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Research Article
Protective Role of *Carissa edulis* Ethanolic Extract Against Dimethoate-induced Hepatotoxicity in Guinea Pigs

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Abstract

Background and Objective: *Carissa edulis* (CE) (Apocynaceae) is distributed in tropical Africa and Asia and commonly used in folk medicine to treat many diseases such as headache, cough, rheumatism and fever. The purpose of this study was to evaluate the protective role of ethanolic extract of CE, a medicinal plant locally called "Al-Arm" in Yemen, against liver injury induced by dimethoate (DM) intoxication in male guinea pigs. **Materials and Methods:** Animals were divided randomly into 5 groups and kept at 5 animals per group. The first group was served as a control group and administered with vehicle orally; the group II administered with DM (14 mg kg^{-1}; 1/25 LD_{50}) orally. Animals of group III, IV and V were administered with 100 mg kg^{-1} of CE extract, 200 mg kg^{-1} of CE extract and 100 mg kg^{-1} Liv-52 orally half hour before DM administration, respectively. All the previous administrations were repeated daily for 21 days. Data were analyzed by one-way ANOVA using SPSS. **Results:** The DM caused a statistically significant increase in the serum level of liver enzymes (AST, ALT, ALP) when compared to control animals, whereas CE and Liv-52 pre-treatment to the DM-intoxicated animals resulted in a significant normalization of the activities of enzymes. Similarly, a significant increase in lipid peroxidation (LPO) level, while induced significant decreases in the activities of liver catalase (CAT) and glutathione-S-transferase (GST). In contrast, co-administration of CE and Liv-52 to DM-treated animals restored most of these biochemical parameters to nearly normal levels. Histopathological examination of intoxicated animals showed many tissues alterations such as; vasodilation, hemorrhage, cytoplasmic vacuolization, inflammation and nuclear pyknosis indicating liver damage, while the animals received CE or Liv-52 showed less pathological effects when compared to animals treated with DM alone. **Conclusion:** The biochemical and histological results confirmed the hepatoprotective effect of ethanolic extract of CE against DM-induced hepatotoxicity in male guinea pigs.

Key words: Dimethoate, hepatotoxicity, *Carissa edulis*, ethanol extract, guinea pigs

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

A natural product is a substance produced by living organisms such as plants, animals and other micro-organisms by primary and secondary metabolism[1]. Medicinal plants are used to treat several diseases by the oldest traditional medicine. The use of these natural products increases all over the world especially in developing countries[2]. These plants are also the sources of many traditional drugs such as morphine and vincristine[3] and play an important role in human and animal health care[4]. Many plants have been documented to have antinociceptive and anti-inflammatory activities, but out of the 250,000-500,000 plant species on earth, only 1-10% has been studied for their potential medicinal values[5]. *Carissa edulis* (CE) belongs to the family Apocynaceae, which consists of about 250 genera and 2000 species and is distributed in tropical Africa and Asia[6]. It is locally called "Al-Arm" in Yemen[7,8]. It is a spiny, branched, small tree or shrub, with a height up to 5 m and milky sap. The leaves are ovate to ovate-elliptic, opposite and flowers are white tinged with purple, red or pink and its fruits are edible, ovoid and red-black containing 2-4 flat seeds[6]. This genus is a rich source of different natural classes of compounds such as; sesquiterpenes, cardiac glycosides, phenolic compound, flavonoids, lignans and chlorogenic acid derivatives[9-12]. The chemical constituents of CE in the literature review indicated the isolation of lignans, flavonoids, phenolic compounds and sesquiterpene[9,11-15]. The CE is a medicinal plant naturally growing at different geographical areas in Yemen with widespread use in traditional medicine and it has been used in different provinces as an oral hypoglycemic agent[7]. It is commonly used in folk medicine to treat many diseases such as headache, cough[16], rheumatism[17], fever, sickle cell anemia, syphilis, helminthiasis and rabies[12,18-20]. In pharmacological studies CE showed antiviral activity[21,22], anticonvulsant[20,23], antiplasmodial[10,24,25], antimicrobial[26], analgesic[19], diuretic[18], as well as hypoglycemic activity[27]. Although, there is no adverse effect reported on CE herbal medicines[12]. The use of chemical pesticides in qat production in Yemen, has been increasing dramatically[28]. So, the farmers use these chemicals in agriculture to enhance food production by eradicating unwanted insects and disease vectors[29]. Organophosphorus (OPs) compounds are the most widely used class of pesticides in agriculture and medicine[30,31]. The primary effect of OPs pesticides is the inhibition of acetylcholinesterase activity[29]. In addition, it has been demonstrated that lipid peroxidation (LPO) mediated by free radicals is one of the molecular mechanisms involved in OPs-induced toxicity[32] which exert their biological effects through an attack on cellular constituents of hepatic and brain tissues[33] through generation of reactive oxygen species (ROS)[34]. In addition, there are many organs and systems that could be affected by OPs pesticides such as; liver[30], kidney[35], brain[36], gonads[31,37], pancreas[38] and immune system[39]. Moreover, it was reported that OPs pesticides have been shown to produce ROS during neuronal damage and seizures[40]. Among OPs pesticides and dimethoate (DM) were the most commonly reported pesticide to be used on farms in Yemen to protect crops and qat farms[41]. A recent study[42] has shown that chronic exposure to DM caused hepatotoxicity and increased liver marker enzymes. Hence, the present study aimed to investigate the protective activity of CE ethanol leaves extract administration for 21 days on some biochemical and histopathological parameters intoxicated with sub-chronic doses of DM in male guinea pigs.

MATERIALS AND METHODS

Location and total time duration of research work: The whole study research is taken 6 months (from September, 2018 until February, 2019) and all experiments were carried out at Department of Biology, Faculty of Science, Ibb University, Yemen.

Chemicals: Dimethoate 40 EC was purchased from local market as a commercial emulsifiable concentrate formulation containing 40% active ingredient. Liv-52 was obtained from Himalaya Drug Company, Bangalore, India. Both the DM and Liv-52 were reconstituted appropriately in 0.5% carboxy methyl cellulose (CMC) for the final concentration immediately prior to use.

Plant material: The leaves of CE were collected from Jeblah district, Ibb Governorate, Yemen. The plant was authenticated by comparison with reference specimens preserved at the Herbarium of Biology Department, Ibb University, Yemen. Voucher specimens were kept in the Herbarium for future references.

Preparation of the ethanol extract: The powdered material of leaves (2000 g) were macerated with 70% ethanol by continuous stirring at room temperature and then evaporated to dryness under reduced pressure and finally yield at 25%. The dried extracts were dissolved in 0.5% CMC and administrated orally when experiments were performed.

Animals maintenance: Adult male guinea pigs (600±200 g) were obtained from the animal house of Biology Department, Faculty of Science, Ibb University, Yemen and kept for one week on a commercial diet in environmentally controlled conditions with free access to diet and water *ad libitum*. The experimental procedure was performed in accordance with the national and international guidelines and regulations approved by the ethical committee of Ibb University. In addition, all administrative approvals were taken and the Ibb University approval paper will be attached.

Experimental design: Animals were randomly divided into 5 groups of five animals each. The control group was given 0.5% CMC suspension by gastric gavage. The animals of groups II were given oral administration of 14 mg kg^{-1} DM (1/25 LD_{50}) dissolved in 0.5% CMC. The animals of groups III were given oral administration of 100 mg kg^{-1} CE ethanol leaves extract plus 14 mg kg^{-1} DM. Animals of group IV were given oral administration of 200 mg kg^{-1} CE plus 14 mg kg^{-1} DM. Animals of group V were given oral administration of 100 mg kg^{-1} Liv-52 plus 14 mg kg^{-1} DM. All the previous administrations were repeated daily for 21 days. At the end of the treatment, the animals of each group were anesthetized with ether and blood was collected directly from the portal vein. The blood sample of animals in each group was divided in two tubes, one of them mixed with heparin to prevent coagulation and the other was allowed to clot at room temperature for 1 h and then centrifuged at 3000 rpm and 4°C for 15 min to obtain sera. The separated serum was sampled into clean tubes and kept in a deep-freezer at -24°C for biochemical analysis.

Estimation of liver function: Serum aspartate aminotransferase (AST) and serum alanine aminotransferase (ALT) were determined using Spinreact Diagnostics Kits (Spain) according to the method of Tietz[43]. While the activity of serum alkaline phosphatase (ALP) was determined using Reactivos GPL Diagnostics Kits (Spain) according to the method of King[44]. The enzyme activity was expressed as U L^{-1}. Glucose concentration was determined using diagnostic kits of Spinreact (Spain) according to the method of Tietz[45].

Measurement of lipid peroxidation: Lipid peroxidation (LPO) was determined based on that of Ohkawa *et al.*[46]. A detailed description of the LPO measurement was previously listed in previously published article[47].

Estimation of antioxidant enzymes: Catalase (CAT) activity was measured by the method of Aebi[48]. Glutathione-S-transferase (GST) activity was measured spectrophotometrically by the method of Habig *et al.*[49]. The total protein content of kidney homogenate was determined by the method of Lowry *et al.*[50]. A detailed description of the LPO measurement was previously listed in previously published article[47].

Histopathological examination: Animals of control and treated groups were put under light ether anesthesia, dissected as quickly as possible and then pieces of livers and kidneys were removed and fixed in 10% neutral formalin for 24 h, then washed by the running tap water and stored in 70% ethyl alcohol, until further processing. Small blocks of about 5×5 mm size were dehydrated, cleared and embedded in paraffin wax. Finally, paraffin sections of 5 microns thickness were cut using rotary microtome (Leica, Germany) and stained with hematoxylin and eosin.

Microscopy and photomicrography: Microscopic slides of liver and kidney were examined carefully under a compound light microscope at Biology Department, Faculty of Science, Ibb University. Slides from the different treated groups were evaluated for any toxic insult compared to slides from the control group. Photomicrographs of selected slides were taken by using (Sony HD, Japan) built-in digital photo camera.

Statistical analysis: Results of the biochemical estimations were reported as mean±SD. To analyze current data, SPSS software version 20 was used. Total variation, present in a set of data was estimated by one-way analysis of variance (ANOVA) and follow up test (LSD). Differences with a p-value was considered as statistically significant at $p<0.05$.

RESULTS

Results of liver function: The ethanol extract of CE was evaluated for its hepatoprotective potential in guinea pigs with DM-induced liver damage. Administration of DM (14 mg kg^{-1}) to male guinea pigs for 21 days increased the serum activities of AST (58.6±4.1 U L^{-1}), ALT (94.2±6.5 U L^{-1}) and ALP (81.6±6.9 U L^{-1}) significantly ($p<0.001$). These values were 35.2±3.8, 68.0±5.6 and 54.8±4.9 U L^{-1} in normal control guinea pigs, respectively (Table 1). The activity of these enzymes was stimulated by 66, 39 and 49%, respectively after DM-intoxication compared with that of the controls. The

Table 1: Activities of AST, ALT and ALP enzymes (Mean±SD), stimulation (%) and inhibition (%) in control and different treated group

	Liver marker enzyme (U L^{-1})		
Treated groups	AST	ALT	ALP
Control	35.2±3.8[a]	68.0±5.6[a]	54.8±4.9[a]
DM	58.6±4.1[b***]	94.2±6.5[b***]	81.6±6.9[b***]
S% versus control	66	39	49
100CE+DM	46.8±5.6[c*]	81.6±4.7[c*]	69.4±5.7[c*]
I% versus DM	20	13	15
200CE+DM	41.8±5.5[c**]	70.8±7.1[c**]	60.6±6.5[c**]
I% versus DM	29	25	26
100 Liv-52+DM	40.2±5.0[c**]	69.2±7.7[c**]	61.0±5.8[c**]
I% versus DM	31	27	25

Each value represents the mean±SD, n = 5. Values marked with asterisks differ significantly from control animals: p<0.05, those marked with the same letter differ insignificantly from control group: p>0.05. *p<0.05, **p<0.01 compared with control, respectively

Table 2: Means±SD of lipid peroxidation, activities of catalase and glutathione-S-transferase, stimulation (%) and inhibition (%) in the liver enzymes of control and different treated group

	Antioxidant enzyme (μmol min mg^{-1} protein)		
Treated groups	LPO	CAT	GST
Control	1.68±0.33[a]	7.48±2.38[a]	40.02±10.88[a]
DM	3.03±0.73[b**]	3.16±2.60[b**]	25.24±10.15[b**]
S% versus control	80	58	37
100CE+DM	2.17±0.34[c*]	3.97±1.51[c*]	33.01±7.54[c*]
I% versus DM	28	26	31
200CE+DM	1.82±0.39[c**]	4.74±1.02[c**]	37.75±9.80[c**]
I% versus DM	40	50	50
100 Liv-52+DM	2.05±0.58[c**]	4.83±0.91[c**]	41.42±8.56[c**]
I% versus DM	32	53	64

Each value represents the mean±SD, n = 5. Values marked with asterisks differ significantly from control animals: p<0.05, those marked with the same letter differ insignificantly from control group: p>0.05. *p<0.05, **p<0.01 compared with control, respectively

results also showed that CE extract (100 and 200 mg kg^{-1}) was able to retain the liver marker enzymes toward the normal levels (20, 13 and 15% and 29, 25 and 26%), respectively (Table 1). Similar results were recorded with Liv-52 and DM co-administration (Table 1).

Results of lipid peroxidation: Levels of LPO were increased (3.03±0.73 nmol mg^{-1} protein) significantly ($p<0.01$) by 80% in the liver homogenates of DM-treated guinea pigs as compared to control animals (1.68±0.33 nmol mg^{-1} protein). However, it was observed that the LPO levels were decreased (2.17±0.34 nmol mg^{-1} protein, 1.82±0.39 nmol mg^{-1} protein and 2.05±0.58 nmol mg^{-1} protein by 28, 40 and 34% in the groups which received DM along with CE (100 and 200) and Liv-52, respectively). The inhibition in LPO levels was statistically significant ($p<0.05$) as shown in Table 2.

Results of antioxidant enzymes: The CAT activity was also found to be inhibited (3.16±2.60 μmol min mg^{-1} protein) significantly ($p<0.01$) by 58% in DM treated group as compared to that of the control group (7.48±2.38 μmol min mg^{-1} protein). However, the activity of CAT was significantly ($p<0.05$) elevated (3.97±1.51 μmol min mg^{-1} protein, 4.74±1.02 μmol min mg^{-1} protein and 4.83±0.91 μmol min mg^{-1} protein) by 26, 50 and 53% in animals which received DM along with the CE (100 and 200) and Liv-52, respectively) as compared to the DM treated group alone (Table 2). The activity of GST was significantly ($p<0.01$) inhibited (25.24±10.15 μmol min mg^{-1} protein) by 37% in DM administered group as compared to the control animals (40.02±10.88 μmol min mg^{-1} protein). However, the activity of GST in animals that received DM administration along with CE (100 and 200) and Liv-52 was significantly ($p<0.05$) elevated (33.01±7.54 μmol min mg^{-1} protein, 37.75±9.80 μmol min mg^{-1} protein and 41.42±8.56 μmol min mg^{-1} protein) by 31, 50 and 64%, respectively as compared to controls (Table 2).

Results histopathological examination: Livers from animals of the control group showed a uniform pattern of the polyhedral hepatocytes. They form cords of hepatocytes around the central vein with normal sinusoidal vessels (Fig. 1a). After 21 days of DM administration, many histopathological changes were observed in the liver sections. These changes were cytoplasmic vacuolization, dilated and congested blood vessels with hemorrhage, ballooning hepatocyte, infiltration with inflammatory cells, nuclear pyknosis, karyorrhexis and sometimes karyolysis indicated liver damage (Fig. 1b and c). The liver sections of the guinea pigs treated with 100 mg kg^{-1} ethanol leaves extract of CE co-administered with DM for 21 days showed little histological changes when compared to animals of DM treated group such as; vacuolization, cellular infiltration and nuclear pyknosis (Fig. 1d and e). The examination of liver sections obtained from guinea pigs co-administered with DM and 200 mg kg^{-1} CE ethanol extract and/or Liv-52 treated group for 21 days showed normal view with just little vacuolization and nuclear pyknosis compared to DM group (Fig. 1f and g).

DISCUSSION

The OPs pesticides are widely used throughout the world especially in developing countries like Yemen. There are recent studies that indicated the incidence of acute

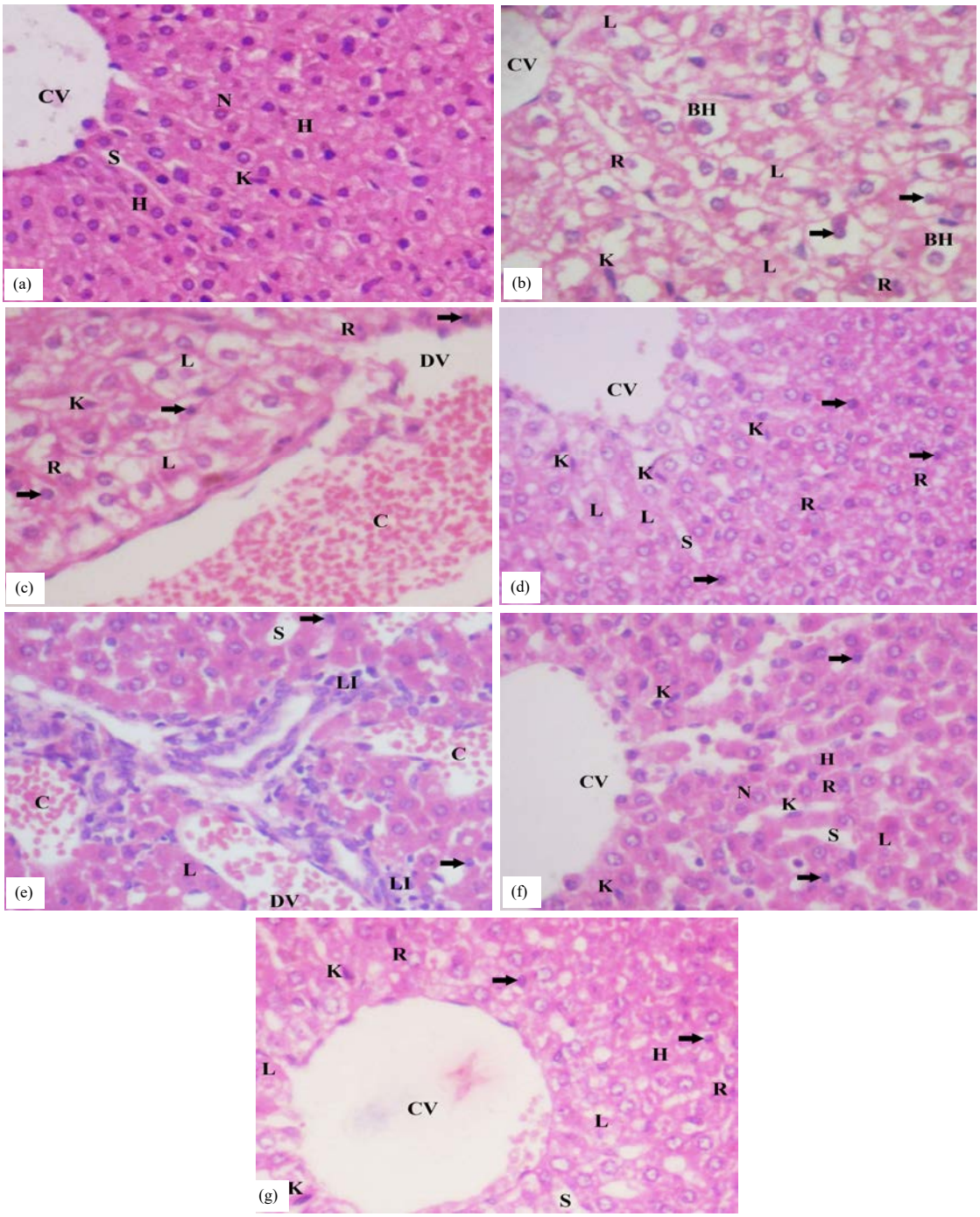

Fig. 1(a-g): Composite image showing hepatoprotective effect of *Carissa edulis* (CE) against DM-induced hepatotoxicity in male guinea pigs. Liver sections were stained with H and E, (a) Control animal showing hepatocytes (H) around central vein (CV) with normal sinusoidal spaces (S), Kupffer cells (K) and nucleus (N), (b and c) DM-treated animals showing vacuolization with ballooning hepatocytes (BH), dilatation of the portal vein (DV), congestion (C), nuclear pyknosis (arrows), karyorrhexis (R) and karyolysis (L), (d and e) CE (100 mg kg^{-1} b.wt.)+DM showing the same histopathological alteration seen in DM group, but with little appearance, (f) CE (200 mg kg^{-1} b.wt.)+DM showing normal view with little histopathological changes and (g) Liv-52 (100 mg kg^{-1} b.wt.)+DM showing normal architecture with little histopathological changes. Magnification 400X

occupational pesticide poisoning among male farmers in Asian countries including Yemen[28,51,52]. The acute effects of OPs pesticides exposure are well known[42,53], but the chronic or long-term effects are still unclear[54]. OPs pesticides produced oxidative stress in many tissues through the formation of ROS[55,56]. All the major macromolecules such as; nucleic acids, proteins and lipids may be attacked by ROS, but lipids are probably the most susceptible[57]. The oxidative destruction of lipids molecules is known as lipid peroxidation and malondialdehyde (MDA) as an end product of lipid peroxidation[56]. The current study, investigated the possible protective effect of CE ethanolic extract against oral subchronic administration of DM, an OPs pesticide, on male guinea pigs. The DM treatment caused a significant increase in the liver marker enzymes which are well known good indicators of liver function and routinely used as biomarkers to evaluate the probable toxicity of drugs and xenobiotics[58,59]. Normally, destruction to the liver parenchymal cells will result in an increase of these enzymes in the blood[60]. These results were in consistence with previous published works[42,47,53] and other related articles[61-66]. The co-administration of CE ethanol extract and Liv-52 with DM to guinea pigs resulted in normalization and restoring of these liver enzymes to normal levels. The present findings complemented the other studies on this plant extract[19,20,26,67-70] and other plants[61,64,65] which is already in common use in the traditional medicine for the management of several diseases and toxins. So, these findings suggested that administration of ethanol leaves extract of CE reduced significantly the hepatotoxicity induced by DM in guinea pigs. This significant reduction in liver marker enzymes suggested the hepatocellular protection of the plant extract[70]. On the other hand, the lipid peroxidation levels in liver homogenate were increased significantly in DM-treated animals. There are several published articles that indicated the elevation of LPO and oxidative stress during OPs pesticides poisoning[33,62,71-73]. In addition, many proposed mechanisms have been reported to explain the oxidative stress induced by DM during liver injury, such as; lipid peroxidation and interaction with membrane molecules resulting from ROS attack on biological tissues[74]. Antioxidants represented the primary defense system that controls the toxicity associated with ROS[75]. Furthermore, DM induced oxidative damage by producing ROS and decreasing the biological activities of some liver antioxidant enzymes, such as; CAT and GST. The present results were in consistence with previous studies which have shown that the acute and subchronic exposure to DM alters the antioxidant status of several tissues in different experimental animals[33,63,64,73,76-79]. Histopathological studies provide supportive evidence for biochemical observations. Animals that intoxicated with DM treatment showed a great impairment in the architecture of the hepatocytes and liver constituents as illustrated in Fig. 1. These observations were in agreement with previous report[63] which demonstrated that rats exposed to DM showed histological changes due interaction of DM with cellular membranes that cause damage and releases of their enzymes into the circulation[80]. Also, another study[81] reported that DM caused histopathological and histochemical changes in the testes of albino rats. Moreover, light microscopic analyses revealed that the DM-treated animals which received CE extract and/or Liv-52 co-administration exhibit little morphological changes compared with that seen in the livers of the DM-treated group. Thus, CE could ameliorate and alleviate the liver damage induced by DM intoxication. There are several reports supported the role of antioxidants in attenuating the toxicity of some pesticides and toxins in experimental animals. For example, a synthetic antioxidant "acetyl gallate derivative" (SAC) showed protective capacity against hepatic oxidative stress and brain DNA damage induced by DM in male rats[33]. In addition, antioxidant quercetin showed protection against DM-induced oxidative stress in human lymphocytes by decreasing lipid peroxidation, protein oxidation and increasing superoxide dismutase and catalase activities[82]. Also, previous investigation revealed a histopathological change in liver tissue of guinea pigs treated with DM and the severity of these lesions was reduced by administration of a combination[47] of vitamin C and vitamin E. Furthermore, the wide range of biological activities of many medicinal plants extract may be due to the presence of different biologically active components such as; flavonoids, saponins, tannins and cardiac glycosides[69,83,84]. Present study adopted the 21 days toxicity study to assess the toxicity of DM, which is a well-accepted method for eliciting any toxicity on long term administration of drug[85]. The present findings were in agreement with the results of previous acute and/or chronic toxicity studies in rat[70,86] and mice[67,87]. These findings illustrated the possible protective capacity of CE extract in mitigating the toxicity induced by DM, but further studies are recommended to explain the exact molecular mechanism by which the plant extract showed its hepatoprotective capacity.

CONCLUSION

According to the presented results of this study, it can be concluded that DM toxicity induced LPO and generation of free radicals in liver tissue leading to oxidative stress and hepatotoxicity. In addition, the combined treatment with CE and/or Liv-52 showed a significant protective effect against

DM-induced liver injury at the biochemical and histological level which provide evidence of the beneficial effect of CE extract in mitigating the subchronic DM intoxication in male guinea pigs.

SIGNIFICANCE STATEMENT

This study discovered the hepatoprotective role of CE ethanol extract against DM-induced hepatotoxicity in male guinea pigs that can be beneficial to overcome the toxicity and side effect associated with liver disorder due to pesticide intoxication. This study will help the researcher to identify the exact mechanism by which the CE extract ameliorates the hepatotoxicity. Thus, a new idea on the use of CE extract to manage pesticide toxicity may be achieved.

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