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Hepatoprotective Effect of Jojoba Oil and Nigella Sativa Seeds in Rats Fed Diet Containing Aflatoxin

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Abstract

Aim: This study aimed to evaluate the hepatoprotective effect of Jojoba oil(J.O.) and Nigella sativa seeds(N.S.) in rats fed diet contaminated with aflatoxin(AF).

Methods: Forty mature male rats were divided into eight groups and treated for 30 days as follows: (1) Untreated control; (2) Jojoba oil 1.25% (low concentration) ; (3) Jojoba oil 2.5% (high concentration) ; (4) Aflatoxin (AF)-contaminated diet (30ug/kg diet); (5) low dose Jojoba oil plus AF; (6) high dose Jojoba oil plus AF; (7) Nigella sativa seeds at 2.5% and (8) Nigella sativa seeds plus AF. Blood samples were collected for biochemical analyses and livers were taken DNA measurement.

Results: The results indicated that Jojoba oil is rich in protein, phenolic compounds, phytic acid, and considerable amounts of simmondsin. Animals fed AF - contaminated diet showed severe biochemical changes and DNA damage. Feeding on ration mixed with Jojoba oil alone at the two tested concentrations or Nigella sativa seeds did not induce significant alterations in all tested parameters. Treatment with Jojoba oil to rats fed AF containing diet protected against hepatotoxicity and DNA damage induced by AF.

Conclusion: It can be concluded that Jojoba oil and Nigella sativa seeds can be incorporated in AF-contaminated feed to protect against hepatotoxicity and DNA damage induced by AF.

Key word: Jojoba oil, Nigilla sativa & Aflatoxin

Introduction

Aflatoxins (AF) are produced as secondary metabolites by the four toxic species of *Aspergillus: A. flavus*, *A. parasiticus, A. nomius* and *A. pseudotamarii*as (**CAST, 2003**). Since their discovery in the 1960s, AF has been demonstrated to be carcinogenic in many animal species, including rodents, non human primates and fish. They have been also suspected to induce hepatocellular carcinoma (HCC) in human. In addition, many species including rat, turkey, duck, trout, and primates, are

susceptible to the carcinogenic effects of AFB1. (**Jackson and Groopman, 1999**). Corn and peanut showed that AFB1 contamination is higher than 50% in Egypt (**Donma and Donma, 2007**). *Nigella sativa*, one of the members of *Ranunculaceae* family, commonly grows in the Middle East, Eastern Europe and Eastern and Middle Asia. *Nigella sativa* and its oil are being used as food additives as well as natural remedies for many ailments for over thousands of years. Many active ingredients have been isolated from *Nigella sativa*, including: thymoquinone, thymohydroquinone, dithymo- quinone, thymol, carvacrol, nigellicine and alpha-hedrin which have been identified, such as antitumour activity, antioxidant activity**,** anti-inflammatory activity, antibacterial activity and immunostimulant (**Salem and Hossain, 2000**).

Long-term studies indicated that AFB_1 is hepatocarcinogenic in rats (**Gelderblom et al, 2001, Abdel-Wahhab et al ,2004 and El-Nekeety et al, 2007**), while another study reported on the nephrocarcinogenicity and cancer promoting activity in rats(**Howard et al ,2001 and Voss et al ,2002**).

Several studies (**Alizadeh et al, 2012 and Sun et al, 2007**) in rodents have shown that FB_1 promotes pre-neoplastic lesions in the liver, suggesting a role for FB_1 -induced genotoxicity (**Karuna and Rao, 2013**). Recently, **Chuturgoon et al, (2014)** reported that FB_1 induces global DNA hypomethylation and histone demethylation in human hepatoma cells that causes chromatin instability and may lead to liver tumourigenesis. FB_1 is resistant to conditions normally used in food processing and, therefore, poses a significant hazard to human and animal health (**Scott, 2012**).

Jojoba (*Simmondsia chinensis* L) is a perennial woody shrub native to semi-arid regions all over the world (**Ranzatoa et al, 2011**). Currently, it is cultivated in the Ismailia Desert in Egypt (**El-Mallah and El-Shami, 2009**). The jojoba plant produces seeds that contain up to 50% liquid wax used as a lubricant additive and in cosmetics (**Wisniak, 1994**). It has been reported that jojoba seeds possess anti-inflammatory activity. Jojoba meal is the protein residue remaining after oil extraction, and it has potential as dietary supplements for animal feeds, as well as for the treatment of overweight animals and humans (**Benzioni et al, 2005**). This protein meal consists mainly of 79% albumins and 21% globulins (**Shrestha et al., 2002**). Previous reports indicated that Jojoba meal contained anti-nutritional compounds known as simmondsins (5-demethylsimmondsin, 4,5-didemethylsimmondsin, simmondsin, and simmondsin 2'-ferulate), (**Vermauti et al., 1994**) which have been identified as the component in jojoba that is most responsible for the inhibition of food intake and for appetite suppression in rodents, rats, dogs, and chickens (**Lievens et al., 2003**). However, the meal also contains several beneficial compounds, such as phytic acid and polyphenols, which shows antioxidant and anti-cancer activity (**Wiseman and Price, 1987**).

Medicinal properties of Nigella sativa seeds (black seeds) have even been mentioned by the Prophet of Islam, Muhammad (Peace be upon him) and its use was recommended for the treatment of many ailments (**Bhatti et al., 2009**).

The aim of this study was to evaluate the hepatoprotective effect of Jojoba oil and Nigella sativa seeds in rats fed Aflatoxin-containing diet.

Material and Method

Animals

Forty mature male albino rats with an average body weight ranging from 160-180 g were obtained from laboratory Animal House Ministry of Health, Helwan,Cairo . Animals were housed in metal cages during the whole experimental period at a temperature of 23 ± 2 "C with free access to water and standard food pellets (El-Nasr Co., Abo-Zaabal, Egypt) and provided with water *ad- libtum*, through the experiment. Rats were acclimatized in our animal facility for at least 1week prior to any experiment. Animals were kept under hygienic conditions, fed on well balanced ration.

Jojoba oil: crude J.O.(its purity100%) were obtained from National Oil Company.

Nigella sativa: They were purified, finely grinded in a mortar and added to the diet along the period of experiment.

Aflatoxin Preparation:

The aflatoxin was produced via fermentation of rice by *Aspergillu sparasiticus* NRRL 2999.The fermented rice was autoclaved, dried and ground to a powder and the aflatoxin content was measured by the use of HPLC(**Hutchins and Hagler,1983**) . The rice powder was incorporated into the basal diet to provide the desired level of 30 ug/kg diet. The diet containing the aflatoxin was analyzed and the presence of parent aflatoxin was confirmed and determined as above.

Methods

Experimental design:

Eight equal groups each of five rats were distributed and subjected to the following:

The first group (G1): Rats kept as a control. The second (G2) and third groups (G3): Rats were feed on ration mixed with Jojoba oil at concentration 1.25% and 2.5% for 30 days, respectively. The fourth (G4) group: Rats fed on ration contaminated with AF (30 ug/kg diet) for 30 days. The fifth (G5) and sixth (G6): groups Rats were fed on ration mixed with Jojoba oil at concentration 1.25% and 2.5% plus AF for 30 days, respectively. The seventh (G7): Rats were fed on ration

mixed with crashed Nigella sativa seeds (black seeds) at 2.5% for 30 days. The eighth (G8): Rats were fed on ration mixed with crashed Nigella sativa seeds at 2.5% plus AF for 30 days. Body weight and food intake were recorded weekly throughout the experiment period.

Samples:

Liver sample

The animals were sacrificed and autopsy performed immediately, liver tissue was removed and divided into 2 portions. The first part was washed with saline solution, then minced and homogenized (10% w/v) on ice-cooled normal saline. The homogenate was centrifuged at 10.000xg for 20 min at 4 C° and the supernatant was used for antioxidant assay **(Chitra et al., 1999).** Lipid peroxidation was determined by estimation of lipid peroxide malonaldehyde (MDA) determined according to **Okhawa et al., (1979).** Reduced glutathione (GSH) was determined chemically using the method described by **Beutler et al., (1963)** which is based on the reduction of 5,5` dithiobis (2 - nitro benzoic acid) (DTNB) with glutathione to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm. The second part was kept at –20 ^{o}C for determination of DNA

Determination of DNA:

The extent of DNA fragmentation (DNA Ladder) has been assayed by electrophoresis genomic DNA samples, isolated from normal as well as experimental rat liver, on agarose /ethidium bromide gel by the procedure described by **Sellins and Cohen (1987)**.

Serum biochemical analyses:

The biochemical assays of serum alkaline phosphatase (ALP) activity according to **Tietz, (1996)**, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) **(Reitman and Frankel 1957),** serum urea level according to **Wybenga et al. (1971**), creatinine according to **Faulkner and King (1976).**

Statistical analysis:

Data were expressed as mean ± S.E. Significant different using student "t" test according to **Snedecor, (1982)**.

Results and Discussions

The effect of different treatments on food intake indicated that the acute toxicity of AF first appeared as a significant decrease in food intake (Table 1). Animals fed AF -contaminated diet showed a significant decrease in food intake throughout the experiment period compared to the control group. Animals treated

with Jojoba oil at both the low and high concentrations also showed a gradual decrease in food intake. The combined treatment of AF and Jojoba oil or Nigella sativa induced a significant improvement in food intake, although the food consumption was still lower than in the control group. It is of interest to mention that the improvement in food intake was pronounced in the group fed AF-contaminated diet and treated with Jojoba oil. These results were in accordance with those reported previously (**Mosaad et al, 2016**) who said that animals treated with Jojoba oil at both the low and high doses and Nigella sativa seeds did not show a decrease in body weight and food intake which may be due to the low levels of simmondsin the ethanol extract (**Ogawa et al., 1997 and Holser and Abbott, 1999**). The slight decrease in food intake and body weight gain may be due to the presence of simmondsin residue which was reported to induce food restriction and growth retardation (**Mohamed et al 2015 and Labib et al., 2012**)

The decrease in body weight gain and food intake reported in this study in the group fed FB_1 -contaminated diet indicated the presence of adverse effects and toxicity in rats caused by ingestion of FB_1. This decrease may indicate protein catabolism, thereby contributing to the observed kidney injury (**Abdel-Wahhab et al., 2004 and El-Nekeety et al., 2007**). Similar decrease in body weight gain and food intake had been reported in rats, ducks (**Ayoub et al, 2011 and El-Nekeety et al., 2007**).

The effect of different treatments on body weight gain of rats is depicted in Table (1). Animals fed FB_1 -contaminated diet failed to gain weight; however, animals treated with Jojoba seed extract showed slight weight gain, although there was a significant difference between these groups and the control. Moreover, animals in the groups treated with the FB_1 and jojoba seed extract did not show any significant increase in body weight, and they were below the normal weight of the control group. Animals receiving combined treatment of FB_1 and Jojoba seed extract showed slightly higher weight gain than those receiving FB_1 alone.

Rats feed on ration mixed with jojoba oil at both the low and high concentrations did not show an acute decrease in body weight and food intake which may be due to the low levels of simmondsin (**Ogawa et al., 1997 and Holser and Abbott, 1999**). The slight decrease in food intake and body weight gain in these groups may be due to the presence of simmondsin residue which was reported to induce food restriction and growth retardation (**Labib et al, 2012; Motawe, 2005 and Abu El-Makarem 2004**). Treatment with Jojoba oil to rats fed AF - contaminated diet improved food consumption and body weight gain which may be due to the withdrawal of the effect of simmondsin (**Cokelaere et al, 2000**). Similar growth retardation was observed in male rats fed defatted Jojoba meal which,

therefore, concluded that the growth retardation seen with defatted Jojoba meal was due to its simmondsin activity through its role in food intake reduction (**Kampf et al, 1986**).

The effect of different treatments on MDA level, reduced glutathione (GSH), and TAC in liver tissue is recorded in Table (2) and revealed that animal fed AF - contaminated diet had a significant increase in MDA accompanied by a significant decrease in GSH. Treatment with Jojoba oil at both concentrations and Nigella sativa seeds did not affect MDA significantly; however, it resulted in a significant increase in GSH level. The combined treatment of FA with Jojoba oil resulted in a significant improvement in the activity of antioxidant enzymes and decreased lipid peroxidation in the liver tissues although they were still significantly different from the control. Treatment with Jojoba oil at large concentration showed the best results of improving antioxidant enzymes activity and at decreasing lipid peroxidation. **Karabela et al , (2011); El-Nekeety et al , (2007) and Abdel-Wahhab et al (2004)** reported that the increase of NO and MDA and the decreased level of SOD and TAC in rats fed with FB_1 suggested that FB_1 administration enhanced the generation of free radicals which directly led to free radical-mediated toxicity. The generation of free radicals is one of the main manifestations of oxidative damage and has been found to play an important role in the toxicity and carcinogenesis induced by many carcinogens (**da Rocha et al, 2014 and Xing et al., 2013**). In this respect, **Hassan et al 2010** reported that liver damage was directly related to free radical mediated toxicity which was known to attack the highly unsaturated fatty acids of the cell membrane and considered a key process in many pathological events induced by oxidative stress. Another mechanism of FB_1-induced injury was suggested by **Pinelli et al.,1999** who stated that FB_1-induced a down-regulation of cytoplasmic phospholipase A2 activity and arachidonic acid metabolism by a mechanism involving prostaglandin production, cyclic adenosine monophosphate synthesis, and protein kinase activation, as well as global DNA hypomethylation and histone demethylation that causes chromatin instability and may lead to liver tumourigenesis (**Voss et al 1996**)

The biochemical results (Table 3) revealed that AF alone induced a significant increase in all biochemical parameters tested. The Jojoba oil alone at both low and high concentrations induced significant changes in ALT, AST, and ALP showed a significant decreased. Animals fed AF-contaminated diet and treated with Jojoba oil and Nigella sativa seeds showed a significant improvement in all biochemical parameters. The observed improvement in all biochemical parameters was more pronounced in the group fed AF and treated with Jojoba oil at low concentration. Our results agreed with that of **Mosaad et al., (2016)** and this study also revealed that treatment with FB_1 resulted in a significant increase in serum CEA, TNF-α, IL-1α, and NO suggesting that FB_1 can induce hepatotoxicity in rats.

Similar results indicated that TNF-α, IL-1α, and NO were produced by macrophages, and they played a vital role in tumor condition (**Choi et al., 2011**). Moreover, TNF-α is an essential factor in tumor promotion(**Abdel-Wahhab et al., 2010**) and is a key factor that regulates the production of other cytokines involved in chronic inflammation and tumor development via the nuclear factor kappa B pathway. (**Suganuma et al., 2000**)

As shown in Table (3) the changes in the serum creatinine concentrations of rats were not significantly different among the treatments, while the mean urea nitrogen concentration of rats on the control diet were lower than the serum urea nitrogen of those fed Jojoba oil or black seed throughout the experimental feeding period except group feed on diet contaminated with AF. The obtained results were in agreement with those of **Gbore and Egbunike, (2009).** Urea is formed in the liver and represents the principal end product of protein catabolism. while creatinine is a metabolic byproduct of muscle metabolism. They are filtered from the blood and excreted in the urine by the kidneys. **Coles**, (1986) reported that there was an increase in the serum creatinine and urea nitrogen when there is chronic nephritis (kidney damage). Results revealed that diets containing ≥5.0 mg FB1/kg significantly increased the serum urea nitrogen compared to those fed the control determination has a reputation of being a more specific test for the diagnosis and prognosis of progressive renal disease than the serum urea nitrogen, as there are fewer non-renal factors that may influence creatinine.

The changes in serum proteins of rats fed dietary AF are shown in Table 4 . The decrease in total serum proteins in Group4 might be contributed to the binding of aflatoxin to DNA. Therefore, aflatoxin hinder transcription and translation in return decrease the protein synthesis**,** as the exo-epoxide product of aflatoxin metabolism reacted with N7-guanine in DNA and contributed to protein adduct formation of aflatoxin (**Cavin et al., 2008**). In regard to group5, 6 and 8 all tested parameters were nearly reverted around normal values this finding might be due to the antioxidant effect of Nigella sativa seeds. These observations are agreed with that of (**Ayoub et al., 2011 and Mosaad et al., 2016**) who used Jojoba oil and Nigella cake protein as feed supplement. Nigella sativa crushed seeds (**Le et al 2004**) and thymoquinone (**Badary and Gamal El-Din 2001**) were significantly decreased serum triacylglycerol level. This result might be due to the antioxidant effect of Nigella sativa against aflatoxin. At the same table (4) the globulin component showed drop in α1, β2, and γ1 and in all the exposure animals with AF, while increase α1, β1 and γ 2globulin as compared with control animals. The results coincided with the tune of total proteins and albumin. This may be attributed to that AF causes hepatotoxicity, nephrosis, hemorrhages (liver and kidneys) **(Ayoub et al., 2011).**

Feeding of rats on diet mixed with Jojoba oil at both concentrations or black seed plus AF find out whether Jojoba oil and Black seeds protect AF-induced DNA damage (Fig.1). DNA fragmentation was examined by agarose gel electrophoresis as demonstrated in Figure (1). Jojoba oil and Nigella treatment found to be effective, to some extent, in preventing the toxin-induced smear formation suggesting that these substances may possess a protective effect for the prevention of liver cells from AF-induced DNA damage and necrotic death.

The present data illustrated that oxidative stress induced by oxygen-derived species can produce a multiplicity of modifications in DNA including base and sugar lesions, strand breaks, DNA–protein cross-links and base-free sites. If left un-repaired, oxidative DNA damage can lead to detrimental biological consequences in organisms, including cell death, mutations and transformation of cells to malignant cells. In order to find out whether fennel and tiger nut protect AF-induced DNA damage, DNA fragmentation was examined by agarose gel electrophoresis fennel and tiger nut treatment found to be effective (to some extant) to prevent the toxin-induced smear formation suggesting that these substances may possess a protective power for the prevention of liver cells from FA-induced DNA damage and necrotic death. At the same time these substances did not induce any fragmentation in DNA when administrated alone.

The same results were obtained by the studies on broiler chicks, Japanese quail and ducks (**Han et al 2008, Toulah, 2007 and Madheswaran et a.l , 2004**) who recorded that the decrease in total serum proteins might be contributed to the binding of aflatoxin to DNA. Therefore, aflatoxin hinder transcription and translation in return decrease the protein synthesis**,** as the exo-epoxide product of aflatoxin metabolism reacted with N7-guanine in DNA and contributed to protein adduct formation of aflatoxin (**Cavin et al. ,2008**).

The antioxidant activity of jojoba oil glucoside was reported by **Mehta et al, (2009). Abdel-Wahhab et al, (2007)** concluded that glucoside decreased DNA damage and hepatocarcinogenesis induced by aflatoxin B_1 by activating the phase II enzymes GSH S-transferase and GSH peroxidase. These results suggest that glucoside is capable of counteracting FB_1 toxicity by suppressing cytochrome P450 mediated bioactivation of FB_1. Jojobenoic acid in jojoba seed extract also has antioxidant activity and has the ability to bind metal ions, representing an additional mechanism underlying their pharmacological effects (**Bouali et al, 2008**). Moreover, **Vermauti et al., (1997**) reported that Jojoba is rich in saponins which were well known to stimulate the cell-mediated immune system, as well as to enhance antibody production (**Oda et al., 2000**). The higher total phenolic content in the extract reported in this study suggested another mechanism for its antioxidant activity. In this respect, **Zheng and Wang (2001)** reported that active polyphenol components such as flavonoids and phenolic acids possess antioxidant activities. Consequently,

the protective effects of Jojoba seed extract against FB_1 -induced biochemical and histological changes in the liver reported herein may be due to the direct mechanism as free radical scavenger, and the indirect mechanism by which the extract may induce its protective effect through the enhancement of the synthesis of antioxidant enzymes in the live (**Yener et al ,2009**)

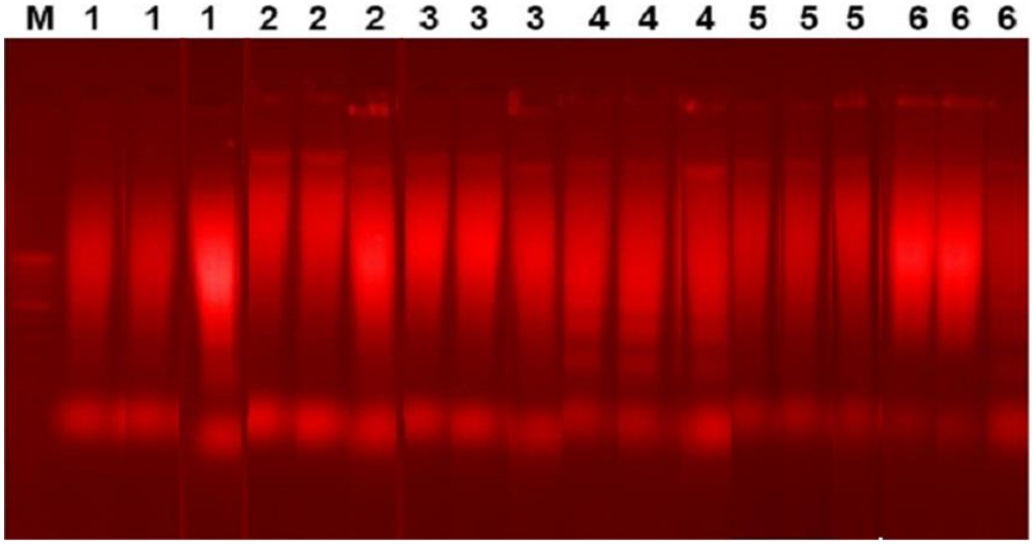

Figure **1**: Agarose gel electrophoresis **of undigested DNA stained by ethydium bromide. The DNA was extracted from liver samples of :(1) control, (2) AF treated, (3) J.O 1.25%, (4) J.O 2.5% (5) J.O 1.25%+ AF and (6) J.O 2.5%%+ AF. (M) 1 kilo base pair marker, 50 bP ladder size.**

Table 1: Feed intake and body weight of control and experimental animals

Groups	Initial body weight/g	Final body weight/g	Body weight gain/ g	Feed intake (g/ period)
Gr1 (ve)	144 ± 2.341	220.8 ± 9.71	72.0 ± 3.077	5000
Gr 2 (J.O 1.25%)	137 ± 3.141	222.2 ± 13.089	91.2 ± 8.03	5000
Gr 3 (J.O 2.5%)	138.2 ± 2.13	232.2 ± 7.28	94.0 ± 5.403***	4.13
Gr 4 (Af)	139 ± 2.91	200.0 ± 5.85	68.8 ± 2.31	3.09
Gr 5 (J.O 1.25%)+AF	135.4 ± 0.6**	208.6 ± 5.99	73.2 ± 6.19	5000
Gr 6 (J.O 2.5%)+AF	138.8 ± 1.39	211.6 ± 3.38	74.8 ± 3.673	4.51
Gr7 (N.S 2.5%)	145 ± 1.87	215.2 ± 3.92	73.0 ± 6.88	3.85
Gr8 (N.S 2.5%)+AF	131.8 ± 3.33*	219.2 ± 9.74	87.4 ± 6.85	3.91

Values represents the mean ± S.E. * P < 0.0 ** P < 0. 01 ***P < 0.001

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Table 2: Mean values (±SE) of liver malondialdehyde and GHS values in rats fed J.O , N.S and AF (n=5)

Groups	Malondialdehyde (mM/100g)	GHS (µmol/mg protein)
Gr1 (ve)	6.78 ± 0.25	16.01 ± 1.28
Gr 2(J.O 1.25%)	6.38 ± 0.26	10.1 ± 0.64**
Gr 3(J.O 2.5%)	5.6 ± 0.152**	12.1 ± 0.597*
Gr 4 (Af)	8.08 ± 0.3*	8.08 ± 0.36***
Gr 5 (J.O 1.25%)+AF	6.78 ± 0.14	7.03 ± 0.34***
Gr 6 (J.O 2.5%)+AF	6.15 ± 0.18	6.13 ± 0.09***
Gr7 (N.S 2.5%)	5.72 ± 0.24*	6.03 ± 0.7 ***
Gr8 (N.S 2.5%)+AF	5.2 ± 0.36**	6.02 ± 0.07***

Values represents the mean ± S.E. * P < 0.0 ** P < 0. 01 ***P < 0.001

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Table(3): Effect of J.O , N.S and AF on some serum biochemical parameters of male rats.

Groups	AST (u/l)	ALT (u/l)	AP (u/l)	Urea (%)	Creatinine (mg/dl)
Gr1 (ve)	22.6 ± 0.75	10.2 ± 1.56	342.4 ±2.87	34.0± 0.55	0.60±0.02
Gr2(J.O 1.25%)	32.4±1.121***	12.72±1.11	339.2±6.0	30.0± 1.64	0.53±0.03
Gr 3(J.O 2.5%)	37.6± 0.98***	19.0± 0.95**	290.2± 14.3**	27.6± 0.98***	0.58± 0.01
Gr 4 (Af)	31.2± 1.02***	14.2± 0.86	333.6± 7.76	40.6± 1.03***	0.67± 0.00014*
Gr5 (J.O 1.25%)+AF	18.4± 2.37	7.2± 0.37	201.4± 2.58***	26.6± 1.25***	0.59± 0.015
Gr6 (J.O 2.5%)+AF	15.2± 1.11***	12.6± 0.51	245.0± 4.11***	29.2± 0.58***	0.624± 0.03
Gr7 (N.S 2.5%)	17.2± 1.46	9.2± 1.07	285.2± 11.05**	30.0± 0.71**	0.62± 0.017
Gr8 (N.S 2.5%)+AF	26.0± 2.47	12.2±1.02	255.0± 11.6***	28.6± 0.81**	0.536± 0.0006**

Values represents the mean ± S.E. * P < 0.0 ** P < 0. 01 ***P < 0.001

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Table(4): Effect of J.O , N.S and AF on serum total protein and electrophoresis pattern of male rats

Groups	T.P	T. Alb	α_1	α_2	Tα	β_1	β_2	T β	γ_1	γ_2	T γ	T. Glo	A/G ratio
Gr1 (ve)	6.784± 0.11	2.082± 0.08	0.616± 0.103	0.326± 0.001	0.942± 0.001	1.216± 0.03	0.612± 0.024	1.828± 0.023	1.324± 0.03	0.616± 0.014	1.886± 0.08	4.656± 0.09	0.450± 0.02
Gr2(J.O 1.25%)	6.76± 0.33	1.854± 0.12	0.952± 0.04*	0.378± 0.001***	1.33± 0.05***	1.20± 0.09	0.590± 0.05	1.788± 0.14	1.19± 0.07	0.620± 0.08	1.81± 0.81	4.920± 0.23	0.374± 0.012**
Gr 3(J.O 2.5%)	6.35± 0.1*	2.02± 0.04	0.58± 0.02	0.290± 0.02	0.85± 0.03*	1.146± 0.06	0.540± 0.04***	1.686± 0.085	1.142± 0.03**	0.60± 0.03	1.728± 0.05	4.264± 0.09*	0.475± 0.0005
Gr 4 (Af)	5.732± 0.11***	1.928± 0.07	0.426± 0.04	0.238± 0.001***	0.664± 0.05***	1.082± 0.04	0.488± 0.04*	1.57± 0.07**	1.10± 0.08**	0.476± 0.03**	1.576± 0.07*	3.792± 0.13***	0.514± 0.03
Gr5 (J.O 1.25%)+AF	6.22± 0.14*	2.04± 0.1	0.288± 0.01*	0.238± 0.01***	0.526± 0.03***	1.66± 0.09**	0.788± 0.05*	2.448± 0.11***	0.632± 0.12***	0.536± 0.06	1.068± 0.13***	4.104± 0.09**	0.50± 0.03
Gr 6 (J.O 2.5%)+AF	6.086± 0.1**	2.04± 0.03	0.318± 0.02*	0.174± 0.01***	0.506± 0.03***	1.656± 0.15*	0.608± 0.16	2.244± 0.08***	0.670± 0.03***	0.620± 0.04	1.290± 0.04***	4.05± 0.08***	0.502± 0.001*
Gr7 (N.S 2.5%)	6.082± 0.09**	1.948± 0.04	0.518± 0.015	0.382± 0.02	0.90± 0.03	1.424± 0.07	0.682± 0.05	2.098± 0.1***	0.616± 0.03***	0.526± 0.03**	1.136± 0.01***	4.134± 0.08**	0.472± 0.01
Gr8 (N.S 2.5%)+AF	5.88± 0.05***	1.712± 0.06**	0.656± 0.03	1.142± 0.07***	1.798± 0.08***	0.402± 0.001***	0.704± 0.04	1.106± 0.04***	0.720± 0.04***	0.542± 0.02**	1.262± 0.04***	4.168± 0.04**	0.412± 0.02

Values represents the mean ± S.E. * P < 0.05 ** P < 0. 01 ***P < 0.001

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