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Secondary Metabolites and Hepatoprotective Activity of *Euphorbia retusa*

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ABSTRACT

Objectives: This study aimed to explore the secondary metabolites from the methanolic extract of *Euphorbia retusa* forssk, as it is known for its use in folk medicine and for being rich in bioactive molecules. **Methods:** The methanolic extract was fractionated by different chromatographic techniques and the structures of the isolated compounds were elucidated by UV, ^{1}H and ^{13}C NMR spectroscopy. Hepatoprotective activity of *E. retusa* extract was evaluated on CCl_4-induced liver damage in rats through biochemical assessment of serum ALT as well as MDA, GSH and NO. It was also evaluated through histopathological study of liver autopsy samples. **Results:** From the aqueous methanolic extract of *Euphorbia retusa* forssk. aerial parts seven known phenolic compounds; kamferol-3-O-*β*-D- glucopyranoside (1), quercetin -3-O-*β*-D- glucopyranoside (2), 3,3`dimethoxy ellagic acid (3), ellagic acid (4), gallic acid (5), kamferol (6) and quercetin (7) were isolated and identified by chromatographic and spectroscopic analysis. Administration of the extract (100 and 200 mg/kg body weight) significantly decreased the AST and ALT levels, inhibited the CCl_4 -induced elevated levels of NO and MDA and increased the level of hepatic GSH. A comparative histopathological study of liver exhibited almost nearly normal architecture as compared to toxicant group. **Conclusion:** *Euphorbia retusa* extract has shown to have hepatoprotective activity on CCl_4-induced liver damage in rats which might be attributed to its phenolic contents.

Keywords: *Euphorbia retusa; flavonoids and hepatoprotective activity*

INTRODUCTION

Euphorbiaceae (Spurge family) is a large family, including 300 genera and over 5000 species[1]. The genus *Euphorbia* is the largest in the family, comprising about 2000 species, a significant percentage mostly those originating in Africa and Madagascar, are succulent[2].Some species of genus *Euphorbia* have been used as medicinal plants in folk medicine for the treatment of skin diseases, gonorrhea, migraine, intestinal parasites and as wart cures[3].Members of genus *Euphorbia* are known for their rich content in secondary metabolites and bioactive molecules such as diterpenes and triterpenes[4]. Diterpenes occurring in plants of the genus *Euphorbia* are the focus of natural product drug discovery because of the wide range of their therapeutically relevant biological activities as antitumor, cytotoxic, anti-viral propertiesand anti-inflammatory activity [5]. Flavonoids and tannins were reported in *Euphorbiaceae,* are known for their anti-tumor, hepatoprotective and antioxidant activities[6]. Also, among *Euphorbia* diterpenes, ingenol 3-angelate, which was approved by the FDA in 2012 and the EMA in 2013 for the treatment of actinic keratosis, a precancerous skin condition[7].

Euphorbia retusa Forssk. locally called as Ghazalah, Idat Al-Haish and Um-labbain, is an endemic and widespread plant in Arabian Peninsula. It is used as anti-asthmatic,anti-microbial, anti-cough and expectorant[8, 9].

MATERIALS AND METHODS

Equipments

^{1}H NMR and ^{13}C NMR spectra were measured on Bruker AMX- 400, Avance 400, and Avance 300 spectrometers with standard pulse sequences operating at 400, 300 MHz in ^{1}H NMR and 100, 75 MHz in ^{13}C NMR, (Bremen, Germany). Chemical shifts are given in δ values (ppm) using tetramethylsilane as the internal standard. UV/VIS: Shimadzu UV-visible recording spectrophotometer model-UV 240. Column chromatography (CC): was carried out on Polyamide6S, (Riedel-DeHaen, Hannover, Germany) and Sephadex LH-20 (Pharmacia Fine Chemicals); PC was carried out on Whatman No. 1 and 3MM paper (46 × 57 cm) using the following solvent systems: (1) BAW (n-butanol: aceticacid: water 4:1:5 upper phase); (2) H_2O and (3) AcOH/H_2O (15:85). Carbon tetrachloride (BDH Chemicals, England), gallic acid, quercetin and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu solution and 95% ethanol were purchased from Merck Co. (Santa Ana, CA, USA). All chemicals and solvents used were of analytical grade.

Plant Material

The fresh arial parts of *Euphorbia retusa*, were collected from Wadi Hagool, Suez city in May 2013.The identity of the plant was kindly confirmed by Prof Dr. Ibrahium El-Garf, Associate Professor of Plant Taxonomy, Department of Botany, Faculty of Science, Cairo University The plant was air dried in shade then powdered. a voucher specimen (# 01Ere) was kept in the herbarium of Faculty of Pharmacy, Helwan University.

Animals

Forty two Adult male Sprague-Dawley rats (120 - 150 g) were purchased from the Animal House, science park European countryside, Misr University for Science and Technology. They were fed a standard diet of commercial rat chow and tap water *ad libitum*. Rats were left to acclimatize to the environment for at least one week prior to inclusion in the experiments. All animal procedures were performed in accordance to the Animal Ethical Committee guidelines of Faculty of Pharmacy, Helwan University (Approval Committee NO 018A 2018).

Extraction and isolation

The aerial parts of *E. retusa* (1.5 kg) were crushed and extracted with 70% methanol by soaking at room temperature and the aqueous methanolic extract was evaporated under reduced pressure and lyophilized (120gm). A sample (100 gm) of the dry extract was fractionated by chromatography on polyamide 6S column. The column was eluted with water then with water–methanol mixture in 20% step wise gradient elution (decreasing polarity). The obtained fractions (500 ml of each fraction) were subjected to paper chromatography using BAW and 15% acetic acid as a developing solvent, and the similar fractions were collected together to give five major fractions (I-V). Separation of fractions II (200 mg) this fraction was subjected to preparative paper chromatography (3MM Whatman filter paper) using 15 % acetic acid in water as eluent. The chromatogram showed one minor and one major band which was cut and eluted with methanol, then evaporated under reduced pressure. The residue was further purified by sephadex LH-20 column chromatography, eluted with methanol to give one spot of pure compound**1** (30 mg). Fraction III (180 mg) have been applied on Sephadex column LH-20 CC eluted with 20 - 40% methanol to yield two collective sub fractions (IIIa and IIIb). Subfraction IIIa was subjected to sephadex LH-20 column, eluted with *n*-butanol saturated with water (upper layer) afforded anothersubfraction which was applied on sephadex LH-20 column and eluted by 30% methanol to afford one spot of compound **2** (20 mg). Subfraction IIIb was subjected to sephadex LH-20 column, eluted with *n*-butanol saturated with water (upper layer) afforded subfraction which was applied on another sephadex LH-20 column, eluted with 40% methanol yielding one spot of compound **3** (40 mg). Elution of fraction IV (150 mg) by *n*-butanol water-saturated; afforded two compounds which were separated by preparative paper chromatography using 15% AcOH as eluent and were further purified on Sephadex LH-20 CC using EtOH-H_2O (1:1) to give pure compounds **4** (25 mg) and **5** (15 mg) respectively. Consecutive CC on Sephadex LH-20 with *n*-butanol-water saturated for elution of fraction V (240 mg) give two sub-fractions which were separated by preparative paper chromatography using BAW (4:1:5) as eluent. These two sub-fractions were further purified on Sephadex LH-20 CC using methanol HPLC to afford the purifiedcompounds**6** (25 mg) and **7** (50 mg).

Determination of total phenolic content

The concentration of total phenolics of the plant extract was determined according to the method described [10]. Five mgs of the methanolic extract of the aerial parts were accurately weighed and dissolved in 5 ml of 50% methanol to prepare a solution of final concentration 1 mg/ml. An aliquot (2 ml) of the extract and standard solution of gallic acid (8-100 µg/ml) was added to 25 ml volumetric flask containing1.5 ml of Folin-Ciocalteu reagent and 4ml of 20% sodium carbonate solution, then the solution was diluted to 25 ml with distilled water. The absorbance of the resulting solution was measured, after 30 min at λ_{max} 765 nm,

against a blank prepared at the same time using 2 ml of distilled water instead of the standard solution. All determinations were carried out in triplicate. The total phenolic concentration was expressed as gallic acid equivalents (GAE).

Determination of Total flavonoid content

Total flavonoid concentration of plant extract and fractions was determined according to the reported procedure[11]. Ten mgs of the methanolic extract were accurately weighed and dissolved in 10 ml of 80% methanol to prepare a solution of final concentration 1 mg/ml. An aliquot (0.5 ml) of the extract and standard solution of quercetin (10-100 µg/ml) was added to 5 ml volumetric flask containing 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate aqueous solution and 2.8 ml of distilled water. The solution was well mixed, and the absorbance was measured spectrophotometrically at 415 nm against blank. All determinations were carried out in triplicate. Total flavonoid content was expressed as mg quercetin equivalent (QE)/g plant extract.

Determination of hepatoprotective activity

Toxicity study

The LD_{50} of the methanolic extract of *Euphorbia retusa* was estimated according to **Karber's procedure**[12]. Male albino rats (120-150 g) were divided into seven groups, each of six animals. Preliminary experiments were carried out to determine the minimal dose that kills all animals (LD_{100}) and the maximal dose that fails to kill any animal. Several doses at equal logarithmic intervals were chosen in between these two doses; each dose was injected in a group of six animals. The number of dead animals in each group, 24 hrs after injection was determined and the median lethal dose (LD_{50}) was calculated.

Experimental Design

Experimental animals were randomly divided into 7 groups (n = 6/group). Groups were arranged as follows: **Group I:** Rats served as normal control and they orally administered saline for 21days. **Group II:** Rats administered only plant extract (100 mg/kg b.wt.,oral) for three weeks. **Group III:** Rats administered only plant extract (200 mg/kg b.wt., oral) for three weeks. **Group IV:** Rats orally administered saline for 15 days followed by administration of CCl_4 (1.5mg/kg b.wt, i.p) twice weekly, for three weeks. **Group V:** Rats administered silymarin (25mg/kg b.wt., oral) for 15 days followed by administration of CCl_4 (1.5mg/kg b.wt., i.p) twice weekly, for three weeks. **Group VI:** Rats administered plant extract (100 mg/kg b.wt. oral) for 15 days followed by administration of CCl_4 (1.5 mg/kg b.wt. i.p) twice weekly and for three weeks. **Group VII:** Rats administered plant extract (200 mg/kg b.wt. oral) for 15 days followed by administration of CCl_4 (1.5mg/kg b.wt., i.p) twice weekly and for three weeks. CCl_4-induced hepatotoxicty study was performed according to the method described[13]. Acute toxicity study was also performed on rats during 24- 72 hrs after the last dose of CCl_4.

Biochemical assessment

By the end of the three weeks experiment, blood samples were obtained from the retro-orbital vein plexuses, under ether anaesthesia. ALT, AST in serum were determined[14]. All animals were sacrificed, then the livers were removed and a part from the liver was homogenated and used for determination of the lipid peroxidation (LPO) which was determined by the estimation of biochemical paramteres like; malondialdhyde (MDA) content[15], the reduced glutathione (GSH)[16]and nitric oxide (NO)[17].

Histopathological studies

Autopsy samples were taken from the liver of rats in different groups and fixed in 10% formalin for twenty four hours. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degrees in hot air oven for 24 hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slidge microtome. The obtained tissue sections were collected on glass slide, deparaffinized, and stained by hematoxylin-eosin stain for routine examination through the light electric microscope[18].

Statistical analysis:

The results were expressed as Mean ± S.E.M. Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer's multiple comparison.

RESULTS AND DISCUSSION

Phytochemical results

Seven known phenolic compounds were isolated from the aqueous methanolic extract of *E. retusa* forssk. aerial parts**.** Four of them were isolated for the first time from this species which were identified as 3,3`dimethoxy ellagic acid (**3**), ellagic acid (**4**),gallic acid (**5**) and quercetin(**7**) and three were previously isolated from the plant kampferol-3-O-*β*-D-glucopyranoside (**1**), quercetin -3-O-*β*-D-glucopyranoside (**2**) and kampferol (**6**).

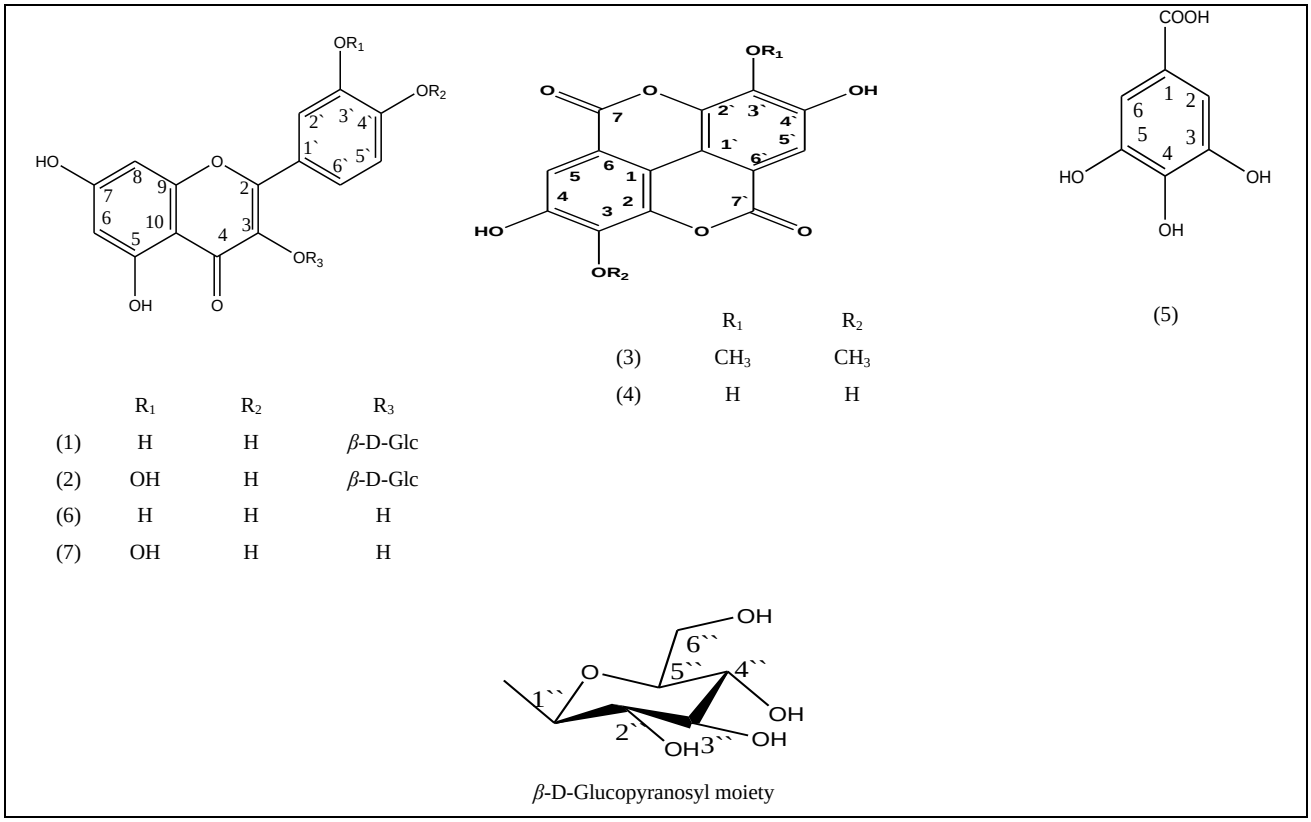

Figure 1. Structures of the isolated compounds

Characterization and identification of the isolated compounds

Kampferol-3-O-*β*-D- glucopyranoside (Astralagin) (1): yellow powder; dark purple under UV light gave bright yellow with ammonia vapor. **R_f** values: 0.52 (1),0.30 (3); **UV λ_{max} nm** (MeOH): 266, 346, +NaOMe: 274, 401, +NaOAc : 274, 393, +NaOAc /H_3BO_3: 267, 352, +$AlCl_3$: 274, 396, +$AlCl_3$/HCl: 274, 394 nm**, ^{1}H NMR** (CD_3OD) : δ (ppm)8.1 (2H, *d*,*J*=8.8 Hz,H-2′, 6`), 6.88(2H, *d*,*J*= 8.8 Hz,H-5′, 3`), 6.21(1H,*d*, *J*=2 Hz, H-6), 6.42 (1H, *d*, *J*=2 Hz,H-8), 5.37(1H, d, *J*=7.4 Hz, H′′),3.07-3.5 (m, sugar protons) ,**^{13}C NMR** (CD_3OD): δ (ppm) 156.96(C-2), 133.68 (C-3), 177.7 (C-4),161.66 (C-5), 99.26 (C-6), 164.6 (C-7), 94.30 (C-8), 157.31 (C-9), 104.43 (C-10), 121.29 (C-1'), 131.34 (C-2'&6`), 115.55 (C-3'&5`), 160.2(C-4'), 101.29 (C-1"), 72.80 (C-2"), 74.59 (C-3"), 72.3(C-4"), 76. 83(C-5"), 61.23 (C-6"). According to chromatographic properties of **compound 1** (R_f value, fluroscence under UV-light and changes in color with ammonia vapor) and UV spectroscopic results it was expected to be kampferol glucoside[19,20,21]**.** Identification of **Compound.1** was achieved by comparison with previously published data [22]**.** The identity of **compound 1** was also confirmed by acid hydrolysis of the compound and gave Kaempferol as the aglycone moiety and glucose as the sugar moiety when compared with authentic samples.

Quercetin-3-O-*β*-D-glucopyranoside (Isoquercetrin) (2): yellow amorphous powder gave dark Purple fluorescent spot turned to yellow on PC with ammonia. Vapor. **R_f** values: 0.6 (1), 0.45(3); **UV λ_{max} nm** (MeOH): 253, 351, +NaOMe: 271, 410, +NaOAc : 273, 375, +NaOAc /H_3BO_3: 262, 377, +$AlCl_3$: 274, 435, +$AlCl_3$/HCl: 275, 403 nm**, ^{1}H NMR** (CD_3OD) : δ (ppm)7.67 (*dd*, *J* = 2.1 Hz & 8.6 Hz, H-6'), 7.53 (*d*, *J* = 2.12 Hz, H-2'), 6.82 (*d*, *J* = 8.62 Hz, H-5'), 6.40 (*d*, *J* = 1.8 Hz, H-8), 6.20 (*d*, *J*= 1.8 Hz, H-6), 5.37 (*d*, *J* = 7.63 Hz, H-1"), 3.28—3.65(overlapped the rest sugar protons) ,**^{13}C NMR** (CD_3OD): δ (ppm) 177.50 (C-4), 164.60 (C-7), 161.60 (C-5), 156.80 (C-2), 156.60 (C-9), 148.80 (C-4'), 145.80 (C-3'), 133.60 (C-3), 122.00 (C-6'),121.60 (C-1'),116.20 (C-5'), 115.80 (C-2'),104.00 (C-10), 98.90 (C-6), 93.80 (C-8), 101.20 (C-1"), 77.70 (C-5"), 74.40 (C-3"), 71.60 (C-2"), 70.02 (C-4"), 61.50 (C-6").According to chromatographic properties of **compound 2** (R_f value, fluorescence under UV-light and changes in color with ammonia vapor) and UV spectroscopic results it was expected to bequercetin glucoside[19,20,21]**.** The identity of **Compound.2** was confirmed by comparison with previously published data [23].

Table 1. Effects of oral administration of *E.retusa*.(100 and 200 mg/Kg) on AST and ALT serum activity in CCl_4 induced hepatotoxicity in rats

Groups	ALT(U/ml)	AST(U/ml)
Normal control	76.67 ± 2.305	216.7 ± 21.65
E.retusa (100 mg/kg)	59.33 ± 5.426^{bc}	156.0 ± 9.462^{bc}
E.retusa (200 mg/kg)	59.00 ± 1.949^{bc}	152.8 ± 5.722^{bc}
CCl_4	355.3 ± 34.64^{a}	442.3 ± 18.78^{a}
Silymarin (25mg/kg)+CCl_4	285.5 ± 44.35^{a}	337.5 ± 21.55^{a}
E.retusa(100 mg/kg)+CCl_4	301.5 ± 68.89^{a}	307.8 ± 51.96^{b}
E.retusa (200 mg/kg)+CCl_4	247.0 ± 24.45^{a}	340.6 ± 33.34^{a}

The results were expressed as mean ± S.E. (n=6). Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer's multiple comparison. Results at $P \leq 0.05$ were considered significant.
a = significantly different from normal control ($P<0.05$).
b = significantly different from CCl_4 group ($P<0.05$).
c = significantly different from Silymarin group ($P<0.05$).

Table 2. Effect of oral administration of *E. retusa* (100 and 200 mg/Kg) on hepatic MDA and GSH and NO in CCl_4-induced hepatotoxicity in rats.

Groups	MDA (ng/g . tissue)	GSH (pg/g. tissue)	NO (Umol/g. tissue)
Normal control	8.400±0.6245	49.47 ± 2.892	7.300 ± 0.6245
E.retusa (100 mg/kg)	29.37 ± 1.650^{abc}	11.57 ± 0.8762^{ac}	28.23 ± 1.519^{abc}
E.retusa (200 mg/kg)	16.57 ± 0.5207^{ab}	21.70 ± 0.8718^{abc}	14.53 ± 0.5207^{ab}
CCl_4	65.27 ± 3.169^{a}	5.367 ± 0.4333^{a}	48.30 ± 2.650^{a}
Silymarin(25mg/kg)+CCl_4	9.800 ± 0.2646^{b}	40.60 ± 0.7638^{ab}	8.800 ± 0.2646^{b}
E.retusa (100 mg/kg) +CCl_4	20.60 ± 20.60^{abc}	15.47 ± 0.5207^{abc}	20.07 ± 0.7881^{abc}
E.retusa (200 mg/kg) +CCl_4	12.20 ± 12.20^{b}	33.60 ± 1.803^{abc}	11.33 ± 0.4910^{b}

The results were expressed as mean ± S.E.M (n=6). Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer's multiple comparison. Results at $P \leq 0.05$ were considered significant.
a = significantly different from normal control ($P<0.05$).
b = significantly different from CCl_4 group ($P<0.05$).
c = significantly different from Silymarin group ($P<0.05$).

3,3`dimethoxy ellagic acid (3): dark violet fluorescent spot under short and long UV light, which has turned to greenish-yellow fluorescence after exposure to ammonia vapour. **R_f** values: 0.3(1),0. 56 (3); **UV λ_{max} nm** (MeOH): 247, 375,**^{1}H NMR** (DMSO-d_6): δ (ppm)7.33 (2H, s, H-5, 5`), 3.99 (6H, s, $OCH_3$ at 3,3`) ,**^{13}C NMR** (DMSO-d_6): δ (ppm)60.19 (3, 3'-OCH_3), 109.33 (C-6, 6`), 111.66 (C-5,5`), 112.96 (C-1,1`), 141.20 (C-2, 2`), 141.59 (C-3, 3`), 152.51 (C-4, 4`), 159.81 (C-7, 7`).According to chromatographic properties of **compound 3** ($R_f$ value, fluroscence under UV-light and changes in color with ammonia vapor) and UV spectroscopic results it was expected to be an ellagic acid derivative[24,25]**.Compound 3** was identified as 3,3`dimethoxy ellagic acid by comparison with previously published data [26]**.**

Ellagic acid (4): shiny buff fluoresence under UV light, which has changed to dull yellow fluoresence after exposure to ammonia vapours. R_f values: 0.34 (1), 0.44 (3); **UVλ_{max}** nm (MeOH): 255, 364,

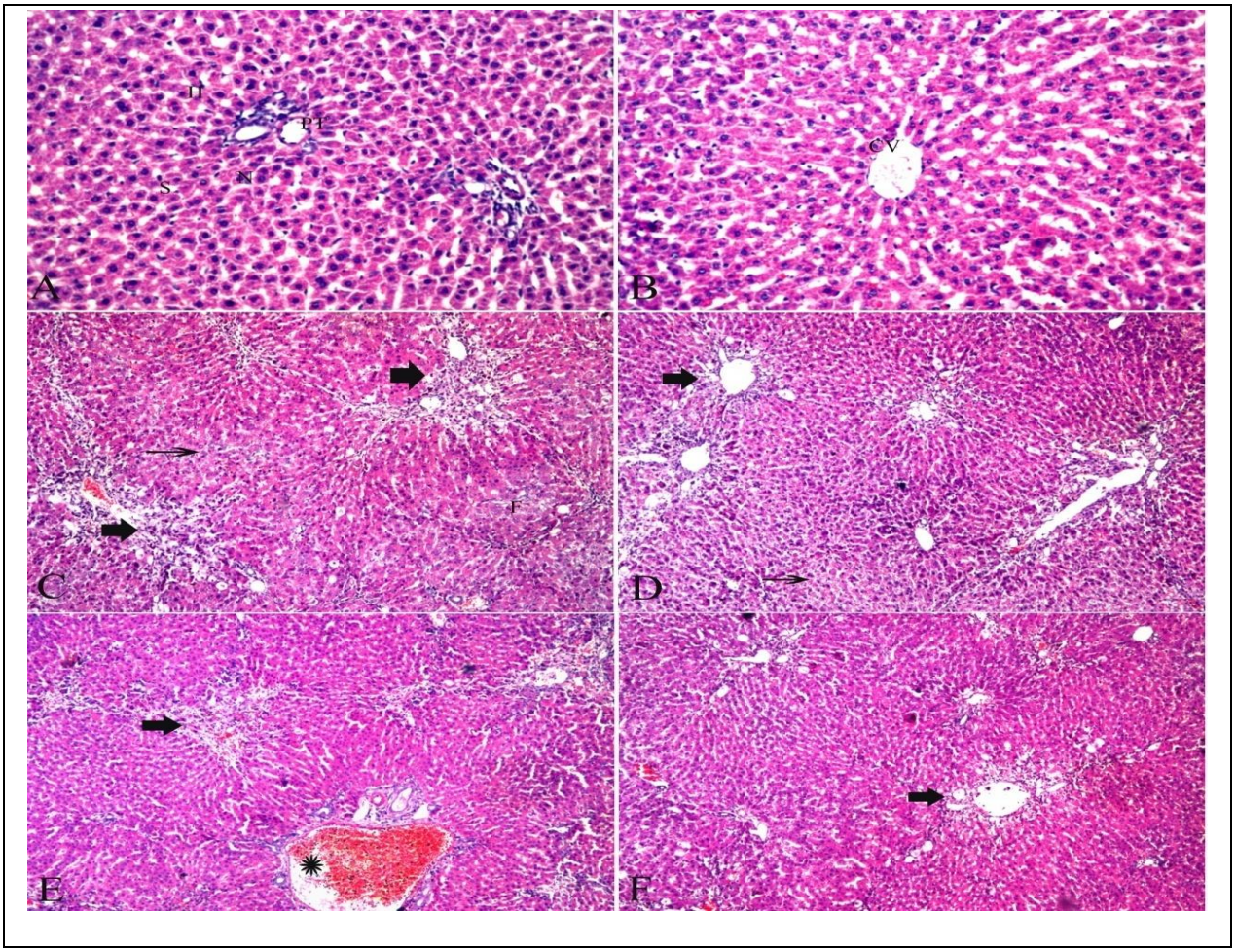

Figure 2. Photomicrographs of Hepatic Histopathology *(H & E. stain, X = 400).*

A. Normal liver section showing portal tract (PT), hepatocytes (H), nucleus (N), blood sinusoids (S). B. Plant group showing normal structure with central vein (CV). C. CCl_4 group showing necrosis with inflammatory cells infiltration (thick arrow) and degeneration of hepatocytes (thin arrow). D. CCl_4 + Silymarin group showing few necrotic cells with inflammatory cells infiltration (thick arrow) and degeneration of hepatocytes (thin arrow). E. CCl_4 + low dose of plant (100 mg/kg) showing necrotic cells with inflammatory cells infiltration (thick arrow) and congested portal tract (star). F. CCl_4 + low dose of plant (200 mg/kg) showing normal hepatic structure with minimally necrotic cells with inflammatory cells infiltration (thick arrow)

^{1}H NMR(DMSO-d_6): δ (ppm)7.46 (2H, s, H-5, 5'),,**^{13}C NMR**(DMSO-d_6): δ (ppm)108.04 (C-6,6`), 110.67 (C-5,5`), 112.39 (C-1,1`), 136.84(C-2, 2`), 140.12 (C-3,3`), 148.59 (C-4,4`), 159.61 (C-7,7`).According to chromatographic properties of **compound 4** (R_f value, fluroscence under UV-light and changes in color with ammonia vapor) and UV spectroscopic results it was expected to be ellagic acid[24,25]. **Compound 4** was confirmed by comparison with previously published data[27, 28].

Gallic acid (5): yellow spot in visible light, which has changed to brown after exposure to ammonia vapours. Under UV light (365 nm) it showed shiny violet fluoresence before and after exposure to ammonia. **R_f values**: 0.67(1),0.54 (3); UV **λ_{max} nm** (MeOH): 272, ^{1}H NMR(DMSO-d_6): δ (ppm)9.17 (brs, three OH at position 3, 4 & 5), 6.92 (2H, s, H-2, H-6), **^{13}C NMR** (DMSO-d_6): δ (ppm)167.96 (C=O), 145.85 (C-3 , C-5), 138.44 (C-4), 120.91 (C-1), 109.20 (C-2, C-6). According to chromatographic properties of **compound 5** and UV spectroscopic results it was expected to be **gallic acid**[29] **Compound 5** was confirmed by comparison with previously published data[30,31].

Kampferol (6): dark yellow colour under UV365 light which was intensified upon exposure to ammonia vapours and has changed to green fluorescence after spraying with aluminium chloride**. R_f values**: 0.82 (1),0.10 (3); UV λ_{max} nm (MeOH): 266, 322, 366, +NaOMe: 280, 428, +NaOAc: 274, 385,

+NaOAc /H_3BO_3: 266, 364, +$AlCl_3$: 278, 423, +$AlCl_3$/HCl: 278, 424 nm**.** According to chromatographic properties of **compound 6** and UV spectroscopic results it was expected to be kampferol[19, 20, 21]**. Compound 6** was achieved by comparison with previously published data[29,32].

Quercetin (7): amorphous yellow powder; a dull yellow color under UV light gave bright yellow with amm.vapor and bright yellow with $AlCl_3$ reagent.**$R_f$values**: 0.33 (1), 0.20 (3); UV λ_{max} nm (MeOH): 259, 374, +NaOMe: 269sh, 432, +NaOAc: 261, 391, +NaOAc /H_3BO_3: 261, 391, +$AlCl_3$: 275 sh, 447, +$AlCl_3$/HCl: 267,326, 423 nm**.** According to chromatographic properties of **compound 7** and UV spectroscopic results it was expected to be quercetin[19,20,21]. **Compound 7** was confirmed by comparison with previously published data[33].

Total phenolic and flavonoid content

Results of the analysis of polyphenols constituents of the methanolic extract showed high concentration of the phenols (33.9 ± 0.65 mg GAE/G extract**)** and flavonoids (37.9 mg QE/G extract) contents.

Toxicity study

Methanolic extract of *Euphorbia retusa* showed LD_{50} = 2g/kg b.wt. and from this result it could be concluded that the methanolic extract was safe and non-toxic

The hepatoprotective assay

CCl_4 administration produced significant elevations of serum ALT and AST compared to the normal control group. The elevated activities of these enzymes are indicative of cellular leakage and liver injury. However, pretreatment of rats with 100 and 200 mg/kg b.wt. of *E. retusa* extract significantly decreased these serum biochemical indices (**Table 1**) as compared with the CCl_4 treatment group which revealed the potency of the hepatoprotectivity of *E. retusa* extract. The hepatic GSH activity in the CCl_4 treatment group was reduced, while MDA and NO activities were increased (**Table 2**) when compared by the normal control group. The hepatotoxicity induced by CCl_4 is due to its metabolite CCl_3, a free radical that alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids, in the presence of oxygen, to produce lipid peroxides, leading to liver damage[34]. The increased value of GSH level in liver tissues of rats treated with two doses of *E. retusa* extract and Silymarin may be due to *de novo* GSH synthesis or GSH regeneration[35]. The increase in MDA levels in liver means that enhanced lipid peroxidation which lead to tissue damage and failure of antioxidant defense mechanisms to prevent the formation of excessive free radicals[36]. Treatment with *E. retusa* extract and silymarin significantly reversed these changes. Several studies have reported the inhibitory effects of flavonoids on lipid peroxidation[37]. Sulfhydryl compounds such as glutathione (GSH) are well known to be an antioxidant substance in organisms, playing a critical role against CCl_4-induced injury by covalently binding to CCl_4. This is considered as the initial reactant in the chain reaction of oxidation, and then result in the lipid peroxidation and the cell membrane disruption[38]. Treatment with *E. retusa* extract (100 and 200 mg/kg) resulted in elevating the content of liver GSH compared with the CCl_4 treated group. Several diseases have been associated with the changes in GSH levels and reduced the resistance to the oxidation stress. The level of GSH was used to monitor the balance of oxidative stress and chemopreventive ability[39]. In our study, *E. retusa* extract exhibited protective effectives against liver damage from CCl_3. Furthermore, the GSH related antioxidant system has been improved. In conclusion, the treatment with *E. retusa* extract could reduce damage induced by CCl_4. The mechanism of protection including the inhibition of lipid peroxidation, increasing the content of GSH, elevating the expression of antioxidant enzymes, all of which result in recuperation of biological parameters.

Biochemical results

The plant extract at a low dose (100 mg/kg) when compared to the standard drug silymarin have significantly changed levels of serum ALT, AST, hepatic MDA, NO by 80 %, 54 %, 200, 220 % respectively and the level of GSH by 72 %. Also, the plant extract at a high dose (200 mg/kg) have significantly changed levels of serum ALT, AST by 80 %, 54 %, respectively and the level of GSH by 47 %, however, showed no significant changes to hepatic MDA, NO. The plant extract at a low dose (100 mg/kg) given concomitantly with CCl4 when compared to the standard drug silymarin have significantly changed levels of ALT, hepatic MDA, NO by 110 %, 128 % respectively and the level of GSH by 62 %, however, showed no significant changes to serum ALT, AST, while The plant extract at a high dose (200 mg/kg) given concomitantly with CCl4 have significantly changed only levels of GSH by 17 %.

Histopathological findings

Hepatoprotective effect of the *E. retusa* extract was further confirmed by the histopathological study of the liver. The liver of control rats revealed normal hepatic architecture central vein, portal tract with distinct hepatic cells, sinusoidal spaces, and prominent nuclei (**Figure 2A**). In the group received plant no evidence of pathological changes were observed (**Figure 2B**). The liver section of CCl_4 treated rats

revealed obvious damage and degenerative changes in the hepatic tissues including inflammatory cells infiltration, hydropic degeneration, a mild degree of fatty change, necrosis .Also, in the CCl_4 group it was observed dilatation and congestion of the central vein and pyknotic nuclei (**Figure 2C**), indicating liver damage and were in agreement with previous findings that CCl_4 causes necrosis, mononuclear cell infiltration, steatosis, degeneration of hepatocytes and increases in mitotic activity[40] in liver. However, in the group treated withCCl_4 and sylimarin showed moderate improvement in histological structure of liver tissues, inflammatory cells infiltration, hydropic degeneration were also observed (**Figure 2D**).

Treatment with CCl_4 and low dose of plant showed mild improvement. Hepatic cords were slightly distorted, mild hepatocyte degeneration, necrotic cells with inflammatory cells infiltration, congestion and dilation of portal tract (**Figure 2E**).

Microscopic examination of liver sections treated with CCl_4 + plant (200 mg/kg) showed nearly normal hepatic architecture. The hepatic lobules appeared with prominent central vein with less sinusoidal dilatation (**Figure 2F**).

Conflict of Interest

There is no conflict of interest to be declared.

CONCLUSION

In conclusion, the results of this study demonstrate that *E. retusa* extract was effective for the prevention of CCl_4-induced hepatic damage in rats. Our results showed that the hepatoprotective effects of *E. retusa* extract may be due to both an increase in the activity of the antioxidant-defense system and an inhibition of lipid peroxidation. However, the protective, curative and antioxidant qualities of *E. retusa* may be attributed to the presence of active principles in the plant extract especially flavonoids and other polyphenolic compounds.

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