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IMMUNOHISTOCHEMICAL EVALUATION OF THE EUPHORBIA INARTICULATA EXTRACT ON LIVER AND KIDNEY TISSUES IN HEPATOCELLULAR CARCINOMA RATS

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ABSTRACT

The study aimed to evaluation of immunohistochemical the Euphorbia Inarticulata Extract on Liver and Kidney Tissues in Hepatocellular Carcinoma Rats. Rats were grouped into six each of six as (1) control received one dose (1 ml 0.9% NaCl/ip) and two weeksafter 2 weeks later receivedgot olive oil (1 ml/sc) (once/week) for 16 weeks, in line with 1% DMSO/p.o. for 18 weeks. (2) 250mg Extract and (3) 400mg Extract groups were received as in the control group and received daily extract (250 mg/kg/P.o.) and (400 mg/kg/P.o.) in DMSO for 18 weeks, respectively. (4) DENA/CCl4 group received one dose of DENA (200 mg/kg/ip) in saline and two after 2 weeksweeks later received (3 ml/kg CCl4/sc) in olive oil (once/week) for 16 sixteen weeks, in line with 1% DMSO/p.o. for 18 weeks. DENA/CCl4 rat model group showed an induced strong positive reaction to Bax immunostaining in the cytoplasm of liver cells and glomeruli and renal tubules. The study suggests that the present model showed hepatic and renal apoptosis that is related to impairing their function and oral administration of E. inarticulata methanol extract, especially in higher dose (400 mg).

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Introduction

Hepatocellular carcinoma (HCC) is among the major primary liver cancer as well as leading source of cancer-related mortality globally [1]. Notably, HCC usually becomes apparent in individuals who have underlying chronic liver disease and cirrhosis that might be caused by viral infections (i.e. hepatitis B & C viruses) [2]. Other risk factors for HCC include environmental toxins, oxidative stress, autoimmune hepatitis, obesity, chronic alcohol use, diabetes mellitus, etc [3, 4]. In Saudi Arabia, liver cancer represents 6.3% of all cancers, with hepatocellular carcinoma accounting for about 90% of all liver cancers [5, 6].

Euphorbiaceae (the spurge family) is a large flowering plant family that comprises about 300 genera and 8,000 species throughout the world, particularly in the arid and semi-arid regions of the tropics and subtropics. *Euphorbia* is the biggest genus of the *Euphorbiaceae* plant family, consisting of close 2 thousand species [7]. The family is highly ecologically diverse; represented by the trees, shrubs, herbs, and even lianas, and described by the occurrence of either white milky latex (such as *Euphorbia*) or watery sap (such as *Ricinus* and *Chrozophora*) which are irritating and more or less toxic and usually protects these plants from browsing animals [8, 9].

Previous surveys in Saudi Arabia showed that a large number of Saudi people are interested in using alternative medicine either alone or along with modern medications [10-12]. The most common medicinal plants found in the region belong to the *Leguminosae*, *Labiatae*, *Compositae*, and *Euphorbiaceae* [13]. *Euphorbiaceae* is widely distributed throughout Saudi Arabia, with about 40 species have been recognized [14, 15]. *Euphorbia inarticulata* Schweinf **(Figure 1)** is a branched spiny, between

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1–2 m high, 3–5-angled succulent, yellow cyathia clustered at the top of the stem, with 3-10 mm long, paired spines [7, 14]. It is common in scattered localities in the southwestern region of Saudi Arabia [16]. Overall, studies on *E. inarticulata* Schweinf are very limited. A previous study revealed that topical use of ethyl acetate extract of *E. inarticulata* on experimentally induced excision injuries in rats presented significant results of wound shrinkage and healing with higher fibroblasts and collagen content in treated animals compared to standard drug-treated groups and control [17]. Nonetheless, the methanol extracts of *E. inarticulata* Schweinf showed no antileishmanial activity [18]. However, studies on the activity of *E. inarticulata* are rare-existent in the literature. Owing to this, the present *in vivo* study aimed to explore the antiapoptotic action of the *E. inarticulata* Schweinf methanolic extract oral supplementation on HCC induced by DENA and CCl4 in male Wistar rats through the immunohistochemical changes in expressions of Bax immunostaining in liver and kidney tissues.

Materials and Methods

Plant Material Collection

The present study collected Euphorbia inarticulata Schweinf. (March 2017, 2018) from Al'Aridah, Jazan region, Saudi Arabia 17°03'45.6"N 43°03'03.7"E https://goo.gl/maps/cXT6m1ppRSo and Photograph from the collection area of the Euphorbia inarticulata Schweinf. Identification of the plant material was kindly verified by Dr. Yahya Masrahi, Department of Biology, Faculty of Science, Jazan University, Saudi Arabia.

Extraction

The dried Euphorbia whole plant (150 gm) was ground and extracted as previously described by Wang *et al.*, (2012) with hexane for 24 hours and then filtered. The residue was extracted by soaking in ethyl acetate for 24 hours and then filtered [19]. The collected residue was extracted by soaking in methanol for 24 hours then filtered and the filtrate was concentrated by a rotary evaporator (BÜCHI, Switzerland). The dried methanol extract was weighed to be used in the study.

Experimental Animals

Thirty-six adult male Wistar rats weigh up to 140-160 g were employed in the current research. Animals were kept under a controlled temperature of 25±2 ºC and 12 hours of light/dark cycle throughout the experiment. The animals were free to use water and a standard commercial pelleted diet. Every effort was made to reduce the number and distress of animals and all animal procedures followed the recommendations of the Institutional Review Board for Scientific Research- Jazan University (Saudi Arabia) with reference No. REC41/1/071.

Hepatocellular Carcinoma Induction

For the induction of hepatocellular carcinoma, rats were given a single intraperitoneal dose of DENA (200 mg/kg) dissolved in saline. After two weeks, the animals received subcutaneous injections of carbon tetrachloride (CCl_4) (3 ml/kg) dissolved in olive oil once weekly for 16 weeks to promote the carcinogenic effect of DENA [20].

Experimental Design

The experimental animals were randomly designated into 6 groups of 6 rats respectively as follows:

Group 1: Control, rats were administered 1 dose of 1ml intraperitoneal injection of 0.9% NaCl. after 2 weeks, the animals received 1 ml olive oil hypodermically (once/week) for sixteen weeks. Furthermore, the rats were administerd 1% DMSO via oral gavage daily for 18 weeks.

Group 2: 250 mg Extract, rats received NaCl and olive oil as Group I. Then, the animals received 250 mg/kg methanol extract daily dissolved in DMSO via oral gavage for 18 weeks (low dose normal group).

Group 3: 400 mg Extract, rats received NaCl and olive oil as Group I. Then, the animals received 400 mg/kg methanol extract daily dissolved in DMSO via oral gavage for eighteens weeks (high dose normal group).

Group 4: DENA/CCl_4, rats received **an** intraperitoneal injection of DENA (200 **mg / kg)** dissolved in 0.9% NaCl. Two weeks later, the animals received 3 **ml / kg of** CCl4 diluted **with** olive oil subcutaneously **once a week** for 16 weeks. In addition, rats received 0.1% DMSO daily for 18 weeks **by gavage** (hepatocellular carcinoma rat model group).

Group 5: DENA/CCl_4 + 250 mg/kg Extract, rats received DENA and CCl_4 as a group (4) and 250 mg/kg extract daily for eighteen weeks (treated group, low dose).

Group 6: DENA/CCl_4 + 400 mg/kg Extract, rats received DENA and CCl_4 as Group (4) and 400 mg/kg extract daily for 18 weeks (treated group, high dose).

Body weights of all animals were measured every week to adjust the doses of the methanolic plant extract and CCl_4 for every kg of body weight over the entire period of the experiment as indicated by any change in the body.

Tissue Sampling

At the end of the experiment, rats were fasted overnight, but were given free water intake and then sacrificed with diethyl ether under mild anesthesia. Liver and kidney tissues were rapidly excised, and specimens of liver and kidney tissues were secured in ten perecent neutral buffered formalin solution for the immunohistochemical staining.

Immunohistochemical Staining for Bax and Bcl-2

The liver and kidney tissues of control and experimental animals used to locate cells expressing bcl2 and Bax proteins were dependent on immunohistochemistry. based on a streptavidin-biotin peroxidase method (Biogenex, San Ramon, CA, USA) [21]. Tissues from every group were secured in buffered formalin (10%), set in paraffin, cut sections (4 μm) with the use of a rotary microtome. It was then located on poly-L-lysine coated clean glass slides, dried at 37°C and used for immunohistochemical studies. Tissue sections embedded in paraffin were deparaffinized and rehydrated with stepwise ethanol diluted with distilled water. Incubation with hydrogen peroxide (3%) blocked Endogenous peroxidase in methanol for 10 min. Microwave helped achieve antigen retrieval in citrate buffer solution (pH 6.0) for 10 minutes, followed by a washing step with Tris-buffered saline (pH 7.6). The tissue sections were then incubated with power Block TM reagent (BioGenex, San Ramon, CA, USA), universal proteinaceous blocking reagent, for 15 min at room temperature to block non-specific binding. Then, these tissue sections were incubated with specific primary antibodies (Bax and Bcl-2, Thermo Scientific, USA) overnight at 4°C. The bound primary antibody was incubated with the secondary antibody conjugated with horseradish peroxidase for 30 min at room temperature. The antigen-antibody complex was detected using 3, 3´- diaminobenzidine (Sigma Fast DAB tablets, D-4293, Sigma St. Louis, MO, USA) and was used as a chromogen after rinsing with Tris-buffered saline. Only cytoplasmic staining was considered for evaluation while nuclear staining was interpreted to be nonspecific staining. The intensity of the staining was scored in ascending order from negligible < mild < moderate < strong intensity staining pattern [22].

Results and Discussion

Effect of E. Inarticulata Methanol Extract on Liver Bax and Bcl-2 Protein

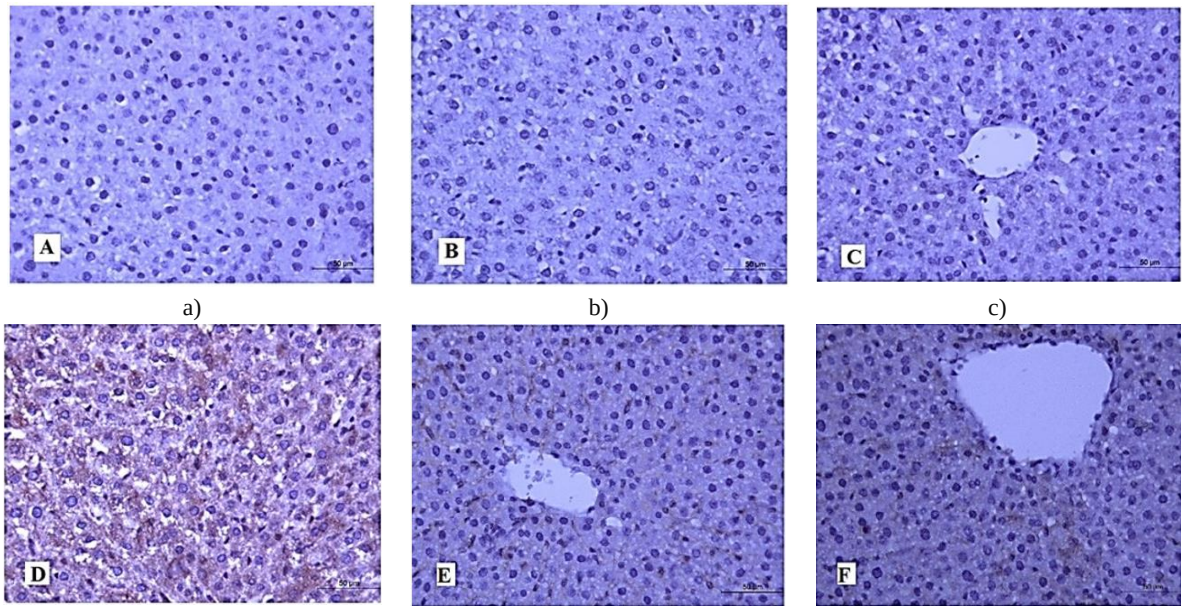

Figure 1. Photomicrographs of liver sections of rats from the control group (a), 250 mg euphorbia (b), and 400 mg euphorbia (c) treated groups, respectively showing negligible Bax immunostaining. A photomicrograph from DENA/CCl4 shows induced Bax immunostaining in the cytoplasm of liver cells (d). A photomicrograph from DENA/CCl4 treated with 250 mg euphorbia showed mild expression of Bax (e) and a photomicrograph from DENA/CCl4 treated with 400 mg euphorbia showed moderate expression of Bax X (f).

Results of liver tissue of Bax protein immunostaining are represented in **Figure 1**. Where the photomicrograph of the normal control group showed negligible Bax immunostaining (a), and in the same manner photomicrographs of the 250mg Extract group (b) and the 400mg Extract group (c) liver sections showed negligible Bax immunostaining. Meanwhile, the photomicrograph of the HCC rat model group (DENA/CCl4) showed a strong positive reaction to Bax immunostaining in the cytoplasm of liver cells (d). Treatment with the *E. inarticulata* methanol extract recorded in photomicrograph (e) that the low dose of 250mg presented a minimal expression of Bax in the cytoplasm of hepatocytes, while the high dose of 400mg presented moderate expression of Bax in the cytoplasm of hepatocytes (f).

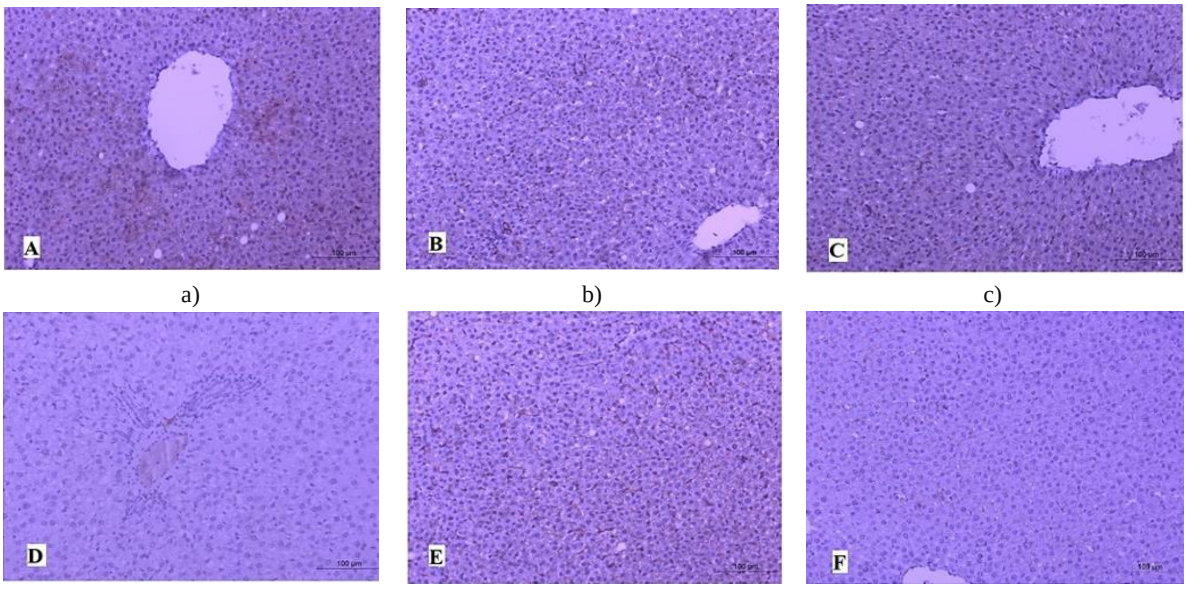

Figure 2. Photomicrographs of liver sections of rats from the control group (a), 250 mg euphorbia (b), and 400 mg (c) euphorbia treated groups, respectively showing a positive reaction to Bcl-2 antibodies mainly in the cytoplasm of hepatocytes. A photomicrograph from DENA/CCl4 rats showed a faint cytoplasmic reaction to Bcl-2 antibodies (d). A photomicrograph from DENA/CCl4 rats treated with 250 mg euphorbia showed moderate expression of Bcl-2 (e) and a photomicrograph from DENA/CCl4 rats treated with 400 mg euphorbia showed mild expression of Bcl-2 (f).

Results of liver tissue immunostaining of Bcl-2 protein are represented in **Figure 2**. The photomicrograph of the normal control group showed a positive reaction to Bcl-2 antibodies mainly in the cytoplasm of hepatocytes (a), as well as in photomicrographs of the 250mg Extract group (b) and the 400mg Extract group (c). However, the liver section of the DENA/CCl4 group showed a faint cytoplasmic reaction to Bcl-2 antibodies (d). Treatment with the *E. inarticulata* methanol extract (e) by the low dose of 250mg showed moderate expression of Bcl-2 in the cytoplasm of hepatocytes, while the high dose of 400mg showed mild expression of Bcl-2 in the cytoplasm of hepatocytes (f).

Effect of E. Inarticulata Methanol Extract on Kidney Bax and Bcl-2 Protein

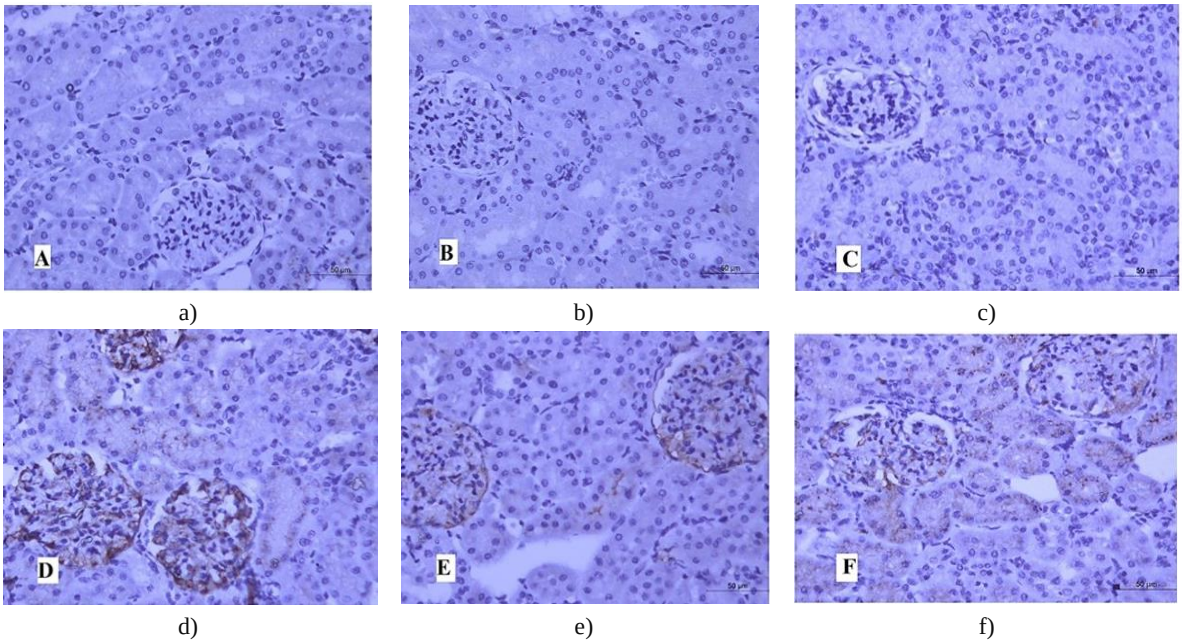

Figure 3. Photomicrographs of kidney sections of rats from the control group (a), 250 mg euphorbia (b), and 400 mg (c) euphorbia treated groups, respectively showing negligible Bax immunostaining. A photomicrograph from DENA/CCl4 treated rats showed a strong positive reaction to Bax in glomeruli and renal tubules (d). A photomicrograph from DENA/CCl4 treated with 250 mg euphorbia showed mild expression of Bax in glomeruli (e) a photomicrograph from DENA/CCl4 treated with 400 mg euphorbia showed moderate expression of Bax in glomeruli and renal tubules (f).

Results of kidney tissue immunostaining of Bax protein are represented in **Figure 3**. Where the photomicrograph of the normal control group showed negligible Bax immunostaining (a), like the already represented in the photomicrographs of the 250mg Extract group (b) and the 400mg Extract group (c). In the meantime, the photomicrograph of the HCC rat model group (DENA/CCl4) showed a strong positive reaction to Bax immunostaining in the glomeruli and renal tubules (d). Treatment with the *E. inarticulata* methanol extract recorded in photomicrograph (e) that the low dose of 250mg showed mild expression of Bax in glomeruli, while the high dose of 400mg showed moderate expression of Bax in the glomeruli and renal tubules (f).

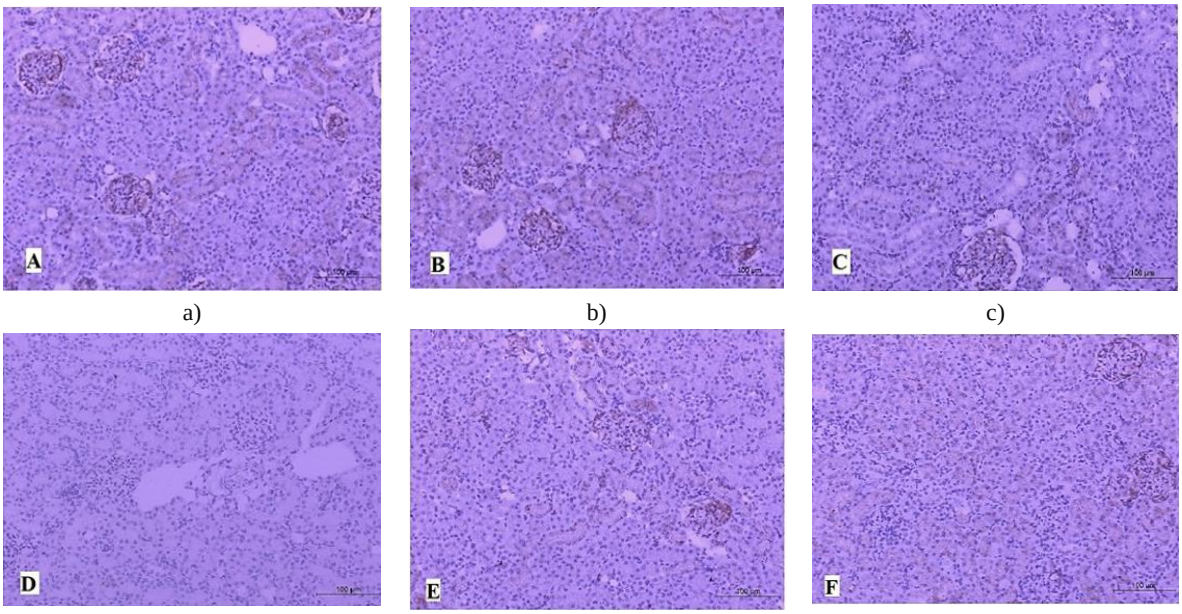

Figure 4. Photomicrographs of kidney sections of rats from the control group (a), 250 mg euphorbia (b), and 400 mg euphorbia (c) treated groups, respectively showing strong Bcl-2 immunostaining in glomeruli and renal tubules. A photomicrograph from DENA/CCl4 rats showed negligible reaction to Bcl-2 immunostaining (d). A photomicrograph from DENA/CCl4 rats treated with 250 mg euphorbia showed strong expression of Bcl-2 in glomeruli and renal tubules (e) and a photomicrograph from DENA/CCl4 rats treated with 400 mg euphorbia showed moderate expression of Bcl-2 in glomeruli and renal tubules (f).

Results of kidney tissue immunostaining of Bcl-2 protein are represented in **Figure 4**. The photomicrographs showed strong Bcl-2 immunostaining in glomeruli and renal tubules in the normal control group (a), the low dose (250mg) Extract group (b), and the high dose one (400mg) (c). However, DENA/CCl4 group showed negligible cytoplasmic reaction to Bcl-2 antibodies (d). Treatment with the *E. inarticulata* methanol extract (e) by the low dose of 250mg showed strong expression of Bcl-2 in glomeruli and renal tubules. Moreover, the high dose group (400mg) presented moderate expression of Bcl-2 in glomeruli and renal tubules (f).

The present study is the first to provide information on the anti-apoptotic activity of *E. inarticulata* Schweinf extract on DENA/CCl_4-induced HCC in rats. Many herbal products have been investigated by *in vitro* and *in vivo* as anti-HCC agents for many years, and certain natural products were found productive in the co-treatment and inhibition of HCC [23]. Hence, effective herbal products have been recommended to be used along with previous chemical drugs and following stoppage of chemical drugs, as a conservational remedy to prevent HCC development [24].

Diethylnitrosamine (DENA) is a notorious chemical with a strong hepatocarcinogenic effect, used to induce HCC in experimental rat models and is similar to human HCC [25]. Also, liver fibrosis was induced in rats by subcutaneous injection of CCl4 which is largely used as a toxic solvent for making animal models of liver fibrosis/cirrhosis [26]. In addition, the hepatorenal toxicity including cell apoptotic induction already been demonstrated in many studies which recorded previously structural integrity of liver and kidney tissues damaged by DENA and CCl4 [27-32]. It was previously concluded that injection of DENA and then repeated prescription of carbon tetrachloride CCl4 could be employed as a model for studying the molecular mechanisms of fibrogenesis and HCC growth and in cancer hazard/chemotherapy testing of drug candidates [33]. The present study results revealed a strong positive reaction to Bax immunostaining and a faint cytoplasmic reaction to Bcl-2 antibodies in the cytoplasm of liver cells which indicates the hepatocyte apoptosis in the rat model is in line with the previously reported in different studies. A previous study used a rat model of HCC induced by DENA recorded increased cytoplasmic staining for Bcl-2 with subsequently decreased cytoplasmic staining for Bax [34], and recently, CCl4 administration was recorded to cause upregulation of pro-apoptotic protein Bax and downregulation of antiapoptotic protein Bcl-2 [26]. It was postulated later that the mitochondrial signaling pathway might be involved in CCl4-induced hepatocyte apoptosis through upregulating Bax and downregulating Bcl2 [35]. The results of the present study revealed that E. inarticulata methanolic extract supplementation recorded negligible Bax immunostaining and positive reaction to Bcl-2 antibodies mainly in the cytoplasm of hepatocytes as

well as in glomeruli and renal tubules. That's in the low (250mg) and high (400mg) doses without any significant change as compared to the control non-treated group that may assess its safety in liver and kidney tissues. Treatment of the rat model revealed a dose-dependent manner of effects that ranged from the mild expression of Bax in euphorbia extract at a low dose to moderate in a higher dose. Meanwhile, the reverse effect recorded for Bcl-2 immunostaining ranged from the moderate expression of Bcl-2 in low doses to mild expression in the higher dose in the cytoplasm of hepatocytes and glomeruli and renal tubules. That is except in the case of kidney tissue as regards Bcl-2 immunostaining in glomeruli and renal tubules which showed more precious effects ranging from the strong expression of Bcl-2 in euphorbia extract low-dose treated group to moderate in the higher dose-treated one. The present study results are consistent with those for many other anti-fibrotic agents that have inhibit apoptosis by upregulating Bcl-2 and downregulating Bax signaling, which is in a dose-dependent manner. A previous study used Ginkgo biloba extract, as a hepatoprotective from CCl4 liver fibrosis indicated from the immunohistochemistry analysis that the extract reduced liver fibrosis by mechanisms that may be associated with inhibiting hepatocyte apoptosis via downregulation of Bax, upregulation of Bcl-2 [35]. That is as in the case of salvianolic acids, which are the main active water-soluble extracts of Salvia miltiorrhiza Bunge. Salvianolic acids were suggested previously to be considered as a potential novel therapeutic agent for the treatment of fibrotic lung diseases and its antiapoptotic activity is represented via Bcl-2 protein decrease in a dose-dependent manner [36] and has previously been demonstrated to block hepatic fibrosis [37]. Also, arsenic trioxide was used on rat experimental hepatocellular carcinoma by DENA and its renal cytotoxicity was studied and recorded that arsenic trioxide didn't show obvious renal toxicity, and that was related to the increased expression of bcl-2 in the renal tubular epithelium, the inhibition of apoptosis and the anti-oxidation effects [38]. Several studies showed that renal toxicity had a close correlation with its oxidized damage to tubules and Bcl-2 is an apoptosis inhibitor and a strong anti-oxidant [39-42]. Inhibition of cell apoptosis via inhibition of Bax molecule expression and increase of Bcl-2 expression is suggested to delay or reverse renal tubular interstitial fibrosis, thus improving the renal function in kidney diseases [43, 44]. A previous study interested in the anti-proliferation activity of Astragalus treatment which is an extract from Radix astragali herbal plant on human HCC was recorded a significant decrease in the expression of anti-apoptotic protein Bcl-2 as compared with the control and the expression of the pro-apoptotic protein Bax was increased significantly by in a dose-dependent manner [45]. The results of the present study are in line with previous studies in stating that Bcl-2 and Bax function as tumor anti-apoptotic and pro-apoptotic factors that might interact with each other in regulating apoptosis and alteration of their levels influence apoptosis. Hence, the immunohistochemical results. Conclusion: The present study suggests that E. inarticulata Schweinf methanolic extract oral supplementation could protect the hepatorenal apoptosis in the HCC induced rat model by DENA and CCl4 via downregulation of the Bax and Bcl-2 protein expressions.

Conclusion

This paper is interested in studying the immunohistochemical evaluation of Euphorbia extract on liver and kidney tissues in mice with liver cancer. TDENA/CCl4 rat model group showed an induced strong positive reaction to Bax immunostaining in the cytoplasm of liver cells and glomeruli and renal tubules, also they were treated with the E. inarticulate methanol extract recorded in photomicrograph (E) that the low dose of 250mg showed mild expression of Bax in glomeruli, while the high dose of 400mg showed moderate expression of Bax in the glomeruli and renal tubules (F). And we still need many studies in this field, and therefore the optimal use.

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Ethics statement: None

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