solidification of the agar medium, the plates were transferred to an incubator at 37^0C for 15 to 20 minutes to dry off the moisture that develops on the agar surface. The plates were inoculated with the

standard bacterial suspensions (as of McFarland 0.5 standard) by help of sterilized glass and allowed to dry for three to five minutes. Dried and sterilized filter paper discs were treated separately with 20µL (250 µg/disc) from 5% phosphate buffered saline (PBS) solution of lectin using a micropipette, dried in air under aseptic condition and were placed at equidistance in a circle on the seeded plate. A control plate was also maintained in each case without any test material. These plates were kept for 4-6 hours at low temperature and the test materials diffused from disc to the surrounding medium by this time. The plates were then incubated at 35 ± 2^0C for 24 hours to allow maximum growth of the organisms. The antibacterial activity of the test agent was determined by measuring the mean diameter of zone of inhibitions in millimeter. Each experiment was repeated thrice. Lactose was used as negative control. All the results were compared with the standard antibacterial antibiotic ampicillin [20 µg/disc, BEXIMCO Pharma Bangladesh Ltd.].

2.10. Antifungal activity

The *in vitro* antifungal activity of the purified lectin was determined by the poisoned food technique (Grover & Moore, 1962; Miah et al., 1990). Potato dextrose agar (PDA) medium was used for the culture of fungi. A required amount of PDA was taken in conical flasks separately and was sterilized by autoclave (121^0C, 15 psi) for 15 minutes. Purified lectin (in PBS solution) was mixed with sterilized melted PDA medium to have 100 µg/mL PDA and this was poured (about 20 mL/plate) in sterilized petridishes. At the center of each plate, 5 days old fungal mycelial block (4 mm in diameter) was inoculated and incubated at 27^0C. A control set was also maintained in each experiment. Linear mycelial growth of fungus was measured after 3-5 days of incubation in triplicate. The average of two measurements was taken as mycelial colony diameter of the fungus in mm. All the antifungal results were compared with the standard antifungal antibiotic Nystatin (100 µg/mL PDA). Lactose was used as negative control. The percentage inhibition of radial mycelial growth of the test fungus was calculated as follows:

$$\% \text{ Inhibition} = (C\text{-}T/C) \times 100$$

Where, C = diameter of the fungal colony in the control petridish and T = diameter of the fungal colony in the treated petridish.

3. Results and Discussion

Sea hare, *Aplysia kurodai* eggs of crude extraction showed strong hemagglutination activity against human and rabbit red blood cells (Table 1) and the activity were cancelled by the presence of saccharides such as galactose and lactose. *A. kurodai* eggs lectin was purified on a lactosyl-agarose column via elution with 50 mM lactose containing TBS (Figure 1). It was shown to be a single polypeptide with molecular masses 56 and 32 kDa under non-reducing (NR) and reducing (R) conditions by SDS-PAGE, respectively (Figure 2). On the other hand, crude extracts of eggs contained various proteins by SDS-PAGE (Figure 2), indicated that the AKL was present as a disulfide-bounded dimeric protein consisting of two 32 kDa polypeptide subunits.

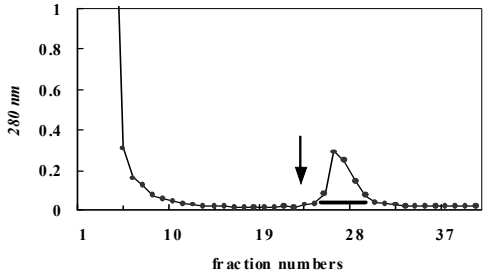

Figure 1. Affinity purification of AKL. Crude extract of *A. kurodai* was applied to a lactosyl-agarose column equilibrated with TBS. The column was washed with TBS and eluted with TBS containing 50 mM lactose (arrow).

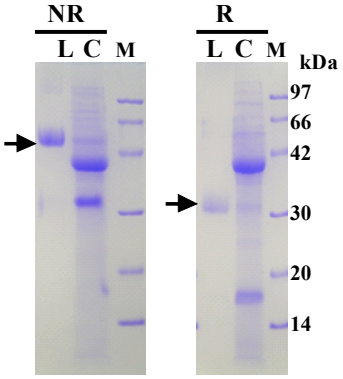

Figure 2. SDS-PAGE pattern of AKL. Purified lectin (L), (10 µg) and crude extract (C), (10 µg) were subjected to SDS-PAGE under non-reducing (NR) and reducing (R) conditions. Standard marker proteins (M) were used as follows, phosphorylase b (97 kDa); bovine serum albumin (66 kDa); ovalbumin (42 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20 kDa); and lysozyme (14 kDa).

Table 1. Hemagglutination of different erythrocytes by AKL

Erythrocytes*	Titer (HU)
Rabbit	2048
Human Type-A	2048
Human Type-B	1024
Human Type-O	1024

Note: *Trypsinized and glutaraldehyde fixed erythrocytes were used

AKL had so similar physicochemical properties with another hexameric 13 kDa lectin (Kamiya & Shimizu, 1981) purified from same species eggs on the affinity to galacturonic acid and the independence from divalent cations for its activity. This result indicated that eggs of *A. kuroai* contain multiple lectins. AKL strongly recognizes both α-galactosides as melibiose (Galα1-6Glc), methyl α-D-galactopyranoside and β-galactosides as lactose (Galβ1-4Glc), *N*-acetyllactosamine (Galβ1-4GlcNAc), methyl β-D-galactopyranoside and fetuin. *N*-acetyl neuraminic acid (Neu5Ac) was not recognized by the lectin since fetuin did not inhibit hemagglutinating activity of AKL and the terminal D-galactose of saccharides was an important for the binding with AKL. From these results, AKL has been characterized as a D-galactose-binding or galactophilic lectin as same as AGL purified from gonad of *Aplysia depilans* (Gilboa-Garber et al., 1985). AKL was suggested to be a glycoprotein containing saccharides as 6-14% molar ratio depending on the sample lots of the lectin detected by the phenol-sulfuric acid method.

Sea hares of *Aplysia* species have been reported to certain some biological active substances, including antibacterial factors (Iijima et al., 2003), toxins (Yamada et al., 2009) and chemical defense substances (Kamiya et al., 2006). Most of these substances are low molecular weight compounds derived from the algae on which the sea hares feed. However, no bioactive high molecular substances except agglutinin have previously been identified in sea hares (Kamiya and Shimizu, 1981). Here our purified lectin was subjected to screening for *in vitro* antibacterial inhibition growth by disc diffusion method (Bauer et al., 1996) against eleven pathogenic bacteria and compared to that of antibacterial antibiotic, ampicilin. AKL (250 µg/disc) exhibited a strong antibacterial activity on the gram-positive bacteria, as *Staphylococcus aureus* and *Bacillus megaterium*. The diameter of zone inhibition by the addition of AKL was significant effective for *Staphylococcus aureus* and *Bacillus megeterium* to be 12 and 11 mm, respectively (Table 2). This result is very similar with antitumor glycoprotein aplysianin E (250 kDa), in the eggs of *A. kurodai* which is inhibited the growth of *Staphylococcus aureus* bacteria (Yamazaki, 1993) and also sea hare, *Dolabella auricularia* (Iijima et al., 2003). However, the lectin has inhibited less effect for *Bacillus cereus* and *Bacillus subtilis.*

Table 2. Antibacterial activity of AKL against gram-positive bacteria

Name of bacteria	Diameter of zone of inhibition in milimeter	
	Lectin (250 µg/disc)	Ampicillin* (20 µg/disc)
Staphylococcus aureus	12±1	21±1
Bacillus megaterium	11±1	20±1
Bacillus cereus	6±1	16±1
Bacillus subtilis	4±1	18±1

Note: *Standard antibacterial antibiotic.

Table 3. Antibacterial activity of AKL against gram-negative bacteria

Name of bacteria	Diameter of zone of inhibition in milimeter	
	Lectin (250 µg/disc)	Ampicillin* (20 µg/disc)
Shigella dysenteriae	9±1	21±1
Salmonella typhi	5±1	19±1
Salmonella paratyphi	5±1	18±1
Shigella sonnei	4±1	20±1
Vibrio cholerae	4±1	15±1
Pseudomonas sp	4±1	14±1
Escherichia coli	0	19±1

Note: *Standard antibacterial antibiotic.

The antibacterial & antineoplastic activity showed against gram-positive bacteria of sea hare, *Aplysia juliana* (Kamiya et al., 1989). However, AKL did not inhibit well against all gram-negative bacteria was tested. *S. dysenteriae* exhibited little sensitivity by the lectin (Table 3), though the control antibiotic, ampicillin inhibited the growth against all gram-negative bacteria. Amongst the gram-positive and gram-negative bacteria, gram-positive bacteria were more effective to the lectin as compared to gram-negative bacteria.

Table 4. Antifungal activity of AKL purified from the sea hare, *A. Kurodai*

Name of fungi	Percentage inhibition of fungal mycelial growth	
	Lectin (100 µg/ml PDA)	Nystatin* (100 µg/ml PDA)
Curvularia lunata	21.53±1	67.86±1
Botryodiplodia theobromae	18.72±1	58.39±1
Macrophomina phaseolina	11.67±1	66.46±1
Alternaria alternata	10.56±1	49.78±1
Colletotrichum corchori	9.63±1	40.57±1
Fusarium equiseti	4.38±1	36.63±1

Note: *Standard antifungal antibiotic.

On the other hand, Aplysianin-A, an antibacterial glycoprotein in the albumen gland of the sea hare *Aplysia kurodai*, inhibited the growth of both gram-positive and gram-negative bacteria (Takamatsu et al., 1995). The growth of inhibition may cause to the presence of H_2O_2 which killed bacteria or halts bacterial growth, for example antibacterial protein aplysianin A (ApA) purified from albumen gland of *A. kurodai* (Jimbo et al., 2003). Recently, a β-galactoside binding pearl shell lectin purified from marine bivalve, *Pteria penguin* (Naganuma et al., 2006) had shown the similar antibacterial activity with AKL, as it effectively inhibited the growth against both gram-positive and gram-negative bacteria. Also rhamnose-binding steelhead trout (*Oncorhynchus mykiss*) eggs lectin inhibited the growth of gram-positive and gram-negative bacteria by recognizing lipopolysaccharide or lipoteichoic acid (Tateno et al., 2002) as same as AKL.

Antifungal activity by AKL was determined against six phytopathogenic fungi with antifungal antibiotic nystatin as positive control. Generally, the lectin had less inhibited the growth of fungal than the case of bacteria. AKL (100 µg/mL in PDA medium) exhibited significant inhibition of mycelial growth against *Curvularia lunata* (21.53%) among all tested fungi. AKL also showed good inhibition of mycelial growth against *Botryodiplodia theobromae* (18.72%) (Table 4). On the other hand, the mycelial growth of *Alternaria alternata*, *Macrophomina phaseolina* and *Colletotrichum corchori* (11.67-9.63%) was moderately inhibited by the lectin. However, the growth of *Fusarium equiseti* was least inhibited by AKL, though the growth of all the six fungi was totally inhibited by antifungal antibiotic Nystatin (100 µg/mL PDA). Although the growth of inhibition effect by AKL against fungi was not strong, some other galactose-, mannose- or fucose-binding lectins have reported (Broekaert et al., 1998) and also sea hare showed antifungal activity (Iijima et al., 1995).

Aplysia kurodai eggs contain a large quantity of AKL but its physiological function is unknown yet. But the egg mass of a sea hare showed antibacterial activity and that the catalytic activity of the eggs decreases during hatching (Kamiya et al., 1984). The presence of the lectins in the eggs of *A. kurodai* led us to consider its possible biological involvement in the defense mechanisms of the species. The wide distribution of lectin with antimicrobial property in the animal kingdom indicates that this lectin has been well conserved during evolution, which is understandable because animals cannot survive unless they can eliminate invading bacteria.

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Urban land use classification and functional zoning of Ulaanbaatar city, Mongolia

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Abstract: As Mongolia moves to a market economy and begins to encourage land ownership and the creation of private land and housing markets, it will need to revise its approach to the spatial planning and control of land uses and development by individuals and businesses. Designed for a regime of exclusive state ownership of land, the existing "target use" restrictions for individual parcels as shown in the detailed Master Plans hinders the exercise of market-driven choices by individuals and businesses wishing to put land and infrastructure to their most productive uses. [Nature and Science 2010;8(2):90-97]. (ISSN: 1545-0740)

Keywords: Urban land use classification, land use zoning and regulation, functional zones

Introduction

Mongolia is no exception to this commonly experienced urbanization pattern. Mongolia experienced rapid urbanization in the second half of the 20th century, and this trend was accelerated during the transition from a centrally planned to a market-based economy. By 2000, the urban population was 1.4 million, equivalent to 58 percent of the national population of 2.4 million. As of 2005, 37.7 percent of the total population of the country resides in Ulaanbaatar region, which covers 0.3 percent of the territory of Mongolia. 94.1 percent of the region lives in Ulaanbaatar city and the remaining 5.9 percent inhabits satellite cities and villages. Since the year 2000, migration to Ulaanbaatar has increased dramatically, and presently, following the official number of registered inhabitants, almost half of the whole Mongolian population lives in the capital.

The main reason for this ongoing migration is the weak infrastructural situation in the rural areas of Mongolia, and the absence of income-alternatives to mobile livestock-keeping, which is the dominant from of livelihood in most of the rural areas of Mongolia. The loss of livestock during the natural hazards of the winters of 1999-2002 in combination with droughts in summer was a major factor which led to massive impoverishment in rural areas and to an increase of migration towards the big cities of the country. In addition to this primary factor, there are several reasons for the rapid population growth by migration. For example, there are the lack of governmental policies for urban management, the existence of many universities and colleges in Ulaanbaatar, etc.

If we admit that any urban and settled areas represent peculiar systems of economic, social, and spatial management that contain in themselves the process of more centralized density within smaller space in the whole of the society, then they form within definite frames of limitations functional and spatial structures with a net internal conformity and on the base of perfecting these structures. It appears natural that, the spatial structures are to be renewed and exposed to evolution pursuant to the spatial demand and requirements of social development. One of the basic issues of urban research study is the problem on how the urban complements, residential quarters, enterprises and plants, warehouses and storages, trade and services, schooling and cultural as well as administrative establishments be spatially located better so as to be ecologically, economically and socially efficient and to satisfy the demands of the sustainable development. As is seen, the internal urban functions in a spatial structure basically find their reflections in the urban land tenure structure. Accordingly, the internal harmony of functional and spatial structures is dependent on the historical specifics, development scope, directions and duties, location and geographic distribution environment of the given city or town. It is also evident from the experiences of countries where the market economy is advanced that it is closely connected with the urban planning and particularly with the urban land tenure or utilization planning, zoning and management policy (B.Chinbat. 2004. p 31).

During the times of the centrally planned economy, the master plan of the capital city development, as was mentioned above, has been elaborated based on

SNIP (Construction Standards and Rules) or by the other plans that were brought closer to the conditions of Mongolia. The development of the master plan of the capital city Ulaanbaatar at a time of centrally planned economy represented not only the urban construction and physical architectural plan, but there is a sufficient proof to consider that it was based on the capital city investment planning and constituted a document that had a legal power to plan and control the urban internal land utilization. The reason for such is that the prevailing majority of industrial plants, economic and infrastructure sites, residential and dwelling apartment quarters were concentrated in the capital city and they had been reflected in the general urban development plan accordingly. On the other hand, everything was based on such a system, where the whole land of the country constituted a common property of the entire people (state), the land had been distributed under the tenure of factories and economic establishments of socialist forms and features and the utilization rules and orders were established by the state power and the tenure had been controlled and inspected by the society via state organs (D. Jamts. 1981. p.48). Judging by this, the stipulations connected with the urban land tenure and distribution, in line with the master plan of the capital city development are witnessed to have been acting as a law on the urban land planning, regulation and control. For instance, the territory of the capital city would be divided into the following zoning in conformity to chapter 60 of section 2 of SNIP (SNIP.II-60-79.1980.p.12):

- Residential zone – it is indicated here that there should be dwelling regions and social centers (administrative, scientific, training and health, sports etc) being distributed and developed as well as the green area facilities of common designation.
- Industrial zone – industries and other manufacturing and production plants and enterprises and sites, related to them.
- Zone of warehouses and storages, common economies – storages, camps, bus parking etc.
- Outer transportation zone – here are the transportation facilities outside the residential zone (passenger and freight stations, airports, forwarding camps, technical services lots etc) location streets, roads and squares.
- Recreation and entertainment zone – entertainment, travel, tourism and sports camps, gardens.
- Other designated zone – this comprises the location and development of water basins, sanitary and conservation of green for health, places and facilities for special designation, agricultural land tenure and so on.

On the bases of this the functional zoning principles were implemented by way of diversifying the capital city into such definite zones as city center, residential area and the felt dwelling quarter and the industrial, storage and warehouses zones were separated from them by a protection stripe. In this connection the capital city construction space includes: 1. city central business district of compact character, centralized in the small ring region the political, administrative, municipal, diplomatic and international, as well as scientific, cultural, higher educational, banking and financial establishments; 2. a transit zone, oriented by the big ring borders which includes operation of offices, apartments, trade and services establishments; 3. outside these internal urban belts there is located the Residential zone with medium and high-rising apartment houses, mainly stretching forward to the west and east alongside the central axis of the city; 4. Industrial and warehousing zone, separated from other zones, located in the southern, western and eastern peripheral edges of the city by the green protection stripe; 5. in the outside of these zones there have been distinguished the development of felt dwelling quarter forming the periphery of the city. At a certain extent, it has created a kind of a Mono-centric urban internal structure.

The radical change of the political, social and economic systems of Mongolia since 1990s had caused a principal modification into the social understanding about the land. There were alterations into the policy, being pursued by the state and government of the country on the land. Not limited by this the role and significance of the land in the society had been changed basically (J. Narantsatsralt. 1998. p 91).

Since the irreversible transfer of the country into the market economy, the urban land use has turned to be based on the market relations and due to the reality that there have appeared no possibilities to regulate further on the land use by the old urban master plan, norms and standards, the course of the transition period the land use within the framework of the capital city has been left with no control and inspection. Regulation has been running in a chaotic competition manner up-to the present. Under these conditions the national specialists of Mongolia, for the first time in history, have attempted independently to work out the Master Plan of the capital city development until the year 2020 and had it approved, but despite this the Ulaanbaatar city land use plan and the land zoning system had not been

even drafted yet. The investment program is not definite, and dependent on these matters the new Master Plan has an abstract feature and represents a mere document of building-architectural physical planning only. In addition, the capital city land utilization Master Plan was projected in 2001 which had attempted to divide the territory of the capital within its margin into the following zones of land policy through legalizing them into the land tenure, regulation, conservation and protection:

- Urbanized and urbanization extension zone.
- Population social revival zone.
- Near-urban intensive Agricultural zone.
- Near-urban traditional or non-intensive Agricultural zone.
- Nature restriction zone
- Conservation of natural rare and picturesque landscapes zone.

The given plan has set forth the boundaries in lines and circles for the urbanized and urbanization zones and it has emphasized the necessity to restrict the chaotic urban outward extension and to increase the urban land use density, but the urban land use classification and the functional zoning issues were left aside considering that these would be regulated by the urban Master Plan. In any case, it is encouraging that such new proposals were reflected in the new Master Plan as to separate the small towns and settlements which are situated in uneven distances in the vicinity of Ulaanbaatar city by green belts and to restrict anew the land use within the green areas of the capital city and so on (B.Chinbat. 2004. p 33).

On the other hand, though such laws of Mongolia as the law on land (2002), on land payment (1997), on land ownership (2002), on urban planning (1998) were adopted and are being implemented duly, they miss basically such important stipulations as the urban land use classification and zoning and this witnesses that a legal environment for the urban land utilization has not been ensured yet.

Materials and Methods

1.It focuses on activities of collecting datas are required in determination of land structure, size and to define the land use purposes in obvious places. This types of research provide with information and gives clear responses to following issues:

- Land privatization, tenure, structure
- Changes in the urban internal structure
- Buildings number and main purpose
- Floor area ratio and building coverage ratio
- Zoning regulation
- Places in ecologically barriers

2. The common grounds, necessary for working out the Ulaanbaatar city functional zoning, were assembled through the comparison of the urban zoning systems of such countries as Japan, Korea and USA with the conditions of Mongolia. The common grounds were assessed and conclusions were drawn thereof.

3. The paper examined new types of land use, emerged from the traditional land uses as well as in the course of the transition period. Field surveys were conducted to estimate the modifications and alterations of the land use structure occurred in the land use structure by the area balance. Ulaanbaatar city is divided into 14 micro regions to represent the functional zones of the city

4. Remote sensing Data: Quick Bird image with a spatial resolution of 0.61 m, Toposheets (scale 1: 5 000), GIS software used Arcview 3.3

Establishment and main purposes of land use zones

For the purpose to identify the features of Ulaanbaatar city land use, we have separated and sorted the following land use zones within the framework of the developed areas of the capital city on the basis of the results of analysis conducted:

Residence zones

RE-1 Single family housing zone
RE-2 Low rise residential zone
RE-3 Middle rise residential zone
RE-4 High rise residential zone
RE-5 “Ger” zone

Main purpose: A Residence zone is to provide a healthy, safe and civil environment for the residents within each zone.

Commercial zones

CO-1 Central business zone
CO-2 Satellite business zone
CO-3 Neighbourhood commercial zone

Main purpose: A commercial zone is to provide adequate space in appropriate locations for retail, service, administrative and recreational development to meet the needs of the citizens.

Industrial zones

IN-1 Danger industrial zone
IN-2 Heavy industrial zone
IN-3 Light Industrial zone

Main purpose: Industrial zone is to provide for the orderly and appropriate growth of industry in the city

Suburban zones

SU-1 Farmer zone
SU-2 Traditional agricultural zone
SU-3 Natural conservation zone

Main purpose: A suburban zone is to provide a agricultural land use, safe and environment for the urban green zone.

Mixed use zones

MU-1 Semi residential zone
MU-2 Semi commercial zone
MU-3 Semi industrial zone

Main purpose: Mixed use zones is to encourage a diversity of compatible land uses that may include a mixture of residential, office, retail, recreational, light manufacturing and industrial and other miscellaneous uses. Development shall be guided by an approved public policy or plan and through the use of planned unit development, special exception or other site plan review process.

Open space zones

OS-1 Green built
OS-2 Avenue, road
OS-3 Restriction zone & area

Main purpose: Open space zones is to protect natural areas and features of the city.

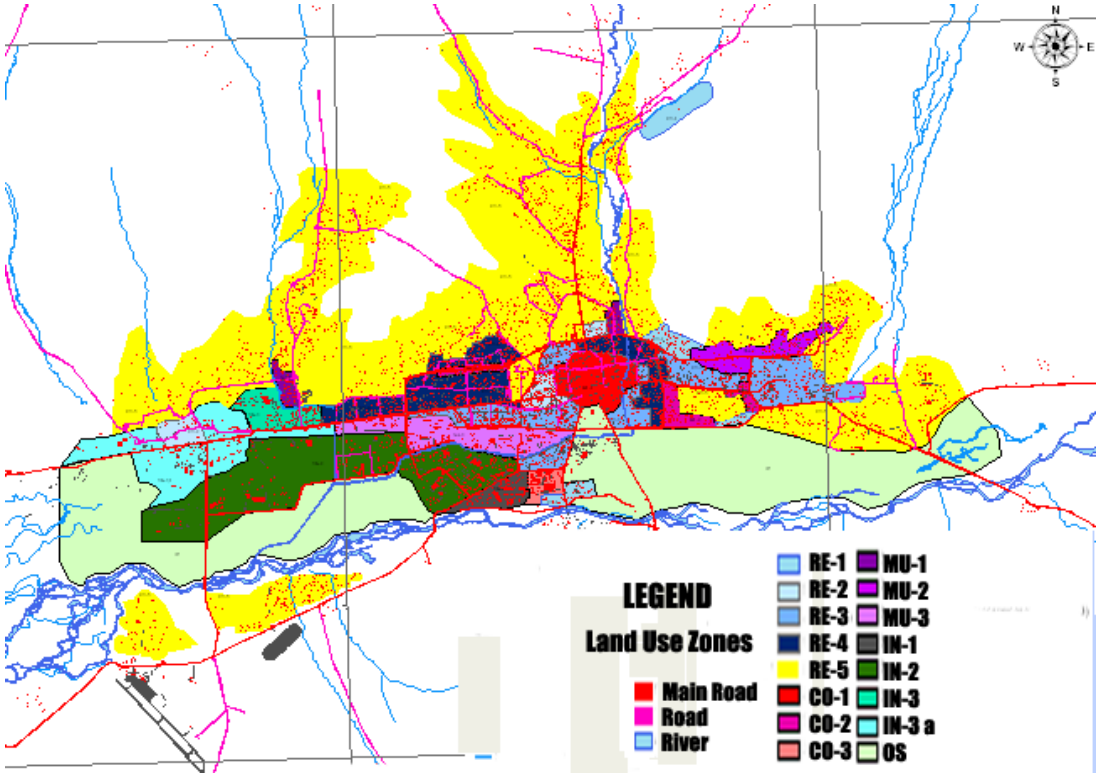

Figure 1. Land use classification and zoning scheme for the Ulaanbaatar

In developing the given list we have made better use of the urban zoning system of the Republic of Korea, the urban zoning systems of such cities of the USA and Japan. The followings (See table 1) are the list of permissible, conditionally permissible and prohibited types of the land use in above mentioned zones. The list represents the initial part of the elaboration of the urban land use zoning scheme for Ulaanbaatar city. But it is clear that the city and construction management standards and norms to be pursued in these zones will represent a heavy work to be established through a thorough investigation study in the field of planning and engineering.

Zoning regulations

The Land Use Zones is the most fundamental system of building control in urban areas. There are 20

categories of Land Use Zones within which the use, density, height, or shape of buildings and so on are regulated in accordance with the basic classifications of the built-up area.

Discussion

There is also noticeable new types of land use that might deteriorate the residential zones of the population, especially those environments which lay near to the residential areas. Accordingly, the demands and requirements for creating the legal environment for and controlling mechanism to plan and regulate the land use in the capital city in conformity to the best interests of the residents of the city are clear.

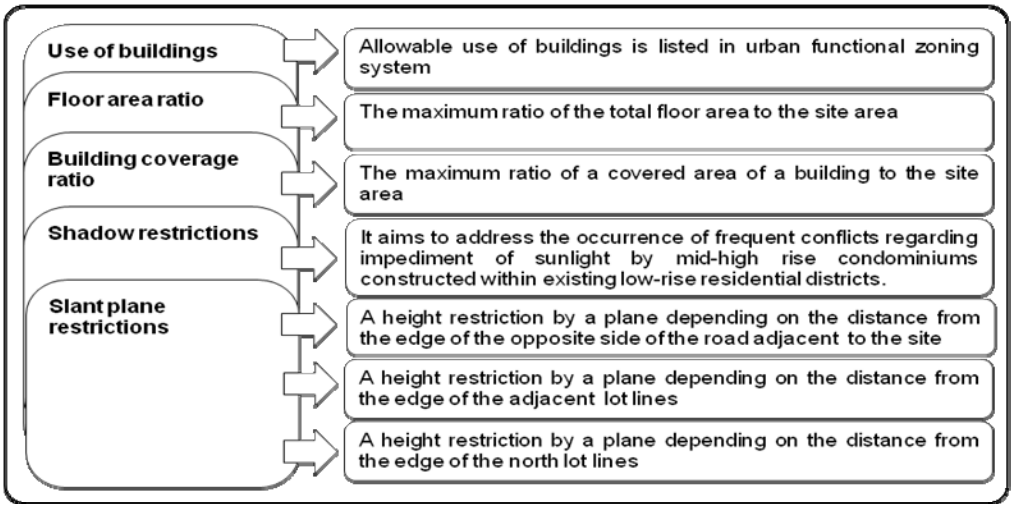

Figure 2.Restrictions on Buildings in the Land Use Zones System

Examples of buildings	Residential zones					Commercial zones			Industrial zones			Suburban zones			Mixed used zones			Open spaces		
	R1	R2	R3	R4	R5	C1	C2	C3	I1	I2	I3	S1	S2	S3	M1	M2	M3	O1	O2	O3
House, houses with other small scale function (store, office, etc)	E	D	C	B	F										D	D				
Kindergarten, Schools (primary, secondary, high)																				
Shrines, temples, churches, clinics											D									
Hospital, University																				
Stores, Cafe, Pub																				
Markets, Restaurant	F	E	E	E	E										E	D	D			
Offices, etc. not specified above						A	B	B												
Hotels, Inns						A	B	B												
Cinema, other entertainment																				
Ger, summer little house																				
Theaters, movie theaters, stores, restaurants, amusement facilities and so on, with more than 10000m² of floor area																				
Bathhouses, Sauna, barbershop, washhouses, beauty salon																				
Auto park, garage, technical market,		F	F	E																
Warehouses of warehousing company, independent garage of other types than specified above	F	F	F	F	F	E	E	E	F	E										
Auto repair shop, Petroleum station										E										
Factory with some possibility of danger or environmental degradation									E	E										
Factory with strong possibility of danger or environmental degradation									E	F										

Legend

R1 Single family housing zone	R5 Ger district	I1 Danger industry zone	S2 Traditional agricultural zone	M3 Semi industrial zone
R2 Low rise residential zone	C1 Central business zone	I2 Heavy industry zone	S3 Natural conservation zone	O1 Green built
R3 Middle rise residential zone	C2 Satellite business zone	I3 Light industry zone	M1 Semi residential zone	O2 Avenue, road
R4 High rise residential zone	C3 Neighborhood com-cial zone	S1 Farmer zone	M2 Semi commercial zone	O3 Restriction zone & area

A	Floor area must not exceed 1000 m²	C	Floor area must not exceed 500 m²	E	Floor area must not exceed 150 m²
B	Floor area must not exceed 700 m²	D	Floor area must not exceed 300 m²	F	Floor area must not exceed 50 m²

Can be built | Conditional can be built | Usually cannot be built

Figure 3. Control of building use by Land Use Zones

Table 1. Use of buildings in land use zones of Ulaanbaatar

	Land use zones	Code	Selection of 14 micro regions		Allowed land uses		Conditional land uses		Prohibited land uses	
			Micro regions name	Research field (m^2)	Num-ber	Area (m^2)	Num-ber	Area (m^2)	Num-ber	Area (m^2)
1	Single family housing zone	RE-1	"Jargalan" town	67472	26	5089	10	9304	-	-
2	Low rise residential zone	RE-2	15th district	455746	58	54097	64	42768	-	-
3	Middle rise residential zone	RE-3	"Urlan" town	64995	16	14082	3	3199	-	-
4	High rise residential zone	RE-4	13th district	237922	39	11583	23	23528	-	-
5	"Ger" zone	RE-5	"7 buudal"	55310	52	3041	11	1891	-	-
6	Central business zone	CO-1	"Baga toirog"	841598	168	171937	51	34107	4	119
7	Satellite business zone	CO-2	Narantuul	54534	31	43560	23	17823	3	872
8	Neighbourhood commercial zone	CO-3	3, 4th district	432120	112	88016	15	3870	1	114
9	Danger industrial zone	IN-1	Aris shir	38902	13	11309	14	5311	6	760
10	Heavy industrial zone	IN-2	4th power station	21371	14	14236	8	7690	2	410
11	Light Industrial zone	IN-3	Suljmel, talh chiher	365354	38	46604	42	19900	-	-
12	Semi residential zone	MU-1	100 ail	374635	43	18011	99	60074	-	-
13	Semi commercial zone	MU-2	Urt tsagaan	72712	11	15580	15	8981	-	-
14	Semi industrial zone	MU-3	Bars and near site	34992	13	3884	15	7726	2	85

Conclusion

1. The urban internal structure, land use classification and zoning are a completely new research direction in the geography of Mongolia and actually in the country there are missing in general researches and surveys, conducted and creative works, published based on the modern urban geographical theory and methodologies of highly market developed nations.

2. The master plan of the capital city Ulaanbaatar development at a time of centrally planned economy represented not only the urban construction and architecture physical plan, but there is a sufficient proof to consider that it was based on the capital city investment planning and constituted a document that had a legal power to plan and control the urban internal land utilization.

3. Since the irreversible transfer of the country into the market economy, the urban land use has turned to be based on the market relations and due to the reality that there have appeared no possibilities to regulate further on the land use by the old urban master plan, norms and standards, in the course of the transition period the land use within the framework of the capital city has been left with no control and inspection, regulation and has been run in a chaotic competition manner up-to the present.

4. On the other hand, though such laws of Mongolia as the law on land (2002), on land payment (1997), on land ownership (2002), on urban planning (1998), and also capital city's land use master plan (2001), Ulaanbaatar city's master plan (2002) were adopted and are being implemented duly, they miss basically such important stipulations as the urban land use classification and zoning and this witnesses that a legal environment for the urban land utilization has not ensured yet.

5. The present land structure of Ulaanbaatar city has all the possible opportunities to be compared with and diversified by according to the land use classification systems being pursued in the advanced countries of the world. In particular, we may note here that the classification of the land use, being applied in the urban planning of the Republic of Korea as identified and stipulated by the urban planning act has more closer identity to the classification of the land use in the capital city of Mongolia.

6. In conformity with this classification we are faced with the necessity to systematize newly the land use of Ulaanbaatar city, to reveal the functional zones according to this classification and to define in detail their boundaries and borders.

7. The land use structure in any belt or zone of the capital city has the opportunity to be enriched with new types of land utilization and there is evident a trend to increase the land use density in the capital.

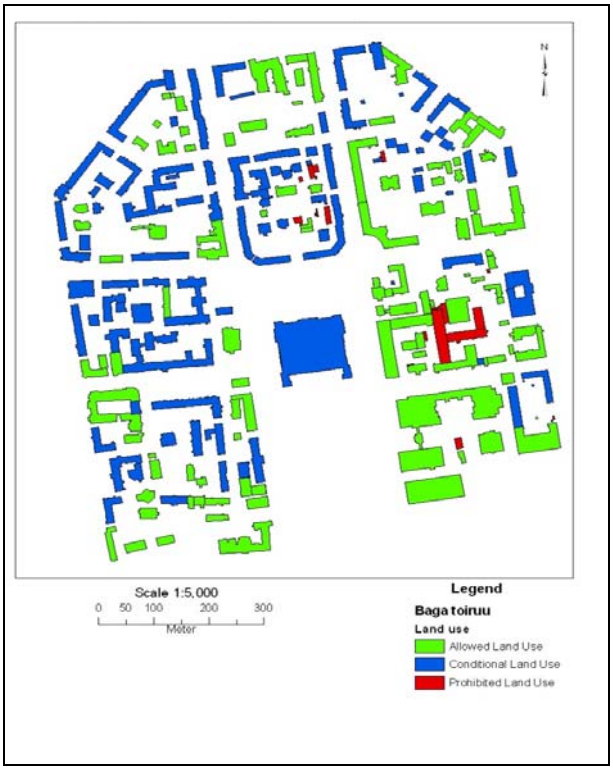

Figure 4.Urban land use zoning regulation of Baga toiruu

8. There are also noticeable new types of land use that might deteriorate the residential zones of the population, especially those environments which lay near to the residential areas. Accordingly, the demands and requirements for creating the legal environment for and controlling mechanism to plan and regulate the land use in the capital city in conformity to the best interests of the residents of the city are clear.

9. To this end we have to identify the Ulaanbaatar city land use functional zones and to clarify more in detail the permissible land use types, conditionally permissible types and the types that prohibit the land use at all, taking into consideration their designation and duty.

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1/20/2010

Studies On Susceptibility Of Methicillin –Resistent Staphylococcus aureus To Some Nigerian Honey

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Abstract: This study was aimed at determining the susceptibility of methicillin-resistant S. aureus (MRSA) isolates to some Nigerian honey. Sixty isolates of S. aureus were obtained from patients attending State Hospital, Jimeta Yola, Adamawa State. Twenty out of the sixty isolates were MRSA which were assessed for susceptibility or resistance to three (one processed and two crude) local honey samples in different concentrations and two commonly used antibiotics namely ciprofloxacin and ofloxacin using disk diffusion assay. All the twenty MRSA were susceptible to undiluted Sardauna plateau honey and its different concentrations of 50%, 25% and 13% (with growth inhibition zone ranging from 13 to 33 mm) but 25% of the isolates were resistant at concentrations of 6%. Against the MRSA isolates, undiluted Hong honey recorded 85% antibacterial activity, followed by 65%, 55%, and 5% respectively for its lower dilutions of 50%, 25% and 13% (with growth inhibition zone 12 or less than 12 mm). The undiluted Abuja honey sample recorded 85% antibacterial activity, followed by 35% and 15% respectively for its lower dilutions of 50% and 25%. Eighty five percent (85%) of the isolates were resistant to ofloxacin and 80% of the isolates were resistant to cipfrofloxacin (growth inhibition zone 20 mm or less for ciprofloxacin and 15 mm or less for ofloxacin, respectively). Values of the minimum inhibitory concentration and the minimum bactericidal concentration of S.P. honey were in the range of 0.4%-0.5% and 0.8 - 1% respectively whereas the values for H. honey and A. honey were in the range of 0.9-0.1% and 1.9-2.0% and 3.5-4.0% respectively. [Nature and Science 2010;8(2):98-108]. (ISSN: 1545-0740).

Keywords: MRSA, honey, methicillin, MIC, MBC

Introduction

MRSA, a major health problem worldwide, is a specific strain of the Staphylococcus aureus bacterium that has become resistant to all penicillin antibiotics such as methicillin and other narrow-spectrum beta-lactam penicillin antibiotics (Schito, 2006). This accounts for MRSA's serious and increasing threat to public health since Jevons first reported on the emergence of the strain in 1961 (Derek et al., 2005). Also, since 1996, vancomycin-resistant S. aureus (VRSA) has emerged against a drug considered the "last line of defense" when all other antibiotics have failed (Guignard et al., 2005). Wyllie et al (2006) reported a death rate of 34% within 30 days among patients infected with MRSA, and 27% death rate among patients infected with methicillin-susceptible Staphylococcus aureus (MSSA).

Studies have shown that MRSA is a growing health problem in many parts of the world, including Europe, America, Africa, the Middle East, and East Asia (Grundmann, 2006). Martha et al. (2009) isolated MRSA isolates from AIDS patients attending some public hospitals in Yola, Adamawa State, Nigeria. In the Netherlands, for instance, the annual number of MRSA strains submitted for epidemiological typing to the National Institute for Public Health and the Environment has risen from less than 200 in the early nineties to about 500 in 2001 (Simon et al., 2008).

A study was conducted on the prevalence and antibiotic susceptibility patterns of MRSA in eight large hospitals (>500 beds) in Africa and Malta from 1996 to 1997. Susceptibility to methicillin (oxacillin) and to other drugs was determined by E test (AB Biodisk, Solna, Sweden, 2001) on a total of 1440 clinical isolates of S. aureus. Methicillin resistance was detected in 213 (15%) of the 1440 isolates tested. The rate of MRSA was relatively higher in Nigeria, Kenya and Cameroon (21–30%), and below 10% in Tunisia, Malta, and Algeria. (Kesah et al, 2003). All MRSA isolates were sensitive to vancomycin, with MICs ≤ 4 mg/L. The isolates were also highly sensitive to ciprofloxacin, except in Kenya, Morocco, and Tunisia, where relative resistance to this drug was noted. Susceptibility to rifampin and fusidic acid seems to be correlated with the clinical use of these compounds. Only 46% of 59 MRSA strains analyzed were susceptible to rifampin, fusidic acid, and

ciprofloxacin. The majority (> 60%) of MRSA strains were multi resistant (Kesah et al, 2003).

Molan (1992) reported that honey has an inhibitory effect on about 60 species of bacteria including aerobes, anaerobes, gram-positives and gram-negatives. Antibiotic-resistant strains of Staphylococcus aureus have been studied and found to be as sensitive to honey as the antibiotic-sensitive strains of the same species. The MIC for 82 epidemic strains of methicillin-resistant Staphylococcus aureus (MRSA) was found to range from 3% to 8% (v/v) (Allen et al., 2000). Another study was conducted on 56 strains of vancomycin-resistant enterococci (VRE) and it has been shown that the MIC values were found to range from 5% to 10% (v/v) for manuka honey with activity due to a phytochemical component and a typical multifloral honey with activity due to hydrogen peroxide. Both the honey samples were collected from New Zealand. In another study, the MIC values for eight strains of MRSA isolated from swabs collected from acute and chronic wounds were all below 10% for honey used as antimicrobial agent (Molan, 1992).

Studies have shown that accurate detection of Oxacillin/Methicillin resistance can be difficult due to the presence of two subpopulations (one susceptible and the other resistant) that may coexist within a culture of Staphylococci (Brown et al.,2005). All cells in a culture may carry the genetic information for resistance, but only a small number may express the resistance in vitro. This phenomenon is termed heteroresistance and occurs in Staphylococci resistant to penicillinase-stable penicillins, such as oxacillin. Cells expressing heteroresistance grow more slowly than the oxacillin-susceptible population and may be missed at temperatures above 35 °C. That is why CLSI has recommended that test isolates against oxacillin, methicillin, or nafcillin be incubated at 33-35 °C (maximum of 35 °C) for a full 24 hours before reading (CLSI, 2005).

Given the threat posed by the growing problem of MRSA to public health, compounded by MRSA increased resistance to almost all new drugs, lack of currently available antimicrobials with rapid cidal activity against MRSA, and the global need for alternative effective anti-MRSA, the continued surveillance and control of MRSA infections, evaluation of honey from various flowery sources, geographic areas and processing against MRSA infection in humans is pertinent, indeed imperative (Kesah et al., 2003). The aim of this study was to compare/ascertain the extent of susceptibility of MRSA isolates to some locally processed or crude honey samples at their varied concentrations.

Materials and Methods

2.1 Collection of antibiotics and honey samples

Ciprofloxacin and Ofloxacin disks each of 5 µg/disk (Tyonex Nigeria Limited, Lagos, 2004) were obtained in a local laboratory consumable store (New Era Medical Diagnostic Laboratories) in Jimeta-Yola metropolis.

Three honey samples for this study were obtained from three local sources: processed honey from Betty Nnadi Farms Ltd, Abuja-FCT and crude honey from traditional bee farmers in Hong LGA, Adamawa State, and on the Mambila or Savannah plateau in Sardauna LGA, Taraba State, Nigeria. The honey samples were stored at room temperature in the dark until they were used for the experiments.

2.2 Collection of Specimens

A total of nine hundred samples were collected from eight pathological sources. Wound biopsy samples both of the needle aspiration and swab types and catheter urine specimens were collected by the clinicians in the ward and sent to the laboratory for isolation purpose. Patients not hospitalized were each given a sterile, wide-necked, leak-proof container for collection of urine samples. Specimens from Genito-Urinary tract were also collected using Swabs. Urethral discharge from male patients was collected using sterile cotton wool swab. Eye swabs were taken using swab sticks as described by WHO (2003) and cultured immediately to prevent enzymatic action killing any microbe present in the samples.

2.3 Isolation and Identification of Staphylococcus aureus

2.3.1 Isolation of S. aureus

To isolate pure colonies, the methods as described in CLSI (2005) were adopted. Two Mannitol Salt Agar (MSA) plates for each specimen were inoculated using the streak method.

2.3.2 Identification of S. aureus

The identification of the Staphylococci isolates was done following the procedures described by WHO (2003) as follows.

(a) Morphological and Cultural Characteristics

Incubated plates were examined for characteristic golden or white staphylococcal growth.

Isolates were examined microscopically using Gram staining procedure for Gram-positive cocci in clusters (GPCC), which suggest the presence of Staphylococcus species.

(b) Biochemical Characteristics

(i) Catalase activity— A wire loop was used to collect a speck of growth from each plate incubated for 24h (section 3.2.1) and emulsify in a drop of 3% hydrogen peroxide (H_2O_2) on

microscope slide. Formation of effervescence indicates presence of Staphylococci strains.

(ii) Coagulase test

Staphytect Plus test method:

Isolates were tested for coagulase production using Staphytect Plus test method. The reagents were purchased from Sanofi Diagnpostic Pasteur, France and the method was followed as instructed by manufacturer. One drop of test latex reagent was dispensed onto one of the circles on the reaction card and one drop of control latex was dispensed onto another circle.

A loop was used to pick up 5 average-size of suspected staphylococcal colonies onto a culture media plate and mix this in the control latex reagent. The colonies were smeared in order to cover the circle. A separate loop was then used to proceed in the same way with the test latex.

The card was rocked for 20 seconds and agglutination was observed under normal lighting conditions.

A result was reported positive if agglutination of the blue latex particles occurred within 20 sec. This identified the strain as a MRSA. A result was reported negative if no agglutination occurred and a smooth blue suspension remained after 20 sec in the test circle.

Coagulase activity using Pastorex Staph Plus®

Isolates were also tested for coagulase production using Pastorex Staph Plus® latex slide agglutination kits as instructed by the maker (Sanofi Diagnnostic Pasteur, France), to differentiate MRSA isolates from MSSA by detection of clumping factor (fibrinogen), Protein-A, and capsular polysaccharides found only in MRSA (Cruickshank et al., 2000).

Result was reported positive if agglutination of the blue latex particles occurred within 20 sec. This identifies strain as a MRSA. Result was reported negative if no agglutination occurred and a smooth blue suspension remained after 20 sec in the test circle. This identified the strain as a MSSA.

Twenty (20) MRSA and 40 MSSA isolates were identified and the MRSA were streaked on different mannitol salt agar slants. These were incubated for growth at 35 ^{O}C for 24h in an incubator and were stored at 4 ^{O}C in a refrigerator.

2.3.3 Antibacterial activity of cloxacilin against the MRSA isolates:

The antibacterial activity of cloxacilin at 5µg/ml against the MRSA isolates were determined using disk diffusion assay: Cloxacillin was selected because it is similar to methicillin in penicillinase resistance (Jawetz et al., 2000; Andrew, 2001). From each slant culture of MRSA isolates, five representative colonies were touched with a sterile loop and were suspended in sterile distilled water. Each MRSA water suspension was diluted in steps of 1:10 to adjust the MRSA suspension to 1x 10^{8} density equal to the 0.5 McFarland standards before inoculation (NCCLS, 1997) and were used for subsequent antimicrobial susceptibility test.

A loopful of culture of each of MRSA grown in MSA at 35 ^{0}C for 24h was surface spread evenly on 4% NaCl supplemented Mueller-Hinton agar (MHA) in Petri dishes. Cloxacilin disks were each picked with a pair of sterile forceps and applied to each uniformly seeded area of the plate, spaced out so that their centers were at least 2 cm apart, incubated aerobically at 35 ^{0}C for 24h.

2.4 Disk diffusion assay to determine zones of growth of inhibition of honey and ciprofloxacin and ofloxacin

The honey samples collected were treated to 40 ^{0}C in a water-bath and various dilutions 50%, 25%, 13%, 6% (v/v) honey were prepared using sterile distilled water. These solutions were used to saturate paper disks for assays to determine zones of inhibition against MRSA growth.

The antibacterial activity of different honey samples and the two selected antibiotics were determined using the procedure as described in section 2.3. The concentrations of the antibiotics used in this study were Ciprofloxacin 5µg/ml and Oflaxacin 5µg/ml. The plates were then incubated at 35 ^{0}C for 24h. For honey samples, a zone diameter that was 13 mm or above was reported sensitive and a zone of diameter of 12 mm or less was reported as resistant. The sensitivity/resistance profile of honey was determined using cloxacillin at 5 µg/ml concentration. For ciprofloxacin and ofloxacin, a zone diameter that was 21 mm or above was reported sensitive and a zone of diameter of 20 mm or less was reported as resistant and for ofloxacin, a zone diameter that was 16 mm or above was reported sensitive and a zone of diameter of 15 mm or less was reported as resistant (WHONET, 2006).

2.5 Determination of MIC of Different Honey Samples

This was done following the procedure as described in NCCLS (1984). Doubled strength (5.0 ml) of MHB were dispensed separately in six test tubes of 10 ml capacity into which the following were added: Graded volumes (0.5ml—4ml) of honey samples, (4.8 ml—0.8 ml) of sterile, distilled water and (0.2 ml) of 24h pure culture of MRSA broth. The MRSA broth was prepared as described in 2.4.1. The test tube which served as a control contained no honey but MHB, distilled water, and the 24h incubated pure culture of MRSA broth. All the test tubes were then incubated at 35 ^{0}C for 24h in a B28 liter Incubator (WTB Binder

Labortechnik GmbH, Germany) and observed visually for growth. The highest dilution of the antimicrobial that showed no visible growth of the organism was taken as the minimum inhibitory concentration.

The same procedure was applied for the other honey samples and the two standard antibiotics.

2.6 Determination of minimal bactericidal concentration (MBC) of honey

A loopful of broth was collected from each of the tubes in the MIC test that showed no visible growth and streaked on Mueller-Hinton agar plates and incubated at 35 ^{0}C for 24h in a B28 liter Incubator and observed for bacterial growth. The MBC was determined by the highest dilution at which there was no visible growth on the solid media.

2.7 Data Analysis

WHONET 5.4, World Health Organization (WHO, 2006) Data-base Software for managing laboratory test results, was used for data analysis to achieve the objective(s) of this study.

Results

3.1 Isolation and identification of S. aureus

Out of nine hundred samples examined, sixty pure cultures of Staphylococcus aureus isolates were obtained from eight pathological sites of patents in Specialist Hospital, Jimeta-Yola, Adamawa State, Nigeria.

S. aureus isolates collected from each of wound swab, urine specimens , ear swab and urethral swab was 17% followed by eye swab (13%), high vaginal swab (13%), wound biopsy (3%) and catheter urine (3%). Table 1 shows the distribution of S. aureus isolates according to their pathological sites.

3.2 Isolation and identification of MRSA

Of the sixty isolates of S. aureus, twenty were identified to be methicillin-resistant S. aureus (MRSA) strains from five pathological sites. Forty percent of MRSA isolates were collected from wound swab followed by eye swab (20%). HVS (20%), UTI(15%) and catheter urine (5%). Table 1 shows the distribution of MRSA isolates according to their pathological sites. The result shows that, out of the sixty isolates of S. aureus, (20) 33.3% were MRSA strains. The diameter of zones for all the MRSA isolates against methicilin were in the range of 6-12 mm.

3.3 Susceptibility Testing

3.3.1 Antimicrobial activity of S.P.Honey and two standard antibiotics against MRSA isolates

The MRSA isolates from eye swabs were highly susceptible to both diluted and undiluted honey from Sadauna Plateau and this was followed by isolates from HVS, wound infection, catheter urine and lastly the wound infection (Table 2).

Undiluted Sardauna Plateau honey sample and its different concentrations (50%, 25%, and 13%) recorded 100% antibacterial activity, followed by 75% for 6% dilution against the MRSA isolates. 85% of the isolates were resistant to ofloxacin and 80% of the isolates were resistant to cipfrfloxacin.

Table 5 shows the percentage resistance profile of MRSA to S.P. Honey and two standard antibiotics.

3.3.2 Antimicrobial activity of Hong Honey and two standard antibiotics against MRSA isolates

Against the MRSA isolates, undiluted Hong honey recorded 85% antibacterial activity, followed by 60%, 55%, and 5% respectively for its lower dilutions of 50%, 25% and 13%. The results are shown in Table 3. Table 5 shows the percentage resistance profile of MRSA to Hong honey and two standard antibiotics.

3.3.3 Antimicrobial activity of Abuja Honey and two standard antibiotics

The undiluted Abuja honey sample recorded 85% antibacterial activity, followed by 35% and 15% respectively for its lower dilutions of 50% and 25%. Table 4 shows the sensitivity of the isolates to the Abuja honey sample and two standard antibiotics, while Table 5 shows the resistance profile of MRSA to Abuja honey and two standard antibiotics.

3.4 MIC and MBC of antimicrobials and their effects on MRSA isolates

Values of the minimum inhibitory concentration and the minimum bactericidal concentration of Sarduna Plateau honey were in the range of 0.4%-0.5% and 0.8 - 1% respectively whereas the values for Hong honey and Abuja honey were in the range of 0.9-0.1% and 1.9-2.0% and 3.5-4.0% respectively. The values of MIC and MBC for ciprofloxacin and ofloxacin against the MRSA isolates were in the range of 0.5-1.0 % respectively.

Table 1: Distribution of S. aureus and MRSA isolates according to their pathological sites

Pathological source	No. of S. aureus	% of S. aureus	No. of MRSA	% of MRSA
Eye swab	8	13.00	4	20.00
Wound swab	10	17.00	8	40.00
Urinary tract infection	10	17.00	3	15.00
Catheter urine	2	3.00	1	5.00
Ear swab	10	17.00	-	-
Urethral swab	10	17.00	-	-
High vaginal swab	8	13.00	4	20.00
Total	60	100.00	20	100.00

Table 2: Growth Inhibition Zone Size (in mm) of S. P. Honey, Ciprofloxacin and Ofloxacin against MRSA isolates

Isolate No.	Specimen site	Growth Inhibition Zone Size (mm) Sardauna Plateau Honey (%) (x_1)	(x_2)	(x_3)	(x_4)	(x_5)	CIP	OFX
1	ey	33	30	27	23	17	21	15
2	ey	30	25	22	15	13	9	15
3	wd	30	25	22	15	13	16	10
4	ey	25	20	17	13	13	16	12
5	ey	32	30	27	22	11	21	12
6	HVS	28	24	23	18	11	21	6
7	wd	26	24	21	16	12	20	6
8	HVS	24	22	19	14	10	9	16
9	HVS	28	24	21	16	10	6	16
10	HVS	25	20	17	13	11	6	6
11	UTI	24	20	17	13	11	10	12
12	wd	24	20	17	13	9	6	6
13	cur	23	19	16	13	10	12	12
14	wd	26	24	21	16	12	15	6
15	UTI	25	20	17	15	10	15	12
16	UTI	24	20	17	16	12	18	8

17	wd	22	19	16	13	11	12	6
18	wd	21	20	17	15	14	22	6
19	wd	26	24	21	17	10	12	10
20	wd	20	18	15	13	12	11	11

Ey (eye swab)
HVS (high vaginal swab)
Cur (catheter urine)
UTI (urinary tract infection)
Wd (wound swab)
(x_1) undiluted Honey
X_2 (50% Honey)
X_3 (25% Honey)
X_4 (13% Honey)
X_5 (6% Honey)
CIP (Ciprofloxacin 5 µg)
OFX (Ofloxacin 5 µg)

Table 3: Diameter of zones (mm) of Hong Honey and two standard antibiotics against MRSA isolates

Isolate No.	Specimen site	Growth Inhibition Zone Size (mm) Hong Honey (%) (x_1)	(x_2)	(x_3)	(x_4)	(x_5)	CIP	OFX
1	ey	14	13	13	13	8	21	15
2	ey	17	13	13	11	10	9	15
3	wd	13	14	13	11	9	16	10
4	ey	24	20	18	12	8	16	12
5	ey	32	20	19	10	8	21	12
6	HVS	16	13	14	8	6	21	6
7	wd	23	20	15	10	10	20	6
8	HVS	16	15	14	6	7	9	16
9	HVS	13	14	13	8	8	6	16
10	HVS	31	14	13	10	8	6	6
11	UTI	24	16	14	9	7	10	12
12	wd	15	13	12	9	9	6	6
13	cur	18	12	11	10	10	12	12
14	wd	23	12	10	10	9	5	6
15	UTI	20	10	12	10	10	15	12
16	UTI	18	12	11	12	10	18	8
17	wd	19	12	11	10	10	18	9
18	wd	10	8	8	6	6	12	6
19	wd	12	10	9	6	6	12	10
20	wd	12	12	10	10	8	11	11

Key:
Ey (eye swab)
HVS (high vaginal swab)
Cur (catheter urine)
UTI (urinary tract infection)
Wd (wound swab)

(x_1) undiluted Honey
X_2 (50% Honey)
X_3 (25% Honey)
X_4 (13% Honey)
X_5 (6% Honey)
CIP (Ciprofloxacin 5 µg)
OFX (Ofloxacin 5 µg)

Table 4: Growth Inhibition Zone Size (mm) of Abuja Honey and two standard antibiotics against MRSA isolates

Isolate No.	Specimen site	Growth Inhibition Zone Size (mm) Abuja Honey (%) (x_1)	(x_2)	(x_3)	(x_4)	(x_5)	CIP	OFX
1	Ey	15	14	13	11	6	21	15
2	Ey	14	14	13	6	6	9	15
3	wd	15	14	13	11	6	16	10
4	Ey	22	18	12	10	10	16	12
5	Ey	30	20	10	9	8	21	12
6	HVS	14	13	10	9	8	21	6
7	wd	20	15	10	9	10	20	6
8	HVS	13	11	10	8	6	9	16
9	HVS	14	10	8	6	6	6	16
10	HVS	18	10	10	8	8	6	6
11	UTI	14	12	10	8	6	10	12
12	wd	19	12	10	8	6	6	6
13	Cur	18	12	11	8	6	12	12
14	wd	15	10	10	8	6	15	6
15	UTI	14	12	10	8	7	15	12
16	UTI	14	11	6	6	6	18	8
17	wd	14	12	10	8	6	18	9
18	wd	10	10	8	6	6	12	6
19	wd	12	10	8	6	6	12	10
20	wd	11	10	8	6	6	11	11

Key: Ey (eye swab)
HVS (high vaginal swab)
Cur (catheter urine)
UTI (urinary tract infection)
Wd (wound swab)
(x_1) undiluted Honey
X_2 (50% Honey)
X_3 (25% Honey)
X_4 (13% Honey)

X_5 (6% Honey)
CIP (Ciprofloxacin 5 µg)
OFX (Ofloxacin 5 µg),,

Table 5: Percentage resistance profile of MRSA to S.P. Honey, Hong honey, Abuja honey and the two standard antibiotics

Resistance profile	% of Isolate		
	S.P	H	A
OFX	10	-	-
CIP OFX	15	-	-
X_5 OFX	15	-	-
X_5 CIP	10	-	-
X_5 CIP OFX	50	10	-
X_4 X_5 OFX	-	5	-
X_4 X_5 CIP	-	10	5
X_4 X_5 CIP OFX	-	30	10
X_3 X_4 X_5 CIP OFX	-	10	10
X_2 X_3 X_4 X_5 CIP	-	5	-
X_2 X_3 X_4 X_5 CIP OFX	-	15	50
X_1 X_2 X_3 X_4 X_5 CIP OFX	-	15	15
X_3 X_4 X_5 OFX	-	-	5
X_3 X_4 X_5 CIP	-	-	5
Total	100	100	100

Key:
X_1 (undiluted Honey)
X_2 (50% Honey)
X_3 (25% Honey)
X_4 (13% Honey)
X_5 (6% Honey)
CIP (Ciprofloxacin 5 µg)
OFX (Ofloxacin 5 µg)

Discussion

The 33.3% of MRSA strains, out of the sixty isolates of S. aureus, isolated from patients attending Specialist Hospital, Jimeta, Yola indicate that there must have been a high prevalence of MRSA infections among patients, who came from Jimeta-Yola and its environs to attend the hospital (Table 1). Kesah et al, 2003 conducted a study on prevalence of MRSA in

large eight hospitals in Africa and Malta from 1996 to 1997 and observed that the rate of MRSA was relatively high in Nigeria, Kenya and Cameroon. These patients may be responsible for hospital acquired and community acquired infection. Hanselmann et al., 2006 reported that recently there was a shift in the epidemiology of MRSA infections to community-associated infections in case of significant illness and death. In view of the search for a solution to the problem of antibiotic resistant strains, this study highlights the potential of honey as a suitable and affordable first-line therapeutic agent against pathogens, particularly MRSA.

Variability in antimicrobial activity of honey samples from different floral and geographic sources against several strains of MRSA known to cause human diseases has been described (Willix et al., 1992). Its antimicrobial activity varies with origin, geographic area and processing against some pathogens, including methicillin-resistant Staphylococcus aureus (MRSA) (Molan, 2001; EARSS, 2004). In this study, all MRSAs tested showed different susceptibilities to each of the test honey samples. The inhibitory action of different honey samples was compared with that of two commonly used antibiotics namely ciprofloxacin and ofloxacin each at 5 µg/ml concentration. Seventy percent of the MRSA isolates tested showed susceptibility to S.P. honey sample at a low concentration of 6% signifying that more than one antimicrobial factor are present in honey (Table 2). Weston et al. 2000 reported that in addition to hydrogen peroxide, non-peroxidase components such as lysozyme, phenolic acids , flavonoids present in honey also contribute to the antibacterial activity of honey. The concentrations of these components differ in different types of honey samples. Carotenoids are also reported to be present in some honey especially the darker honey (Frankel et al, 1998). Some componens mainly 3,4,5-trimethoxy benzoate, methyl-4-hydroxy-3,5-dimethoxy benzoate and 3,4,5 trimethoxy benzoic acid responsible for the exceptionally high antibacterial activity of manuka honey were isolated by testing fractions of the honey for activity against S. aureus (Russel, 1983).

The anti-MRSA activity recorded in this study for honey was consistent with earlier reports on its curative and antibacterial properties (Willix et al., 1999). MRSA showed a susceptible level of 100% to the undiluted honey sample from Sardauna Plateau, followed by 75% susceptible level at 6% concentration. In this study, zones of growth inhibition of 13 mm or more for undiluted honey and 50% honey dilutions in disk diffusion proof the basis for offering some honey from some flora as a unique treatment in MRSA infections (Allen et al., 2000; Derma Sciences, 2008).

MRSA showed the highest susceptible level of 100% to the undiluted S.P. honey sample followed by 85% susceptible level at 6% concentration and is suggested as the treatment of choice against the pathogens, considering the huge impact of the strains as agents of nosocomial infections worldwide The present study has shown that compared with the honey samples and their 50% to 6% concentrations, ciprofloxacin and ofloxacin used in concentrations of 5µg/ml were less active on the MRSA isolates. The resistance levels among the MRSA isolates were 85% and 80% for ciprofloxacin and ofloxacin respectively, compared with 25% resistance levels at 6% concentration of honey from Sardauna Plateau and 15% and 16% resistance levels for undiluted Hong and Abuja honey samples respectively. These results find an analogy in an earlier report (Molan, 2000) of more effective treatment of bacterial infected burn wounds with honey than with silver sulphadiazine, a recognized antibacterial ointment.

The values of MIC ands MBC of the antimicrobials showed that their initial inhibition of the MRSA isolates did not stop only at prevention of growth (bateriostasis), but also extended to killing of the bacteria (bactericidal) activity. Three of the MRSA isolates showed total resistance both to Hong and Abuja honey samples, and to the two standard antibiotics. The isolates, however, were susceptible to S. P. honey.

Based on the results obtained in this study, it can be concluded that some honey from some flora has the potential as a suitable and affordable first-line therapeutic agent against pathogens, particularly MRSA. It may, however, be recommended that further understanding of the active species in the potency of honey is needed to optimize their selection and use as first- or -second line antimicrobials in various diseases caused by MRSA.

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The relationship between serum adiponectin and steatosis in patients with chronic hepatitis C genotype-4

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Abstract: The mechanisms underlying steatosis during hepatitis C virus (HCV) infection are complex and multifactorial. The aim of our study was to assess whether host metabolic factors influence the degree of hepatic steatosis and fibrosis in patients infected with hepatitis C virus genotype 4 by investigating the role of adiponectin, leptin and insulin resistance. **Methods:** Adiponectin and leptin levels, HCV genotypes, HCV-RNA, IR (HOMA-IR), body mass index and liver steatosis and fibrosis were assessed in 74 chronic patients with HCV genotype 4. **Results:** Chronic HCV patients with steatosis showed lower serum adiponectin levels and higher levels of leptin, HOMA, alanine aminotransferase, γ glutamiltransferase and fibrosis scores. Low adiponectin levels were independently associated with grades of steatosis and HOMA-IR. Adiponectin levels showed significant inverse correlation between adiponectin and steatosis grade, BMI, HOMA and fibrosis stage. The multivariate analysis of factors showed that steatosis was significantly associated with low adiponectin concentration while, leptin, Insulin, HOMA, ALT, γ-GT and cholesterol were positively associated with steatosis. **Conclusion:** This study stated that Egyptian patients with HCV genotype-4 suffering from steatosis had lower adiponectin level that is inversely correlated with insulin resistance. These data support a role for adiponectin in protection against liver injury and that hypoadiponectinemia may contribute to hepatic steatosis progression. Further molecular and genetic studies with larger numbers of patients are required to confirm these results. [Nature and Science 2010;8(2):109-120]. (ISSN: 1545-0740).

Key words: Adiponectin, steatosis, hepatitis C, leptin

1. Introduction

Infection with hepatitis C virus (HCV) is a leading cause of chronic liver disease worldwide **(Alter and Seeff, 2000)** Genotype 4 is the most common genotype of HCV in Egypt and its response to treatment is still a controversy **(Zekri et al., 2000).** Steatosis is a common histological feature of hepatitis C virus (HCV) infection but the relative importance of host and viral factors remains unclear **(Tsochatziz et al.,2009)**. Hepatic steatosis is considered to be mostly associated with viral factors in genotype 3 namely viral steatosis while host factors seem to play the major pathogenic role in HCV genotype non-3 infection, namely metabolic steatosis **(Tsochatziz et al., 2007).**

Non-alcoholic fatty liver disease (NAFLD) represents a spectrum of liver diseases characterized mainly by macrovesicular steatosis that occurs in the absence of alcoholic consumption. The hepatic histology varies from isolated hepatic steatosis alone "first hit" to fatty liver accompanied by hepatocellular damage plus inflammation known as steatohepatitis "second hit" which is followed by the development of fibrosis. Steatosis is associated with risk factors for nonalcoholic steatohepatitis (NASH), particularly obesity, rather than with alcohol consumption (**Monto et al., 2002).** Obesity is a well-recognized risk factor for the development of steatosis and of fibrosis in HCV infected patients **(Lonardo et al., 2004).** Visceral fat distribution rather than body mass index (BMI) proved to be associated with HCV related steatosis. Adipose tissue has traditionally been considered an energy storage organ, but over the last decade, a new role has emerged for the adipose tissue as an endocrine organ.. It secretes variety of hormones including adiponectin and leptin, which may contribute to the development of metabolic abnormalities **(Adinolfi et al., 2001).**

There are few data on adipocytokines and liver function. There are some controversial data about the relationship between serum leptin levels and HCV-related steatosis **(Giannini et al., 2000).** Regarding adiponectin, its levels are associated in healthy humans with plasma concentrations of

various liver function tests; however, there is no data about the secretion of adiponectin during hepatitis C infection **(Lopez-Bermejo et al., 2004)**. Although the deleterious association between obesity and HCV infection is well recognized, it has not been ascertained whether adipocytokines and, in particular, adiponectin may have a role in the development of steatosis in chronic hepatitis C. Adiponectin modulates hepatic fat content and has an antiseatotic effect on liver. Moreover, adiponectin is a hepatic insulin sensitizer and has anti-inflammatory and antifibrotic effect in experimental murine models of liver damage **(Kamada et al., 2003).**

Leptin is an adipocyte-derived antiobesity hormone that in rodents prevents "lipotoxicity" by limiting triglyceride accumulation and also regulates matrix deposition (fibrosis) during wound healing **(Chitturi et al., 2002).** Leptin has been suggested to play a role in the pathogenesis of hepatic steatosis and steatohepatitis in the absence of viral infection **(Tungtrongchitr et al., 2006).** Leptin and adiponectin have been implicated in the pathogenesis and progression of non-alcoholic steatohepatitis (NASH) and chronic hepatitis C **(Tsochatzis et al., 2008).**

Insulin resistance is a frequent feature of chronic hepatitis C. Whether insulin resistance could be the cause or consequence of steatosis and fibrosis is unknown **(Fartoux et al., 2005),** the interaction between insulin resistance (IR), steatosis and genotype to fibrosis in chronic hepatitis C virus (HCV) infection has not been comprehensively assessed. In chronic hepatitis C (CHC), there is a close association between insulin resistance (IR), hepatic steatosis **(Moucari et al., 2008),** progression of fibrosis **(Camma et al., 2006)**, adipocytokine profile **(Hui et al., 2003)** and a lower rate of sustained virological response (**Conjeevaram et al., 2007).**

The objective of this study was to assess whether host metabolic factors influence the degree of hepatic steatosis and fibrosis in patients infected with hepatitis C virus genotype 4 by investigating the role of adiponectin, leptin and insulin resistance.

2. Patients and methods

Seventy four patients were enrolled in this study, they were admitted to division of Internal Medicine and Hepatology Ain shams University hospital with a diagnosis of chronic hepatitis C (CHC) genotype 4. All patients were evaluated with physical examination, laboratory tests and underwent pretreatment liver biopsy. Entry criteria included patients aged 42–63 year, with elevated alanine aminotransferase (ALT) levels for at least 6 months on at least 3 occasions, positive for HCV-RNA and liver histology compatible with chronic hepatitis C. Patients were divided into two groups according to presence or absence of steatosis.

We excluded patients with: (a) Other causes of chronic liver disease, Wilson's disease, haemochromatosis, autoimmune hepatitis and alcoholics, (b) Decompensated liver disease (c) History of heart failure, diabetes, thyroid diseases, abnormal renal function and cancer (d) Previous treatment with antiviral agents, metformin, vitamin E, or thiazolidinedione and (e) The use of drugs known to induce liver steatosis (corticosteroids, amiodarone, tamoxifen, valproic acid) within the last 6 months. The study protocol had been approved by the Ethics Committee and subjects gave written consent to participate in the present study.

2.1 Clinical, laboratory and virological parameters:

(1) Clinical Assessment:

Complete clinical evaluation was performed for all patients. Baseline characteristics collected at the time of liver biopsy included the age, ethnicity, height, weight and waist circumference.

Body mass index (BMI; kg/m2) and waist circumference were calculated.

BMI= body weight (kilograms) / the square of height (meters).

Visceral obesity was defined as a waist circumference $\geq$102 cm in males and $\geq$88 cm in females.

(2) **Liver Histopathology:**

Liver biopsy was done for all patients for diagnostic purposes. Liver specimens were formalin-fixed and paraffin-embedded for histological evaluation. Stained sections were evaluated according to a scoring system that includes the semi-quantitative assessment of liver disease grading and staging **(METAVIR study, 1994, Brunt et al., 1999).**

(3) Grading:

Steatosis was graded by percentage of cells showing fatty changes. Grading was made according to macrovesicular steatosis and necroinflammatory activity Macrovesicular steatosis was graded as:

- Grade 0: Absent or minimal (less than 5% of hepatocytes involved)

- Grade 1: Mild (5-30% of hepatocytes involved)
- Grade 2: Moderate (30- 60% of hepatocytes involved)
- Grade 3: Severe (60% of hepatocytes involved)

- Necroinflammatory activity. Grade 1: mild; Grade 2: moderate; Grade 3: severe.

(4) Staging:

According to the METAVIR system [20], fibrosis was staged on a scale from F0 to F4, as follows;

- F0: no fibrosis;
- Stage 1(F1): portal fibrosis, without septa.
- Stage 2(F2): few septa.
- Stage 3(F3): many septa without cirrhosis.
- Stage 4(F4): cirrhosis.
-

The stages of fibrosis were modified to two categories as follows: mild fibrosis (stages 0 to 2) and advanced fibrosis (stages 3 and 4).

- Blood assays: At the time of liver biopsy, assays were carried out on plasma and serum samples collected after overnight fasting and stored at -70°C until use. Routine blood tests were performed at University hospital laboratory.

A) Virology

All serum samples were tested by ELISA for the presence of a hepatitis C antibody (anti-HCV). Serological testing for anti-HCV was carried out using a commercial microparticle enzyme immunoassay {a line immunoassay (INNO-LIA HCV Ab III; Innogenetics, Ghent, Belgium)}.

- **HCV RNA**

All ELISA anti-HCV-positive samples were submitted to RNA extraction, reverse transcription and a nested PCR with primers complementary to the conserved area of the 5'_ NC region of HCV, essentially as described by **(Ginabreda et al., 1997).** HCV genotyping was determined in all HCV RNA-positive samples. A line probe assay (Inno-LiPA HCV II; Innogenetics) was used to determine the genotype in the amplicons of the 5_ NC region according to the procedure described by the manufacturer.

- **HCV genotyping**

In order to ascertain the presence of HCV genotype 4 in clinical specimen, HCV-RNA isolated from each HCV-infected patient was amplified in the 5′-untranslated region (UTR) by nested reverse transcription–polymerase chain reaction (RT-PCR) using previously described primers and conditions **(Holland et al., 1994).** The amplified HCV-RNA PCR products were purified using the Wizard SV GEL and PCR Clean-Up System kit (Promega Corporation, Madison, WI, USA), and the nucleotide sequences were then determined by direct double-strand DNA cycle sequencing using the DTCS Quick Start master mix (Beckman Coulter) with each of the internal HCV primers, according to the manufacturer's instructions. Electrophoresis and analysis of DNA sequence reactions were done using the CEQ 8000 sequencer (Beckman Coulter). The derived sequences were then analyzed using the CEQ8000 software (Beckman Coulter), and compared to sequences available from the GenBank database. Only patients with HCV genotype 4 infection were included in this study.

B) Adipocytokines

Serum adiponectin and leptin concentrations were measured by using commercial ELISA (human adiponectin ELISA kit and human leptin ELISA kit; Quantikine, R&D Systems, Wiesbaden, Germany).

C) Serum Insulin levels and HOMA-IR calculation

- **Plasma glucose** concentration was measured by God-PAP enzymatic colorimetric method using Biomerieux test kit, Cat. No. 5127.
- **Serum levels of insulin** were assayed by commercially available radio-immunoassay (Abbott IMx Insulin assay) which is a micro-particle Enzyme Immunoassay for the quantitative measurement of human insulin

The degree of insulin resistance was calculated according to the homeostasis model assessment for insulin resistance [HOMA-IR] measured by multiplying fasting serum insulin (microunits per milliliter) and fasting plasma glucose (micromoles per liter) divided by 22.5 **(Matthews et al., 1985).**

3. Statistical analysis

Statistical analyses were performed with STATISTICA software. All text and table values are expressed as means ± S.D. For analysis of parameters, analysis of variance (ANOVA) was used to address differences between groups. Univariate analysis was made by chi-square test for frequencies and by Mann–Whitney rank-sum test for means. For multivariable analysis, when steatosis was used as dependent variable (i.e.

absence vs. presence of steatosis), we considered as possibly independent variables as body mass index (BMI), HOMA score, plasma adiponectin level, leptin level, ALT, γ-GT, cholesterol and liver fibrosis scores. The sensitivity (Sn), specificity (Sp), positive predicative value (PPV), negative predictive value (NPV) and accuracy calculated for adiponectin, leptin and HOMA by using ROC curves. Pearson's correlation coefficients were used to test the correlation between variables. (P values less than 0.05 were considered to be statistically significant).

4. Results

The baseline characteristics of the study population are shown in Table 1; univariate analysis of factors associated with steatosis showed that there was no significant difference in the age between patients with and without steatosis. Patients with steatosis showed significant increase in BMI, HOMA index, ALT, γ-GT and cholesterol when compared with patients without steatosis.

Patients with steatosis showed significantly lower serum adiponectin and significantly higher serum leptin levels compared to those without steatosis. Serum levels of adiponectin and leptin showed no significant differences between male and female in both studied groups, also, there was no significant difference in the levels of these adipocytokines between CHC patients with mild and moderate steatosis (grade 1 &2) or between different stages of fibrosis. (Fig. 1, 2, 3, 4).

Table 2 shows the sensitivity (Sn), specificity (Sp), positive predicative value (PPV), negative predictive value (NPV) and accuracy calculated for adiponectin, leptin and HOMA. At adiponectin value of 11.6 (ug/ml), the sensitivity and NPV values were (100%) but the specificity (84.1%) and PPV (40.5%) decreased. At the leptin value of 7.5 (pg/ml), the sensitivity was further increased to 90%, but the specificity 84.1 and PPV (79.4%) decreased. In ROC analysis, the area under the receiver characteristic curve for HOMA index (0.997+0.003) was greater than for adiponectin values (0.988+0.009) or Leptin values (0.936+0.029) indicating a greater ability of HOMA index for distinguishing steatosis from non-steatosis group(Fig5a-c).

Table 3 shows the correlation between serum adiponectin and other parameters in patients with steatosis, adiponectin levels showed significant inverse correlation with steatosis grade, BMI and HOMA. It was poorly associated with leptin, insulin, ALT, γGT, cholesterol and triglyceride.

The analysis of factors independently associated with the presence of steatosis according to HCV genotype4 is presented in Table 4.The multivariate analysis of factors showed that steatosis was significantly associated with low adiponectin concentration while, leptin, Insulin, HOMA, ALT, γ-GT and cholesterol were positively associated with steatosis.

Table (1) Univariate analysis of the factors associated with liver steatosis in 74 non diabetic chronic hepatitis C genotype 4 patients

Patients characterization	With steatosis (n =30)	Without steatosis (n = 44)	P-value
Age(mean±SD)	52±0.5	54.5±0.2	0.471
BMI(mean±SD)	26±1.2	23±0.2	0.0001*
No. Male sex	21	33	
No. Female sex	9	11	
Grade of Steatosis ❖ *Macrovesicular steatosis* (no. &%) • Grade 0: (no steatosis) • Grade 1: steatosis up to 30% • Grade 2: steatosis between 30 and 60%; ❖ *Necroinflammatory activity* • . Grade 1: mild • Grade 2: moderate • Grade 3: severe	 0 20(66.7%) 10(33.3%) 20(66.7%) 10(33.3%) 0	 44 (100%) 0 0 40(91%) 4(9%) 0	
Stage of Fibrosis (no. &%) • mild fibrosis (stages 0 to 2) • advanced fibrosis (stages3 to 4)	 24(80%) 6(20%)	 39(88.6%) 5(11.4%)	
HOMA-IR(mean±SD)	4.67	2.59	0.003*
ALT (IU/L)(mean±SD)	97	73	0.0001*
γ-GT (IU/L) (mean±SD)	88	45	0.001*
Cholesterol (mg/dL) (mean±SD)	230	188	0.001*
Triglycerides(mg/dL) (mean±SD)	125	122	0.103

* Significant p<0.05, BMI: body mass index; ALT, alanine aminotransferase; -γGT:gamma-glutamyltransferase

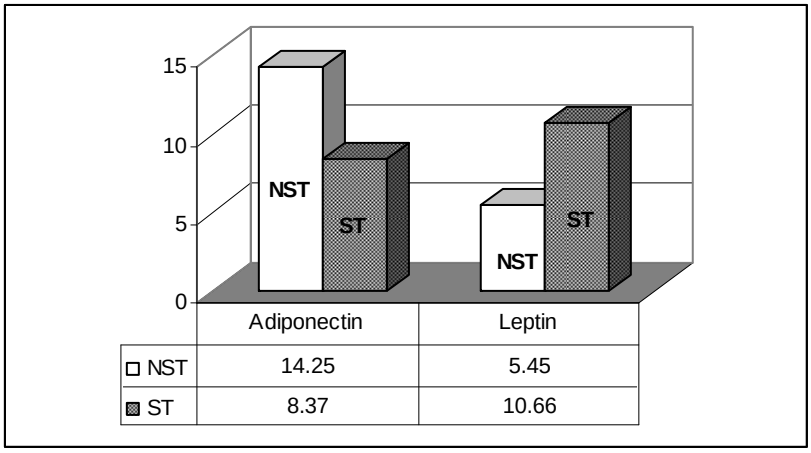

Fig (1) Serum levels of Adiponectin (ug/ml) and leptin (pg/ml) among chronic hepatitis C patients with (ST) and without steatosis (NST)

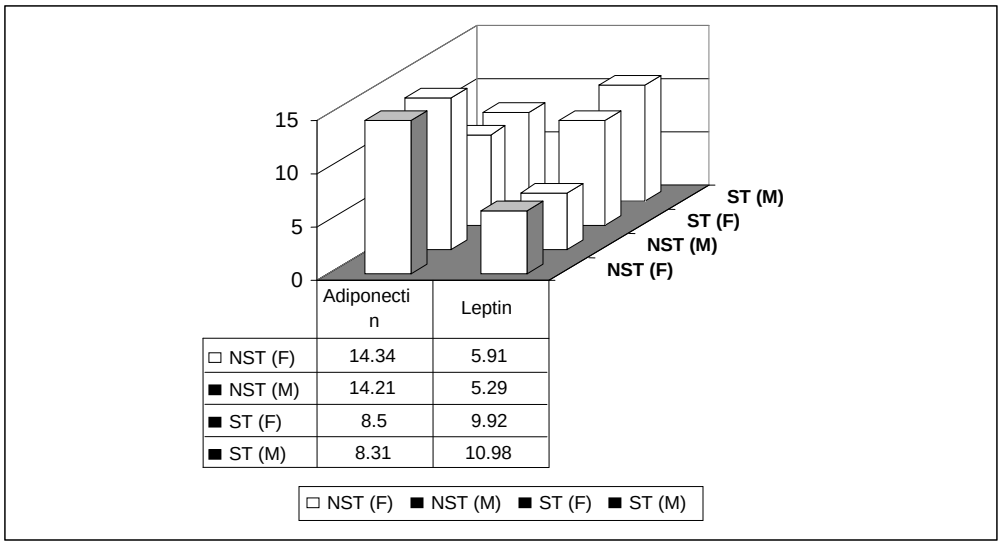

Fig (2) Serum levels of Adiponectin (ug/ml) and leptin (pg/ml) in different Sex (female & Male) among chronic hepatitis C patients with (ST) and without steatosis (NST)

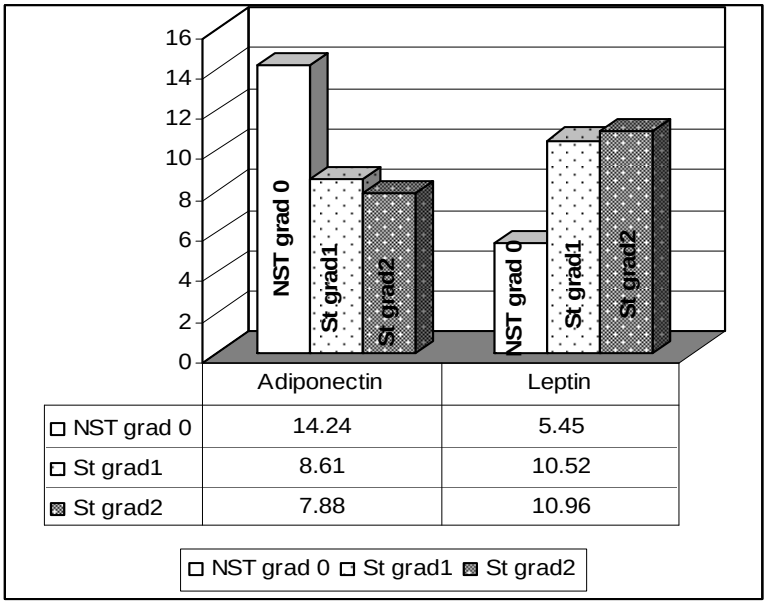

Fig (3) Serum levels of Adiponectin (ug/ml) and leptin (pg/ml) among chronic hepatitis C patients with and without steatosis (According to grade of Steatosis)

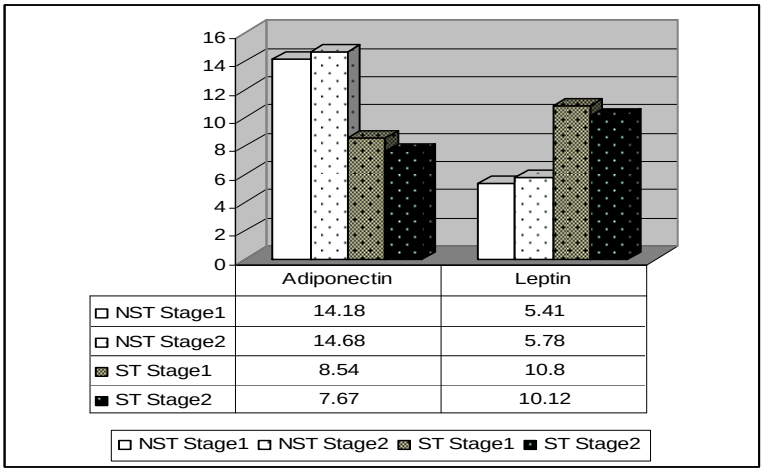

Fig (4) Serum levels of Adiponectin (ug/ml) and leptin (pg/ml) among chronic hepatitis C patients with and without steatosis (According to stage of fibrosis)

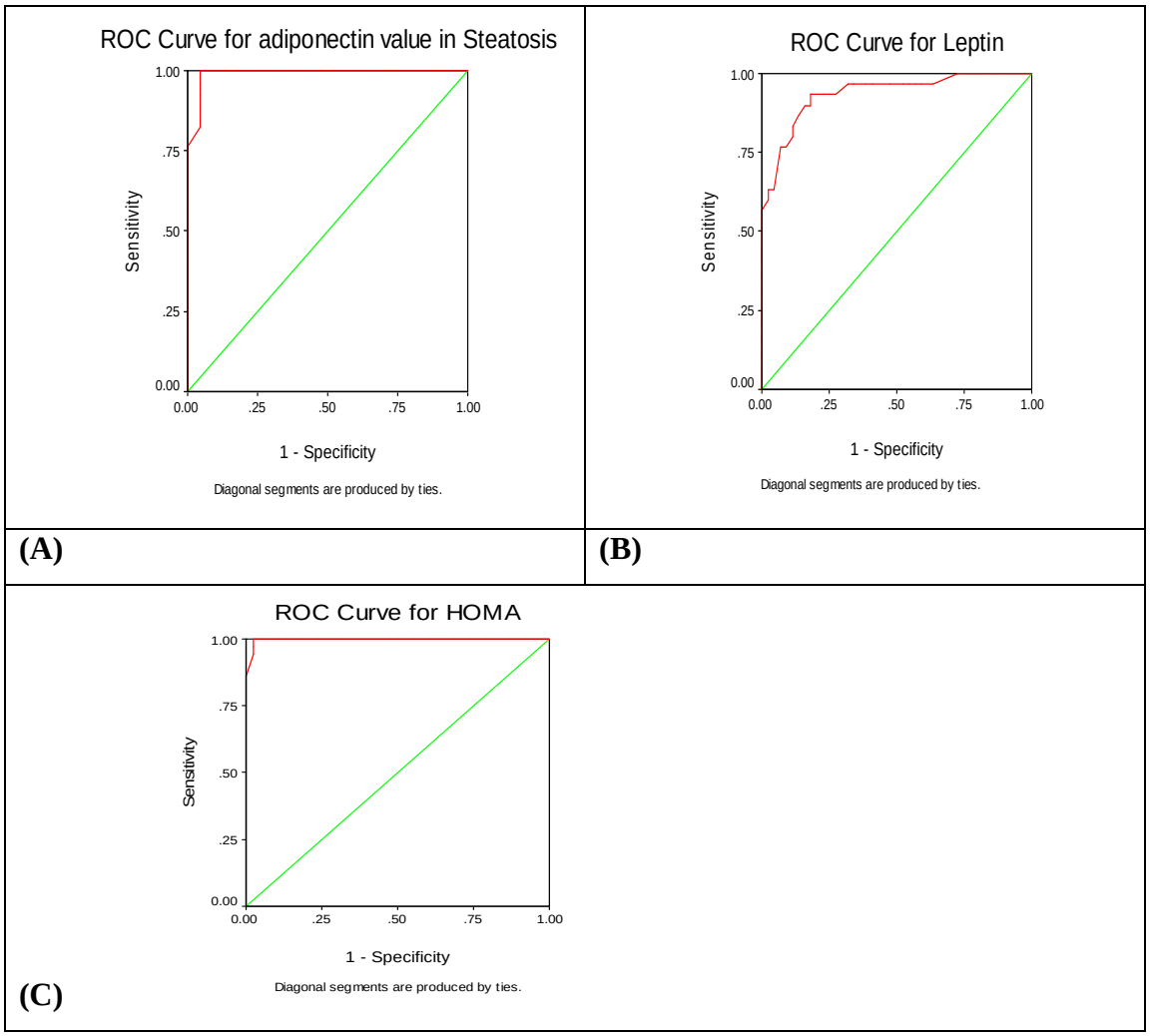

Fig (5) Receiver operating characteristic curve for Adiponectin (A), receiver operating characteristic curve for Leptin (B) and receiver operating characteristic curve for HOMA index (C)

Table (2) Sensitivity, Specificity, PPV, NPV, and Accuracy According to Steatosis

Parameter	Sensitivity	Specificity	PPV*	+NPV	Accuracy
(1)Adiponectin	30/30(100%)	37/44(84.1%)	40.5%	50%	90.5%
(2)leptin	27/30(90%)	37/44(84.1%)	79.4%	92.5%	86.5%
Combined (1)&(2)	30/30(100%)	36/44(81.8%)	78.9%	48.6%	89.2%
(3)HOMA	18/30(60.0%)	26/44(59.1%)	50.0%	68.4%	59.5%
Combined (1)&(3)	30/30(100%)	37/44(84.1%)	81.1%	100%	90.5%
Combined (2)&(3)	27/30(90%)	37/44(81.1%)	79.4%	92.5%	86.6%

Data expressed in percentages *PPV=Positive predicative value, +NPV= Negative predicative

Table (3) Correlation between adiponectin and other variables in patients with steatosis

	Serum adiponectin	
parameters	**r**	**P value**
Age	-0.162	0.394
BMI	-0.334	0.001*
Fibrosis	-0343	0.063
Steatosis grade	-0.372	0.001*
Leptin	0.066	0.729
Insulin	0.019	0.922
HOMA	-0.341	0.001*
ALT	-0.256	0.172
γ GT	-0.221	0.240
Cholesterol	-0.276	0.139
Triglyceride	-0.240	0.201

* Significant p<0.05

Table (4) Multivariate analysis of factors independently associated with steatosis in chronic hepatitis C patients

Dependent Variable	(I) steot.	(J) steot.	Mean Difference (I-J)	Std. Error	95% Confidence Interval for Difference[(a)]	Sig.
BMI	**ST**	**NST**	1.840*	.320	1.202/ 2.478	.000
Adiponectin	**ST**	**NST**	-6.329*	.706	-7.738 /-4.920	.000
Leptin	**ST**	**NST**	4.866*	.798	3.273 /6.458	.000
Iinsluin	**ST**	**NST**	3.581*	.792	2.001/5.161	.000
HOMA	**ST**	**NST**	.928*	.438	5.429E-02/1.801	.038
Cholesterol	**ST**	**NST**	10.308*	4.784	.766/19.850	.035
Triglyceride	**ST**	**NST**	3.040	4.399	-5.734/11.814	.492
ALT	**ST**	**NST**	41.317*	5.929	29.492/53.141	.000
γGT	**ST**	**NST**	40.003*	3.739	32.545/ 47.460	.000

ST; steatosis group, NST; non-steatosis group

* The mean difference is significant at the .05 level.

5. Discussion

Steatosis is an established risk factor for disease progression in chronic hepatitis C. The reported prevalence of steatosis in patients with chronic hepatitis C varies between 40% and 80%, this figure represents an approximately 2-fold increase compared to the prevalence of steatosis in another common chronic liver disease like hepatitis B (20%). This evidence suggests that HCV may directly cause steatosis, at least in some patients **(Monto et al., 2002).**

Cytokines are mediators of cellular communication produced by multiple liver cell types such as Kupffer cells, stellate cells, hepatocytes and endothelial cells. Cytokines can directly induce necrosis or apoptosis. There are also beneficial cytokines, such as adiponectin, which is one of the beneficial cytokines, made outside the liver and appear to protect against liver damage. Adiponectin is specifically secreted by adipocytes that circulate at relatively high levels in the bloodstream **(Kershaw and Flier, 2004).**

The results of this study demonstrated that CHC patients with steatosis had reduced serum levels of adiponectin, with significant inverse correlation between adiponectin level and steatosis grade, HOMA index, BMI and fibrosis stage. These results were in agreement with **Petit et al., 2005** who reported an association between serum levels of adiponectin and HCV related steatosis, they stated that adiponectin is a cytokine secreted by adipocytes with antilipogenic effects that may protect nonadipocyte tissues, such as liver from fat accumulation. Chronic hepatitis C patients have the lowest levels of adiponectin that inversely correlated with steatosis which lead to increased serum free fatty acids, which are then taken up by hepatocytes. In a study done by **Lopez-Bermejo et al., 2004** they found that adiponectin is inversely correlated with BMI, intraabdominal fat and indices of insulin resistance. Two receptors of adiponectin have been cloned; adiponectin receptor 1 is abundantly expressed in skeletal muscle, whereas adiponectin receptor 2 is predominantly expressed in the liver **(Yamauchi et al., 2003).** Growing evidence suggests that adiponectin can regulate lipid and glucose metabolism and lipid fat content in hepatocyte **(Yamauchi et al., 2001).**

Tsochatzis et al, 2007 declared that hepatic steatosis in genotype 4 is mostly associated with metabolic factors, similarly to those in genotype 1 CHC patients and that the actions of adiponectin on the liver are to oppose fatty acid synthesis, and promote mitochondrial oxidation, these actions are exerted through activation of the cyclic-AMP dependent protein kinase (AMPK). Adiponectin also exerts anti-inflammatory effects by opposing the synthesis and release of TNFα from macrophages within adipose tissue. In addition, hypoadiponectinaemia is independently associated with IR and this, in turn, is strictly associated with the development of steatosis. **Dixon et al, 2001** stated that whether hepatic steatosis is a consequence of hepatic or peripheral insulin resistance or whether hepatic steatosis causes hepatic insulin resistance remains unclear. It is likely that excess free fatty acid flux due to peripheral insulin resistance may induce hepatic steatosis. On the other hand, excess fat deposition in the liver may render hepatocytes less sensitive to insulin action and lead to hepatic insulin resistance which occur in early stages of course of HCV infection before the development of cirrhosis **(Petit et al.,2001)**

In our study the result of ROC curve indicated greater ability of HOMA index for distinguishing steatosis from non-steatosis group, studies supported this association suggesting that IR enhances progression to fibrosis by inducing steatosis, implying a complex mechanism in which inflammatory activity and modified cytokine profile have a distinct role **(Lo Iacono et al., 2007),** other studies were not able to demonstrate this association **(Hsu et al., 2008, Papatheodoridis et al., 2006).**

Insulin resistance in chronic HCV infection could be caused by an interplay between viral and host factors. HCV infection per se generates multiple defects in hepatic insulin signaling pathways. The major role of HCV in IR development is also supported by the identification of IR in patients with normal BMI and without significant fibrosis **(Yaneda et al., 2007).** Recently, a study was able to demonstrate a direct role of viral replication in IR development, establishing a significant correlation between HOMA-IR and HCV-RNA levels even after adjustment for age, gender and BMI as known factors which might be confounders **(Pittas et al., 2004).**

In this study serum leptin showed significant increase in patients with steatosis compared with patients without steatosis, also there was no correlation between leptin levels and adiponectin levels. Several studies have evaluated the role of leptin in HCV steatosis. However, some controversial data were obtained; leptin was found associated with steatosis in some studies but not all (**Giannini et al., 2000**, **Manolakopoulos et al., 2007). Giannini et al., 2000** found no relationship

between leptin levels and severity of steatosis. In contrast, another study observed that hepatic steatosis was associated with leptin, BMI, percentage of body fat, and visceral obesity **(Manolakopoulos et al., 2007**). Serum leptin levels were found increased in proportion to the severity of steatosis **(Testa et al., 2000, Lin et al., 2002).** It has been proposed that the liver becomes refractory to the 'anti-steatotic' effects of leptin, a state of 'hepatic leptin resistance' that accompanies hepatic insulin resistance rather than correcting it **(Testa et al., 2000).** There is also a possibility that the relationship of adipose tissue disorders including regional and generalized obesity and lipodystrophies to hepatic steatosis may be due to reduced central or peripheral actions of leptin, an adipocyte derived hormone. High plasma levels of leptin have been related to liver steatosis and steatohepatitis in the obese and nonobese patients [38-39] **(Tobe et al., 1999, Uygun et al., 2000).** In these subjects, leptin resistance may occur centrally or at the level of liver. Patients with severe generalized lipodystrophies who have reduced blood leptin levels are also susceptible to hepatic steatosis. Hepatic steatosis is also observed in *ob/ob* and *db/db* mice that have leptin and leptin receptor mutations, respectively. Similarly, patients with congenital leptin deficiency due to leptin mutations and those with leptin resistance due to leptin receptor mutations should also have marked hepatic steatosis, however, none of the patients described so far have been reported to have liver enlargement or hepatic steatosis **(Ozata et al., 1999)**

In conclusion, our study reported that Egyptian patients with HCV genotype-4 suffering from steatosis had a lower adiponectin level that is inversely correlated with insulin resistance. These data support a role for adiponectin in protection against liver injury and that hypoadiponectinemia may contribute to hepatic steatosis progression. Further molecular and genetic studies with larger numbers of patients are required to confirm these results.

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Comparative studies on the impact of Humic acid and formalin on ectoparasitic infestation in Nile tilapia *Oreochromis niloticus*

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Abstract: Naturally infested *Oreochromis niloticus* (*O.niloticus*) were collected and examined for external parasites (*Trichodina* and *Cichlidogyrus*).The aim of present study was to investigate the efficacy and difference in treatment of formalin as a chemical and Humic acid as a natural product in treatment of ectoparasitic infestation of *O.niloticus*. The prevalence of isolated parasites showed high infestation rate of *Trichodina* (100%) and *Cichlidogyrus* (65%). The main clinical sings of infested fish were slimy dark skin with signs of asphyxia, rapid opericular movement, aggregation of fish on the water surface with gulping the atmospheric air. Flashing of fish, detached scales with frayed fins with presence of hemorrhagic lesions on the skin, fins, gills with congested gills, sluggish movement, finally off food and loss of escape reflex. Hematological parameters of infested fish showed significant decrease in red blood cell counts, hemoglobin percentage and packed cell volume. Total WBCs showed non significant difference. Serum total protein and cholesterol were significantly decreased, while, AST, ALT, alkaline phosphatase, urea sodium and potassium were significantly increased. Chemical treatment using formalin 20 ppm for 24h. As a long bath and natural treatment using Humic acid 3 ppm for 24h. As along bath showed complete eradication of *Trichodina* and *Cichlidogyrus.* [Nature and Science 2010;8(2):121-125]. (ISSN: 1545-0740).

Key words: *Oreochromis niloticus- Trichodina – Cichlidogyrus*- Humic acid and formalin

1. Introduction

Ectoparasitic infestation causes mass mortality and great economic losses such mortalities which are due to the break down of epithelial integrity, resultant loss ionic and osmotic balance. Furthermore, open lesions in the dermis allow fungal and bacterial infections to establish. These together with anorexia, leading to high mortality (Noga 1996 and Eissa 2002).

All fish drugs are toxic to fish fortunately it usually takes higher concentrations of the drug to harm the fish than it does to harm the pathogen. In recent years considerable changes have been happened in attitude towards the use of chemical parasitisides in aquaculture and fish diseases with increasing public awareness concerning the quantities and types of chemicals used and their potential impact on the environment. There is a need for alternative measures to control diseases among farmed fish. Treatment of fish diseases is limited because of only few chemicals approved for use in aquaculture and there are not effective for all pathogens. Chemical treatment may be linked to side effects such as toxic stress (Meinelt *et al* 2000 and 2001). Application of chemical treatment by using formalin is widely used against many protozoan and monogenetic trematodes, showed a great success for eradication of such ectoparasites (Ebtsam and Yonuis (2003), However, as formalin is reducing agent on fish culture. A trial for treatment for such parasites using natural substances as humic acid and Humates is considered of great interest and preferable. Therefore present study was carried out to investigate the prevalence and intensity of ectoparasites, Trichodina, Cichlidogyrus in naturally infesting *Oreochromis niloticus* monitoring their effect on hematological parameters and certain serum constituents with comparative treatment trials of them with either chemical treatment as formalin and natural treatment as Humic acid.

Materials and methods

Fish:

A total number of 120 naturally infested freshwater cultured *O.niloticus* of average weight 80-100 g were obtained alive from private fish farm in Kafr El-Sheikh Governorate in May 2009. Another group 20 apparently healthy and free from parasites was collected from El-Wafaa fish farm. all fish were held in glass aquaria with aerated chlorine free tap water thermostatically adjusted at 24+1^{o}c.fish were fed commercial ration once a day , subjected for parasitic examination and blood examination and treatment with formalin and Humic acid.

Drugs:

Formalin: Obtained from El- Gomheria Company Egypt
Humic acid: (Biofarm) from Grand Vet Company .Egypt

Experimental design:

A group of 120 naturally infested *O. niloticus* fish were examined according to the method described by Paperna (1996) for the presence of external protozoa and monogenetic flukes with recording the prevalence and intensity of each parasite. The infested fish were continuously examined for gross clinical signs and lesions according to Noga (1996). After recording the prevalence and intensity of each parasite, the groups of infested fish were divided into 6 groups of 20 fish each. First 5 groups were used for treatment with formalin and Humic acid for investigating the hematological and biochemical studies, whereas the 6 th group was left without treatment for studying the effect of parasites and mortality rate. Another group of 20 apparently healthy (parasite free) 7 th group was kept as control for hematological and biochemical examination. Blood samples were collected from caudal vein (Lucky, 1977) for estimating total erythrocytic count, total leucocytic count, packed cell volume (PCV) and hemoglobin content according to method described by Lucky, 1977. Prepared serum samples were analyzed for estimation total protein (Wotten and Freeman, 1982), total cholesterol (Richmond, 1973), serum aspirate and aniline transaminases (AST and ALT) (Reitman and Frankel, 1957), Alkaline phosphatase (Kilchling and Feribury, 1951), serum urea (Fawcett and Scott, 1960), serum creatinine (Husdon and Rapoport, 1968) and finally sodium and potassium (Colline and Palkinthome, 1952). The degree of infestation of the parasite was determined by counting the parasites per microscopic field pre and post treatment according to Nahla (1993). Mortality rate pre and post treatment was also recorded. Soon after blood sampling, the first 5 infested groups were subjected to treatment, first group treated by using commercial grade of formalin 20 ppm as long bath for 24 h. 2 nd and 3 rd groups were subjected to treatment with Humic acid at 1 ppm for 48 h and 2 ppm for 24 h. respectively. The 4 th group subjected to treatment with Humic acid with 3 ppm for 60 min. the 5 th group subjected to treatment with Humic acid for 30 min. Smears from skin and gills were taken just before and after treatment for judging the anti-parasite effect for each drug of treatment. The 6 th group was kept as control without treatment for parasitic infestation. Another blood sample was taken from the completely cured infested group after 2 weeks and the 7 th parasite free control group for studying the effect of formalin and Humic acid on the hematological and biochemical parameters .Blood was collected from the caudal vein of five fish in each group at the beginning and at the end of the experiment. The blood sample was divided into two portions. The first portion was kept as a whole blood in heparinized tubes for hematological examination. Serum was separated from the second portion for biochemical analysis.

Clinical examination:

Clinical examination for *O.niloticus* performed according to Austin and Austin (1986)

Results

Clinical signs of *O.niloticus* infested with external parasites:

The study revealed that naturally infested *O.niloticus* with *Trichodina sp*. and *Cichlidogyrus* showed slimy dark skin with signs of asphyxia, rapid opericular movement, aggregation of fish on the water surface with gulping the atmospheric air. Flashing of fish, detached scales with frayed fins and presence of hemorrhagic lesions on the skin, fins **Fig (A)** and gills and congested gills, sluggish movement and finally off food and loss of escape reflex.

Micoscopical examination :

Microscopical examination of skin, fins and gill smears from infested fish showed *Trichodina sp*. **Fig (B)** and *Cichlidogyrus* sp. **Fig (C).** High infestation rate with *Trichodina* 100% and *Cichlidogyrus* 65% .The intensity of infestation of *Trichodina* was higher than that of *Cichlidogyrus*. The effect of formalin and Humic acid treatment, the degree of parasite infestation and mortality rate were compared and recorded in **table (1)** from the obtained results that formalin 20 ppm for 24 h. as long bath was highly effective against ectoparasitic infestation, on the other hand the use of Humic acid at the dose of 3 ppm for 24 h. showed complete eradication of *Trichodina* and *Cichlidogyrus*. Concerning the effect of ectoprotozoal infestation and monogenetic trematodes on the hematological picture **table (3)** showed significant decrease in the red cell counts (RBCs), hemoglobin (Hb) and hematocrite value (PCV) in infested fish in comparison to parasite free control group. There were no significant alteration in total leucocytic counts, there were disturbance in serum constituents of infested fish expressed by decrease of total protein and cholesterol and significant increase in the activity of AST, ALT, Alkaline phosphatase, urea, sodium and potassium levels as shown in **table (3)** serum creatinine level showed non – significant alteration in both infested and non infested.

Table (1) effect of formalin and humic acid on infestation in *O.niloticus*

	Infest. fish pre treat.	Infest. fish treat with formalin		Infest. fish treat with humic acid		Infest. non treated fish
		5 Ppm /48h.	20 ppm/24h.	1 ppm/48h.	3 ppm/24h.	
Trichodina	++++	+	-	-	-	++++
Cichlidogyrus	+++	++	-	++	-	+++
Mortality	12%	14%	19%	1%	2%	25%

*light infestation=protozoa, 1-10 monogenea 2-5
*moderate infestation =protozoa, 12-15 monogenea 8-10
*heavy infestation =protozoa, 15-20 monogenea 10-20

Table (2) effect of formalin and humic acid on hematological picture of *O.niloticus* infested with *Trichodina* and *Cichlidogyrus*.

Group / Parameter	Control non infested fish	Infested Fish before treated	Infested two weeks post treat. with formalin	Infested two weeks post treat. with humic acid
			20 ppm/24h.	3 ppm/24h.
RBCs count	1.63±0.081	1.22±0.061	1.39±0.069	1.62±0.081
Hb	6.3±0.315	5.7±0.285	5.9±0.295	6.3±0.315
PCV%	22.4±1.120	17.0±0.850	19.3±0.965	21.3±1.065
WBCs	4.5±0.225	4.63±0.231	4.35±0.217	4.12±0.206

Table (3) effect of formalin and humic acid on some serum constituents of *O.niloticus* infested with *Trichodina* and *Cichlidogyrus*.

Group / Parameter	Control non infested fish	Infested fish before treated	Infested two weeks post treated with formalin	Infested two weeks post treated with humic acid
			20 ppm/24h.	3ppm/24h.
Total protein	4.8±0.24	4.4±0.22	4.6±0.23	4.7±0.23
Cholesterol	142±6.20	119±5.95	132±6.60	138±6.90
AST	95±4.75	112±5.60	1.5±0.075	97±4.87
ALT	26.3±1.31	29.2±1.46	28.1±1.40	27.3±1.36
Alkaline phosphatase	19.3±0.96	25.3±1,26	22.3±1.11	20.2±1.01
Urea	2.61±0.13	3.7±0.18	2.70±0.13	2.67±0.13
Creatinin	0.57±0.028	0.60±0.03	0.59±0.02	0.58±0.02
Sodium	132±6.60	138±6.90	135±6.75	133±6.65
Potasium	6.2±0.31	6.8±0.34	6.5±0.32	6.3±0.31

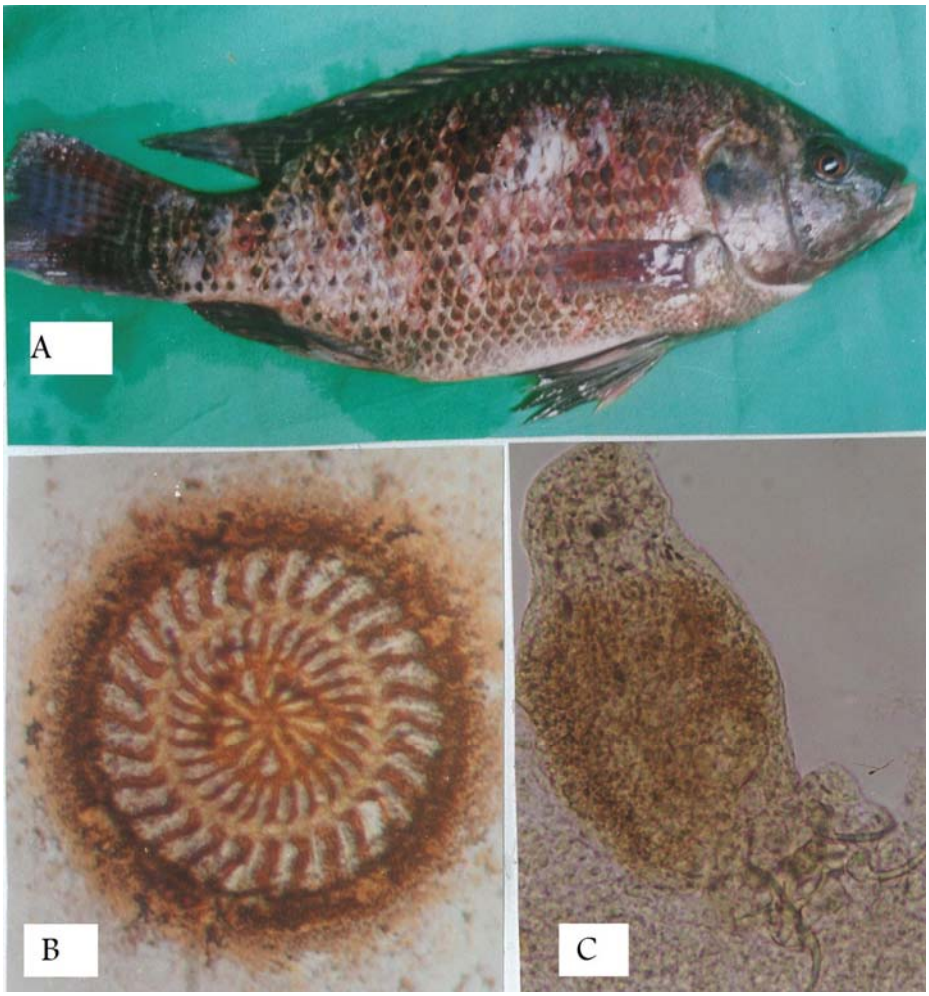

Fig. (A) : Showing skin and fin hemorrhage in tilapia ,(B)*Trichodina* sp and (C) *Cichlidogyrus*

Discussion

Present study aimed to investigate the difference of by chemicals treatment represented by formalin and natural products treatment represented by Humic acid to ectoparasitic infestation, *Trichodina* and *Cichlidogyrus* in *O.niloticus*. The main clinical signs observed were slimy dark skin ,asphyxia with aggregation of fish near the water surface these clinical sings coincide with the finding of Osman (2001) and Eissa (2002).Scale sloughing, skin and fins hemorrhages could be attributed to continuous irritation of adhesive discs of *Trichodina* and movements with feeding activity of *Cichlidogyrus* Eissa (2002). Asphyxia and respiratory dysfunction may be attributed to damaged gill filaments which caused by Cichlidogyrus infestation (Osman 2005).Concerning the effect of formalin and Humic acid it appears from the results that formalin was highly effective against ectoparasitic infestation at the dose of 20 ppm for 24 h. these results nearly agreed with finding given by Woo (1995) and Ebtsam and Younis (2003), on the other hand the use of Humic acid at the dose of 3ppm for 24h. was highly effective in eradication *Trichodina* and *Cichlidogyrus* these results nearly agreed with the results met by Nesreen (2008). The use of natural treatment for controlling parasitic diseases is safer than chemical treatment as formalin which have side effects on fish and water as it is reducing agent lowers oxygen level in water, toxic to fish and of public health importance in food fish when there is residues in fish musculature Brown (2000). Concerning the effects of ectoprotozoal infestation and monogenetic trematodes on hematological picture, significant decrease in the mean red cells counts, hemoglobin percent and main hemochrite value in infested fish in comparison to parasite free control group these results may be due to hemorrhage occur in damaged and destructed skin and gill filaments as the results of parasitic movements and feeding activity also,

due to off food of infested fish (Schaperclaus,1992; Eissa, 2002;Osman, 2005).There were significant alteration in total WBCs count in infested fish. These results may be attributed to stimulation of hemobiotic organs and immune system (Roberts,1989;Ebtsam and Younis ,2003).Disturbance in serum constituents of infested fish was represented as decrease in serum total protein and cholesterol as well as progressive significant increase in activity of AST,ALT, Alkaline phosphatase, urea, sodium and potassium levels, these may be due to progressive irritation and destruction caused by *Trichodina* and *Cichlidogyrus* in case of heavy infestation. Serum liver enzymes (AST and ALT) increased as the results of injury and inflammation of tissues and gills due to parasitic infection. Serum urea significantly increased, these may be attributed to increased ammonia level as a result of disturbance in excretory and respiratory functions of destructed gill lamellae which leads to self poisoning with ammonia (Schaperclaus,1992 and Ebtsam and Younis ,2003).Serum creatinine level showed non significant change in both infested and non infected *O.niloticus*.

Significant osmo-regulatory disturbance was also recorded and expressed as marked increase in serum sodium and potassium levels, which was represented clearly in mortalities rate of infested fish. These may be attributed to destruction of skin epidermis and gill lamellae caused by parasitic infestation. After two weeks of treatment, progressive changes in blood picture and serum constituents were some what improved and returned towards control group. In conclusion, that ectoparasitic infestation in fish should be diagnosed and treated as quick as possible to avoid fish motilities and economical losses and using natural treatment such as Humic acid is recommended and preferable than chemical treatment in fish ectoparasitic infestation as their application is cheap , more safe, and of little side effects on fish.

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