

Immunohistopathological and Biochemical Study of the Effects of Dead Nettle (*Urtica Dioica*) Extract on Preventing Liver Lesions Induced by Experimental Aflatoxicosis in Rats

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ABSTRACT

Objective: Aflatoxicosis is a mycotoxicosis infection with an acute or chronic course that forms due to aflatoxins (AFs) in humans and animals. Aflatoxins primarily affect the liver and can lead to histopathological necrosis, fibrosis and hepatocarcinogenesis of the organ. This paper studied the preventive effects of dead nettle leaf (*Urtica dioica* leaf; UDL) extract on liver lesions that were induced by experimental aflatoxicosis in rats.

Methods: A total of 30 rats were separated into three groups of 10 rats each. Experimental group A (control) received normal rat food, experimental group B (AFB1) received 2 mg/kg of AF, and experimental group C (AFB1 + UDL extract) received 2 mg/kg of AF + 2 ml/rat/day of UDL extract. After three months of experimentation, blood and tissue samples were taken from the rats by necropsy to perform chemical and histopathological analyses.

Results: According to the biochemical and histopathological findings, antioxidant system activity increased and lipid peroxidation and liver enzyme levels decreased in the group that received UDL extract.

Conclusion: The extract of UDL had hepatoprotective effects against aflatoxicosis.

Keywords: Aflatoxicosis, extract of dead nettle leaf, liver lesions, rat

Estudio inmunohistopatológico y bioquímico de los efectos del extracto de la ortiga mayor (*Urtica dioica*) en la prevención de lesiones hepáticas inducidas por aflatoxicosis experimental en ratas

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RESUMEN

Objetivo: La aflatoxicosis es una infección por micotoxinas con un curso agudo o crónico producido por aflatoxinas (AF) en seres humanos y animales. Las aflatoxinas afectan principalmente el hígado y pueden conducir a necrosis histopatológica, fibrosis o hepatocarcinogénesis del órgano. En este trabajo se estudiaron los efectos preventivos del extracto de la hoja de ortiga mayor (*Urtica dioica* l; UDL) sobre las lesiones hepáticas inducidas por aflatoxicosis experimental en ratas.

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Métodos: Un total de 30 ratas se separaron en tres grupos de 10 ratas cada una. EL grupo experimental A (control) recibió comida normal de ratas; el grupo experimental B (AFB1) recibió 2 mg/kg de AF; y el grupo experimental C (AFB1 + extracto de UDL) recibió 2 mg/kg de AF + 2 ml/rata/día de extracto de UDL. Después de tres meses de experimentación, se tomaron muestras de sangre y tejidos de las ratas en una necropsia encaminada a realizar análisis químicos e histopatológicos.

Resultados: Según los hallazgos bioquímicos e histopatológicos, la actividad del sistema anti-oxidante aumentó, y la peroxidación del lípido y los niveles de la enzima del hígado disminuyeron en el grupo que recibió el extracto de UDL.

Conclusión: El extracto de UDL tuvo efectos hepatoprotectores contra la aflatoxicosis.

Palabras clave: Aflatoxicosis, extracto de hoja de ortiga mayor, lesiones hepáticas, rata

West Indian Med J 2018; 67 (3): 255

INTRODUCTION

Aflatoxins (AFs) are metabolites that are synthesized by fungi in food. Ingesting these metabolites can cause cancer and acute or chronic infections in humans and animals (1). Many studies have examined this subject due to the role of AFs as one of the strongest carcinogens that can lead to acute and chronic toxications in both humans and animals (1–3). Contamination of various food products with mycotoxins is a major problem. Aflatoxins are especially problematic because they can produce toxic metabolites from the *Aspergillus* fungi genus and the isolated mycotoxins that most commonly affect human and animal health. Among AFs, AFB1 is the most frequently identified mycotoxin that yields the strongest toxic effects (4). The mechanisms of mycotoxins, especially AFs, occur by inhibiting deoxy-ribonucleic acid (DNA), ribonucleic acid (RNA) and protein synthesis (5). Aflatoxins cause disorders such as anorexia, liver damage, immunosuppression, regression in body growth, increases in mortality rate, decreases in egg production, loss of strength against infections, and disorders in protein and carbohydrate metabolism (6).

Detoxification (elimination) of AFs in food is important for both human and animal health. The main method for elimination is through the deactivation of AFs in food. This is achieved by binding food with chemical materials. While in the 1990s, aluminum silicates were widely mixed with food, interest in elimination methods increased after the year 2000, and bacteria (such as bread yeast, lactobacillus and yeast cell components) were tested and achieved positive results. Researchers who used melatonin and various plants also obtained positive results (6–8).

Dead nettle (*Urtica dioica* L)

Dead nettle belongs to the *Urtica dioica* species of the Urticaceae family and has been used for the treatment of diseases in folk medicine. Its roots, seeds and leaves have been commonly used to treat a variety of ailments (9). According to previous studies, the plant produces anti-tumoral, anti-hypertensive, hepatoprotective and immunostimulatory effects (10–14); it also offers analgesic, antiulcer, antioxidant and antimicrobial benefits (15). Using biochemical and histopathological methods, this study aimed to reveal the hepatoprotective effects of dead nettle leaf (*Urtica dioica* leaf; UDL) extract on rats afflicted by experimentally induced aflatoxicosis.

SUBJECTS AND METHODS

Experimental animals and scheme of the experiment

Thirty Sprague Dawley rats were separated into three groups: A, B and C. Group A (control) was fed normal rat food, group B (AFB1) was given food with an AF, and group C (AFB1 + UDL extract) was given food with an AF and an UDL extract mixture (2 ml/rat/day). Groups B and C were the experimental groups, and experimentation was planned to take place over a period of 90 days.

Adding aflatoxin into food

Pellet food was prepared by adding 2 mg/kg dosages of AFB1 (Sigma-Aldrich A6636 from *A. flavus*) into a commercial food that was determined to be AF-free according to laboratory tests. The amount of AF was determined according to information gathered from the existing literature (2). To develop liver cancer in rats, a reported minimum of 100–150 mg/kg/day of AFB1

could be administered with a minimum toxic dose of 30–40 mg/kg/day (2). For this reason, 2 mg of AF added to 1 kg of rat food after estimation of food production amounted to 20 g total for a single rat.

Plant material

Dead nettle (*Urtica dioica* L) has frequently been used for its plant material. Methanol can be extracted from the plant with an electric grinder and an extraction tool (10).

Performed analyses and research

Biochemical analyses

On the 90th day of the study, the rats were anaesthetized with diethyl ether, and their blood was taken by an intracardial route into blood tubes. Erythrocyte packages were extracted from the blood samples, and enzyme activities, such as malondialdehyde (MDA), superoxide dismutase (SOD), alanine transaminase (ALT), aspartic transaminase (AST), glutathione peroxidase (GPx) and glutathione S-transferase (GST), were determined with test kits that were built in accordance with the procedures of the test. Liver tissue was also obtained to determine the levels of MDA, SOD, GST and GPx. After taking the blood from the heart and centrifuging it at 3000 revolutions per minute for 15 minutes at +4°C, serum enzyme levels were established with the blood sera in the test kit (DPC; Diagnostic Products Corporation, CA 90045, USA) and an autoanalyser (BM/HITACHI-911).

Histopathological analyses

After the rats were euthanized on the 90th day of the study, necropsies were performed, and the macroscopic findings were recorded. Collected tissue samples were fixed

in a buffered 10% formalin solution, embedded in paraffin blocks and sectioned in 4 µm thick. The sections were examined microscopically after being stained by Sudan black, haematoxylin-eosin and Masson's trichrome.

Statistical analyses

Biochemical analysis results and average and standard deviation ($\bar{X} \pm SD$) were calculated according to the standard methods of relevant software (Minitab for Windows). Differences between group averages were calculated according to the Mann-Whitney U test, and discrepancies between live weights were calculated according to the Duncan's new multiple range test. Statistical significance was set at $p < 0.05$ for each of the tests.

RESULTS

Biochemical findings

Significant changes were determined in the tissue and erythrocyte of rats during GPx, MDA, GST, SOD, and serum enzyme (ALT, AST) activities after the test (Table 1).

Macroscopic findings

No deaths were observed in the control and experimental groups until the 90th day of the study. A localized, pale colour was observed in the livers of the rats from group B (AFB1). It was the only macroscopic change in organs that was discerned during the study.

Histopathological findings

Group A (control)

Microscopically, normal histopathological views of the liver were observed (Fig. 1).

Table 1: The effects of aflatoxin and variety of additives on biochemical parameters in the rats' blood and liver tissue

Parameters	Control group (mean \pm standard deviation)	Aflatoxin-treated group (mean \pm standard deviation)	AFB1 + UDL extract (mean \pm standard deviation)
Serum aspartic transaminase U/L	161.5 \pm 39.2	248.5 \pm 66.6*	223.0 \pm 45.0
Serum alanine transaminase U/L	43.0 \pm 9.1	115.5 \pm 27.7*	73.2 \pm 17.8*
Erythrocyte glutathione mg/ml	39.5 \pm 0.84	38.8 \pm 1.0	42.5 \pm 0.5
Erythrocyte malondialdehyde nmol/ml	0.39 \pm 0.08	0.69 \pm 0.2*	0.54 \pm 0.02
Erythrocyte glutathione S-transferase U/ml	0.62 \pm 0.16	0.75 \pm 0.39	0.61 \pm 0.19
Erythrocyte superoxide dismutase U/ml	428.6 \pm 10.5	443.5 \pm 3.5	444.5 \pm 6.8
Tissue glutathione mg/g	27.5 \pm 2.1	21.8 \pm 0.5*	24.1 \pm 2.2
Tissue malondialdehyde nmol/g	35.4 \pm 304	73.7 \pm 13.8*	35.5 \pm 6.9
Tissue glutathione S-transferase U/g	8.8 \pm 1.2	8.7 \pm 2.7	8.5 \pm 2.04
Tissue superoxide dismutase U/g	1879.7 \pm 36.8	1451.9 \pm 275*	1879.7 \pm 36.8

* $p < 0.05$

Group B (AFB1)

In the livers of group B's rats, similar findings were observed. In the lobelets of the livers, particularly in the periarterial and midzonal regions, pale focal swellings, or vacuolar-hydropic degeneration, occurred. The nuclei of the hepatocytes were generally pyknotic and dark stained. These degenerative foci were significantly distinct from the surrounding hepatocytes and exhibited diffuse necrosis that covered half or all of the lobulets. In the cytoplasm of some of the hepatocytes, vacuoles of varying sizes were observed in the cell bodies, particularly those that had degenerated. Through specific lipid staining, the relationship between these vacuoles and lipoidosis was dismissed. While solid hepatocytes were typically noted in the periportal regions and in the parenchyma between the degenerative foci, numerous focal areas of necrosis had formed in the periportal regions over time (Figs. 2, 3). In the periportal regions, the nuclei of certain cells were pyknotic, exhibited karyorrhexis, and contained cytoplasm that had been stained dark during lysis. The hepatocytes' nuclei varied in size and exhibited multiple degrees of staining. While certain hepatocytes had two nuclei, other hepatocytes had between two and four nuclei. In the periarterial regions, enormous and abnormal nuclei (megalocytosis) and cytoplasm were commonly observed in degenerative hepatocytes (Fig. 4). Increases in the bile ducts of certain portals were verified in two of the three cases (Fig. 5), and increases in the connective tissues of portal gaps were confirmed. However, proliferation from these regions to the parenchyma had yet to be observed.

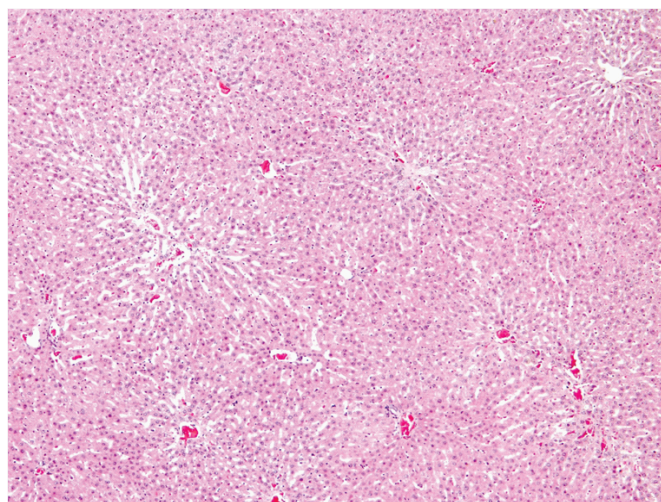


Fig. 1: Normal histopathological views of the liver of a group A (control) rat were observed (haematoxylin-eosin, X 160).

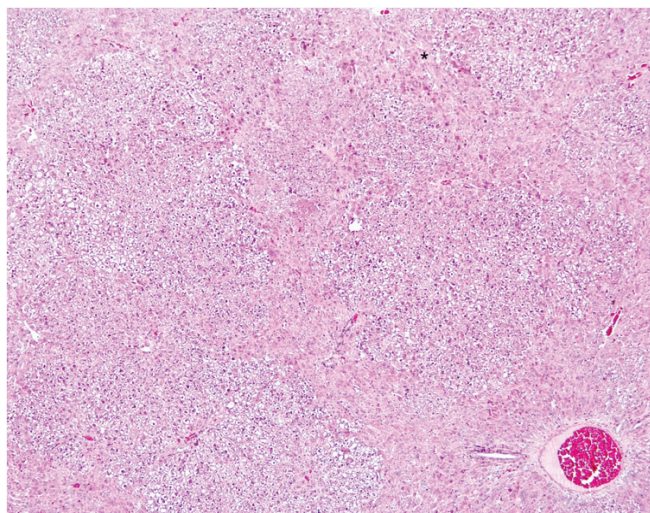


Fig. 2: Liver from group B AF-treated rats revealed extensive degenerative and necrotic changes in the hepatocytes (haematoxylin-eosin, X 80).

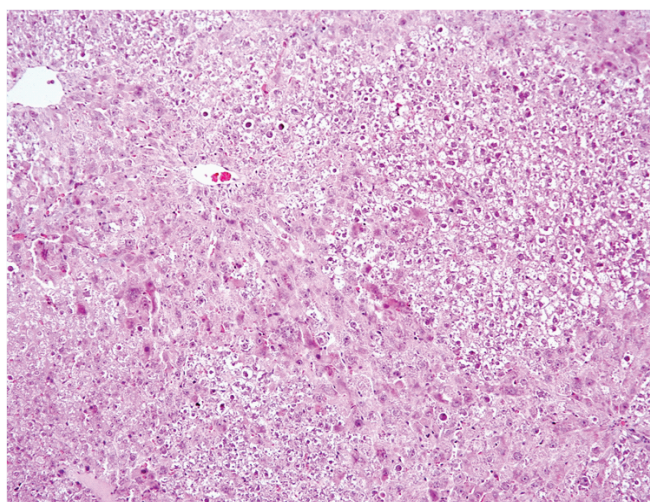


Fig. 3: Group B (AFB1) – a magnified photomicrograph of the area marked * in Fig. 2 (haematoxylin-eosin, X 300).

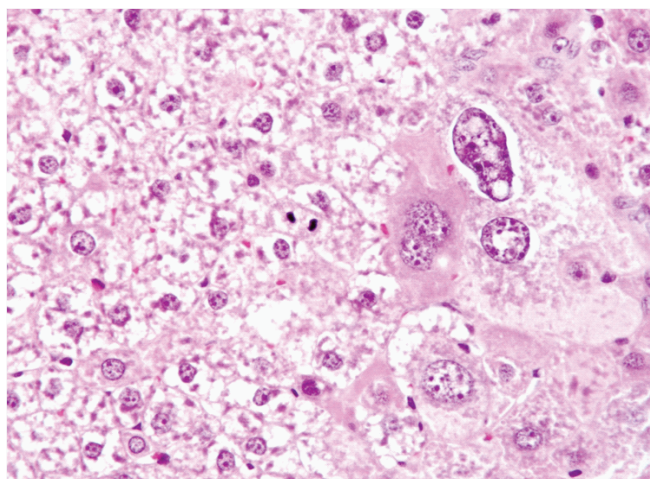


Fig. 4: Group B (AFB1) – dysplastic hepatocytes, nucleus of which demonstrates an enlarged, hyperchromatic and pleomorphic appearance with a coarse chromatin pattern, their cytoplasm distinctly large (megalocytosis) (haematoxylin-eosin, X 400).

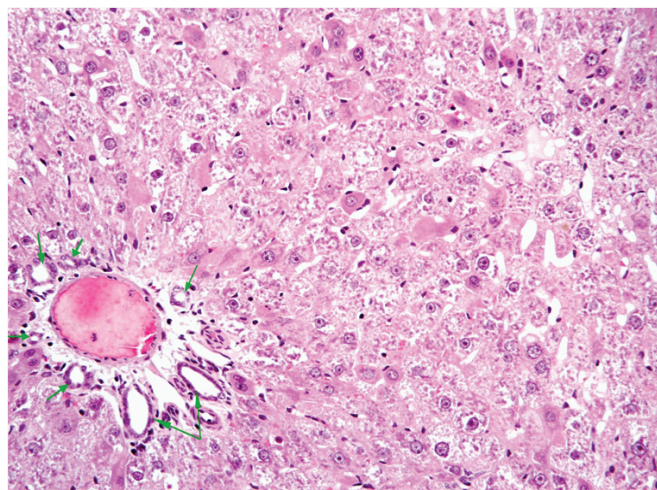


Fig. 5: Group B (AFB1) – bile-duct hyperplasia (arrows) (haematoxylin-eosin, X 360).

Group C (AFB1 + UDL extract)

In all cases, degenerated cellular bodies were observed in the livers. However, the number of these cellular foci ranged between two and four. Hepatocyte groups usually consist of 5–10 or 20–30 cells. These groups often contain vacuolar or severe hydropic degenerative cells. Degenerated hepatocyte groups are primarily localized in the midzonal regions. Within these semi-normal hepatocytes that are localized between the foci, slightly degenerated hydrophic and light-coloured hepatocytes and abnormal, dark stained hepatocytes with pyknotic nuclei and eosinophilic cytoplasm have been observed (Fig. 6). Hepatocytes that contained large cytoplasm, nuclei of varying sizes and marginal hyperchromasia were also recorded. However, compared to group B, these cells appeared at a significantly lower frequency. Substantial proliferation and hypertrophy in perisinusoidal cells also occurred. Table 2 presents the microscopical findings.

Table 2: Effects of *Urtica dioica* L and aflatoxin on the hepatic structure

Changes/lesions in liver	Control group	Aflatoxin-treated group	Aflatoxin + UDL extract-treated group	<i>p</i> -values
Enlargement and paleness	0/6	3/6	1/6	> 0.05
Hydropic degeneration/or fatty changes	0/6b	6/6a	2/6b	< 0.01
Dysplastic hepatocytes	0/6b	6/6a	0/6b	< 0.01
Bile-duct proliferation	0/6b	4/6a	0/6b	< 0.01
Periportal fibrosis	0/6b	4/6a	0/6b	< 0.01

Values with different letters (a and b) in the same row are significantly different ($p < 0.01$).

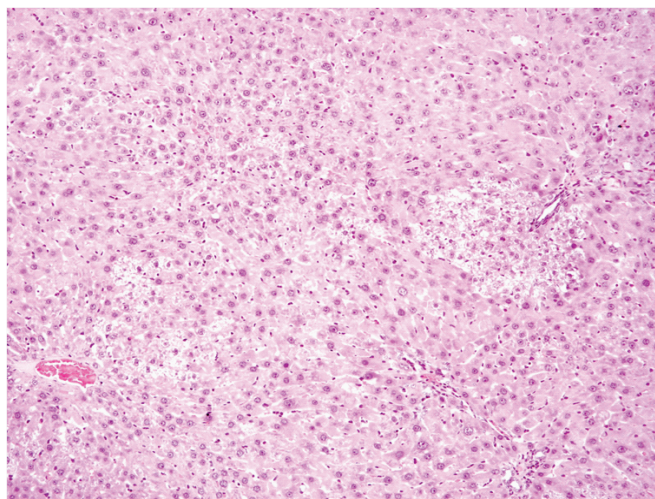


Fig. 6: Group C (AFB1 + UDL extract) – focus of degenerative cells (arrows), apoptotic and degenerative changes in the hepatocytes (haematoxylin-eosin, X 270).

DISCUSSION

Free radicals are molecules that regularly form in the body. While these are typically removed by the body's antioxidant defence systems, interruption of this balance can lead to an increase in free radicals and cellular damage (16). When free radicals form at a high frequency, they can exceed the protective effect of cellular defences. This can prove harmful to lipids, which are the most sensitive components. Unsaturated cholesterol and phospholipids are present in the structure of cellular membranes and can easily react with free radicals to create lipid peroxidation (17). Lipid peroxidation is responsible for ageing, mutagenesis, carcinogenesis and arteriosclerosis. For diseases such as these, oxygen and free radicals are well known for their important role in tissue injury (18). For these reasons and to reduce tissue injury induced by active oxygen and free radicals, foods rich in antioxidants and antioxidant supplements should be taken (19).

According to *in vitro* studies on the action mechanisms of AFs, AFB1 increases the formation of free radicals in reactive oxygen types that cause chromosomal damage (20). Due to the tissue and cellular damage induced by aflatoxicosis, biochemical changes occur with increases in ALT, AST and MDA levels and with decreases in the enzyme activities of SOD and glutathione reductase (17). Moorthy *et al* asserted that the presence of catalase, deferoxamine and SOD could prevent AFB1's lethal effect on liver cells (21). Lipid peroxidation is an oxidative degradation of unsaturated fatty acids that is associated with changes in membrane

structure and enzymatic inhibition. High levels of lipid peroxidation that are associated with AFB1 intake can lead to hypersensitivity and cellular destruction in cellular membranes (22).

The main source of glutathione, which plays an important role in the detoxification of free radicals, is amino acids. Glutathione is a strong antioxidant that removes harmful products formed through lipid peroxidation and increments in free radicals by reacting with them. Glutathione is converted into glutathione oxide by reacting with lipid peroxidation products that result from oxidative injury. According to research on glutathione levels that are changed by free radical damage and lipid peroxidation, decreases in glutathione levels and increments in lipid peroxidation products have yet to be found (18). In the present study's measurements of GPx tissue, group B's (AFB1) glutathione amounts were statistically low, and group C (AFB1 + UDL extract) showed low glutathione amounts that were not considered statistically significant. Glutathione and erythrocyte tissue data made no statistically significant changes in any of the groups ($p > 0.05$). These results confirmed that AFB1 caused liver toxicity and that UDL extract was preventive of this particular type of toxication.

According to the data collected on MDA and erythrocytes, the rats in group B (AFB1) had higher MDA levels in their livers and erythrocyte tissue than those in groups A (control) and C (AFB1 + UDL extract). This difference was considered to be statistically significant at $p < 0.05$. By contrast, while group C (AFB1 + UDL extract) had slightly higher levels of MDA and erythrocytes than group A (control), the differences between these findings were not considered to be statistically significant ($p > 0.05$). In the study of the effect of UDL extract on aflatoxicosis, increases in the MDA levels of the liver and the erythrocytes were prohibited to the point that group C's (AFB1 + UDL extract) levels were almost normal. Liver damage indicated by MDA increases suggested that UDL extract protected the liver.

According to the data that recorded GST and erythrocyte activity levels, both experimental study groups (B and C) lacked significant increases in either of these regions. While the GST and erythrocyte enzyme levels were higher in group B (AFB1) than in group A (control), this difference was not statistically significant. The roles of GST include cellular transport, detoxification and intracellular binding. As a catalyst for change, GST neutralizes electrophilic zones and makes products more soluble in water by binding xenobiotics to GPx groups that belong to cysteine amino acid within GPx.

These GPx conjugates can be either extracted from the organism or further metabolized (23). As the centre of detoxification, this kind of influence is typical of the liver. There were also no significant GST level increases in the livers of the control group. In spite of the high erythrocyte enzyme levels in group B (AFB1), the differences between groups A and B were not considered to be statistically significant. The cytotoxic and carcinogenic effects of AFB1 were likely caused by the 8, 9-epoxide form, aflatoxicol's 8, 9-epoxide metabolites, and likely, the AFs Q1, P1 and M1 (24).

Alanine transaminase and AST are enzymes that are commonly used as diagnostic indicators of liver damage. Increases in their levels of production are caused by the cytotoxic effects of AF. In the cases of degenerative and necrotic hepatocellular changes, these enzymes are secreted into blood circulation (21). In the present study, increases in AST and ALT levels were due to the toxic effects of AFB1, which caused changes in the metabolic activities of the livers.

According to the analyses of the livers, while group B's (AFB1) SOD activity level saw significant decreases from the levels recorded in group A (control) ($p < 0.05$), the SOD and erythrocyte activity levels increased in groups B and C. However, the differences in SOD enzyme levels were not considered to be statistically significant ($p > 0.05$). Initial increases in SOD can remove these harmful effects. Increases in cellular SOD activity in the cell are caused by adaptations caused by the extent of oxidative stresses and the dosages of the stressors. In this study, decreases in SOD activity levels that were generated by antioxidant defensive enzymes suggested indirect signs of induced superoxide radical formation. Because of the excessive production of superoxide radicals, increases in SOD activity may occur due to the activation of the enzyme's inactive form. For instance, group C's superoxide radical formation was suppressed by antioxidant activity.

Signs, such as petechial, pallor, enlargement and blunted edges of the liver, and ecchymotic haemorrhaging due to high dosages of toxication, were reported in the livers of the experimental aflatoxicosis cases (25). Macroscopically, mild changes in the organs were found only in the livers of rats that received AFB1 (group B). While no macroscopic changes in the livers of group C's rats (AFB1 + UDL extract) could be detected due to the protective effect of UDL extract on the liver, the degenerative and necrotic liver changes to the rats in group B could not be prevented.

It has been implied that epoxides are formed by metabolizing specific AF molecules in complex oxygenase enzyme systems that are bound to hepatic microsomal cytochromes P-450. This process plays a very significant role in the toxic and carcinogenic effects of AFB1 (26). By binding to macromolecules such as nucleic acids and nucleoproteins, these epoxide metabolites cause an inhibition of enzyme and protein synthesis that results in damage to cellular unity (26, 27). Hepatic microsomal cytochromes P-450 are localized in the hepatocytes of the pericentral areas with the highest concentrations in the liver (28). Pathological changes were detected in hepatocytes such as paleness, swelling, focal necrosis and hydropic degeneration. These findings were initially common in the pericentral regions of the liver and later in the intermediary regions of the liver. Lesions were primarily localized in the intermediary and centrilobular areas of the liver (21, 25). Findings reported in the cases of aflatoxicosis have included binucleic hepatocytes, marginal hyperchromasia, large numbers of nucleoli and enlarged hepatocyte nuclei (25). In the present study, similar hepatocytes were found in each group, and they were especially common in group B (AFB1). This case may be associated with increases in RNA and DNA that were caused by the AF's suppression of cell division (29). In other studies, proliferation in the bile ducts and hyperplasia in the epithelium of the liver have been found (30, 31). These changes are associated with the dosages and duration of AFB1. In addition, these changes occur subsequent to necrotic changes in the liver, which are one of the reactions of the liver to necrotic damage (28). In the present study, proliferation in bile ducts and hyperplasia in the epithelia of the livers were noted only in group B (AFB1).

The current study also drew attention to some of the microscopical findings that had been observed in group B but had not formed, or only slightly formed, in group C. This is thought to be associated with the hepatoprotective effects of UDL extract on liver injuries that have been induced by AFB1. The extract of UDL can prevent lipid peroxidation and activate immune systems to aid the prevention of liver damage. Other therapeutic characteristics have also been known to contribute to these hepatoprotective effects. In conclusion and in accordance with the biochemical and histopathological findings of the present study, UDL extract could produce hepatoprotective effects against aflatoxicosis.

ACKNOWLEDGEMENTS

This study was summarized from the PhD thesis of the same title. This project was supported by Turkish Scientific and Technological Research Council of Turkey (project number: 104 V 139).

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