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	effects on rumen fermentation characteristics degradability and consequently lambs performance. Seven concentrates feed mixtures (CFM), contained soybean meal was replaced with untreated Jatropha meal (UJM) by 0%, JMU (CFM^0), 25% JMU (CFM^1), 50% JMU (CFM^2), or heated Jatropha meal (JMH) by 25% (CFM^4) and 50% JMH (CFM^5) or biological Jatropha meal (JMB) by 25% (CFM^{10}) and 50% JMI (CFM^{11}), were formulated to study their degradation kinetics in the rumen, concentration of anti-nutritive compounds and performance of lambs fed tested rations. Biological treated (BJM) was more effective in decreasing anti-nutritive compounds than heat treatment. These were reflecting on the degradation kinetics, where DM and OM and their effective degradability (ED) were higher in (BJM) than (HJM). No significant differences were detected for daily gain of lambs fed rations contained Basel or that contained 50% BJM. Economic cash return was more profit for BJM ration than the Basel ration. Under the conditions of the present experiment, could be concluded that the bacterial treated JCMB could be replaced up to 50% JMB with Soybean meal at CFM. [Abo El-Fadel .M.H., Hussein, A.M. and Mohamed, A.H. Incorporation Jatropha Curcas Meal on Lambs Ration and It's Effect on Lambs Performance. Nature and Science 2011;9(2):15-18]. (ISSN: 1545-0740). http://www.sciencepub.net. **Keywords:** *Jatrofa curcas* meal, biological treated heated treated, degradability and daily gain		
4	**Possibility of Symbiosis between Some Gram-negative Bacteria and *Legionella pneumophila*** [1]H. T. El Zanfaly, [2]H. Rüden and [2]K. Weist [1]Water Pollution Control Dept., National Research Center, Dokki, Cairo, Egypt, [2]Institute of Hygiene, Freie University, 12203 Berlin, Germany zanfalywater@yahoo.com **Abstract**: One of the biotic factors that affect *Legionella* survival and multiplication is the presence of other organisms. Most documents mentioned to the intracellular proliferation of *Legionella* in amoebae and ciliates. It is important to define the relationship that may exist between *Legionella* and other bacteria and the possibility of growth extracellulary in unsterile tap water. The basic experiments involved a comparison for the changes in numbers of *Legionella pneumophila* that was inoculated alone in sterile dechlorinated tap water with that resulted from culturing the same strain in the presence of by-products of culturing four different gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 15142; *Proteus mirabilis* ATCC 14153; *Escherichia coli* ATCC 14229 and *Acinetobacter baumannii* ATCC 19606) separately in sterile tap water. The results revealed somewhat variable stimulation effect for bacteria by-products on *Legionella pneumophila.* The qualitative as well as quantitative variations in the bacterial by-products as a function of variations in strain used and the period allowed to produce the by-products are the variables that affect the results. The first day by-products supporting ability can be arranged in the following descending order: *Prot. mirabilis – Ps. aeruginosa – A. baumannii*. *E. coli* by-product has no supporting activity. From the second day till 25th day the descending order appeared as: *Ps. aeruginosa – E. coli – A. baumannii – Prot. mirabilis.* [H. T. El Zanfaly, H. Rüden and K. Weist, Possibility of Symbiosis between Some Gram-negative Bacteria and *Legionella pneumophila.* Nature and Science 2011;9(2):19-28]. (ISSN: 1545-0740). http://www.sciencepub.net. **Key words:** *Legionella pneumophila*. Gram-negative bacteria. Symbiosis. Bacterial byproducts	Full Text	4
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	Nigeria, to determine the concentrations of pollutant gasses in livestock buildings in order to establish baselines for exposure limits in the context of animal and human welfare in tropical environments. The concentrations of aerial ammonia, nitrous oxide, methane, carbon monoxide, hydrogen sulphide and sulphur dioxide in selected intensively managed poultry pens in Port Harcourt area of Rivers State, Nigeria were measured during the month of November, 2007. Studies reveal that overall mean aerial concentrations of carbon monoxide CO (19.1±1.35 ppm) was the highest mean value recorded and was followed by the 1.06 ± 0.16 ppm and 0.89±0.14 ppm recorded for flammable gas (methane) and ammonia respectively, while the 0.12±0.07 ppm recorded for nitrous oxide was lowest. The study showed that these figures are lower than limits recommended for animals in Europe. [Chidi Nwagwu, Promise N. Ede, Ifeanyi C. Okoli, Okwunna K. Chukwuka and Grace C. Okoli. Evaluation of aerial pollutant gases concentrations in poultry pen environments during early dry season in the humid tropical zone of Nigeria. Nature and Science 2011;9(2):37-42]. (ISSN: 1545-0740). http://www.sciencepub.net. **Keywords:** Pollutant gases, poultry pen, aerial environment, humid tropics, Nigeria		
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15	**Influence of Magnesium and Copper Foliar Application on Wheat Yield and Quality of Grains under Sandy Soil Conditions** El-Saady A. M[1].; F.E.Abdalla[1]; A.E. EL-Metwally[2]; S.A.Safina[2] and Sara, S. El-Sawyi[1]. [1].Fertilization Technology Dept., National Research Centre (NRC), Dokii – Cairo - Egypt [2.] Agronomy Dept., Faculty of Agriculture, Cairo University **ABSTRACT:** Two field experiments were conducted during the winter seasons of 2007/2008 and 2008/2009 at Ismailia Experimental Station, Agriculture Research Center, Ismailia Governorate, to study the influence of foliar feeding with magnesium , copper either as single nutrient or in combination on yield ;yield components and grains quality of wheat (*Triticum aestivum* L.) cv. Sakha 94. Nine treatments were applied: two levels of Mg, two levels of Cu and four combined treatments (Mg + Cu), in addition to control treatment. Results showed that positive significant effect on plant height (cm), tillers number/m^2, spike number/m^2, spike length (cm), spike weight (g), grains number/spike, grains weight /spike (g), 1000-grain weight (g), grains yield/fed. and straw yield/fed. were achieved by spraying the of copper and magnesium treatments. However, the highest significant increment in grain yield was obtained by spraying the highest Cu level (1.68 kg Cu/fed.), while spraying the lowest Cu level (0.84 kg Cu/fed.) gave the highest straw yield. On the other hand, combination treatment (6.72 kg Mg + 1.68 kg Cu/fed.) showed the highest values for protein, N, Mg, Cu and Zn contents. However, spraying wheat plants with low Cu level (0.84 kg Cu/fed.) gave the highest value of grain carbohydrate percentage. [El-Saady A. M.; F.E.Abdalla; A.E. EL-Metwally; S.A.Safina and Sara, S. El-Sawyi. **Influence of Magnesium and Copper Foliar Application on Wheat Yield and Quality of Grains under Sandy Soil Conditions.** Nature and Science 2011;9(2):109-115]. (ISSN: 1545-0740). http://www.sciencepub.net. **Keywords**: Wheat, Magnesium, Copper, Foliar application, Yield, Quality and Sandy soil	Full Text	15

Hepatoprotective activity and antioxidant effects of El Nabka *(Zizyphus spina-christi)* fruits on rats hepatotoxicity induced by carbon tetrachloride

Heba Ezz El- Din Yossef, Abeer A. Khedr* and Mohamed Z. Mahran

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Abstract: The present study was designed to evaluate the protective effect of the El Nabka (Zizyphus *spina-christi)* fruits as an antioxidant against carbon tetrachloride (CCL_4) induced oxidative stress and hepatotoxicity in Albino Wistar rats was investigated. Subcutaneous injection of CCL_4, produced a marked elevation ($P<0.05$) in the serum levels of aspartate transaminase(AST), alanine transaminase(ALT) and alkaline phosphatase(ALP). Daily dietary containing powder of ZSCF at 2.5, 5, 10, and 15% of basal diet for 6 weeks produced a reduction in the serum levels of liver enzymes. ZSCF has also restored normal levels of malondialdehyde and retained control activities of endogenous antioxidants such as Superxide Dismutase (SOD), and Glutation Peroxidase (GSH). Therefore, it is concluded that ZSCF can protect the liver against CCL_4-induce oxidative damage in rats, and the hepatoprotective effect might be correlated with its antioxidant and free radical scavenger effects.

[Heba Ez. Youssif, Abeer A. Khedr and Mohamed Z. Mahran. **Hepatoprotective and antioxidant effects of *Zizyphus spina-christi* fruits on carbon tetrachloride induced hepatotoxicity in rats.** Nature and Science 2011;9(2):1-7]. (ISSN: 1545-0740). http://www.sciencepub.net.

Keywords Zizyphus, hepatotoxicty, antioxidants- and malondialdehyde

1. Introduction:

The liver is one of the most important organs, owing to its biological functions such as drug metabolism, amino acid metabolism, lipid metabolism and glycolysis. Acute and chronic liver diseases constitute a global concern, and medical treatments for these diseases are often difficult to handle, and have limited efficiency **(Lee *et al.,* 2007)**. Therefore, there has been considerable interest in role of complementary and alternative medicines for the treatment of liver disease. Developing therapeutically effective agents from natural products may reduce the risk of toxicity when the drug is used clinically.**(Shen *et al.,* 2009).** Chemicals such as carbon tetrachloride (CCL_4) catabolised radicals induced lipid peroxidation, damage the membranes of liver cells and organelles, causes the swelling and necrosis of hepatocytes and result to the release of cytosolic enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphates (ALP) into the circulating blood. (**Singh *et al.,* 1998; Xiong *et al.,* 1998)**. The major component of the antioxidant system in mammalian cells consists of three enzymes, namely, Superxide Dismutase (SOD), Catalase (CAT) and glutathione peroxidase. These enzymes work in concert to detoxify superoxide anion and hydrogen peroxidase (H_2O_2) in cells. Therefore, reducing oxidative stress may be an effective therapeutic strategy for preventing and treating hepatic fibrosis **Amin and Ghoneim (2009).**

Zizyphus *spina-christi* belongs to the family Rhamnaceae and grows throughout Upper Egypt and Sinai . Zizyphus has a common name ‘‘Nabka”, Arabs used it to maintain a healthy lifestyle and used for soothing properties **(Adzu *et al.,* 2002)**. Zizyphus species are commonly used in folklore medicine for the treatment of various diseases such as digestive disorders, weakness, liver complaints, obesity, urinary troubles, diabetes, skin infections, loss of appetite, fever, pharyngitis, bronchitis, anemia, diarrhea, and insomnia **(Han and Park, 1986; Kirtikar and Basu, 1984).** The genus Zizyphus is known for its medicinal properties as hypoglycemic, hypotensive anti-inflammatory, antimicrobial, antioxidant, antitumour,

and liver protective agent and as an immune system stimulant **(Said *et al.*, 2006).** They widespread in the Mediterranean region, Africa, Australia, and tropical America. Previous phytochemical studies on the different species of the genus Zizyphus led to the isolation and characterization of cyclopeptide alkaloids, flavonoids, sterols, tannins, and triterpenoid saponins **(Ikram *et al.*, 1981; Nawwar *et al.*, 1984)**. Phytochemical studies of the genus Zizyphus have revealed that peptide and cyclopeptide alkaloids, flavonoids, sterols, tannins, betulinic acid and triterpenoidal saponin glycosides have been isolated and chemically identified**. (Ikram *et al.*, 1981; Higuchi *et al.*, 1984; Nawwar *et al.*, 1984; Han *et al.*, 1990; Barboni *et al.*, 1994; Abu-Zarga *et al.*, 1995; Cheng *et al.*, 2000; Shahat *et al.*, 2001; Tripathi *et al.*, 2001).**

The aim of this work was to evaluate the role of Zizyphus spina-christi fruits to reduce the hepatotoxicity in rats that induced by CCL4.

2. Material and Methods:

Plant material

The fruits of Zizyphus *spina-christi* were collected from Sinai, Egypt in January 2010, and the plants were identified by the corresponding author. The fruits were cleaned and washed under tap water .Seeds were separated, dried at 40 ◦C under-vacuum oven and then crushed to a fine powder.

Total phenolic content

The total phenolic conten was estimated using the Folin–Ciocalteu's reagent **(Obanda and Owuor, 1997; Singletion and Rossi, 1965)**. A calibration curve of gallic acid (ranging from 5 mg/ml to 30 mg/ml) was prepared and the results were determined using gallic acid standard curve, and expressed as milligram of gallic acid equivalents per gram of the extract.

Animals

Thirty six adult male albino rats, weighing (150-170) g each, were obtained from Medical Insects Research Institute, Doki, Cairo, Egypt. Rats were housed in wire cages under normal laboratory conditions and were fed on standard diet for one week as an adaptation period. Diet was introduced to rats in special food cups to avoid scattering of food. Also, water was provided to rats by glass tubes projecting through the wire cages from an inverted bottle supported to one side of the cage. Food and water were provided ad-labium and checked daily. Standard diet was prepared from fine ingredients according to **AIN, (1993).**

Experimental groups:

Male Wister rats were randomly divided into two main groups, the first, negative control group (n=6), fed on basal diet and the second hepatotoxic groups (n=30), which were subjected to subcutaneous injection of a single dose of 0.3 ml/kg CC14 mixed with equal volume of corn oil on the 7th day. **(Saraswat *et al.*, 1993) .**

Hepatotoxic groups (n=30) were divided into 5 subgroups 6 rats per group (1) positive control fed on basal diet, group (2) fed on basal diet containing 2.5% of ZSCF, group (3) fed on basal diet containing 5% of ZSCF, group (4) fed on basal diet containing 10% of ZSCF powder and group (5) fed on basal diet containing 15% of ZSCF powder. Food intake was calculated daily and rats were weighed weekly. Feeding and growth performance were carried out by the determination of food intake and body weight gain

Blood and tissue collection:

At the end of the experiment (8 weeks) rats were starved for 12h then sacrificed under general anesthesia (ether). Blood samples were collected into clean dry centrifuge tubes, stored at room temperature for 15 minutes, put into a refrigerator for one hour; centrifuged at 3000 rpm for 10 minutes to separate serum. Serum was carefully aspirated and transferred into dry clean Wassermann tubes using Pasteur pipette and then kept frozen at - 20˚ C till analysis.

Biochemical assays:

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) , gamma glutamine transferase (GGT), alkaline phosphates(ALP) and total bilirubin (T.B) were determined using commercially available kits (Alkan Medical, Cairo, Egypt) according to the manufacturer's instructions.

The most prominent product of lipid peroxidation (LP) is malondialdehyde (MDA) which is used as an indirect index of LP in biological system **(Sorg, 2004).** The method of **Uchiyama and Mihara (1978)** was used to determine MDA, based on its reaction with thiobarbituric acid to form a pink complex with maximum absorption at 535 nm. The superoxide dismutase (SOD) enzyme activity was determined according to the method described by **Sun and**

Zigman (1978) .This method was based on the ability of SOD to inhibit the auto-oxidation of epinephrine at alkaline pH to adrenochrome and other derivatives, which were easily monitored in the near-UV region of the absorption spectrum.

The glutation peroxidase (GSH-Px) activity was assayed by the method of Rotruck ***et al.*** **(1973)**.

Serum total cholesterol, triglyceride (TG) and high density lipoprotein (HDL-c) were determined using the methods described by of **Allain *et al.*,(1974), Fossati and Prencip (1982) and Lopez-virella (1977)** respectively. The determination of low density lipoprotein cholesterol (LDLc) and very low density lipoprotein cholesterol (VLDLc) were carried out according to the methods described by **Lee and Nieman (1996)** calculation was as follows:

$$\mathbf{VLDL_c = \frac{TG}{5}} \quad \mathbf{And\ LDL_c = Total\ cholesterol - (HDL_c + VLDL_c).}$$

Statistical analysis

SPSS (version 10) (SPSS Inc., Chicago, IL, USA) was used to carry out a one-way analysis of variance (ANOVA) . When significant differences were detected by ANOVA, analyses of differences between the means of the treated and control groups were performed using Dennett's t-test.

3. Results and Discussion

The proximate chemical composition of dry ZSCF was presented in **table (1)**. The results indicated that carbohydrate content (83.18%) was high in dry ZSCF while protein (4.7%), moisture (5.4%) and fat (0.94%) contents were low. These results are in agreement with those obtained **Berry-Koch *et al.*** **(1990)** and **Abdelmuti (1991)**. Also, **Saied *et al.*** **(2007)** observed that the flesh of ZSCF is rich in carbohydrates. Also, **Nour *et al.*** **(1987)** and **Abdelmuti (1991)** found that one hundred gram dried fruit pulp contains 314 calories, 4.8 g protein and 0.9 g fat .

The present results also indicated that ZSCF contained high level of total phenolic compounds (7.55mg /g as gallic acid) .Other investigators reported that ZSCF has many compounds such as peptide and cyclopeptide alkaloids, flavonoids, sterols, tannins, butulinic acid and triterpenoidal saponin glycosides (**Ikram *et al.*, 1981; Higuchi *et al.*, 1984; Nawwar *et al.*, 1984; Han *et al.*, 1990; Barboni *et al.*, 1994; Abu-Zarga *et al.*, 1995; Cheng *et al.*, 2000; Shahat *et al.*, 2001; Tripathi *et al.*, 2001).**

Data in **table (2)** illustrates the mean value of liver functions for normal and hepatotoxic rats. Levels of ALT and AST in serum were used as biochemical markers to evaluate the hepatic injury. The serum ALT, AST, GGT and ALP levels and T.B content were significantly higher ($P \leq 0.05$) in hepatotoxic groups than that in normal group. However the values of ALT, AST, GGT and ALP for 10% and 15% ZSCF levels were significantly lower than that of positive control. Serum T.B content level in 5, 10 and 15% ZSCF groups were significantly lower ($P \leq 0.05$) than that of positive control. Carbon tetrachloride (CCL_4) induced a severe hepatic damage, which represented in markedly elevating activities of ALT and AST in serum **(shen *et al.*, 2009).** Similar results were obtained by **Amin and Ghoneim (2009)** who found that serum AST and ALT levels were significantly increased in the fibrosis group compared to that in the normal group, but were significantly decreased in the ZSCF treated groups . Compared to that of normal rat, serum GGT activity increases 6.90 times after 8 weeks of CCL_4 induction; serum AlP content decreases 66% after 8 weeks of CCL_4 induction. ZSCF restores normal levels of both AlP and GGT in serum reduced the CCL_4-induced levels of ALT and AST. **Shen *et al.*** **(2009)** concluded that CCL_4 induced a severe hepatic damage, which represented in elevating markedly activities of ALT and AST in serum.

Results in **table (3)** represent the mean values of lipid profile in normal and hepatotoxic rats. It was clear that serum cholesterol, triglyceride, LDL and VLDL were significant ($P<0.05$) higher in hepatotoxic positive control groups compared to that in normal group (negative control group). The hepatotoxic groups were significantly improved by addition of ZSCF especially at 15% level which

showed similar in serum cholesterol triglyceride, LDL and VLDL levels to negative control group.

HDL in hepatotoxic groups were improved by addition of ZSCF specially at 15% level. It is important to note that peptide and cyclopeptide alkaloids, flavonoids, sterols, tannins, butulinic acid and triterpenoidal saponin glycosides have been isolated and chemically identified from ZSCF **(Ikram *et al.*, 1981; Higuchi *et al.*, 1984; Nawwar *et al.*, 1984; Han *et al.*, 1990; Barboni *et al.*, 1994; Abu-Zarga *et al.*, 1995; Cheng *et al.*, 2000; Shahat *et al.*, 2001; Tripathi *et al.*, 2001).**

Data in **Table (4)** indicated the effect of adding different portions of ZSCF to hepatotoxic rats diet on antioxidant defense system.

MDA is commonly used as marker of free radical mediated lipid peroxidation injury **Amin and Ghoneim (2009)**. MDA level in serum of hepatotoxic positive control group was significantly ($P \leq 0.05$) elevated compared to that in normal group .Addition of ZSCF to hepatotoxic rats diet reduced ($P<0.05$) MDA levels compared to positive control group. Increasing the levels of addition of ZSCF to the hepatotoxic rats diet groups resulted in significant decrease in MDA levels. **Amin and Ghoneim (2009)** showed that MDA level in CCL4-induced animals increased significantly by two-fold compared to control level.

SOD is the first line of define to oxidative stress, and GSH is an intracellular reluctant that play major roles in catalysis, metabolism and transport (**Amin and Ghoneim, 2009)**. The mean values of SOD and GSH activity of liver in hepatotoxic group were significantly lower than that of control group , while the 15% level of ZSCF group was recovered significantly ($P \leq 0.05$) compared to the other concentration of ZSCF groups, followed by 10%, 5% and 2.5% ZSCF groups respectively. **Amin and Ghoneim (2009),** reported that pretreatment of ZSCF increased the activity of tested antioxidant enzymes SOD and CAT. The antioxidant properties of flavonoids from various plant extracts reveal their stimulatory action on antioxidative enzymes **(Nagata *et al.*, 1999; Sreelatha** et ***al.*, 2009). Amin and Ghoneim (2009),** showed that ZSCF exerts a therapeutic effect on CCL_4-induced liver fibrosis in rats, possibly through its antioxidant action. **Shen *et al.* (**2009**)** observed that CCL4-induced generation of peroxy radicals and O2•− results in inactivation of CAT and SOD, and they showed that CCL_4 challenge significantly decreased the activities of SOD and CAT in liver.

Zizyphus jujube administration inhibited lipid peroxidation at higher level after CCL_4 treatment. Interestingly, FZJ 200mg/kg was able to increase the activities of endogenous antioxidant enzymes (SOD, CAT, and GSH-Px) and levels of GSH in hepatic tissue. FZJ pretreatment demonstrated to inhibit MDA of the reactive oxygen radical production **(Shen *et al.*, 2009)**.

Data in **table (5)** show the effect of adding different portions of ZSCF to hepatotoxic rats diet on body weight in rats. Before inducing hepatotoxicity, there was no significant difference in the body weight between groups After the period of treatment it is clear that weight of normal group was increased significantly ($P<0.05$) compared to the positive control groups, followed by the weight of 15, 10, 5 and 2.5% of ZSCF groups respectively. The relative body weight gains were increased by 33.97, 30.53, 22.32, 21.57% for 15, 10, 5 and 2.5% of ZSCF groups respectively as compared to positive control group which increased by 15.42% .

Table (1). Proximate chemical composition of dry ZSCF

Protein (%)	Moisture (%)	Fat (%)	Carbohydrate(%)	Ash (%)	T. phenolic mg /g gallic
4.7±0.8	7.4±0.2	0.94±0.3	83.18±029	3.79±0.4	7.55±0.33

Values in the table were expressed as means ± SD

Table (2) Effect of adding different portions of ZSCF to hepatotoxic rats diet on liver functions.

	Control(-)	Control (+)	Levels of ZSCF				LSD
			2.5%	5%	10	15	
ALT (IU/l)	$91.1^{d} \pm 4.03$	$170.75^{a} \pm 1.7$	$125.47^{b} \pm 2.98$	$123.69^{b} \pm 2.36$	$106.56^{c} \pm 0.35$	$103.58^{c} \pm 2.3$	3.8
AST (IU/l)	$44.22^{d} \pm 4.03$	$67.84^{a} \pm 1.70$	$59.75^{b} \pm 2.98$	$57.74^{b} \pm 2.36$	$48.8^{c} \pm .35$	$47.76^{c} \pm 2.3$	3
GGT (IU/l)	$17.36^{e} \pm 0.75$	$65.13^{a} \pm 2.76$	$36.66^{b} \pm 3.63$	$33.72^{b} \pm 1.21$	$27.62^{c} \pm 1.08$	$24.05^{d} \pm 0.65$	3.001
ALP (IU/l)	$34.5^{d} \pm 2.5$	$112^{a} \pm 7.5$	$56.4^{b} \pm 6.2$	$50.0^{bc} \pm 3.3$	$48.15^{c} \pm 2.5$	$37.79^{d} \pm 1.8$	6.68
T.B (mg/ /dl)	$0.37^{d} \pm 0.02$	$0.625^{a} \pm .03$	$0.615^{ab} \pm 0.02$	$0.557^{bc} \pm 0.05$	$0.56^{bc} \pm 0.03$	$0.525^{c} \pm 0.01$	0.048

Values in the table are expressed as means ± SD
Different letters in the same row are significantly different ($P \leq 0.05$)

Table (3). Effect of adding different portions of ZSCF to hepatotoxic rats diet on lipid profile:

	Control (-)	Control (+)	Levels of ZSCF				LSD
			2.5%	5%	10%	15%	
Cholesterol (mg/dl)	$96.15^{d} \pm 3.4$	$143.65^{a} \pm 7.7$	$115.4^{b} \pm 4.7$	$108.35^{c} \pm 3.3$	$101.8^{cd} \pm 2.4$	$96.4^{d} \pm 4.77$	6.9
Triglyceride (mg/dl)	$50.13^{d} \pm 4.2$	$111.1^{a} \pm 6.2$	$70.23^{b} \pm 3.1$	$63.4^{bc} \pm 7.2$	$62.4^{bc} \pm 5.7$	$56.8^{cd} \pm 3.5$	7.8
HDLc (mg/dl)	$52.1^{a} \pm 3.7$	$27.5^{f} \pm 2.3$	$33.33^{e} \pm 1.6$	$38.4^{d} \pm 2.2$	$42.65^{c} \pm 1.7$	$48.3^{b} \pm 1.8$	3.5
LDLc (mg/dl)	$34.1^{e} \pm 1.7$	$93.94^{a} \pm 6.8$	$68^{b} \pm 5.9$	$57.3^{c} \pm 4.7$	$46.7^{d} \pm 0.95$	$36.62s^{e} + 6.6$	7.4
VLDLc (mg/dl)	$10^{d} \pm 0.82$	$22.21^{a} \pm 1.24$	$14^{b} \pm 0.78$	$12.7^{bc} \pm 1.4$	$12.5^{bc} \pm 1.3$	$11.37^{cd} \pm 0.71$	1.56

Values in the table were expressed as means ± SD
Different letters in the same row are significantly different ($P \leq 0.05$)

Table (4). Effect of adding different portions of ZSCF to hepatotoxic rats diet on antioxidant defense system .

`	Control(-)	Control(+)	Levels of ZSCF				LSD
			2.5%	5%	10%	15%	
MDA(nmol/ml)	$7.9^{e} \pm 0.75$	$34^{a} \pm 2.2$	$24.75^{b} \pm 1.7$	$23.5^{b} \pm 0.56$	$13.85^{c} \pm 10$	$10.375^{d} \pm 0.27$	1.88
SOD(unit/prot)	$7.3^{a} \pm 0.28$	$2.71^{f} \pm 0.35$	$3.63^{e} \pm 0.75$	$4.4^{d} \pm 0.45$	$5.3^{c} \pm 0.3$	$6.45^{b} \pm 0.39$	0.68
GSH (mg/dl)	$31.55^{a} \pm 2.9$	$16.93^{f} \pm 2.9$	$20.2^{e} \pm 1.8$	$22.9^{d} \pm 2.5$	$25.1^{c} \pm 3.8$	$27.95^{b} \pm 2.3$	4.12

Values in the table were expressed as means ± SD
Different letters in the same row are significantly different ($P \leq 0.05$)
MDA (molondialdehyde), SOD(Superoxide dismutase enzymes), GSH(glutathione peroxides)

Table (5) . Effect of adding different portions of ZSCF to hepatotoxic rats diet on body weight in rat.

	Control (-)	Control(+)	Levels of ZSCF				LSD
			2.5%	5%	10%	15%	
Initial weight (g)	$162.25^{a}\pm2.22$	$162.25^{a}\pm3.86$	$157.75^{a}\pm4.19$	$160.25^{a}\pm2.87$	$159.75^{a}\pm3.20$	$160.5^{a}\pm3.69$	5.06
Final weight (g)	$233.25^{a}\pm4.27$	$187.25^{e}\pm3.30$	$191.75^{de}\pm4.78$	$196^{d}\pm3.37$	$308.5^{c}\pm2.65$	$215^{b}\pm4.08$	5.66
Relative gain (%)	$43.75^{a}\pm1.69$	$15.42^{e}\pm1.23$	$21.57^{d}\pm2.34$	$22.32^{d}\pm1.78$	$30.53^{c}\pm1.50$	$33.97^{b}\pm0.84$	2.43

Values in the table were expressed as means $\pm$ SD
Different letters in the same row are significantly different ($P\leq0.05$)

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Incorporation *Jatropha Curcas* Meal on Lambs Ration and It's Effect on Lambs Performance

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Absract: This study was conducted to determine the effect of heat (HJM), or biologically with lactobacillus bacteria (BJM), treatments of Jatropha ***curcas*** meal with on concentrate ion of anti-nutritive compounds. In order to replacement of costly imported soybean meal and find out their effects on rumen fermentation characteristics degradability and consequently lambs performance. Seven concentrates feed mixtures (CFM), contained soybean meal was replaced with untreated Jatropha meal (UJM) by 0%, JMU (CFM^0), 25% JMU (CFM^1), 50% JMU (CFM^2), or heated Jatropha meal (JMH) by 25% (CFM^4) and 50% JMH (CFM^5) or biological Jatropha meal (JMB) by 25% (CFM^{10}) and 50% JMI (CFM^{11}), were formulated to study their degradation kinetics in the rumen, concentration of anti-nutritive compounds and performance of lambs fed tested rations. Biological treated (BJM) was more effective in decreasing anti-nutritive compounds than heat treatment. These were reflecting on the degradation kinetics, where DM and OM and their effective degradability (ED) were higher in (BJM) than (HJM). No significant differences were detected for daily gain of lambs fed rations contained Basel or that contained 50% BJM. Economic cash return was more profit for BJM ration than the Basel ration. Under the conditions of the present experiment, could be concluded that the bacterial treated JCMB could be replaced up to 50% JMB with Soybean meal at CFM.
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Keywords: *Jatrofa curcas* meal, biological treated heated treated, degradability and daily gain.

INTRODUCTION

In Egypt sheep and goats industry is the least developed compared to other livestock industries. Feeds costs in sheep production the highest cost of the production requirements and may account 70-80% of costs. Nutrition is an important factor in sheep development, and a variety of nutrients are involved in proper growth and reproductive maturation. On contrast, it is well known that in Egypt, there is a serious shortage in rations and many oil crops had by-product which represent a real problem. jatropha is oil crops belonged to family was known for its toxicity. The toxicity of jatropha was related to contain anti- nutritional compounds, which can effect on animal performance. several study found that addition of 5% detoxified of castor meal in the diet has not been caused adverse effects or nutritive problems on lactating dairy cows, beef cattle and sheep (Alexander, 2008). Moreover, these authors found that calves fed milk from test cows showed neither apparent muscle residue accumulation nor abnormality organs. the heat treatment in combination with the chemical treatment of sodium hydroxide and sodium hypochlorite has also been reported to decrease the anti- nutritional compounds level in jatropha to 75% (Hass and Mittelbach, 2000). Egypt was planted jatropha curcas in different areas (luxor, ismailia, suez and giza). the hectare is yield up to 5 tons seed given about 1.85 tons of oil in the year (El-Gamassy, 2008). the protein quality of the meal obtained from shelled jatropha seeds is high with 1-2% residual oil has a crude protein (cp) content of between 58–64%. The available information on the toxic principles of jatropha is very scanty with feeding. The purpose of this study to investigate the effect of heat or biological treatments on degrading anti-nutritional compounds and their effects on lamb's performance.

MATERIALS AND METHODS

The present study was carried out at el-sero experimental station, belonging to the Animal Production Research Institute, Agricultural Research Center.

Detoxification methods

Heat treatment:

Jatropha curcas meal sample which left after extraction of oil, was heated in boiling water for 15 min to inactivate the anti-nutritional

Lactic acid bacteria (LAB) treatment:

Jatropha meal was treated with lactobacillus acidophilus, at rate of 1g/100kg JM, stored in plastic containers for 21 days at room temperature, then dried to reach about 6% moisture and was ground to pass a 2 mm screen.

Anti-nutritional compounds analysis:

Trypsin inhibitor activity was determined essentially in untreated and treated Jatropha meal samples, according to Smith et al., (1980). Analysis of Lectin content was conducted by haemagglutination assay described by Gordon and Marqardt (1974). Total saponin

(triepennid and steroidal) content was determined using a spectrophotometric method described by Hiai et al., (1976). Phytate content was determined by a colorimetric procedure described by Vairtrash and Laptera (1988). Seven concentrate feed mixtures (CFM's) were formulated to be iso-nitrogenous iso-energetic, by replacing soybean meal contained in the concentrate feed mixture (CFM*), with 25 or 50% of untreated Jatropha meal UJM, for CFMU[1], CFMU[2], respectively. Mixtures of (CFM*), where soybean meal was replaced with 25, or 50% of heated HJM, for CFM[3], CFM[4] mixtures, respectively, or 25 or 50% of treated meal with lacto bacillus bacteria BJM, for CFM[5] or CFM[6] mixtures, respectively. Representative samples of different concentrate feed mixtures, were analyzed according to A.O.A.C, (`1999). Chemical composition of UJM and BJM are shown in Table (1).

Degradability of different nutrients

Nylon bags technique was applied to determine degradability of DM, OM and CP for CFM's as described by Orskov and McDonald (1979). The degradability kinetics of DM, OM and CP were estimated (in each bag) by fitting the disappearance values to be equation $P= a+b (I-e^{c})$ as proposed by Orskov and McDonald (1979), were P represents the disappearance after time I least squares estimated of soluble fractions are defined as the rapidly degraded fraction (a), slowly degraded fraction (b) and the rate of degradation (c). The effective degradability (ED) for tested rations was estimated from equation of McDounald (1981).

Feeding trial was conducted by using twenty male growing male lambs, (18.9 $\pm$1.20kg and 5–6 months). Animal were divided into tow similar (10 animals each). Feeding trials lasted 150 days and animals were fed according to NRC (1994). The control group (R1) received basel ration composed concentrate fed mixture (CFM)50% and fresh berseem (FB)40% and rice straw 10% and, respectively. Meanwhile tested group (R2) received CFM where soybean meal was replaced by 50% of (BJM). Animals were weighed (biweekly). Economical evaluation was calculated for the tested rations according to the prevailing prices of feeds during the time of the experiment. The data were statistically analyzed to test the significant using one way analysis of variance according to SAS, 2004, and Duncan's multiple range test was applied to test significant among means **(Duncan, 1955).**

ESULTS AND DISCUSSION

Chemical analysis of untreated and treated Jatropha meal.

Treatment of JM with lactobacillus (Lac) was resulted in a decrease in CF content by about 18.8%, meanwhile other treatments had quite similar for CF content.

Table (1): Chemical composition (%) of Jatropha meal and anti-nutritional compounds.

Ingredients	Untreated	Treated	
	JM	HJM	BJM
Chemical Composition (%)			
OM	92.76	92.87	92.48
CP	40.83	40.07	43.60
CF	10.77	11.24	8.25
EE	9.45	10.33	9.21
NFE	31.71	31.13	31.92
Ash	7.24	7.13	7.52
Anti-nutritional compounds			
Trypsin inhibitor mg/g	23.30	8.84	4.20
Lectin mg/ml^{-1}	55.41	12.17	7.35
Phytate g/100g	6.50	3.40	2.75
Saponnin %	4.50	3.50	2.40

*JM :untreated Jatropha meal
*HJM : Treated Jatropha meal with heat
*BJM : Treated Jatropha meal with Bacteria

On the other hand CP content was increased by about 6.8%, while other treatment was resulted in a decrease in CP content by about 1.63% and 1.84% (with heat) (Table 1). Ash content was increased by about 4% with biological treatment. Data in Table (1), showed that both treatments had a positive effect on decreasing concentration of anti-nutritional compounds, which consider as inhibitors and negative had effect on animals appetite (Ahmed and Adam, 1979 and Hajos et al., 1995). Bacteria treatment with lactobacillus (LB) decreased concentration of Trypsin inhibitors and lectin by about 82% and 86.7%, respectively. Meanwhile, heat

treatment decreased the concentration of Trypsin inhibitor and lectin by about 75.54% and 83%, respectively.These results are in agreement with Haas and Mittelbach (2000) and Harinder et al., (2008) who reported that heat treatment has a positive effect on reducing Trypsin inhibitor and lectin concentration in JCM. On the meantime, phytic acid concentration was decreased. Saponins concentration of JCM was less affected by different treatment methods, these results agreed with those of Rakshit et al., (2008) who have reported that Saponins was the lowest anti-nutritional compound affected with different treatment methods. So, lactobacillus (LB) treatment had higher effect on reducing anti-nutritional compounds as compared with heat treatment, which had lower effect. On the meantime, Martinez-Herrera et al., (2008) and Belewu et al.,(2010) observed that biological treatment was more effective on decreasing anti-nutritional compounds than heat treatment.

Ruminal degradation kinetics contents (a,b and c) for DM, OM and CP of concentrate feed mixtures (CFM's) are presented in Table (2). It illustrated that washing loss fraction (a) degradable fraction (b) rate of degradation (c) and effective degradability (ED) of DM and OM were less ($P<0.05$) for untreated (UJM with 25% & 50%) levels as compared with the control mixture (CFM).

TABLE (2): Degradation kinetics of DM, OM and CP for experimental concentrate feed mixtures

Ingredients	Experienced concentrate feed mixtures							
	CFM^0	CFM^1	CFM^2	CFM^3	CFM^4	CFM^5	CFM^6	±
DM								
A	28.27^a	26.12^b	23.85^c	27.32^a	26.15^{ab}	28.13^a	27.32^a	1.07
B	55.28^a	52.15^b	49.82^c	54.42^a	53.48^{ab}	55.20^a	54.62^{ab}	1.36
C	0.045	0.042	0.038	0.041	0.038	0.040	0.038	0.004
EDDM	54.46^a	50.39^b	45.52^c	52.78^{ab}	50.16^b	53.84^{ab}	52.71^b	6.58
OM								
A	26.78^a	24.36^b	22.57^c	25.68^a	24.72^b	25.82^a	25.43^a	0.88
B	56.72^a	52.63^b	50.65^c	53.72^b	52.67^b	56.16^a	55.44^a	0.67
C	0.052	0.048	0.042	0.051	0.049	0.052	0.050	0.006
EDDM	56.90^a	52.21^b	47.74^c	53.72^b	52.67^b	56.24^a	54.94^{ab}	7.62
CP								
A	23.42^a	22.62^{ab}	21.53^5	23.18^a	22.92^{ab}	23.28^a	23.12^a	0.53
B	64.46^a	60.82^b	58.33^b	62.18^{ab}	60.18^b	64.32^a	63.63^a	0.65
C	0.054	0.051	0.046	0.053	0.052	0.054	0.053	0.005
EDDM	57.47^a	50.72^b	45.80^c	53.67^{ab}	52.35^b	55.83^a	54.68^a	1.43

A,b and c means in the same raw for each parameters with different superscripts are significantly different ($P<0.05$).

Also, washing loss fraction (a) degradable fraction (b) rate of degradation (c) and effective degradability (ED) of DM and OM were higher ($P<0.05$) for biological treatment as compared with untreated one. Lower soluble fraction (%) and rate of degradation were noticed with untreated JM ration for DM and OM degradation compared to the control. The treatment with bacteria increased DMD and OMD slightly higher than treatment with heat treatment. The decrease of degradability of CFMs containing untreated UJM may be due to the negative effect of Trypsin inhibitor and lectin on ruminal microorganisms. Ahmed and Adam (1979) and Rakshit et al .,(2008) concluded that Trypsin inhibitor content of JM as well as other anti-nutritional compounds are affecting digestibility. The digestibility of CP for CFMs contained untreated UJM was lower than digestibility of CP for CFMs contained treated JM as a result to the high content of Trypsin inhibitor on UJM. On the mean time, the degradability of CP with bacteria treatment was higher than heat treatment, may be as a result to the over protection with heat treatment.

Average daily feed intake, daily gain and economic return for lambs fed experimental rations are shown in Table (3). There were no significant differences between experimental rations concerning the average daily feed intake. There were no significant differences between experimental rations among average daily gain. These results could be due to the positive effect of the biological treatment. These results are in agreement with Belewu, et al, (2010) who reported that treated Jatropha meal has not a negative effect on both daily gain and feed intake.

Results of economical evaluation are shown in Table (3). As a result of replacement 50% Soybean meal with BJM, the average daily feed cost in D2 was decreased by 17.24% than the control group. At the

same time, both economic return and economic efficiency was improved by 4.11 and 19.32%, respectively for D2 as compared with control ration. Under conditions of the present experiment, could be concluded that bacterial treated BJM could be replaced up to 50% of soybean meal in CFM without any adverse effect on lambs performance.

Table (3): Effect of experimental diets on feed intake, daily gain and economic efficiency

Items	Experimental Rations	
	R1	R2
No of animals	10	10
Days of Experiment	150	150
Av. Initial B.W. kg	19.10	18.70
Av. Final B.W. kg	49.5	48.5
Total B.W. gain kg	30.4	29.8
Av. Daily gain g	203	199
Av. feed intake	1234	1206
Av. Daily Feed cost (LE)	1.45	1.20
Price of daily gain LE	5.10	5.00
Economic Return	3.65	3.80
Economic Efficiency	3.52	4.20

LE= Egyptian

a, b, c Means in the same raw having different significantly differ (P<0.05)

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Possibility of Symbiosis between Some Gram-negative Bacteria and *Legionella pneumophila*

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Abstract: One of the biotic factors that affect *Legionella* survival and multiplication is the presence of other organisms. Most documents mentioned to the intracellular proliferation of *Legionella* in amoebae and ciliates. It is important to define the relationship that may exist between *Legionella* and other bacteria and the possibility of growth extracellulary in unsterile tap water. The basic experiments involved a comparison for the changes in numbers of *Legionella pneumophila* that was inoculated alone in sterile dechlorinated tap water with that result from culturing the same strain in the presence of by-products of culturing four different gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 15142; *Proteus mirabilis* ATCC 14153; *Escherichia coli* ATCC 14229 and *Acinetobacter baumannii* ATCC 19606) separately in sterile tap water. The results revealed somewhat variable stimulation effect for bacteria by-products on *Legionella pneumophila*. The qualitative as well as quantitative variations in the bacterial by-products as a function of variations in strain used and the period allowed to produce the by-products are the variables that affect the results. The first day by-products supporting ability can be arranged in the following descending order: *Prot. mirabilis – Ps. aeruginosa – A. baumannii*. *E. coli* by-product has no support activity. From the second day till 25th day the descending order appeared as: *Ps. aeruginosa – E. coli – A. baumannii – Prot. mirabilis*.

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Key words: *Legionella pneumophila*. Gram-negative bacteria. Symbiosis. Bacterial byproducts

INTRODUCTION

Water and moist environments may be the natural habitat for *Legionella pneumophila*, the causative agent of Legionnaires' disease. The principal route of these bacteria transmission is thought to be by inhalation of contaminated aerosols [1, 2]. One of the important factors that should be considered for studying the spread of pathogens through water is the survival of the causative agent which in turn depends on many abiotic factors such as pH, temperature, and nutrients availability [3]. Some pathogens known to survive in low-nutrient waters include *Pseudomonas cepacia* [4], *Pseudomonas aeruginosa* [5], *Legionella pneumophila* [6], *Salmonella typhimurium, Yersinia enterocolitica, Shigella* sp., and enteropathogenic *Escherichia coli* [7, 8]. A study on suspension of *Legionella pneumophila* in sterile distilled and tap water showed longterm survival but no evidence of multiplication [9]. On the other hand, it was demonstrated that naturally occurring *L. pneumophila* multiplied in tap water at 32^{o}, 37^{o} and 42^{o}C [10, 11]. Furthermore, it was reported that *Legionellae* is unable to proliferate in an aquatic environment without their hosts or perhaps complex diverse biofilms. They require preformed amino acids as carbon and energy sources [12].

Concerning the role of biotic factors that may support the growth of *L. pneumophila* in water, Tison *et al.* [13] concluded that the high rate of multiplication of *L. pneumophila* was dependent on active photosynthesis of cyanobacteria. Accordingly, under darkness conditions, which occur in plumbing systems, the cyanobacteria may not be able to support the growth of *L. pneumopila*. Factors other than cyanobacteria photosynthesis may be involved in providing the nutrients necessary for the growth of *Legionellae* in tap water as well as in plumbing systems. Several studies have shown that aquatic protozoa, especially amoebae, can provide the intracellular environment required for the replication and persistence of *Legionellae* [14-18]. It might be that the biofilm formed on pipe walls support the survival and growth of *Legionellae* outside a host cell [19]. *Legionella* resistant to high temperature and entrapment in the biofilm give it an advantage to survive in the hot water pipe lines as well as water storage tanks at homes and hospitals. Static water in building networks is often at warm-water temperatures that stimulate growth in the accumulated sediments [20]. The study of Murgan *et al.* [21] using a biofilm reactor suggested that *L. pneumophila* may persist in the absence of amoebae, but in a model potable water system, the amoebae were required for multiplication of the bacteria.

Only a few studies have attempted to characterize the interactions between water bacteria and *Legionella* in such diverse habitats as free water and biofilms [17]. The satellite growth study demonstrated that

Flavobacterium breve can support growth of a subculture of *L. pneumophila* on an L-cysteine deficient medium [22]. In another study by the same authors [23], suspensions of different density of isolates mixture of non-*Legionellaceae* bacteria appear to enhance the survival or cryptic growth of agar grown *L. pneumophila.* High density (10^8 CFU/ml) of non-*Legionellaceae* caused a decline in *L. pneumophila* numbers within the first week of incubation. Naturally occurring *L. pneumophila* was multiplied in the presence of associated bacteria.

Such information may aid in the design of control measures aimed at preventing or elimination *Legionella* multiplication and spread of Legionnaires' disease and add basic knowledge concerning the ecology of *Legionella*. In the present study, we examined the role of the by-products resulted from sterile water cultured with four gram-negative bacterial species in supporting the multiplication of agar grown strain of *Legionella pneumophila* type 1 (ATTC 33152).

MATERIALS AND METHODS

Four ml of 24 hr broth cultures of *Escherichia coli* (ATCC 14229); *Proteus mirabilis* (ATCC 14153); *Acinetobacter baumanii* (ATCC 19606) and *Pseudomonas aeruginosa* (ATCC 15142) were centrifuged at 4000 r.p.m for 10 min. The sediment of bacteria was then re-suspended in phosphate buffer and inoculated separately in 3 liter sterile flasks contained 2 liter of autoclaved tap water and kept on a shaker at room temperature (18 – 20^{o}C). At intervals, 50 ml sample of each flask were filtered through 0.2 µm pore size membrane filter (Sartoriüs A.G.W 3400, Göttingen-Germany). The sterile filtrate resulted from every strain was transferred to a sterile 100 ml screw cap bottles. Each bottle was inoculated with 0.5 ml of *Legionella pneumophila* type 1 (ATCC 33152) suspension which resulted from picking five isolated colonies from cultured Buffered Charcoal Yeast Extract (BCYE) agar, Oxoid plates, suspended in 5 ml of phosphate buffer and vortex mixed. The same inoculum's of *L. pneumophila* was held in 50 ml sterile tap water as a control. All the inoculated bottles were incubated on a shaker at room temperature. The changes in *L. pneumophila* counts were checked by periodically transfer a 1.0 ml from the inoculated bottles to 9.0 ml phosphate buffer, serially diluted and from each of three dilutions, 2.0 ml was subcultured on BCYE agar plates, incubated at 37^o C for 24 -72 hr and *Legionella* colonies on the un-crowded plates were counted as mean figures and expressed as a colony forming units (CFU) / ml. At the same time, and as controls, 0.2 ml of each of the four bacterial species stock water culture was subcultured on pre-prepared McConkey agar (Oxoid) plates, and counted after incubation at 37^o C for 24 hr.

RESULTS

The possibility of supporting the multiplication of agar grown *Legionella pneumophila* strain was investigated in the presence of bacteria by-products resulted from inoculation of agar grown four laboratory stock cultures of gram-negative bacteria separately in sterile tap water. The changes in *L. pneumophila* counts resulted from inoculation in bacteria by-products were determined. Along the study period, there are some evidence supporting the phenomenon of *L. pneumophila* multiplication. The ability of bacteria by-products to support *Legionella* growth was varied and depends on bacterial species, and the age of by-product used. The first day filtrates resulted from the four tested bacterial species, except *E, coli*, could support *Legionella* multiplication (Tables 1-5). Subcultures from the inoculated first day by-products begin to show the multiplication of *Legionella* at the fifth day in case of *Ps. aeruginosa* and *A. baumannii* and at the twelfth day in case of *Prot. mirabilis.* According to the results at the end of sub-culturing period (after 25 days), *Prot. mirabilis* showed superiority in supporting *Legionella* multiplication followed by *Ps. aeruginosa* and finally *A. baumannii* (Tables 1, 2, 4 and 5). The age of bacteria by-products (depend on how long the bacteria stayed in water before membrane filtration) used to represent another factor in determining the ability of the tested species to support *L. pneumophila* multiplication (Tables 1 - 4). To consider this factor in the evaluation of the by-products activity of the four species used, it was supposed that beginning by the by-products of the second day and all over the period of study, the species that could give much more samples with higher counts of *Legionella* than the control is the most active ones. So, if this evaluation proposal is agreeable, it is possible to arrange the bacterial species used in the following descending order: *Ps. aeruginosa – E. coli – A. baumannii – Prot. mirabilis.* This set of experiments also demonstrated the drop in count of the bacteria including *Legionella* (Table 5) and non-*Legionellaceae* (Tables 1- 5), when inoculated in sterile tap water, (as control), and kept at room temperature. *L. pneumophila* survival was extended to 19–25 days (Table 5). In case of other bacteria strains used, the drop in count was followed by a slight increase or stability in numbers (Tables 1 – 5).

DISCUSSION

Legionella is difficult to grow in the laboratory requiring a specific combination of nutrients in the medium. Their nutritional requirements seemed to contradict the widespread distribution of *Legionella* in freshwater environments where nutrient levels are low. Through this study, a new methodology was provided for studying the possibility of using bacterial by-

products, via a nutritional symbiosis system, for *L. pneumophila* multiplication in water at room temperature. The four bacterial species used in this study are normally existing in water and especially in the biofilm formed on pipe walls and plumbing system at home, hospitals and network. The possibility of by-products constituents' differences by the time was considered by using the filtrate resulted from membrane filtration for bacteria cultured in sterile tap water as a medium for culturing *L. Pneumophila* and checking the changes in numbers by time.

When Yee and Wadowsky [10] demonstrated the possibility of *L. pneumophila* growth in tap water at 37 to 42^{o}C, they mentioned that other investigators [9] were not succeeded to demonstrate this phenomenon through inoculating agar maintained strain of *L. pneumophila* in sterile tap water which incubated at room temperature. In addition, Stout *et al*. [24] findings mentioned to inability of *L. pneumophila* to multiply in a low-nutrient aqueous environment. The present study concentrated specifically on four bacterial species that normally exist in water and associated with biofilm formed on water pipes and plumbing materials that can support bacterial growth. We tried to put the light on the possible role that can be played by some bacterial species for supporting *L. pneumophila* multiplication in water. Room temperature was used to incubate the seeded sterile tap water in order to simulate the usual conditions in pipes of cold tap water. The results confirmed that bacteria by-products may be different in composition from bacterial species to another and also by time elapsed between inoculation the bacteria in tap water and membrane filtration to get the growth by-product.

Considering the ability of the four bacterial species to support *L. pneumopila* multiplication, *Prot. mirabilis* by-products of the first day showed the highest function as multiplication supporter, while by the time the by-product showed weak function. The previous character may be due to the inability of *Prot. mirabilis* to grow and the rapid cells viability losses in sterile tap water (Table 2). The data on the ability of *Ps. aeruginosa* as the supporter for multiplication put it in the second position between the four examined bacterial species (Table 4). The superiority of *Ps. aeruginosa* was confirmed by the work of Stout *et al.* [24]. *A. baumannii* comes in the third position as multiplication supporter (Table 3), while *E. coli* by-products showed no effect (Table 1). It was mentioned that *E. coli* isolated from respiratory infection was not able to stimulate *L. pneumophila* growth as satellite colonies when tested in nutritionally deficient agar media [11]. The high multiplication rate that was observed by previous investigators for *legionella* [25-28] may be due to the presence of different microorganisms and slime materials on water pipes that may provide *Legionella* with essential nutrients to proliferate. The first day by-products may contain proteins affected by the extracellular proteases of *L. pneumophila* [11] producing the amino acids needed for supporting the multiplication.

The sequence of the tested bacteria according to their activities from the second day till the 25th day was varied from that appeared for the first day by-products due to the extension in time and the possibility of more variations in their by-products composition. The by-products after the 12th days lose most of their abilities to support *Legionella* multiplication which may be due to lowering the metabolic activities as survival strategy of these microorganisms. We would like to attract the attention that the failure in detection the cultured strain of *Legionella pneumophila,* whether in the absence or the presence of other bacteria may be due to its changes, by time, to non-culturable form. Pathogen proliferation potential exists in nearly all water systems. Many factors are involved but most importantly are the presence of microbial biofilms, the degree of microbial diversity, and the availability of nutrients. Managing the microbial fouling process to reduce the risk of Legionnaires' disease principally consists of controlling biofilms and limiting microbial diversity within the entire system. Delineation of the factors which are involved in the multiplication of *L. pneumophila* in aquatic habitats may aid in the formation of practical procedures or protocols necessary for the elimination or prevention of its multiplication in water. Other gram-negative, gram-positive and non-culturable bacterial species, which are not included in the present study, may have much growth supporting effects and needs much more studies. Some studies should be carried on the chemical composition of bacteria by-products that produced in sterile tap water to but a clear explanation for the differences between species of bacteria as a multiplication or survival supporter for *L. pneumophila*. Special attention should be given to the hospital distribution system as a source of water contamination by other bacteria that can support *Legionella* survival and proliferation. High population number of heterotrophic bacteria in the hospital tap water should be controlled by achieving the free residual chlorine at levels that ensure safety for patients.

T A B L E 1. *L. pneumophila* behaviour as a result of inoculation in *E.coli* filtrate.

Date / Organism	Changes in *E.coli* and *L.pneumophila* counts (cfu/ml)																			
	26.01	27.01	28.01	29.01	30.01	31.01	01.02	03.02	05.02	07.02	08.02	0.9.02	10.02	12.02	14.02	15.02	16.02	18.02	20.02	22.02	24.02
E.coli	$6.4X10^8$	$1.6X10^6$	$3.2X10^6$	$1.5X10^5$	$2.6X10^5$	$2.5X10^5$	$2.2X10^5$	$2.1X10^6$	$1.4X10^5$	$1.1X10^5$	$2.1X10^5$		$3.2X10^5$	8.0X84	$6.0X10^4$		$2.5X10^4$	$1.0X10^4$	$1.8X10^3$	$2.0X10^3$	
Filtrate with *Legionella*			$2.0X10^6$	$1.2X10^6$	$1.4X10^6$	$4.6X10^5$	$1.3X10^6$	$4.0X10^6$	$1.1X10^6$	$6.0X10^5$	$3.9X10^5$	$5.5X10^5$	$6.8X10^4$	$1.3X10^6$	1.7X103		$3.0X10^4$	$2.0X10^3$	<100	<10	
			$1.2X10^6$	$1.6X10^6$	$3.7X10^5$	$1.3X10^6$	$8.2X10^5$	$8.2X10^5$	$4.3X10^5$	$4.8X10^5$	$4.2X10^5$	$6.2X10^5$	$4.8X10^6$	<100			$1.0X10^5$	$1.2X10^5$	$3.8X10^3$	$5.0X10^3$	
				$1.8X10^6$	$4.8X10^5$	$1.0X10^6$	$1.0X10^6$	$1.1X10^6$	$4.1X10^5$	$5.5X10^5$	$7.2X10^5$	$7.9X10^5$	$2.8X10^6$	<100			$5.0X10^5$	<1000	<100	$5.0X10^1$	
					$2.6X10^4$	$1.0X10^4$	$2.0X10^4$	$8.6X10^3$	$1.8X10^4$	$6.0X10^5$	$1.4X10^6$	$1.4X10^6$	$1.2X10^6$	$1.2X10^6$			$9.5X10^5$	$1.9X10^6$	$1.1X10^6$	$1.1X10^6$	
						$1.0X10^4$	$1.0X10^4$	$7.2X10^3$	$7.1X10^3$	$5.1X10^3$	$6.2X10^3$	$8.4X10^3$	$3.1X10^3$	$7.0X10^3$			<100	<1000	<100	<10	
							<100	$8.0X10^2$	$1.0X10^2$	$2.0X10^2$	$2.0X10^2$	$8.0X10^2$	$3.1X10^5$	$6.0X10^4$			$1.2X10^6$	$1.1X10^4$	$1.1X10^5$	$1.1X10^5$	
								$1.2X10^5$	$8.0X10^4$	$7.0X10^4$	$2.0X10^4$	$3.0X10^4$	$1.0X10^4$	<100			$1.1X10^3$	<100	<100	<10	
									$2.0X10^4$	$2.9X10^4$	$4.7X10^4$	$7.3X10^5$	$1.3X10^6$	$8.0X10^5$			$2.6X10^3$	$9.0X10^3$	<100	$4.1X10^2$	
										$5.8X10^3$	$5.6X10^3$	$1.8X10^4$	$1.6X10^6$	$7.8X10^5$			$1.0X10^2$	<100	<100	<10	
											$2.5X10^3$	$2.1X10^3$	$1.1X10^6$	$1.5X10^6$			$9.0X10^5$	$7.0X10^3$	$8.0X10^3$	<10	
												<100	$2.4X10^3$	<100			<100		<100	<10	
													$1.2X10^4$	<100			$1.8X10^3$	$1.0X10^3$	<100	<10	
														<100			$1.9X10^5$		<100	<10	<10
																	$7.2X10^4$	$2.6X10^5$	<1000	<100	$3.9X10^4$	<10
																		<1000	<100	<10	<10	
																				$8.1X10^5$	$7.8X10^4$	$1.6X10^3$
																				$2.0X10^1$	<10	
																					<10	

T A B L E 2. *L.pneumophila* behaviour as a result of inoculation in *Prot. mirabilis* filtrate

Date / *Organism*	Changes in *Prot. mirabilis* and *L.pneumophila* counts (cfu/ml)																			
	26.01	27.01	28.01	29.01	30.01	31.01	01.02	03.02	05.02	07.02	08.02	0.9.02	10.02	12.02	14.02	15.02	16.02	18.02	20.02	22.02	24.02
P.mirabilis	$1.8X10^8$	$5.0X10^4$	$7.0X10^4$	$9.0X10^4$	$5.2X10^4$	$2.5X10^3$	$8.0X10^2$	$3.5X10^4$	$6.1X10^4$	$4.8X10^4$	1.0X103		6.1X104	<100	<100			<100	<100	<100	
Filtrate with *Legionella*			$1.6X10^6$	$9.0X10^5$	$1.3X10^6$	$4.8X10^5$	$1.0X10^6$	$2.0X10^6$	$1.0X10^6$	$4.8X10^5$	$2.1X10^6$	1.2X106	$7.8X10^5$	$4.8X10^6$	$9.6X10^5$		$3.1X10^5$	$1.0X10^3$	<100	$2.8X10^2$	
			$1.3X10^6$	$1.8X10^6$	$3.4X10^5$	$1.1X10^6$	$9.0X10^5$	$1.1X10^6$	$5.3X10^5$	$1.9X10^6$	1.4X10^5		$4.8X10^5$	$1.6X10^6$	$1.5X10^3$		$8.0X10^4$	$2.6X10^4$	$1.3X10^3$	$2.2X10^4$	
			$1.7X10^6$	$2.9X10^5$	$7.8X10^5$	$7.1X10^5$	$1.0X10^6$	$3.8X10^5$	$4.3X10^5$	u.c.			$6.8X10^5$	$6.1X10^6$	<100		$4.6X10^3$	<1000	<100	$1.2X10^2$	
				$1.4X10^4$	$1.0X10^4$	$<10^4$	$7.2X10^3$	$1.0X10^4$	$3.0X10^4$	$3.0X10^4$			9.3X103	$2.6X10^5$	$5.0X10^2$		u.c.	<1000	<100	<10	
					$1.0X10^4$	$2.0X10^4$	$8.6X10^3$	$2.8X10^3$	$4.1X10^3$	$3.8X10^3$				$2.1X10^5$	u.c.		<100	<1000	<100	$3.0X10^{10}$	
					$2.0X10^4$	$6.3X10^3$	$5.8X10^3$	$2.1X10^3$	$2.6X10^3$				$3.6X10^3$	$2.1X10^6$	$1.6X10^3$		$3.0X10^2$	<100	<100	<10	
					$8.0X10^4$	$1.3X10^4$	$6.1X10^4$	$4.0X10^4$					$2.0X10^4$	$1.0X10^4$	$7.0X10^2$		$1.3X10^3$	<100	<100	<10	
					$6.9X10^3$	$1.9X10^4$	$1.7X10^4$						$1.2X10^6$	$2.6X10^6$	$2.0X10^6$		$2.1X10^3$	$3.0X10^5$	<100	<10	
					$7.1X10^3$	$7.2X10^3$							$6.9X10^3$	$2.0X10^6$	$1.1X10^6$		$5.0X10^2$	<100	<100	<10	
					$3.1X10^3$	$4.2X10^3$								<100			$3.0X10^2$	<100	<100	<10	
													$9.8X10^3$	$1.0X10^4$	$5.0X10^2$		<100		<100	<10	
														$8.3X10^3$	100		$1.4X10^3$	$1.2X10^3$	<100	<10	
															<100		$2.1X10^5$		<100	<10	<10
																$1.1X10^5$	$9.0X10^4$	$3.0X10^3$	<100	<10	<10
																		<1000	<100	<10	<10
																				$1.1X10^4$	$1.0X10^3$
																				$2.3X10^3$	<10
																					<10

u.c.: Un countable

TA B L E 3. *L.pneumophila* behaviour as a result of inoculation in A *baumannii* filtrate

Date / Organism	Changes in A. *baumannii* and *L.pneumophila* counts (cfu/ml)																			
	26.01	27.01	28.01	29.01	30.01	31.01	01.02	03.02	05.02	07.02	08.02	09.02	10.02	12.02	14.02	15.02	16.02	18.02	20.02	22.02	24.02
A.baumannii	$4.9X10^7$	$4.0X10^4$	$1.9X10^6$	$2.6X10^5$	$2.8X10^5$	$4.4X10^5$	$5.0X10^5$	$1.5X10^5$	$7.8X10^4$	$5.4X10^4$	$1.8X10^5$		$9.0X10^4$	$6.5X10^4$	$1.1X10^5$		$1.1X10^5$	$2.0X10^4$	$1.4X10^3$	$2.1X10^4$	
Filtrate with			$1.7X10^6$	$1.5X10^6$	$2.0X10^6$	$5.1X10^5$	$1.2X10^6$	$1.0X10^6$	$1.2X10^6$	$6.3X10^5$	$1.9X10^6$	$5.2X10^5$	$6.3X10^5$	$2.4X10^6$	$7.8X10^3$		$7.0X10^4$	$3.0X10^3$	$4.0X10^2$	$2.0X10^1$	
Legionella			$1.1X10^6$	$1.9X10^6$	$3.1X10^6$	$1.3X10^6$	$1.2X10^6$	$1.0X10^6$	$7.0X10^5$	$7.3X10^5$	$4.1X10^5$	$5.8X10^5$	$2.8X10^6$	$3.4X10^3$			u.c.	$2.5X10^4$	4.0X85	$4.3X10^4$	
			$1.7X10^6$	$3.9X10^5$	$7.2X10^5$	$9.1X10^5$	$1.2X10^6$	$3.5X10^5$	$5.1X10^5$	$6.1X10^5$	$9.1X10^5$	$5.2X10^6$	$1.9X10^3$				$5.9X10^5$	$7.0X10^5$	<100	$8.5X10^2$	
				$2.3X10^4$	$3.0X10^4$	$1.0X10^4$	$1.4X10^4$	$1.2X10^4$	$3.8X10^3$	$6.0X10^3$	$4.3X10^5$	$6.7X10^5$	< 100				$2.0X10^3$	<1000	<100	<10	
					$1.0X10^5$	$3.0X10^4$	$8.5X10^3$	$6.5X10^3$	$4.7X10^3$	$4.4X10^3$		$3.2X10^3$	<100				$2.0X10^2$	<1000	<100	<10	
						$3.0X10^4$	$7.1X10^3$	$5.1X10^3$	$8.3X10^3$	$4.8X10^3$	$5.1X10^3$	$3.2X10^5$	$9.0X10^4$				$1.1X10^3$	<100	<100	<10	
							$3.1X10^5$	$9.0X10^3$	$1.6X10^4$	$2.0X10^4$	$3.0X10^4$	$1.1X10^4$	$1.0X10^2$				$7.0X10^2$	<100	<100	<10	
								$4.0X10^4$	$7.1X10^5$	$1.9X10^5$	$7.5X10^5$	$1.4X10^6$	$5.0X10^5$				$2.8X10^4$	$5.0X10^3$	$7.1X10^4$	$2.9X10^4$	
									$8.1X10^3$	$6.4X10^3$	$4.8X10^5$	$1.1X10^6$	$1.0X10^6$				$1.1X10^3$	$1.0X10^3$	<100	<10	
										$2.7X10^3$	$2.2X10^3$	$1.8X10^3$	$1.0X10^2$				$4.0X10^2$	<100	<100	<10	
												$9.2X10^3$	$1.0X10^4$	<100			$1.0X10^3$		<100	<10	
													$9.0X10^3$	$1.0X10^2$			$3.1X10^3$	$1.0X10^3$	<100	<10	
														<100			u.c		<100	<10	<10
																$4.2X10^5$	$4.2X10^5$	<1000	<100	<10	<10
																		<1000	<100	<10	<10
																			<100	<10	<10
																				<10	<10
																					<10

u.c.: Un countable

TA B LE 4. *L.pneumophila* behaviour as a result of inoculation in *P.aeruginosa* filtrate

Date / Organism	Changes in *P.aeruginosa* and *L.pneumophila* counts (cfu/ml)																			
	26.01	27.01	28.01	29.01	30.01	31.01	01.02	03.02	05.02	07.02	08.02	0.9.02	10.02	12.02	14.02	15.02	16.02	18.02	20.02	22.02	24.02
P.aeruginosa	$2.3X10^9$	$2.0X10^6$	$1.1X10^7$	$6.1X10^7$	$5.4X10^6$	$5.8X10^6$	$5.2X10^6$	$1.2X10^7$	$3.7X10^6$	$4.1X10^6$	$1.5X10^7$		$2.1X10^6$	$2.1X10^6$	$1.2X10^6$		$1.4X10^6$	$2.1X10^6$	$1.0X10^6$	$1.1X10^6$	
Filtrate with *Legionella*			$1.8X10^6$	$1.5X10^6$	$1.1X10^6$	$3.8X10^5$	$1.4X10^6$	$1.0X10^6$	$1.2X10^6$	$6.1X10^5$	$4.8X10^5$	$6.8X10^5$	$5.1X10^5$	u.c.	$1.2X10^4$		$2.4X10^5$	$2.1X10^4$	<100	$2.0X10^3$	
			$1.0X10^6$	$1.7X10^6$	$4.5X10^5$	$4.0X10^6$	$1.1X10^6$	$9.0X10^5$	$5.3X10^5$	$1.1X10^6$	$1.8X10^5$	$5.1X10^5$	$1.9X10^6$	$1.0X10^4$			$1.4X10^5$	$1.0X10^3$	$4.0X10^4$	$2.0X10^4$	
				$1.9X10^6$	$4.6X10^5$	$1.0X10^6$	$9.3X10^5$	$7.4X10^5$	$5.6X10^5$	$4.1X10^5$	$4.4X10^5$	$6.1X10^5$	$1.6X10^6$	$2.9X10^3$			$2.6X10^5$	<1000	$9.0X10^3$	$2.5X10^3$	
					$2.1X10^4$	$2.0X10^4$	$1.0X10^4$	$8.5X10^3$	$1.3X10^4$	$1.8X10^5$	$1.2X10^5$	$5.1X10^5$	$4.8X8^5$	$7.6X10^5$			$1.6X10^6$	$3.8X10^6$	$2.1X10^6$	$2.2X10^6$	
						$7.0X10^4$	$4.0X10^4$	$8.1X10^3$	$4.1X10^3$	$4.9X10^3$	$1.1X10^4$		u.c.	<100			$2.0X10^2$	<1000	<100	<10	
							$2.0X10^4$	$1.0X10^3$	$7.6X10^3$	$6.3X10^3$	$8.1X10^3$	$5.9X10^3$	$6.8X10^1$	$6.0X10^3$			$1.8X10^3$	<100	<100	<10	
								$1.3X10^5$	$9.0X10^4$	$8.2X10^4$	u.c.	$3.0X10^4$	u.c.	<100			$1.2X10^3$	<100	<100	$8.3X10^2$	
									$4.0X10^4$	u. c.	u.c.	$2.2X10^5$	$3.0X10^5$	$4.0X10^5$			$4.9X10^3$	$2.0X10^5$	$8.0X10^4$	$1.1X10^5$	
										$5.8X10^3$	$6.9X10^3$	$5.2X10^3$	$2.3X10^6$	$1.6X10^6$			$3.9X10^3$	$1.0X10^3$	<100	$8.0X10^2$	
											$2.4X10^3$	$3.3X10^3$	$1.8X10^3$	<100			<100	<100	<100	<10	
												$1.3X10^3$	$1.9X10^4$	<100			<100		<100	<10	
													$1.5X10^6$	$1.8X10^4$			$4.0X10^4$	$9.0X10^4$	$7.0X10^4$	$9.8X10^4$	
														<100			$2.4X10^5$		<100	<10	<10
																$3.8X10^4$	$1.6X10^5$	$2.0X10^3$	<100	$3.0X10^1$	<10
																	$1.3X10^4$	$4.0X10^5$	<10	$1.8X10^4$	
																		$3.0X10^3$	<10	<10	
																			$7.0X10^3$	<10	
																				<10	

u.c.: Un countable

TA B LE 5. Survival of *L.pneumophila in* sterile drinking water

Date / Organism	Changes in *L.Pneumophila* counts (cfu/ml)																			
	26.01	27.01	28.01	29.01	30.01	31.01	01.02	03.02	05.02	07.02	08.02	09.02	10.02	12.02	14.02	15.02	16.02	18.02	20.02	22.02	24.02
Legionella in sterilized drinking water	$1.4X10^6$	$7.9X10^5$	$1.3X10^6$	$2.2X10^6$	$5.2X10^5$	$1.8X10^6$	$1.4X10^3$	$1.2X10^6$	$5.0X10^5$	$6.5X10^5$	$8.5X10^5$	$6.1X10^5$	$5.2X10^5$	$6.0X10^3$			$2.0X10^4$	$1.0X10^3$	$1.0X10^3$	u.c.	
	$5.2X10^6$	$1.6X10^6$	$2.7X10^6$	$6.5X10^5$	$1.1X10^6$	$3.1X10^6$	$1.3X10^6$	$5.3X10^5$	$2.5X10^6$	$6.7X10^5$	$7.3X10^5$	$1.5X10^6$	$7.1X10^3$				$3.1X10^5$	$8.0X10^3$	$4.0X10^2$	<10	
	$2.4X10^6$	$2.1X10^6$	$6.1X10^5$	$1.4X10^6$	$1.2X10^6$	$1.1X10^6$	$6.5X10^5$	$3.8X10^5$	$3.2X10^5$	$2.1X10^6$	U.C.	$1.5X10^4$					$5.2X10^5$	$6.5X10^4$	$9.0X10^4$	u.c.	
	$2.0X10^4$	$1.5X10^4$	$4.0X10^4$	$1.0X10^4$	$7.9X10^3$	$2.0X10^4$	$4.9X10^5$	$5.1X10^5$	$7.8X10^5$	$1.1X10^6$	$9.0X10^4$						$7.0X10^4$	$2.8X10^4$	$4.5X10^3$	<10	
	$8.6X10^5$	$2.9X10^4$	$6.0X10^4$	$7.2X10^3$	$1.2X10^5$	$2.8X10^4$	$4.0X10^4$	$1.8X10^4$	$2.0X10^4$	$7.0X10^3$							$1.1X10^5$	$3.0X10^5$	$3.2X10^5$	$3.0X10^5$	
	$4.0X10^4$	$3.2X10^4$	$7.3X10^3$	$4.8X10^3$	$2.6X10^3$	$2.8X10^3$	$2.6X10^3$	$2.0X10^4$	$4.1X10^4$								$7.0X10^2$	<100	<100	<10	
	$3.3X10^5$	$1.9X10^5$	$9.0X10^4$	$6.0X10^4$	$3.8X10^4$	$1.0X10^4$	$2.0X10^4$	$7.5X10^3$									$2.0X10^4$	<100	<100	<10	
	$4.0X10^4$	$7.9X10^3$	$4.3X10^3$	$3.8X10^4$	$6.1X10^5$	$8.3X10^5$	$8.8X10^5$										$5.2X10^3$	$4.1X10^4$	<100	<10	
	$1.1X10^4$	$5.3X10^3$	$6.0X10^3$	$6.5X10^4$	$1.5X10^6$	$9.2X10^5$											<100	<100	<100	<10	
	$3.1X10^3$	$3.0X10^3$	$2.6X10^3$	$1.2X10^3$	<100												<100	<100	<100	<10	
	u.c.	$5.0X10^4$	$9.0X10^4$	$1.9X10^4$													$6.0X10^4$		<100	$3.2X10^2$	
	$9.0X10^4$	$3.0X10^5$	$2.1X10^5$														$7.0X10^4$	$1.3X10^3$	<100	<10	
	$1.2X10^6$	$4.0X10^4$															$8.9X10^5$	$1.0X10^3$	<100	<10	<10
	$8.1X10^5$	$1.2X10^5$	$1.2X10^5$														<1000	<100	<10	<10	
	$2.1X10^5$	$3.0X10^3$															<100	<10	$3.1X10^3$		
	$1.1X10^5$	$4.6X10^4$	$1.0X10^4$	$1.1X10^4$																	
	$5.0X10^3$	<10																			
	<10																				

u.c. : Un countable

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Antifungal Effects of Colloidally Stabilized Gold Nanoparticles: Screening by Microplate Assay

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Abstract: Collidally stabilized gold nanoparticles NPs having sizes in the range of 3-20 nm have been prepared by citrate chemical reduction methods. The gold nanoparticles were characterized employing transmission electron microscopy TEM. The *in vitro* release kinetics and associated antifungal effects were investigated for *Pencillium*. Micro plate reader analyses were utilized for monitoring the antifungal effects. The results provided strong evidence that could warrant the consideration of gold nanoparticles as antifungal material. Such treatment could circumvent the side and passive immune effects of other antifungal material. Also, the nanoparticles thus prepared have the potential and ability of targeting specific sites.

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Key words: gold nanoparticles; colloidally stabilized; antifungal; microplate assay; release kinetics

1 .Introduction

When the size of a material is reduced to the nanometer length scale, its electronic and thus chemical properties manifest a pronounced change [1-3]. In nanometals, the properties of the surface become dominant and this lead to new characteristic properties [2]. In noble metals, the coherent collective oscillation of electrons in the conduction band induces large surface electric fields that greatly enhance the radiative properties of gold and silver nanoparticles during their interaction with resonant electromagnetic radiation [3]. The synthesis of noble metal nanoparticles has attracted an increasing attention due to their new and different characteristics as compared with those of the macroscopic condensed phase. As a result, nanoparticles are applied in a multitude of applications in various fields, such as: medicine, biotechnology, optics, microelectronics, catalysis, information storage, and energy conversions [1]. Gold nanoparticles have larger surface area and higher dispersion owing to their very small size (<20 nm). Moreover, colloidal gold solutions are the subject of an increased interest for investigation of their cytotoxicity properties for applications in pharmacology, medicine, food industry, and water purification, *etc.* Interaction of metal nanoparticles with microorganisms from fungi to viruses, *e.g.* HIV, is an expanding field of research [4]. In addition, Au^{+} ions from gold-based solutions have long term lasting biocidal effects and are known for their thermal stability and low volatility.

In this paper, we focus attention on the preparation and characterization of gold Nanoparticles and the study of their antifungal effects on *Pencillium* fungal species depending on preparation gold Nanoparticles without capped polymer to benefit from a unique property of these nanoparticles. Micro plate based assays, is a rapid and sensitive method for detection, will be utilized for monitoring fungal growth and inhabitation.

2. Materials and methods

2.1. Materials

Tetra aureate chloride hydride $HAuCl_4$, tri-sodium citrate $Na_3C_6H_5O_7$, were provided by Sigma Aldrich, Germany. Hydrogen chloride HCL, Nitric acid HNO_3, and deionized water were provided by El Gomhoria Co., Egypt. All purchased Chemicals were used without additional purification.

2.2. Synthesis of Gold Nanoparticles.

The gold nanoparticles have been synthesized via Ferns method by the citrate reduction of $HAuCl_4$ [6]. A 100-mL 3-neck round bottom flask was cleaned in aqua regia (3 parts HCl, 1 part HNO_3) and rinsed with deionized water. An amount of 100 mL of 1 mM $HAuCl_4$ solution were heated to boiling and refluxed while being constantly stirred. Then,

10mL of a 38.8, 40 of trisodium citrate solution were added quickly. The color of solution had been noticed to change from yellow to black and finally to deep red. After the color change, the solution has been refluxed for an additional 15 minutes. The heat was then turned off and the solution had been stirred until it cooled to room temperature.

2.3. Characterization of Gold Nanoparticles

2.3.1. Transmission Electron microscopy

The size and morphology of the gold were studied using transmission electron microscopy TEM.A Jeol microscope, Japan, operating at an accelerating voltage of 80 kV. The gold samples were first diluted (1:10) in distilled water, and a 20 µL aliquot was applied onto a carbon coated grid. The solution was then left for 1 minute, and the excess was removed from the grid by blotting with a filter paper. The grids were placed in the grid box for tow hours to allow for drying before imaging.

2.3.2. Kinetics of the release study

Gold nanoparticles NPs were placed in a dialysis bag with a cut off of Mw=12 kDa. The bag was suspended in a phosphate buffer saline PBS solution (30 mL, pH 7.4). A continuous release of gold nanoparticles was measured over a period of 10 h. The release rate and order of kinetics were calculated to find the kinetic pattern, similar to the customary patterns observed for the release of many other drugs. The concentration of gold released was determined by atomic absorption spectrophotometry (Z-5000, Hitachi, Japan), Briefly the solution of gold nanoparticles was processed at 120 ºC in concentrated nitric acid in an oil bath for two hours [11-12]. The percentage of gold released was calculated using equation (1).

% cumulative gold released= [Wt / Wc] × 100 (1)

Where Wc is the total gold content in the dialysis bag and Wt is the gold content in the PBS medium at a time.

2.3.3. Micro-Plate Assay Anti-fungal effect.

Pencillium LB111 was purchased from the Department of Microbiology, Al-Azhar University, and Cairo, Egypt. The antibiogram method was used for fungal cell growth on mycotoxins (aflatoxin) [7]. Petri vessels with agarized Czapek Dox medium were inoculated by spreading fungi medium in a 96 well micro plates and were incubated for 24 hrs, at 37 °C. The micro-plate cells were exposed to solutions of gold nanoparticles having different concentrations ranging from 10 µM up to 140 µM. Growth kinetics' in the 96 well micro plate has been optically monitored using a micro plate reader [Tecn Infinity M200]. Again, the turbidity had been measured at wave lengths between 400-700 nm, in order to avoid any interference with the characteristic gold absorbance peak at 520 nm. The growth of fungi in the culture had been monitored by measuring the optical density OD at 700 nm. Samples were analyzed in replicates, at 37 °C. The plate had been exposed to constant shaking between measurements, with the data being recorded every 30 minutes. Survival was calculated from the last point in the growth curve, relevant to a control value.

3. Results and Discussions

The size and morphology of the gold NPs were studied using transmission electron microscopy TEM. The particle size has been obtained using image analysis. The size has been reported as the mean diameter as shown in figure 1. It is clearly apparent that the gold nanoparticles have spherical form with a well-controlled particle size. Also and as would be expected, the particle size strongly depends preparation conditions. To get the distribution of particle size, the particle diameter of some particles which were selected at random from the different visions with each sample was measured. The distribution of particles size of gold became narrower and the average particle size became smaller. An average particle size about 10 nm can be observed with the samples us thprepared.

In Figure 2, the release kinetic for S1and S4 is represented. The two samples were having a particle size of 5 and 20 nm, respectively. The colloidal gold NPs has been placed in a dialysis bag and released into the release medium took place through dialysis membrane [13-15]. The measurements for each sample were conducted using atomic absorption due to the low concentration of gold released in the early stages of the experiments. The method was proven valid and very sensitive in the recognition of the low percentage of the gold released; more particularly in the early stages of the experiment. Continuous release of gold has been observed over the period of the study of 10 hours. The rate of dissolution and the concentration of gold was obeying the zero order kinetics with r^2>0.96. The gold nanoparticles that had been released to the medium are expressed as the cumulative per cent of the drug released versus time. Our results are similar to the patterns observed with other drugs Margalit *et al.* have illustrated the mechanism of release of metal nanoparticles[13-15]. The dissolution profile showed that the rapid release of the gold nanoparticles sample 1 in comparison to the release of sample 4. About 90% of the gold nanoparticles S1 were released throughout the duration of the experiment i.e. after 8

hours. About 80% of the gold nanoparticles S2 were released throughout the duration of the experiment, *i.e.* after 10 h. This rapid gold release was correlated with the particle size and increasing surface area of the gold nanoparticles. Therefore, small nanoparticles underwent more rapid release kinetics than the bigger nanoparticles due to higher diffusability of the small particles. However, there was a difference in the final cumulative drug released throughout the study, the difference was insignificant at $p<0.05$. Therefore, both the sample 1 and sample 2 might be rapidly in release when incorporated in a dosage form and rapidly absorbed when applied in vivo. The above results suggest that the gold nanoparticles would be stable and rapidly released when applied at the infection sites.

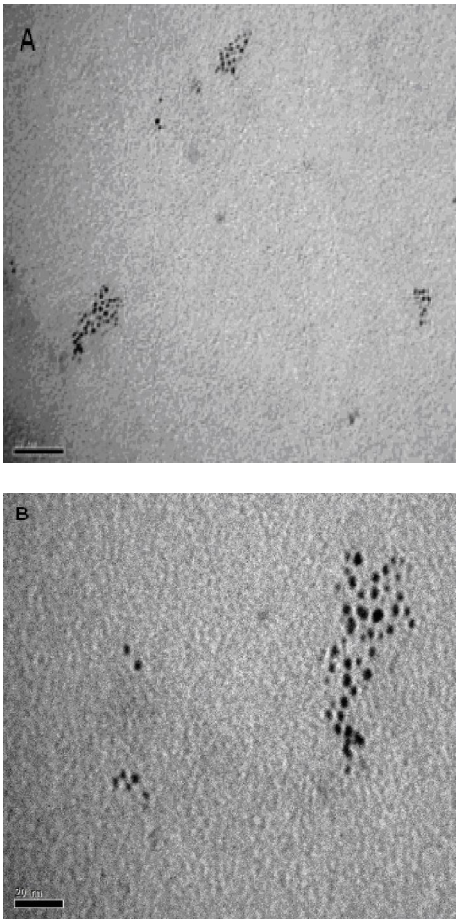

Figure 1. Image of spherical Gold nanoparticles using Transmission electron microscope (Jeol, Japan) operating at an accelerating voltage of 80 kV with scale bar 100nm (A) and with scale bar 20 nm (B).

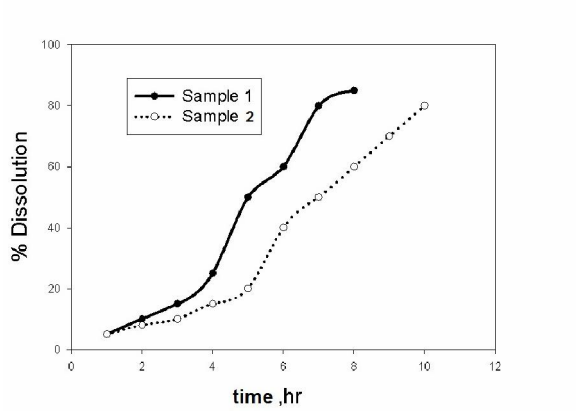

Figure 2. Cumulative amount of per cent gold released versus time using atomic absorption spectrophotometer20. The particle sizes were 5 and 20 nm, in the same order for Samples 1 and 2.

The colloidal gold nanoparticles were tested for their antifungal properties. The antifungal properties of gold nanoparticles against fungal species; namely: *Pencillium* are shown in Figure 3. The growth inhibitory concentration measurements were measured using fractional concentration ranging from10 to 130 µM. The antifungal activity of Gold ions could be described as following reasons: disruption of transmembrane energy metabolism and membrane electron transport chain by formation of insoluble compounds in the cell wall, the formation of insoluble compound may be due to the inactivation of cell wall sulphhydryl group, Gold ions can create mutation in fungal DNA by displacing the hydrogen bonds, gold ions can dissociate the enzyme complexes which are essential for respiratory chain and membrane permeability, disruption of membrane bounded enzymes and lipids could cause the cell lysis [4,5].

Pencillium growths were completely inhibited upon treatment with 130 µM of gold. Beyond any doubt; gold nanoparticles are lethal to fungi species owing to their smaller sizes. The lack of capping polymers on the surface of gold could contribute to the enhancement the NPs lethality. Intuitively this could owe to exposure to the unique properties of the unmodified surface of the nanometal particles [1-3]. It has been generally believed that the mechanism of the antibacterial effects of gold ions Au^{+} involves their absorption and accumulation by the bacterial cells that would lead to shrinkage of the cytoplasm membrane or its detachment from the cell wall [4]. Overall, effects of the gold nanoparticles on fungi are attributed to genome islands encoding a lot of toxins [8].

The complete inhibition of *Pencillium* growth that has been observed here is contrary to the finding of Zharov *et al.* [9]. They could not observe noticeable inhibition of bacterial growth upon treatment with surface modified gold NPs. It is our belief that the inhibition of the growth of microorganisms is not related to penetration of outer cell wall but is also dependent on the size of gold. Additional factors, albeit not completely understood, could come into play; e.g. the energetic of the gold NPs, the kinetics and mechanism of permeability. Nanosize. The interaction of the surface modified nanoparticles with the peptide glycol layer of the cells has a remarkable effect on the inhibition of growth of microorganisms [10]. The result thus obtained clearly indicates that the unmodified nanoparticles with their smaller size and unique surface properties are more potent [1-3]. In addition, the nature of the surface treatment plays a paramount role in the ability of the nanoparticles to inhibit the growth of microorganisms.

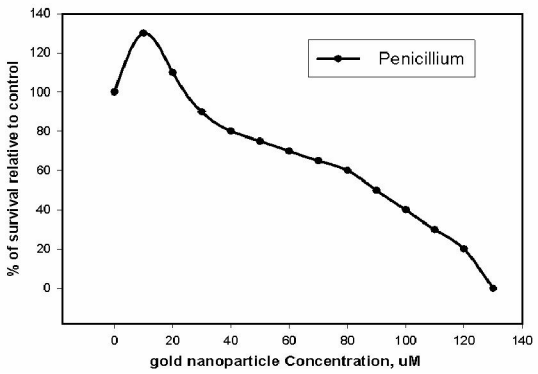

Figure 3. Survival Curve of fungal Strains Using Gold nanoparticles conc. and control, optical density using micro plate reader

4. Concluding Remarks

This work suggests that gold nanoparticles having sizes ranging from 5-20 nm.The results thus obtained lend strong evidence that could warrant the consideration of gold nanoparticles as antifungal agent that could circumvent the side and passive immune effects of other biocidal medications. The kinetic release data indicates that the release of the gold nanoparticles is inversely correlated with the size of the nanoparticles i.e. the release increased with smaller particles. The gold nanoparticle has the capacity to be used in the injection, spray, or lotion form on the infecting site. As such, a gold nanoparticle has a wide range of potential applications in medical applications, therapeutics, sterilization, general hygiene, etc. The unmodified nanoparticles with their smaller size and unique surface properties are more potent in the inhibition of growth of microorganisms. In addition, our results lend additional support to the fact that the nature of the surface treatment plays a paramount role in the ability of the nanoparticles to inhibit the growth of microorganisms.

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The New Cosmic Theory: The Universal Compartments and The Universal Vital factor

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Abstract: The ever expanding universe with limitless frontiers and with the accretions of the cosmic stars, plants and the natural cosmic matters all swim in the matrix of the universe with 'vital force' holding and controlling every thing in the universe. The many theories of the universe have fallen short of convincing the scientific communities, of the nature of the invisible cosmic web and vital factor that's keeping every thing in the universe in an organized unpredictable equilibrium. The first ever principle of a quantitative equation to calculate the 'vital factor' in any vital biological and vital non biological matters in the universe, is explained in this theory. The Universe is composed of limitless, ever increasing and expanding units or blocks that make up the universe called Universal compartments which are composed of invisible and imaginary irregular boundaries that hold on to each other by the Vital force. These forces also control any natural matter in the Universe. An example equation to more easily illustrate this point, can be seen in this quantitative equation: Fvf = EUr, where Fvf is the vital Force, E is the differential energy and Ur is the rate of natural accretion, the rate of intussusceptions, the rate of growth or the rate of changes that occurs in any full form of natural matters in the Universe whether vital biological or vital non biological.
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Key Words: Matters, biological, stars, universe, accretion, changes, radius, gravity, universe, genes, cosmos, earth, galaxy, extraterrestrial, evolution, theory, vital force, planets. black hole, singularity, Planck Era, theory of relativity.

Introduction

Human beings have been wondering about the origin of the universe, this origin and who has created all biological and non biological matters in the universe since life has come into existence. Science myths, Epics, scriptures, old and modern theories had provided us with a different perspective into the nature and origin of the universe and the very variable dates of the appearance of the first biological materials on our planet.

The Sumerians regarded the Universe as consisting of Heaven and earth. Earth was seen as a flat disc surrounded by a hollow space. There was a cosmic explosion at a point which created time, space, force and matter. The Babylonian creation myth, *Enûma Eliš*, which was recovered by Austen Henry Layard in 1849 (in fragmentary form) in the ruined of the library of Ashurbanipal at Nineveh (Mousal, Iraq) and published by George Smith 1876[1]. The Myth is composed of seven tablets, dated ca. 1100B.C.E(2), and described the universe as a primordial formless and empty world. Day and night precede the creation of luminous bodies (*Enûma Eliš* 1:38). Water was the primordial fundamental substance for the creation of the Universe. Since the first theory of Human creation published by the Sumerian 18th Century B.C.

The Eridu Genesis and the Deluge flood, written by Sumerian language on a single tablet , published in 1914 by Arno Poepel(1881-1958) in the companion volume PBS IV (PP.9-70), translated by Thorkild Jacobson (1904-1993), the tablet dated about 18th century B.C. The myth talked about the creation of Mankind, it covers the creation of the world and that the Universe was created from the sea. The Greeks, Egyptians, Persians, Romans, have modeled their Cosmology on early Cosmological discoveries by the Sumerians and Babylonians.

Cosmology and Cosmogony studied and researched for thousands of years and yet many ancient and modern theories still did not answer the many serious questions or provide satisfactory proof about the origin and the mysterious force that keeps the order of the universe. Many modern theories are derived from the ancient's myths like the Big Bang, but still do not answer many important questions. The universe has been expanding from initial singularity, Bid Bang was proposed by Alexander Friedman 1922 and as did George Lemaitre 1927 (3).

The Big Bang model occured roughly 13.7 years ago when the hot and dense universe suddenly exploded and expanded. According to Hubble's Law, Galaxies are known to be moving away from earth at a speed almost proportional to their distance (4). The factor that leads to this sudden expansion according to universal compartments theory is the vital factor which lead and started the Big Bang as well as still having an effect to day and beyond. The vital factor is the factor behind the many minis Bangs continuing in the universe, the accretions, the ever continuous expansion and the infinite order and direction of the Universe.

Albert Einstein's general theory of relativity and the advances of the astrological discovery and observation of the very distant objects have made it possible for the scientific community to speculate about the formation and origin of the Universe and

has allowed the scientists to reach the most accepted idea of the origin of the Universe the Big Bang Theory as the most possible cosmological theory.

Many theories try to explain the origin of the universe from the Big Bang, the Steady State Universe Theory, the Oscillating Universe Theory, the Nebular hypothesis, the fission Theory, the inflation Theory, the capture Theory, the Accretion theory, Planetary Collision Theory, Stellar Theory, Gas Cloud Theory and many other intelligent and random chance models.

The Theory at Time Zero.

The smoky emptiness of space was not empty, it was filled with the universal ingredients "the Universal Smoke" which contain the inert gases and the inert energy in and around the smoke, later condensed and at the centre of the smoke there was a super dense and hot center of the smoke. The smoke and its center point were enclosed in a colossal black hole. A vital factor was the trigger for the explosion of the condensed energy and matter. The vital factor was the spark for the ignition and the super Big Bang cosmology (5) (6) (7), which started the formation of the universe. The Universe is formed of endless and ever increasing Universal compartments.

The Universe is continually expanding since creation from the smoke and gases captured in a colossal black hole 13.7-15 billion years ago. The Big bang is a by-product of the condensation of the Smoke in the deep labyrinth of the Universe ignited by the "Vital factor". Interaction between the particles in the condensed smoke resulted in intense condensation of the power which built up slowly until it reached the precise moment when the big bang become inevitable. Grand explosion followed by massive expansion of the universe. The vast space formation after the Big Bang is formed from different and endless, expanding compartments. These countless compartments have invisible boundaries of immense power "compartment energy" that keeps the minute and massive matter or energy and any thing within this compartment in a controlled mini universe of its own.

The energy that governs and controls one compartment may be the same, close to or different from the energy of nearby or surrounding compartments and when these laws are different the supernova explosion or a black hole will be created and the black hole will disappear, fragmented or changed when the law of physics of the nearby compartment equalized or overcome the that of the black hole continue if the laws of physics continue to be different.

The universe and every thing in it are controlled by a tuned and self destructive program which is the living space program. This program is a living force that existed in time zero which is the time of the big bang.

The compartments of the Universe are composed of layers which interact with each other for exchange of energy. The compartments react with each other in a vital controlled mechanism. The compartments have a cycle of change and stages of evolution. Each compartment is born, lives and dies and ends up in darkness like the cycle of birth and end of a vital matter.

The compartments in the Universe are grouped in layers that interact with each other for exchange of energy and matter. The compartments react with each other like any vital matters. The compartment has a cycle of change and stages of evolutions like active living matter. The compartments, are born, live and eventually will die and end up in different matters or empty darkness, like the cycle of birth and the end of living matters.

Every compartment has a different life and age depending on when it was formed.

All compartments in the Universe are located within a special web of matrix of energy which is the one which keeps stabilizing all matters and energy in all the compartments and the whole universe. All compartments swim in a Universal web of the matrix. This web of matrix is the source of the vital energy to refuel every compartment of the universe.

The Vital force could be measured in the principle of this equation as Fvf equivalent to E (energy) divided by the Ur which is the rate of growth or changeable rate of the matter which could increase or decrease in size.

The Universe has and still expanding and getting faster which defy all the physical laws that dictate that an object in motion will eventually slow down. With time The expansion of matters and energy of the universe will get bigger and faster with increase in its accretion and energy due to the increase universal vital factor which is always on the increase but not infinitely as long as the rate of growth and change of the Universe is not taken over by other Universal forces which if overwhelming may lead to Universal collapse.

Energy of the Universal web which keeps and stabilized all the compartments of the universe. This web of matrix is the source of energy to refuel every compartment of the universe.

Another illustration of a possible measuring technique of the Universal Vital could be seen as follows:

$$Fvf = EUr$$

Fvf (Vital force) equivalent to E (energy) divided by the Ur which is the rate of growth or changeable rate of the matter which could increase or decrease in size.

Conclusion

The Universe originated with a Big Bang. This Big Bang was ignited by the Vital Force which led to the explosion of the super condensed gases in the centre of the inert colossal black hole. The Vital matter is limitless and is always in the Universe energizing, forming, speeding and expanding the matters and energy in the Universe. The Vital Force is an endless form of power that controls the whole Universe. The speed by which particles and matters travel in the universe depend on few factors among which is the vital factor which could enable any matters to travel over the speed of light in special conditions.

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Evaluation of aerial pollutant gases concentrations in poultry pen environments during early dry season in the humid tropical zone of Nigeria

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Abstract: A study was conducted at Port Harcourt, in the humid tropical rainforest zone of Nigeria, to determine the concentrations of pollutant gasses in livestock buildings in order to establish baselines for exposure limits in the context of animal and human welfare in tropical environments. The concentrations of aerial ammonia, nitrous oxide, methane, carbon monoxide, hydrogen sulphide and sulphur dioxide in selected intensively managed poultry pens in Port Harcourt area of Rivers State, Nigeria were measured during the month of November, 2007. Studies reveal that overall mean aerial concentrations of carbon monoxide CO (19.1±1.35 ppm) was the highest mean value recorded and was followed by the 1.06 ± 0.16 ppm and 0.89±0.14 ppm recorded for flammable gas (methane) and ammonia respectively, while the 0.12±0.07 ppm recorded for nitrous oxide was lowest. The study showed that these figures are lower than limits recommended for animals in Europe.
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1. Introduction

The poultry production system in the tropics is essentially categorized into extensive, semi-intensive and intensive production systems. The intensive system usually involves commercial production of high performance exotic breeds of livestock. This system is resource driven and requires the operator to be in control of the housing, nutritional and health needs of the livestock (Williamson and Payne, 1978). The relative success of commercial poultry production in the tropics (Delgado *et al*., 1998; FAO, 2000) has made these livestock business ventures very attractive in most developing countries. The intensification of modern poultry production systems in the tropics is however increasingly regarded as a source of air pollutants, which could be both aggravating and environmentally harmful. Aerial pollutants in livestock buildings include organic and inorganic dusts, pathogens and other microorganism as well as gases (Whyte, 1993; Carpenter, 1996; Wathes *et al*., 1997).

Most gaseous pollutants found in such environment originate from the breakdown of fecal matter, thus, their concentrations will at least in part, depend on the ventilation efficiency and rate, as well as the stocking density and movements of the animals (Wathes *et al*., 1983; MAFF, 1987). Over 100 gaseous compounds are found in the air of livestock building in the temperate zone (Hartung, 1988), and include aerial ammonia, carbon monoxide, sulphur oxide, hydrogen sulphide, nitrous oxide and flammable gas (methane), among others. Most are simple odorants, which may give rise to complaints among neighbors, while some are green house gases. In this zone, concentrations of most of the gases are usually in the range of parts per million (ppm) or lower with the exception of carbon dioxide which may record concentration levels 5 to 10 times higher than the ambient (Wathes, 2001).

Aerial pollutants have economic and public health importance in livestock production (Okoli *et al*., 2006). Their concentration levels and emission rates in livestock buildings when high eventually result in health problems among housed animals. Their public health importance is predicated on the diseases they may cause in livestock workers, when their levels become high in the livestock pens (Okoli *et al*., 2004). Most studies of noxious gases in livestock pens have focused upon ammonia probably because of its' toxicity and role in acid rain formation. However, intensive livestock production contributes to global emission of other important aerial pollutants such as volatile (VOC) and reactive organic compounds (ROC) that impact adversely upon the countryside and has contributed many defects on the ozone layer (Nahm, 2000). Human and animal respiratory health may be compromised

indoors by pollutants such as gases, dust, microorganisms and endotoxins, also addressed as bioaerosols (Hamilton *et al.,* 1993; Hartung, 1994; Hartung *et al.,* 2002).

Specifically, particulate emissions such as dust and microorganisms from buildings play a role in respiratory affections in people living in the vicinity of animal enterprises (Müller and Wieser, 1987; Seedorf, 2004). Interest in air quality in livestock buildings has grown substantially among agricultural, environmental and animal scientists, engineers and veterinarians over the past decade (Okoli *et al*., 2006). Research on the concentrations and emission rates of aerial pollutant gases in tropical livestock buildings is therefore needed in order to establish baselines for exposure limits in context of animal and human welfare in the tropical environments.

This study reports recent field measurements of the concentrations of aerial ammonia, nitrous oxide, methane, carbon monoxide, hydrogen sulfide and sulfur dioxide in selected poultry pens in the Port Harcourt area of Rivers State, in the humid tropical zone of Nigeria, during the month of November 2007.

2. Materials and methods

2.1 Study area: The study area, Port Harcourt, is the capital of Rivers State Nigeria. It is located in the South-south humid geopolitical zone of Nigeria; bounded by latitude 4^o 44` to $4^o$ 52` North of the equator and longitudes 6^o 56` to $7^o$ 07` East of the Greenwich meridian. The climate falls within the sub-equatorial climate belt. It has a mean yearly temperature 30^oC and relative humidity of 80% to 100% and a mean yearly rainfall of about 2327mm (Port Harcourt Master Plan, 1975).

A survey of various farms engaged in commercial layer and/or broiler production was carried out in Port Harcourt. Four farms were selected based on the following criteria, the age of the farm, age of birds, breed type, size of the flock, length of exposure in the industry, proximate to residential areas etc. The husbandry system practiced in farm 1, 2, and 3 were deep litter system except in farm 4 were battery cage and deep litter system with other livestock such as pig, fish, and goat were reared. The age of the litter in the deep litter system ranged from 2 to 20 weeks, while the flock size ranged from 300 to 8000 birds. A random sampling of poultry litter was made in each of the poultry pens and the moisture content analysed within 24 hours of collection according to AOAC (1990) method.

2.2 Husbandry methods employed in the various poultry farms: Table 1 shows the husbandry methods employed in the various poultry farms in early dry season. Flock age in different farms studied ranged from 5 to 8 weeks in broiler farm, 23 to 58 weeks in layer farm and 9 to 20 weeks in pullet farm respectively. Age of litter also ranged from 4 to 6 weeks in broiler farm, 3 to 7 weeks in layer farm and 3 to 12 weeks in pullet farm, flock size ranged from 650 – 1450 in broiler farm, 500 – 3000 in layer farm and 350 – 8000 in pullet farm as shown below.

Table 1: Husbandry methods employed in poultry pens during early dry (November)

Farms	Pens	Litter	Age of litter	Type of birds	Age of bird	Flock size	Roofing method
	FA1	D.L	4WKS	B	5WKS	650	I.R
	FA2	D.L	6WKS	B	7WKS	1450	I.R
Broiler	FA3	D.L	5WKS	B	8WKS	1000	I.R
	FA4	D.L	5WKS	B	7WKS	1000	I.R
	FA5	D.L	5WKS	B	7WKS	1000	I.R
	FB1	D.L	5WKS	L.H	42WKS	500	I.R
Layer	FB2	B.C	7WKS	L.H	58WKS	3000	I.R
	FB3	B.C	7WKS	L.H	58WKS	3000	I.R
	FB4	B.C	3WK	L.H	23WKS	3000	I.R
	FC1	D.L	5WKS	P	9WKS	350	I.R
Pullet	FC2	D.L	14WKS	P	20WKS	7800	I.R
	FC3	D.L	14WKS	P	20WKS	8000	I.R

FA in the above row means poultry pens, D.L = Deep litter, B.C = Battery cage, L.H = Laying hens, P = Pullets, B = Broilers, WKS = Weeks, I.R = Corrugated iron sheets.

2.3 Structural measurements of poultry farms for aerial pollutant gases: Table 2 shows the building measurements of poultry houses used for the study. The mean value obtained were 21.95m, 3.88m, 10.23m and 0.65m for the length of the wall, height of the roof, width of the wall and sidewall, respectively. The highest value recorded in length of wall was 49.98m obtained from FC2 and FC3 respectively while the lowest value 5.79m was obtained from FA1 and FB1 respectively.

2.4 Measurement of environmental factors of poultry farms: The temperature readings were taken in the morning (9 – 11am) and afternoon (1 – 3pm) in each poultry pen. Both inside and outside temperature were determined with a hygrometer (Praziosonshtgro Multithern model). The measurement was carried out every 6 minutes at a height of 2m upward of the poultry house. The wind speed of the area was measured hourly using the Beaufort wind scale.

Table 2: Structural measurements of poultry houses for aerial pollutant gas measurements

Farm	*Pens*	*Length (m)*	*Height (m)*	*Width (m)*	*Sidewall (m)*
	FA1	5.76	1.67	4.26	0.30
	FA2	20.00	4.57	10.00	0.76
Broiler	FA3	20.00	4.57	10.00	0.76
	FA4	20.00	4.57	10.00	0.76
	FA5	20.00	4.57	10.00	0.76
	FB1	5.76	1.67	4.25	0.30
Layer	FB2	20.00	4.57	10.00	0.76
	FB3	65.62	4.57	10.00	0.76
	FB4	20.00	4.57	10.00	0.76
	FC1	11.94	2.13	4.26	0.30
Pullet	FC2	49.98	4.57	20.00	0.76
	FC3	49.98	4.57	20.00	0.76
	MEAN	21.95	3.88	10.23	0.65

2.5 Measurement of concentration of aerial pollutants in the poultry pen: Measurement of the concentration of aerial ammonia, nitrous oxide, methane, carbon monoxide, hydrogen sulphide and sulphur dioxide were made in four poultry farms, equally divided between broilers reared on deep litter, pullets reared on deep litter and layers reared on both deep litter and battery cage. The buildings were chosen to be representative of their type. Each house was monitored once at 6 hours in the early dry season.

The procedure described by Wathes *et al.* (1997), which involves taking representative reading at different locations in a pen, was adopted. Six of the sampling locations were within the birds or human's breathing zone 0.5m and 1.5m above the floor respectively. The factors considered included proximity to the open side wall, middle of the pen as well as sampling height. Such representative readings from each were later pooled to obtain the mean of each pen.

Concentration of gasses were measured in part per million (ppm) as well as lower emission limit (LEL) in the case of flammable gas methane using the Gasman hand held personal gas detector (Crowcon, Instruments Ltd, England). During the gas measurements, these hand held equipment were held at about 0.3m above the litter and the readings were recorded within 10 seconds. Gas detector was calibrated for zero and span before and after reading.

2.6 Data analysis: Both the descriptive and inferential methods were adopted in the analysis of data. The descriptive statistics include the use of mean, standard deviation and coefficient of variation (ANOVA). Where significant differences were observed, mean were separated using Least Square Difference method (Steel and Torrie, 1980). Computer software used was statistical package for social science (SPSS, 2003).

3. Results

3.1 Environmental factors measurements: Table 3 showed the measurements of environmental factors in the study. The mean air temperature within the pen during the period of the study ranged from 32.2 to 32.3^{O}C, while mean relative humidity was in the range of 83 to 83.8%. The mean moisture contents of the litter in the various poultry pens were 18.04, 38.07 and 25.78% for broilers, layers and pullets, respectively. The mean wind speed during the period of the study was 4.22m/s, 4.35m/s and 3.33m/s for broilers, layers and pullet pens, respectively.

Concentration of gases measured in different pen in early dry season: Table 4 showed the concentrations of aerial pollutant gases in the different pens in early dry season (November). In the broiler pen, CO recorded the highest mean concentration (18.8 ± 1.36ppm) followed by CH_4 (1.06 ± 0.16 LEL) and NH_3 (0.89 ± 0.14ppm), while the 0.57 ± 0.10, 0.16 ± 0.06 and 0.06 ± 0.03ppm recorded for SO_2, H_2O and N_2O respectively were lowest. The range of 0.66 ± 0.51 to 1.00 ± 0.00ppm of SO_2 obtained in FA1, FA3 and FA4 were significantly higher (Pp< 0.05) than those of other pens.

Overall mean concentration of CO (19.1 ± 1.35ppm) was highest followed by the 2.12 ± 0.72ppm, 1.87 ± 0.54 LEL and 1.10 ± 0.44ppm recorded for NH_3, CH_4 and SO_2 respectively while 0.38 ± 0.12ppm and 0.12 ± 0.07ppm recorded H_2S and N_2O respectively were lowest in the layer pen as shown Table 5. The range of 3.16 ± 0.75 to 2.83 ± 1.60ppm of NH_3

obtained in FB3 and FB4 were significantly higher (p< 0.05) than those of other pens. Again, the 1.60 ± 0.54 to 0.91 ± 0.20ppm of SO_2 obtained in FB3, FB4 and FB1 were significantly higher (P< 0.05) than those of other pens. Similarly, 0.63 ± 0.20 to 0.53 ± 0.15ppm and 2.6 ± 0.63 to 2.16 ± 0.75ppm of H_2S and N_2O obtained in FB3, FB4 respectively were significantly higher (Pp< 0.05) than those other pens.

Table 3: Measurement of environmental factors in various poultry pen during early dry season (November)

Farm	Pens	Moisture of litter (%)	Relative humidity %	Temperature (^oC)	Wind speed m/s
	FA1	27.67	83.00	32.00	1.60
	FA2	25.31	83.00	32.00	3.30
Broiler	FA3	34.14	85.00	33.00	5.40
	FA4	21.74	85.00	32.00	5.40
	FA5	27.74	83.00	32.50	5.50
	Mean	18.04	83.80	32.20	4.22
	FB1	43.21	83.00	32.00	3.30
Layer	FB2	48.21	83.00	32.00	3.30
	FB3	48.17	85.00	33.00	5.40
	FB4	42.87	83.00	33.00	5.40
	Mean	38.07	83.50	32.20	4.35
	FC1	27.48	83.00	32.00	3.30
Pullet	FC2	28.24	83.00	32.00	3.30
	FC3	21.64	83.00	33.00	3.40
	Mean	25.78	83.00	32.30	3.33

Table 4: Concentration of aerial gases in different broiler pins in early dery season (November)

Pens	NH_3 (ppm)	H_2S (ppm)	SO_2 (ppm)	CO (ppm)	N_2O (ppm)	CH_4 (LEL)
FA1	0.91±0.20	0.21±0.07	1.00^a±0.05	19.80±1.32	0.08^a±0.04	1.16±0.40
FA2	1.00±0.00	0.16±0.08	0.66^a±0.51	20.5±1.22	0.01^b±0.04	1.00±0.00
FA3	1.00±0.00	0.11±0.04	0.10^b±0.00	19.8±1.47	0.06^a±0.05	1.16±0.40
FA4	0.83±0.25	0.13±0.05	0.10^b±0.51	20.0±1.78	0.10^a±0.00	1.00±0.00
FA5	0.75±0.27	0.20±0.06	1.00^a±0.00	14.3±1.03	0.03^b±0.05	1.00±0.00
MEAN	0.89±0.14	0.16±0.06	0.57±0.10	18.8±1.36	0.06±0.03	1.06±0.16
SEM	0.04	0.01	0.20	1.15	0.01	0.03

^{a,b}: Means in the same column with different superscript are significantly different (p<0.05).

In the pullet pen (Table 6), overall mean concentration of CO (19.8 ± 1.16ppm) was highest followed by the 0.77 ± 0.35 and 0.55 ± 0.47ppm of CH_4, NH3 and SO_2 respectively, while the 0.17 ± 0.35 and 0.15 ± 0.08ppm for H_2S and N_2O were lowest. The range of 20.8 ± 1.16 to 20.3 ± 1.03ppm of CO obtained in FC3 and FC2 were significantly higher (P < 0.05) than those of other pens. Similarly, 1.00 ± 0.00ppm and 1.00 ± 0 .00 LEL of NH_3 and CH_4 obtained in FC3 were significantly higher (p<0.05).

Table 5: Concentration of aerial pollutant gases in different layer pens in early dry season (November)

Pens	NH_3 (ppm)	H_2S (ppm)	SO_2 (ppm)	CO (ppm)	N_2O (ppm)	CH_4 (LEL)
FB1	1.00^b±0.00	0.21^b±0.07	0.91^a±0.20	18.6±1.36	0.06^b±0.05	1.00±0.00
FB2	1.50^b±0.54	0.18^b±0.07	0.33^b±0.51	19.6±1.36	0.03^b±0.05	1.66±0.81
FB3	3.16^a±0.75	0.63^a±0.20	1.50^a±0.54	18.1±0.75	0.21^a±0.07	2.66±0.63
FB4	2.83^a±1.60	0.53^a±0.15	1.60^a±0.54	20.3±1.96	0.23^a±0.12	2.16±0.75
MEAN	2.12±0.72	0.38±0.12	1.10±0.44	19.1±1.35	0.12±0.07	1.87±0.54
SEM	0.51	0.11	0.28	0.49	0.05	0.25

^{a,b}: Means in the same column with different superscript are significantly different (p<0.05)

Table 6: Concentration of aerial pollutant gases in different pullet pens in late rainy season (October)

Pens	NH_3 (ppm)	H_2S (ppm)	SO_2 (ppm)	CO (ppm)	N_2O (ppm)	CH_4 (LEL)
FC1	0.66±0.51	0.20±0.08	0.33^{b}±0.51	18.50±1.04	0.21±0.16	0.50^{b}±0.54
FC2	0.50±0.54	0.20±0.08	0.33^{b}±0.51	20.30±1.03	0.13±0.05	0.33^{b}±0.51
FC3	1.00±0.00	0.15±0.05	0.83^{a}±0.25	20.80±1.16	0.11±0.04	1.00^{a}±0.00
MEAN	0.72±0.35	0.17±0.07	0.55±0.42	19.80±1.16	0.15±0.08	0.77±0.35
SEM	0.14	0.01	0.16	0.69	0.03	0.20

^{a,b}: Means in the same column with different superscript are significantly different (P<0.05).

The 0.83 ± 0.40, 0.21 ± 0.16 and 0.20 ± 0.08ppm of SO_2, N_2O and H_2S obtained in FC3, FC1 and FC2 respectively were significantly higher (p< 0.05) than those other pens.

4. Discussion and Conclusion

The mean environmental factors readings such as temperature and humidity in the pens during the period of study were 32^{o} and 83.5% respectively and were much higher than the optimal levels for efficient poultry production (Ferguson, 1986). The present results revealed that the concentrations of the various aerial pollutant gases in the poultry pens were relatively low during the month of November. For example, the mean concentration of ammonia in this study ranges from 0.72 to 2.12ppm, while that of CO was 18.8 to 19.8ppm. These figures are much lower than the current exposure limits recommended for animal welfare in Europe or the averages of 12.3 ppm and 24.2 ppm obtained in poultry houses in the UK during winter and summer months (Wathes *et al*., 1997; CIGR, 1992). The relatively low concentration of aerial pollutant gases in this study confirms the findings of Okoli *et al*. (2004) in the month of August in nearby Imo State. Similar values were also reported by Vucemilo *et al*. (2005) in an earlier study of intensive poultry breeding facilities.

The present study showed variations in the gas concentration across the farms, which could be attributed to the breed effect of the birds, age and type of litter management, type of feed, type of housing design and individual farm attributes. It would be seen from the study that environmental factors associated with high temperature, relative humidity and wind speed during the period of study may have helped in moving gases generated inside the poultry pens to the outside. Taken together, these measurements demonstrate relatively high standard of air quality in the poultry pens.

However, there is hardly any attempt at enforcing standards in livestock building design and construction in Nigeria either for benefit of the health of the operators or for the welfare of the animals. There is need for further studies on the actual impact of polluted environment on human and animal health. Further studies of gas concentration during other months of the year and other livestock species and facilities will be needed to generate detailed data for policy formulation on the management of pollutant gases in livestock production.

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High Altitude Nainital Zoo- A Unique Characteristic of Ecotourism

A Concept Paper

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Abstract: The Nainital High Altitude Zoo one and only Zoo in the newly born state of Uttarakhand is situated at an elevation of 2100 m above mean on the hill of Sher- Ka- Danda. Zoo ecotourism is regarded as being more than tourism to natural areas and should be viewed as a means of combining the goals of resource conservation and local development through tourism in a synergistic fashion.

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Introduction

Many wild species both plants and animals have become extinct from their natural habitats, because of destruction of forests as well as the other natural unit areas by the anthropogenic pressure.

Therefore the remaining species are not in a position to cope with the changing conditions of the environment. Expending human population resulted in to expanding needs of man with the scientific progress and technology development; man started utilizing natural resources at a much larger scale. Man is primarily responsible for destruction of nature's gifts. Conservation and management of wildlife not only preserving the remaining flora and fauna but also helping in promoting economic activities that brings money through tourism. It also contributes towards maintenance of biodiversity of landscape. Many wildlife such colorful birds, animals and other forms of life in the important in maintaining the ecosystem. Destruction of forests or its reduction will cause disappearance of much wild life. Therefore it imperative to conserve and manage the forests as well as wildlife, which are in endangered and threatened condition, this also needs the both the conservation and management programme side by side, due to continuous increase in the number of endangered species of flora and fauna steps have been taken to protect and manage the wild life of the country.

The Ex- situ conservation carried out natural habitat for perpetuating, Sample Population, Genetic Resource Centers, Zoo, Botanicals Gardens, Culture Collection etc. or in the form of gene pools and Gametes Storage for fish, Germless banks for seeds, Pollen, Ova cells etc.

The origin of Zoo may be said to have commenced with the opening of the London Zoo in 1828, most of the older Zoos in North America and Europe were founded in the later part of the 19th century after 1870. During the period animal species were being regularly discovered and the various Zoos were keen to collect as many different kinds of animals as possible for public display or in en – closure having compounds, which did not differ much from the old menageries of the mediaeval times (Sharma, 2000).

The Zoo movement in India is also one of the oldest in the world. The first Zoo was setup in Madaras in the year 1855, which are seen followed by Trivandrum (1857), Bombay (1863), Hyderabad (1959) and Assam state at Guahati (1960). As wildlife biodiversity is an important attribute in the eco-tourism with abundance and variety of flora and fauna are attracting the more tourists (Singh, 2002). Ecotourism is viewed as a means of protecting natural areas through the generation of revenues, environmental education and the involvement of local

people. In such ways both conservation and development are being promoted in sustainable forms.

Zoo Ecotourism

Zoo ecotourism is regarded as being more than tourism to natural areas and should be viewed as a means of combining the goals of resource conservation and local development through tourism in a synergistic fashion. This means that care should be taken to ensure that the goal of tourism development do not interfere with the goals of protecting natural areas and biodiversity local population may become advocates for protection of their natural resources and take pride in the unique surrounding which attract outsiders (Kandari & Chandra, 2004).

Increasing income, providing employment, increasing government revenues and earning foreign exchange. Social reasons of encouraging crest cultural exchange using tourism to help achieve environmental and cultural conservation objectives for which resources are not otherwise available(Dixit, 2001).

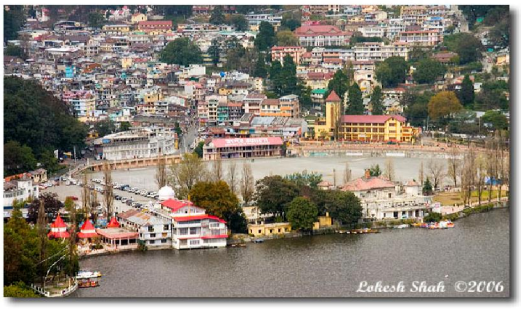
Tourism in a wildlife area should be subordinate to the main objective of wildlife conservation. The primary responsibility of wildlife tourism should be to promote awareness amongst the general public for wildlife conservation. Our wildlife habitats are comparatively small, having varying degree of protection. For creating awareness among the public, park interpretation centers, guide services, reading material and facilities for conducted trek inside the habitat should be provided (Kandari & Chandra, 2004).

Zoo Ecotourism in Nainital

Nainital is referred to in the 'Manas Khand' of the 'Skanda Purana' as the Tri-Rishi-Sarovar, the lake of the three sages, Atri, Pulastya and Pulaha who were reputed to have arrived here on a penitential piligrimage and finding no water to quench their thirst dug a hole and siphoned water into it from Mansarovar the sacred lake inTibet.The Second important mythological reference to Nainital is as one of 64 'Shakti Peeths'. These centres were created wherever parts of charred body of Sati fell, when Lord Shiva was carrying around her corpse in grief. It is said that the left eye (Nain) of Sati fell here and this gave rise to patron deity of town Nainital. It is said that the lake is formed in the emerald eye shape. Naina Devi temple is located at the northern end of the lake. Thus name of Nainital derivated from Naina and the tal (Lake).

Nainital is a glittering jewel in the Himalyan necklace, blessed with scenic natural spledour and varied natural resources. Dotted with lakes, Nainital has earned the epithet of **'Lake District' of India** . The most prominent of the lakes is Naini lake ringed by hills. Nainital has a varied topography. Nainital's unending expense of scenic beauty is nothing short of a romance with awe-inspiring and pristine Mother nature.

After the British Occupation E. Gardiner was appointed as the commissioner of Kumaun Division on May 8th 1815. In 1817 the second commissioner of Kumaun Mr. G.W. Traill has conducted the second revenue settlement of Kumaun, Mr. Traill was the first European to visit Nainital but he did not popularize his visit in respect for the religious sanctity of the place. In the year 1839 an English businessman from Rosa, Mr. P. Barron a sugar trader and his friend an avid hunter strayed into the hills while hunting they got lost and while finding their way back chanced on the wondrous spot. So enamored was Barron with the vision of the placid lake that he left the sugar business and build a European Colony on shores of the lake. In 1841, Nainital appeared in issue of the 'Englishman Calcutta' announcing the discovery of a lake in the vicinity of Almora. According to the earliest data available on tourist in Nainital by 1847, it had become popular hill resort. On 3rd October 1850, the Nainital Municipal Board was formally constituted. It was the second Municipal Board of North Western Provinces. To catalyze the formation of a town the administration transferred land to the wealthy Sah community of Almora, on condition that they build houses on the land. In 1862, Nainital became the summer seat of the North Western Provinces. After it was made the summer Capital, a remarkable expansion of the town occurred with the growth of magnificent bungalows all around and construction of facilities such as marketing areas, rest houses, recreation centers, clubs etc. together with the secretariat and other administrative units. It also became an important centre of education for the British who wanted to educate their children in the better air and away from the disco formats of the plains.



A Bird Eye View of Nainital

The Nainital High Altitude Zoo one and only Zoo in the newly born state of Uttarakhand is situated at an elevation of 2100 m above mean on the hill of Sher- Ka-Danda, where the mountain quail was last seen in 1876, this is also known as Pandit Govind Ballabh Pant High Altitude Nainital Zoo.

The Nainital Zoo is situated about 2 Km. from Tallital bus Station and connected by motor road. The Zoo was established in 1984 and is spread over in area of 4.693 ha. The Zoo was declared open to public on Ist June 1995 and is managed by "The Bharat Rana Pandit Govind Ballabh Pant High Altitude Zoo Management Society" from Ist March 2002.

The main objectives of Zoo is to conserve the High Altitude Himalayan birds and animals which all endemic and endangered and to create awareness about our rich Himalayan fauna among the general public.

Flora of High Altitude Nainital Zoo

The Zoo has also been created to facilitate the research and coordinate breeding of endemic and endangered Himalayan fauna and flora of many species of trees, shrubs and herbs. Among the flora there are species of trees, shrubs and herbs and grasses. The common tree species are *Quercus leuchotrichophora, Quercus floribunda, Rhododendron arboretum, Cupress torulosa, Cedrus deodara, Populous ciliate, Lyonia ovalifolia, cornus macrophycla, Prunus ceracoides, Aesculus indica, Litcia ambrosia, Machilus odratissima, Quercus sirreta, Grevillea robusta, Arundinaria falcate and Rosa moschata.* Besides these tree species, the following under canopy plant species were present in the Zoo area these are *Viburnum colinifotium, Thalictrum foliolosum, Desmodium tiliaefolium, Indigofera pulchelle, Crateagus crenulat, Rubus elliptic, Berberis aristata, Pennisetum candestinum, Cenchrus ciliasi, Saccharum spomtemenum, Eriphorum comosum, Lolium perenne, Roscoea purpure, Artemisia vulgari, Drosera lunata, Arundenaria nepalans, Setaria homonyma, Capillepedium parvifloru, Apluda mutica, Andropogon tristis, Festura gigantean, Sporobolus indicus, Bromus uniloide, Arundinella setasa, Cymbopogon distans, Themeda triandra, Ciyprus alopccurides, Eragrostes nigra, Tripogen filiformis, Polypoen pugex.*

Fauna of High Altitude Nainital Zoo

To understand the different characteristics of the Zoo we made a broad outline work for the study to know the diversity of animals in Zoo, management of these animals, people participation and ecotourism activities.

In Nainital Zoo, carnivores, omnivorous, primates, herbivores, birds like pheasants, parakeets, praying birds, and small birds are present. In the category of carnivores Siberian tiger (*Panthera tigris*), Snow leopard (*Panthera pardus*), Leopard cat (*Felis bengalensis*), Tibetan wolf (*Canis lupuschanco*) and Hill fox (*Valpus valpus montana).* In omnivorous category there are Himalayan Black Bear *(Selenarctos hribetanus*), Palm Civet Cat (*Pagumo larvata*), Himalayan Civet *(Martes flavigula)* and Himalayan Yellow Throated Marten. In Primates category Japanese Macaque *(Macaca fuscota)* and Bonnet Macaque *(Macca radiate).* In herbivorous category Sambar *(Cervus unicolar),* Sika Deer *(Cervus Nippon),* Barking Deer *(Muntiacus muntijak),* Goral *(Nemorhaedus goral),* Serow *(Carpriconis sumatraensis*), Rodents and Wild Hare.

Zoo Management

Zoo management is very complicated task, it needs continuous watch and supervision of Zoo animals and birds in the context of their diets and health care and sanitation works. The zoo authority and concerned zoo staff performed all these tasks regularly. For the animals and birds diets, there is a kitchen with all the essential

equipment for cooking, chopping and cleaning works. Apart from this there are some essential works such as cheaking of flesh for carnivores, food material of herbivores and omnivores, sanitation of cages (enclosures), cages of carnivores, cages of herbivores, sanitation of deer cages, and sanitation work for birds, facilities regarding to animal healts etc for animals performed by Zoo staff.

Ecotourism Activities

Among the tourists visited the Zoo children (5-12 years age) and adult (> 12 years) were 20.75% and 79.3% respectively. Total number of tourists visited in the Zoo during 2004-2005 was 94,884. The maximum tourists (26.6%) were visited in the month of June, 2005 and followed by the month May, 2005 (15.9%), however, very small number of tourists came in the month of February 2006.

As far as economy is concerned, Zoo authority earned total rupees seventeen lakhs and one thousand fourteen only (Rs. 17, 01040.00), out of them 88.4 and 11.6% received from adults and children. 26.3% amount earned during June 2005 out of them 29.8 and 25.8% came from children and adult tourists respectively.

Conclusion

It is concluded that the conservation and management of Zoo species needed a proper planning and expenditure. Nainital high altitude Zoo is one of the unique characteristic of Zoo ecotourism in India. This provides us the information of our nature gift of wild life and promotes the ecotourism activities; this requires new research planning for better management and sustainability.

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Effects of Aqueous Purslane (*Portulaca Oleracea*) Extract and Fish Oil on Gentamicin Nephrotoxicity in Albino Rats

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Abstract: Nephrotoxicity is of critical concern when selecting new drug candidates during the early stage of drug development. Because of its unique metabolism, the kidney is an important target of the toxicity of drugs, xenobiotics, and oxidative stress. Gentamicin (GM) is an antibiotic induced nephrotoxicity as it induces conspicuous and characteristic changes in lysosomes of proximal tubular cells consistent with the accumulation of polar lipids (myeloid bodies). These changes are preceded and accompanied by signs of tubular dysfunctions or alterations (release of brush border and lysosomal enzymes; decreased reabsorption of filtered proteins. The effect of gentamicin (80 mg/kg Bw/day) without or with oral administration of aqueous purslane *(Portulaca oleracea*) extract (400mg/kg BW/day) and fish oil (5mg/kg BW/day) co-treatments for 15 days was evaluated in adult male rats (80-120g). Plasma urea, uric acid and creatinine levels were assayed. Lipid peroxidation (indexed by MDA) and antioxidants enzymes like GSH, SOD and CAT were assessed. There was a decrease in plasma levels concentration of urea, uric acid and creatinine, In addition to decreasing in activities of GSH, SOD and CAT as well as an increasing in MDA concentration in the kidney as a result of gentamicin injection. Co-administration of aqueous purslane extract and fish oil was found to improve the adverse changes in the kidney functions with an increase in antioxidants activities and reduction of peroxidation.We propose that dietary fish oil or purslane extract supplementation may provide a cushion for a prolonged therapeutic option against GM nephropathy without harmful side effects.

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Key words: purslane,fish oil,nephrotoxicity and antioxidants

1. Introduction:

The kidney is a complex organ consisting of well-defined components that function in a highly coordinated fashion. A number of drugs, chemicals, heavy metals have been shown to alter its structure and function. Both acute and chronic intoxication have been demonstrated to cause nephropathies with various levels of severity ranging from tubular dysfunctions to acute renal failure (Barbier.*et al*., 2005). Nephrotoxicity is of critical concern when selecting new drug candidates during the early stage of drug development Because of its unique metabolism, the kidney is an important target of the toxicity of drugs, xenobiotics, and oxidative stress (Uehara *et al*., 2007). The role played by antioxidants during drug-mediated toxicity was determined if they can reduce the oxidative stress induced by reactive intermediates produced by various chemicals and drugs (Sohn *et al* .2007 and Wu. *et al.* 2007).

Aminoglycosides are nephrotoxic because a small but sizable proportion of the administered dose is retained in the epithelial cells lining the S1 and S2 segments of the proximal tubules (Vandewalle *et al*., 1981) after glomerular filtration.Aminoglycosides accumulated by these cells are mainly localized with endosomal and lysosomal vacuoles but are also localized with the Golgi complex (Sandoval, *et al*., 1998). They elicit an array of morphological and functional alterations of increasing severity, aminoglycosides induce conspicuous and characteristic changes in lysosomes of proximal tubular cells consistent with the accumulation of polar lipids (myeloid bodies) (Begg, *et al*., 1995). These changes are preceded and accompanied by signs of tubular dysfunctions or alterations (release of brush border and lysosomal enzymes; decreased reabsorption of filtered proteins.

The *P. oleracea* was a rich source of omega-3-fatty acids, which was important in preventing heart attacks and strengthening the immune system (Simopoulos, 2004). Several biological properties have been attributed to *P .oleracea*:antiseptic, antispasmodic, diuretic, vermifuge (Xiang, *et al*., 2005), anti-scorbutic, antibacterial, wound-healing (Lim and Quah, 2007), analgesic, anti-inflammatory activities and skeletal muscle relaxant, bronchodilator, anti-ascorbic, antipyretic, anti-asthma, and antitussive effect (Islam, *et al*., 1998).

2. MATERIALS AND METHODS

1. Chemicals and drugs

Gentamicin and fish oil were purchased from Sigma Company (United Kingdom),Purslane was purchased from local market. Billirubin (total ,direct) kit, ALP kit, urea kit, uric acid kit and creatinine kit from Diamond Diagnostics (Egypt), total protein kit and albumin kit from Spinreact Company (Spain) , ALT and AST kit from Biomerieux chemical company and chemicals used in measurement of antioxidants from Sigma chemical company.

2. Plant extract

The aqueous extract of the purslane herb were boiled in the traditional way. Briefly, herbs were minced and seeped in boiling water in the proportion of 1:10 (w/v) for 3 h. This was repeated two additional times for 3 h of boiling. After boiling, the resulting crude extract was filtered and the filtered extract was evaporated to dryness under reduced pressure at 40 °C and a yield of 24–28% (w/w) was obtained. The dried powder was kept at 4 °C for future use (Hongxinga *et al*., 2007).

3. Experimental animals and design:

White male albino rats (Rattus norvegicus) weighing about 140-180g were used as experimental animals in the present investigation. They were obtained from the animal house of Research Institute of Opthalmology, El-Giza, Egypt. They were kept under observation for about 15 days before the onset of the experiment to exclude any intercurrent infection. The chosen animals were housed in plastic cages with good aerated covers at normal atmospheric temperature (25±5oC) as well as 12 hours daily normal light periods. Moreover, they were given access of water and supplied daily with standard diet of known composition and consisting of not less than 20% proteins, 5.5% fibers, 3.5% fats and 6.5% ash and were also supplied with vitamins and mineral mixtures.

The considered rats were divided into four groups containing six animals for each. These groups were:

Group 1: It was regarded as normal animals which were kept without treatments under the same laboratory conditions and was regarded as normal control group for other ones.

Group 2 (toxic group): The animals in this group were received intraperitoneal injection of single nephrotoxic dose of gentamicin for 15 days (80 mg/kg body weight) (Priyamvada *et al*., 2008). This group was considered as control for the remained groups.

Group 3 (Toxic treated with purslane aqueous extract): The rats in this group were administrated aqueous extract of purslane by gastric intubation after injection with gentamicinat at dose level of 400mg/kg b.wt for 15 days (Fayong Gong *et al*., 2009).

Group 4 (Toxic treated with fish oil): The rats in this group were administrated fish oil by gastric intubation after injection with gentamicinat at dose level of 5mg/kg b.wt for 15 days (Ali and Bashir, 1994).

All the treatments were performed orally and daily between 8.00 and 10.00 a.m .

By the end of the experimental periods, normal, control groups and treated rats were sacrificed under diethyl ether anesthesia. Blood samples were taken and centrifuged at 3000 r.p.m. for 30 minutes. The clear non- haemolysed supernatant sera were quickly removed, divided into three portions for each individual animal, and kept at -20 oC till used.

4. Phytochemical analysis of purslane

4.1. Samples preparation

For fatty acid analysis, crude oil was obtained from samples extracted with petroleum ether (b.p. 40–60 °C) in a Soxhlet apparatus; the remaining solvent was removed by vacuum distillation. For organic acids and phenolics determination and antioxidant capacity assay, an aqueous extract was prepared: three powdered sub samples (~ 5 g; 20 mesh) were extracted with 250 mL of boiling water for 45 min and filtered through Whatman no. 4 paper. The resulting extract was lyophilized in a freeze dried apparatus (Ly-8-FM-ULE, Snijders, Holland) and yields were calculated for Q. Sta Apolónia (leaves: 23.06 ± 1.16%; stems: 27.64 ± 1.56%), Q. Pinheiro Manso (leaves: 29.27 ± 0.65%; stems: 25.61 ± 0.14%), S. Bartolomeu (leaves: 21.21 ± 2.17%; stems: 22.03 ± 0.46%), and Samil (leaves: 25.88 ± 1.43%; stems: 25.31 ± 0.46%). The lyophilized extracts were kept in an exsicator, in the dark (Oliveira, *et al*, 2009). For the characterization and quantification of the phenolic compounds by HPLC/DAD, each lyophilized extract was redissolved in water. For organic acids determination they were redissolved in sulphuric acid 0.01 N prior to analysis by HPLC/UV.

4.2. Fatty acid composition

Fatty acids were determined by gas chromatography(DAN1 model) with flame ionization detection (GC-FID) capillary column based on the following *trans*-esterification procedure: fatty acids were methylated with 5 mL of methanol:sulphuric acid:toluene 2:1:1 (v/v), during at least 12 h, in a bath at 50 °C and 160 rpm; then 5 mL of deionized water was added, to obtain phase separation; the FAME were recovered with 5 mL of diethyl ether by

shaking in a vortex, and the upper phase was passed through a micro-column of sodium sulphate anhydrous, in order to eliminate the water; the sample was recovered in a vial, and filtered through a 0.2 µm nylon filter (Milipore) before injection. The fatty acid profile was analyzed with a DAN1 model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID) and a Macherey–Nagel column (30 m × 0.32 mm ID × 0.25 µm d_f). The oven temperature program was as follows: the initial temperature of the column was 50 °C, held for 2 min, then a 10 °C/min ramp to 240 °C and held for 11 min. The carrier gas (hydrogen) flow rate was 4.0 mL/min (0.61 bar), measured at 50 °C. Split injection (1:40) was carried out at 250 °C. For each analysis 1 µL of the sample was injected in GC. The results were recorded and processed using CSW 1.7 software (DataApex 1.7) and expressed in relative percentage of each fatty acid, calculated by internal normalization of the chromatographic peak area. Fatty acids were identified by comparing the relative retention times of FAMEs peaks from samples with standards (Oliveira *et al.*, 2009).

4.3. Analysis of phenolic compounds by HPLC/DAD

Twenty microliters of lyophilized purslane leaves and stems extracts were analyzed using a HPLC unit (Gilson) and a Spherisorb ODS2 (25.0 × 0.46 cm; 5 µm, particle size) column. The purslane leaves and stems lyophilized extracts were analyzed using a mixture of formic acid 5% (A) and methanol (B), with a flow rate of 0.9 mL/min, as follows: 0 min—5% B, 3 min—15% B, 13 min—25% B, 25 min—30% B, 35 min—35% B, 39 min—45% B, 42 min—45% B, 44 min—50% B, 47 min—55% B, 50 min—70% B, 56 min—75% B, 60 min—100% B.Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the range of 200–400 nm, and chromatograms were recorded at 330 nm. The data were processed on Unipoint system Software (Gilson Medical Electronics, Villiers le Bel, and France). Peak purity was checked by the software contrast facilities. Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. The compounds were quantified as 5-caffeoylquinic acid (Oliveira *et al.*, 2009).

5. Preparation of tissue homogenates

After the completion of the experiment, the kidneys were removed, decapsulated and kept in ice-cold buffered saline (154 mM NaCl, 5 mM Tris–HEPES, pH 7.5). The cortex was carefully separated from medulla as described earlier (Khundmiri etal., 2004). A 15% (w/v) homogenate was prepared in 0.1 M Tris–HCl buffer pH 7.5 using Potter-Elvehejem homogenizer (Remi motors, Mumbai, India); by passing 5 pulses. The homogenate was centrifuged at 3000*g* at 4 °C for 15 min to remove cell debris and the supernatant was saved in aliquots and stored at −20 °C for assaying the enzymes of carbohydrate metabolism, free-radical scavenging enzymes and for estimation of total-SH and lipid peroxidation.

6. Assay of kidney and liver function:

ALT (E.C. : 2.6.1.2.) Activity in serum was determined according to the method of Reitman and Frankel (1957) using reagent kits purchased from BioMerieux Chemical Company (France). AST (E.C.: 2.6.1.1.) activity in serum was determined according to the method of Reitman and Frankel (1957) using reagent kits purchased from Randox Company (United Kingdom). Bilirubin level in plasma was determined according to the method of Jendrassik et al., (1938) using the reagent kits purchased from Diamond Diagnostics (Egypt). Alkaline phosphatase activity in serum was determined according to the method of Rec. GSCC (DGKC) (1972) using the reagent kits purchased from Diamond Diagnostics (Egypt). Serum total proteins concentration was determined according to the method of Peters (1968) using reagent kits purchased from Spinreact Company (Spain). Serum albumin concentration was determined according to the method of Doumas et al. (1971) using reagent kits purchased from Spinreact Company (Spain). Urea concentration in serum was determined according to the method of Patton and Crouch (1977) using the reagent kits purchased from Diamond Diagnostics (Egypt). Uric acid concentration in serum was determined according to the method of Fossati et al., (1980) using reagent kits purchased from Diamond Diagnostics (Egypt). Creatinine level in serum was determined according to the method of Henry (1974) using the reagent kits purchased from Diamond Diagnostics (Egypt).

7. Assay of enzymatic and non-enzymatic antioxidant parameters

They were conducted chemically using chemicals purchase from Sigma chemical company and using Jenway spectrophotometer (Germany),Superoxide dismutase (SOD) was assayed by the method of Kar and Mishra (1976)., Catalase as described by Cohen *et al.*, (1970) and glutathione peroxidase (GSH-Px) by the method of Van Dam *et al.* (1999). Lipid peroxidation (LPO) determined according to methods of Ohkawa *et al.*, 1979 and vitamin C determined according to methods of Kyaw, 1978. Ascorbic acid concentration

liver homogenate was determined at spectrophotometrically at 700 nm using acid phosphotungstate (Kyaw, 1978).

8. Statistical analysis

The Statistical Package for the Social Sciences (SPSS for WINDOWS, version 11.0; SPSS Inc, Chicago). Results were expressed as mean ± standard error (SE) and values of P>0.05 were considered non-significantly different, while those of P<0.05 and P<0.01 were considered significantly and highly significantly different, respectively (Levesque, R., 2007).

3-Results

The fatty acids profile is composed by twenty four fatty acids, with all samples presenting a similar constitution, although with some variations (Fig.1). Palmitic (C16) acid was the most abundant one in all samples, which contains 31.80% in sample. Oleic (C18:13n9c+t) acid was the second in order of importance, which contains 27.17% in sample followed by Butyl phenol, which containes 9.70% in sample. For the remaining fatty acids only stearic (C18) (2.02%), linoleic (C18:2n6c) acids (2.70%) and Linolenic (C18:3n3) acid (2.42) were present in considerable amounts. Three phenolic compounds were identified and quantified: Gallic Acid, Ferulic Acid and Caffeic Acid (Fig.1 and 2). The three phenol compounds have different concentration .gallic acid has the highest concentration (16685.09 ug/mlx7) then caffeic acid which has concentration (467.02 ug/mlx7), then ferulic acid which has concentration (167.91 ug/mlx7).

The gentamicin-induced rats exhibited a very highly significant decrease (P< 0.001) of body weight gain as compared to the normal ones (fig. 3). The injection with gentamicin gives -267.19% percentage changes in body weight gain. The oral treatment of gentamicin rats with extract of purslane after gentamicin administration exerted a very highly significant increase (P< 0.001) in body weight gain fish oil to gentamicin- intoxicated rats caused a very highly significant increase (P< 0.001) in body weight gain (fig. 3).

The gentamicin -induced rats exhibited a non-significant decrease of kidney weight gain as compared to the normal ones(fig.4).The oral treatment of gentamicin rats with extract of purslane and fish oil after gentamicin administration exerted a non-significant increase in kidney weight gain as compared to the gentamicin-control group (fig 4). The gentamicin-induced rats exhibited a very highly significant decrease (P< 0.001) of liver weight gain as compared to the normal ones (fig. 5). The oral treatment of rats with extract of *purslane* after gentamicin administration exerted a non-significant increase in liver weight gain as compared to the gentamicin-control group, while the administration of fish oil to gentamicin- intoxicated rats caused a very highly significant increase (P< 0.001) in liver weight gain (fig. 5).

The gentamicin intoxicated rats showed a highly significant increase (P < 0. 01) in serum level of urea, creatinine and uric acid as compared to normal control group (fig.6,7&8).The treatment with purslane extract of *Portulaca oleracea* to gentamicin intoxicated rats showed a significant decrease (P < 0.05) in urea and highly significant in creatinine and uric acid level (P < 0.01) as compared to gentamicin control group.The treatment with fish oil to gentamicin intoxicated rats showed a significant decrease in serum urea (P < 0.05) and highly significant in creatinine and uric acid level (P < 0.01) as compared to gentamicin control one.Treatment of gentamicin nephritic rats with fish oil and purslane give percentage changes in creatinine (-73.96%) and (-67.74%) respectively as compared with control ones.Treatment gentamicin nephritic rats with fish oil and purslane give percentage changes in urea (-51.50%) and (-52.95%) respectively as compared with control ones.Treatment gentamicin nephritic rats with fish oil and purslane give percentage changes in uric acid (-68.39%) and (-57.77%) respectively as compared with control ones (fig. 6,7&8).

The serum ALT , AST and ALP activities in gentamicin intoxicated rats showed a very highly significant increase (P < 0.001) as compared to the normal rats(fig. 9,10&11). The oral treatment with purslane extract exerted a very highly significant decrease (P< 0.001) in serum ALT, AST and ALP activities with apercentage change of -80.37%, -89.23% and -73.37% respectively as compared to gentamicin control group.While the oral treatment with fish oil exerted a highly significant decrease (P< 0.01) in serum ALT activity with apercentage change(-51.42%) as compared to the gentamicin control rats (fig.9). While, the oral treatment of gentamicin rats with fish oil exerted a significant decrease (P< 0.05) in serum AST activity as compared to the gentamicin control rats. Treatment with fish oil gives percentage changes in serum AST -46.22% as compared to the control ones (fig. 10). The treatment with fish oil to gentamicin intoxicated rats showed a very highly significant decrease (P < 0.001) in serum ALP activity as compared to gentamicin control ones.Treatment with fish oil give percentage changes in serum ALP -54.25% as compared to the control ones (fig. 11).

The nephritic rats induced by gentamicin exhibited a very highly significant decrease (P<

0.001) of serum total protein , albumin and globulin concentrations as compared to the normal rats(fig.12,13&14). The oral treatment of nephritic rats with purslane extract exerted a very highly significant (P< 0.001) in serum total protein and albumin concentration as compared to the nephritic control ones. While the treatment with fish oil showed a highly significant (P< 0.01) increase in gentamicin intoxicated rat as compared to gentamicin control group.While the treatment with fish oil to gentamicin intoxicated rats showed a very highly significant increase (P < 0.001) in serum albumin as compared to gentamicin control group (fig.13). On the other hand, The oral administration of purslane extract to nephritic rats showed a very highly significant increase (P< 0.001) in the serum albumin concentration as compared to the nephritic control rats(fig. 13). While the treatment with fish oil to gentamicin intoxicated rats showed a significant increase in serum albumin (P< 0.05) as compared to gentamicin control group.The gentamicin intoxicated rats showed a highly significant increase (P < 0.01) in plasma total bilirubin as compared to normal control group (fig. 15).The treatment with purslane extract showed a significant decrease (P < 0.05) in total bilirubin as compared to gentamicin control group.While the treatment with fish oil showed a highly significant decrease (P < 0.01) in total bilirubin as compared to gentamicin control group(fig. 15). The gentamicin intoxicated rats showed a significant increase (P < 0.05) in plasma direct bilirubin as compared to normal control group.The treatment with purslane extract showed a (P < 0.05) significant decrease in direct bilirubin as compared to gentamicin control group.While the treatment with fish oil showed (P< 0.01) highly significant decrease in direct bilirubin as compared to gentamicin control group.The gentamicin intoxicated rats showed a very highly significant increase (P< 0.001) in plasma indirect bilirubin as compared to normal control group(fig. 16). The treatment with purslane extract showed a (P < 0.01) a highly significant decrease in indirect bilirubin as compared to gentamicin control group.While the treatment with fish oil showed (P < 0.05) a significant decrease indirect bilirubin as compared to gentamicin control group (fig. 17).

The gentamicin intoxicated rats showed a very highly significant (P<0.001) increase in kidney MDA level as compared to normal control group.The treatment with purslane extract to gentamicin intoxicated rats showed a highly significant (P<0.01) decrease in kidney MDA level as compared to gentamicin control group (fig 18).The treatment with fish oil extract to gentamicin intoxicated rats showed a non- significant decrease in kidney MDA level as compared to gentamicin control group(fig. 18). The gentamicin intoxicated rats showed a very highly significant (P<0.001) decrease in kidney vitamin C content as compared to normal control group.The treatment with purslane extract to gentamicin intoxicated rats showed a very highly significant (P<0.001) increase in kidney vitamin C content as compared to gentamicin control group.The treatment with fish oil extract to gentamicin intoxicated rats showed a significant increase (P>0.05) in kidney vitamin C content as compared to gentamicin control group (fig. 19).

The gentamicin intoxicated rats showed a very highly significant (P<0.001) decrease in kidney catalase, SOD and reduced glutathione activities as compared to normal control group(fig.20, 21&22).The treatment with purslane extract showed a very highly significant (P<0.001) increase in kidney catalase activity as compared to gentamicin control one (fig. 20).The treatment with fish oil showed a very highly significant increase in kidney catalase activity (P<0.001) as compared to gentamicin control one. The treatment with purslane extract showed a very highly significant (P<0.001) increase in kidney SOD activity as compared to gentamicin control one (fig. 21).The treatment with fish oil showed a highly significant increase (P<0.01) in kidney SOD activity as compared to gentamicin control one.The oral treatment of gentamicin rats with extract of purslane to gentamicin intoxicated rats exerted a highly significant increase (P< 0.01) in kidney reduced glutathione activity as compared to the gentamicin -control group.The oral treatment of gentamicin rats with fish oil to gentamicin intoxicated rats exerted a significant increase (P< 0.05) in kidney reduced glutathione activity as compared to the gentamicin -control group (fig. 22).

The gentamicin intoxicated rats showed a significant (P<0.05) increase in hepatic MDA level as compared to normal control group (fig. 23).The treatment with purslane extract to gentamicin intoxicated rats showed a highly significant (P<0.01) decrease in hepatic MDA level as compared to gentamicin control group.The treatment with fish oil extract to gentamicin intoxicated rats showed a non significant decrease in hepatic MDA level as compared to gentamicin control group(fig. 23). On other hand, the gentamicin intoxicated rats showed a highly significant (P<0.01) decrease in ascorbic acid contents.The treatment with purslane extract to gentamicin intoxicated rats showed a very highly significant (P<0.001) increase in vitamin C content as compared to gentamicin control group (fig. 24).The treatment with fish oil extract to gentamicin intoxicated rats showed a significant (P<0.05) increase in vitamin C content as compared to

gentamicin control group (fig. 24).

The gentamicin intoxicated rats showed a very highly significant (P<0.001) decrease in hepatic catalase activity as compared to normal control group (fig. 25).The treatment with purslane extract showed a very highly significant (P<0.001) increase in hepatic catalase activity as compared to gentamicin control one.The treatment with fish oil showed a highly significant increase in hepatic catalase activity (P<0.01) as compared to gentamicin control one.The gentamicin intoxicated rats showed a very highly significant (P<0.001) decrease in hepatic SOD activity as compared to normal control group (fig. 26).The treatment with purslane extract and fish oil showed a highly significant (P<0.01) increase in hepatic SOD activity as compared to gentamicin control ones. On other hand, the gentamicin -induced rats exhibited a highly significant decrease (P< 0.01) of hepatic reduced glutathione activity as compared to the normal control group.The oral treatment of gentamicin rats with extract of purslane and fish oil to gentamicin intoxicated rats exerted a highly significant increase (P< 0.01) in hepatic reduced glutathione activity as compared to the gentamicin (fig. 27).

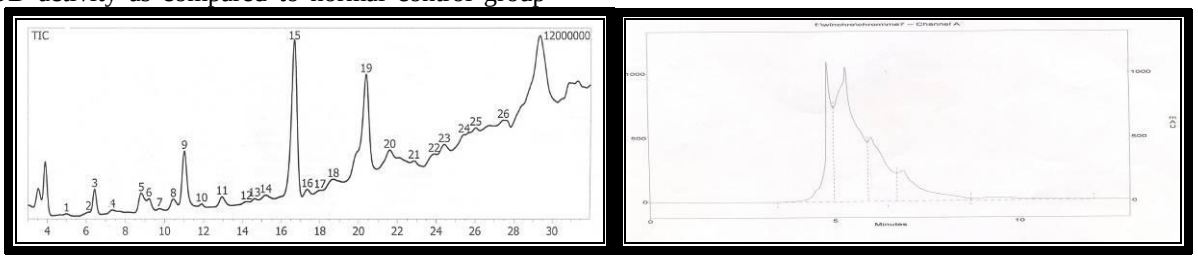
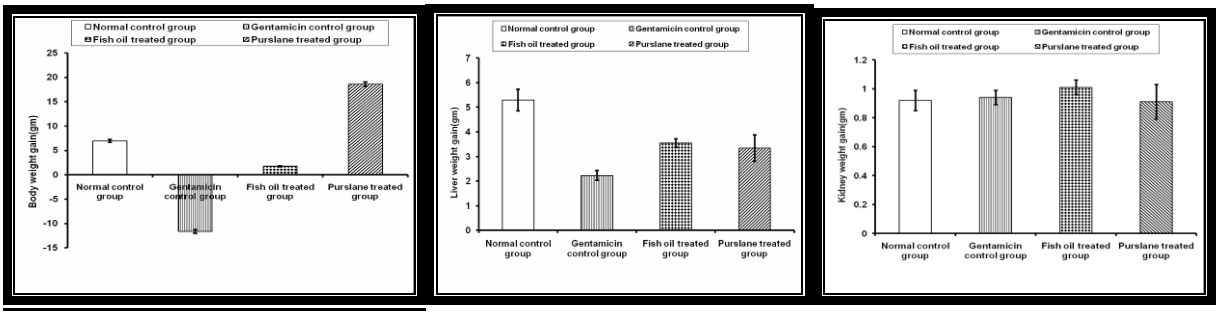


Fig (1) Phytochemical analysis of purslane by HPLC

Fig(2)Phytochemical analysis of purslane by GC



Fig(3) : Effect of purslane extract and fish oil on Kidney weight on gentamicin nephritic rats.

Fig (4): Effect of purslane extract and fish oil on body weight gain on gentamicin nephritic rats.

Fig(5): Effect of purslane extract and fish oil on liver weight gain on gentamicin nephritic rats

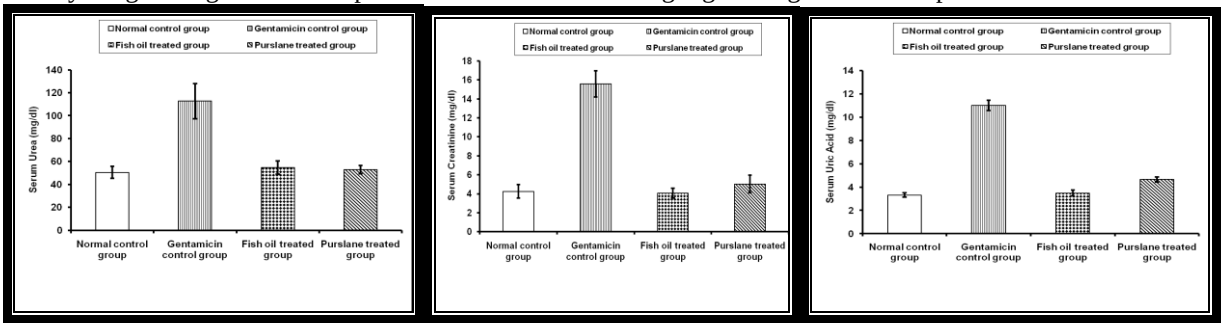


Fig (6): Effect of purslane extract and fish oil on serum urea level on gentamicin nephritic rats

Fig (7): Effect of purslane extract and fish oil on serum creatinine level on gentamicin nephritic rats.

Fig (8): Effect of purslane extract and fish oil on serum uric acid level on gentamicin nephritic

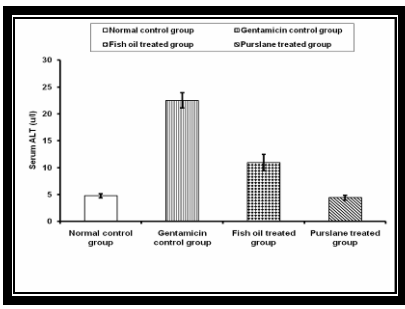


Fig (9): Effect of purslane extract and fish oil on serum ALT level on gentamicin nephritic rats

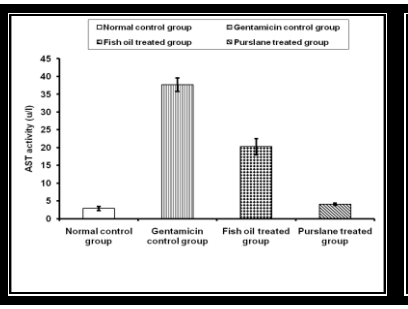


Fig (10): Effect of purslane extract and fish oil on serum AST level on gentamicin nephritic rats.

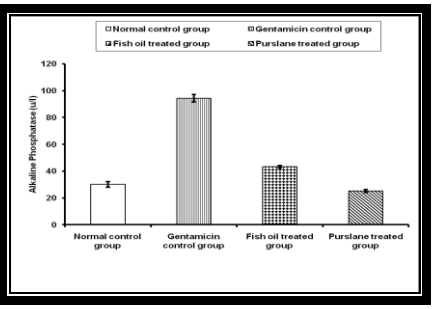


Fig (11): Effect of purslane extract and fish oil on serum ALP level on gentamicin nephritic

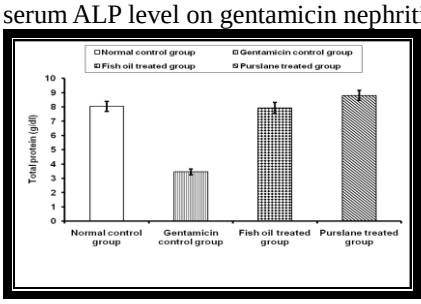


Fig (12): Effect of purslane extract and fish oil on serum total protein level on gentamicin nephritic rats

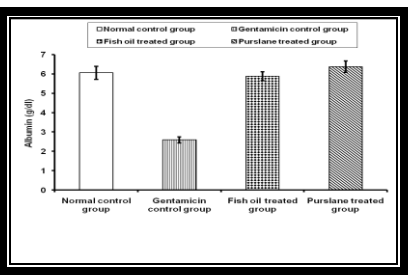


Fig (13): Effect of purslane extract and fish oil on serum albumin level on gentamicin nephritic rats.

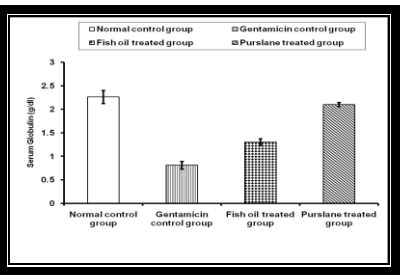


Fig (14): Effect of purslane extract and fish oil on serum globulin level on gentamicin nephritic

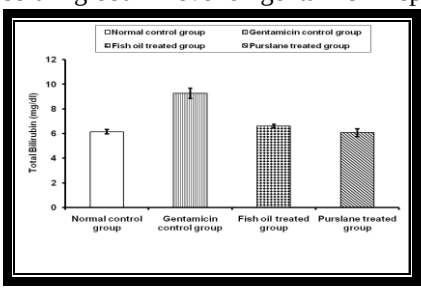


Fig (15): Effect of purslane extract and fish oil on serum direct Billirubin level on gentamicin nephritic rats.

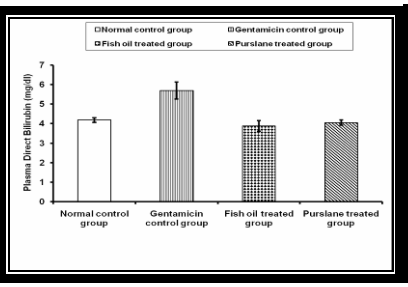


Fig (16): Effect of purslane extract and fish oil on total Billirubin level on gentamicin nephritic rats

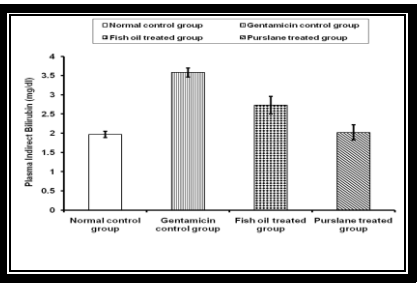


Fig (17): Effect of purslane extract and fish oil on serum total indirect Billirubin level on gentamicin nephritic

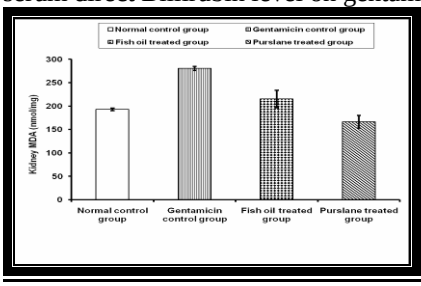


Fig (18): Effect of purslane extract and fish oil on Kidney MDA level on gentamicin nephritic rats

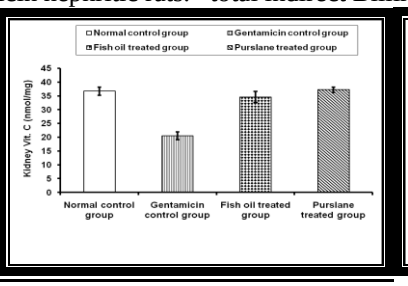


Fig (19): Effect of purslane extract and fish oil on kidney Vit.C level on gentamicin nephritic rats.

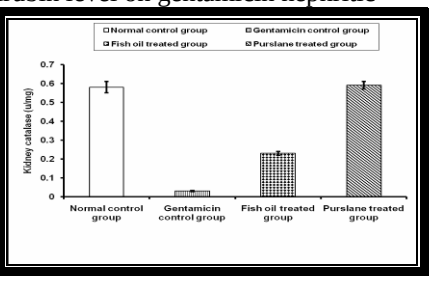


Fig (20): Effect of purslane extract and fish oil on Kidney catalase level on gentamicin nephritic rats

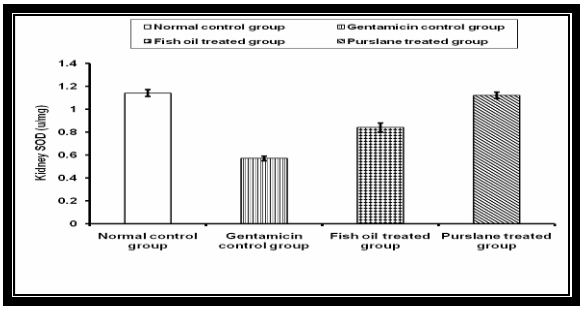


Fig (21): Effect of purslane extract and fish oil on Kidney SOD level on gentamicin nephritic rats

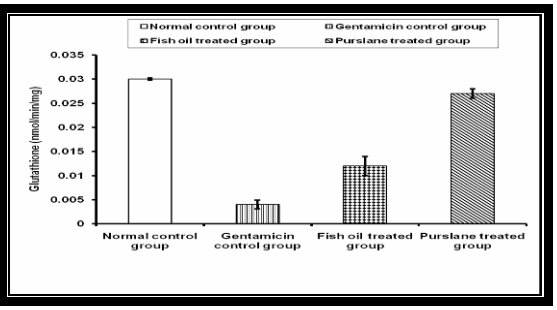


Fig (22): Effect of purslane extract and fish oil on kidney Glutathione level on gentamicin nephritic rats

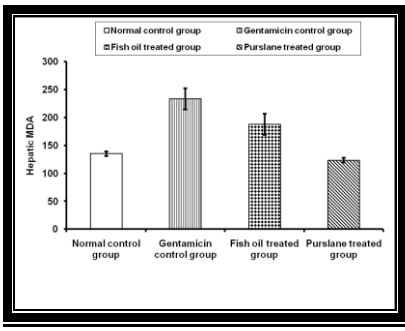


Fig (23): Effect of purslane extract and fish oil on liver MDA level on gentamicin nephritic rats

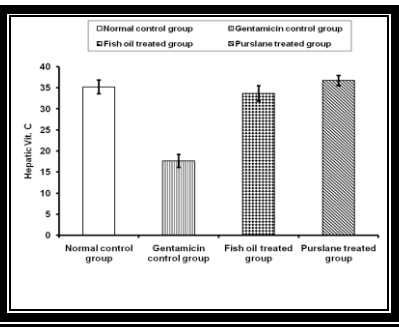


Fig (24): Effect of purslane extract and fish oil on liver Vit.C level on gentamicin nephritic rats.

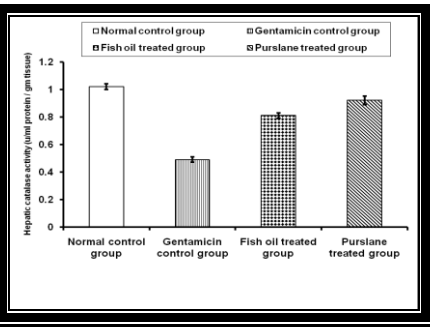


Fig (25): Effect of purslane extract and fish oil on liver catalase level on gentamicin nephritic rats

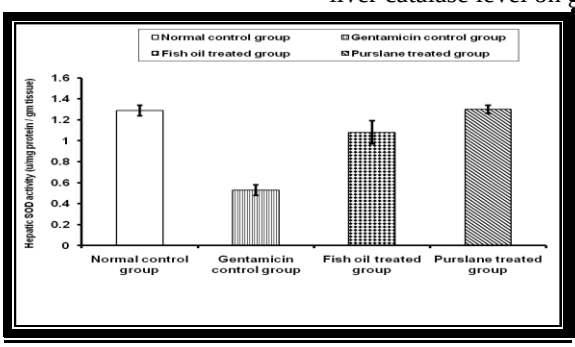


Fig (26): Effect of purslane extract and fish oil on liver SOD level on gentamicin nephritic rats

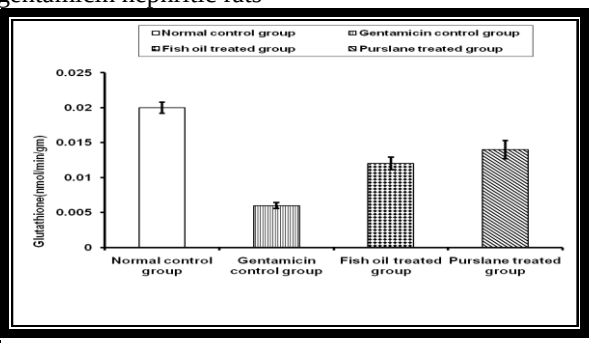


Fig (27): Effect of purslane extract and fish oil on liver Glutathione level on gentamicin nephritic rats

4. Discussion:

The kidney is a complex organ consisting of well-defined components that function in a highly coordinated fashion. A number of drugs, chemicals, heavy metals have been shown to alter its structure and function.In the present study gentamicin was selected as a nephrotoxicant to induce kidney damage. Fish oil (FO) enriched in ω-3 fatty acids has profound beneficial health effects against various pathologies including cardiovascular diseases, respiratory diseases, diabetes, depression, cancers, inflammatory and immune renal disorders. Reports showed that FO prevents gentamicin -induced nephrotoxicity (Vandewalle *et al.*,1981). Recent research demonstrated that purslane is a good source of compounds with a positive impact in human health. Those compounds include omega-3 fatty acids and β-carotene, vitamins and essential amino acids, α-tocopherols, ascorbic acid, and glutathione, as well as phenolics, and coumarins. Organic acids are also present and alkaloids have been reported to be important chemical constituents of this species (Simopoulos 2004). In our result Purslane presented high amounts of fatty acids as omega-3 and omega-6 PUFA, which are essential dietary fatty acids that cannot be synthesized by humans but have to be

ingested. These acids play an important role in human growth, development and disease prevention.

Epidemiological and clinical studies suggest that omega-3 PUFA, found predominantly in marine organisms, may have beneficial effects in the prevention of several cardiovascular diseases (Davis *et al*, 2007), and in treatment of nephrotoxicity (Priyamvada *et al*., 2008) According to the above mentioned, purslane could be regarded as an alternative source of these nutrients for human consumption. In general terms, the obtained results were in agreement with those observed in previous works (Odhav *et al*. 2007 and Oliveira *et al*., 2009). A few bibliographic data were available concerning to phenolic composition of *P. oleracea*. (Cai *et al*, 2004) referred that the most representative phenolic compounds of purslane were flavonols and flavones, but neither identification nor quantification was presented. (Xueqin *et al*., 2006) developed an analytical procedure for flavonoids identification in *P. oleracea*, but only apigenin was quantified in significant amount, higher in leaves than in stems, and kaempferol was also present just in one sample. Recently, (Spina *et al*., 2008) identified and quantified some benzoic acids and flavonoids in methanolic extracts of wild and cultivated purslane. In our results three phenolic compounds were identified and quantified (Gallic acid,Ferulic acid &Caffeic acid)which they are antioxidant agents that can be used in treatment or prevent several diseases The obtained results were in agreement with (Oliveira *et al*., 2009).

Body weight is frequently the most sensitive indicator of adverse effects of xenobiotics. So, it is considered as a determinant parameter of toxicity testing. Increased catabolism, seen in acute renal failure, results in acidosis which is accompanied by anorexia. Hence, oral food intake decreases and this causes body weight loss (Ali *et al*., 1992). In the present study, gentamicin was used to induce kidney failure in rats. A gradual decrease in food intake and growth rate was observed in gentamicin treated rats. A very highly significant decrease in body weight gain observed in gentamicin intoxicated control rat. These results were in agreement with (Erdem *et al*., 2000 and Bello &Chika, 2009). On the other hand, our study showed marked ameliorations on body weight gain for gentamicin intoxicated rats as compared to gentamicin control group. This effect could be associated to alterations in nutrient absorption and metabolic utilization after treatments. Our results in agreement with (Erdem *et al*., 2000) who showed that gentamicin caused a severe loss in body weight that was inhibited by taurine administration in gentamicin group. It was found that carbon tetra chloride (CCl_4) induced renal disorders in rat due to presence of abnormally high levels of BUN in serum, urobilinogen in urine and creatinine both in urine and serum are possible indicators of hepatic and/or kidney injuries induced through CCl_4 treatment (Muhammad *et al*., 2009). Gentamicin induced toxic effects in the kidney (Fouzia Rashid *et al*., 2005). The renal dysfunction due to gentamicin treatment was manifested by a very highly significant increase in serum urea, creatinine and uric acid levels as compared to the normal group of rat. This is in agreement with the results of Saleemi *et al*., 2009 and Polat, *et al*., 2006. It was reported that treatments with gentamicin produces nephrotoxicity (Atessahin *et al*., 2003) as a result of reduction in renal functions which was characterized by an increase in serum creatinine and serum urea level accompanied by impairment in glomerular functions. Serum creatinine level was more significant than the urea levels in the earlier phase of the renal damage. In the present study, it was shown that treatment with gentamicin alone to rats caused nephrotoxicity, which was correlated with increased creatinine, and urea levels in plasma (Karahan, *et al*., 2005). Our result showed that the treatment of gentamicin intoxicated rats with fish oil made decreasing in serum creatinine, serum uric acid and serum urea level due to its ability to treatment nephrotoxicity. This is in agreement with the results of Karahan, *et al*., 2005. Also, our result showed that the treatment of gentamicin intoxicated rats with purslane extract made decreasing in serum creatinine and serum urea level. This is in agreement with the results of Nitha *et al*. 2008.

There are various drugs that may cause side effect such as Cisplatin (cis-diamminedichloroplatinum II, CP) that is a major antineoplastic drug for the treatment of various forms of cancers (Nakashima *et al*., 1990) and (Taguchi *et al*., 2005). However, CP and its analogs accumulate in the kidney causing nephrotoxicity ($Khan_1$, *et al*., 2009).Since the BBM contains a number of hydrolytic enzymes and transport systems, the effect of CP was determined on the activities of BBM enzymes and on Pi transport to assess damage caused by CP administration. CP significantly decreased the activities of Alkaline phosphatase(ALP), γ-glutamyl transferase (GGTase), and leucine aminopeptidase (LAP), BBM marker enzymes, in cortical homogenates and isolated BBM vesicles. A similar decrease was observed in medulla, suggesting an overall CP-induced damage to the kidney. The CP-induced decrease in BBM enzymes suggested a severe damage to the structural architecture of the BBM affecting its transport functions as these enzymes were shown to be directly or indirectly involved in the transport of various solutes ($Khan_1$ *et*

al., 2009). The decrease in BBM enzyme activities was in fact due to the loss/release of enzymes and other proteinic components from damaged BBM into the lumen that later appear in the urine as demonstrated previously for CP and other toxicants (Anees *et al*., 2008 and $Khan_2$ *et al*., 2009). In contrast to CP, GT consumption, however, significantly increased the activities of BBM enzymes in the homogenate and BBM, indicated an overall improvement in renal BBM integrity as shown earlier (Khan *et al*., 2007 and $Khan_2$ *et al*., 2009). A variable increase in the activity of ALP/GGTase in the renal cortex and medulla can be considered due to their localization in the thickness of BBM (Yusufi *et al*., 1994) or due to differential access/accumulation of GT in these tissues.GT consumption in combination with CP treatment resulted in the reversal of CP-induced alterations in the activities of certain BBM enzymes in the renal tissues. The activities of ALP and GGTase in renal BBMV remained significantly higher in TCP compared to CP-treated renal BBM preparations, indicated a marked reversal of CP-induced effect by GT consumption on these enzymes. CP-induced decrease in LAP was also reduced by GT in renal BBM preparations ($Khan_1$, *et al*., 2009). The results convincingly demonstrate that GT consumption not only prevented the CP elicited decrease in the activities of certain enzymes, but they remained significantly higher in TCP compared to control and much higher than CP-treated rats as shown earlier ($Khan_2$, *et al*., 2009) . The activity of lysosomal enzyme, ACPase was significantly increased in the cortex and medulla by CP treatment. Alterations in ACPase activity demonstrate CP-induced loss of lysosomal function (Courjault-Gautier *et al*., 1995 and Kuhlmann *et al*., 1997).

Elevated activities of serum ALT, AST and ALP and levels of bilirubin (total & direct) after gentamicin intoxication in agreement with the result of El-Daley (1996). ALT is an enzyme used as an indicator of GM hepatic damage to rat hepatocytes (El-Tawil and Abdel-Rahman, 2001).AST presents two isozymes, one located in the cytoplasm and the other in the mitochondria.The presence of these enzymes outside the cell represents damage to the hepatic cell.Alkaline phosphatase is an ectoenzyme of the hepatocyte plasma membrane; an increase in serum alkaline phosphatase activity has been related to damage to the liver cell membrane (Kaplan, 1986).In view of the present results, it was found that gentamicin causes a highly significant elevation in serum activity of ALP. These results are in agreement with Fouzia Rashid *et al*., (2005) and $Khan_1$, *et al*., (2009).Serum bilirubin is one of the most sensitive tests employed the diagnosis of hepatic diseases. Bilirubin, is a chemical breakdown product of hemoglobin, is conjugated with glucuronic acid in hepatocytes to increase its water solubility. The increases of plasma total and direct bilirubin levels by gentamicin ensure that gentamicin is a toxic agent for liver which agree with (Abd Elzaher *et al*., 2007 and Abd Elzaher *et al*, 2008) .The above increases might be attributable to the excessive production of bilirubin as a result of excessive break down of red blood cells and the inability of animals to excrete bilirubin due to obstruction, either extra hepatic (from tumors or stones) and /or intrahepatic due to damaged liver cells (Abd Elzaher, 2008). Albumin and globulin are two key components of serum proteins. Because albumin is synthesized in the liver, it is one element that is used to monitor the liver function (Friedman *et al*., 1980). The present study results revealed that total serum proteins and albumin showed a marked significant decrease in gentamicin control rats as compared with the normal ones. These results are in accordance with the results of Kumar *et al*., (2004) and Natarajan *et al*., (2006) who showed a decrease of total protein content due to destruction of protein synthesizing subcellular structures.The decrease of total protein content in serum of gentamicin control rats was due to several reasons like increased free radical production by gentamicin. In the present study treatment with fish oil and purslane shows their ability to restore the normal functional status of the poisoned liver,that observed in gentamicin reduced animals and also to protect against subsequent gentamicin nephrotoxicity.The mechanism by which the fish oil induces its nephroprotective activity is not certain. However, it is possible that omega-3, a constituent of fish oil, is at least partly responsible for the protective activity against gentamicin nephrotoxicity (Priyamvada *et al*., 2008).An additional and important factor in the nephroprotective activity of any drug is the ability of its constituents to inhibit the aromatase activity of cytochrome P-450, thereby favoring liver regeneration. On that basis, it is suggested by Speck and Lauterburgh, (1990) which fish oil could be a factor contributing to its nephroprotective ability through inhibition of cytochrome P-450 aromatase. The serum activities of ALT; AST; ALP and γ-GT and plasma level of bilirubin in treated animals with fish oil after gentamicin intoxication in agreement with El-Daley (1996) `who showed the protective effect of fish oil on gentamicin–induced nephrotoxicity in rats.Also, our result showed that the treatment of gentamicin intoxicated rats with purslane extract decreased serum activities of ALT ; AST; ALP; and plasma level of bilirubin because it contain omega-3 ,omega-6 and phenolic compounda as antioxidants (Oliveira *et al*.,2009). In the present

study the aqueous extract of purslane show improvement in biochemical parameters as a result of hepatotoxin challenge, indicating improvement of the functional status of the liver. Significant changes in classical enzymes such as ALT, AST and ALP, exclusively,as well as GGT suggest liver impairment ,since these are reliable indices of liver toxicity ,which are in agreement with (Omoniyi *et al*., 2006). An increase in ALP level may be attribute able to altered metabolism of the skeletal muscle (Olagunju *et al*., 2000). The protective effects due to treatment with purslane extract strongly indicated the possibility of the extract being able to prevent and/or mitigate any leakages of marker enzymes into circulation condition the hepatocytes to accelerate regeneration of parenchymal cells and preserves the Integrity of the plasma membranes and hence restores these enzymes levels (Al-Howiriny *et al*., 2004).

The aminoglycoside antibiotic gentamicin elicits renal tubular toxicity and cell death. Previous *in vivo* and *in vitro* studies suggested the mediation of reactive oxygen species in the tubular effects of gentamicin. In *vivo* animal models, reactive oxygen species have been identified as mediators of proximal tubular necrosis and acute renal failure caused by gentamicin (Walker, *et al*., 1999). Reactive oxygen species have been consistently demonstrated to be involved in the development of gentamicin -induced acute renal failure. It has been reported that gentamicin increases lipid hydroperoxide and suppresses superoxide dismutase, catalase and glutathione peroxidase activities (Martínez-Salgado *et al*., 2007).The present results have clearly demonstrated the ability of gentamicin to induce oxidative stress in rat liver and kidney, as evidenced by the very highly significant rise of lipid peroxidation product;and a very highly significant decline of endogenous antioxidants GSH, SOD and CAT. These findings are in agreement with other reports as Parlakpinar *et al*., (2005); Polat *et al*., (2006) and Yaman and Balikci, (2010).

On the other hand, the decrease in GSH level in liver might be attributed to the inhibition of its regeneration enzyme GSH-Rx (glutathaion reductase) by gentamicin treatment (Polat, *et al*., 2006).GSH is synthesized from oxidized glutathione (GSSG) and NADPH through the action of GSH-Rx (glutathaion reductase) (Akbay, *et al*., 1999). Also, a highly significant decrease in GSH activity was reported in this study, these observations are in agreement with those of Pedraza-Chaverri *et al*. (2000) and Farombi *et al*., (2006).

It was found that gentamicin administration to rats enhances the production of H_2O_2 in renal cortical mitochondria as a result of the increase in the production of superoxide anions. Superoxide anion and H_2O_2 may interact to form a reactive and unstable radical, namely a hydroxyl radical. This radical is formed by the reaction between H_2O_2 and Fe^{2+}(Shah and Walker, 1992).

Fe^{2+} appeared to play an important role in the production of reactive oxygen radicals in gentamicin nephrotoxicity and when oxygen radicals begin to accumulate, renal cells exhibit a defensive mechanism by using various antioxidant enzymes; such as catalase, SOD and glutathione peroxidase activities(Obatomi and Plummer, 1993). Reduced activity of one or more antioxidant systems, due to the direct toxic effect of gentamicin or volume depletion due to gentamicin administration, leads to an increase in lipid peroxidation.The decreased amount of intracellular glutathione and the accumulation of H_2O_2 and hydroxyl radicals are the triggering factors in gentamicin nephrotoxicity.Also, a highly significant decrease SOD and catalase activity was reported in this study, these observations are in agreement with those of, Yaman and Balikci *et al*, (2010). It has been reported that GM suppresses antioxidant defense enzymes and increases lipid peroxidation in the kidney (Parlakpinar *et al*., 2005). The present results confirm earlier findings (Yazar *et al*., 2003) and show that GM administration to normal rats caused severe damage to renal tissues most likely by ROS mediated mechanism as evident by decreased activities of above antioxidant enzymes and total SH levels that led to increased lipid peroxidation (LPO). Also, a highly significant increase of lipid peroxidation activity was reported in this study, these observations are in agreement with those of (Anees.*et al*, 2008). In the present results, it was found that the hepatic vitamin C content showed a highly significant decrease in gentamicin untreated rats as compared to normal rats. Our results are in accordance with those of previous investigators (Kalayarasan *et al*, 2009).The observed decrease in the levels of ascorbic acid may be due to their increased utilization for scavenging gentamicin and/or oxygen derived radicals. Vitamin C plays an important role in the tissue defense system against the oxidative stress (Wefers and Sies, 1988). Decreased activities of vitamin C were found in the kidney of rats treated with gentamicin, indicating an increase in lipid peroxidation levels of these animals (Kalayarasan *et al*., 2009). A number of investigations have demonstrated that diet supplemented with fish oil (FO) enriched in ω-3 fatty acids has profound beneficial health effects against various pathologies (Simopoulos 1991) including cardiovascular diseases, respiratory diseases, diabetes, depression, cancers, inflammatory and immune renal disorders (Thakkar *et al*., 2000).

Reports showed that FO prevents gentamicin and cyclosporine-A-induced nephrotoxicity (Thakkar *et al.*, 2000). However, the biochemical mechanism or cellular response by which FO protects against UN nephrotoxicity has not been examined. The present work was undertaken to study detailed biochemical events/cellular response/mechanisms of gentamicin nephropathy and its protection by fish oil (FO) .We hypothesized that fish oil would prevent gentamicin -induced nephrotoxicity due to its intrinsic biochemical and antioxidant properties that would lead to improved metabolism and antioxidant defense mechanism in the kidney. The results of the present study demonstrate marked amelioration of gentamicin -induced nephrotoxicity parameters by dietary FO supplementation most likely by improving energy metabolism, BBM integrity and antioxidant defense (Priyamvada*et al.*, 2008).The protection against GM effect by FO can be attributed to its intrinsic biochemical and natural antioxidant properties. As can be seen from the results, feeding of FO alone caused significant increase of SOD, catalase and GSH-Px activities accompanied by lower LPO in renal tissues. Thus, it appears FO enriched in ω-3 fatty acids enhanced resistance to free radical attack generated by GM administration similarly as demonstrated in lupus nephritis and other pathologies (Chandrasekar *et al.*, 1994). Dietary FO supplementation has also been shown to strengthen antioxidant defense mechanism in the plasma of normal rats (Erdogan *et al*. 2004).Recently, dietary FO has been shown to protect against ethanol-induced gastric mucosal injury (Leung,. 1992) in rats, a number of inflammatory diseases including lupus nephritis (Chandrasekar *et al*., 1994), IgA nephropathy (Donadio, 2001) and murine AIDS (Xi and Chen., 2000).Preliminary reports also showed partial protection by dietary FO/ω-3 fatty acids against cyclosporine/GM-induced nephrotoxicity (Thakkar *et al*., 2000 and Ali and Bashir., 1994); however, the mechanism involved was not studied in detail. Our results support that ω-3 fatty acids enriched FO may be effective dietary supplementation in the management of GM nephrotoxicity and other pathologies in which antioxidant defense mechanism are decelerated. The enzymes of oxidative carbohydrate metabolism and gluconeogenesis; Bruch bordered membrane (BBM), antioxidant defense mechanism and ^{32}Pi transport capacity appeared to be severely affected by GM treatment (Priyamvada. *et al*, 2008). The present results have clearly demonstrated the ability of fish oil to decrease oxidative stress in rat liver, as evidenced by the very highly significant decrease of lipid peroxidation product; and a very highly significant rise of endogenous antioxidants GSH, SOD and CAT These findings are in agreement with other reports (Choi-Kwon *et al*., 2004 and Priyamvada *et al*., 2008).

5. Conclusion:

We conclude that while GM elicited deleterious nephrotoxic effects by causing severe damage to renal mitochondria, BBM and other organelles and by suppressing antioxidant defense mechanism, dietary supplementation with fish oil enriched in ω-3 fatty acids caused improvement in nutrition/energy metabolism, BBM integrity, ^{32}Pi transport capacity and antioxidant defenses and thus prevented GM-induced various deleterious effects. However, purslane enriched in ω-3, ω-6 fatty acids and phenolic compound caused highly improvement than fish oil in GM-induced nephrotoxicity parameters. Based on our present observations and already known health benefits, we propose that dietary fish oil or purslane extract supplementation may provide a cushion for a prolonged therapeutic option against GM nephropathy without harmful side effects.

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Closteridia as an Etiological Agent of Mucoid Enteropathy in Rabbits.

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Abstract: This study was carried out to investigate the anaerobic bacteria as a causative agent of Mucoid enteropathy in Rabbits. Thirty - three isolates of *Clostridia perfringens (C. perfringens*) were isolated mainly from caecum of 225 specimens of forty- five diseased and freshly dead rabbits. Genotyping of *C. perfringens type A* using Multiplex PCR revealed that alpha toxin genes are detected in 8 isolates. The pathogenicity of isolated clostridia in rabbits was carried out. The clinical signs, morbidities, mortalities and body weight gain were recorded for experimentally infected rabbits. Reisolation of *C. perfringens type A* from freshly dead and/or sacrificed experimentally infected rabbits has been done. Histopathological study of intestine of affected rabbits was carried out.

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Keywords: Mucoid enteropathy, rabbits, Closridia perfringens

1. Introduction:

During recent years, rabbits industry became well established in Egypt. Rabbit meat is used as a good source of animal protein, and some breeds are reared for fur production as well as for medical and biological purposes (Ragheb et al., 1999). Enteritis remain one of the major problems facing rabbitaries, causing high mortalities mainly after weaning which is of economic importance. Mucoid enteropathy in rabbits is a multifactorial disease associated with several factors, one main factor is changes in immature caecum at weaning (Lelkes, 1987). High carbohydrate and low fiber diet, these types of diet can cause production of bacterial toxins agents like C*lostridia perfringens, Clostridia defficile* and *Clostridia spiriforme* (Butt et al., 1994). Investigation were conducted on Mucoid enteropathy outbreaks in different rabbit farms in Egypt and Clostridia perfringens type A was mainly incriminated and could be isolated from caecum of rabbits which died suddenly after short illness with sever diarrhea. Clostridia organisms are widely distributed in nature and considered to be normal inhabitant in intestinal tract of man and animal. Under certain circumstances usually stress factors and disease conditions, these organisms may become active and produce toxins which are responsible for a variety of diseases in animals (Smith and Williams 1984). Also alpha toxins of *C. perfringens type A* could be detected in the caecal content of dead rabbits (Diab et al., 2003). Therefore, the aim of this study was to monitor the current bacterial agents responsible for enterotoxaemia in rabbits through isolation and identification of the prevalent clostridia anaerobic bacteria from field affected rabbits; typing and identification of C. *perfringens* toxins genes by multiplex PCR and finally histopathological studies of experimentally infected rabbits.

2. Materials and methods

A. Materials

A.1. Specimens:

Two hundreds fifty five specimens from liver, spleen, caeci and intestine were collected from forty five diseased and /or freshly dead rabbits of different breeds and from different localities at Sharkia and Dakahlia Governorates with an average 4-12 weeks- old. All rabbits were subjected to clinical and / or postmortem examination, specimens were subjected to bacterial isolation and identification.

A.2. Bacterial media:

A.2.1. Blood agar base

A.2.2. Cooked meat media

A.2.3. Neomycin sheep Blood agar medium (Willis., 1977):

A.2.4. Brain heart infusion broth

It was used for enrichment and preservation of pure isolates *C. perfringens* and for further identification to extract DNA for PCR.

A. 2.5. Peptone water broth

A.2.6. Production Medium (Roberts et al., 1970).

It was used for production of *C . perfringens* exotoxins

A.2.7. Gas generating kit and Gaspak anaerobic jar.

A.2.8. Enriched egg yolk agar medium (Cruickshank et al., 1975.

A.2.9. Semi-Solid agar medium

A.2.10. Gelatin Medium (Smith and Holdeman, 1968):

A.2.11. biochemical identification:

- Lactose, Glucose, Maltose and sucrose used for identification and differentiation of anaerobes with 0.0018% phenol red
- Christensen's urease agar slants
- Indol test media

A.3. Chemicals, reagents, stains and solutions:

The following chemicals and reagents were prepared according to (Cruickshank et al., 1975; Koneman et al., 1992 and Baron et al., 1994)

1- Hydrogen peroxide 3% ($H_2 O_2$) freshly prepared for catalase test.
2- Kovak's reagents for indole test.
3- 0.02% methyl red solution for methyl red.
4- Bromocresol purple for detection of lecithinase activity.
5- Phosphate Buffered saline (PBS).
6- Phenol red for sugar fermentation test as 0.0018% and urea utilization test as 0.0012%.
7- Natural buffered formalin 10% for fixation and preservation of the affected organs for histopathological examination.
8- Gram stains which was used to differentiate between the organisms into Gram-positive or Gram negative.

A.4. Antibiotics:

1- Neomycin sulphate used to obtain separate colonies of clostridium and prevention the growth of other anaerobes.
2- Streptomycin and Rivampicin for preparation of resistant strain of inoculated microorganisms (*C. perfringens type A*) for experimental infection.

A.5. Experimental rabbits:

Thirty - two native breed rabbits were obtained from private farm in Sharkia Governorate for experimental infection. Rabbits were fed on ration obtained from (El Marwa Company) containing: (protein 18.5%, fiber 15.5%, fat 3.4% and calories 26500). The ration contains Clazurel as anticoccidial drug and not contain antibiotics.

A.6. Material used for polymerase chain reaction

A.5.1 Materials, buffers and reagents used for multiplex PCR after (Yoo et al., 1997)

- Tris boric EDTA (TBE) 5x: Tris base-Boric acid-EDTA. It was used for DNA extraction and as buffer for visualization of PCR products in agarose.
- 10x PCR buffer (Gibco/ BRL, Grand Island, N.Y.).
- $MgCl_2$ (2.5 mM) applied biosystem PCR mix, USA)
- dNTPs (deoxy nucleotide triphosphate) 10mM) (Gibc6)
-Taq thermostable DNA polymerase (Biometra) (2U).
-Template DNA (*C.perfringens* alpha, beta and epsilon toxin genomes
-Reference strains of (alpha, beta and epsilon toxin genomes) were obtained from National Laboratory for Veterinary Quality Control (NLVQP), Animal Health Research Institute (AHRI), Dokki, Giza, Egypt.
- Oligonucleotide primers (100 pmol).

Primers for the four toxin genes (alpha, beta, epsilon and iota) of *C. perfringens* were selected after (Yoo et al., 1997) as shown in table (1).

Table (1) shows Primers for the four toxins genes of *C. perfringens* type

Primer name and direction	Nucleotide sequence	Size of amplified products (bP)
Cpa(alpha toxin genes Forward Reverse	5' GTT GAT AGC GCA GGA CAT GTT AAG3' 5' CAT GTA GTC ATC TGT TCC AGC ATCC3'	402
Cpb(beta toxin genes Forward Reverse	5' ACT ATA CAG ACA GAT CAT TCA ACC3' 5' TTA GGA GCA GTT AGA ACT ACA GAC 3'	236
Cpe(epsilon toxin genes Forward Reverse	5' ACT GCA ACT ACTACT CAT ACT GTG 3' 5' CTG GTG CCT TAA TAG AAA GAC TCC3'	541
Cpi (iota toxin genes Forward Reverse	5' GCG ATG AAA AGC CTA CAC CAC TAC3' 5' GGT ATA TCC TCC ACG CAT ATA GTC3'	317

A.5.2. Agarose gel electrophoresis buffers and reagents (Piattir et al., 2004)

a- Agarose gel 1.5%
b- Agarose 1.5gm
c- Electrophoresis buffer (TAE or TBE 100 ml)
d- TBE (Tris Boric EDTA).
Composed of 0.5 x (0.04 µl Tris-borate and 1 mM EDTA, pH 8.0
e- Ethidium bromide solution
Ethidium bromide powder 10 mg
Distilled water (D.W.) 1 ml

It mixed and put into melted agarose to reach to a final concentration of 1.0- o.5 mg /ml. It was used as a fluorescent dye to stain the DNA during examination by UV transilluminator.

f- Gel loading buffer
Bromo phenol blue 0.25%
Xylene cyanol 0.25%
Glycerol 30.0%

They are dissolved in sterile D.W. and covered with aluminum foil to be stored at room temperature. DNA ladder (100 bp, pharmacia, USA).

B. Methods

B.1 Clinical and postmortem examination:

Clinical examination of diseased rabbits was carried out for recording clinical signs. Faecal samples were examined microscopically for excluding coccidiosis. Post mortem examination of both freshly dead and sacrificed rabbits were carried out to recording the post mortem lesions. Specimens from liver, spleen, ceacum and intestine were collected in separate sterile container for bacteriological examination. In addition specimens of affected intestine from dead and sacrificed rabbits were collected in 10% buffered formalin for histopathological examination.

B.2 Bacterial isolation:

Specimens of liver, spleen, caeci, and intestine were cultivated on sterile freshly prepared cooked meat media in two tubes, one tube was heated in water bath at 80^{o}C for 15-20 minutes to kill any other vegetative form of the bacteria than clostridia spores, the other tube was left without heating then incubated anaerobically in anaerobic jar that contained catalyst and gas generator kits at 37^{o}C for 24-48 hours. A loopful from previously incubated tube was streaked into surface of 10% sheep blood agar with neomycin sulphate plate (100µg/ml). Then the plate was incubated anaerobically at 37^{o}C for 24-48 hours. Suspected colonies of Clostridia were picked up and examined for their morphological and cultural characters then subculture on brain heart infusion broth tubes for preservation. (Koneman et al., 1992 and Baron et al., 1994).

B.3. Bacterial identification.

B.3.1 Morphological identification

Bacterial smears were prepared from the suspected colonies and stained with Gram stain and examined microscopically for morphological characteristic. The colonial appearance was also studied to investigate their structure, surface edge and colour.

B.3.2. Biochemical identification.

Suspected colonies of isolated microorganisms were identified by a series of biochemical tests according to Smith and Holdeman (1968), Willis (1977) and Koneman et al., (1992).

- Catalase test using few drops of freshly prepared 3 % H_2O_2.
- Indol test using tubes with 2 % peptone water.
- Gelatinase – activity test using nutrient gelatin media.
- Methyl red test using glucose phosphate peptone.
- Lactose, glucose, sucrose and maltose fermentation test using 1% peptone broth.
- Lecithinase reactions test using egg yolk agar plate.

B.3.3. Molecular identification.

B.3.3.1. DNA extraction, crude cell lysate (Daube et al., 1994)

One to five pure colonies of C. *Perfringens* that showed double zone of haemolysis on blood agar were grown overnight in 10 volumes of brain heart infusion (BHI) supplemented with 1% (W/V) sodium thioglycolate under anaerobic conditions at 37^{o}C for 16-24hours. The cells were harvested by centrifugation (5000Xg, 15min, 4^{o}C). Take 1 ml of culture then were washed twice with 1 ml of phosphate buffer saline (PH 7.2) then pellet was suspended in 50 ml of TE buffer. The mixture was mixed by vortex apparatus, boiled for 10 min. for cell lyses, centrifuged at 13000xg for 2 min. and 5 ml of the supernatant was used as template.

B.3.3.2. PCR amplification and cycling protocol: (Yoo et al., 1997).

PCR was performed in thermocycler in a total reaction volume of 50 ml containing 5 ml of 10 x PCR buffer, 4 µl of $Mgcl_2$, 1 µl of dNTPs, 0.5 ml of each primers, 5 ml template DNA, 1 µl of Taq – polymerase and up to 50 µl of distilled water. The following program was used: initial denaturation at 94^{o}C for 5 min, followed by 30 cycles consisting of denaturation at 94^{o}C for 1 min, annealing at 55^{o}C for 1 min, and extension at 72^{o}C for 1 min.

B.3.3.3. Detection of PCR products.

Ten µl of amplified PCR products were mixed with gel loading buffer and electrophoresed in 1.5% agarose gel as shown in the following: The prepared agarose was melted by using microwave oven, let the solution cool down to about 60^{o}C at room temperature, then ethidium bromide was added by 0.5 µg/ml and mix thoroughly. It poured directly on gel casting try after proper installation of the desired comb at one side of the gel, about 5-10 mm from the end of the gel and after the gel was solidified at room temperature, carefully remove the comb. The holes that remain in the gel are the wells. Electrophoresis buffer (TBE or TAE buffers) that used for preparation of the gel was added into the tank to a level of 1-2 mm above the gel layer. Then the samples and a 100bp DNA ladder (market) were mixed with proper gel loading buffer then loaded in the wells, the cover of the tank was closed and the power supply was attached. A current of 60 -80 V for 1 hour was passed on the electrophoresis unit and bromophenol blue was allowed to run to run 2/3 of the gel length before terminating the run. Stopped the run and the gel was transferred to ultraviolet

transillumination to observe the specific amplicons, compared with the marker and photographed by digital camera.

B.4. Preparation of resistant strains.
C. Perfringens type A. was treated with streptomycin and Rifampicin for obtaining resistant serotype. It was subcultured for 24-48 hours anaerobically in 5 successive brain heart infusion broth containing increased concentration of streptomycin and Rifampicin (0.01gm,1 µg to 1 gm, 50µg) respectively, then subcultured on blood agar containing 1 gm streptomycin and 50 mg Rifampicin per litter.

B.5. Titration of inoculum:
Clostridia strain was prepared by selection one colony from blood agar to be cultivated on brain heart infusion broth and incubated anaerobically for 48hours. Serial dilution of microorganism in sterile PBS. The viable cell concentration of the inoculum was determined by colony count on blood agar. The infected dose was adjusted to $1x10^9$CFU.

B.6. Experimental design:
To study the pathogenicity of *Clostridia perfringens type A* in rabbits. Thirty- two native breed rabbits were divided into two main groups according to age. First group (G1) containing 16 rabbits aged 4-6weeks. Second group(G2) containing 16 rabbits aged 8-10weeks. Each group of rabbits were subdivided into two subgroups (1a, 1b) and (2a, 2b) each subgroup containing 8 rabbits. Subgroup (1a) rabbits were infected orally with C. *Perfringens* type C in a dose of 1.0 ml containing $1x10^9$CFU. Meanwhile, subgroup (1b) was remained as a negative control. Subgroup (2a) rabbits were infected orally with C. *Perfringens* type A in a dose of 1.0 ml containing $1x10^9$CFU. Meanwhile, subgroup (2b) was remained as a negative control. All experimental rabbits were observed for 2 weeks before experimental infection to be examined to exclude coccidia, *E coli* and clostridium infection. Clinical signs, morbidities, mortalities of all experimentally infected rabbits were recorded for 3 weeks post infection, an histopathalogical examination was carried out.

B.7. Histopathological examination:
Histopathalogical examination was carried out according to Schauer et al., 1998). The necropsy was performed and samples were collected from the affected intestine and fixed in 10 % buffered neutral formalin solution. Five - micron thick paraffin sections were prepared, stained with hematoxyline and eosin and then examined microscopically for histopathological finding.

3. Results
3.1 Clinical findings
Clinical examination of collected diseased rabbits was shown that watery brownish and/or yellowish diarrhea, or white mucoid discharges staining the hair around the anal, and the hind quarters, belly swollen, impacted ceacum and off food. In late stages of the disease, examined rabbits showed dehydration, emaciation and deaths among all ages especially young rabbits 3-12 weeks age.

3.2. Post mortem findings
Post mortem examination of both freshly dead and scarified rabbits revealed congestion of the liver; heart and kidneys; caecum filled with watery to mucoid contents and gases; distention of the small intestine with watery fluid contents; congested mesenteric blood vessels, in addition, some examined rabbits revealed ceacum containing pasty feted odor contents and colon filled with mucoid material instead of hard pellets.

3.3. Bacterial isolation.
Specimens from liver, spleen, intestine and ceacum were collected from rabbits involved in enteric problems and cultivated on cooked meat media for enrichment of clostridia species and incubated an aerobically at 37^oC for 18-24 hours. Aloopful from previously enriched culture were streaked on neomycin sulphate sheep blood agar and incubated anaerobically at 37^oC for 24-48h. Colonies were rounded, raised, smooth, opaque glistening, showed double zones of haemolysis and 2-4mm in diameter.

Table (2) Shows Prevalance of bacterial and non bacterial isolates among examined rabbits.

number	Total no. of C. perfringens	Ceacal Coccidiosis	Hepatic coccidiosis	Round nematodes
45	33	19	2	27
Percentages	73.33%	42.2%	4.4%	60%
Site	Ceacum	Ceacum	Liver	Ceacum

Table (3) bacterial isolates according to different ages:

Age	No of rabbits	Clostridium		
		No	Percentage	
1M	8	4	50%	
2M	27	20		74%
3M	10	9	78,4%	90%
Total	45	33		

C .perfringens, at 2-3 months old rabbits were 78.4%

3.4. Bacterial identification.

3.4.1 Morphological identification

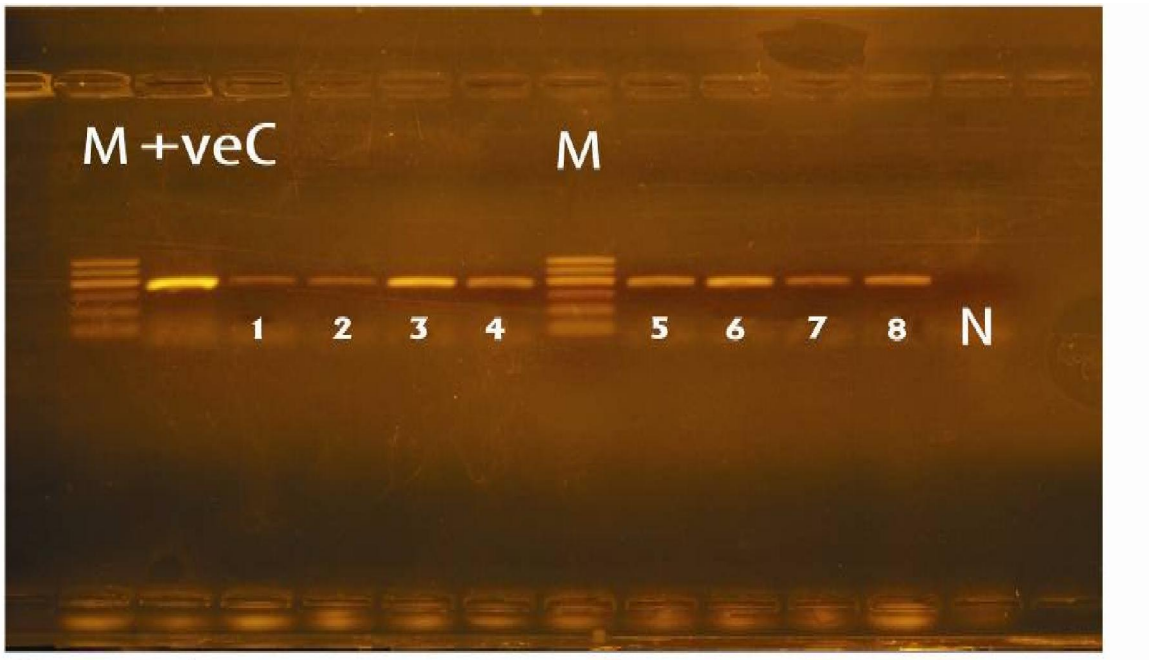
Smears from the colonies were stained with Gram's stain for microscopical examination. Clostridium isolates were Gram positive, short bacilli, straight with parallel sides and rounded ends. Some strains kept in refrigeration for 4-5 months showed central oval non bulging spore.

Biochemical identification

Clostridium isolates were positive with Gelatinase activity, Lecithinase reaction, Sugar fermentation test (Lactose, Glucose, Sucrose, Maltose), Methyle red test and Indol test and negative with Catalase test.

3.4.2- Molecular identification by Polymerase chain reaction:

PCR used for identifying and typing of toxin genes of *C. perfringens* that were isolated from diseased or freshly dead rabbits. C. perfringens *type A* identified by presence of alpha toxin genes and gave at 402bp fragment that were shown in eight strains as in picture no. A.



Picture no. A

Lane "M": 100 bP DNA ladder (Marker).
Lane "C": Positive (+ ve) control of *C. perfringens type "A".*
Lane "1, 2, 3, 4, 5, 6, 7, 8" were only alpha toxin genes producing $_{C}$*. perfringens type "A"* field isolates.
Lane "N" Negative (- ve) control.

3.6 Clinical finding of rabbits experimentally infected with *C. perfringens type A.*

The most clinical findings in experimentally infected rabbits with *C. perferingens type A.* were depression, ruffled fur anorexia, slight swollen belly, watery brownish to yellowish diarrhea, decrease feed intake and sometimes, faeces admixed with mucus material. The mortalities of experimentally infected rabbits begin 5 days post infection with an incidence percentage 0f 25% . The most sever symptoms were observed in experimentally rabbits aged 8-10 weeks – old.

3.6.2. Post-mortem findings of rabbits experimentally infected with *C. perfringens type A.*

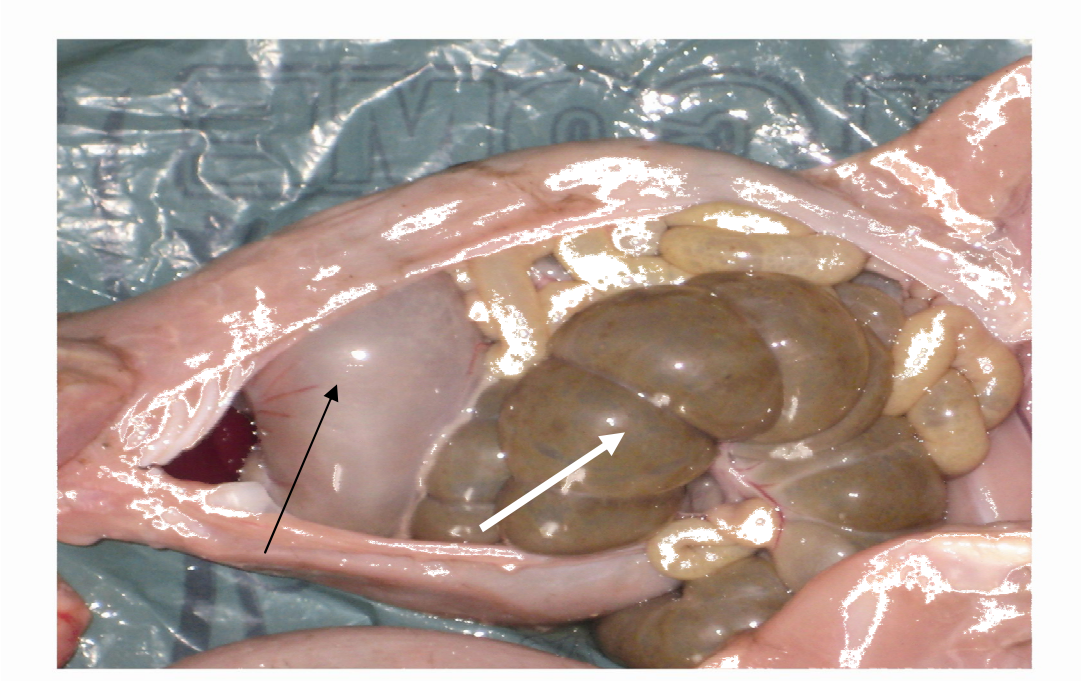
The main gross lesions were congestion of liver, spleen, heart and engorgement of subcutaneous blood vessels. fully distended stomach with fluids and gases, also small intestine and ceacum was distended with watery to mucoid contents and gases. Catarrhal enteritis in ceacum in some cases. Fig. (1&2).

3.6.3. Reisolation trial of *C. perfringens type A* from experimentally infected rabbits.

Reisolation of *C. perfringens type A* was performed in cocked meat medium containing 1.0 gm/liter streptomycin.

Table (4) Shows result of morbidity and mortality percentage of in experimentally infected rabbits.

Group number	Inoculums	Age	Morbidity		M Mortality	
			NO	Percentage	NO×	Percentage
1a	*C. perfringens type A*{	wks4-6	86/	75.%	2/8	25 25%
1b	-ve	wks4-6	3 3/8	37. 37.5%	0/8	0%
2a	*C. perfringens type A*	8-10 wks	6/8	75.%	2/ 2	25 25%
2b	-ve	8-10 wks	/83	37 37.5%	0/ 0	0%



Fig, (1): Caecum of rabbit experimentally infected orally with *C. perfringens type A* at 4-6 weeks –old showing ceacum filled with watery mucoid material and gases, distention in the jejunum and ileum with watery fluid content and congestion of liver. (sacrificed experimental case 7d after infection).

Fig, (2) showing intestine of rabbit experimentally infected orally with *C. perfringens type A* at 4-6 weeks – old with presences of desquamated epithelium. (sacrificed 7 days after infection

3.6.4. Histopathological results of experimentally infected rabbits.

Intestinal lumen of rabbit inoculated with *C. peferingens type A* had excess mucus casts mixed with a few leukocytes with villous destruction and sever inflammatory reaction, mainly macrophages and lymphocytes in both mucosa and submucosa Fig. (3). Other rabbits that infected with *C. peferingens type A* revealed proliferation of intestinal glands, submucosal edema and leukocytic infiltration with regeneration attempts in the surface epithelium Fig (4). On the other hand the intestinal coats of non infected rabbits revealed normal intestinal coats Fig. (5).

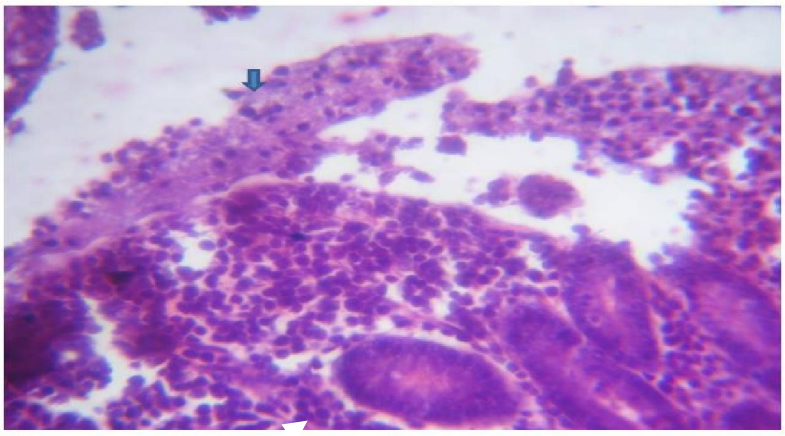


Fig. (3). Small intestine of rabbit infected with *C.perferingens type A,* showing mucus casts with a few leukocytes (arrow) inside intestinal lumen. H&E x 1200.

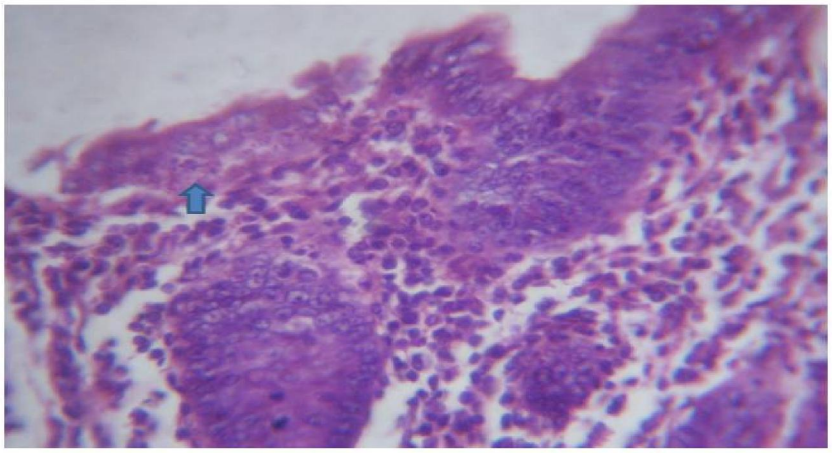


Fig. (4). Large intestine of rabbit infected with *C.perferingens type A* showing proliferation of surface and glandular epithelium. H&E x 1200 .

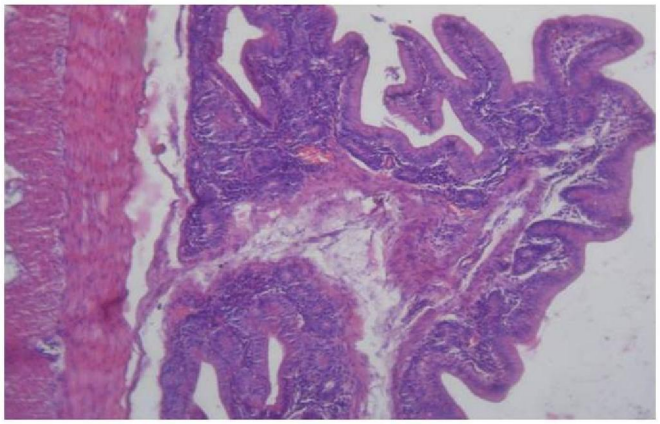


Fig. (5). Small intestine of rabbit showing normal intestinal coats H&E x 300.

4. Discussion:

Rabbit farming enterprises in Egypt have experienced serious losses in weaned animals due to gastrointestinal problems. Investigations had confirmed that these animals suffered from Mucoid enteropathy caused by *C. perfringens type A* Diab et al., (2003). In the present study, isolation and characterization of some anaerobic pathogens causing Mucoid enteropathy in rabbits was carried out. Mucoid enteropathy syndrome (MES) or Epizootic rabbit enteropathy (ERE) have emerged and disseminated in different farms causing great

economic losses during the past years all over the world including Egypt. Enteric diseases are responsible for high morbidity characterized by growth depression, poor food conversion rate or mortality specially in young rabbits_Licois., (2004). In this study samples from liver, spleen, caeci and intestine were collected from rabbits either showing signs of diarrhea or freshly dead rabbits suffering diarrhea from different localities at sharkia and Dakhalia governorates. The isolated anaerobes were clostridia and identified from intestinal samples were 73.33% . The high incidence of clostridia infection at 2-3 month - old rabbits (78.4%) these means that the bacterial isolates were more common at the weaning age rather than suckling age. This results agreed with Mcpherson., (2000) who recorded that enterotoxaemia disease most commonly seen in weaning rabbits. Moreover, Patton et al. (1978) and Carman and Borriello (1984) recorded that the predisposition of rabbits to anaerobic infections is increased by stress factors, bacterial infection, parasitic infection, dietetic disorder and excessive antibiotic administration. The biochemical finding suggested that all isolates of Clostridia recovered from intestinal samples of examined rabbits were *Clostridia perfringens*. This result was confirmed by multiplex PCR. This result agreed with Ali et al., (1994) who isolated *C. Perfringens* (81%) from total of 120 rabbit samples and types toxigenic strains (22 strains) into type A, D and E. The type A was the most predominant one (16 strains). In addition Cocchi et al., (2008) showed that *C. Perfringens type A* was most commonly recorded type from ceacum of diseased rabbits and represented by 99.33 % which agree with our results. PCR was used for identification and confirmation of the causative agents, PCR is very sensitive and specific technique for detection of genes encoding alpha, beta, epsilon and iota exotoxins of *C. perfringens* (Nillo., 1980: Titball et al., 1989: Daube et al., 1994 and Yoo et al., 1997). In the present study, multiplex PCR was very sensitive test for genotyping of *C. perfringens* isolates. The recorded results revealed that the tested strains were identified and typed as eight *C. perfringens* according to presence of alpha toxin gene and give a

Characteristic band at 402 bp. This result was agreed with results obtained by Yoo et al., (1997) who developed multiplex PCR assay for determination the toxin genes of *C. perfringens* that give similar characteristic band. Experimentally infected rabbits, aged 6-8 weeks and 8-10 weeks-old, with *C. perfringens type A* showed depression, ruffled fur, anorexia slight swollen belly, watery brownish to yellowish diarrhea staining hind quarter decrease feed intake and sometimes, faeces admixed with mucus material. Mortality reach was the same, as it reach to 25. The main gross lesions were congestion of liver, spleen, heart and fully distended stomach with fluid and gasses, catarrhal enteritis, ceacum and colon were distended with watery mucoid contents and gases. Histopathological findings revealed that the intestine of rabbits infected with *C. perfringens type A* had excess mucous, casts mixed with a few leucocytes with villous destruction and sever inflammatory reaction, mainly macrophages and lymphocytes in both mucosa and submucosa. Intestine of other rabbits infected with *C. perfringens type A* revealed proliferation of intestinal glands, submucosal odema and leucocytic infiltration with regeneration attempts in the surface epithelium. Similar findings were recorded by Percy et al., (1993) and Wilber., (1999).

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Response Of Sunflower To Environmental Disparity

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Abstract: Sunflower crop has an evolutionary benefit of being able to maintain high level of viability in a variety of environments. Field experiments, one each in spring and autumn were executed at Pir Mehr Ali Shah, Arid Agriculture University Rawalpindi, Pakistan for two years (2007& 08) to document the effect of growing degree days on performance of sunflower hybrids. Four Sunflower hybrids, Alisson-RM, Parasio-24, MG-2 and S-278 were planted in Randomized Complete Block Design with four replications during spring and autumn. The data on yield and yield attributes of sunflower like number of achenes per head, hundred achenes weight, biological and achene yield along with achene oil content was recorded. All parameters were influenced by prevailing temperature. Amongst hybrids, MG-2 produced the maximum values for all parameters during both the seasons (spring & autumn). Overall, spring planted crop exhibited significantly higher values for achenes per head, biological yield, achene yield and oil content in comparison with autumn planting, which may be attributed to accumulation of more growing degree days during the season.

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1. Introduction

Temperature is a basic factor that affects the course of life, specifically the physiological time. Thermal time gives a measure of physiological time as it relates to many poikilothermic species of plants (Trudgill *et al.* 2005). Most crop species are adapted to particular temperature ranges which is major environmental factor influencing their distribution (Atkinson and Porter, 1996), but a key factor which influences plant growth, development and productivity (Kaleem *et al.*, 2009). Expression of different yield and yield attributes under varying seasons is considered due to the different climatic conditions those are based on temperature prevailing during the crop life cycle (Killi and Altanbay, 2005).

Although sunflower is a temperate zone crop but it can perform well under various climatic and soil conditions. A number of plant's developmental, morphological and physiological adaptations to the environment, influence sunflower yield and oil (Hassan *et al*., 2005). World wide cultivation under very hot and cold conditions might have developed the unique properties of sunflower tolerance to both low and high temperature (Khalifa *et al*., 2000). Sunflower is a C_4 plant having higher physiological activity but is sensitive to cold temperatures and called as warm season plant as compared to C_3 plants (Brouder *et al*., 2008).

Environmental disparity alters morphological, physiological, quantitative and qualitative expressions of sunflower which are affected by extreme growing conditions. All physio-morphic developments occurring in plant are markedly influenced by temperature as the primary factor governing the growth (Chan *et al.*, 1998). Having wide adaptability, different sunflower hybrids require different total number of cumulative degree-days or growing degree days for growth, development and maturity (Qadir *et al.*, 2007). The most common temperature index used to estimate plant development is growing degree days (GDD). A linear relationship between GDD and rate of plant development has been reported by Lu *et al*. (2001). GDD can be used to classify plants for their flowering, estimate maturity/harvest and to predict the duration between two stages (Bonhomme, 2000). Sur and Sharma (1999) recorded that the total growing degree days decreased from 1731 to 1621 with delay in planting, as the late sown crop experienced lower temperature during the seed filling period. However, Kaleem, *et al.*, (2009) concluded that lower yield associated with late planting in sunflower was due to warmer temperature during the early growth period, which accelerated stem growth and early switching over from vegetative to reproductive stage.

Reproductive phase of sunflower crop is more sensitive to cold condition resulting in floral abortion,

infertile pollens, poor seed set and empty seeds with reduced seed size that affects the total output (Clarke and Siddique, 2004). Autumn sowing with high temperatures and low relative humidity at the time of pollination, affects pollen health and vigor, causing poor pollination, produces less weight, empty and sterile achenes, thus influencing sunflower head fertility and achene yield (Miralles *et al.*, 1997). Sunflower biomass production is positively correlated with temperature and photoperiod. Biological and achene yield was affected at maturity due to temperature and photoperiod during development under different seasons as higher biomass and seed yield was recorded in spring sowing than in autumn sowing (Villalobos *et al*., 1996). Spring and autumn sowings of sunflower performed differently. Seed yield along with growth and development was significantly affected with delayed sowing because of lesser efficiency of components contributing in sunflower yield (Kaleem *et al*., 2009).

Planting sunflower crop in different seasons causes temperature variations in the field, thus crop will grow in different environmental variables like temperature, sunshine, rainfall and relative humidity. Sowing in two season (spring & autumn) created difference in temperature for growth, development and maturity, thus a wide range of temperature may be encountered from sowing till maturity during both the seasons. The present investigation was thus contemplated to investigate relationship of growing degree days with yield and yield components of sunflower crop sown during spring and autumn seasons.

2. Materials and methods

Field experiments were conducted at Pir Mehr Ali Shah, Arid Agriculture University, Rawalpindi, Pakistan, located at 33^o and 38^o N and 73^o and 04^o E, during 2007 and 2008 (spring and autumn in each year). The soil of experimental site was loam type in texture having sand 43%, silt 46% and clay 11%, pH 7.4 and EC 0.66 m S cm^{-1}. Available NPK status in the soil before sowing was 300, 5.00 and 140 mg kg $^{-1}$ respectively. The particular experimental site was winter fallow prepared for sowing by giving one soil inverting plough, thereafter, ploughed thrice with tractor mounted cultivator and planked with last ploughing. Recommended dose of fertilizer of 80 kg Nitrogen and 60 kg P_2O_5 per hectare was applied in the form of Urea and DAP at the time of last ploughing. Spring crop was sown on 18th March for each year while autumn crop was sown on 18th August for each year. Four sunflower hybrids (Alisson-RM, Parasio-24, MG-2 and S-278 were sown by using seed @ 5kgs / ha. Seeds were sown with dibbler by putting two seeds at each pre-marked spot. Plant to plant distance was maintained 25 cm and row to row 75 cm in net plot size of 5x3 m^2. After complete emergence, one plant was maintained per hill. Weeds were kept under control by hand weeding through out crop life cycle.

The cumulative growing degree days from emergence till maturity were calculated from meteorological data obtained from Meteorology Department located near experimental site (Table I) through out crop life cycle by the equation of Dwyer and Stewart (1986).

$$CHU = \sum^{t_1}_{t_2}[(T_{max}+T_{min})/2-8]$$

where $[(T_{max}+T_{min})/2-8]>0$

$T_{max}+T_{min}$ were daily maximum and minimum air temperatures in degree centigrade and t_1 and t_2 were the time intervals. Base temperature for sunflower development is 8°C (Sadras & Hali, 1988).

To record the data for number of achenes head^{-1}, ten heads were taken at random from each plot. The number of achenes were counted and the average was worked out. Similarly, to record HAW (hundred achenes weight), five samples of hundred achene were taken randomly from the total seed lot of each plot, then weighed using digital electronic balance and average was worked out. Two central rows of 5m length were harvested after complete maturity from the each plot for biological yield (on 8.7.2007, 5.7.2008 during spring and 14.11.2007, 21.11.2008 during autumn. Harvested plants were sundried for 15 days during autumn, 9 days during spring season and weighed with the help of spring balance to obtain biological yield per plot and then per hectare yield was computed. For achene yield, plants already harvested from two central rows and then sundried for different days according to season were threshed manually. Achene yield per plot was recorded which was converted into kg ha^{-1}. Similarly, achene oil concentration was determined by using the NMR (Nuclear Magnetic Resonance system), Model MQA-7005, Oxford Institute, USA, as described by Granland and Zimmerman, (1975). The equipment was standarderized with six different oil contents having the samples previously analyzed.

2.1 STATISTICAL ANALYSIS

The collected data were subjected to statistical analysis by applying MSTATC, separately for both the years (Freed and Eisensmith, 1986). Analysis of Variance Techniques were employed to test the significance of data. Least Significant Difference Test at 5% probability was used to compare the means **(**Montgomery**,** 2001).

3. RESULTS

3.1 Number of Achenes Head^{-1}

Hybrids under evaluation exhibited statistically significant differences for yield and yield components

during both the seasons. As regards number of achenes head^{-1}, the hybrids differed significantly during spring & autumn seasons (Table 3). The hybrid MG-2 produced the maximum (1182.50, 641.37) number of achenes head^{-1} during both seasons respectively. The least number of achenes head^{-1} were observed from Parasio-24 (761.62, 452.12) which was statistically ($p< 0.05$) at par with hybrid Alisson-RM (801.75). Comparison of years showed non significant differences during spring while statistically significant differences were achieved during autumn (Table 3). The interaction of hybrids x years was statistically significant. The maximum (1326.50) number of achenes head^{-1} were produced by the hybrid MG-2 during spring 2007 and autumn 2008 (715.00) while Parasio-24 gave the minimum (742.25) number of achenes head^{-1} during spring 2007 and 387.50 number of achenes head^{-1} during autumn 2007.

3.2 Hundred Achenes Weight

The differences among hybrids for HAW showed statistically non significant differences during the both (spring and autumn) seasons. Comparison of years and interaction of hybrids x years were also statistically non significant (Table 4).

3.3 Biological Yield

Non significant differences among hybrids for biological yield was observed during spring season (Table 5). However, during autumn hybrids exhibited statistically significant differences. The hybrid MG-2 produced the highest (10162.25 kg ha^{-1}) biological yield which was statistically ($p<0.05$) significant from rest of the hybrids, whereas, the lowest (5842.75 kg ha^{-1}) biological yield was recorded from hybrid Parasio-24 during autumn. Comparison of years for biological yield exhibited statistically non significant differences during spring while statistically significant differences during autumn season (Table 5). The interaction of hybrids x years were statistically significant for both the seasons (spring and autumn). The maximum biological yield (14752.75 kg ha^{-1}) was obtained from MG-2 during spring 2007 and biological yield of 10604.50 kg ha^{-1} was recorded from same hybrid during autumn 2008 while Parasio-24 gave the minimum biological yield (10710.00 kg ha^{-1}) during spring 2007 and 5463.00 kg ha^{-1} during autumn 2007 was observed from same hybrid.

3.4 Achene yield

Similarly, statistical differences among hybrids for achene yield were recorded during spring & autumn (Table 6). The highest achene yield (4360.72, 1984.00 kg ha^{-1}) was obtained from the hybrid MG-2 during two seasons respectively. The lowest achene yield (3303.25, 1311.55 kg ha^{-1}) produced by Parasio-24 during both the seasons respectively. Comparison of the years showed statistically significant differences during both, spring and autumn seasons (Table 6). The interaction of hybrids x years were statistically significant for both the seasons (spring and autumn). The maximum achene yield (4725.25 kg ha^{-1}) was recorded from MG-2 during spring 2007 while achene yield of 2171.72 kg ha^{-1} was recorded from same hybrid during autumn 2008. The hybrid Parasio-24 gave the minimum achene yields of 2199.45 kg ha^{-1} and 1059.90 kg ha^{-1} during spring 2008 and autumn 2007 respectively.

3.5 Oil contents

In present study, statistical differences among hybrids for oil contents were recorded during the both spring and autumn seasons (Table 7). The maximum oil content (48.39, 46.46 %) was exhibited by hybrid MG-2 during spring and autumn, respectively. Comparison of the years showed statistically non significant differences during spring while significant differences were exhibited during autumn season (Table 7). Oil content recorded during autumn 2008 was 3.10 % higher as compared to those observed during autumn 2007. The interaction of hybrids x years was statistically significant for both the seasons (spring and autumn). The maximum (49.2, 47.2 %) oil content was recorded from the hybrid Parasio-24 during spring 2007 and from MG-2 during autumn 2007, respectively, while the minimum (39.18, 38.20 %) was recorded from Alisson-RM during spring 2008 and autumn 2007, respectively.

Table 1: Meteorological data of two years, Spring, Autumn 2007 and Spring, Autumn 2008

SPRING 2007						SPRING 2008				
MONTH	Temperature (°C)		Rain fall (mm)	RH (%) (Mean)	Sun shine (Hours) (Mean)	Temperature (°C)		Rain fall (mm)	RH (%) (Mean)	Sun shine (Hours) (Mean)
	Max (Mean)	Min. (Mean)				Max. (Mean)	Min. (Mean)			
March	23.1	9.0	143.2	47.0	7.4	29.7	11.8	19.1	57.0	7.9
April	34.0	15.9	18.0	44.0	10.7	29.7	15.8	92.9	59.3	7.7
May	37.0	19.8	80.6	42.0	10.0	37.2	20.8	10.1	40.0	9.9
June	37.6	23.0	22.3	51.0	9.5	35.6	22.3	225.0	62.4	7.5
July	35.2	21.5	262.5	68.0	9.3	35.0	22.8	432.5	69.6	7.4
AUTUMN 2007						**AUTUMN 2008**				
Aug	34.2	21.8	485.0	72.0	8.3	33.3	23.0	221.0	66.6	7.5
Sep	32.9	19.4	201.0	68.0	7.8	32.3	19.7	66.0	51.8	8.1
Oct	31.5	12.6	0.00	54.0	9.6	31.0	15.4	24.0	43.8	7.9
Nov	26.0	8.2	10.0	71.0	7.0	25.2	8.1	18.0	50.5	8.5
Dec	-	-	-	-	-	20.8	5.5	71.7	55.9	6.4

Table 2: GDD accumulated during spring and autumn seasons (Means of two years)

S#	Growth Weeks	GDD accumulated (Spring Season)		GDD accumulated (Autumn Season)	
		during the week	Total GDD	during the week	Total GDD
1	1	67.60	67.60	116.95	116.95
2	2	92.45	160.05	137.25	254.2
3	3	90.65	250.7	125.00	379.2
4	4	112.25	362.95	139.00	518.2
5	5	132.35	495.30	134.00	652.2
6	6	128.25	623.55	116.3	768.5
7	7	143.00	766.55	104.6	873.1
8	8	140.60	907.15	93.5	966.6
9	9	150.25	1057.4	106.15	1072.75
10	10	125.50	1182.9	101.22	1173.97
11	11	149.70	1332.6	88.15	1262.12
12	12	162.35	1494.95	77.65	1339.77
13	13	163.90	1658.85	47.86	1387.63
14	14	151.80	1810.65	-	-
15	15	142.11	1969.7	-	-
16	16	159.05	2128.75	-	-
Grand Total GDD			**2128.75**		**1387.63**

Table 3: Number of Achenes Head $^{-1}$ of sunflower hybrids during two seasons of 2007 and 2008

Hybrids	Spring			Autumn		
	2007	**2008**	**Mean**	**2007**	**2008**	**Mean**
Alisson-RM	756.50 c	847.00 c	**801.75 B**	442.50 c	647.50 a	**545.00 B**
Parasio-24	742.25 c	781.00 c	**761.62 B**	387.50 c	516.75 b	**452.12 C**
MG-2	1326.50 a	1039.00 b	**1182.50 A**	567.75 b	715.00 a	**641.37 A**
S- 278	1269.00 a	873.00 bc	**1071.00 A**	387.75 c	653.75 a	**520.75 BC**
Mean	**1023.56**	**884.81**		**446.37 B**	**633.25 A**	

*Any two means not sharing a letter in common differ significantly at 5% probability level

Table 4: Hundred Achene weight (HAW) (g) of sunflower hybrids during two seasons of 2007 and 2008

Hybrids	Spring			Autumn		
	2007	**2008**	**Mean**	**2007**	**2008**	**Mean**
Alisson-RM	5.39 NS	5.25	**5.32 NS**	4.60 NS	4.88	**4.74 NS**
Parasio-24	5.63	5.30	**5.47**	4.98	5.04	**5.01**
MG-2	5.42	5.24	**5.33**	4.96	5.08	**5.02**
S- 278	5.46	5.20	**5.33**	4.89	4.96	**4.92**
Mean	**5.47**	**5.24**		**4.85**	**4.99**	

*Any two means not sharing a letter in common differ significantly at 5% probability level

Table 5: Biological yield (kg ha^{-1}) of sunflower hybrids during two seasons of 2007 and 2008

Hybrids	Spring			Autumn		
	2007	**2008**	**Mean**	**2007**	**2008**	**Mean**
Alisson-RM	12720.50 ab	12885.00 ab	**12802.75**	6099.00 c	9185.00 b	**7642.00 B**
Parasio-24	10710.00 b	11505.00 ab	**11107.50**	5463.00 c	6222.00 c	**5842.75 C**
MG-2	14752.75 a	13980.00 ab	**14366.37**	9720.00 b	10604.50 a	**10162.25 A**
S- 278	14172.50 a	13012.50 ab	**13592.50**	6540.00 c	9275.00 b	**7907.50 B**
Mean	**13088.93**	**12845.62**		**6955.50 B**	**8821.75 A**	

*Any two means not sharing a letter in common differ significantly at 5% probability Level

Table 6: Achene yield of sunflower hybrids during two seasons of 2007 and 2008

Hybrids	Spring			Autumn		
	2007	**2008**	**Mean**	**2007**	**2008**	**Mean**
Alisson-RM	3419.05 cd	3487.10 cd	**3453.07 BC**	1260.00 de	1928.50 ab	**1594.25 AB**
Parasio-24	3207.00 d	3399.50 cd	**3303.25 C**	1059.9 e	1563.20 bcd	**1311.55 B**
MG-2	4725.25 a	3996.20 bc	**4360.72 A**	1796.10 abc	2171.72 a	**1984.00 A**
S- 278	4215.75 ab	3718.05 bcd	**3966.90 AB**	1387.80 cde	2050.72 a	**1719.26 AB**
Mean	**3891.76 A**	**3650.21 B**		**1375.98 B**	**1928.53 A**	

*Any two means not sharing a letter in common differ significantly at 5% probability Level

Table 7: Achene oil contents (%) of sunflower hybrids during two seasons of 2007 and 2008

Hybrids	Seasons					
	Spring			Autumn		
	Years			Years		
	2007	2008	Mean	2007	2008	Mean
Alisson-RM	44.25 ab	39.18 b	**41.71C**	38.20 f	41.70 de	**39.95 B**
Parasio-24	49.20 a	46.74 a	**47.97B**	44.52 bc	45.22 ab	**44.87 A**
MG-2	47.90 a	48.88 a	**48.39A**	47.22 a	45.70 ab	**46.46 A**
S- 278	47.07 a	46.94 a	**47.00B**	40.00 ef	42.60 cd	**41.30 B**
Mean	**47.10**	**45.43**		**42.48 B**	**43.80 A**	

*Any two means not sharing a letter in common differ significantly at 5% probability level

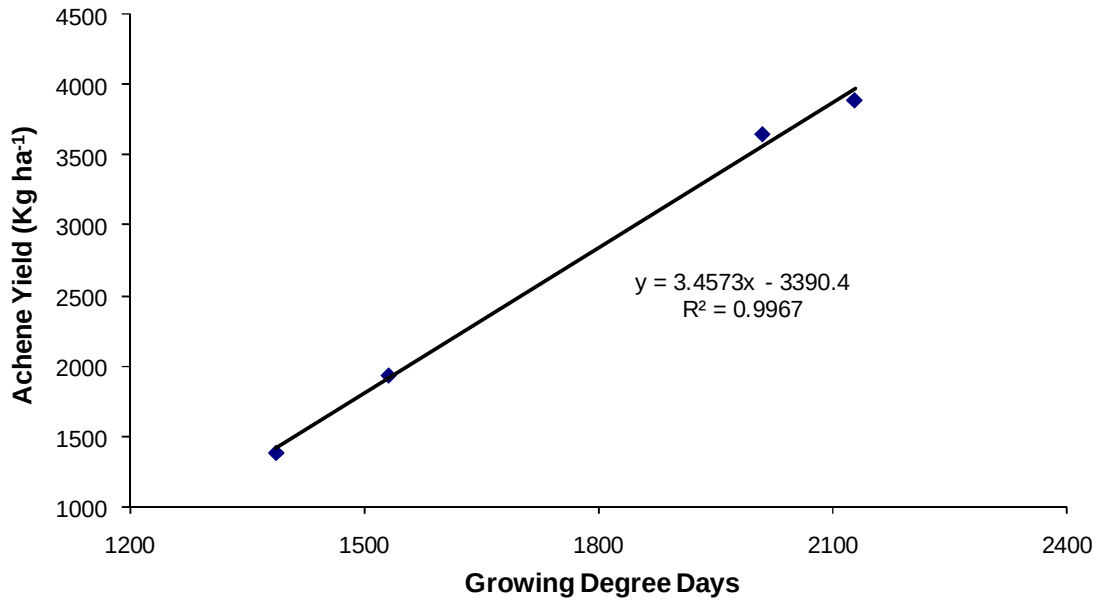

Fig. 1: Relationship between growing degree days and achene yield (Means of two years)

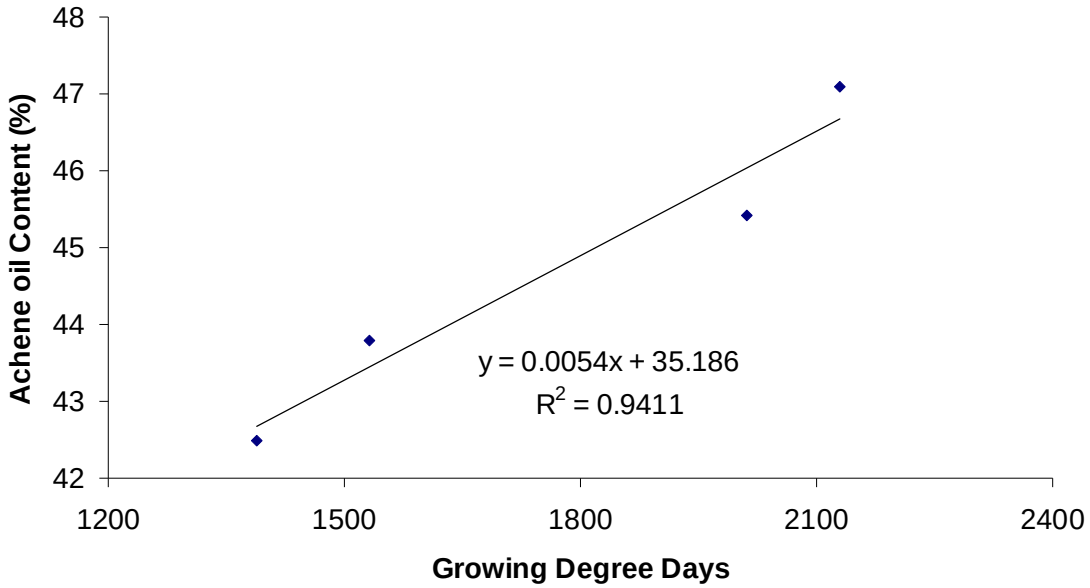

Fig. 6: Relationship between growing degree days and achene oil content (%) (Means of two years)

4. DISCUSSION

Sunflower productivity largely depends on the prevailing weather conditions throughout the life cycle of the crop. The primary factor governing crop growth rate is temperature (Baydar and Erbas, 2005). Temperature is a major environmental factor that determines the rate of plant growth and development (Qadir *et al.* 2007). Higher GDD accumulated for spring planting during both the years (Table 2) provided the clue that the best sowing time of a particular crop is spring planting to have good yield (Kaleem *et al.* 2010a). Environmental factors, especially temperature during the period of achene development and maturity, might have affected achene yield, yield attributes and oil content (Kaleem *et al.* 2010b). The accumulation of GDD determines the maturity of crop, yield and yield components. Sur and Sharma (1999) observed that the total GDD decreased from 1731 to 1621 with delay in planting, as the late sown crop experienced lower temperature during the seed filling period. In present study, 2128.75 GDD during spring and 1387.63 during autumn were accumulated during two seasons those may have caused the differences in out put of different parameters. Linear relationship between GDD and yield components have been reported by Lu *et al.* (2001), Agele (2003), Clarke and Siddique (2004) and Qadir *et al.* (2007).

Results presented in table 5 exhibited higher biological yield for hybrids during spring than that from autumn season. The differences among the hybrids for biological and achene yield may be due to genetic potential of the hybrids which showed its results under prevailing environmental conditions, accumulating more GDD during spring than during autumn induced higher yield out put in spring. Lower temperature at reproductive stage of the crop during autumn might have depressed assimilate utilization and greater restriction on biomass production and reduction in the duration of seed filling resulted reduced assimilate partitioned to seeds, thus lesser yield from autumn crop. The results of present study are in agreement with the findings of Villalbos *et al.* (1996) who concluded that sunflower biomass production was positively co-related with accumulated heat units. Baydar and Erbas (2005) also concluded that higher achene production is attributed to interaction of environmental factors, those partitioned photosynthates in achenes. These results are in conformity with those of Sumangala and Giriraj (2003) who concluded that favorable growing conditions during flowering and seed setting period characterized by optimum temperature and more sunshine hours for spring crop resulted in maximum achene yield. Kumar *et al.* (2008) also found that higher prevailing temperatures contribute the positive correlation with the seed yield regarding oil seed crop. Linear relationship (Fig. 1) between GDD and achene yield during both the seasons i.e. spring and autumn are in line with above findings.

Differences among hybrids for achene oil content in varying seasons may be attributed to their genetic potential as well as interactive effects of environmental

variables during achene development and crop physiological maturity. More heat units accumulation along with long sunshine hours during spring, increased oil content than during autumn. Our results are in line with those of Demurin *et al.* (2000) who found an increase in achene oil content with increase in temperature during flowering to maturity in sunflower and reported that 1^{o}C increase in temperature increased achene oil content by 1% in sunflower. Similarly, Weiss, (2000) concluded that crops maturing at higher temperature would accumulate higher oil content. Qadir *et al.* (2006) concluded that temperature is a major environmental factor that determines the rate of development as well as oil accumulation in sunflower and recorded higher achene oil content from spring sunflower crop which matured at higher temperature, ultimately accumulating more heat units. Linear relationship (Fig. 2) between GDD and achene oil content during both the seasons i.e. spring and autumn is also supportive to the above findings.

5. CONCLUSION

It is concluded from present results that economically successful sunflower crop should be planted during spring. However, autumn crop can be sown in case of failure of any summer crop (July-August) as an alternate crop with half of recommended inputs.

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Removal of Ag^{+}, Co^{++} and Cs^{+} From Wastewater Using Porous Resin Blend (Epoxy/PVA)

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Abstract: In this study, removal of silver, cobalt and cesium from aqueous solutions under different experimental conditions using a prepared porous resin blend (Epoxy/Polyvinyl alcohol) was investigated. Blending of Epoxy with PVA and thereafter foaming in a viscous state were carried out to attain the optimum hydrophilicity. Gamma rays were used in the preparation process to control the granular size and the compatibility of the blend**.** Characterization of the blend after milling was reported using thermogravimetric analysis (TGA), Fourier transform infra red (FTIR) and scan electron microscopy (SEM). The adsorption of Ag(I), Co(II) and Cs(I) ions from aqueous solution by the prepared porous resin blend was examined by batch equilibration technique. The effects of initial ion concentration, temperature, pH and shaking time on the adsorption of metal ions were investigated. The adsorption amount of ions increased with the increase of shaking time, temperature, metal ion concentration and pH of the media. The results showed that metal ion adsorption followed the order $Ag^{+}>Co^{++}>Cs^{+}$. The amount of metal ion adsorbed at equilibrium for Ag^{+}, Co^{++} and Cs^{+} at pH 5 was 9.8, 9.4 and 9.1 mg/g. It was found that the adsorption isotherm of the ions fitted Langmuir isotherms. [Nature and Science 2011;9(2):82-89]. (ISSN: 1545-0740).

Key Wards; Blend, γ- irradiation, metal ion, porous resin, adsorption, contaminated water

1. Introduction:

Heavy metals are often problematic environmental pollutants, with well-known toxic effects on living systems. Nevertheless, because of certain useful physical and chemical properties, some heavy metals, including silver, cobalt, and cadmium are intentionally added to certain consumer and industrial products such as batteries, electroplating. Cobalt was once widely used in pharmaceutical products and agricultural chemicals while cesium has taken more importance after nuclear reactor construction has been expanded.[1,2] Large amount of any of them may cause acute or chronic toxicity.[3-5] Heavy metals in human body tend to bioaccumulate, which may result in damaged or reduced mental and central nervous function, and damage to blood composition, lungs, kidneys and liver. Notably, it has been declared that the regulatory levels of health metals in drinking water level often not exceed 10 μg/L[6-8] .

The removal of heavy metal ions from aqueous solutions, either for pollution control or for raw material recovery, has been taking on increasing importance in recent years. Different treatment techniques, such as chemical precipitation, coagulation–precipitation, adsorption and ion exchange, have been progressively developed to remove heavy metals from contaminated water [9–11]. Coagulation–flocculation and chemical precipitation are perhaps the most widely used, however, they both have the drawbacks of difficult sludge disposal and more importantly the diminished effectiveness when treating water with low heavy metal levels[12].

Membrane filtration and reverse osmosis were also reported [13]. However, these methods usually involve expensive materials and high operation costs. Other methods, such as electrodialysis, membrane electrolysis and electrochemical precipitation, have also been investigated, however, their applications have been limited due to the high energy consumption [14] On the other hand, as a cost effective method, ion exchange process normally involves low-cost materials and convenient operations, and they have been proved to be very effective for removing contaminants from water such as ammonia and heavy metals [15-17]. Moreover, ion exchange is particularly effective for treating water with low concentration of heavy metals which is very common in practice [18]. The development of potential low-cost adsorbents with high exchanging levels is essential to facilitate the application of ion exchange processes for heavy metal removal. Various materials, including natural and synthetic zeolites and polymeric resins, have been studied for this purpose [19].

The aim of the present work is the preparation and characterization of cationic porous resin by blending a functional polymer, namely polyvinyl alcohol with epoxy, acting as carrier. Batch technique was used to study the sorption of Ag^{+}, Co^{++} and Cs^{+}. Different parameters, such as temperature,

pH of the solution, different concentrations of metal ion and effect of shaking time were manifested.

2. Experimental Approach:

2.1. Materials

The samples were prepared using propyl epoxy commercial grade and partially hydrolyzed polyvinyl alcohol and a commercial hydrogen gas was used in foaming the blend purchased from Optco, Egypt. The PVA powder was dissolved into magnetically stirred distilled water for 3 hours at 80 ^{o}C getting a solution with the concentration of 20 %. Cobalt chloride, silver chloride and cesium chloride were used as a simulated waste; all salts employed were of analytical grade purchased from Merck Co. bidistilled water was used for the simulated waste solutions: 200 ppm, 100 ppm, 50 ppm and 25 ppm.

2.2. Scientific Equipments

A Fourier transform infrared (FTIR) spectrophotometer from Mattson 1000, Pye-Unicam, England was used to analyze the chemical and/or physical interactions in the wave number range 400–4000 cm−1. The concentrations of the metal ions were measured with an Ati Unicom (Model 929) atomic absorption spectrophotometer. The pH values of the buffer solutions were determined with a Ø50 pH meter (Beckman, Beckman Instrument). TGA - 50, Japan was used to characterize the polymer resin thermally. Atomic absorption Spectrophotometer (Perkin-Elmer 2380) using lamps for Ag, Co and Cs were used for measuring all adsorption process. Merck atomic absorption standard solutions of these metals were used for calibration process.

2.3. Methods of preparation

PVA/Epoxy was mixed to realize the optimum ratio which has good structure and best hydrophilic character: 5%, 10%, 20% and 30 % by weigh of PVA were mixed with Epoxy. Heating of the mixture to reach the maximum compatibility and then reduce the temperature of the mixture blend till reach a very viscous state. Foaming of the blend was for more than 5 hours till solid form performed to achieve a highly porous blend. Irradiation of different doses; 5, 10, 20 and 2 KGy by Gamma cell (^{60}Co) was carried out to attain better porosity and best hydrophilicity. Milling of the solid porous blend using agate mortar and pestle was performed to obtain small bead with different diameter.

2.4. Water uptake

Water uptake/swelling behavior of different resins formed was studied in water as a function of the applied dose for different composition. Swollen polymers were wiped off with tissue paper to remove surface water and then weighed immediately to know the percent swelling/ percent water uptake, which was calculated as:

$$\text{Water uptake \%} = [(W-W_0)/W_o]x100 \quad (1)$$

Where, W, Wo are the weight of swollen blend resin and dry blend resin, respectively.

2.5. Adsorption experiments

Adsorption experiments were carried out using a batch wise method. Dried samples (0.1 g each) of blend resin were added into 100 cm^3 Erlenmeyer containing volume of 30 cm^3 of each metal ion solution (50 ppm) and adjusted to desired pH. The mixture solution was stirred at 25 ^{0}C. After filtration of the solution, the ion concentration of the filtrates was analyzed with an atomic adsorption spectrophotometer. The amount of metal ions adsorbed on the adsorbent at adsorption equilibrium, q_e (mg/g) was calculated according to the following equation

$$q_e = (C_o - C_e)V/W \quad \ldots\ldots\ldots \quad (2)$$

Where C_o and C_e are the initial and equilibrium metal ion concentration (mg/l), respectively, V is the volume of solution (l), and W is the weight of the resin (g).

2.6. Desorption experiments

The elution of heavy metals is the most common elimination method of the exhausted sorbent, allowing both recovery of metal ions at higher concentration and recycling of the sorbent for subsequent uses. For an effective recycling process, adsorbed metal ions should be easily desorbed without destroying the adsorbent materials under the suitable conditions. The adsorption was performed with the same procedure mentioned above. On the other hand, desorption was examined as follows: 1 g of the blend resin loaded by Ag(I), Co(II) and Cs(I) from the previous metal adsorption study was put into a 250 ml Erlenmeyer flask containing 100 ml (0.1 N HCl). The mixture was then shaken with the shaker at 100 rpm at room temperature. Samples of the solution were taken every 5 min until steady state (about 30 min) was achieved. The metal concentration was then determined by the Atomic absorption Spectrophotometer (Perkin-Elmer 2380). The adsorption and desorption processes were repeated for three cycles using the same adsorbent to evaluate the effect of regeneration on the capacity of re-adsorption of Ag(I), Co(II) and Cs(I) on the prepared epoxy blend resin.

3. Results and Discussion:

3.1. Characterizations of the resin

3.1.1 FTIR

Evidence of blending and network formation has been provided by the characterization of the synthesized porous resin. The use of IR spectroscopy is a well-known method for the identification of the chemical groups. The IR spectra of the synthesized porous resin, prepared by blending propyl epoxy, commercial grade, with partially hydrolyzed polyvinyl alcohol, are given in Fig. 1. Polyvinylalcohol is produced by the reaction of polyvinylacetate with methanol. The spectrum should be a fairly simple combination of methylene and hydroxyl vibrational peaks. The resulting hydrogen bonding between hydroxyl groups produces a wide hydroxyl stretch at 3400 cm^{-1}. Three successive peaks at 670, 1300 and 1650 cm^{-1} were recorded which manifest the presence of epoxy.

The C-O stretch appears at 1095 cm^{-1}, is typical for secondary alcohols. Sometimes polyvinylacetate peaks are seen at 1739 and 1239 cm^{-1} when hydrolysis is not complete. Thus bands at 1746 and 1246 cm^{-1} due to methyl acetate by-products are sometimes discernable.

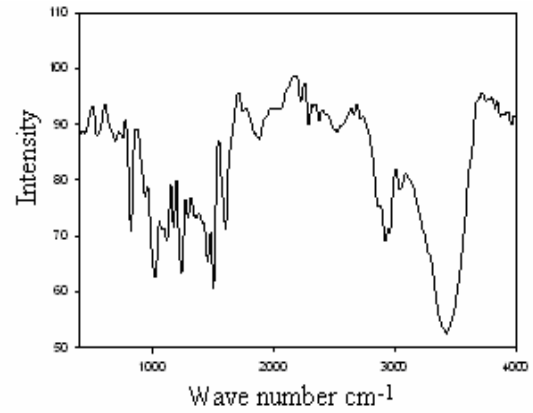

Figure (1): FTIR of Epoxy/PVA blend resin

3.1.2 TGA

Temperature at which the loss in weight occurs is considered to be the thermal resistance of polymer [21, 22]. An improvement in the thermal characteristics was detected as a result of blending PVA with epoxy. The blend showed high thermal resistance up to 360 °C above which loss of weight could be detected while complete decay was noticed close to 400 °C. From ambient to 350°C, a gradual weight decrease was reported, this loss of weight does not exceed 10% evidencing the evaporation of non bounded water. Meanwhile, the abrupt change in weight, was recorded within the 55%, temperature range 350 - 400 °C. The third division, as shown in Fig.2 exhibits the change in weight (loss of 17%) over the temperature raise up to 580 °C. No step peak has been seen manifesting the polymer-polymer compatiblization (foam component) all over the applied range of temperature from ambient to 580 °C.

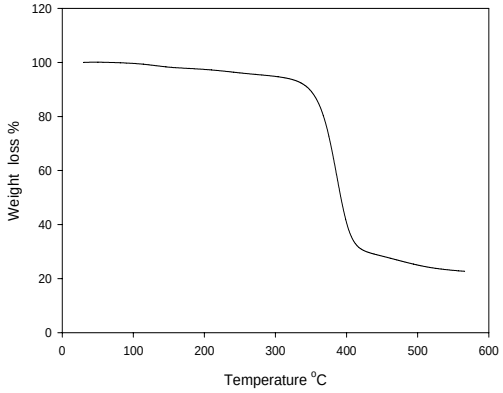

Figure (2): Thermogravimetic analysis of porous resin blend (Epoxy/PVA)

3.1.3. SEM

Scan electron microscopy was applied to distinguish between primary textures due to the synthesis process and those due to subsequent treatment of surface, [23]. Figure 3 showed scattered gaps as a result of foaming the blend, forming large cavities distributed in a regular manner. This may provide an evidence of forming homogenously distributed porous resin upon synthesis.

3.2. Water uptake

Water uptake of the porous blend resin was studied as a function of composition and gamma irradiation. 20 % PVA blend showed maximum water uptake, as shown in Fig. 4. It was found that a dose of 10 KGy was the proper dose for at most water uptake as obviously demonstrated by Fig. 5. Foaming of blend remarkably improved water uptake. This may be accounted for an increase in pore number, pore size and/or increase in dimensions of the formed foam, i.e. increase in the allowed surface area of the net matrix.

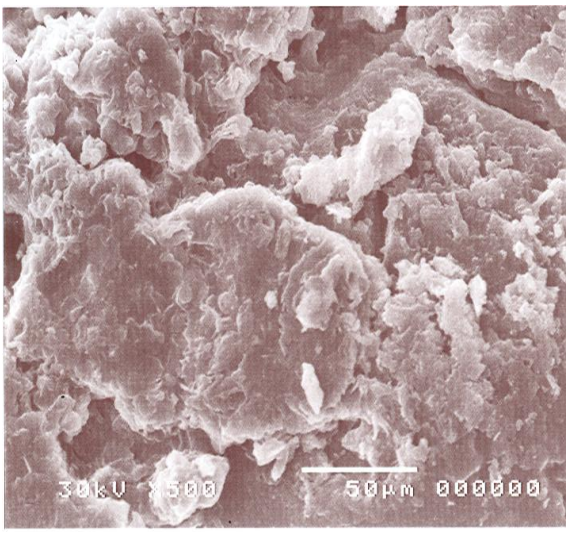

Figure 3: Scan Electron Microscope of Epoxy/PVA blend foam

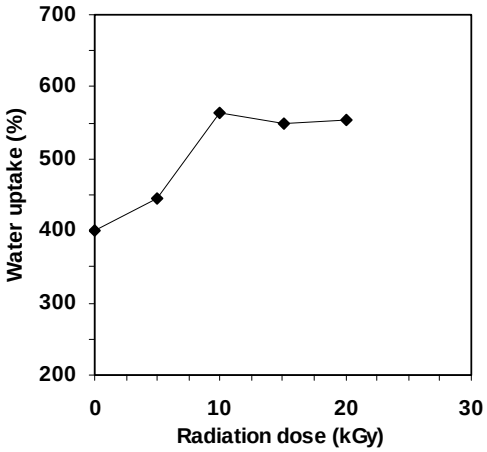

Figure (5): Effect of radiation dose on water uptake of Epoxy/PVA blend resin; PVA 20 wt. %

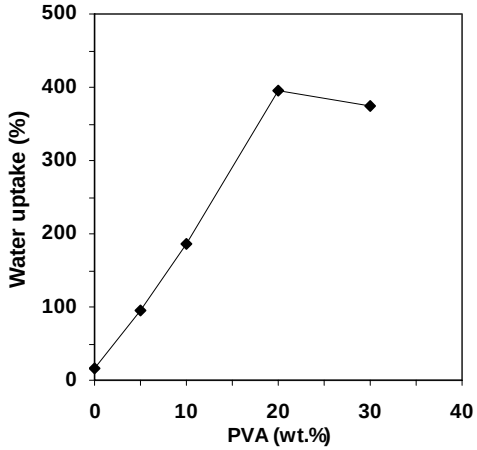

Figure: (4) Effect of Epoxy wt.% added to PVA on water uptake

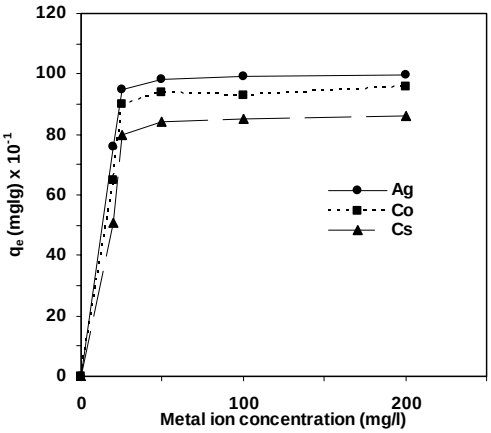

Figure (6): Effect of metal ion concentration (mg/l) on the removal of silver, cobalt and cesium from their aqueous solution using porous resin blend (Epoxy/PVA); pH 6; 30 °C; 2h.

3.3. Adsorption of metal ion

3.3.1 Effect of metal ion concentration:

The adsorption of metal ion by the synthesized porous resin blend was conducted in different initial concentrations ranging from 25 ppm to 200 ppm. Figure 6 shows the relationship between the initial concentration of metal ion and the adsorption amount. It is clear from the figure that, the adsorption amount of metal ion increased with increasing initial ion concentration untill reached a plateau value at a concentration of ~100 ppm. This is attributed to the chelating sites capacity of the prepared (Epoxy/PVA) porous resin blend which becomes saturated as concentration nearly reached 100 ppm. The removal percent can be arranged in the following manner $Ag^{+}>Co^{++}>Cs^{+}$, which can be largely accounted for the ionic radius and charge of the metal ion.

3.3.2. Effect of temperature

The effect of the temperature on the adsorption amount of ion onto the prepared porous resin blend was studied within the range 25–60 ^{0}C, where the initial concentration being kept at 50 ppm at pH 5, the results are shown in Fig. 7. From this figure it is clear that with increasing temperature, the adsorption amount of metal ion slightly increased. This may be either due to accelerating of some originally very slow adsorption steps or due to creation of some new active sites on the surface of adsorbent [24]. Also, it is quite possible that a diffusion process takes place and is partly contributing to the rate of adsorption [25].

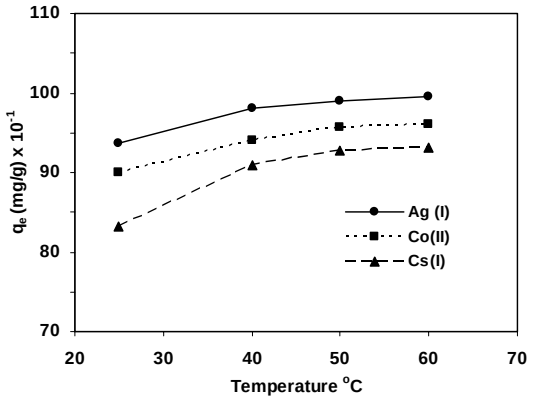

Figure (7): Effect of temperature on the removal of silver, cobalt and cesium ions from their aqueous wastes by porous resin blend (Epoxy/PVA); pH 5, concen. 50 ppm; t= 2h.

3.3.3. Effect of pH

The effect of the initial pH value on metal ion removal by the prepared porous resin was investigated within the pH range 2-6 taking into account the precipitation pH value of metal ions [26,27]. At different pH values, the protonation and deprotonation behaviors of acidic and basic groups would be influenced. The surface structure of the porous resin blend and the metal ions would exist in different forms. The experimental results for the effect of pH on the non-competitive adsorption of metal ions are shown in Fig. 8. It can be seen that adsorption significantly increased with the increase of pH (within pH 3–5), reaching a value of 9.8, 9.4 and 9.1 mg/g, for Ag(I), Co(II) and Cs(I), respectively. At low pH values, the high hydrogen ion concentration at the interface electrostatically repels the positively charged metal ions and prevents their approach to the resin blend surface [28].

3.3.4. Effect of contact time

Removal of Ag(I), Co(II) and Cs(I) from their aqueous solutions by Epoxy/PVA resin blend with time was carried out at pH 5 and the effect of contact time on the sorption of these metal ions are presented in Fig. 9. As may be seen from Fig. 9, the binding of Ag(I), Co(II) and Cs(I) on Epoxy/PVA resin blend from the solution increases very rapidly with agitation time. The investigations indicated that the binding of Ag(I), Co(II) and Cs(I) was found to be about 7.3, 6.6 and 6.2 mg/g, respectively within the initial 15 min. Later, the percentage sorption of Ag(I), Co(II) and Cs(I) reached about 9.8, 9.4 and 9.1 mg/g respectively and attained equilibrium by the end of a contact period of 60 min. The increase of contact time further from 60 to 120 min had no significant effect on the percentage sorption of all metal ions. Therefore, a contact time of 60 min was applied for all other subsequent experiments. Initial rapid binding of Ag(I), Co(II) and Cs(I) occurs initially due to easily available exchangeable sites located on surface of the Epoxy/PVA resin blend. The subsequent slow process suggests that intrapore diffusion was also involved in the sorption process. It was previously stated that the plateau portion of the curve corresponds to pore diffusion where as the linear portion of the curve reflects surface layer diffusion [29].

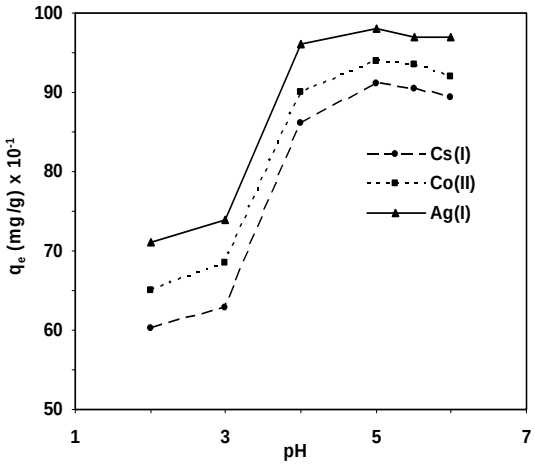

Figure (8): Effect of pH on the adsorption amount of Ag(I), Co(II) and Cs(I) from aquous waste using porous resin blend (Epoxy/PVA); metal ion conen. 100 ppm; t=2h; temp. 30 ^oC.

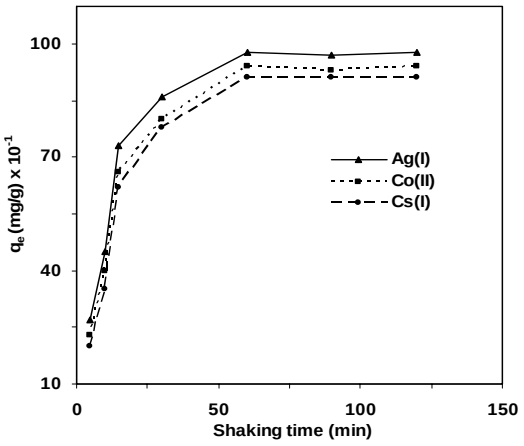

Figure (9): Effect of shaking time on the adsorption amount of Ag(I), Co(II) and Cs(I) from aquous solution usings porous resin blend (Epoxy/PVA); metal ion conc. 100 ppm; pH 5; 30 ^oC

3.4.5. Adsorption kinetics

The equilibrium adsorption isotherm is fundamental in describing the interactive behavior

between solute and adsorbent. The popularly used Langmuir isotherm is expressed by the linear Equation 3 [30, 31]:

$$C_e/q_e = C_e/Q_o + 1/bQ_o \quad \ldots\ldots\ldots\ldots \quad 3$$

where q_e is the amount of metal ion adsorbed per unit mass of adsorbent (mg/g), C_e is the equilibrium concentration of metal ions(mg/l), Q_o is a measure of adsorption capacity of adsorbent (mg/g) and b is the Langmuir isotherm constant (l/mg) related to the energy of adsorption. A plot of C_e/q_e versus C_e exhibits a straight line of slope 1/ Q_o and intercept $1/bQ_o$ as shown in Figure 10.

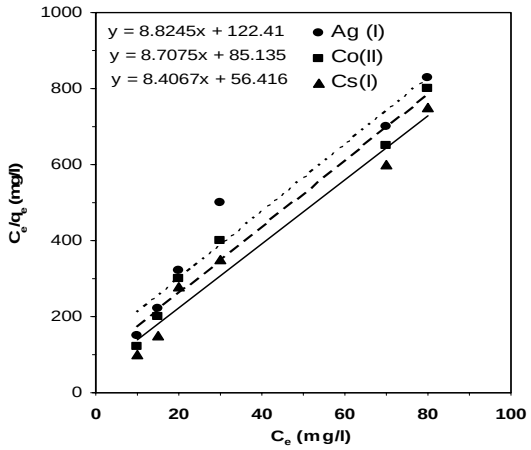

Figure (10): Linearized Langmuir for the adsorption of metal ions (Ag^+, Co^{++} and Cs^+) using Epoxy/PVA blend resin

From the value of the Langmuir constant and the coefficient obtained, it can be concluded that the Langmuir equation gives better fit to the experimental data. The theoretical Langmuir isotherms of Ag^+, Co^{++} and Cs^+ on porous resin are depicted in Figure 10. The q_o values calculated from Langmuir model are fairly close to the experimental results.

3.4.6. Desorption experiment

Table 1 shows the Ag(I), Co(II) and Cs(I) adsorption and desorption of intact blend resin compared with regenerated blend resin adsorbent. The adsorption capacity of regenerated blend resin decreased with increasing cycle times. This may be explained by the fact that esopores of adsorbent prevent metal ions from desorption which results in reduction in the number of active sites and consequently in the adsorption capacity.

Table 1. Adsorption and desorption of Ag(I), Co(II) and Cs(I) per mass of Epoxy/PVA blend resin at each cycle of regeneration

Cycle number	Ag(I)		Co(II)		Cs(I)	
	Adsorbed (mg/g)	Leached (mg/g)	Adsorbed (mg/g)	Leached (mg/g)	Adsorbed (mg/g)	Leached (mg/g)
1[a]	9.8	9.1	9.4	8.5	9.1	8.1
2	9.4	8.5	8.7	7.7	8.5	7.4
3	8.9	8.0	8.3	7.2	7.9	6.8
4	8.2	7.1	7.7	6.5	7.4	6.3

1[a] Intact blend resin adsorbent was used in cycles number 1 whereas regenerated blend resin adsorbent was used in cycle number 2, 3, and 4 consecutively.

4. Conclusions:

A porous resin blend (Epoxy/Polyvinyl alcohol) was synthesized with PVA at different weight ratios for removing Ag(I), Co(II) and Cs(I) ions from aqueous solutions. The FTIR spectra reveal the hydrogen bonding between hydroxyl groups represented by a wide hydroxyl stretch at 3400 cm^{-1}. Three successive peaks at 670, 1300 and 1650 cm^{-1} were recorded which manifest the presence of epoxy. All the adsorption processes tended toward equilibrium after a contact time of 60 min and the percentage sorption of Cs(I), Co(II) and Ag(I) reached 9.1, 9.4 and 9.8 mg/g, respectively. The adsorption increased significantly with the increase of pH (within pH 3–5), reaching the values 9.8, 9.4 and 9.1 mg/g, for Ag(I), Co(II) and Cs(I), respectively. By increasing adsorption temperature, the adsorption amount of metal ion slightly increased. Furtherly, adsorption amount of metal ion increased with increasing initial ion concentration and reached a plateau value at a concentration of~100 ppm.

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Immunostimulant Effect of Different Fractions of *Nigella sativa* L. Seeds against Rabies Vaccine

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ABSTRACT: Interest in new methods of potentiating the immune response against vaccine antigens has increased considerably over the past decade for improving existing vaccines. The present study was designed to evaluate the immunostimulant effect of oils, n-hexane and methanol fractions of *Nigella sativa L.* seeds in combination with vitamin E and selenium as new adjuvant compared with aluminum hydroxide (alum) as established adjuvant against rabies vaccine in male Swiss albino mice. Inoculation was done intraperitoneally in the form of two doses, two weeks apart. Five samples of sera were collected for every two weeks beginning from two weeks after the last vaccination till the 12th week and the antibody were detected using indirect ELISA technique. Our results revealed that both methanol and volatile oil fractions of *Nigella sativa L.* seeds can improve the immune response against rabies vaccine save and suggested that they could be used as an alternative adjuvant to alum in rabies vaccine.
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Key words: rabies vaccine; adjuvant; immunostimulant effect; *Nigella sativa L.* seeds; vitamin E and selenium.

INTRODUCTION

Vaccines were designed to protect against diseases by inducing specific immunity. Immunization is a proven tool for controlling and even eradicating diseases. The densely populated countries especially in Africa and Asia, the rabies is endemic and remains a major health problem. Rabies is a viral disease of mammals and is most commonly transmitted through the bite of a rabid animal. Rabies disease is caused by a neurotropic virus belonging to the family *Rhabdoviridae*. Rabies is one of the oldest and most devastating viral diseases affecting humans and animals. It was recognized in Egypt before 2300 B.C. and was described by Aristotle in ancient Greece. It is the most lethal of all infectious diseases and has the widest host range of any virus **(Fenner and White, 1994)**. Globally, according to WHO Fact Sheet, an estimation of 10 million people receive post exposure antiserum treatment each year, after being exposed to rabies-suspect animal (**Gómez et al. 2010**).

Adjuvants are highly valuable additions to vaccines. Adjuvants may modulate the quality and quantity of the immune response following vaccination. Most of the cell culture rabies vaccines commercially produced for animals and sometimes for human was adjuvanted principally with aluminum salts (alum), which held the antigen at its site of deposition, delaying its adsorption and subsequently released antigen in a deduced secondary response (**Glenny et al. 1931 and Nakashima et al. 1981)**.

Nigella sativa Linn. (a dicotyledon of *Ranunculaceae* family), commonly known as black seed or black cumin, is a grassy plant grows in temperate and cold climate areas and has green to blue flowers and small black seeds. It is an annual herbaceous plant native to Asia, and cultivated and naturalized in Europe and North Africa. In Egypt, *Nigella sativa* has been steadily increasing for the strong demand to volatile oils for pharmaceutical purpose **(Tohamy et al., 2010)**. It has been traditionally used for culinary and medicinal purposes as a natural remedy for a number of illnesses and conditions that include diuretic, appetitive, hemorrhagic and anti-dandruff therapy, asthma, hypertension, diabetes, inflammation, cough, bronchitis, headache, eczema, fever, dizziness, and influenza a carminative, lactagogue, and vermifuge, as well as in food as a spice or condiment (**Baytop, 1999; Ali and Blunden, 2003; Isik et al. 2010**).

The general chemical composition of the *Nigella sativa* seeds is fats (31-35.5%w/w), proteins (16-19.9%w/w), carbohydrates (33.9%), fibers (4.5-6.5%) and moisture (5-7%) **(Ansari and Sadiy, 1989)**. The fatty component of the seed consists mainly of fixed oil and volatile oil. The fixed oil consists of high percentage of unsaturated fatty acids (74.4-82.5%), including arachidonic and eicosadienoic acids, while saturated fatty acids are only 14.9-17.3%. Steam distillation of the whole oil yields 1.4-1.9% volatile oils based on the weight of total oil extracted **(Rathee et al. 1982)**. This is equivalent to 0.40-0.45% w/w of the weight of the

seeds used to extract the oil **(Juknevicience et al. 1977)**. This oil is also rich in fatty acids (oleic, linoleic and linolenic acid) and carotene (**Al-Jassir, 1992**). The active constituent of the volatile oil, nigellone was first isolated by **Mahfouz and El Dakhakhny (1960)** and thymoquinone which was then isolated. When thymoquinone exposed to air, dimerization occurs with the formation of dithymoquinone **(El Dakhakhny, 1965). El Alfy et al. (1975)** isolated a white crystalline compound identified as thymohydroquinone, which is probably a reduction product of thymoquinone. Also, *Nigella sativa* seeds contain monotropens such as *p*-cymene and α-pinene **(El-Dakhakhny, 1965)**, nigellidine **(Atta and Malik, 1993)**, nigellimine **(Atta and Malik, 1985)** and a saponine **(Ansari and Sadiy, 1989)**. The chemical composition of the plant was summarized in a recent review (**Labib, 2005**). Consequently, the present study was aimed to evaluate the effect of different fractions of *Nigella sativa* seeds on the immune response against rabies vaccine in Swiss albino mice.

MATERIALS AND METHODS

Plant material:

Dried seeds of *Nigella sativa* L. (black seed) were purchased from a local market, and authenticated by botanists from faculty of Science, Cairo University.

Nigella sativa fixed oil:

Nigella sativa fixed oil, the natural oil of the black seed, was purchased from the local market. It was intra-peritoneally injected in a dose of 2.06 mg/Kg body weight (**Zaoui, et al. 2002)**.

E-SELEN:

E-SELEN, a mixture of vitamin E acetate 150 mg/ml and sodium selenite 1.67 mg/ml, produced by MAM Egypt, M.O.H.Reg. No.: 2597/2005 was diluted depending upon their LD_{50} of sodium selenite in mice (0.9 mg/Kg body weight) according to **(Toxic Report Series, 1994)** and vitamin E acetate (100 mg/kg body weight) according to **(Toutain et al. 1992)** was mixed with the inactivated rabies vaccine in a ratio of 1:1 to make a homogenous mixture.

Extraction of volatile oils from Nigella sativa L. seeds:

According to **Samsam-Shariat and Moatar, (1996),** 500 grams of crushed seeds of *Nigella sativa* seeds were placed in a rounded flask of a quick fit steam distillation apparatus in which steam was passed from steam generator and sufficient water was added. A 30 ml of glycerin was added to the content of the flask to raise the boiling point. The content of the flask were boiled gently until all the volatile oil has been distilled, saving guard against any charring of the material in the flask. The oil fraction (F1), being lighter than water, was separated by a separating funnel, dried over anhydrous sodium sulphate and kept in a dark away from light and moisture.

Extraction of n-hexane and methanol fractions from Nigella sativa L. seeds:

According to **Boskabady et al. (2008),** 300 ml of n-hexane was added to 500 grams of the chopped, dried *Nigella sativa L.* seeds and the solution was kept at room temperature for 48 hours. The solution was decanted and the solvent was evaporated and this represented the n-hexane fraction (F2). Methanol was added to the remaining powder and the mixture was allowed to remain at room temperature for 48 hours. The solution was then decanted and the solvent was evaporated and this represented the methanol fraction (F3) of the *Nigella sativa* seeds.

Rabies vaccine:

Aluminum salts have become the reference preparations for evaluation of new adjuvants for human vaccines. Therefore, it is important that aluminum adjuvants be used optimally to allow correct evaluation of the experimental adjuvant **(Gupta and Siber, 1995)**. Therefore, two types of Rabies vaccines for human vaccination against rabies virus infection were used:

1) Inactivated, purified and adsorbed rabies vaccine prepared on VERO cells and adsorbed onto aluminum hydroxide salts was used as control (VC).
2) Inactivated and purified rabies vaccine prepared on VERO cells and produced by Sanofi Pasteur, France. According to the method described by **Madbouly et al (2006)**, this inactivated rabies vaccine was mixed with the new adjuvants as follows: Four parts of the water phase (vaccine and vitamin E and selenium combination is mixed thouroughly with 1% tween 20) was mixed with one part of the oil phase (one part of span 80 was thouroughly mixed with 9 parts of the *Nigella sativa* fraction).

Animals:

Male Swiss albino mice weighing 20-25 gram were supplied by National Organization for Drug Control and Research (NODCAR). Animals were kept under standard laboratory conditions of light/dark cycle (12/12h.), temperature (25 ± 2°C)

and fed on normal laboratory diet and water ad libidum. They were acclimatized for a week in the new environment before initiation of experiment.

Experimental Design:

A total of 150 mice were assigned into 6 groups (each containing 25 animals). Each group was intraperitoneally vaccinated by 0.5 ml per mice contained different Rabies vaccine adjuvants in the form of two doses, two weeks apart as follows:

Group 1: Rabies vaccine mixed with aluminum hydroxide salts (VC- group).

Group 2: Rabies vaccine adjuvanted with E-SELEN (VE- group)

Group 3: Rabies vaccine adjuvanted with a mixture of E-SELEN and *Nigella sativa* fixed oil (VEFO-group).

Group 4: Rabies vaccine adjuvanted with a mixture of E-SELEN and volatile oils fraction of *Nigella sativa* seeds (VEF1- group).

Group 5: Rabies vaccine adjuvanted with a mixture of E-SELEN and n-hexane fraction of *Nigella sativa* seeds (VEF2- group).

Group 6: Rabies vaccine adjuvanted with a mixture of E-SELEN and methanol fraction of *Nigella sativa* seeds (VEF3- group).

Blood samples were collected every two weeks beginning from two weeks after the first vaccination dose and continue for 3 months. Then the antibody titres in the serum were evaluated using ELISA technique.

ELISA Technique:

The indirect enzyme-linked immunosorbent assay (ELISA) technique, a sensitive and simple method was used for quantitative determination of antibodies. The 96-well micortitre plates coated with inactivated rabies antigen were incubated with diluted antisera (1:100) followed by incubation with an enzyme labeled preparation of anti-immunoglobulin. The color change in each well was estimated spectrophotometrically at 490/630 nm according to the method described by **Hubschle et al. (1981).**

Statistical Analysis:

The data are expressed as means ± S.E. The enhancement effects of the different adjuvants were statistically analyzed by One-way Analysis of Variance (ANOVA) followed by Dunnet's tests for multiple comparisons with the level of significance accepted at $P<0.05$.

RESULTS

The use of an adjuvant to enhance antibody titer is an attractive option to improve the performance of an existing vaccine. Figure 1 illustrates the results of the indirect ELISA for the measurement of antibodies using inactivated rabies antigen coated plates with antisera collected from different vaccinated groups. After the second doses, the result showed that there is no significant difference between the immune response of rabies vaccine adjuvanted with alum (VC) and mixture of E-SELEN (VE). While, significant ($P<0.001$) increase specific immune response and was observed in the groups (3-6) which received the formulation with the novel adjuvant systems; all *Nigella sativa* fractions were generally effective in increasing the antibody against the rabies vaccine. Among the *Nigella sativa* seed fractions, the highest antibody was detected in the group treated with rabies vaccine adjuvanted with a mixture of E-SELEN and methanol fraction (VEF3) followed by VEF1, VEF2 and VEFO groups, respectively.

Table (1) : The results of the optical density (O.D.) for (1:100) dilution of the collected sera of the inoculated groups measured at wave length of 490/630 nm .

Group name	**4th week**	**6th week**	**8th week**	**10th week**	**12th week**
VC	0.462	0.809	0.621	0.569	0.465
VE	0.471	0.828	0.655	0.575	0.483
VEFO	0.733	0.908	0.923	0.896	0.596
VEF1	0.913	1.244	1.040	0.995	0.735
VEF2	0.815	0.956	0.951	0.920	0.733
VEF3	1.014	1.340	1.247	1.115	0.960

Table (2): The standard errors and the significance and non-significance (ns) between groups.

Group name	4th week	6th week	8th week	10th week	12th week
VC	0.462 ± 0.002	0.809 ± 0.005	0.621 ± 0.005	0.569 ± 0.004	0.465 ± 0.010
VE	0.471 ± 0.004ns	0.828 ± 0.003ns	0.655 ± 0.005ns	0.575 ± 0.005ns	0.483 ± 0.008ns
VEFO	0.733 ± 0.002***	0.908 ± 0.002***	0.923 ± 0.006***	0.896 ± 0.003***	0.596 ± 0.006***
VEF1	0.913 ± 0.004***	1.244 ± 0.003***	1.040 ± 0.004***	0.995 ± 0.004***	0.735 ± 0.005***
VEF2	0.815 ± 0.002***	0.956 ± 0.006***	0.951 ± 0.006***	0.920 ± 0.004***	0.733 ± 0.006***
VEF3	1.014 ± 0.003***	1.340 ± 0.009***	1.247 ± 0.007***	1.115 ± 0.002***	0.960 ± 0.005***

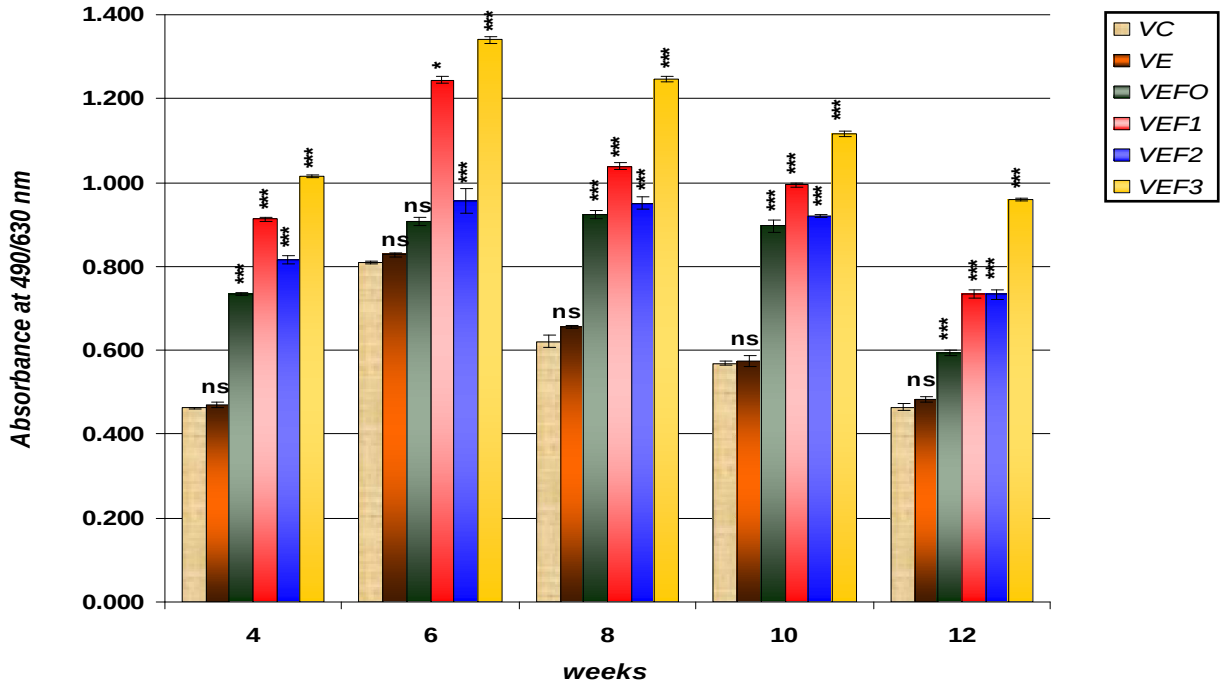

Figure 1 the mean immune response of different groups against rabies vaccine detected by indirect ELISA technique

DISCUSSION

Vaccines require optimal adjuvants including immuno-stimulants and delivery systems to offer long term protection from infectious diseases in animals and man. The current study was aimed to investigate the immunostimulant effect of different fractions of *Nigella sativa L.* seeds against rabies vaccine.

The ideal method for controlling rabies virus in both man and animals is by using active immunization through injecting either live attenuated or inactivated rabies vaccines. The WHO's experts **(WHO, 1973)** recommended that "No vaccines containing living virus could be used in man". The U.S. authorities recommended the use of inactivated vaccines in animals **(CDC, 1978)** because of the residual neuro-virulence of the virus which is very dangerous due to its auto-interference phenomena which could occur at non detectable level and the undesirable effect in case of using the living attenuated viral vaccines in immune-suppressed animals where it may act as avirulent strain **(Wachendorfer, 1976)**.

Although the inactivated cell culture rabies vaccines cause no or fewer reactions than the nervous tissue or the chicken embryo derived vaccines, they are relatively free from aggregates that could contain ineffective virus particles, but the virus titre obtained requires further concentration **(Sokol, 1973)**. Thus choose of natural immune-stimulants as adjuvant in this study based upon:

1) Its effective stimulation of the immune cells like macrophage **(Basil and Erwa, 1993)** and T-lymphocyte **(El-Kadi et al. 1990)** as shown in *Nigella sativa* adjuvant vaccines **(Madbouly et al. 2006).** Moreover, the *Nigella sativa* oil has shown potent antioxidant and anti-inflammatory effects in several inflammation-based models, including experimental encephalomyelitis, colitis, peritonitis, edema, and arthritis, through suppression of the inflammatory mediators' prostaglandins and leukotrienes. Also, the oil and certain active ingredients showed beneficial immunomodulatory properties, augmenting T-cell and natural killer cell-mediated immune responses (**Salem, 2005; Terzi et al. 2010**).
2) The powerful role of the combination of vitamin E and selenium as antioxidant protecting the sensitive rapidly proliferating cells of the immune system from oxidation damage, an immunopotentiating agent (**Yasunaga et al. 1982)**, increasing cell-cell interaction by membrane alteration **(Tengerdy and Lacetera 1991)** and significant enhancement for the formation of IgM & IgG in contrast to alum **(Inagaki et al. 1984).** *Nigella sativa* oil is so beneficial due to its content of over 100 components (such as aromatic oils, trace elements, vitamins, and enzymes) and contains about 58% of essential fatty acids, including omega-6 and omega-3. These are necessary for the formation of prostaglandin E1 that balances and strengthens the immune system, enabling it to prevent infections and allergies and control chronic illnesses (**Terzi et al. 2010**).
3) To overcome the disadvantage that associated with the aluminum hydroxide salts (alum) in rabies vaccines. **Redhead et al. (1992)** reported a transient rise in the level of brain tissue aluminum that peaks around the second and third day after intraperitoneally injection of alum adsorbed vaccines into mice that not observed in saline control group and with vaccine not containing aluminum salt. Also **Jefferson et al. (2004)** noticed that alum adsorbed vaccine associated with local pain lasting up to 14 days in older children administered such vaccines. Moreover, **Verdier et al. (2005)** observed histopathological lesions similar to the Macrophagic Myofascitis (MMF) described in humans, and was still present 3 months after aluminum phosphate and 12 months after aluminum hydroxide adjuvanted vaccine administration.

Throughout history, black seed has been one of the most revered medicinal seeds. The Islamic Prophet, *Muhammad* (Sal Allahu Alayhi Wasallam) recommended its use over 1400 years ago. Regarding to the previous studies that revealed that *Nigella sativa* oil and seeds are scientific techniques, a number of pharmacological actions of *Nigella sativa* have been investigated including immunostimulant, anti-inflammatory, antioxidant (**Labib, 2005**). Black seed oil and its derivatives inhibit eicosanoid generation in leukocytes and membrane lipid peroxidation **(El-Dakhakhny et al., 2002)**. Besides, the seeds contain eight essential amino acids that improve natural immune system activity **(Omar et al., 1999).**

In the current study, fixed oil, volatile oils, n-hexane and methanolic fractions of *Nigella sativa L.* seeds were studied as new adjuvants to the rabies vaccine in the presence of vitamin E and selenium. As compared with control group (VC), there was a significant ($P<0.001$) increase in the antibodies against rabies antigen in all vaccines adjuvanted with *Nigella sativa* fractions. The most superior effect of the used adjuvants on the immune response of mice against rabies vaccine was belonged to the groups vaccinated with the methanol and volatile oils fractions, respectively. Our results are in agreement with that of **Kanter et al., (2005)** who showed that the thymoquinone which is the major active principle of *Nigella sativa* has immuno-potentiating activities via increasing neutrophil percentage and hence increasing the defense mechanism of the body against infection. Concerning the first and second groups the first group was vaccinated with the alum (VC) and the second one (VE) was vaccinated with vitamin E and selenium (E-SELEN) adsorbed rabies vaccine, we noticed that the immune response in both groups were quite similar. But regarding to the side effect of the alum reported by **Verdier et al. (2005)**, the use of both vitamin E and selenium is safer than alum. This result is in agreement with that obtained by **Madbouly et al. (2006).** Also the immune stimulant effect of the methanol fraction to rabies antigen was showed to be more potent than that of the n-hexane fraction and whole oils which means that lipid soluble ingredients of the *Nigella sativa.* seeds are mainly responsible for their immunostimulant effect. Our results indicate that the new formulations were safe, well-tolerated, and immunogenic and promote

more rapid and prolonged protection against rabies infection.

CONCLUSION

The use of an adjuvant to enhance antibody production is an attractive option to increase the vaccine efficacy. A potent immunostimulant effect of mainly methanol and volatile oil fractions of *Nigella sativa* L. seeds in combination with vitamin E and selenium allow them to be used as new adjuvants for rabies vaccine. More studies are still needed in order to isolate and identify the effective compounds in the *Nigella sativa* L. seeds that responsible for the immunostimulant effect.

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Improving Growth and Productivity of Fennel Plant Exposed to Pendimethalin Herbicide: Stress–Recovery Treatments

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Abstract: The present experiments were carried out during two successive winter seasons (2008/2009 and 2009/2010) in the green house of the Botany Department, National Research Centre of Egypt. The present study aimed to improve the growth and productivity of fennel plant under physiological stress of the herbicide pendimethalin. A three hour-pre-sowing seed treatment in methionine, tryptophan and pyrimidine derivative substance (SG93) each at 100 and 500mg/l was applied. Whereas, the herbicide pendiemthalin (8.5ml/l) was supplied as pre-sowing soil corporation. The results indicated that the herbicide caused significant reduction in growth parameters of the fennel plant estimated as shoot length (cm) and fresh and dry weight per plant at the age of 84 and 119 days. The stress of the pendiemthalin herbicide was reflected in the significant decreases in the photosynthetic pigment contents of fennel leaves at both stages and in the content of total protein. Significant increases in total phenolic and free amino acids were recorded as well. The herbicide exposure, however, had led to a decline in plant productivity in the measured yield components. But oil percentage or quality were not influenced. Noticeable counteraction effects on growth and productive capacity of fennel were achieved by the pre-sowing-seed soaking treatment in the amino acids methionine and tryptophan each at 100mg/l and in the pyrimidine derivative SG93 at 500mg/l. Interestingly better performance was obtained in case of the dual treatments, i.e. with the seed treatment under the exposure to the herbicide as pre-emergence soil application.

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Key words: Fennel, growth, pendimethalin, photosynthesis, productivity, stress-recovery.

1. Introduction:

Fennel (*Foeniculum vulgare* Mill. *Apiaceae*) is a perennial hemicryptophyte plant inhabits the Mediterranean basin. It is known as a medicinal aromatic herb as its fruit is used in the remedy against digestive disorders. Bitter fennel is used as food flavor, in liqueurs and in the perfumery industry (Tanira, *et al*., 1996). The major volatile (essential) oil constituents of the plant are anethole and fenchone (Simandi, *et al*., 1999). Fennel extracts proved to have anti-inflammatory, antispasmodic, carminative, diuretic, expectorant, laxative, analgesic, stimulant of gastrointestinal mobility and are used in treatment of nervous disturbances (Choi and Hwang, 2004). Anand *et al*. (2008) reported the anticancer activity of anethole of fennel seeds.

Pendimethalin (stomp) is a dinitroaniline herbicide; applied as pre-emergence soil corporation; is generally used for selective weed control in different economic crops. It acts on cell division by blocking mitotic division and causing accumulation of abnormal microtubular structures (Fennell *et al*., 2006). It is known to be persistant in soil with different crops up to 75 day after sowing (Asha and Tomar, 2008).

In this respect, it has been found that the inhibitory stress effects of several herbicides on crop plants during weed control could be minimized and / or alleviated through exogenous application of some growth agents (Hassan *et al*., 1996). In other cases, nutrient elements (Nalewaja and Matysiak, 1991), amino acids, pryimidine and purine bases exerted similar protective effects

(Hassan and Gad, 2003; Hassan *et al*., 2006; El-Awadi, 2007).

The application of these substances act, however, as protectants to crop plants against herbicide damage without reducing activity on the target weed species as explained by Davies (2001). Davies *et al*. (1998) reported that these protectants acted through increased activities of cytochrome P_{450}, glutathione-s-transfereases (GSTs) or via raising glutathione levels

In this connection, the adverse effects of the dinitroaniline herbicide (butralin) on chromosomes of both somatic and germ cells of mice were reversed by using thiola (N- (2-mercaptopropionyl) glycine) prior to the herbicide treatment (Abd El-Aziz and Hassan, 1994). Forgacs *et al*. (2000) pointed that the amino acids arginine, histidine, lysine, ornithine, phenylalanine and tryptphan bind to 2, 4-D and the binding process is of a saturation character. More recently, in his study, El-Awadi (2007) proved the stress-recovery actions of the amino acids tryptophan and methionine on the damagable effects of butralin and pendimethalin on both physiological and cytological levels in faba bean and wheat. Meanwile, the pyrimidine derivative substance SG93 was found to modulate plant growth response of different plant species under other abiotic stress conditions (Hassasn *et al*., 2006)

In the present study we aimed to test the stress-recovery actions of the amino acids methionine and tryptophan in addition to the pyrimidine derivative substance SG93 as protectants to the medicinal plant *(Foeniculum vulgare* Mill.) under the exposure to the dinitroaniline herbicide pendimethalin. In this, a pre-sowing seed soaking treatment is applied. Our objective is to improve growth and productivity of fennel plant under the stress of such an herbicide.

2. Materials and Methods

Pot experiment was carried out during two successive winter seasons (2008-09-2009-10) in the wire house of Botany Department in the National Research Centre of Egypt.

I-Cultivation and treatments:

Fennel seeds were selected, sterilized in sodium hypochlorite solution (1%) for 15 minutes, washed thoroughly with distilled water, and then soaked in the following solutions for 3 hours:

1- Distilled water (control).
2- In methionine
3- In tryptophan and
4-In the pyrimidine derivative (SG93- Fig.1, constructed by the Department of Pharmaceutical Industry of the National Research Centre).

All at 100 and 500mg/l. The pots contained equal amounts (about 12Kg) of sieved soil (clay and sand; 2: 1 v/v) and were divided into 14 groups. The soil was pre-plant incorporated in seven groups in case of pendimethalin. At 2cm depth, each 10 seeds were sown in each pots (30-cm diameter) at the 17th of Nov. Stomp (pendimethalin) was applied as soil application (pre-emergence) at the day of sowing. Treatment was carried out in the early morning. The amount of the herbicide stomp applied per pot was calculated according to the surface area as related to the area of a feddan (4200m^2). Standard agricultural practices were carried out as recommended. Each treatment included 5 replicates = 50 plant. The pots / treatments were distributed following a complete randomized design of distribution.

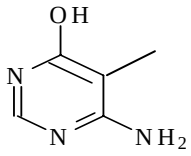

Figure 1: Chemical formula of SG93

II- Growth and yield measurements:

Plant samples were taken at the juvenile (age - 84 days) and at the fruiting (age- 119 days) from sowing. Plant height, number of leaves, number of branches, fresh and dry weight of the shoots were recorded at both stages.

Yield of fennel plant was recorded as the number of umbels per plant, the number of seeds per umbel and weight of seeds per umbel and per plant at the end of experiment.

III- Biochemical analyses:

Photosynthetic pigments were estimated in fresh tissues of fennel leaves according to (Wettstein, 1957). Protein percentage was determined according to

A.O.A.C. (1990). Total Free amino acids were determined using the ninhydrin colorimetric method defined by Plummer (1978). Following the method reported by Snell and Snell (1952), total phenolic contents were estimated.

Seed fixed oil content, was determined as reported in the (A.O.A.C., 1990) with Soxhelt apparatus using petroleum ether (40-60^{o}C).

The essential oil was extracted from the dry seeds and estimated referring to the British Pharmacopoeia (1980), dehydrated over anhydrous sodium sulfate and then kept at refrigerators (-4^{o}c) till GC analysis.

Essential oil of fennel seeds was analyzed by GC using an Agilent Technologies, (6890N Network GC system, U.S.A.) using capillary column HP 5% (30 m x 320 Um), 0.25um film thickness. Oven temperature was programmed at 70°C for 2 min. from 700°.190°C at rate of 4 ml/min. and finally 250°C (15 min) with N2: H2: Air at 30:30:300 ml/min. The temperature of the detector (FID) was maintained at 280°C. Identification of the oil components was based on the comparison of the Rts of the separated compounds with those of standard compounds that injected under the same conditions and confirmed for the major compounds by their relative retention indices.

IV- Statistical analysis:

A complete experimental randomized block design with 5 replicates was adopted. Combined results` analysis of the average values of the two seasons was carried out and the values of LSD were calculated as described by Snedecor and Cochran (1980).

3. Results:

a. Effect of pendimethalin, amino acids and pyrimidine derivative on fennel growth

Table (1) show that pre-sowing application of pendimethalin caused a significant decrease in the length of shoots, number of leaves, number of branches per plant and in fresh and dry weight (g) of shoots of fennel plants, at both growth stages of 84 and 119 days as compared to the control.

Pre-soaking fennel seeds in the low concentration (100mg/l) of methionine or tryptophan and the high concentration (500mg/l) of pyrimidine derivative (SG93) significantly increased the length of shoots, number of leaves and number of branches per plant and fresh and dry weight (g) of shoots as compared to control at both growth stages. Other treatments appeared no considerable influence on growth promotion.

Application of pendimethalin herbicide in interaction with the pre-sowing growth factor seed treatments, i.e. methionine, tryptophan and pyrimidine derivative substance SG93 significantly improved growth parameters of the fennel plant (Table 1). Significant increases in shoot length, number of leaves and number of branches per plant and fresh and dry weight (g) of shoots were recorded as compared to sole treatments of the growth substances. In this respect, maximum effect was recorded in response to the lower concentration treatment methionine in combination with the pendimethalin.

b. Effect of pendimethalin, amino acids and the pyrimidine derivative on fennel yield:

Data presented in Table (2) indicated the inhibitory action of the pendimethalin herbicide on fennel plant productivity. Significant reduction in number of umbels per plant, number of seeds per umbel and in the weight of seeds per umbel and per plant were recorded as compared to the control. The productive capacity was, however, restored under the influence of the pre-sowing seed treatment. Thus, at the low concentration of methionine and tryptophan, and with the high concentration of pyrimidine derivative substance SG93 significant increases in the number of seeds per umbel and weight of seeds per umbel and per plant were obtained.

Additive augmentation of the productivity was achieved in respect to the dual interaction treatments, i.e under exposure to the herbicide in combination with the growth factors` seed treatments. Therefore, number of umbels per plant, number of seeds per umbel and in weight of seeds per umbel and per plant increased significantly in comparison to sole growth factor treatments (Table 2). From the results, the amino acid methionine treatment showed the most prominent positive effects on productivity of the fennel plant, followed by that of tryptophan and pyrimidine derivative SG93.

Table 1: Effect of soil application of pendimethalin, amino acids and pyrimidine derivative on the growth of fennel at 84 and 119 day-old plants.

		Shoot length (cm)		No. of leaves		No. of branches		Shoot FW (g)		Shoot DW (g)	
	mg/l	A	B	A	B	A	B	A	B	A	B
Control	**0**	51.45	64	8.09	20.31	5.09	8.76	11.96	16.49	1.56	3.3
Pendimethalin	**8.5ml/l**	43.44	57.64	6.74	17.25	4.26	7.32	10.03	14.61	1.32	2.83
Methionine	**100**	56.83	79.93	10.11	25.37	5.88	11.16	13.22	22.20	1.72	4.44
	500	54.29	71.97	8.66	22.34	5.19	10.12	12.63	18.89	1.64	3.79
Tryptophan	**100**	55.17	71.3	8.91	22.36	5.37	10.52	12.83	19.76	1.67	3.95
	500	54.48	69.85	8.45	21.21	5.15	9.58	12.67	17.46	1.64	3.51
Pyrimidine derivative	**100**	53.89	66.12	8.97	22.52	6.26	12.7	12.53	17.92	1.63	3.58
	500	55.65	71.31	9.09	22.81	5.49	10.81	12.94	19.80	1.68	3.96
Methionine + Pendimethalin	**100**	59.07	82.17	11.22	28.17	5.73	11.36	13.94	23.61	1.81	4.72
	500	55.49	72.4	9.0	22.59	5.41	10.00	12.90	19.93	1.68	3.99
Tryptophan + Pendimethalin	**100**	56.11	73.16	9.52	23.90	6.26	12.7	13.05	20.92	1.69	4.19
	500	55.31	71.36	8.86	22.25	5.49	10.81	12.86	18.44	1.67	3.69
Pyrimidine derivative + Pendimethalin	**100**	54.91	72.93	9.22	23.14	5.59	10.36	12.81	18.62	1.66	3.72
	500	57.06	75.20	9.74	24.53	6.09	11.57	13.28	21.61	1.72	4.32
L.S.D	**1%**	2.51	3.99	0.61	2.09	0.48	1.11	0.85	1.87	0.11	0.38
	5%	1.78	2.82	0.43	1.48	0.34	0.78	0.60	1.32	0.08	0.27

Table 2: Effect of soil application of pendimethalin, amino acids and pyrimidine derivative on the yield of fennel.

	mg/l	No. of umbels/ plant	No. of seeds/ umbel	Wt. of seeds/umbel	Wt. of seeds /plant
Control	**0**	9.68	114.33	1.43	13.89
Pendimethalin	**8.5ml/l**	8.34	85.33	0.93	7.78
Methionine	**100**	10.38	129.46	1.85	19.28
	500	9.97	119.74	1.59	15.88
Tryptophan	**100**	10.13	122.36	1.68	17.05
	500	9.69	116.37	1.52	14.8
Pyrimidine derivative	**100**	9.90	118.33	1.56	15.47
	500	10.25	127.86	1.78	18.41
Methionine + Pendimethalin	**100**	11.43	147.6	2.35	26.85
	500	10.38	125.85	1.75	18.31
Tryptophan + Pendimethalin	**100**	11.03	139.95	1.67	21.78
	500	10.15	118.58	1.92	16.62
Pyrimidine derivative + Pendimethalin	**100**	10.20	124.09	1.68	17.23
	500	10.73	136.92	1.99	21.32
L.S.D	**1%**	0.95	15.23	0.21	3.70
	5%	0.67	10.77	0.15	2.62

c. Effect of pendimethalin, amino acids and pyrimidine derivative on the some biochemical constituents of fennel

1-Photosynthetic pigment contents:

The results indicated in Table (3) show that the pre-emergence application of the herbicide pendimethalin caused significant reduction in the photosynthetic pigment contents of fennel leaves, throughout the duration of the experiment. Such inhibitory effect under the stress of the herbicide was significantly counteracted via the seed exposure treatments. However, low concentration with the amino acids and the high concentration of SG93 had resulted in significant increases in leaf chlorophyll a, b and cartonoids. In this respect, the maximum increase was detected with the amino acid methionine. On the other hand, seed exposure to higher concentrations of the amino acids and the low concentration of pyrimidine derivative as well showed an insignificant effect.

From the same table, the combined treatments of the amino acids and the pyrimidine derivative under the exposure of the herbicide pendimethalin induced extra elevation of the photosynthetic fennel leaf pigmentation. Therefore, the estimated chlorophylls a, b, and carotenoid contents had exceeded that in the corresponding levels under the single treatments with either the amino acids or the pyrimidine derivative substance as well. This trend was observed at both growth stages of the plant, i.e. 84 and 119 age-days. Seed exposure to the low concentration of each of the amino acids and the high concentration of the pyrimidine derivative substance (SG93) had favored other treatments (Table 3).

2-Total phenolic content:

As shown in Table (3), soil application of pendimethalin caused significant increase in total phenolic contents in fennel plants, as compared to the untreated control. Similarly sole treatments with both concentrations of the amino acid tryptophan, pyrimidine derivative SG93 and high concentration of the amino acid methionine induced significant increases in total phenolic contents. On the contrary, low concentration of the amino acid methionine caused non-significant effect in the contents of total phenolic contents.

The combined treatments of pre-sowing seed-soaking in both concentrations of the amino acid methionine and in the pyrimidine derivative substance under the exposure to the pendimethalin herbicide resulted in an increase in the total phenolic contents as compared to the control (Table 3).On the other side, combined treatment with the amino acids tryptophan at both concentrations showed insignificant effect on the total content phenolic contents.

3-Total free amino acid content:

Soil corporation of the dinitroaniline herbicide pendimethalin caused significant increase of total free amino acids content in the fennel plant as compared to the control. Except with the pyrimidine seed treatment at 500mg/l, the estimated total free amino acid contents were elevated over the control in response to the single amino acids and to the pyrimidine substance SG93 seed treatments as well.

On the other hand, dual treatments with both the amino acids and the pyrimidine derivative in interaction with the herbicide pendimethalin resulted in high total free amino acids than that estimated in the control, with an exception of the amino acid methionine at its low concentration 100mg/l (Table 3).

4. Total protein content:

As shown in Table (3), total protein content was significantly reduced in fennel plant due to the exposure to the pendimethalin herbicide in comparison to the control. Such inhibitory effect was reversed via the pre-sowing seed-soaking treatment in the growth factors under test. This was true at all of their concentrations except that of the tryptophan at its high one (500 mg/l). In comparison to the control, the latter resulted in significant reduction in the total protein content of fennel as compared to the control.

The combined treatments of pre-sowing fennel seed soaking treatment with both concentrations of the growth substances; methionine, tryptophan and pyrimidine derivative with soil corporation of the pendimethalin herbicide showed an enhancement effect on total protein

accumulation in fennel plant as compared to the control (Table 3).

Table 3: Effect of soil application pendimethalin, amino acids and pyrimidine derivative on photosynthetic pigment contents, total phenolic content, total free amino acids content and total protein content of fennel plants.

	mg/l	Ch. A (mg /g f. wt.)		Ch. B (mg /g f. wt.)		Cart. (mg /g f. wt.)		Total phenolic content (mg/g d. wt.)	Total free amino acids (mg/g d. wt.)	Total protein (mg/g d. wt.)
		A	B	A	B	A	B			
Control	**0**	0.659	0.791	0.198	0.233	0.302	0.415	33.07	32.38	13.44
Pendimethalin	**8.5ml/L**	0.484	0.699	0.175	0.203	0.261	0.367	47.03	35.91	11.88
Methionine	**100**	0.706	0.794	0.242	0.259	0.342	0.453	31.49	48.97	15.83
	500	0.679	0.821	0.205	0.238	0.32	0.425	36.65	36.56	18.13
Tryptophan	**100**	0.698	0.842	0.226	0.249	0.326	0.438	39.87	48.30	18.33
	500	0.663	0.791	0.208	0.242	0.315	0.408	39.17	47.60	7.50
Pyrimidine derivative	**100**	0.667	0.824	0.204	0.235	0.315	0.418	47.03	37.98	20.00
	500	0.688	0.824	0.232	0.248	0.333	0.438	36.10	27.85	15.21
Methionine + Pendimethalin	**100**	0.768	0.907	0.262	0.304	0.381	0.515	41.87	28.68	15.42
	500	0.723	0.873	0.223	0.259	0.340	0.434	40.96	53.07	16.67
Tryptophan + Pendimethalin	**100**	0.725	0.896	0.233	0.280	0.351	0.477	34.82	53.56	14.58
	500	0.701	0.829	0.215	0.250	0.325	0.427	33.78	41.42	15.21
Pyrimidine derivative + Pendimethalin	**100**	0.683	0.843	0.223	0.263	0.326	0.425	40.91	37.10	15.00
	500	0.737	0.862	0.238	0.270	0.355	0.441	40.01	36.83	16.56
L.S.D	**1%**	0.026	0.053	0.020	0.022	0.028	0.027	3.18	1.56	0.80
	5%	0.018	0.036	0.014	0.015	0.019	0.019	2.25	1.11	0.56

A- Juvenile growth stage 84 days B- Fruiting growth stage 119days

5- Effect of pendimethalin, amino acids and pyrimidine derivative on the oil content and composition:

From the data presented in Table (4), the exposure to the pendimethalin herbicide resulted in significant increase in the fixed oil percentage of the yielded fennel seeds as compared to the control. Whereas methionines at both its concentration- seed treatments and the pyrimidine substance at its low one caused significant decreases in the fixed oil percentage. Tryptophan at 100 and 500mg/l, and pyrimidine derivative (500mg/l) treatments tended to elevate the fixed oil percentage to high significant levels in comparison to other treatments (Table 4). From the same table, the pendimethalin herbicide combination treatments with either methionine at both its concentrations or with the prymidine derivative substance at its higher one increased significantly the fixed oil percentage in the yielded fennel seeds as compared to the control. The effect of the other treatments was more or less proximal with the control.

The essential oil of the produced fennel seeds in all treatments were subjected to fractionation using gas chromatography (GC). Anethol, 1, 8 cineol and fenchone are recorded as the main components of the

essential oil of fennel seeds (Table 4). As compared to the control, anethole percentage ranged from 85.61 to 87.58 % while 1, 8 cineol and fenchone ranged from 3.70 to 5.72% and 3.7% to4.47 respectively. The highest percentage of anethole was obtained in the treatment 100mg/l methionine-seed–treated plants. Whereas, a considerable increase in the percentage of 1.8 cineol was recorded in response to the pyrimidine derivative material-seed-treatment (Table 4). On the contrary, the application of tryptophan and pyrimidine substance (SG93) both at the 500mg/l concentration resulted in remarkable decreases in the percentage of fenchone (Table 4).

In the dual treatments with pendimethalin herbicide combined with the growth factors` seed-soaking treatments, the absence of estragol was recorded. The main component of the analyzed essential oil was also anethole (85.6%-90.60%), 1,8cineol (4.86%-6.73%) and fenchone (3.62-7.73). Both concentrations of methionine and tryprophan seed treatments had induced the highest percentage of anethole, whereas the methionine produced the essential oil with a high percentage of 1, 8 cineol in the yielded seed of fennel plant (Table 4).

Table 4: Effect of soil application pendimethalin, amino acids and pyrimidine derivative on fennel oil content and composition.

	mg/l	Essential oil %	Fixed oil %	Major essential oil constituents (%)							
				αPinene	D Limonene	1,8 Cineol	Fenchone	Anethol	Estragol(Methyl chavicol)	Known	Unknown
Control	**0**	0.79	5.82	0.37	0.07	5.09	4.13	86.11	0.05	4.18	95.82
Pendimethalin	**8.5 ml/l**	1.09	7.78	0.40	-	6.51	3.93	89.14	-	99.58	0.42
Methionine	**100**	0.89	4.80	0.36	0.02	4.93	4.13	87.58	0.53	97.55	2.45
	500	0.95	5.46	0.39	0.11	4.50	4.12	85.61	0.84	95.57	4.43
Tryptophan	**100**	0.78	6.05	0.23	0.11	5.72	4.10	87.53	0.18	97.87	2.13
	500	1.09	6.49	0.33	0.13	6.22	3.70	87.14	0.23	97.75	2.25
Pyrimidine derivative	**100**	1.03	5.28	0.34	0.09	3.97	4.47	86.22	0.73	96.63	3.37
	500	1.06	6.68	0.48	0.17	4.38	3.80	86.77	0.67	96.27	3.73
Methinione + Pendimethalin	**100**	1.10	7.00	0.50	0.21	6.10	3.62	89.44	-	99.37	0.63
	500	0.94	6.70	0.60	0.21	6.73	4.60	87.86	-	99.40	0.60
Tryptophan + pendimethalin	**100**	0.97	5.39	0.42	0.16	4.86	4.77	87.9	-	97.69	2.31
	500	1.26	5.92	0.48	0.17	5.87	7.73	85.6	-	99.37	0.63
Pyrimidine derivative + Pendimethalin	**100**	1.08	5.86	0.35	-	5.35	3.73	90.60	-	99.68	0.32
	500	1.25	6.70	0.36	-	5.33	3.78	90.51	-	99.62	0.32
L.S.D	**1%**	0.04	0.33	-	-	-	-	-	-	-	-
	5%	0.03	0.24	-	-	-	-	-	-	-	-

4. Discussion:

Effect of the single application of the herbicide pendimethalin on fennel:

In the present result, the adverse effects of the dinitroaniline herbicide pendimethalin on the growth of fennel plant were detected in the reduction of shoot length, number of leaves per plant, number of branches per plant and fresh and dry matter accumulation. These results are supported with those obtained by Meena and Mehta (2009). Similar observations were previously mentioned on garlic by Adam *et al*. (1996). The growth-induced inhibition of the herbicide was, however reflected on the productivity as yield component characteristics which were markedly declined. Similar to the present observations ; Kothari (2002) and Meena and Mehta 2009 had reported that pendimethalin reduced yield components, i.e. the number of umbels/plant number of umbellate/umbel, number of seed/umbellate seeds and straw yields of fennel. In this respect, one may attribute the decline in fennel yield in response to the exposure to the herbicide pendimithilin to the disturbance effects on the photosynthetic process due to the suppression of the biosyntheses of the photosynthetic pigments (Shabana *et al*., 2001).

Starratt and Lazarovits (1999) recorded an increase in free amino acids in melon seedlings in response to exposure to dinitroaniline herbicides. In the present results, similar observations are noted on the fennel plant. This may explain the significant reduction observed in total protein content. In this connection, another dinitroaniline herbicide butralin caused remarkable reduction in protein, carbohydrate, leghaemoglobin and ureide in the nodule fraction of soybean (Mahmoud *et al*., 1996). In contradiction to these results are those reported by Panneerselvam *et al*. (1998) on soybean, as a result of pendimethalin application.

It is reported herein that soil application of pendimethalin which caused significant increase in the total phenolic contents over the control; could be compared with those obtained by Abd El Wahed *et al*. (2003) due to the herbicide thiobencarb influences on rice.

In the yielded fennel seeds, while significant increases in essential and in fixed oil percentages were recorded as compared to the control, no changes in the fractions were obtained in response to the herbicide. These percentages were 89.14% anethol, 6.51 - 1, 8 cineol and 3.93 fenchone, but with the absence of estrigol and D lominene. These results are supported by the findings of Chaudhary (2000) and Kothari, *et al.* (2002) on fennel and on rose-scented geranium. They also reported that pendimethalin herbicide had not impaired the quality of essential oil.

Herbicide stress-recovery via pre-sowing seed-soaking treatments:

In the present study, seed amino acid treatments induced noticeable counteraction of the pendimethalin-induced-growth inhibition in fennel. Thus, shoot elongation, total number of leaves and branches, as well as fresh and dry matter accumulation were significantly enhanced, particularly at low concentrations of methionine and tryptophan as well as at the high concentration of the pyrimidine substance SG93. In case of the dual treatments with the herbicide, such influence was sustained reaching higher level of growth promotiom. Such growth promotion led to an improvement in yield components of the fennel plant. Such observations are in agreement with the alleviation of the adverse effects of herbicides due to amino acid treatments (Devine and Preston, 2000) and to a pyrimidine derivative substance (Hassan and Gad, 2003).

The positive significant protecting effects of the pre-sowing seed-soaking treatments with the growth factors under investigation on leaf photosynthetic pigments content either as sole treatment or under exposure of pendimethalin are proved herein. The lower concentration treatments of the amino acids and the higher one of the pyrimidine substance SG93 exerted the best resultsmin this respect. Similar trends were obtained on the total phenolic content, total free amino acids and protein content. On the other hand, a synergistic effect had resulted in the interaction treatments, i.e. with growth factors` application under the exposure to the herbicide pendimethalin. Thus revealed extra elevations in total phenolic, total free amino acid and in total protein content, as compared

to those of sole treatments. Similarly an enhancement effect of the growth factors in interaction with herbicide exposure was obtained in the estimated percentage of the fixed and essential oil percentages in the yielded fennel seeds. This implicated the results obtained by El-Awadi and Hassan (2010) on fennel and by Talaat (2005) on *Pelargonium graveolens* L. In addition to their role in protein synthesis, amino acids are the basic molecules in biosynthesis of primary and secondary metabolites in plants (Coruzzi and Last, 2000).Therefore, their exogenous application might compensate the metabolic stress process achieved by a given herbicide, mainly through rendering higher availability of energy resources. In the present work, the amino acids methionine and tryptophan applied as pre-sowing seed soaking treatment had achieved a stress-recovery action against the dinitroaniline herbicide pendimethalin.

This herbicide is found to act as a mitotic inhibitor (Fennell *et al*., 2006).

Thus methionine counteraction effect could be either via its functions in the process of mRNA translation to begin protein synthesis (Lodish *et al*., 2000) or as a regulatory molecule in the form of S-adenosylmethionine (SAM) (Hesse and Hoefgen, 2003). Also methionine enhanced the polyamine accumulation which are controlled by stress signaling (Panicot *et al*., 2002), furnishing aspartate levels (Hesse *et al*., 2004) and linking with growth hormones such as cytokinins, auxins and brassinosteroids (Maxwell and Kieber, 2004), which interfere with cell division.

The amino acid tryptophan is known to participate in pathways relevant to biosynthesis of auxins and other natural products. In this connection its application was reported to enhanced growth rates, photosynthetic pigment, total sugars and free amino acid contents in geranium plants (Talaat (2005). This effect was attributed to its action as a precursor for IAA biosynthis (Normanly *et al*., 2004).

In the present results a stress-recovery action of the pyrimidine derivative substance SG93 against the induced-inhibitory effect of the pendimethalin herbicide was achieved. This could be interpreted in the light that pyrimidines are playing variable roles in biological processes in plants. They are the building blocks for nucleic acid synthesis, energy sources, synthesis of sucrose, polysaccharides as well as production of important secondary metabolites (Stasolla *et al*., 2003).

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Influence of Magnesium and Copper Foliar Application on Wheat Yield and Quality of Grains under Sandy Soil Conditions

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ABSTRACT: Two field experiments were conducted during the winter seasons of 2007/2008 and 2008/2009 at Ismailia Experimental Station, Agriculture Research Center, Ismailia Governorate, to study the influence of foliar feeding with magnesium , copper either as single nutrient or in combination on yield ;yield components and grains quality of wheat (*Triticum aestivum* L.) cv. Sakha 94. Nine treatments were applied: two levels of Mg, two levels of Cu and four combined treatments (Mg + Cu), in addition to control treatment. Results showed that positive significant effect on plant height (cm), tillers number/m^2, spike number/m^2, spike length (cm), spike weight (g), grains number/spike, grains weight /spike (g), 1000-grain weight (g), grains yield/fed. and straw yield/fed. were achieved by spraying the of copper and magnesium treatments. However, the highest significant increment in grain yield was obtained by spraying the highest Cu level (1.68 kg Cu/fed.), while spraying the lowest Cu level (0.84 kg Cu/fed.) gave the highest straw yield. On the other hand, combination treatment (6.72 kg Mg + 1.68 kg Cu/fed.) showed the highest values for protein, N, Mg, Cu and Zn contents. However, spraying wheat plants with low Cu level (0.84 kg Cu/fed.) gave the highest value of grain carbohydrate percentage.

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Keywords: Wheat, Magnesium, Copper, Foliar application, Yield, Quality and Sandy soil.

1. Introduction

Wheat (*Triticum aesitivum L.*) is cultivated worldwide primarily as a food commodity. Because of its importance in the Egyptian diet. Wheat is considered a strategic commodity. Calderini and Slafer (1998) noticed that only 3 of 21 countries (Egypt, Germany and India) exhibited a clear continuous increase in wheat yield (per unit area) during the last decade. Cropping intensity in Egypt accompanied with shortage in fertilization led to a serious depletion of both macro- and micronutrients from soil, especially sandy soil.

Magnesium has major physiological and molecular roles in plants, such as being a component of the chlorophyll molecule, a cofactor for many enzymatic processes associated with phosphorylation, dephosphorylation, and the hydrolysis of various compounds, and as a structural stabilizer for various nucleotides. Studies indicated that 15 to 30% of the total magnesium in plants is associated with the chlorophyll molecule. Magnesium has functions in protein synthesis that can affect the size, structure, and function of chloroplasts (Marschner 1995). Hanna and Abdel Mottaleb (1998) concluded that the magnesium fertilization as foliar application tended to increase grain, straw yield and 1000-kernel weight, number of grains/spike and grain content of Mg , P, K, and crude protein. El-Amry *et al.* (2001) reported that maximum grain yield and chemical composition (protein, ash, oil, fiber, total carbohydrate, and phytic acid) of wheat were obtained by application of Mg with 5 or 10 kg /feddan.

Copper is an essential micronutrient for higher plants that required for the functioning of more than 30 enzymes, all of which are either redox catalysts (e.g., cytochrome oxidase, nitrate reductase) or dioxygen carriers (*e.g.*, haemocyanin). Consequently, their importance is pronounced on the health and diseases of plants, animals, and human being, as they are dependent on trace elements needed from food (Mohamed and Taha, 2003). Dobermann and Fairhurst (2000) found that copper affected metabolic processes like photosynthesis and respiration reduction in pollen viability and increase in spikelet sterility and many unfilled grains. El-Magid et al. (2000) reported that the application of Fe, Cu, Zn and Mn increased grain and straw yields of wheat. Zeidan and Nofal (2003) showed that application of micronutrients only (iron, manganese, zinc and copper) or with adding 1% urea on growth and yield of wheat caused significant increases in wheat grain protein content, yield and quality of wheat. El-Maghraby (2004) found that application of micronutrients in wheat plants had

highly significant effects on the uptake of macronutrients (N and K) and micronutrients (Fe, Mn, Zn and Cu) by straw and had highly significant effects on the uptake of macronutrients (N, P an K) and micronutrients (Fe, Mn and Zn) by grains. Karamanos *et al.* (2004**)** reported that maximum grain yield of wheat was obtained by foliar Cu application.

The purpose of this study was to explore the response of wheat to magnesium and copper levels either alone or in combination to acheive the highest values of yield and its components as well as grains quality under sandy soil condition.

2. Material and Methods

Two field experiments were carried out in Ismailia Experimental Farm, Agricultural Research Center, Ismailia governorate, during the 2007/2008 and 2008/2009 growing seasons to study the influence of copper, magnesium and their interaction on yield and its components and quality of grain. The experimental design was randomize complete block (RCBD) with six replicates and nine treatments.

Treatments

The experiment contained nine foliar spray treatments as follows:

1- control (water spray)
2- 3.36 kg Mg/feddan
3- 6.72 kg Mg/feddan
4- 0.84 kg Cu/feddan
5- 1.68 kg Cu/feddan
6- 3.36 kg Mg + 0.84 kg Cu/feddan
7- 3.36 kg Mg + 1.68 kg Cu/feddan
8- 6.72 kg Mg + 0.84 kg Cu/feddan
9- 6.72 kg Mg + 1.68 kg Cu/feddan

Wheat plants were sprayed with the aforementioned treatments two times, the first was 45 and the second was 60 days after planting. The sprayed solution volume was 350 and 400 L/fed. in the first and second spray, respectively.

Soil was ploughed using a chisel plough and divided into experimental units, 2.0 m long and 3.0 m wide. Every plot contained 15 rows each of 20 cm width. Wheat grains were sown on November 22 th and 13 th in 2007/2008 and 2008/2009 seasons; respectively at the rate of 60 kg/feddan by hand drilling in rows.

Soil Analysis:

Representative soil samples were taken after soil preparation and before fertilization from the experimental sites (0-30 cm depth) for physico-chemical characteristics (Table 1).

Nitrogen, phosphorus and potassium were added at rate of 106 kg N/fed, 37 kg P_2O_5/fed., and 24 kg K_2O/fed.. Nitrogen was applied as ammonium sulfate (20.6 % N) in three equal splits (at planting, 30 and 50 days after sowing) in both seasons. Phosphorus was applied as a single super phosphate (15.5 % P_2O_5) during soil preparation. Potassium was applied as Potassium sulphate (50 % K_2O) at 30 days after sowing. The whole experimental plots were also sprayed with mixed iron, manganese and zinc in EDTA form two times (45 and 60 days after planting) at rate of 0.5 g/L. from each nutrient.

Table 1: Physico-chemical characteristics of soil (0 – 30 cm) in 2007/2008 and 2008/2009 seasons.

Characteristics	2007/2008	2008/2009
Physical Properties		
Sand (%)	88.4	90
Silt (%)	4.0	3.2
Clay (%)	7.6	6.8
Texture	Sand	Sand
E.C dS/m	0.20*	0.35*
pH	8.95****	9.15****
Chemical Properties		
$CaCO_3$ %	1.20*	1.84*
Organic Matter %	0.54*	0.09*
Exchangeable macronutrients (mg / 100 g soil)		
P	0.36*	0.62*
K	7.6*	4.24*
Na	18.0*	41.4***

Ca	240	308
Mg	9.0*	3.64*
Determined micronutrients (ppm)		
Fe	3.7*	3.83*
Mn	2.8*	1.13*
Zn	0.22*	0.15*
Cu	0.1*	0.25*

*** = low ** = Adequate *** = High **** = Very high**

Data of soil analysis was evaluated according to Ankerman and Large (1974); texture according to Bauyoucos (1954); pH & E.C according to Jackson (1973); $CaCO_3$ according to Black (1965); Organic matter according to Walkely and Black (1934) ;K, Na, Ca & Mg according to Jackson (1973); P according to Olsen *et al*. (1954) and Fe, Mn, Zn & Cu Lindsay and Norvell (1978)

Plants were irrigated at 6 days interval using sprinkler system.Weeds were controlled by hoeing.

Plant sampling

Wheat grains after harvest were taken to determine macro- and micronutrients, protein and carbohydrate. Grains were washed in sequence with tap water, 0.01 N HCl- acidified distilled water and distilled water, and then dried in a ventilated oven at 70 ^{o}C till constant weight was obtained.

Yield and its components:

At maturity, i.e. 160 days after planting the plants were harvested. Samples from wheat were taken to determine the following characteristics: Plant height (cm), tillers number/m^2, number of spikes per m^2, number of grains per spike, spike length, in (cm), 1000 grains weight (g), Grain yield [ardab /fedden, (one Ardab = 150 kg.)] and straw yield (tons /feddan). Grain and straw yields were determined in one m^2 then, converted to feddan.

Measurements and determinations:

Nitrogen was determined using Micro – Kjelahl method (Markaham, 1942), using boric acid modification as described by Ma and Zuazage (1942), and distillation was done using Gerhardt apparatus. Phosphorus was photometrical determined using the molybdate-vanadate method Jackson (1973). Potassium, sodium and calcium were measured using Dr. Lang -M8D Flame-photometer. Magnesium, Fe, Mn, Zn and Cu were determined using the Atomic Absorption Spectrophotometer (Perkin-Elmer 100 B). Protein calculated as (N %) × 6.25. Carbohydrate percentage in grains was determined according to the method adapted by Shaffer and Hartmann (1921).

Statistical analysis:

Collected data were subjected to the proper statistical analysis with the methods described by Snedecor and Cochoran (1967). Since the data in both seasons took similar trends and variances were homogeneous according to Bartllets test, the combined analysis of both seasons was done. LSD test was applied at 5 % level for comparing the numerical averages Waller and Duncan (1969).

3. Results and Discussion

Yield and its components:

Foliar application with the applied magnesium and/or copper had a significant effect on plant height (cm), tillers number/m^2, spike number/m^2, spike length (cm), spike weight (g), grains number/spike, grains weight /spike (g), 1000-grain weight (g), grains yield ardab/fed. and straw yield ton/fed. (Table 2). Spraying wheat plants with 3.36 kg Mg + 0.84 kg Cu /fed. produced the greatest increase for tillers number/m^2, spike length and spike weight as compared with control and other treatments. While, the highest values for grains number/spike, grains weight /spike and 1000-grain weight were determined by the magnesium and copper application of 6.72 kg Mg + 0.84 kg Cu /fed. Moreover, foliar application with 6.72 kg Mg/fed. and 1.68 kg Cu/fed. gave the highest value for plant height. Moreover, high level of copper (1.68 kg/fed.) produced the greatest increase for grains yield, while the lowest level of Cu (0.84 kg/fed.) gave the highest value for straw yield but, the differences between treatments did not reach to the level significance. The lowest values of grain and straw yields and yield components were resulted from the

untreated treatment (control). Such effects of foliar application with Mg and/or Cu might be due to their critical role in crop growth, involving in photosynthesis processes, respiration and other biochemical and physiological activates and thus, their importance in achieving higher yields. These results are similar to those obtained with copper application on wheat plants by El-Badry (1995), Negm (1998), Zeidan and Nofal (2003), El-Maghraby (2004) and Kumar *et al.* (2009). On the other hand, Hanna and Abdel Mottaleb (1998), El-Amry *et al.* (2001), and Hussain *et al.* (2005) showed that grains yield of wheat plants was affected positively by application of magnesium.

Grain chemical compositions:

Chemical composition of grains i. e, N, Mg, Cu and Zn concentrations showed significant response to magnesium and copper foliar application treatments (Table 3). Foliar application with high level of magnesium (6.72 kg/fed.) mixed with high level of copper (1.68 kg/fed.) gave the highest value of wheat grains N, Mg, Cu and Zn concentration compared with other treatments and control. This might be in part attributed to the favorable effect of copper and magnesium to form vegetative plant materials which in turn increase N, Mg, Cu and Zn uptake by plants (Marschner, 1995). In this respect, Hanna and Abdel Mottaleb (1998) concluded that magnesium fertilization as a foliar application tended to increase grain content of Mg , P and K. Also, Negm (1998) showed that N and Cu contents of wheat grains were significantly increased by foliar application of copper at 100 ppm, while Zn grain content was not affected by copper treatments.

Table(2): Plant height (cm), tillers number, yield and yield components of wheat plant as affected by copper and magnesium foliar applications (combined analysis of 2007/2008 and 2008/2009 seasons)

Parameters / Treatments	Plant height (cm)	Tillers no./m^2	Spike no./m^2	Spike length (cm)	Spike weight (g)	Grains no./ spike	Grains weight /spike (g)	Grains weight /m^2 (g)	1000- grains weight (g)	Grain yield ardab /fed.	Straw yield ton/fed
Control (water foliar spray)	84.50	693.3	581.30	7.72	1.67	22.33	0.77	280.5	30.63	7.86	2.63
3.36 kg Mg /fed.	88.00	690.7	621.30	8.77	2.33	31.50	1.15	363.5	32.97	10.16	4.35
6.72 kg Mg /fed.	89.33	734.0	680.50	9.17	2.45	33.50	1.27	384.4	36.23	10.77	4.59
0.84 kg Cu /fed.	87.67	767.3	733.30	9.03	2.30	29.83	1.18	467.9	39.10	13.09	5.01
1.68 kg Cu /fed.	89.50	826.7	773.30	8.80	2.53	36.50	1.25	518.3	40.38	14.51	4.45
3.36 kg Mg + 0.84 kg Cu /fed.	90.33	840.0	768.00	9.13	3.02	35.67	1.40	469.7	37.02	13.15	4.70
3.36 kg Mg + 1.68 kg Cu /fed.	90.83	778.3	678.00	9.13	2.60	34.83	1.13	441.0	37.57	12.35	4.80
6.72 kg Mg + 0.84 kg Cu /fed.	89.83	757.0	691.20	9.08	2.62	36.50	1.45	435.4	41.12	12.19	4.32
6.72 kg Mg + 1.68 kg Cu /fed.	94.83	826.7	757.30	8.90	2.58	32.67	1.25	494.3	40.93	13.83	4.78
LSD at 0.05	3.40	77.38	48.93	0.75	0.49	3.84	0.20	67.3	3.81	1.88	0.91

Grain quality:

Quality parameters of wheat grains (protein and carbohydrates) showed significant response to magnesium and/or copper foliar application treatments (Table 3).

Spraying wheat plants with mixture of magnesium and copper at rate of 6.72 kg Mg + 1.68 kg Cu/feddan resulted in the highest significant value of grain protein content (16.65 %) compared with other foliar application treatments. Magnesium has functions in protein synthesis that can affect the size, structure, and function of chloroplasts (Marschner, 1995). But, magnesium and copper sufficient levels increased protein content indirectly through its role in nutrients balance in plant tissues. The present results are in the same direction with those reported by Zeidan and Nofal (2003) with copper application on wheat plants. Moreover, Hanna and Abdel Mottaleb (1998) and El-Amry *et al.* (2001) found that grain protein of wheat increased markedly by Mg application.

Also, the results in Table (3) indicated that 0.84 kg Cu /fed. gave the highest carbohydrate in grains (69.33 %) as compared with untreated plants and other treatments. This indicates that there is no value to increase copper and magnesium level more than 0.84 kg Cu and 3.36 kg Mg/fed. for obtaining high carbohydrate content of grains. Even it is contributed in photosynthesis and enzymes controlling carbohydrate formation, the minimum requirements of copper and magnesium were enough to accumulate suitable carbohydrate contents. Migahid and Sadek (1994) they found that carbohydrate content in wheat plants was significantly increased by copper application. Also, El-Amry *et al.* (2001) reported that maximum grain total carbohydrate of wheat was obtained by application of Mg with 5 or 10 kg /feddan.

Table (3): Protein (%), carbohydrate (%), N (%), Mg (%), Cu (ppm) and Zn (ppm) in grains of wheat plant as affected by copper and magnesium foliar applications (combined analysis of 2007/2008 and 2008/2009 seasons)

Parameters / Treatments	Protein %	Carbohydrate %	N%	Mg%	Cu ppm	Zn ppm
Control (water foliar spray)	6.9	61.7	1.1	0.26	3.3	34.8
3.36 kg Mg /fed.	12.8	63.7	2.1	0.27	4.4	39.7
6.72 kg Mg /fed.	13.8	65.3	2.2	0.29	4.5	42.4
0.84 kg Cu /fed.	13.0	69.3	2.0	0.29	5.3	45.8
1.68 kg Cu /fed.	13.8	68.2	2.2	0.29	7.2	48.7
3.36 kg Mg + 0.84 kg Cu /fed.	14.6	67.8	2.3	0.32	5.4	46.9
3.36 kg Mg + 1.68 kg Cu /fed.	15.2	67.3	2.4	0.33	7.5	48.3
6.72 kg Mg + 0.84 kg Cu /fed.	16.2	67.2	2.6	0.35	5.6	50.9
6.72 kg Mg + 1.68 kg Cu /fed.	16.7	67.3	2.7	0.35	7.9	54.4
LSD at 0.05	0.5	0.8	0.2	0.01	1.0	3.6

It could be concluded that under sandy soil condition foliar application of magnesium and copper with 6.72 kg Mg/feddan and 1.68 or 0.84 kg Cu/feddan could be used to obtain high yield and yield components of wheat plants. Also, to obtained wheat grains with high N, Mg, Cu, Zn and cured protein percentage, magnesium and copper application should be applied at 6.72 kg Mg/feddan in combination with 1.68 kg Cu/feddan as foliar application. While, to obtained wheat grains with high carbohydrate percentage copper application should be applied in 0.84 kg Cu/feddan as foliar application.

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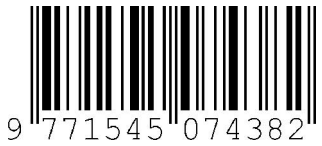

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