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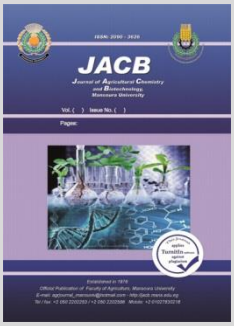
Antioxidant Activity of Some Natural Compounds in Alleviating the Hepatotoxicity Effects Induced by Emamectin Benzoate in Male Mice

Sherifa F. M. Dawoud [1]; T. M. M. Al-Akra[2] and Amina M.G. Zedan[1*]

[1]Biological and Environmental Sciences Dept. Faculty of Home Economic, Al-Azhar University
[2]Agric. Zoology and nematology Dept. Faculty of Agric., Al-Azhar University



ABSTRACT

Emamectin benzoate (EMB) is a biopesticide that is used in agriculture as an insecticide. Due to its wide range of use, it is easier to reach ecologically and affects human health. This study aims to evaluate the protective effect of natural compounds against EMB-induced hepatotoxicity. It is the first study quantifying the hepatoprotective effect of these extracts against EMB effects. Biochemical analysis includes MDA, CAT, SOD, ALT and AST analysis. However, genetic studies include the gene expression of Mgst1, CYP2E1, caspase3, IL-1β and DNA fragmentation, as well as, histopathological investigations were performed. Male mice were distributed into five groups: G1: the negative control, G2: EMB group (5mg/kg diet), G3: EMB+*Boswellia serrata* (90 mg/kg diet), G4: EMB+*Cinnamomum zeylanicum* (600mg/kg diet), G5: EMB+ powder of the snail (600mg/kg diet), and the experiment continues for eight weeks. The results appeared that EMB induced oxidative stress in the liver by increase the activity of MDA. The biomarker of liver injury ALT and AST were elevated with antioxidant enzymes inhibition. EMB produced several histopathological changes in the liver. Relative expressions of Mgst1, CYP2E, caspase-3 and IL-1β genes elevated in the liver. The increase in DNA damage was noticed as recorded by an increase in tail length, tail DNA% and tail moment. Co-treatment with natural compounds reduced the toxicity of EMB. They reduce the abnormal biochemical, histopathological, gene expression and DNA damage by increasing antioxidant capacity. Therefore, the natural compounds used in this study act as potent hepatoprotective agents against EMB induced hepatotoxicity in mice.

***Keywords*:** Emamectin benzoate; hepatotoxicity; gene expression; DNA fragmentation; antioxidants

INTRODUCTION

Pesticides used in public health or in agriculture sector have various actions on metabolic mechanisms. It affects on non-target animal and human health (El-Bialy *et al.*, 2020). One of macrocyclic lactone is emamectin benzoate (EMB). It is developed as a pesticide formed by avermectins fermentation. The chemical structure contains 10% avermectin B1b and 90% avermectin B1a (Hayes and Laws 2013). In the human central nervous system, γ-aminobutyric acid-reactive neurons are limited so EMB was considered safe to human (Roberts *et al.*, 1984), but the EMB-lipophilicity makes it penetrates the membranes of the cell. Consequently, it creates toxicity in animals and humans (Wolterink *et al.*, 2011). Although the data about the effect of EMB on antioxidant status is insufficient. Many studies confirmed that avermectins insecticides created oxidative stress in intoxicated animals. Similarly, it created the kidney and liver oxidative stress in offspring and mothers after lactational exposure (Mossa *et al.*, 2017). One of the mainly valuable medicinal plants is *Boswellia serrata* (BS) (Mark, 2018). The treatment with BS significantly leads to a decrease in liver fibrosis. BS has the ability for recovering endogenous antioxidant mechanisms. Subsequently, it scavenges free radicals and allows hepatocyte regeneration (Eltahir *et al.*, 2019). Cinnamomum zeylanicum (CZ) prevents and treats many diseases (Hussain *et al.*, 2019). Cinnamon is utilized extensively as an herbal drug (Morgana *et al.*, 2014). It has been applied to the remedy of gastric diseases and inflammatory disorders (Shen *et al.*, 2012). Phytochemical study of cinnamon has detected a lot of coumarins, flavonoids, alkaloids, glycosides, steroids, anthraquinone, terpenoids and tannins (Shihabudeen *et al.*, 2011). Cinnamon's hepatoprotective impact has been reported by Eidi *et al.* (2012). Garden snails are one of invertebrates. To date, it has given a wide variety of natural products. It is including terpenes, aliphatic hydrocarbons, alkaloids, steroids, amino acids, peptides, and carbohydrates (Leal *et al.*, 2012). The natural products of snails have a wide array of remedial properties including; antimicrobial, anticoagulant, immune-modulating, wound healing, anticancer, antioxidant, antihypertensive and anti-inflammatory (Perdicalis *et al.*, 2013).

Consequently, the aim of this study was to evaluate the protective role of some natural compounds against oxidative stress and hepatotoxicity induced by EMB in mice.

MATERIALS AND METHODS

Emamectin benzoate (EMB) have a trade name called Speedo (5.7% WG) was purchased from EL-Shoura chemicals Co., was used in this study as insecticide.

Plant material and extraction

For preparing methanol extract of *Boswellia serrata* and *Cinnamomum zeylanicum*, the methods of Yazdanpanahi, *et al.* (2014) and Ojarudi**,** *et al.* (2020) were used respectively with some modifications belong to the concentrations. The cinnamon bark and gum resin of *Boswellia serrata* were obtained from the local market.

* **Corresponding author.**
E-mail address: AminaZedan1948.el@azhar.edu.eg
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They were crushed into small pieces. Two hundred grams were placed in a flask and macerated with 600 mL methanol in a water bath with a shaker for 2 hours at 50 °C. Then, the mixture was filtered with Whatman No. 1 filter paper more than once and dried at room temperature. The crude extract was stored in a well-closed container, protected from light, and kept at 4°C.

Preparation snail's powder

The garden snails (*Eobania vermiculata*) were dried in the oven at 50-60 ^{0}C for two hours. Then ground in a blender to a very fine powder**.**

Chemical Composition

The chemical composition of natural extracts and snail's powder was estimated. Bioactive compounds are measured by GC/MS System (Thermo Scientific TRACE 1310 Gas Chromatograph, USA) attached with an ISQ LT single quadrupole mass spectrometer according to Hadi and Hameed (2017) at The Institute of Marine Biology research, The National Research Center

Animals and groups

In this experiment, 45 male albino mice with an approximate weight of 19 gm were used. The animals were maintained in the standard condition (temperature 22 ± 2°C, with a light/dark cycle of 12 hr) in stainless steel cages in an artificially illuminated. After compatibility, the animals were divided randomly into five groups, each containing nine animals for each treatment. They were treated as follows; G1: mice are given a diet without any additives, G2: mice are treated with EMB at a dose of 5 mg/kg (National Registration Authority for Agricultural and Veterinary Chemicals, 2011) diet (equivalent to 1/10 LD50), G3: mice are treated *Boswellia* (90 mg/kg diet) (Yassin *et al*., 2013) with EMB, G4: mice are treated *Cinnamomum* (600 mg/kg diet) (Prasanna and Anand 2019) with EMB, G5: mice are treated powder of the snail (PS) (600 mg/kg diet) with EMB (this is the first study on PS). After eight weeks mice have fasted overnight. They were sacrificed after anesthetized using diethyl ether and their organs (liver, kidney, spleen, heart, and testis) were removed and immediately weighed after sacrifice. The liver was quickly removed and stored in liquid nitrogen until transferred to -80 and then used for genetic studies. Another portion of liver stored in formalin 10% for histological studies.

Biochemical studies

Blood samples were taken in a glass tube without EDTA and left for two hours for coagulating at room temperature. It was centrifuged at 4000 rpm for 20 min to obtain sera samples. Serum samples were kept at −20°C until used for biochemical assays. According to the technique of Reitman and Frankle (1957) AST and ALT activity were measured. Malondialdehyde (MDA) was investigated according to the technique of Ohkawa *et al. (*1979). Catalase (CAT) was estimated according to Aebi (1984). Superoxide dismutase (SOD) was estimated according to Nishikimi *et al.* (1972).

Histopathological examination

Small parts of the liver were fixed in formalin solution (10%). They dehydrated in ethanol from 70% to 100%. Then, they cleared in xylene and transfer to paraffin. The sections of liver stained with Eosin and Hematoxylin dyes (Suvarna *et al.*, 2013).

Molecular analysis

The RNeasy kit (Qiagen) was used for isolated total RNA from the liver as described by Abd-Allah *et al.* (2015). The purity and integrity of RNA were assessed by Nanodrop, and 1% agarose gel electrophoresis, respectively. The Quantiscript reverse transcriptase was used in RNA reverse transcription to cDNA. Real-time PCR reaction contains cDNA as a template in the presence of QuantiTect SYBR Green qPCR Master Mix and gene-specific primers, designed by the Primer 3 web-based tool based on the published mouse sequence (Table 1), along with Step One Plus real-time PCR system (Applied Biosystem, USA) and reaction cycles as described by Khamis *et al.* (2018). The critical threshold (Ct) quantities for the target genes were normalized with quantities of the Ct of the internal control (*β-actin*).

Table1. Primers used for real-time PCR.

Gene	Forward primer (/5 ------ /3)	Reverse primer (/5 ------ /3)
Mgst1	TTTTGCCAACCCGGAAGACT	GAGGCCGATACCGAGAAAGG
Cyp2E1	CTCCTCGTCATATCCATCTG	GCAGCCAATCAGAAATGTGG
Caspase 3	GGTATTGAGACAGACAGTGG	CATGGGATCTGTTTCTTTGC
IL1b	CACCTCTCAAGCAGAGCACAG	GGGTTCCATGGTGAAGTCAAC
β-actin	AAGTCCCTCACCCTCCCAAAAG	AAGCAATGCTGTCACCTTCCC

Comet assay

Comet assay was performed according to Eldamaty *et al.* (2021). In brief, a weight of 1 gram of crushed samples was transferred to 1 ml ice-cold PBS. This suspension was stirred for 5 min and filtered. 100 µ1of cell suspension was integrated with 600 µ1 of agarose (low-melting, 0.8% in PBS). This mixture (100 µ1) was spread on slides. The slides were immersed in lysis buffer for 15 min. Then they were placed in the electrophoresis chamber containing the same lysis buffer without SDS. The conditions of electrophoresis were 100 mA and 2 V/cm for 2 min. Staining with ethidium bromide. The DNA fragment migration patterns were evaluated with a fluorescence microscope at a magnification of 40x and with excitation filter 420-490nm. Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpoo1, UK) attached to a CCD camera. It was used to assess the qualitative and quantitative extent for DNA damage by calculates the DNA-migration length and the percentage of migrated DNA.

Ethics approval

This experiment was carried out under Egyptian ethical codes for studies on experimental animals and approved by the Ethics Committee of Al-Azhar University. The experimental protocol was approved by the Biological and Environmental Sciences Department, Faculty of Home Economics, Al-Azhar University, Egypt.

Statistical analysis

All the data were expressed as means ±SD. The statistical significance was evaluated by one-way ANOVA

(analysis of variance) using SPSS version 20 software, and the individual comparisons were obtained by Duncan's multiple range test (DMRT). Values were considered statistically significant when $p<0.05$ (Bryman and Cramer, 2011).

RESULTS AND DISCUSSION

GC-MS analysis

The chemical compositions of methanol extract for *Boswellia serrata* are shown in Table 2. Fourteen compounds were identified. The major comprised organic compounds were 1,6,10-Dodecatrien-3-ol,3,7,11 trimethyl-, (E)- (16.19%) at RT 17.9 that acts as antidiabetic, hepatoprotective and anti- inflammatory activities. Also it contains 1-Heptatriacotanol that has antioxidant, anticancer, anti inflammatory and sexhormone activity (Shareef *et al.*, 2016) and this compound present in BS, CZ and PS (Kalairasan *et al.*, 2011). The major components of the essential oils of the Boswellia found in this study is Isopropyl-1,5,9-trimethyl-15 oxabicyclo[10.2.1]pentadeca-5,9 dien-2-ol (Isoincensole) with concentration 58.30% (Awoke and Joshi, 2021).

Table (3) illustrated that there are twenty one of bioactive compounds in Cinnamon, the major are 1-[1-(2,2 Dichlorovinylimino)-2,2-di methylpropyl]-3-(p tolyl)thiourea (7.30%), 1-Heptatriacotanol (8.51%) and 24-Norursa-3,12-diene (8.43%). The bioactive compounds presented in Cinnamomum that played a major role in reducing the side effect of EMB as Oleic acid that found in CZ and PS and have anti-inflammatory, anti-androgenic, anti-cancer, preservative and hypocholesterolemic characters (Sreekumar *et al.*, 2014). PS also contain bioactive compounds that have antiinflammatory, hypocholesterolemic, cancer preventive, and hepatoprotective effects such as 12,15-Octadecadienoic acid, methyl ester, (Z,Z,Z)- (Rehana and Nagarajan 2013). The bioactive compounds of PS are showing in Table (4). There are twenty bioactive compounds, the major compounds are 2-(2 butoxyethoxy) ethyl acetate (11.49%) and benzene, 1,2,4-trimethyl- (21.39%).

Table 2. Bioactive compounds identified in methanolic extract of *Boswellia serrata*

n	RT	Compound Name	Area %	Peak Area	Formula	MW
1	7.48	Cyclopropane, pentyl	2.52	1711415172.16	C8H16	112
2	10.20	1-HEXANOL, 2-ETHYL-,ACETATE	4.55	3091011881.26	C10H20O2	172
3	17.90	1,6,10-Dodecatrien-3-ol,3,7,11 trimethyl-, (E)-	16.19	10998543245.16	C15H26O	222
4	18.27	Dodecanoic Acid, Ethyl Ester	0.98	662240817.76	C14H28O2	228
5	24.32	1,3,6,10 Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1 methylethyl)-, [S-(E,Z,E,E)]-	1.01	686677160.95	C20H32	272
6	24.85	(R,1E,5E,9E)-1,5,9-Trimethyl-12-(prop-1-en-2-yl)cyclotetradeca-1,5,9-triene	2.40	1630904366.91	C20H32	272
7	25.31	Hexadecanoic acid, ethyl ester	0.81	550653815.15	C18H36O2	284
8	25.75	(S,E)-8,12,15,15-Tetramethyl-met hylenebicyclo[9.3.1]pentadeca-7,11-diene	1.27	859650907.34	C20H32	272
9	26.45	Thunbergol	6.72	4564371557.29	C20H34O	290
10	27.00	1-Heptatriacotanol	1.25	845938343.56	C37H76O	536
11	27.91	(3E,7E,11E)-1-Isopropyl-4,8,12trime thylcyclotetradeca-3,7,11-trienol	1.16	789102248.63	C20H34O	290
12	28.17	Isopropyl-1,5,9-trimethyl-15 oxabicyclo[10.2.1]pentadeca-5,9 dien-2-ol	58.30	39595790847.85	C20H34O2	306
13	30.12	Nerolidol-Epoxyacetate	1.90	1287616100.01	C17H28O4	296
14	37.70	15,17,19,21 Hexatriacontatetrayne	0.94	641545071.66	C36H58	490

Table 3. *Cinnamon zeylanicum*-bioactive compounds identified in methanolic extract

n	RT	Compound Name	Area %	Peak Area	Formula	MW
1	4.14	1-[1-(2,2 Dichlorovinylimino)-2,2-di methylpropyl]-3-(p tolyl) thiourea	7.30	60352088.42	C15H19Cl2N3S	343
2	4.23	12,15-Octadecadiynoic Acid, Methyl Ester	5.95	49209036.43	C19H30O2	290
3	4.78	Acetic acid, Octyl Ester	2.64	21792402.94	C10H20O2	172
4	4.87	1-Deoxy-d-arabitol	5.27	43599137.66	C5H12O4	136
5	5.38	Benzene, 1-Ethyl-3-Methyl-	4.24	35021407.31	C9H12	120
6	5.94	Benzene, 1,2,3-Trimethyl-	5.19	42936038.66	C9H12	120
7	6.49	Butanoic acid,2-amino-4 (methylsulfinyl)-, (ñ)-	3.10	25661984.56	C5H11NO3S	165
8	7.73	2-Myristynoyl pantetheine	3.79	31359300.08	C25H44N2O5S	484
9	9.11	2-t-Butyl-5-propyl [1,3]dioxolan-4-one	4.28	35417344.02	C10H18O3	186
10	11.84	Isobornyl thiocyanoacetate	2.79	23044475.82	C13H19NO2S	253
11	24.32	9-Octadecenoic acid (Z)-	4.67	38621464.83	C18H34O2	282
12	27.04	9-Octadecenoic acid (Z)-	6.98	57753324.42	C18H34O2	282
13	28.12	Tetraneurin - A -DIOL	5.50	45458012.84	C15H20O5	280
14	28.48	9-Octadecenoic acid (Z)-	3.25	26853996.60	C18H34O2	282
15	28.55	12-Methyl-E,E-2,13-octadecadien-1-ol	1.25	10295788.30	C19H36O	280
16	28.61	1,2,3-propanetriyl ester, (E,E,E)-	4.10	33897683.33	C57H104O6	884
17	30.14	2-Hydroxy-3-[(9E)-9-Octadec Enoyloxy]propyl	3.33	27495030.34	C39H72O5	620
18	30.50	Ethyl iso-allocholate	4.00	33058044.47	C26H44O5	436
19	30.74	1-Heptatriacotanol	8.51	70329474.00	C37H76O	536
20	30.86	24-Norursa-3,12-diene	8.43	69688437.10	C29H46	394
21	31.13	Methyl Commated	5.45	45039447.46	C31H50O4	486

Table 4. chemical compounds identified in powder of snails

n	RT	Compound Name	Area %	Peak Area	Formula	MW
1	4.11	Benzene, 1,2Dimethyl-	4.38	111174139.15	C8H10	106
2	4.28	12,15-Octadecadiynoic acid, methyl ester	1.20	30377255.91	C19H30O2	290
3	4.42	Benzene, 1,2Dimethyl-	5.98	151704650.28	C8H10	106
4	4.73	2-(2 Butoxyethoxy)Ethyl Acetate	11.49	291626987.71	C10H20O4	204
5	4.98	Benzene, 1-Ethyl-3 Methyl-	1.91	48417446.59	C9H12	120
6	5.10	Benzene, 1-ethyl-3-methyl-	2.20	55904135.75	C9H12	120
7	5.95	Benzene, 1,2,4-trimethyl-	21.39	542893530.62	C9H12	120
8	6.46	Benzene, 1,3,5-Trimethyl-	4.47	113545991.43	C9H12	120
9	6.93	Para tolyl Acetaldehyde	1.53	38957845.11	C9H10O	134
10	7.02	4,6-Decadiyne	1.44	36515281.63	C10H14	134
11	7.08	Benzene, 1,2-Diethyl-	2.33	59222824.33	C10H14	134
12	7.72	2-Oxazolamine,	1.22	30939888.78	C17H17N3O3	311
13	8.40	10,13-Octadecadiynoic acid, methyl ester	1.92	48795220.27	C19H30O2	290
14	9.10	7,10 Pentadecadiynoic Acid	1.49	37826282.48	C15H22O2	234
15	24.30	Cyclopropanebutanoic acid,	1.75	44394544.24	C25H42O2	374
16	27.05	9-Octadecenoic Acid (Z)-	5.13	130272311.97	C18H34O2	282
17	28.13	01297107001 Tetraneurin - a -Diol	2.09	52957950.46	C15H20O5	280
18	30.74	24-Norursa-3,12-diene	2.97	75388425.64	C29H46	394
19	30.86	1-Heptatriacotanol	3.26	82663638.05	C37H76O	536
20	31.13	Ethyl iso-allocholate	1.97	50084419.16	C26H44O5	436

EMB effects

The alterations in the body weight (BW) of the mice of all groups and weight of organs are shown in Table. 5. Mice in EMB groups showed a significant decrease ($p < 0.05$) in the body weight. These results agreed with Khaldoun *et al*. (2015) and El-Sheikha and Gala (2015), they found a decrease in the body weight after treatment with EMB. These decrease due to overstimulation of cholinergic. It causes rise in gastric motility and a reduction in food absorption. These decrease due to reduction in food intake, low palatability of food, or increased lipid and protein degradation due to toxicity related to treatment (Mansour and Mossa, 2010). Treatment with natural compounds effectively alleviate EMB-induced body weight decrease and improves body weight due to they have antioxidant, anti-inflammatory and antifibrotic characteristics. The treatment with powder of snail alleviates the side effects of EMB that induce body weight decrease. Snails has to date produce a wide diversity of products. These products include alkaloids, terpenes, steroids, aliphatic hydrocarbons (Leal *et al*., 2012). Significant decrease was obtained in the weight of kidney when the mice treated with EMB if compared to the negative control and other treatments. The negative control recorded the highest weight of kidney. These results indicated that treatment with EMB and other materials did not affect the mean weight of most organs (liver, testes and heart). The weight of spleen was significantly decreased in the treatments if compared with the negative control.

Table 5. Effect of EMB alone or combined with the natural compounds on the body and organs weight of mice (M ± SD).

Treatment	Body weight (gm)	Organs weight				
		Kidney(gm)	Tests(gm)	Liver(gm)	Spleen(gm)	Heart(gm)
G1	33.78±3.25^{a}	0.29±0.04^{a}	0.13±0.02	1.89±0.36^{a}	0.28±0.02^{a}	0.19±0.03^{ab}
G2	24.08±3.61^{c}	0.21±0.05^{c}	0.12±0.03	1.23±0.49^{b}	0.18±0.04^{b}	0.16±0.03^{b}
G3	30.44.± 5.17^{ab}	0.28±0.04^{ab}	0.13±0.01	1.73±0.50^{ab}	0.16±0.05^{b}	0.22±.0.01^{a}
G4	27.82.±2.12^{b}	0.24±0.02^{b}	0.14±0.01	1.28±0.17^{b}	0.16±0.07^{b}	0.19±0.04^{ab}
G5	29.95±1.90^{b}	0.25±0.02^{ab}	0.13±0.01	1.48±0.18^{ab}	0.11±0.01^{b}	0.16±0.01^{b}
SIG	0.00	0.00	0.48	0.48	0.00	0.06

G1: the negative control, G2: EMB, G3: EMB+ extract of Boswellia *serrate*, G4: EMB+ extract of Cinnamomum *zeylanicum* L, G5: EMB + powdered of the snail, sig: significant at $p < 0.05$, the values with different letters in each column showed a significant difference.

Antioxidants and oxidative markers

In approximately all living cells exposed to oxygen, SOD and CAT played a central role as antioxidant protection. In the current study, EMB-treated mice showed inhibition of SOD and CAT activity (Figure 2, 3). This may be due to a creation of ROS, namely H_2O_2 and superoxide anion. The accumulation of H_2O_2 and superoxide anion can activate some signaling pathways and lead to oxidative stress (Djordjevic *et al.*, 2011). On the other side, EMB significantly amplified MDA concentration (Fig 1) at 60 days of exposure. The production of MDA is a biomarker that determined the level of oxidative stress (Pryor and Stanley, 1975). This increase in MDA agrees with the decrease in CAT and SOD when the mice are treated with EMB. MDA is an outcome of peroxidation for polyunsaturated fatty acid which resulted during degradation by ROS. This supports the incidence of cell toxic stress. Besides, the elevated level of MDA interacts with DNA causing potentially mutagenic effects (Del Rio *et al*., 2005). Subsequently, DNA damage obtained herein was compatible with MDA results (Fig.1). These findings are parallel with those recorded by Zhu *et al.* (2013), who found that EMB inhibited the activity of SOD and increased the level of MDA. These results also agreed with some studies that indicated administration of EMB to mice or rats produces a significant increase of MDA while it decrees activities of CAT and SOD (El-Sheikh and Galal, 2015; Meligi and Hassan, 2017; Mossa *et al.*, 2017). There are many substances that can ameliorate ROS induction by EMB. Abou-Zeid *et al.* (2018) used pumpkin seed oil with EMB with the mice that ameliorated the toxic effects including oxidative stress. This present study revealed that co-administration of EMB with BS, CZ and PS ameliorated the toxic effects of MDA, CAT, and SOD. Boswellic has anti-inflammatory and antioxidant properties. The intake of boswellic acid (an active compound of *Boswellia* species)

protected liver antioxidative and inflammatory injury induction by acetaminophen (Chen *et al.*, 2016). Boswellic acid reduces the formation of MDA and increased SOD and CAT activity (Zhang *et al.*, 2016). Oxidative stress is also inhibited by BS treatment with overall antioxidant potential which increased in the liver (Eltahir *et al.*, 2019). MDA in these results decreased, meanwhile, CAT and SOD increased with co-administration with CZ. These results agreed with El Fadil *et al.* (2020) and Ojarudi *et al*. (2020). They stated that cinnamon extract had a defensive effect against oxidative stress produced by substances. The mechanism involves an increase in antioxidant enzymes (SOD and CAT) and a decrease in MDA levels. The defending action of cinnamon may be due to its inhibition effect on ROS generation during the production of flavonoids and phenolic compounds (Azab *et al.*, 2011). These authors demonstrated indicate the importance of snail's products, which possibly lead to anti-inflammatory agents. Reviews also indicated that there is a lack of reports that have studied the molluscs anti-inflammatory activity (Ahmad *et al*., 2018). The treatment of EMB+PS increased the activity of antioxidants (CAT, SOD) and decreased MDA. This agreed with the previous literature about antioxidants and ROS relationship (Mossa, *et al.*, 2017; Abou-Zeid, *et al.*, 2018).

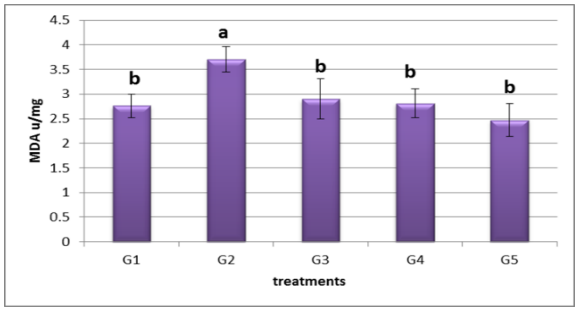

Figure 1. Effect of EMB alone or combined with natural compound on MDA level in mice.

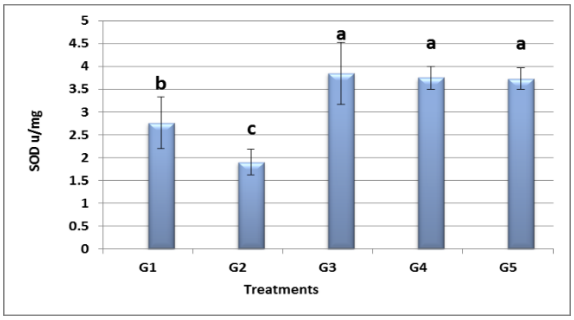

Figure 2. Effect of EMB alone or combined with natural compound on SOD activity in mice

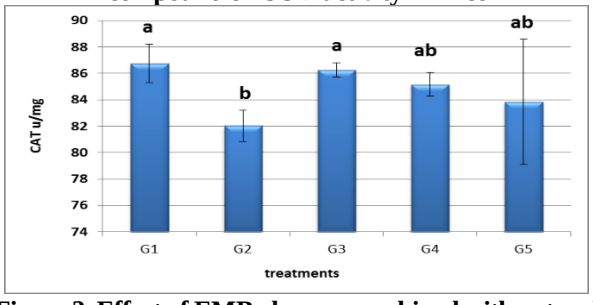

Figure 3. Effect of EMB alone or combined with natural compound on CAT activity in mice.

Biochemical analysis findings

Elevated plasma ALT and AST levels have been reported like a sign of liver cell damage (El-Shenawy and Abdel-Rahman, 1993). With a hepatocellular injury, the secretion of these enzymes increased in the blood. ALT and AST noticeably increase with EMB. This increase is sustaining the hypothesis that exposure to pesticide resulted in toxicity against biochemical liver (Hernández *et al.*, 2013). The results diagrammatic in Figure (4) agreed with Khaldoun *et al.* (2017), who reported a markedly elevation of AST and ALT levels in the plasma of EMB-treated rats. These alterations of AST and ALT activity may be due to necrotic-changes of hepatic tissue that appear in histopathological examination (Figure. 7). Also, these results agreed with El-Sheikh and Galal (2015). Production of ROS caused damage to the diverse membrane components of the cell. This damage leads to infiltrate of cytoplasmic enzymes (Bagchi *et al.*, 1995). Amelioration of the adverse effect of EMB on ALT and AST appears when BS, CZ and PS are treated with EMB (Figure 4). The findings in this respect are in agreement with that obtained by Chen *et al.* (2016), who found inhibition in serum levels of AST and ALT in boswellic acid-treated mice. In addition, histological obtained in this study data further supported the AST and ALT results. Eltahir *et al. (*2019) reported that BS markedly reduces CCl4-induced increases in ALT and AST levels. These results suggested that with its anti-inflammatory, antifibrotic and antioxidant characteristics, BS has hepatoprotective effects against toxic-substances cause liver injury. On the other hand, the treatment of EMB-treated mice with cinnamon ameliorates the adverse effect of EMB. Cinnamon reduces AST and ALT increase that induced by EMB. These results agreed with Hussain *et al. (*2019), who found that the mice treated with cinnamon markedly inhibited acetaminophen-dependent increases in the levels of AST and ALT. In addition, PS has a positive effect against the harmful effects of the insecticide on ALT and AST. It may be due to snail's products which have a wide array of healing properties. These therapeutic properties including antimicrobial, anticoagulant, immune modulating, wound healing, anticancer, antioxidant, antihypertensive, and anti-inflammatory (Perdicalis *et al*., 2013).

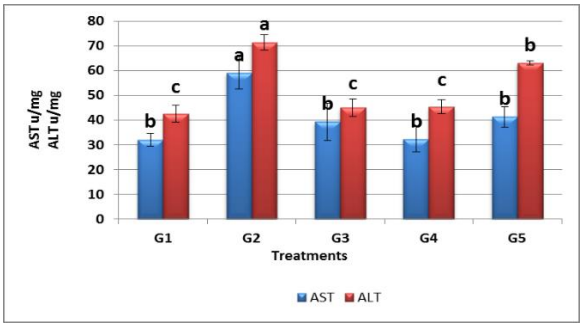

Figure 4. Effect of EMB alone or combined with natural compounds on enzyme biomarkers in the liver of mice. ALT: Alanine aminotransferase, AST: Aspartate aminotransferase

Molecular analysis

The obtained qPCR results showed a significant upregulation in the expression levels of *Mgst1*, *Cyp2E1*,

Caspase3 and *IL1b* genes in the mice liver treated with EMB (G2) as compared to the negative control group (G1). This elevation expression was significantly downregulated following treatment with EMB+BS (G3), EMB+CZ (G4) and EMB+ PS (G5) (Fig.5). Microsomal glutathione S-transferase 1 (Mgst1) gene is responsible for the decrease of lipid hydroperoxides. It binds electrophiles with glutathione. The protein of this gene is a component of the outer membrane of the mitochondria and endoplasmic reticulum. It keeps these membranes of oxidative stress (Johansson *et al.,* 2010). The cytochrome P450 (CYP450) is monooxygenases and catalyzes a lot of reactions implicated in the breakdown of toxic carcinogens. This enzyme contains subcategories like CYP2E1 subtype. CYP2E1 metabolizes both exogenous and endogenous substances (Lewis *et al.,* 2003). Changes in the expression of CYP2E1 gene are involved in much pathology found by toxic substances (Zhou *et al.,* 2009). This alteration may be due to its responsibility in procarcinogens metabolism and drugs as activator enzymes (Wang and Chou, 2010). These findings revealed that subchronic treatment of EMB to mice produced a significant upregulation in the expression levels of Mgst1and Cyp2E1genes in the liver. This elevated expression was significantly ($P \leq 0.05$) downregulated following the treatment with natural compounds (Figure.4). These results agree with Abou-Zeid *et al.* (2018), who reported that the Mgst1 and CYP2E1 genes expression markedly increased in the liver of emamectin-treated mice. In addition Cárcamo *et al.* (2011) and Cárcamo *et al.* (2017) found that the CYP gene expression was upregulated by the treatment with EMB in trout fish. As a result, this suggested that EMB changes the transcriptional process of proteins implicated in the distribution, metabolism and elimination of xenobiotics (Abou-Zeid *et al.* 2018). Similarly, the gene activity increased with treatment by many toxicants (Aniya *et al.* 2000). Mgst1 activation may be contributed to the depletion of glutathione and oxidized glutathione accumulation. The different measurements refer to activation of ROS related to the activation of Mgst1. This correlates with the results obtained herein where EMB activated the CYP2E1 expression and subsequently increased the Mgst1 expression. In accordance with these findings, the activation of CYP2E11, that causes extensive ROS release in a hepatoma cell line, was shown to be improved the expression and activation of Mgst1 (Marí and Cederbaum, 2001). Caspase-3 is the major apoptosis effector protein that is induced by a caspase initiator as caspase-9. The results indicated that EMB-induced cell apoptosis through activation of both caspase-9 and caspase-3 decay intracellular proteins and carry out the program of cell death *(*Zhang *et al.,* 2017). These findings revealed that subchronic treatment of EMB to mice caused a markedly upregulation in the caspase3 gene expression levels in the liver. These results agreed with Azoz *et al.* (2020) who observed that the caspase-3 gene expression obviously increased after administrated rats with EMB. The apoptosis is activated by EMB linked to ROS generation. In turn, ROS triggers the activation pathway of the mitochondrial-dependent intrinsic. It leads to disruption function of mitochondria and consequent potential mitochondrial membrane breakdown to be released cytochrome-c (Zhang *et al.,* 2017). Cytokines are considered essential players in disorders associated with inflammation throughout the body. The quantity of inflammation affects the long term result of liver disease. The pro-inflammatory cytokines Interleukin (IL)-1 play a vital role in several stages of liver diseases. (Niederreiter and Tilg 2018). On the other hand, insecticides are linked with direct or indirect alteration of vital inflammatory activation and immune mechanisms (Miller *et al.,* 2009). Celik-Ozenci *et al.* (2011) and Banerjee *et al.* (2001) established the poisoning of pesticides resulted in the creation of free radicals in organisms and inducing oxidative stress. The free radicals increasing perhaps make modifications in the cell structures and affect the immune function (Liu *et al.,* 2014). Exposure to many insecticides accelerates the formation of IL-1b cytokines (Duzguner and Erdogan, 2010). Liu *et al.* (2014) reported that the creation of cytokines perhaps acts like a kind of compensatory response after undesirable stress effects on an organism. Therefore, IL-1b cytokine is a desirable indicator for studies belong to immune-suppression and related to poisons. The results revealed that mice treated with EMB produces a significant upregulation in the *IL1b* gene expression levels in the liver. These outcomes are in parallel agreed with that reported by Liu *et al.*, (2014), who found that avermectin caused immune suppression by increasing the *IL-1 β*-mRNA levels in the pigeon. Also, Duzguner and Erdogan, (2014), found that imidacloprid stimulated *IL-1b* expressions in the liver. On the other hand, treatment with natural compounds led to a downregulation the expression of genes as a result of reducing the harmful effects of the insecticide. Boswellic acid administration inhibited the elevation of cytokines and CYP2E1. Consequently, it lowered ROS production and reduced oxidative stress in the mice liver (Chen *et al.,* 2016). Gayathri *et al. (*2007) demonstrated that extract of *Boswellia serrata* downregulated *IL-1β* cytokines in peripheral blood mononuclear cells. The cinnamon extract has an immune stimulant outcome on the proliferation of human lymphocytes and *IL-1β* construction by monocytes (Shan *et al*., 1999). It regulates immune function via regulating pro and anti-inflammatory mediators and the gene expression in macrophages (Cao *et al.,* 2008). These explain the cinnamon role in adjustable gene expression in EMB treated mice. The data revealed that treatment with snail powder caused a significant downregulation in the expression levels of studied genes in the liver to be compared with the positive control. This agreed with Sarkar *et al.* (2015), who support the anti-inflammatory function of the Indian freshwater snail.

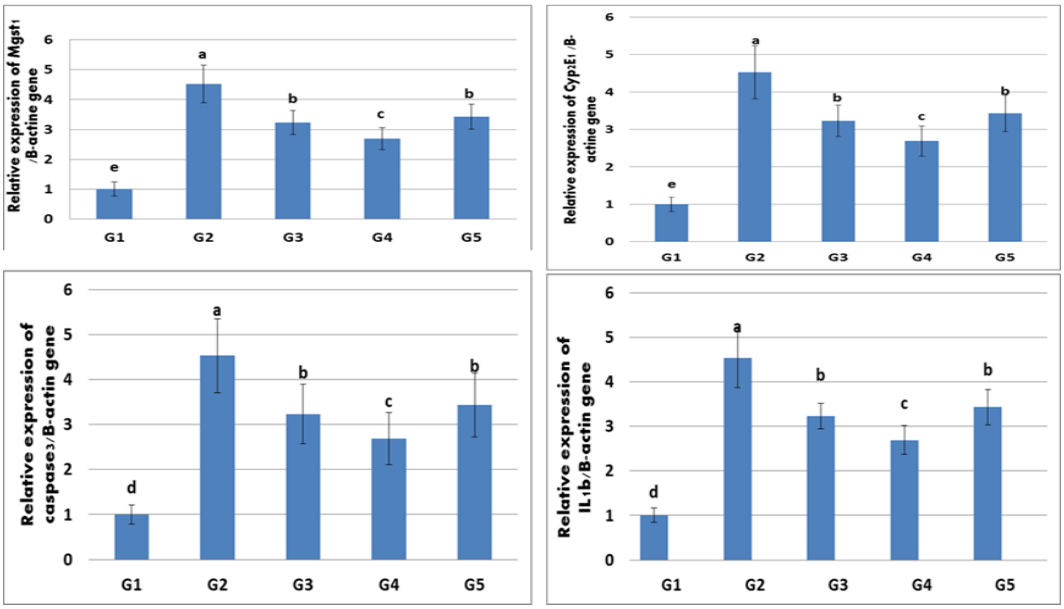

Figure 5. Relative expression of *Mgst1, CYP2E1, Caspase 3* and *IL1b* (compar to β-actin as internal control) genes in the liver of mice treated with EMB alone or combined with natural compounds.

Comet assay

Emamectin administration to mice induced a marked increase in liver DNA fragmentation (Figure 6 and Table 6).

These results agreed with Zhang *et al.* (2017) and Yun *et al.* (2017), who detected apoptosis in cells of EMB human liver exposure by enhanced caspase- 3 activities and increased DNA fragmentation. This agreed with Azoz *et al.* (2020), who found that EMB oral intake by rats caused a markedly increase in DNA damage. Insecticides exposure is reported to produce DNA break that leads to the process involved in cells genotoxic effect (Dusinska and Collins 2008; Mater *et al.*, 2014). The fragmentation of nuclei and condensation of chromatin were noticed resulting from EMB exposure in Sf-9 cells (Wu *et al.*, 2016). On the other, treatment with BS reduce the tail length, tail DNA%, and tail moment. This agreed with Rajabian *et al.* (2016), who found that DNA damage in BS-treated cells with glutamate was markedly decreased if compared with glutamate-treated cells. Moreover, consistent with these results *B. serrata* also inhibited the oxygen radicals (Ammon, 2010). Alcohol and aqueous extract of BS has high activity in scavenging free radicals (Sharma *et al.*, 2011; Azemi *et al.*, 2012). Regarding cinnamon extract, Sağlam *et al.* (2012) demonstrated that the length of comet tail was decreased in the diabetic group treated with cinnamon. Karadağlı (2014) showed that cinnamon is a protective agent in inhibiting damage induced by oxidative stress. The ability of cinnamon inhibited DNA damage may be due to its containing of high flavonoid and polyphenol compounds (Kumar *et al.*, 2012).

Table 6. Comet parameters in the liver of mice treated with EMB alone or combined with natural compounds.

Group	Tailed %	Untailed %	Tails length µm	Tail DNA%	Tail moment
G1	1.75	98.25	1.45±0.53 ^{d}	1.36	1.97±0.69^{d}
G2	32	68	9.63±1.27 ^{a}	7.41	71.36±9.10^{a}
G3	18	82	7.44±0.93 ^{b}	5.60	41.66±7.41^{b}
G4	14	86	6.21±0.90 ^{c}	4.22	26.21±3.57^{c}
G5	20	80	8.03±1.11 ^{b}	6.16	49.46±7.14^{b}

Different superscript letters in the same column of tail length showed significance difference at P< 0.05

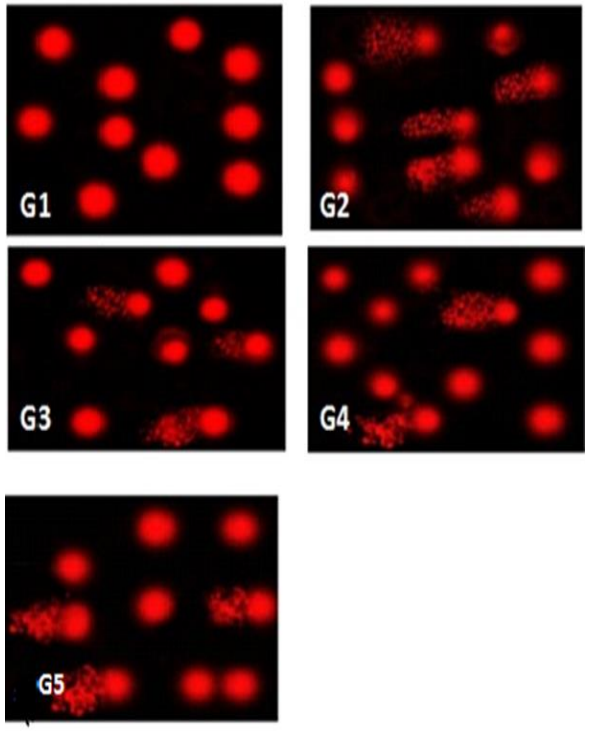

Fig. 6. Representative images of the cells assayed by the comet assay. Where G1 = negative control; G2 = mice treated with EMB;G3= mice treated with EMB+BS ; G4 = mice treated with EMB+CZ; G5 mice treated with EMB+ PS.

Histopathological findings

The most histological effects related to the treated mice with EMB are shown in Figure.7 (G2 a and b). The treatment *Boswellia* with EMB enhanced the architecture of liver. It showed slight activation of kupffer cells, slight infiltration of lymphocytes, karyolysis of some nuclei and normal central vein (Figure 7, G3). The same results appeared with the treatment by methyl extract of *Cinnamomum*. It revealed hepatocytes recovering with minimal vacuoles. Also, it can be seen that the normal

central vein and hepatocytes (Figure 6 G4. The treatment with PS decreased the side effects induced by EMB in mice liver (Figure 6 G5).

The results belong to histopathological studies with EMB treatment agreed with El-Sheikh and Galal (2015), Khaldoun *et al.* (2017) and Abou-Zeid *et al.* (2018), who found that emamectin administration produced pathological changes in the liver. These changes include blood vessel congestion with infiltration of lymphocytes, necrosis with hepatic parenchyma infiltration, also, dilated sinusoids. The EMB-side effects caused by the production of ROS that induced many changes in the cell. This clearly appears in icreasing MDA as seen in Figure 1. ROS generated by EMB can damage membrane components of the cell and lead to the leakage of cytoplasmic enzymes (Bagchi *et al.*, 1995). This appeared in ALT and AST activites (Figure 4). Also the oxiditive stress can lead to apoptosis (Temiz, 2020). This cleary appeared in gene expression of caspas3 (Figure. 5). Khaldoun *et al.* (2017) suggested that EMB toxicity is possibly caused by oxidative stress. The BS treatment combined with EMB leads to recovery and return to the normal appearance of hepatocytes and central vein. The most histological effects showed slight lymphocyte infiltration and karyolysis of some nuclei (Figure 6, G3). These results agreed with Eltahir *et al*. (2019), who reported that BS has antifibrotic characteristics, anti-inflammatory and antioxidant against CCl4 that induced degeneration in the liver. Also, it inhibits the biosynthesis of leukotrienes that considered pro-inflammatory mediators (Zhang *et al.*, 2013). Leukotrienes increased cell permeability. Also, boswellic acid inhibits transcription factor NF-κB. It is a vital downstream mediator for cytokines during inflammation (Cuaz-Pérolin *et al.*, 2008) that can also concurrently reduced oxidative stress (Umar *et al.*, 2014). Cinnamon reduced the side effect of EMP in liver tissue. It removed the histopathological changes through anti-oxidant activity (Hussain *et al.*, 2019). The cinnamon also has anti-inflammatory activity and free radicals-scavenge ability. So, a supplemented diet with cinnamon increased the protection against anti-toxic exposure (Bellassoued *et al.*, 2019). Ziconotide, first isolated from the snail Conus magus venom significantly blocks N-type voltage-gated calcium channels (Schroeder *et al.*, 2004). It is effective for the healing chronic pain. There is no sufficient studies took place about snail powdered and its role in eliminating the damage in the liver induced by toxic substances.

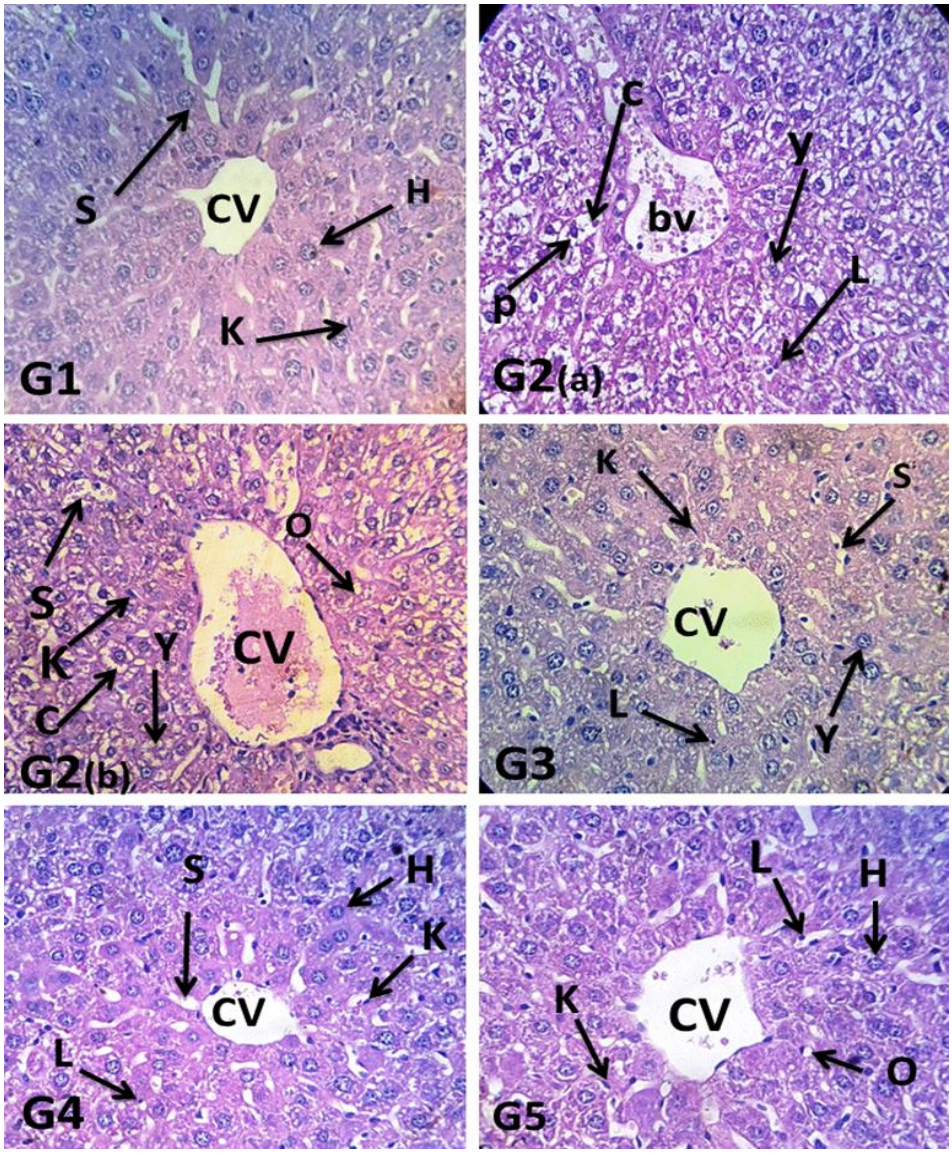

Figure 7. Histopathological changes

CONCLUSION

It can be concluded that EMB induced changes in the gene expression of *Mgst1*, *CYP2E*, *caspase-3* and *IL-1β*. Also, it is induced DNA fragmentation with increasing MDA, AST, and ALT. In contrast, it decreased both CAT and SOD levels. The treatment with natural extracts can remove the side effects of EMB. The findings belong to EMB and natural compounds are new in this filed beside there is no sufficient studies on the PS belong to this topic. So it can be used these natural materials to avoid the side effects of insecticide and other toxin substances.

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النشاط المضاد للأكسدة لبعض المركبات الطبيعية في التخفيف من آثار السمية الكبدية التي يستحثها الإيمامكتين بنزوات في ذكور الفئران

شريفة فتح الله محمد داود[1]، طارق محمد مصطفي الأقرع[2] و أمينة محمد جاد زيدان[1]

[1]قسم العلوم البيولوجية والبيئية- كلية الاقتصاد المنزلي- جامعة الازهر

[2]قسم الحيوان الزراعي والنيماتودا – كلية الزراعة – جامعة الازهر

الإيمامكتين بنزوات (EMB) هو مبيد حيوي يستخدم في الزراعة كمبيد حشري. ونظرًا لنطاق إستخدامة الواسع ، فمن السهل وصوله إلي البيئة وتأثيره على صحة الإنسان. لذلك تهدف هذه الدراسة إلى تقييم التأثير الوقائي للمركبات الطبيعية ضد السمية الكبدية التي يسببها EMB. هذه هي الدراسة الأولى التي تحدد التأثير الوقائي لهذه المستخلصات ضد تأثيرات EMB. تم عمل تحليلات كيمائية شملت قياس نشاط MDA ، CAT، SOD ، انزيمات الكبد ALT، AST ، كما شملت الدراسات الوراثية التعبير الجيني لـجينات *Mgst1*، *CYP2E1*، *caspase3*، *IL-1β* كما شملت دراسة قياس درجة DNA fragmentations ، كما تم إجراء الفحوصات النسيجية الخاصة بالكبد. تم توزيع ذكور الفئران على خمس مجموعات: المجموعة الاولي : المجموعة الضابطة ، المجموعة الثانية : (EMB 5 ملجم / كجم غذاء), المجموعة الثالثة : EMB + مستخلص اللبان المر (90 ملجم / كجم غذاء), المجموعة الرابعة: EMB + مستخلص القرفة (600 ملجم / كجم غذاء), المجموعة الخامسة: EMB + مسحوق القواقع (600 مجم / كجم غذاء) وإستمرت التجربة لمدة 8 أسابيع. تشير النتائج إلى أن EMB تسبب في زيادة الإجهاد التأكسدي عن طريق زيادة نشاط MDA والعلامات الحيوية لإصابة الكبد ALT و AST مع تثبيط الإنزيمات المضادة للأكسدة. كما أنتجت EMB العديد من التغيرات النسيجية المرضية في الكبد. بالإضافة إلي إرتفاع التعبيرات النسبية لجينات *Mgst1*، *CYP2E*، *caspase-3*، *IL-1β*. كما لوحظ زيادة في تلف الحمض النووي وتم تسجيلها من خلال زيادة طول الذيل والنسبة المئوية لـ Tail DNA، Tail moment . قللت المعاملة بالمركبات الطبيعية من سمية EMB من خلال تقليل الموشرات الكيميائية الغير طبيعية والتغيرات النسيجية المرضية ، والتعبير الجيني ، وتلف الحمض النووي وذلك عن طريق زيادة القدرة المضادة للأكسدة. وبالتالي ، فإن المركبات الطبيعية في هذه الدراسة تعمل كعوامل وقائية لحماية الكبد ضد السمية الكبدية التي يسببها EMB في ذكور الفئران.