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Ameliorative Effect of Rosemary Extract (*Rosmarinus Officinalis L.*) Against Ethion Bound Residues Induced Alterations in Experimental Animals

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Abstract

Fatal poisoning is widespread upon human exposure to toxic agents such as pesticides, heavy metals and environmental pollutants. In this regards, the application of the natural dietary agents as antidotes has engrossed a substantial attention. One of the ancient known traditional medicines and spices with an arsenal of metabolites of several reported health benefits is Rosemary (*Rosmarinus officinalis L.*). The current study was implemented to demonstrate the protective and nutritional impact of rosemary leaves extract (1% w/w) in experimental rats fed on maize cake containing ethion bound residues (1.6 ppm). Feeding animals with treated cake containing ethion bound residues caused elevation in serum alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase activities ($P<0.01$, $P<0.001$ and $P<0.01$), respectively. Whereas total protein and albumin were significantly decreased ($P<0.01$ and $P<0.05$), respectively.

As well as, concomitant elevation in serum creatinine and urea levels ($P<0.01$). Also, a significant ($P<0.01$) increase in cholesterol, triglyceride and low density lipoprotein and a significant ($P<0.001$) decrease in high density lipoprotein content as compared to the control were obtained. In addition a decrease in acetyl cholinesterase, catalase, and glutathione-S-transferase activity was observed as compared with controls. Furthermore, acute ethion bound residues intoxication caused histopathological changes in liver, kidney and brain. Administration of rosemary shows better effect in the activity of liver enzymes, kidney function markers, lipids contents as well as, protein profiles and antioxidant status. Rosemary also exhibited some improvement in the histological architecture of liver, kidney and brain.

Keywords: Ethion bound residues; Rosemary; Hepatotoxicity; Nephrotoxicity; Lipid profile; Rat

Abbreviations: Ops: Organophosphorus; CBs: Carbamates; PYs: Pyrethroids; OPIs: Organophosphorus insecticides; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; ALP: Alkaline Phosphatase; TC: Total Cholesterol; TG: Triglycerides; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein; GST: Glutathione-S-Transferase.

Introduction

Pesticides and agrochemicals, in general, became an important component of worldwide agriculture systems during the last century, allowing for a noticeable increase in crop yields and food production. Exposure to either natural or synthetic chemicals represents a worldwide public health problem. Humans or animals could be exposed to pesticides in air, soil, food, fruits, vegetables, or even pharmaceutical and cosmetic products via inhalation, ingestion, or direct contact. Among the many classes of pesticides to which humans are exposed to via the diet are the organophosphorus (OPs), carbamates (CBs) and pyrethroids (PYs), which mechanisms of actions to their target organism also occur in human and other mammalians. At the same time, during the last decades we realized that agrochemical residues did spread in the environment, causing significant contamination of terrestrial ecosystems and poisoning human foods [1-5].

Maize crop is one of the food crops that have several uses, whether as a food for man or as animal feed due to its high nutrition value. The use of pesticides can result in the accumulation of their residues in seeds, oil and cake .The residues in cake reach the milk through lactating animals and eventually into human body. Since consumers could be at risk from the pesticide residues which may be present in cake, therefore it is important to develop a procedure for the removal of pesticide residue from cakes. Ethion (O,O,O',O'-tetraethyl-S,S'-methylene-bis phosphorodithioate) is one of the widely used organophosphorus insecticides (OPIs), which have been identified as contaminants in many component of the global ecosystem. It is used to kill aphids, mites, thrips, leafhoppers and maggots on crops, ornamentals and animals. Ethion is converted in the liver to its active oxygenated analog, ethion mono-oxon, via desulfuration by cytochrome P-450 enzymes [6-10].

Natural herbs are widely consumed by humans on a daily basis; these natural products have many biologic and pharmacologic properties. Rosemary (*Rosmarinus officinalis L.*) (RM) is one of the most widely used and well- documented medicinal plants in the world, it is used as flavouring agent for food, beverages, and cosmetics preparations. The phytochemical screening of this plant has revealed its richness in beneficial active molecules such as polyphenolic compounds. For this reason, rosemary extracts exhibit many beneficial health effects as antiviral, radioprotective, anti-microbial, neuroprotective, anticancer, antidiarrheal and anti-inflammatory properties, hypolipidaemic agent, hepatoprotective (and nephroprotective [11-23]. Rosemary (*Rosmarinus officinalis L.*), contains antioxidants such as rosmarinic acid, diterpenoids like carnosic acid, carnosol, rosmanol, and epirosmanol as well as tocols and carotenoids. The antioxidant activities of Rosemary (*Rosmarinus officinalis L.*) extract have been associated with phenolic diterpenes that scavenge singlet oxygen, hydroxyl radicals and lipid peroxyl radicals [24-27]. The protective role of rosemary extract against ethion bound residue - induced hepatic, nephro, and neurotoxicity is not fully explored. Therefore, the present study was planned to evaluate protective effects of rosemary aqueous extract against ethion bound residue - induced toxicity by examining hematological alterations, histopathological changes and oxidative stress.

Materials and Methods

^{14}C-Labeled ethion

^{14}C-ethion labeled in the ethoxy groups (Figure 1) was prepared in a single vessel reaction using ^{14}C-ethyl alcohol (Sp. Act. 37 MBq, Amersham, England) according to Abdel-Gawad et al [28]. ^{14}C-Ethion had a specific activity of 7.4 MBq/g and the radiometric purity was 98 %. The R_f values of ethion in different solvent systems (Toluene: Xylene 20: 20; Dioxane: Xylene: Petrolum ether 10: 20: 20 and n-Hexane: Ethyl acetate 98: 2) are 0.75, 0.8 and 0.76, respectively. The absorbance of ethion was measured by UV spectrophotometer (JASCO) at λ=290 nm.

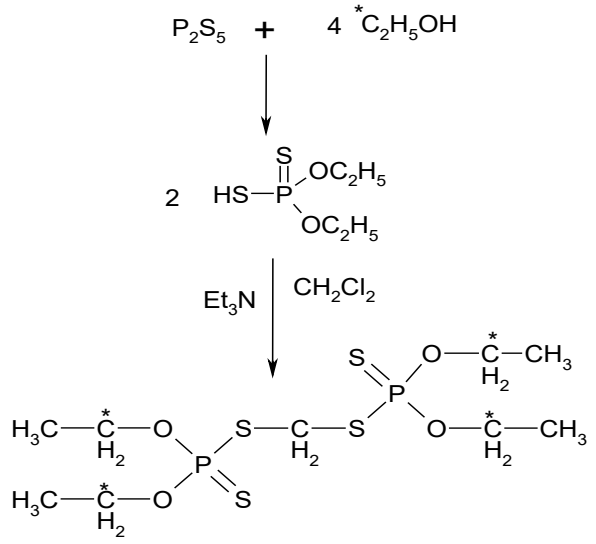

Figure 1: Pathway of ^{14}C-ethion synthesis.

Plant material and experimental design

Seeds of maize were cultivated under normal field conditions in a controlled, isolated field area as practiced in the field. Shortly at blooming stage, leaves of plants were treated twice, 15 days apart, with ^{14}C-ethion at the dose of 4 mg/plant. Maize cobs were collected at harvest time (30 days) after the second spray of labeled ethion, and dried for preparation of

the cake and determination of radioactivity.

Extraction of Maize Seeds

Seeds were crushed and extracted through soxhlet with n-hexane for 24 hrs. The cake after evaporation of hexane was further extracted with methanol for 6 h.

Radioactivity Measurements

The ethion bound residue in cake used in the experiment was determined as follows: 100 mg of cake was digested, using [1 mL Solusol (tissue and gel solubilizer), 1 mL 30 % H_2O_2 and 70 µL glacial acetic at 40–50ºC]. The radioactivity was counted by Liquid Scintillation Counting (LSC) and it was 1.6 ppm.

Preparation of Aqueous Extract Rosemary Leaves

Fresh rosemary leaves were collected from local market. The leaves were washed carefully, and then air dried in shade at room temperature, grinded to fine powder. Each 500 g of fine powdered herb was then refluxed in 2 liters of 70% ethanol at 60°-70°C for 36 hrs in a continuous extraction (soxhlet) apparatus. The ethanol extract was filtered and concentrated under reduced pressure at 60°C using a rotary evaporator. Ten grams of rosemary extract were dissolved in 100 mL of distilled water and incorporated in 1 kg of control and maize cake then mixed manually for at least 30 min. to ensure complete distribution.

Toxicological Studies

Twenty male Wister rats, aged three months old and weighing 150 ± 20g, were obtained from the animal house of the National Research Centre, Dokki, Giza. Animals were maintained under controlled conditions of temperature at 25 ± 2ºC, relative humidity of 50 ±15%, and normal photoperiod (12h dark: 12h light) for one week giving them the standard rat pellet and water. They were provided with tap water and balanced diet ad libitum. All animals have received human care in compliance with the state authorities following the Egyptian rules of animal protection. The rats were randomly distributed into four groups of five rats each as follows:

Group 1: Animals fed on maize cake free from ethion residues or rosemary extracts (Control).

Group 2: Animals fed on maize cake free from ethion residues and rosemary extracts (1% w/w).

Group 3: Animals fed on maize cake containing ethion bound residues (1.6 ppm) (Treated).

Group 4: Animals fed on maize cake containing ethion bound residues (1.6 ppm) and rosemary extracts (1% w/w).

Blood Sampling and Biochemical Analysis

At the end of the experimental period (30 days) the blood samples were collected from the eye plexuses of the animals. Then, it is divided into two parts, the first part was put into a clean centrifuge glass tube with EDTA to prepare plasma and the second part was put in a dry clean centrifuge glass tube without any coagulation to prepare serum. The samples were left for 15 minutes at normal of the temperature of room, after that the tubes were centrifuged for another 15 minutes at 3000 rmp for analysis of biochemical parameters. Cholinesterase activity was determined according to Ellman method [29] as modified by Gorun et al [30]. The liver parameters [alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, and albumin], kidney parameters (urea and creatinine), lipid profile, [total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL)] and antioxidant parameters [catalase (CAT) and glutathione-S-transferase (GST)] were determined using Kits purchased from Bio-diagnostic Co., Egypt.

Histopathological Effect of Ethion on Selected Tissues

Selected tissues (testes, liver, kidney and brain) were isolated and fixed in 10% neutral buffered formalin for 24 hours, rinsed with water, dehydrated in alcohols, cleared in xylene, and embedded in paraffin. Tissue blocks were sectioned at 4-5 micron thickness and routinely stained with Haematoxylin and Eosin (H&E) stain [31] and examined by light microscopy.

Statistical Analysis

The obtained data from serum biochemical and enzymes analysis were expressed as mean ± standard error. Data were analyzed by using Student's t-test. $P < 0.05$, $P < 0.01$ and $P < 0.001$ values were used [32].

Results and Discussions

Ethion, an organophosphorus pesticide, used world widely causing public health concern [33]. Compared with control rats, the present results of the estimated biochemical parameters indicated that ethion bound residues caused a significant inhibition in the plasma acetylcholinsterase ($P< 0.01$) (Table 1). The inhibitory effect on acetylcholinsterase was expected, as the organophosphate compounds are anti cholinesterase agents for RBC, and plasma ChE, Such findings have been reported since the 1950's by many investigators [34,8,35]. Meanwhile, significant inhibition ($P< 0.01$) was continued when RM supplemented to ethion bound residues treated animals.

Groups	Plasma-Cholinesterase level (µ mole/mL)
	Means a ± SD
Group 1	1.38 ± 0.040
Group 2	1.31 ± 0.055
Group 3	1.26 ± 0.034 ***
Group 4	1.242 ± 0.031***

Table 1: Effect of ethanolic extract of rosemary on cholinesterase activities of rats treated with ethion bound residues for 30 days. a: results are expressed as mean ± SD for five samples, ***: Significance at P< 0.01.

The liver, as an organ of metabolism and excretion, is endowed with the task of detoxification. Toxicants such as viruses, fungal products, bacterial metabolites, pollutants and chemotherapeutic factors could cause different liver disorders [24]. Sensitive indicators of liver cell injury are serum transaminases such as AST, ALT and ALP. Our results have shown that oral administration of cake contaminated with ethion bound residues induced hepatotoxicity. The effects of subchronic administration of ethion bound residues and the protective effects of RM on serum biochemistry are shown in Table 2. Compared with control rats, activities of hepatic enzymes, ALT, AST, and ALP were significantly increased (P<0.01, P<0.001 and P<0.01), respectively (Group 3). Whereas the other two parameters, the total protein and albumin were significantly decreased (P<0.01 and P<0.05), respectively. In contrast, RM-treatment significantly suppressed elevation of ALT, AST and ALP and increasing of the total protein and albumin concentrations (Group 4).

The liver is considered as one of the target organs affected by pesticides toxicity owing to its storage in the liver after pesticides exposure. In the current study, the liver enzymes assays indicated that ethion bound residues treatment induced significant elevation of serum AST, ALT, and ALP activities. It has been shown that organophosphorus insecticides can elevate the enzymatic activities of ALP, ALT, AST, and LDH [17,26]. Supplementation with RM leaves extract was able to diminish significantly the ethion-induced changes in all the mentioned parameters. RM leaves extract acts as an antioxidant with hepatoprotective effect which has been confirmed by Mehmetcik et al. These results are run in parallel with the results of [36] who found that, a significant improving effect of pre-treatment with rosemary on the altered activities of serum ALT, AST, GGT and ALP induced by Pb-acetate intoxication. Similarly, results of the study by Sotelo-Félix, et al. [20] and Gutiérrez, et al. [37] showed that methanolic extract of rosemary could restore the elevated ALT and AST activities in both acute liver damage and liver cirrhosis experimental models. Also, these results are in agreement with Albasha and Azab [38] who reported that aqueous extract of rosemary has significantly decreased the release of AST and ALT.

	ALT	AST	(ALP)	T. P	ALB
Groups	(U/L))	(U/L)	(U/L)	(g/dL)	(g/dL)
	Means a ± SD	Means a ± SD	Means a ± SD	Means a ± SD	Means a ± SD
Group 1	50 ± 8.09	110.4 ± 7.7	185.8 ± 15.5	4.25 ±0.5	2.27 ± 0.46
Group 2	44.0 ± 6.16	108.6 ± 9.32	183.6 ± 8.88	4.27 ± 0.23	2.11 ± 0.15
Group 3	71.8 ± 5.26***	156.8 ± 8.35****	239.6 ± 5.73***	3.1 ± 0.1***	1.53 ± 0.1*
Group 4	51.0 ± 7.35***	104.8 ± 5.76****	199.2 ± 9.65***	4.17 ± 0.14****	2.06 ± 0.13***

Table 2: Effect of ethanolic extract of rosemary on liver function of rats treated with ethion bound residues for 30 days. a: results are expressed as mean ± SD for five samples, *: Significance at P< 0.05, ***: Significance at P< 0.01, ****: Significance at P< 0.001.

The elevated serum levels of urea and creatinine indicate reduced ability of the kidney to eliminate the toxic metabolic substances [39]. Many pesticides can cause some toxic and adverse effects on the kidney tissues [29]. Kidney is one of the target organs of experimental animals attacked by OP compounds and can alter plasma urea, uric acid, and creatinine levels [40]. The levels of urea and creatinine in the plasma of rats are measured as indicators for kidney function [6]. In this work, exposure to cake containing ethion bound residues (group 3) produced a significant increase (P< 0.01) in the values of urea, and creatinine concentrations relative to the control group (Table 3) confirming a pronounced impairment in kidney function.

Elevated serum creatinine level and blood urea were noticed in mice receiving ethion residues for three months [41]. These results agreed with the results of Abdel-Gawad, et al. [35] who illustrated that the exposure to ethion residues in soybean seeds increases the level of serum urea and creatinine. On the other hand, rats administered RM alone revealed insignificant change in the mentioned parameters as compared to the control group. The treatment with RM recorded significant (P<0.001) decrement in urea and creatinine in comparison with cake containing ethion bound

residues treated group (Table 3). These results suggest the ability of RM extract to restore impaired renal excretory function. This is in accordance with results of the study by Sakr and Lamfon [42], indicating that water extract of rosemary could reduce the levels of urea and creatinine in serum of rats, when compared to CCl_4-treated group of animals.

Group	Urea	Creatinine
	(mg %)	(mg %)
	Means a ± SD	Means a ± SD
Group 1	59.0 ± 4.0	1.06 ± 0.64
Group 2	49.4 ± 5.73	1.002 ± 0.018
Group 3	76.8 ± 5.36***	1.55 ± 0.20***
Group 4	45.6 ± 4.77****	0.98 ± 0.038***

Table 3: Effect of ethanolic extract of rosemary on kidney function of rats treated with ethion bound residues for 30 days. a: results are expressed as mean ± SD for five samples,***: Significance at P< 0.01, ****: Significance at P< 0.001.

Considering that liver damage could result in a change of the lipid content in serum, cholesterol, triglycerides, HDL and LDL in serum were determined. Treatment with cake containing ethion bound residues (group 3) caused a significant ($P < 0.01$) increase in cholesterol, triglyceride and LDL, while caused a significant decrease ($P < 0.001$) in HDL content as compared to the control (Table 4). This is in agreement with recent studies on CCl_4 poisoning [43]. RM extract supplementation also resulted in the significant attenuation in the level of serum, cholesterol, triglycerides, HDL and LDL in serum, toward the control level which again strengthens the hypolipidemic effect of this extract. Several studies have demonstrated a significant increase in the serum-lipid constituents in the experimental animals, treated with different OPI [44-46]. The hypolipidemic effects of RM in our studies were previously reported by Ozkol, et al. [47] and Labban, et al. [48], respectively. Other reported investigations are consistent with our results. Also, treatment rosemary oil in rats fed high fat diet modulated the elevation of lipids parameters [49-50].

Groups	Cholesterol	Triglycerides	HDL	LDL
	(mg %)	(mg %)	(mg %)	(mg %)
	Means a ± SD	Means a ± SD	Means a ± SD	Means a ± SD
Group 1	76.8 ± 5.07	71.0 ± 5.57	34.5 ± 1.73	14.0 ± 1.0
Group 2	71.6 ± 5.22	63.0 ± 2.45	40.2 ± 1.60	10.04 ± 0.31
Group 3	107.6 ± 7.58***	95.4 ± 5.55***	30.88 ± 1.6****	24.9 ± 1.67***
Group 4	72.2 ± 4.92****	61.6 ± 3.21****	36.66 ± 0.96****	15.26 ± 0.32****

Table 4: Effect of ethanolic extract of rosemary on lipid profile of rats treated with ethion bound residues for 30 days. a: results are expressed as mean ± SD for five samples,***: Significance at P< 0.01, ****: Significance at P< 0.001.

CAT and GST, constitute a mutually supportive team of defense against reactive oxygen species (ROS). CAT helps in the removal of H_2O_2 formed during the reaction catalyzed by SOD. GST catalyzes the formation of the thiol group of glutathione to electrophilic xenobiotics. These antioxidant enzymes can, therefore, alleviate the toxic effects of ROS. In general, pesticide treatment produces oxidative stress by the generation of free radicals and induced tissue lipid peroxidation in mammals and other organisms. Reduced activates of antioxidant enzymes (CAT, SOD) after treatment of pesticides are important indicators for the toxicity of these chemicals [51-53]. The results obtained clearly indicated that treatment with ethion bound residues for 30 days resulted in a significant decrease in the activities of CAT and GST as compared to control animals (Table 5). However, rats treated with rosemary extract showed increase in CAT and GST as compared to control rats. When rosemary extract administrated with ethion bound residues, it has capable of recovering the activities of CAT and GST to the normal values. Our results are in agreement with those found by Khalil, et al. [54] who reported that Serum enzymatic GST, (CAT), and (GPx) were found to be increased by the administration of R. officinalis in animals induced by Streptozotocin (STZ). Moreover, Abdel-Gawad, et al. [35] demonstrated that CAT and GST were found to be decreased in animals treated with ^{14}C-Ethion residues and increased by the administration of artichoke leaf powder.

Groups	CAT (U/l)	GST(U/mL)
	Means a ± SD	Means a ± SD
Group 1	348 ± 15.03	3006 ± 99.52
Group 2	355 ± 15.17	3056 ± 70.88
Group 3	312 ± 6.57 ***	2523 ± 63.77****
Group 4	345 ± 11.51 ***	2922 ± 49.96 ****

Table 5: Effect of ethanolic extract of rosemary on Antioxidant activities of rats treated with ethion bound residues for 30 days. a: results are expressed as mean ± SD for five samples, ***: Significance at P< 0.01,****: Significance at P< 0.001.

Histopathological Effect of Ethion on Selected Tissues

The histopathological examination of control liver sections showed normal histological structure (Figure 2 A,B). animals exposed to bound ethion showed mild congestion of central veins associated with mild mononuclear inflammatory cells infiltration (Figure 2C), which is apparently improved when using Rosemary (Figure 2 D) The histopathological examination of liver revealed severe congestion of central veins and hepatic sinusoids, associated with severe necrosis, vacuolar degeneration of hepatocytes and mononuclear inflammatory cells infiltration due to pesticide treatment as documented previously. The exposure to the pesticides impair the liver function and to cause injury to the hepatic tissues. The drastic alterations observed in the liver tissues due to pesticide exposure suggested hepatotoxic effects as reported by Iseri, et al. [55-57].

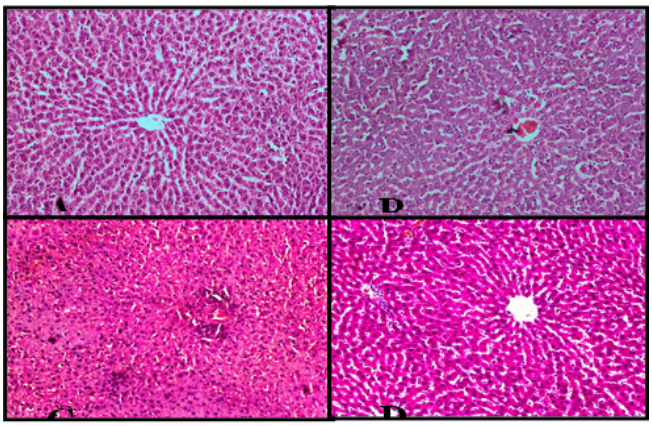

Figure 2: Photomicrographs of H and E stained Liver sections. (Figure A): Control rat shows normal histologic structure of liver (X 200). (Figure B): shows mild congested central veins (Arrows) (X 100). (Figure C): Shows mild congestion of central veins with mild inflammatory cells infiltration in sections exposed to Bound Ethion (X 100). (Figure D): shows apparent normal hepatic sections exposed to both Bound Ethion and Rose Mary extract. (X 200).

Control kidney sections showed normal histological features (Figure 3A). Examined sections of male rats treated with bound ethion showed mild hemorrhage within renal tissue (Figure 3B) as well as mild degenerative changes (Figure 3C) Which appeared as control ones (Figure 3D). The most prominent histopathological alterations of total ethion treated rats showed severe interstitial nephritis, hemorrhage and edema, associated with severe infiltration with inflammatory cells as previously concluded by Mamun et al [58-61]. Sections from control and Rosemary treated groups showed no histopathological alterations in the cerebral cortex (Figure 4A) Treatment with bound ethion showed mild congestion of cerebral blood vessels associated with mild gliosis (Figure 4B, C). Brain of rat treated with bound ethion and Rosemary showing significant improvement with slight congestion of cerebral blood vessels (Figure 4D).

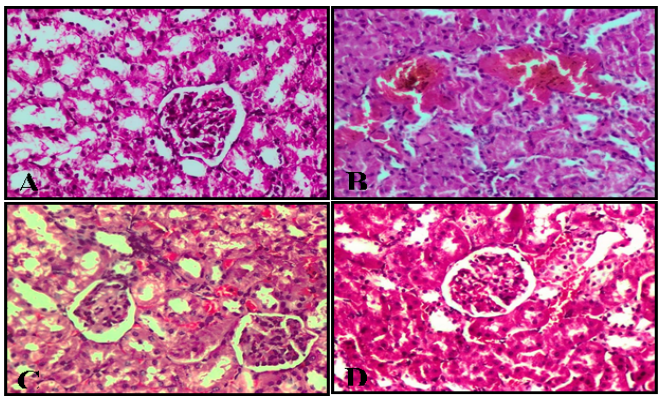

Figure 3: Photomicrographs of H and E stained Kidney sections. (Figure A): Control rat shows normal histologic structure of kidney. (Figure B): shows mild hemorrhage within renal tissue**.** (Figure C): shows mild degenerative changes in sections exposed to Bound Ethion. (Figure D): shows apparent normal kidney sections exposed to both Bound Ethion and Rosemary extract. (X 200).

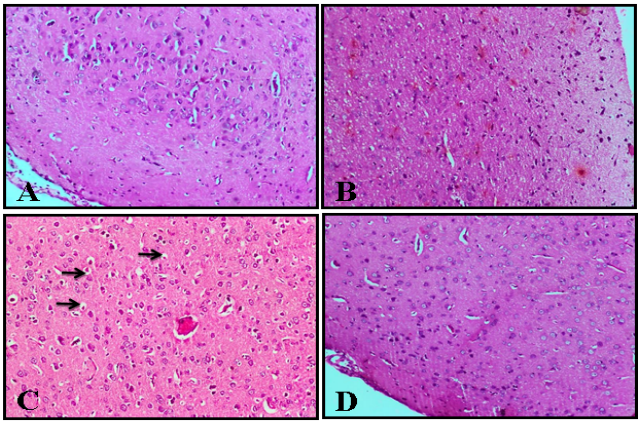

Figure 4: Photomicrographs of H and E stained Brain sections. (Figure A): photomicrograph of **c**ontrol rat brain shows nohistopathologicstructure in cerebral cortex. (Figure B): shows mild congestion of cerebral blood vessels. (Figure C): Shows vacuolar degeneration in brain exposed to bound Ethion (Arrows)**.** (Figure D): brain of rat treated with bound Ethion and Rosemary showing significant improvement with slight congestion of cerebral blood vessels (H& E X 100).

Ethion showed important degenerative changes in brain

tissue in the form of congestion of cerebral blood vessels, edema vacuolations as mentioned by Abdel-Salam, et al. [59] who stated that treatment with only OPIs causes' neuronal damage in the cerebral cortex and degeneration of some Purkinje cells in the cerebellum. The oxidative stress increased in the brain tissue suggesting increased generation of free radicals leading to neuronal injury [62-66] as well as decreased antioxidant enzyme activities [67-69]. The administration of Rosemary has been reported to attenuate and ameliorate the alterations caused by ethion in male rat.

Conclusion

In conclusion, the present data revealed that RM extract had free radical scavenging capacity. Ethion bound residues administration induced liver and kidney damage as well as hyperlipidimic effects as evidenced from elevated hepatic, kidney and lipidemic markers. However, coadministration of RM extract markedly ameliorated the deleterious effects of ethion bound residues mainly through their antioxidant activity. Histopathological studies on liver, kidney and brain tissues confirmed our data. It is possible that RM extract may be useful as adjuvant due to reduce some of the adverse effects of the pesticide bound residues. Thus, this study suggests the potent role of rosemary in management of injury-induced by ethion bound residues exposure and this effect may be attributed to its antioxidant activity.

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