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Ameliorative Effects of Ethanolic Neem Extract on Dietary Aflatoxin Induced Hematological Damage and Hepatotoxicity in Mice

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Abstract: The aqueous ethanolic extract of *Azadirachta indicia* bark was evaluated for its ability to ameliorate aflatoxin induced damage to blood cells and hepatotoxicity in albino mice. Six groups (I-VI) of mice were used in this study. Mice in group I were exposed to aflatoxin-free feed only while those in group II were fed with aflatoxin contaminated feed only for 3 weeks. Groups III-V were exposed to aflatoxin contaminated feed for three weeks each and then treated interperitonially with 0.25 mL of 50, 100 and 150 mg/kgbwt aqueous ethanolic extract of *A. indica*, respectively, at one dose daily for 7 days. Group VI was fed aflatoxin-free feed for three weeks and then administered with 150 mg/kgbwt *A. indica* extract to ascertain the effect of this concentration of the extract on the mice. Aflatoxin was immunosuppressive, leading to a reduction in PCV, WBC, neutrophile and lymphocyte counts in group II mice as compared to other groups. Mice in group II had the highest significantly different ($p<0.05$) mean lesion score as compared to the control group. However with an increase in extract concentration a strong capacity to boost hematological parameters was recorded for the extract at the expense of compromising hepatocytes integrity. This study showed that low concentration of aqueous ethanolic extract of *A. indica* bark is safe and therapeutic for ameliorating hematological damage and hepatotoxicity induced by dietary aflatoxin.

Key words: Aflatoxin, *Azadirachta indica*, blood, hepatotoxicity, neem

INTRODUCTION

Aflatoxins especially aflatoxin B_1 (AFB_1) have been reported to induce carcinogenicity, immunosuppresion, mutagenicity, teratogenicity and genotoxicity in mammals (Abdel-Wahhab *et al*., 2002; Choi *et al*., 2010; Madrigal-Santillan *et al*., 2006). Aflatoxin mediated toxicity has been related to its pro-oxidant potential due to the generation of Reactive Oxygen Species (ROS) during the metabolic processing by liver enzymes. When this happens, oxidative stress is created by increasing lipid peroxidation and decreasing enzymatic and non-enzymatic antioxidants (Choi *et al*., 2010; Choudhary and Verma, 2005). In an attempt to counteract the adverse effects of aflatoxin-induced hepatotoxicity, many traditional plants have been tested due to their naturally occurring antioxidants (Vinitketkumnenu *et al*., 1999; Choudhary and Verma, 2005; Verschaeve *et al*., 2004; de Boer *et al*., 2005; Jodynis-Liebert *et al*., 2006; Preetha *et al*., 2006; Naaz *et al*., 2007; Bluma *et al*., 2008; Abdel-Wahhab *et al*., 2010; Velazhahan *et al*., 2010). However, the search continues since phytotherapy is revolutionalizing traditional medicine.

Neem (*Azadirachta indica* Juss.), prevalent in Asia, Africa and other tropical parts of the world is an ever green tree known for its potent pesticide and medicinal properties, probably due to the presence of diterpenoids, limonoids, C-secomeliacins, C-Seco-limonoids, phenols in the bark, flavonoids, flavonolglycosides, dihyrocholcones and tannins in the leaves, fruits, flowers and bark (Bandyopadhyay *et al*., 2002; Dasgupta *et al*., 2004; Winkler *et al*., 2007). It has been reported to have specific anti-inflammatory, antipyretic and hypoglycemic activity, and also exhibits antimicrobial and anticancerous properties. Other remedial properties include reduction of paracetamol-induced liver damage, enhancement of hepatic glutathione and glutathione-dependent enzyme, and *in vitro* antiviral activity (Panda and Kar, 2000; Parida *et al*., 2002; Dasgupta *et al*., 2004; Farah *et al*., 2006; Senthil-Nathan *et al*., 2009). It is also on record that

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neem bark aqueous extract has potent anti-secretory, anti-ulcer and immunomodulatory activity in experimental animal models (Bandyopadhyay *et al*., 2002; Bandyopadhyay *et al*., 2004; Chaube *et al*., 2006). Consequently, the bitter extract obtained from parts of this tree is used as tonic, astringent and useful in relieving fever, thirst, nausea, vomiting and skin diseases among the indigenous African and Asian people. The chemopreventive potential of dietary neem flowers has also been demonstrated in inhibiting AFB_1 and 9, 10 dimethyl-1,2-benzanthracene (DMBA) induced liver and mammary gland carcinogenesis in rats (Farah *et al*., 2006). However, there are very limited reports on the ameliorative effects of ethanolic extracts of neem bark on aflatoxin induced hepatotoxicity and hematological damage in experimental mammals. Therefore this study aimed at evaluating the ameliorative effect of *A. indica* bark against aflatoxin-induced hepatotoxicity. The effect of the extract was also tested on the hematological parameters of experimental mice.

MATERIALS AND METHODS

Plant material: Neem (*Azadirachta indica*) bark obtained from Babcock University in June 2009 was rinsed with distilled water and then air dried. The dried samples were ground, extracted in 80% aqueous ethanol for 72 h and concentrated at 40°C in a rotary evaporator. The dried extract was then stored at 4°C throughout the period of the study.

Determination of Total Phenolic Content (TPC) in crude neem extract: The total phenolic content was estimated by the Folin-Ciocalteu colorimetric method according to Singleton and Rossi (1965). Fifty (50 mg crude extract was mixed with Folin-Ciocalteu reagent (0.5 mL) and deionized water (7.5 mL). The mixture was kept at room temperature for 5 min, and then 10 mL of 7% sodium carbonate was added and incubated for 90 min at room temperature. After incubation, the absorbance against the reagent blank was determined at 760 nm. The total phenolic content of the bark's extract was expressed as Gallic acid equivalent (mg/g dry weight). The sample was analyzed in triplicates.

Determination of Total Flavonoid Content (TFC) in ethanolic extract of neem: The TFC was measured using spectrophotometer according to the method of Dewanto *et al*. (2002). One mL of the extract (containing 100 µg/mL) was diluted with 4 mL water in a 10 mL volumetric flask. Initially, 5% $NaNO_2$ solution (0.3 mL) was added to the volumetric flask. At 5 min, 10% $AlCl_3$ (0.3 mL) was added and at 6 min 1.0 M NaOH (2 mL) was added. Water (2.4 mL) was then added to the reaction flask and mixed well. Absorbance of the reaction mixture was read at 510 nm. TFC was determined as quercetin equivalent (mg/g of dry weight). Three readings were taken and the results averaged.

Determination of 2, 2-diphenyl- 2- picrylhydrazyl (DPPH) radical scavenging activity: The DPPH radical scavenging activity was performed according to the assay system of Mensor *et al*. (2001). One mL of a 0.3 mM DPPH methanol solution was added to a 2.5 mL solution of the extract and allowed to react at room temperature for 30 min. The absorbance (Abs) of the resulting mixture was measured at 518 nm and converted to percentage antioxidant activity (AA%), using the formula:

$$AA\% = [(Abs_{control} - Abs_{sample})/Abs_{control} \times 100]$$

Determination of inhibition of lipid peroxidation: A modified Thiobarbituric Acid Reactive Substance (TBARS) assay was used to measure the lipid peroxide formed (Ruberto *et al*., 2000). Egg yolk homogenate was used as the lipid-rich medium. Egg homogenate (0.5 mL, 10 % v/v) and 0.1 mL of the extract were mixed in a test tube and made up to 1mL with distilled water. Then 0.05 mL of $FeSO_4$ (0.07 M) was added to induce lipid peroxidation and incubated for 30 min. This was followed by the addition of 1.5 mL of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 mL of 0.8% (w/v) TBA in 1.1% sodium deodecyl sulphate and 20% TCA. The resulting mixtures were vortexed and heated at 95°C for 60 min. After cooling, 5.0 mL of butan-1-ol was added to the tube and then centrifuged at 3000 rpm for 10 min. The absorbance (Abs) of the organic layer was measured at 532 nm. The percentage inhibition of lipid peroxide formation by the extract was calculated by the formula:

$$\%Inhibition = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$$

Feed formulation: The rat chow used for this study was formulated according to Fapohunda *et al*. (2009). The source of the aflatoxin ingested by the mice was from aflatoxin-contaminated maize and groundnut cake purchased from a feed mill at Ijebu-ode, Ogun state, Nigeria. The complete ration had a contamination level of 78 parts per billion (ppb) when tested using the Enzyme-linked Immunosorbent Assay (ELISA) AgraQuant Total aflatoxin assay 4/40 kit (Romer Labs®, Singapore).

Experimental design: Swiss albino mice were purchased from the Department of Zoology, University of Ibadan, Nigeria. The mice were acclimatized in the animal house of the Department of Biosciences and Biotechnology, Babcock University, Nigeria, for 2 weeks with uninterrupted food and water supply. Six experimental groups (5 mice per group) were utilized for this study: Group I was exposed to feed free of aflatoxin (negative control); Group II were fed with aflatoxin contaminated feed alone for three weeks (positive control); Group III-V

Table 1: Hematological parameters in mice treated with ethanolic neem bark extract after 3 weeks exposure to dietary aflatoxin.

parameters (mean ± SE)	Group I	Group II	Group III	Group IV	Group V	Group VI
PCV (%)	43±4*	27±0.7	30±0.3*	34±2.3*	40±0.5*	38±1.6*
WBC (mm^3)	2086±37	1728±76	2352±123*	2232±168*	2416±216*	2157±80*
Neutrophils (%)	74±2.3	69±6.6	76±8.1	78±6.0	82±2.3	84±2.2
Lymphocytes (%)	21±0.7	16±3.5	32±1.9*	2.8±0.7*	27±1*	34±0.9*

*: $P<0.05$ represents significant differences compared to aflatoxin fed mice (Group II)

were fed with aflatoxin contaminated feed for three weeks each and then treated intraperitonially for 7 days with 0.25 mL of 50, 100, 150 mg/kgbwt aqueous ethanolic extract of *A. indica*, respectively. Group VI was fed with aflatoxin-free feed for three weeks and then administered with 150 mg/kgbwt *A. indica* extract alone to ascertain the effect of this concentration of the extract on the mice.

Hematological analysis: At the completion of the exposure duration, blood samples were obtained from the jugular vein of the mice into anticoagulant bottle. The blood samples were analyzed for hematological parameters (PCV and differential WBC count) according to the procedure reported by Oyewole *et al*. (2009).

Histological study: Liver was excised from the experimental mice after the exposure duration and washed in cold saline for histopathological examination. All specimens were fixed in 10% formal saline, routinely embedded in paraffin, cut into 6 μm thick sections and stained with hematoxylin and eosin (H&E) (Drury *et al*., 1967). The lesions were scored according to a modification of the Brunt system of scoring liver anormalies (Merat *et al*., 2010). A non-alcoholic steatohepatitis activity index was defined which scored the grade of disease activity between 0 and 12 according to four histologic features: steatosis, hepatocyte balloning, inflammation and fibrosis.

This study was carried out at Babcock University between January and September 2010.

Statistical analysis: Data were expressed as mean ± standard error (SE) after analysis using SPSS version 15.0. One way ANOVA test was performed to analyze the difference between different groups and Duncan's multiple range test (DMRT) was used for mean separation. Pearson Correlation analysis was also utilized to determine the relationship between hematological parameters and histological studies.

RESULTS

Polyphenolic content and antioxidant activity of neem extract: The TPC, TFC and antioxidant activity of aqueous ethanol extract of *A. indica* stem bark are shown in Fig. 1. The aqueous ethanolic extract yielded 85.7 mg GAE/g of phenol and 97.4 mg QE/g of flavonoid. The result showed that 2,2-diphenyl- 2- picrylhydrazyl (DPPH) free radical scavenging activity of the extract was 84% while inhibition of lipid peroxidation was 43%.

Table 2: Lesion scoring of the liver of mice treated with ethanolic extract of neem bark after 3 weeks exposure to dietary aflatoxin

Parameters	Steatuses	Ballowning inflammation	Lobular	Fibrosis	Mean±S.E.
Group I	3	1	3	0	0.58±0.15
Group II	7	4	6	0	1.42±0.31*
Group III	3	1	4	0	0.67±0.22
Group IV	4	2	4	0	0.83±0.21
Group V	5	4	5	0	1.17±0.27
Group VI	6	4	4	4	1.50±0.15*

*: $p<0.05$ represents significant differences compared to group I (Negative control)

Hematological studies:

PCV: The exposure of mice to aflatoxin contaminated feed alone induced a significant ($p<0.05$) reduction in PCV as compared to the negative control (Table 1). The administration of different concentrations of the extract to the experimental mice induced significant ($p<0.05$) concentration-dependent increase in PCV in all treated groups except group III when compared to the positive control group (II). The administration of 150 mg/kgbwt of the extract alone (Group VI) did not show any significant difference in PCV ($p<0.05$) compared with the negative control.

WBC/differentials: The results of the WBC and differential counts (Table 1) showed that dietary-aflatoxin (Group II) reduced the WBC count compared to the negative control (Group I). However, there was significant ($p<0.05$) increase in WBC in the groups treated with neem extract as compared to the positive control (Group II). A similar trend was observed for neutrophils, with Groups III, IV and V having increased neutrophil levels of 76, 78 and 82%, respectively as compared to the positive control (69%). However, there was no significant difference for all the groups. The administration of the extract to aflatoxin fed mice boosted the lymphocyte levels significantly ($p<0.05$) as compared to the negative and positive controls.

Lesion scoring of the liver: The results in Table 2 showed that dietary aflatoxin (Group II) and the neem extract alone (Group VI) induced significant ($P<0.05$) increase in mean lesion than the negative control (Group I). Although there was numerical reduction in

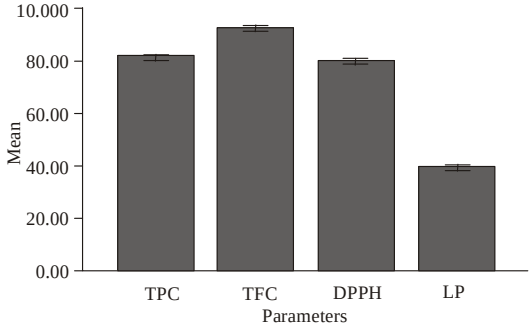

Fig. 1: Total phenolic content (mg GAE/g), Total flavonoid content (mg QE/g), DPPH free radical scavenging activity (% DPPH) and lipid peroxidation (% LP) inhibition of aqueous ethanolic extract of *A. indica* bark.

mean lesion in the neem extract treated groups (Groups III-V), the result was not statistically significant when compared with the positive control (Group II).

The commonest lesions noticed in this study were disrupted histoarchitechture, dilated sinusoids, congested central vein, lobular inflammations, necrosis and macro- and microsteatosis, mild fibrosis and mild regeneration of hepatocytes (Fig. 2b-h). It was observed that the independent administration of aflatoxin and neem extract alone induced severe hepatic anormalies histologically while the administration of 0.25 mL of 50 mg/kgbwt ethanolic neem extract daily for 7 days significantly ($p<0.05$) reversed the damages mostly by the formation of binucleated hepatocytes in the periportal zone (zone 3).

DISCUSSION

Antioxidants are known to protect against Reactive Oxygen Species (ROS) toxicity by the prevention of ROS formation, interruption of ROS attack, scavenging the reactive metabolites and converting them to less reactive molecules. They also act by enhancing the resistance of sensitive biological targets to ROS attack, facilitating the repair caused by ROS and providing co-factors for the effective functioning of other antioxidants (Choi *et al*., 2010). In this study, the flavonoid content was more in the extract than phenols while inhibition of DPPH free radical was significantly ($p<0.05$) different from the inhibition of lipid peroxidation. The high DPPH free radical scavenging activity of the extract suggests that the antioxidants in the extract contributed to the therapeutic effect on the test mice. However, the antioxidant activity of the ethanolic neem extract could have been through any of the mechanisms mentioned above.

The low percentage PCV of the group fed with aflatoxin alone (Group II) may have been due to aflatoxin induced hemolysis arising from lipid peroxidation of the plasma membrane (Verma *et al*., 2001). In contrast, the concentration-dependent increases in PCV in the extract treated groups (III-V) confirmed the capacity of

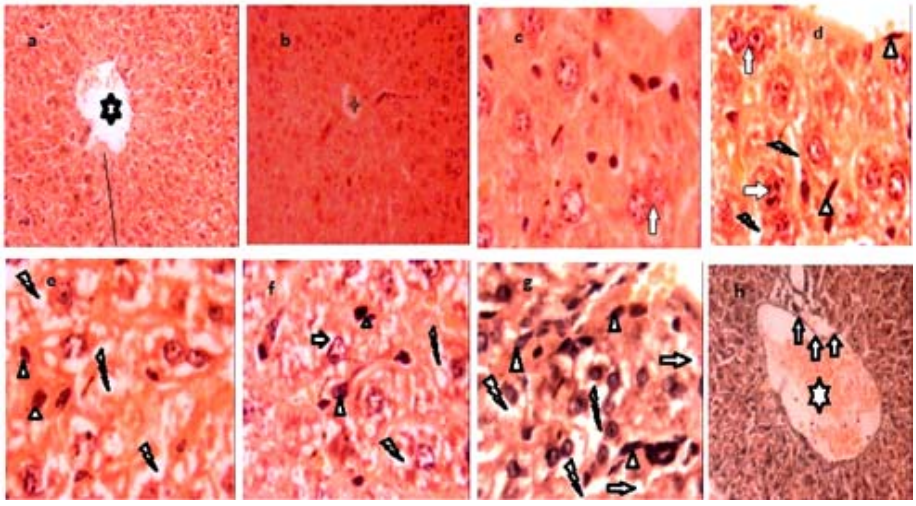

Fig. 2: Photo micrographs in a liver section of (a) control mice showing normal hepatocyte architecture radiating{ line} from the central vein {six point star}(H&E,X 100), (b&c) group III mice showing constricted central vein with surrounding radiating hepatocytes {four point star} and bilobed nuclei {up arrow} at zone 3 of the porta acinus (b: H&E, X100; c: H&E, X400), (d) group IV mice showing increased binucleate hepatocytes {right & up arrow} at zone 3 of the porta acinus, some pyknotic cells {triangles} and steatosis {lightening} (H&E, X400), (e) group V mice showing distorted histo-architecture, increased pyknotic cells {tringles} and macro- and microsteatosis {lightning} and coagulative necrosis (H&E, X400), (f) group II mice showing disrupted histo-architechture, diffused micro- and macrosteatosis {lightning}, coagulative necrosis {right arrow}, lobular inflammations and pyknotic cells {triangles}(H&E, X400), (g & h) group VI mice showing diffused pyknotic cells {triangles}, coagulative necrosis {right arrow}, fibrosis {up arrow}, distorted histo-architechture, dilated and congested central vein {six point arrow} (g: H&E, X400; h: H&E, X100).

antioxidants in the extract to convert aflatoxin B_1 and G_1 to their inactive forms (B_2 and G_2) and scavenge reactive oxy-radicals formed during aflatoxin attack (Verma *et al*., 2001).

The low WBC of the mice exposed to only dietary aflatoxin suggests that aflatoxin acted as an immunosuppressant in this study. This corroborates the findings of Abdel-Wahhab *et al*. (2002) who reported low levels of hematological parameters in animals fed with aflatoxin contaminated feed alone. In contrast, our findings contradict the report of Fapohunda *et al*. (2009), who stated that the presence of aflatoxin increased the WBC of mice. In literature, different doses of aflatoxins can either stimulate or suppress the immune system (Mogilnaya *et al*., 2010). It was however noted that the extract treated groups had increased WBC; an indication of the capacity of the extract to act as an immune booster.

The induction of severe hepatic damage observed in groups II (mice fed with 78 ppb total dietary aflatoxin alone for 3 weeks) and VI (mice administered 150 mg/kgbwt extract alone for 7 days) may have been due to oxidative stress caused by the continuous detoxification of aflatoxin and *A. indica* extract in the liver. It is on record that aflatoxin is a hepatotoxin via this mechanism (Gong *et al*., 2002; Fapohunda *et al*., 2007). On the other hand, the presence of azadirachtin, a bioactive compound found in neem (Khan and Awasthy, 2003; Dasgupta *et al*., 2004; Senthil-Nathan *et al*., 2005; Senthil-Nathan *et al*., 2007; Srivastava and Raizada, 2007) may have been responsible for the effect on group VI. By structural analysis, azadirachtin is known to possess a biophore (-O-CH =) in its furan moiety which is responsible for its toxic potentials (Khan and Awasthy, 2003). Aflatoxins also have this biophore in their structure; a supposed cause of same kind of hepatic damage in group II. This result is converse to that of PCV and WBC. As the independent administration of toxin (Group II) and 150 mg/kgbwt extract alone (Group VI) induced severe hepatic necrosis, group VI increased PCV and WBC while group II decreased these parameters.

The treatment of aflatoxin-induced damage with 50 mg/kgbwt (Group III) extract improved the alignment of hepatocytes compared to 100 mg/kgbwt and 150 mg/kgbwt extract concentration. Our deduction from the observations in this study is that the least extract concentration administered to group III mice may have bound to the aflatoxin such that both substances could not exert their adverse effect on the cells. However as the concentration of the extract increased, the liver produced more free radicals which led to the exertion of damage on the hepatocytes; though it improved the PCV and WBC of the groups (III-VI). Our explanation above was further authenticated by the very strong positive correlation (r = 0.986) observed between PCV and lesion scoring of groups III-V mice. A weak positive correlation (r = 0.59) was also observed between WBC and lesion scoring of the liver for same groups. This suggests that the PCV and liver lesions increased with an increase in the concentration of the extract, thus indicating more damage induction in the liver. Similar trend can be associated with the WBC of these test groups. Therefore, treatment with 50 mg/kgbwt of the extract indicated the most effective therapeutic dose in this study.

The regeneration of hepatocytes observed in the liver obtained from group III and group IV mice was evidenced by the formation and presence of binucleate hepatocytes resulting from cell division which took place in the periportal zone (zone 3). The regenerative process observed in group III mice is indicative of the potential of aqueous ethanolic neem bark extract to counteract the damage induced by 78 ppb total dietary aflatoxin on hepatocytes. This was similar for group IV mice however; there were levels of architectural distortions observed than in group III. In pathological conditions, dead liver cells are replaced by proliferation of surviving hepatocytes, Kupffer cells, endothelium, bile ducts and vessels, provided the supportive reticular framework is preserved. If however, the reticulum is damaged, healing can be accomplished only by scar formation, "fibrosis", which may produce more damage by inducing rearrangement of the blood circulation, leading to cirrhosis (Chitturi and Farrell, 2001; Charlton *et al*., 2002; Bradbury and Berk, 2004; Farrell and Larter, 2006). Since fibrosis was obviously absent in the liver of group III-V mice, it can be concluded that the extract induced regeneration of hepatocytes by assisting in the preservation of the reticular framework from further damage and enhancement of rapid proliferation of surviving liver cells.

In summary, 78 ppb dietary total aflatoxin decreased PCV and WBC and induced many lesions on hepatocytes of albino mice. However, the increase in tested concentrations of aqueous ethanolic extract of *A. indica* bark boosted the PCV and WBC of the mice but exerted great damage on hepatocytes. The least tested concentration of the extract (50 mg/kgbwt) counteracted the most the hepatotoxic damage induced by aflatoxin. Therefore, therapeutic effects of a concentration of any intended drug or plant extract must be carefully monitored because it may cause serious deleterious effect on vital organs of the body (Thermer *et al*., 2003). This study showed that low concentration of aqueous ethanolic extract of *A. indica* bark is safe and therapeutic for ameliorating hematological damage and hepatotoxicity induced by dietary aflatoxin.

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