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Hepatoprotective Impact of Boldo (Peumus Boldus) Extract against Azoxystrobin Induced DNA Damage, Gene Expression Modulation, Biochemical and Histopathological Alterations Mediated-ROS Generation in Male Rats

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Abstract

Peumus boldus Molina, ordinarily mentioned to as 'boldo', is expended in folk medicine to cure gastrointestinal and hepatic diseases. This plant is wealthy in several antioxidant compounds. The current research evaluated the ameliorative effect of boldo leaves extract on lipid peroxidation, liver antioxidant enzymes, ROS generation, DNA damage, gene expression, and histopathological changes induced by Amistar fungicide in male rats. The rats were allocated in four groups treated with boldo extract (50 mg/Kg b.w) and/or with Amistar (1/20 of LD50). As likely, the treatment with fungicide significantly ($P<0.05$) decreased the antioxidant enzymes activities, while increasing the liver biomarker parameters, lipid peroxidation, ROS generation, DNA damage, and hepatic tissue lesions as well as altered the expression of stress and antioxidant related genes in comparison with control rats. Pretreatment with boldo extract significantly improved the negative effects induced by Amistar on the liver cells. The research results indicate that the boldo extract acts as a defender against the oxidative hepatic injury produced by fungicide. The defensive capability of boldo extract could be owing to the existence of natural antioxidants and anti-inflammatory constituents' likes gallic acid, ellagic acid, querectin, daidzein, ferulic acid and rutin.

Keywords: *Peumus boldus*; Amistar; Lipoperoxidation; Antioxidants; ROS, DNA damage

Introduction

A large number of natural products and dietary ingredients, including plant extracts, have been studied for their antioxidants powerful capacity (Pohl and Lin 2018). There are few widely reliable and accessible natural products used for the common liver diseases treatments. Globally, the complementary and alternative medicine (CAM) is growing as the safest medical branch **[1]**. Researchers have used several scientific techniques to determine the effects of plants used in conventional CAM for the treatment of liver ailments in recent years **[2]**.

Clinical trials have confirmed the medicinal plants mechanisms and modes of action, as well as their powerful therapeutic efficacy in many cases. So far, hundreds of plants have been investigated, but only a few have been analyzed in details. Medicinal plants are becoming more common as a result of their perceived usefulness in the treatment and prevention of disease with very low side effects. Furthermore, since most countries do not have stringent controls on herbal preparations, access to these forms of therapies is nearly unregulated **[3]**.

There are numerous plant extracts used in traditional medicine and there are grown scientific interest in understanding their mode of actions. Boldo (Peumus boldus), a Moni-miaceae tree native to Chile, has been used for medicinal purposes **[4]**. Boldo is

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actually commonly used in folk medicine in Latin America and also North Africa and is known in pharmacopeia as a medicinal herb. It is advised to be used as hepatic function regulator, cholagogue, antispasmodic, digestive stimulant and nervous sedative. As a leaf extract that is consumed after feeding, it is most commonly taken **[5]**. Boldo leaves are abundant in flavonoids, alkaloids, and basic oil **[5]**.

Pesticides are commonly used to preserve yields of economically important crops in order to prevent harvest losses to meet the rising demands of the world food supply **[6]**. Because of inappropriate disposal and improper use, accidental leaks and leakages pose a great danger to the environment and human health, the marketing of pesticides is subject to strict regulatory limits **[7]**. Azoxystrobin is a strobilurin fungicide that possesses fungicidal activity by binding cytochrome-b sites that obstruct the transfer of electrons between cytochrome b and cytochrome c1. This interaction activity of azoxystrobin is elevating ROS generation in the mitochondria **[7]**. Globally the Amistar, whose active component is azoxystrobin, turned into one of the leaders of fungicides in the last three years **[8]**. The potential Azoxystrobin bioaccumulation or biomagnification values are scarce **[9]**. However, the nephrotoxic and hepatotoxic effects of agro-pesticides were in the focus of the scientific studies carried on laboratory animals **[10]**. So, these scientific studies were taken into consideration as a powerful link between the toxicity of agro-pesticides and liver and kidney impairment indicators **[11]**.

In view of the fact that most metabolism pathways take place in the liver, it is the primary detoxification organ. The liver is at great risk of injury because of its role in the biotransformation of xenobiotics, as the contaminants can be accumulated with high intracellular concentrations **[12]**. It is well known that biotic and abiotic stresses lead to oxidative stress through the rapid generation of ROS "reactive oxygen species" **[13]**. Excessive ROS accumulations create membrane oxidation and DNA protein damage as well as the alteration in the gene expression patterns **[14]**. In order to boost the protective properties of individuals against environmental oxidative stress, the exogenous antioxidants supplementation must be raised **[15]**. So, there is a great interest in bioactive natural compounds due to their protective properties against both the toxicity of various toxins and other pathogenic factors, especially ROS **[16]**. The plant extracts do their preservative impact through free radicals scavenging and the antioxidant defense mechanism modulating **[10]**. Therefore, the current study was undertaken to estimate the effectiveness of boldo extract against Amistar-induced biological alterations in male rats.

Materials and Methods

Fungicide and chemicals

Amistar fungicide (39% SC) was obtained from Elzyat pesticide company Egypt. The kits used of for biochemical analyses were obtained from Bio-diagnostic Co., Dokki, Giza, Egypt. Invitrogen (Germany) kits were used for molecular biological analyses.

Animals

Adult male Sprague Dawley rats weighing 120 ± 5 g, 8–9 weeks old were getting from Animal Breeding House (ABH) of the National Research Centre (NRC), Dokki, Giza, Egypt. The rats were distributed in Polypropylene cages (ten rats each cage). All of the rats were held in accordance with the Guide for the Care and Use of Laboratory Animals, which was licensed by the NRC Local Ethical Review Committee **[17]**.

Plant extraction preparation

Plant fresh materials (Boldo leaves) were collected from a private Farm (Haraz company, Cairo, Egypt), immediately transferred to the laboratory at NRC. Afterward, the plant samples were dried and stored in the refrigerator up to use. Dried plant materials (150 grams) were utilized for extract preparation according to **Cordero-Pérez et al. [18]** by using ethyl alcohol (70%). The collected plant materials after extraction were kept at 4 °C until use.

HPLC analysis

HPLC analysis was done by Agilent 1260 series. The separation was done by Eclipse C18 column (4.6 mm x 250 mm i.d., 5 µm). The mobile phase formed from water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate 0.9 ml/min. The mobile phase was programmed sequentially in a linear gradient as following: 0 min (82% A); 0–5 min (80% A); 5-8 min (60% A); 8-12 min (60% A); 12-15 min (82% A); 15-16 min (82% A) and 16-20 (82%A). The multi-wavelength detector was adjusted at 280 nm. Each injected sample was 5 µl. The column temperature was adjusted to 40 °C.

Treatment design

Forty adult male rats were randomly bifurcated into four groups (n=10). Group I: animals were given water and filled in as a control. Group2: animals received boldo extract orally at concentration 0.1 mg/kg. b.wt for 35 days **[18]**. Group3: animals received Amistar fungicide in drinking water at a concentration 1/20 of LD50= 5000 mg/kg b. wt. for 35 days **[19]**. Group4: rats received boldo extract orally at concentration 0.1 mg/kg. b. wt. plus Amistar fungicide in drinking water at a concentration 1/20 of LD50= 5000 mg/kg b. wt. for 35 days. All doses were fresh prepared, and animals were monitored for any toxicity or mortality signs all the experiment duration.

Blood and liver sampling

After last day of treatment animals were fasted overnight and blood samples were aspirated. Animals were then sacrificed by cervical dislocation and liver

samples were then collected for analysis. Collected blood was centrifuged at 3000 rpm for 10 min at 4 °C and serum samples were separated and held at -20 °C for biochemical analysis. The liver was extracted, washed, weighed, and bifurcated into two parts: the first part was preserved in formalin (10%) for histopathological analysis, while the other part was used for biochemical and molecular analyses.

Serum function biomarkers

According to the approaches of **Reitman and Frankel [20]** and **Young *et al*. [21]** serum biomarkers such as transaminase (AST, ALT), and ALP were used. Additionally, other biomarkers such as total Albumin and bilirubin concentration in serum were measured according to the protocol, which is detailed in the kit's guidance booklet.

Oxidative stress markers in liver tissue

The methods of **Nishikimi *et al*. [22]**, **Aebi [23]** and **Satoh [24]** were used to assess oxidative stress biomarkers such as SOD, CAT and lipid peroxide (LPO).

Comet Assay

Determination of the DNA damage in the animals liver tissues using comet assay was carried out according to **Blasiak *et al.* [25]**. The DNA damage in the tail was evaluated in 3 categories from class 1 to class 3.

ROS generation assessment

The ROS generation in hepatic tissues was estimated with a flow cytometer using a fluorescent probe according to **Khalil and Abdu [26]**. Estimation of the signals of ROS formation was done at 525 nm emission and 488nm excitation.

Expression profile stress and antioxidant-related genes

Total RNA of hepatic tissues was extracted using TRIzol® reagent according to the extraction manual of the reagent. The isolated RNA was used to synthesis the cDNA immediately after RNA isolation using first Strand cDNA Synthesis Kit *via* reverse transcription reaction **[27, 28]**. The synthesized cDNA were used for Real-Time reaction using SYBR® Premix Ex TaqTM kit. The C_T values obtained from qRT-PCR reactions using specific primers (Table 1) were normalized on the C_T values of housekeeping (*β-actin*) gene using $2^{-\Delta\Delta CT}$ method.

Histopathological examinations

The liver tissue dehydrated in alcohol and embedded in paraffin wax, and thick sections were cut then stained with hematoxylin and eosin (H&E). The liver slides were examined for any histopathological variations using a light microscope (Olympus BX50) with a digital camera (Olympus E-410) **[29]**. The liver histopathological changes were scored as follows: normal appearance (−), mild (+), moderate (++) and severe (+++).

Table 1: Primers sequence used for gene expression

Gene	Primer sequence (5′-3′)	NCBI Reference
IL-2	F: TCA AGC CCT GCA AAG GAA AC	M22899.1
	R: TCC AGC GTC TTC CAA GTG AA	
TNF	F: TTC GGA ACT CAC TGG ATC CC	BC107671.1
	R: GGA ACA GTC TGG GAA GCT CT	
CYP2E1	F: TGT TTC TGT GAC TTT GGC CG	NM_031543.2
	R: GCA CCA CAG CAT CCA TGT AG	
Gpx1	F: GAC CGA CCC CAA GTA CAT CA	NM_030826.4
	R: GCA GGG CTT CTA TAT CGG GT	
GRx	F: GCG TGG AGG TGT TGA AGT TC	H33579.1
	R: TAG AAT TTG GGT CCC GTC CA	
Beta actin	F: CTA CAA TGA GCT GCG TGT GG	AW862650.1
	R: AGG CAT ACA GGG ACA ACA CA	

Statistical analysis

All data values were stated as means ± standard error (S.E.M). The data were analyzed with the Statistical Package for Social Sciences (SPSS 0.25) for windows. The results were examined using one-way analysis of variance (ANOVA) followed by Duncan's test for comparison between different treatment groups. Statistical significance statements were set at $P \leq 0.05$.

Results

Analysis of the Boldo (*Peumus boldus*) extract by HPLC

HPLC chromatograms have affirmed ten marker components existing in ethanol extract of Boldo (Peumus boldus) (Fig. 1). These phenolic components have been identified as gallic acid (Rt:: 3.378 min; conc: 250.63 µg/ml), chlorogenic acid (Rt:: 4.183 min; conc: 364.52 µg/ml), coffeic acid (Rt:: 4.183 min ; conc: 110.69 µg/ml), syringic acid (Rt:: 6.168 min; conc: 33.28 µg/ml), rutin (Rt:: 7.937 min; conc: 83.62µg/ml), ellagic acid (Rt:: 8.868 min; conc: 1672 µg/ml), ferulic acid (Rt:: 10.234 min; conc:118.96 µg/ml), naringenin (Rt:: 10.475 min; conc: 239.12 µg/ml), daidzein (Rt:: 12.285 min; conc: 184.48 µg/ml), and querectin (Rt:: 12.646 min; conc: 71.63 µg/ml) by their retention time and UV absorbance of purified standards.

Body and relative organ weights

Administration of 1/20 LD50 doses of Amistar fungicide for 45 days resulted in a significant decline ($P < 0.05$) in body weight gain in comparison with the control group (Fig. 2A). Simultaneously, a significant increment ($P < 0.05$) was observed in the relative liver weight of Amistar exposed rats in comparison with the control group (Fig.2B). However, treatment of Amistar-exposed rats with boldo extract increased considerably the body weight and decreased the liver weight in comparison with those in rats exposed to Amistar only (Fig.2A & B).

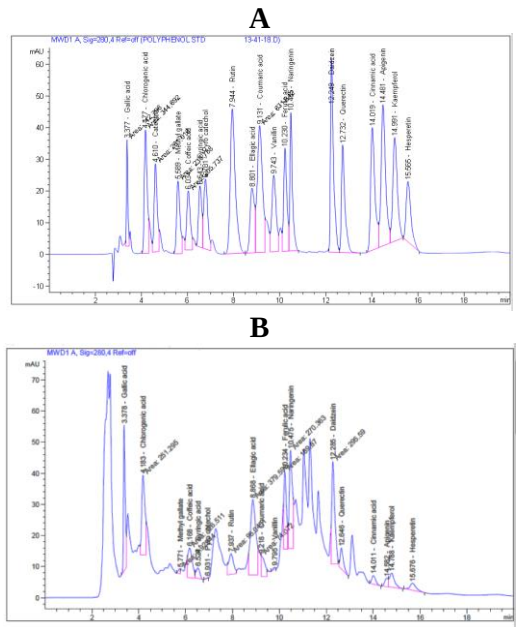

Fig. 1: HPLC chromatogram of standards (A) and Boldo *(Peumus boldus)* extract (B) at 280 nm.

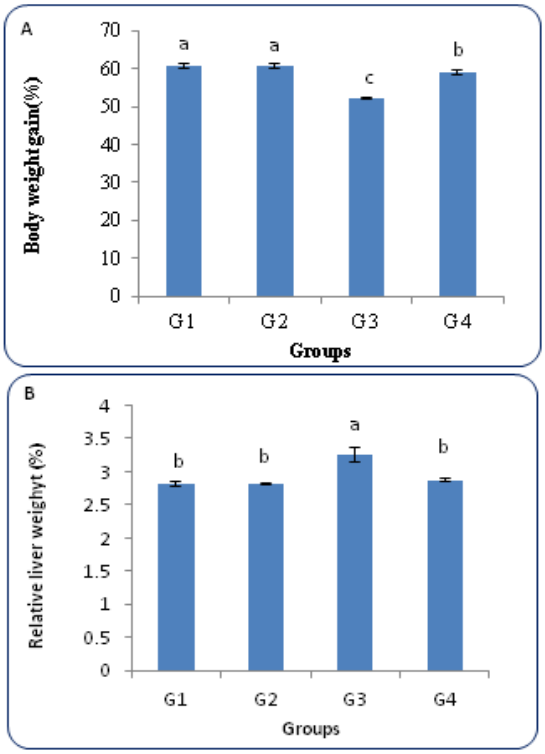

Fig. 2: The body weight percentage gain (A) and the relative liver percent (B) of rats exposed to Amistar fungicide for 45 days. Bars represent the group means of 5 rats ± SEM. Bar values not sharing superscripts letters (a, b, c, d) vary significantly at P < 0.05. (G1) control, (G2) boldo extract group, (G3) Amistar group (G4) Amistar + boldo extract group.

Liver function parameters

Rats exposed to Amistar exhibited considerably high levels of AST, ALT, and ALP as compared to control rats (Fig. 3A to 3C). Total Albumin and bilirubin concentration in serum of rats exposed to Amistar did not alter significantly as compared to control rats. In contrast, the liver enzymes levels were reduced in the rats treated with Amistar and boldo co-administration as compared to their levels in the rats exposed to Amistar only (Fig. 3A to 3C).

Antioxidant enzymes

Amistar exposed rats showed significant (P<0.05) diminishing in SOD (Fig. 4A) as well as CAT activities (Fig. 4B), where, the activity values of SOD and CAT were 4.73 µmol/mg protein and 34.87 u/mg protein, respectively in Amistar- exposed rats as compared to control rats (6.97 µmol/mg protein, 41.88 u/mg protein, respectively). Amistar-exposed rats treated with boldo extract showed recovery in the SOD and CAT activities, while the SOD and CAT activities values were 6.93 µmol/mg protein and 39.71 u/mg protein, respectively, as compared to animals exposed to Amistar only.

Lipid peroxidation

Exposure of rats to Amistar drove a significant increment (P < 0.05) in lipid peroxidation (LPO) as evidenced by the rising in serum LPO levels by 1.45 as compared to the control group (Fig. 4C). However, the serum LPO levels were reduced in the rats treated with Amistar and boldo co-administration, the augmentation in serum LPO levels was 1.34% as compared to those in rats exposed to Amistar only (Fig. 4C).

DNA damage

Amistar-exposed rats appeared a significant (P < 0.01) elevation in the rate of DNA damage in the liver tissues compared to control rats (Table 2). However, rats treated with boldo extract exhibited a low rate of DNA damage similar to those in control rats. Moreover, the rats exposed to both the Amistar and boldo extract showed a significant decline in the DNA damage rate as compared to those exposed to Amistar only (Table 2).

Table 2: Effect of Boldo extract against Amistar induced DNA damage in liver tissues

Treatment	No. of cells		Class[¥] of comet				DNA damaged cells (mean ± SEM)
	Analyzed	Total comets	0	1	2	3	
G1: Control	500	33	467	25	8	0	6.64±0.81[c]
G2: Boldo extract	500	31	469	28	3	0	6.23±0.58[c]
G3: Amistar	500	113	387	38	32	43	22.58±1.29[a]
G4: Amistar + Boldo extract	500	74	426	29	27	18	14.84±0.67[b]

[¥]: Class 0= no tail; 1= tail length < diameter of nucleus; 2= tail length between 1X and 2X the diameter of nucleus; and 3= tail length > 2X the diameter of nucleus.(*): No of cells analyzed were 100 per an animal. Data are presented as mean ± SEM. [a,b,c,d] Mean values within treatment with unlike superscript letters were significantly different (*P*< 0.05).

ROS formation

Generation of ROS levels in liver samples of rats exposed to Amistar was increased considerably (P <

0.05) in comparison with control rats (Fig. 5). On the other hand, animals treated with boldo extract showed relatively similar ROS levels to those in control rats. While, the rats exposed to both the Amistar and boldo extract appeared significant reduction in the ROS levels as compared to those exposed to Amistar only (Fig. 5).

Expression of stress and antioxidant-related genes

Expression levels of stress-related genes (IL-2, TNF-α and CYP2E1) in the rats' liver tissues exposed to Amistar were considerable ($P<0.05$) increased as compared to those in control rats (Fig. 6A to 6C). Moreover, rats exposed to Amistar only showed a significant reduction in the expression levels of antioxidant-related genes (GPx and GR) as compared to control rats (Fig. 6D and 6E). On the other hand, the expression levels of IL-2, TNF-α and CYP2E1 genes were considerably reduced in the animals exposed to Amistar and boldo extract simultaneously as compared to those exposed to Amistar only (Fig. 6A to 6C). Also, expression levels of GPx and GR genes were considerably elevated in the animals administrated simultaneously with Amistar and boldo extract as compared to those exposed to Amistar only (Fig. 6D and 6E)

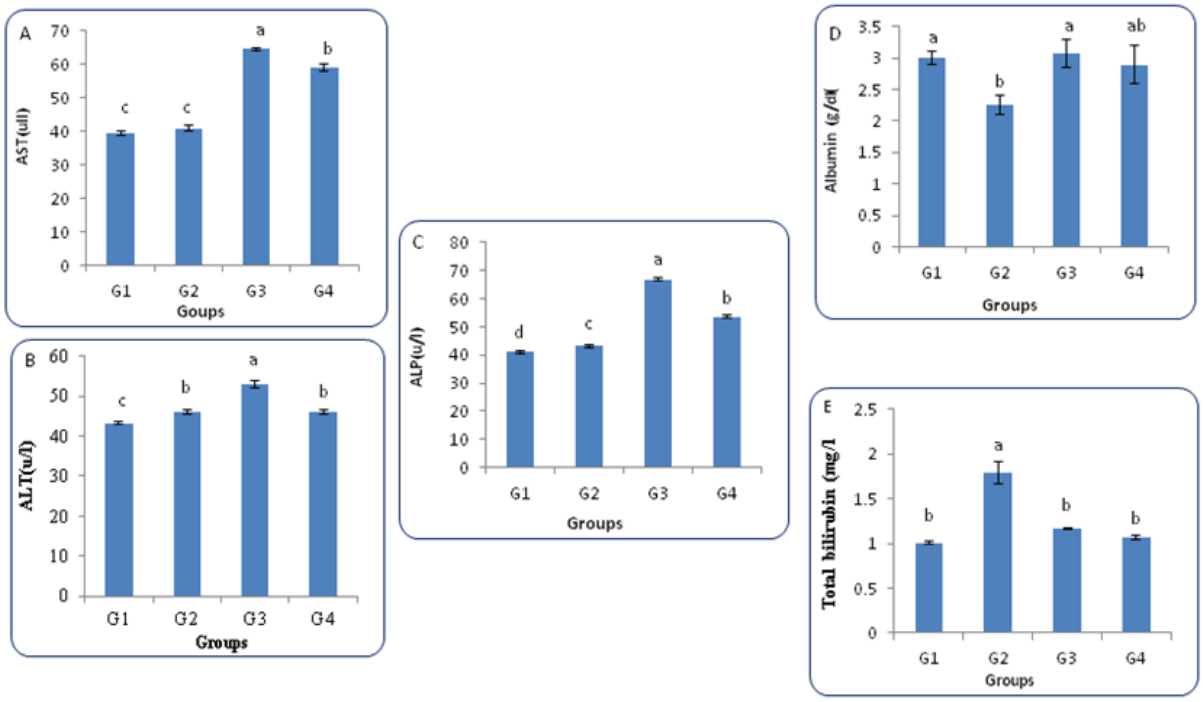

Fig. 3: Effect of boldo extract on Amistar fungicide induced alteration in aspartate (A) AST, (B) ALT, (C) ALP, (D) Albumin total and (E) bilirubin concentration in the sera of female rats. Each value is a mean of 5 rats ± SEM; a, b c values are not sharing superscripts letters vary significantly at $P \leq 0.05$, (G1) control, (G2) boldo extract group, (G3) Amistar group (G4) Amistar + boldo extract group.

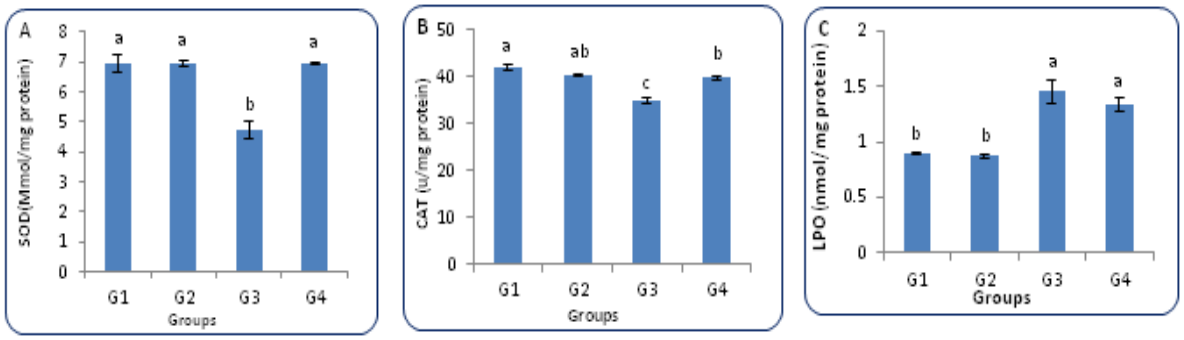

Fig. 4: Effect of boldo extract on Amistar fungicide induced alteration in (A) superoxide dismutase, (B) catalase and (C) lipid peroxidtion in the tissue of female rats. Each value is a mean of 5 rats ± SE; a, b, c values are not sharing superscripts letters vary significantly at $P \leq 0.05$, (G1) control, (G2) boldo extract group, (G3) Amistar group (G4) Amistar + boldo extract group.

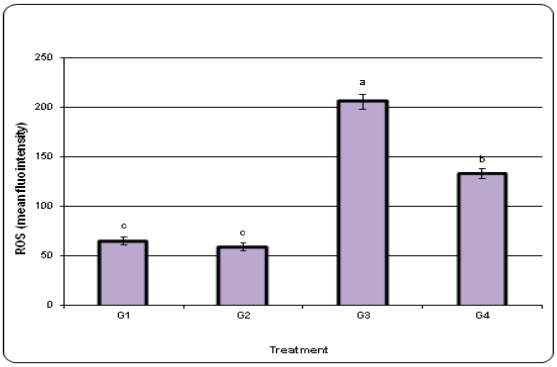

Fig. 5: The influence Boldo extract against Amistar induced alterations in intracellular ROS generation levels in liver tissues. G: Control; G2: Boldo extract; G3: Amistar; G4: Amistar + Boldo extract. Results have appeared as the mean ± SD. a,b,c Mean with different letters, within the tissue, differ significantly (P< 0.05).

Histopathological alteration

Histological investigations of liver sections from rats of control and boldo extract groups show the normal hepatic lobules architecture. The central veins lie at the center of the lobules embraced with hepatocytes cords. The hepatic sinusoids are present between the strands of hepatocytes (Fig. 7A & 7B). In the case of rats given Amistar, microscopic examination showed the disturbance of the hepatic lobule. In the blood sinusoids, the focal necrosis of some hepatocytes, and cell debris were found. Also, some nuclei showed hyperchromasia and other showed pyknosis (Fig. 7C). On the other hand, Amistar plus boldo extract-treated group were showing a dilated and congested vein (Fig. 7D).

Grading of the histopathological alterations like hepatic lobule disturbance, hepatocytes focal necrosis, cell debris in sinusoids, and hyperchromasia that occurred in the liver of rats in different tested groups was presented in Table 3. Rats administered with Amistar exhibited severe disturbance of the hepatic lobule, moderate focal necrosis of hepatocytes, as well as cell debris in the blood sinusoids, mild nuclei hyperchromasia, and others showed pyknosis. However, rats were given Amistar plus boldo extract showed a mild dilated and congested vein was present (Table 3).

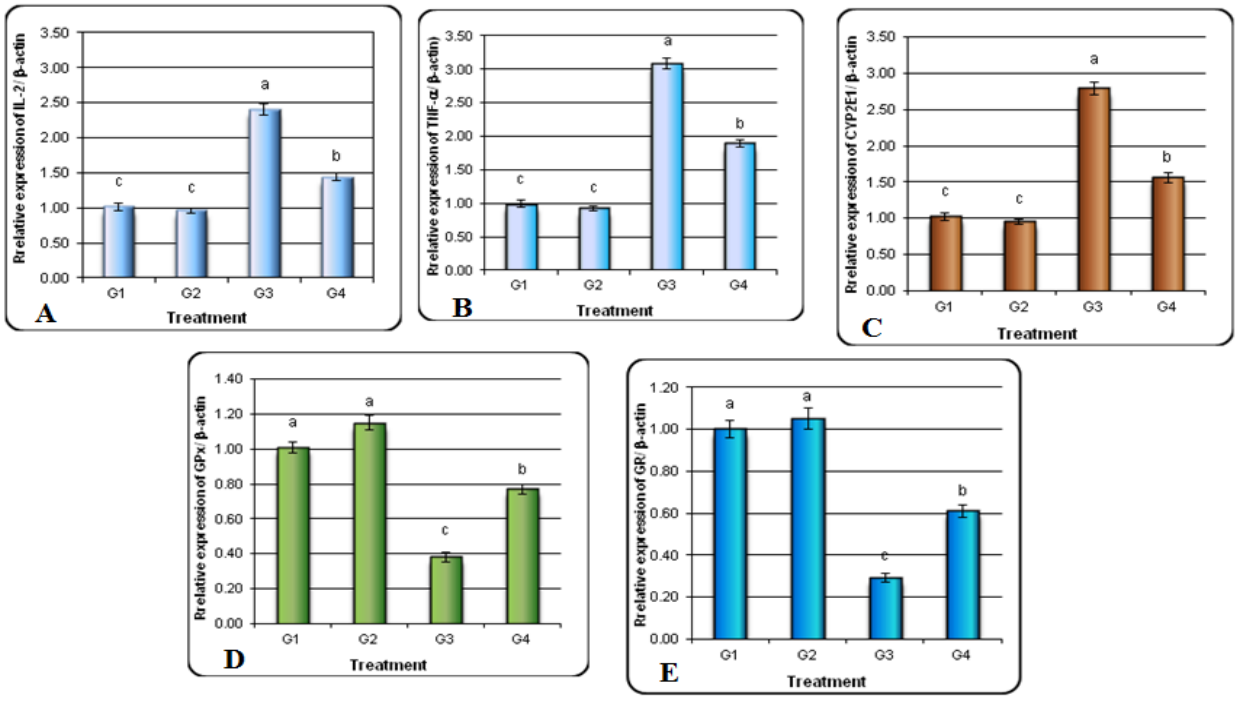

Fig. 6: Alteration in the expression levels of (A) *IL-2*, (B) *TNF-α*, (C) *CYP2E1*, (D) *GPx*, and (E) *GR* genes in liver tissues of rats treated with Boldo extract and/or Amistar. G: Control; G2: Boldo extract; G3: Amistar; G4: Amistar + Boldo extract. Data are shown as mean ± SEM. [a,b,c] Mean values within tissue with unlike superscript letters were significantly different (P< 0.05).

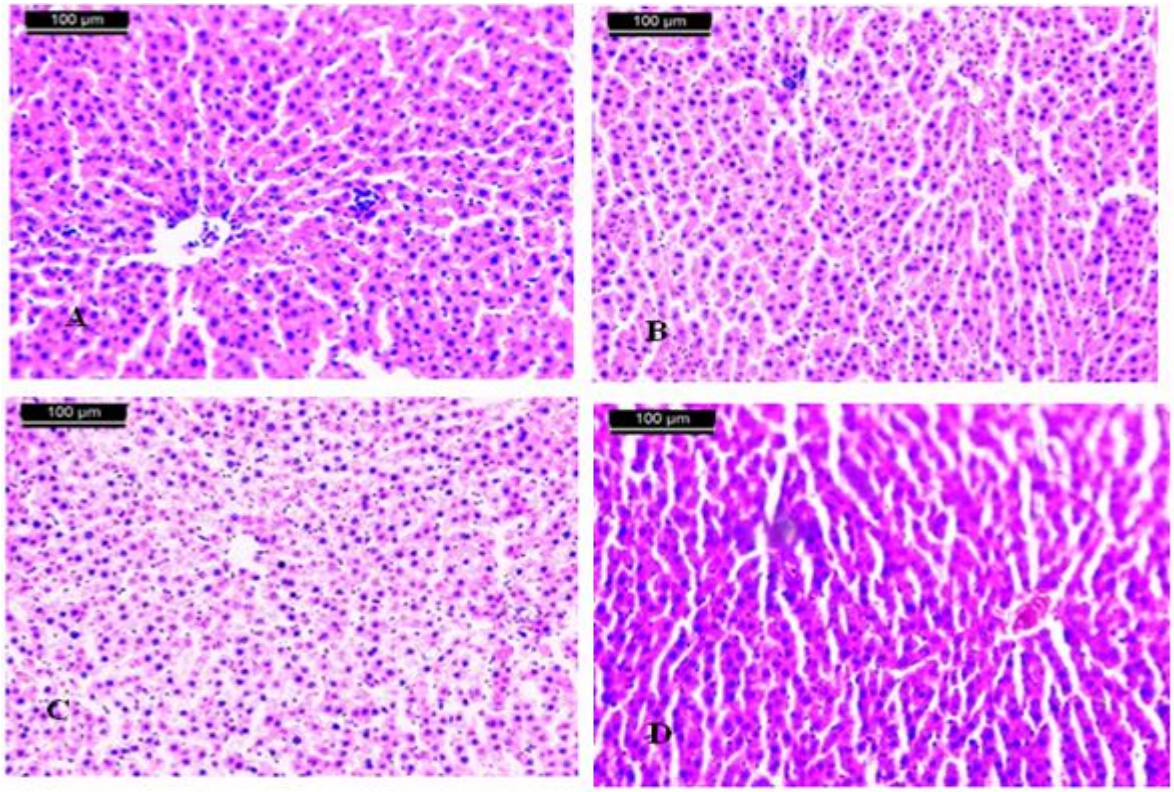

Fig. 7: Photomicrographs of liver sections from (A, B) normal liver tissue in the control group and boldo extract showing the normal architecture of the hepatic lobule. The central vein (CV) appears at the center of the lobule surrounded by cords of hepatocytes (HC). Between the strands of hepatocytes, the hepatic sinusoids are presented (HS), (C) Amistar at a high dose showing the disturbance of the hepatic lobule. Notice the focal necrosis of some hepatocytes, the presence of cell debris in the blood sinusoids. Some nuclei showed hyperchromasia and other showed pyknosis. (D) Amistar plus boldo extract shows a dilated and congested vein (H & E stain, Scale bar 100 µm).

T**able 3:** Grading of the histopathological alterations in the liver of rats, exposed to Amistar fungicide and Boldo extract.

Histopathological injury	G1	G2	G3	G4
Hepatic lobules disturbance	-	-	+++	+
Nuclei hyperchromasia	-	-	+	+
Nuclei pyknosis	-	-	+	-
Cell debris in sinusoids	-	-	+	
Focal necrosis	-	-	++	-
Dilated and congested vein	-	-	-	+

None (–), mild (+), moderate (++), severe (+++).
(G1) control, (G2) boldo extract group, (G3) amistar group (G4) Amistar+ boldo extract group.

Discussion

The widespread application of fungicides in agriculture leads to increased sub-chronic or chronic toxic effects on organisms and environmental pollution. Therefore, when animals or humans are exposed to contaminated food with fungicides, it has negative public health effects, especially on the digestive system. Some studies have reported that excessive fungicides exposure leads to an increase in the incidence of liver and colon cancer **[30]**.

The liver plays a vital role in the xenobiotics and harmful compounds' detoxification and digestion. Therefore, hepatotoxicity and health risks are produced by some form of change in its function. Liver biomarker enzymes such as AST, ALT, and ALP are good indicators for liver disease and liver damage **[10]**. Therefore, hepatic biomarkers can be used as an early warning in the growth of diseases. Hepatocytes contain AST, ALT, and ALP enzymes that play altered metabolic roles, and their elevation is considered as hepatic injury biomarkers **[10]**. In the current study, the integrity of the liver is controlled by serum ALT, and AST markers. The present results revealed a significant rise in ALT and AST. ALT is a cytosolic enzyme predominantly expressed by hepatocytes, which involves liver cell lysis and releases of the enzyme into the blood, thus letting the agrochemicals cytotoxically affect the liver **[10]**.

Exposure of the cells to chemical pollutants can possibly increase the production of ROS and probably produce a change in the balance of cellular redox levels ultimately resulting in more oxidative damaged biomolecules. Changing the normal redox

balance can change the different enzymes activity and cell signaling pathways in tissues and can therefore be an important mechanism for exercising intoxication of different xenobiotics and resolving the pathogenesis of many diseases **[31]** .

The free radicals production, especially ROS, proposes the most excellent mechanism to clarify pesticide-induced toxicity at a high dose **[10, 32]**. To the finest of our findings that ROS generation increased considerably in rats exposed to Amistar compared to control rats. Associate this suggestion, azoxystrobin caused oxidative damage as a result of the creation of the free radicals that persuaded injury to cell components, such as lipids and DNA damage as well as the alteration in the gene expression patterns **[33]**. The present study proved that Amistar fungicide was able to increase the DNA damage rate and alter the stress expression and antioxidant-related genes in male rats compared with control rats. In the same line, **Cobanoglu et al. [34]** reported that treatment of human leukocytes with fungicide (signum) in vitro induced significant DNA damage at different doses. Moreover, micronucleus frequency and DNA damage were elevated in erythrocytes exposed to fungicides **[35]**.

Numerous studies have illustrated the embroilment of ROS and oxidative stress in fungicide toxicity, and it is generally clarified that ROS induced DNA damage **[36, 37]**. Other studies exhibited that fungicide may prompt oxidative stress and that ROS could have a risky role in fungicide-induced genotoxicity in vitro **[38, 39]**. **Hashem et al. [40]** presented that oral exposure to fungicide stimulated DNA damage in the liver of female rats and clarified the mechanism through oxidative stress elevation and antioxidant defense system suppression.

The present study exhibited that treatment of male rats with Amistar fungicide induced expression alteration in stress-related genes (IL-2, TNF-α and CYP2E1) and antioxidant (GPx and GR) genes in liver tissues. Little information is available regarding the effect of fungicides on gene expression changes. **Arabi et al. [41]** proved that treatment of Cochliobolus sativus with fungicides induced elevation in the expression levels of CYP genes. They reported that fungicides affecting the expression levels in CYP genes could be credited to increase the oxidative stress mediated-ROS generation in the cells.

Boldo active constituents (gallic acid, ellagic acid, querectin, daidzein, ferulic acid, and rutin) possess suppression effects on azoxystrobin-induced liver damage. The influence of gallic acid and querectin is derived from free radical scavenging and the anti-inflammatory effects of TNF-α as well as suppressing IL-2 and CYP2E1 genes **[42-46].**

Medicinal plants are highly important to public health, so they are commonly employed as antioxidants and free radical scavengers **[47]**. Boldo leaves contain a variety of alkaloids and flavonoids. Boldene is the most important alkaloid compound found in boldo plant. It belongs to the aporphene class. This compound is used in folk medicine, especially in the treatment of stomach disorders and as a metabolic stimulant **[48]**. Additionally, boldine, which is found in the boldo leaves, has been shown to be the primary contributor to antioxidant activity **[48]**. Furthermore, catechin is one of the natural phenolic compounds found in boldo. It belongs to the family of flavonoids **[48]** and has the highest antioxidant potential **[49]**. Within our experiment, rats pretreated with a boldo extract and subsequently treated with a fungicide showed significantly diminishing lipid peroxidation in the hepatic tissue than rats treated only with the fungicide Amistar. This data proves that a boldo extract could avoid the oxidative damage caused by Amistar fungicide. The results of this study show that rats pretreated with boldo extract have significantly lower lipoperoxidation than rats only treated with Amistar fungicide. The existence of boldine and catechin substances in the boldo extract could be act as free radical scavengers due to the high antioxidant activity of these compounds **[48]**. Boldine efficacy is attributed to the creation of phenoxy radicals and other free radical species as a result of the oxidation of boldine's aporfinic structure and catechin's polyphenolic structure which degrade superoxide anions, hydrogen peroxides, and hydroxyl radicals **[49]**.

Pretreatment of rats with boldo extract followed by exposure to Amistar increased the antioxidant activities of SOD and CAT as well as elevated the antioxidant genes expression (GPx and GR). This action could be credited to Boldine, which has also been shown to restore the function of antioxidant enzymes. SOD and CAT work together to eradicate superoxide radicals from cells. SOD converts hydroxyl radicals to hydrogen peroxide (H_2O_2) and O_2, and CAT, as well as GPx, catalyzes the decomposition of H_2O_2 to H_2O. Additionally, GR is activating the glutathione molecules by catalyzing the reduction of glutathione disulfide to glutathione. However, the diminished activity of CAT and SOD, as well as reduction of the expression levels of GPx and GR due to Amistar exposure, is caused by the reinforced production of superoxide radicals that restrain catalase activity **[50]** leading to increase H2O2 levels that suppress SOD because reflect overproduction of free radicals **[51]**. Additionally, the boldo extract activity is chiefly a result of the catechin presence, which is consistent with the elevation free radical scavenging capacity of the infusion attributed to catechin **[52]**. The action mechanism of the active ingredients of boldo extract augmented the potential that protecting the DNA structure from damage in liver tissues is probably due to enhancement of the production of the antioxidant enzymes which inhibiting the generation of ROS molecules.

The disturbances in liver marker enzymes and oxidative stress in male rats in the current study were distinctive of initial hepatic cell injury that showed in liver sections of histopathological examination in the Amistar-treated group. The histological variations may be due to the creation of free radicals that caused liver tissue deterioration as appeared by promoted hepatic LPO, which has a critical role in hepatic cellular membrane injury. The current results are inconsistent with plentiful studies, which reported that fungicide induced some histopathological alterations in the liver **[53]**. They indicated that these toxic impacts of fungicides on the liver and gastrointestinal tract could be due to the genotoxic effects prompted by fungicides, which were detected in the current experiment and supported by the findings from precedent studies.

We showed that after administration of boldo extract, the amelioration in liver enzymes and antioxidant enzymes compared to the control group. Because we have little influence over the quantities of endogenous antioxidants, it would be sensible to augment the exogenous antioxidants (primarily by ingestion) to boost individuals' protective properties against environmental oxidative stress **[54]**. According to current studies, improving the concept of dietary supplementation with natural antioxidants would significantly increase the protection against infections.

In conclusion, the data obtained in our study let us recommend utilize of boldo leaf extract as an active chemoprotective agent as a result of its influence that avoids the oxidative damage originated by Amistar fungicide on the hepatic tissues. Future studies can exactly decide if boldo extract's activity results only in its capacity to function against free radicals or if it also contains a restoring activity at the levels of the oxidative enzymes.

Author contribution

-All authors were complicated in the design of the research paper and analyses as well as the collection of data. WKBK, MGE, SMK and HFB have performed genotoxicity and molecular biological analyses. ARHF has performed histopathology. AAR and ABS performed the HPLC and biochemical analysis as well as interpretations of the data obtained from this study and were major contributors in writing the manuscript. All authors have contributed to writing the manuscript, read and approved the final version.

Data availability and Declarations

-The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

- Declarations Ethics approval of the experiments was completed under the standard conditions in the Animal Breeding House (ABH), of the National Research Centre (NRC), Dokki, Cairo, Egypt. The Local Ethics Committee at the National Research Centre (NRC) approved the work under the number (14114052021).

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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