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Protective Effect of Cinnamon Zeylanicum, Berberis Vulgaris and Ulva Lactuca Extracts on Hepatocellular Toxicity Induced by Aspergillus Flavus Intake in Rats

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- Berberis Vulgaris
- Ulva lactuca

Abstract

Background: Aflatoxin B1 produced by the fungus *Asperagillus flavus* causes great economic losses and poses health hazards to human and animals through its toxic biological effects on liver, kidney and lungs. **The aim of this work** was to study the potential protective effect of *Cinnamomum zeylanicum, Berberis vulgaris and Ulva lactuca* extracts on hepatocyte toxicity induced by *A. flavus* intake in rats. We also investigated the effect of *A. flavus* and the studied extracts on liver and kidney structure and function and the potential modulation of p53 and ICAM-1 gene expression as well as the liver antioxidant status. **Our results** showed a damaging effect of *A. flavus* intake on both liver and kidney as reflected by liver histopathological examination and the impaired liver and kidney functions measured by ALT, AST, Albumin, Urea, creatinine and glucose. Cinnamon and *Berberis and Ulva* pre-treatment kept these parameters to almost its normal levels compared to the induced unprotected animals. All tested extracts reduced the oxidative stress status and increased the antioxidant status by lowering TBARS and increasing NO levels significantly but had no significant effect on SOD activity. *A.flavus* intake caused a significant decline in both P53 and ICAM-1 gene expression; however, administration of Cinnamon or *Berberis* caused a significant increase in expression with Cinnamon causing the highest increase in p53 and *Berberis* causing the highest increase in ICAM-1. **In conclusion**, we recommended the use of *cinnamon Zeylanicum* or *Berberis vulgaris* as protective natural antioxidants against hepatocellular aflatoxin induced toxicity.

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Introduction

Aflatoxins are a group of closely related toxins that are widely distributed in nature in different agricultural communities. It has been demonstrated that the fungus *Asperagillus flavus* (*A.flavus*) can infect corn, producing Aflatoxin B1 (AFB1), a potent hepatotoxic and hepatocarcinogenic secondary metabolite (1-4). Hepatocellular carcinoma (HCC) is the most common type of primary cancer in the liver with the main risk factors including hepatitis B virus, environmental, metabolic factors and dietary habits (5), specifically, direct or indirect intake of diet contaminated with aflatoxin, a significant risk factor for HCC in less developed countries (6). AFB1 is metabolized in the liver to a reactive AFB1 epoxide that is very reactive and binds to cellular macromolecules such as DNA leading to the formation of AFB1-DNA adduct which is highly correlated to the carcinogenic effect in both animal and human (7). p53 is the most commonly mutated tumor suppressor gene in cancers (8). Aflatoxin B1 intake induces a G to T transversion in codon 249 of the P53 gene (9), a mutation that is proven to inhibit the p53-dependent apoptosis and is correlated to HCC as well as non-malignant liver disease associated with aflatoxin B1 intake (10, 11). Impaired *p53* is commonly found in HCC patients in countries with high dietary aflatoxin dietary exposure (12). On the other hand, Intracellular adhesion molecule (ICAM-1) is a useful marker for the determination of the severity of liver disease, fibrosis, HCC progression and monitoring the response of disease to treatment (13, 14). Despite the ongoing wide spread pharmaceutical and medical research efforts for combating liver disease, the current outcome is still considered insufficient and many of the drugs used today against liver diseases have proven to be intolerable with many drug-induced Hepatotoxicities and side effects (15). Therefore the use of natural extracts from medicinal plants is considered as safe and effective sources of new drugs against liver diseases. Phenolic substances found in certain plants exert a wide range protective role against several human diseases through its strong antioxidant, anticancer and anti-inflammatory (16, 17), antidiabetic (18) as well as Hepatoprotective actions (19). Barberry *(Berberis vulgaris)* is a plant that grows in different regions of the world; it is extensively used as a medicinal plant in traditional medicine as well as a food additive (20). Berberin, the major active constituent of Barberry have demonstrated various pharmaceutical effects including: Cardiovascular, immunomodulatory, antimicrobial, anti-inflammatory, Cytotoxic and many other effects (21). Cinnamon is isolated from the inner part of the plant tree *Cinnamomum zeylanicum* and is commonly used as a spice and a food additive by different regions all around the globe. However Cinnamon is also commonly used in traditional medicine and has proven numerous beneficial medical effects (22). On the other hand seaweeds such as the green alga *Ulva lactuca* has been used as rich sources of proteins, carbohydrates, vitamins, trace minerals and many other bioactive compounds with various good health effects (23).

The aim of this study was to investigate the potential protective effects *Cinnamon Zeylanicum*, *Berberis vulgaris and Ulva lactuca* extracts on hepatocellular toxicity induced by the intake of *A.*

flavus producing AFB1 in rats. The p53 and ICAM gene expression as well as the alterations in liver antioxidant status by the extracts was also assessed.

Subjects and methods:

All chemicals and reagents were of the highest quality available and purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Real time PCR kits were purchased from Fermentas, Canada.

Plants and alga extract preparations:

Cinnamuom Zeylanicum, and *Berberis Vulgaris* were purchased from local markets and *Ulva lactuca* green algae was collected from the Abu Kir coast and identified by Prof. Dr. Samy Shaalan, Microbiology and Botany Department, Faculty of Science, Alexandria University, Egypt. *Ulva lactuca* was washed by distilled water and then dried on fresh air at room temperature and 250 g of dried algae were soaked in 500 ml methanol. On the other hand, 250 g of powdered *Cinnamuom Zeylanicum* or *Berberis vulgaris* were separately soaked in 500 ml absolute ethanol for 3 days at 25°C in a shaker incubator and supernatants were collected by filtration using Bückner filter and evaporated under vacuum to sticky oil solution which was lyophilized.

Animals and experimental design:

Animal treatment was conducted in accordance with the standard guidelines for the care and use of experimental animals by the medical research ethics committee, Medical Research Institute, Alexandria University, Egypt. A total of forty eight female rats, 12 weeks of age, 120-150g body weight were used for this study and were divided into six groups (8 rats / group) as following:

Group I: Control, fed on a regular diet.

Group II: Induced untreated, this group was orally administrated with *A. flavus* water suspension prepared by dissolving slant of *A.flavus* with 2ml distilled water and rats were orally injected with 0.15 ml for 4 weeks and fed on the same as control group diet.

Group III: Cinnamon extract treated group, rats were given a suspension of Cinnamon-DMSO extract (20mg-0.25mL/ 100g body weight) for two weeks then received *A.flavus* for extra 4 weeks with regular diet.

Group IV: *Berberis* extract treated group, was given suspension of *Berberis* -DMSO (20 mg-0.25 ml/ 100g body weight) for two weeks then received *A. flavus* for extra 4 weeks with regular diet.

Group V: *Ulva* treated group, animals were given suspension of Ulva-DMSO extract (20 mg-0.25 ml/ 100g body weight) for two weeks then received *A. flavus* for extra 4 weeks with regular diet.

Group VI: DMSO treated group, animals were given 0.25 ml of DMSO/100 gm body weight for 2 weeks then received *A. flavus* for extra 4 weeks with regular diet.

After the indicated treatment periods, rats were fasted for 2 days and decapitated to collect the blood for serum isolation and liver which were quickly washed in cold saline then cut into pieces.

One gram of liver was homogenized with 9 volumes of potassium phosphate buffer, 0.1M, pH 7.4, then centrifuged at 3000 rpm for 15 minutes and the supernatant was stored at -80ºC to be used as a liver homogenate.

Biochemical measurements: Serum thiobarbituric acid-reactive substances (TBARS) was measured by the method of Tappel and Zalkin (24). Serum Liver Alanine Amino Transferase (ALT) and Aspartate Amino Transferase (AST) activities (25). Serum urea and creatinine (26, 27) respectively. Glucose concentration (28), Superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities (29, 30), finally the level of nitric oxide (NO) was measured (31).

RNA isolation and qPCR for the determination of p53 and ICAM gene expression:

Total RNA was isolated from liver samples by RNeasy total RNA isolation kit (Qiagen GmbH, Hilden Germany), according to the manufacturer's instructions. Quality and quantity of RNA were confirmed phtometrically.

Five micrograms of total RNA were added to 0.5 µg random hexamers primers and 4 µl of 5X reaction buffer, 0.5 µl RNase inhibitor, 1mM dTNP and finally 1µl of reverse transcriptase was added and the mixture was incubated for 60 min at 42 ºC for transcription and the reaction was stopped at 70 ºC for 10min. qPCR reaction master mix was prepared by adding 12.5 µl Maxima probe master mix 2X, 0.3 µM Forward primer, 0.3 µM Reverse primer of P53 or ICAM-1 (Table 1) and 0.2 µM probe and 500 ng/ reaction template DNA was added to the individual PCR tubes and completed to 25ul with free nuclease water.

Finally, the PCR was carried out as following ; initial denaturation for 10 minutes at 95 ºC, and 40 cycle of denaturation for 15 seconds at 95 ºC, annealing for 30 seconds at Tm-5 ºC for each primer and extension for 30 seconds at 72 ºC.

Primers		primer sequence 5′- 3′	A.T ºC
P53	**forward**	CGTCGAAGAAAA	60
	reverse	TCCAAGGCCTCATTCAGCTC	
ICAM-1	**forward**	CTGCACGTGCTGTATGGTCCT	65
	reverse	AGGGGGTCCAGGCAGGAGTC	

Table 1. Primer sequence and annealing temperatures for PCR

Histological studies:

A tissue sample from rat liver was fixed in 10% formaline-saline and embedded in paraffin blocks. A representative 4 µm thin section was then stained using hematoxylin and eosin (H&E) stain and photomicrographs were taken at 400 x. (32).

Statistical analyses;

Data were analyzed by one-way analysis of variance (ANOVA) using Primer of Biostatistics (Version 5) software. Significance of means ± SD was detected groups by the multiple comparisons Student-Newman-keuls test at $P \leq 0.05$.

Results:

Liver and kidney function in response to *A.flavus* and crude extracts protection:

A significant increase in serum AST and ALT activities, creatinine and glucose levels and a significant reduction in Albumin and Urea levels was observed in *A. flavus* induced rats compared to control group. On the other hand, Cinnamon, *Berberis* and *Ulva* extracts successfully decreased AST and ALT activities and albumin level than that of induced untreated group Table (2). Moreover, Cinnamon and *Berberis* administration significantly increased the urea and normalized creatinine levels and caused a reduction in glucose levels than the control. On the other hand, *Ulva* administration caused a significant reduction in glucose levels and a slightly increased urea level compared to the induced untreated group but did not affect the creatinine level compared to the induced untreated group, Table (2).

Table 2. Effect of natural extracts on liver (ALT, AST and albumin), kidney (Urea and Creatinine) functions and glucose levels during prevention of A.flavus induced hepatotoxicity.

Groups	ALT (U/L)	AST (U/L)	Albumin (g/dL)	UREA (mg %)	Creatinine (mg %)	Glucose (mg %)
Control	89±14.4	184.8±13.1	4.88±0.41	45±2.21	0.65± 0.16	70.8± 8.11
Induced untreated	120.6±7.5#	260.6±10.1#	4.297±0.88#	20±4.2#	0.82±0.06#	101.7± 9.46#
Cinnamon treated	92.66±8.6*	190.7±10.8*	4.99±0.36*	67.6±10.6*#	0.67±0.08*	42.5± 7.9*#
***Berberis* treated**	94±10*	191.1±17.7*	4.86±0.23*	55±3.63*#	0.69±0.07*	51.3± 3.03*#
***Ulva* treated**	97.4±10*	198±15.8*	4.99±0.52*	30±4.14#*	0.79±0.1#	27.9± 2.4*#
DMSO treated	125±6.4#	263.6±10.5#	4.381±0.3#*	10±4.14#*	0.27±0.03#*	71.7± 10.8*

or * A significant difference with control group or induced untreated group mean (respectively). $P \leq 0.05$

The effect of *A.flavus* and crude extract administration on antioxidants status:

A. flavus administration stimulated oxidative stress as reflected by the significant increase in the TBARS level accompanied by reduction in NO level and GPx activity compared to control group. All tested extracts reduced oxidative stress status by lowering TBARS level significantly compared to both control and induced untreated group and increasing NO levels significantly higher than control and induced untreated group as well, $p<0.05$. Finally no statistical difference was observed in SOD activity between any of the studied groups Figure (1).

p53 and ICAM-1 gene expression:

A. flavus intake significantly decreased both p53 and ICAM-1 gene expression while treatment with Cinnamon and Berberis significantly increased both levels than the induced untreated animals. Cinnamon caused the highest increase in p53 while Berberis caused the highest increase in ICAM-1 levels Table (3).

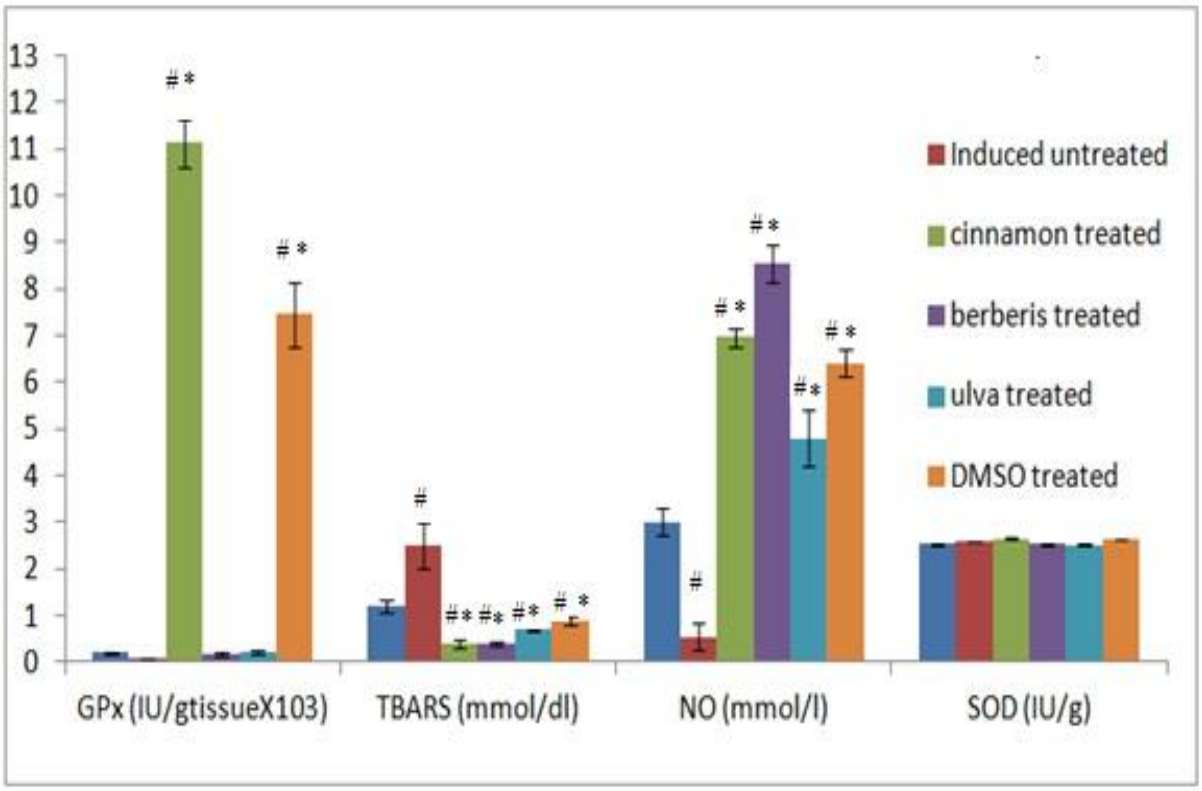

Figure 1. The effect of *A.flavus* on antioxidants status in rats pretreated with different natural extracts; # or * means a significant difference with control group or induced untreated group means, respectively. $P \leq 0.05$

Table 3. p53 and ICAM-1 gene expression in rats orally injected with *A.flavus* and treated with Cinnamon or *Berberis*.

Fold change in gene expression				
Gene	**control**	**Induced**	***Cinnamon Zeylanicum***	***Berberis vulgaris***
P53	100%	87%	410.5%	269.3%
ICAM-1	100%	65.3%	100.03%	139.05%

Histopathological examination:

Histopathological examination of Liver sections of the control animals showed normal cellular architecture with distinct hepatic cells (Figure 2A). Liver sections of rats intoxicated with A. flavus (AFB1 group) showed disturbed lobular architecture, vacuolar degeneration with hydropic degeneration in hepatocytes, local hyperemia in the area surrounding the central veins, degenerative changes and focal necrosis, marked sinusoidal contraction, and a few hepatocytes with pyknotic nuclei in lobules were noticed (Figure 2B). In the AFB1-Cinnamon and Berberis groups, an almost normal architecture of the liver was observed, indicating the protection afforded by the two plant extracts. However, the appearance of the Cinnamon treated group was quite similar to that of the control group and tissue damage and necrosis were of less extent compared to Berberine

treated group (Figure 2C and 2D). The AFB1-Ulva extract group showed no curative effect on acute liver damage (data not shown)

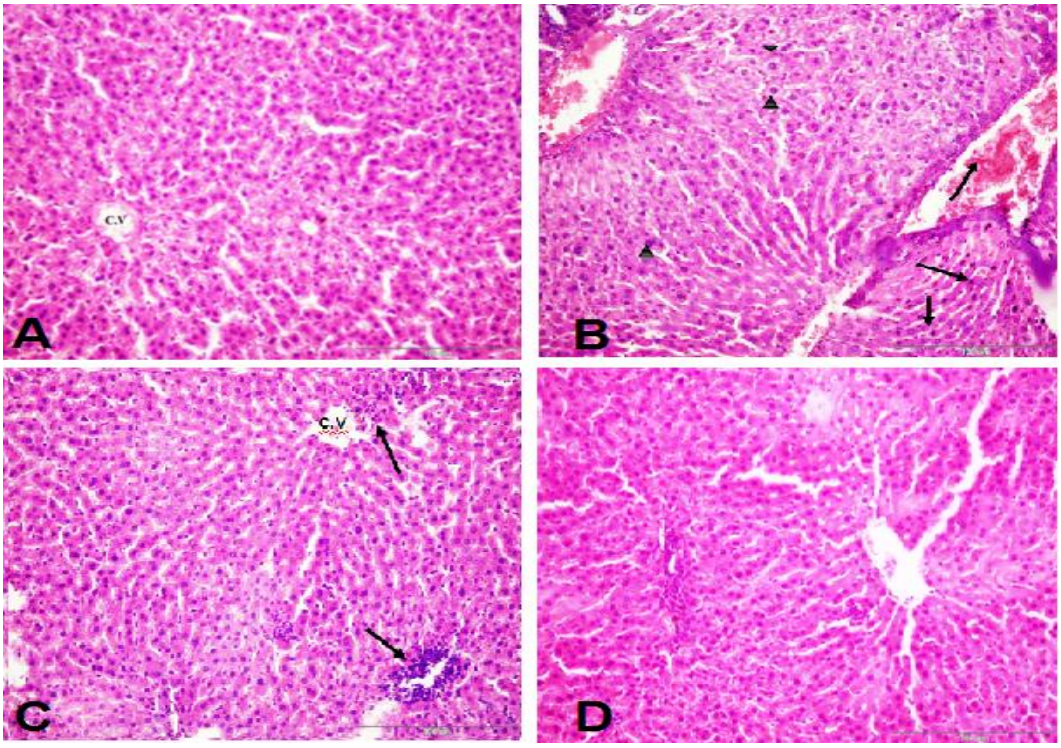

Figure 2. Microscopic examination of rat liver showing: (A) Control group showing normal liver histological appearance, C.V: central vein; (**B)** induced untreated animals fed with *A. flavus (AFB1 group)* showing severe hydropic hepatocyte degeneration (arrowheads) and hepatocytes with pyknotic nuclei (arrow); **(C)** AFB1-Cinnamon treated group showing similar appearance to that of the control group, mononuclear cell infiltration in portal areas (arrows); **(D)** Animals with AFB1- berberine extract (Hx & E x400).

Discussion:

The liver plays an essential role in different physiological processes; most importantly the detoxification of endogenous and exogenous compounds, a process that can be affected by certain nutrients or food supplements (33, 34). Aflatoxins are carcinogenic secondary metabolites produced by certain strains of the *Aspergillus* fungi. The ideal way for minimizing their hazardous health effects is primarily to avoid food contamination. However this is not always achievable, therefore the effective protection against these compounds can be crucial in fighting liver diseases associated with Aflatoxin contamination in food stuff. Our aim was to investigate the potential protective role of *Cinnamon Zeylanicum, Berberis Vulgaris and Ulva Lactuca* extracts on hepatocellular toxicity induced by AFB1 produced by *Aspergillus Flavus* Intake in Rats. Our study confirmed that the intake of *A.flavus* producing AFB1 in rats caused liver damage reflected by the significant increase in the AST and ALT activities, creatinine and glucose levels as well as the significant decrease in urea and albumin levels compared to the control group. These results together with the histopathological examination of the liver confirmed the hepatocytes damage and the liver and kidney malfunction. High serum ALT and AST are usually indicative of liver damage (35) due to the increased membrane permeability and/or cell necrosis and

enzyme leakage into the serum (36-37). Furthermore, the increased level of serum creatinine in response to aflatoxin intake indicates altered protein catabolism and/or renal dysfunction that may be secondary to hepatocytes destruction (38, 39). The increased levels of glucose in damaged hepatocytes in untreated animals indicates a metabolic alteration in their ability to utilize glucose as in normal hepatocytes metabolism causing an increase in glucose levels in this group of animals. Cinnamon, *Berberis* and *Ulva* extracts administration before exposure to *A.flavus* normalized AST and ALT activities, albumin, creatinine and glucose levels indicating a protective role for these extracts against induced liver aflatoxicosis. Our results accords with Aravind *et al 2003,* that in chronic and sub-clinical aflatoxicosis, changes in biochemical parameters may occur before any clinical symptoms develop (40). To investigate the possible mechanisms involved in cell damage in response to Aflatoxin intake, we measured the alterations in reactive oxygen species (ROS) and antioxidant status. It is difficult to directly measure ROS due to its short half-life (41), it can be measured indirectly through products such as TBARS released as result of the increase in lipid peroxidation and cellular damage caused by oxidative stress (42) and Nitric oxide (NO) that acts in many reported cases as an antioxidant through scavenging (ROS) and dropping dramatically in the case of elevated oxidative stress (43). Moreover, Glutathione peroxidase (GPx) and superoxide dismutase (SOD) are two major antioxidant enzymes that are used as metabolic markers for oxidative stress (44). Our data showed that *A.flavus* intake increased (TBARS) and decreased the antioxidant NO levels and antioxidant enzyme (GPx) with no effect on (SOD) activities. Therefore confirming that aflatoxin induced liver cell necrosis is due to the increase in oxidative stress status. It is well known that oxidative stress increases when prooxidant production increases accompanied by a reduction in the antioxidant scavenger system. Our results are in the same line with another study showing increased lipid peroxidation and decreased non-enzymatic antioxidants such as glutathione, ascorbic acid and enzymatic antioxidants such as (GPx) (45, 46). Therefore, our findings confirm the major role of oxidative stress in hepatotoxicity caused by AFB1. To further investigate the mechanisms involved in Aflatoxin induced cytotoxicity, we studied the effect of *A.flavus* administration on a major cell cycle regulating gene, we measured the p53 expression and our results showed a significant decrease in p53 expression after the intake of *A.flavus* which could be due to the direct effect of oxidative stress on p53 as was shown in our data and reported elsewhere (47). Cinnamon and *berberine* administration normalized the p53 expression indicating protection of normal cell cycle control upon administration of these natural extracts before AFB1 exposure, therefore suggesting a potential protective role for Cinnamon and *berberine* extracts against Aflatoxins and potentially p53-mediated tumorigenesis. The severity of liver damage by *A.flavus* and the protection by Cinnamon and *berberine* was also examined by ICAM-1 expression analysis and a significant decrease in expression was detected upon *A.flavus* administration, an effect that was reversed by *berberine* and *Cinnamon*, therefore, confirming the protective effect of these two

extracts on liver structure. The increase in ICAM-1 expression by *Cinnamon* and *berberine* is also considered as protective way against cancer proliferation through the modulation of immune response by ICAM-1 (48). We performed liver histopathological examination to assess liver structural damage induced by *A.flavus* administration and the potential protection by *cinnamon* and *berberine.* Our histopathological data confirmed all the previously described biochemical parameters that Cinnamon extract markedly reduced the toxicity of AFB1 and preserved the architecture of liver tissue to near normal followed by *Berberine* extract but to a lesser extent; On the other hand, *Ulva* did not have any protective action.

CONCLUSION

Our results suggest that Cinnamon, Berberine and to a lesser extent Ulva extracts may act as hepatoprotective agents against AFB1 induced hepatotoxicity, they increased the antioxidant status, restored liver enzymes to normal levels and increased p53 and ICAM-1 expression therefore restoring cell-cycle control and cell immune response. Therefore, we recommended the usage of Cinnamon Zeylanicum and Berberis vulgaris as protective compounds against aflatoxin toxicity.

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