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Antioxidant and Hepatoprotective Effects of Methanolic Extracts of *Zilla spinosa* and *Hammada elegans* Against Carbon Tetrachloride-induced Hepatotoxicity in Rats

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Abstract: The detoxification, metabolism, and excretion of various endogenous and exogenous materials occur mainly in the liver. Liver diseases are a global concern, and classified as chronic hepatitis, cirrhosis, and hepatosis. The development of safe hepatoprotective agents remains an unmet need. Therefore, we investigated the antioxidant effects of methanolic and *n*-hexane fractions of *Zilla spinosa* (ZSM and ZSH, respectively) and *Hammada elegans* (HEM and HEH, respectively) against carbon tetrachloride (CCl_4)-induced liver toxicity in rats. Antioxidant activity was studied by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The rats were divided into 11 groups (n=6)—group 1 (control), group 2 (CCl_4 only), group 3 (CCl_4+silymarin 10 mg/kg), group 4 (CCl_4+HEM 250 mg/kg), group 5 (CCl_4+HEM 500 mg/kg), group 6 (CCl_4+HEH 250 mg/kg), group 7 (CCl_4+HEH 500 mg/kg), group 8 (CCl_4+ZSM 250 mg/kg), group 9 (CCl_4+ZSM 500 mg/kg), group 10 (CCl_4+ZSH 250 mg/kg), and group 11 (CCl_4+ZSH 500 mg/kg). Serum levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma glutamyl transferase, and total bilirubin were measured. The extent of hepatic injury was histopathologically assessed. Treatment with ZSM and ZSH at 250 and 500 mg/kg did not significantly affect biochemical results compared with the CCl_4 only group. However, treatment with both HEM and HEH at 250 and 500 mg/kg provided significant ($p<0.001$) results compared with the CCl_4 only group. These results were consistent with histological findings. HEM and HEH at 250 μg/mL significantly inhibited DPPH radical formation by 38.16 and 35.65%, respectively. However, antioxidant effects of ZSM and ZSH were insignificant.

Keywords: *Z. spinosa*; *H. elegans*; hepatoprotective.

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List of abbreviations

ALP, alkaline phosphatase
ALT, alanine aminotransferase
AST, aspartate aminotransferase
CCl_4 carbon tetrachloride
DPPH, 2,2-diphenyl-1-picrylhydrazyl
GGT, gamma glutamyl transferase
HDL, high-density lipoprotein
HEM, *Hammada elegans* methanolic extract
HEH, *Hammada elegans n*-hexane extract
MDA, malondialdehyde
NP-SH, non-protein sulfhydryls
LDL, low-density lipoprotein
SE, standard error
VLDL, very low-density lipoprotein
ZSM, *Zilla spinosa* methanolic extract
ZSH, *Zilla spinosa n*-hexane extract

1 Introduction

Oxidative stress influences the progression of liver diseases [1]. By attenuating oxidative stress, cellular antioxidant systems prevent liver diseases [2]. Excessive oxidative stress damages cell membranes, proteins, and DNA. Consequently, it induces toxicity, such as hepatic cancers and muscular dystrophy. Currently available synthetic antioxidants are associated with adverse effects. Therefore, herbal antioxidants are being considered due to their enhanced safety and effectiveness [3-6].

Zilla spinosa belongs to the family Cruciferae or Brassicaceae, which comprises 3,709 species and 375 genera [7]. Some species of this family are edible. Many species of this family possess anticancer, anti-rheumatic, antidiabetic, antifungal, insecticidal, and antibacterial activities [8]. *Z. spinosa* is traditionally used for the treatment of kidney and gall bladder stones [9]. It also exerts anti-thyroid effects due to progoitrin and goitrin [10]. It is rich in natural compounds, such as triterpenoids, sterols, progoitrin, flavonoids, goitrin, carbohydrates, and glucosinolates of free sinapine [10]. Owing to these components, different fractions of *Z. spinosa* exert antiviral, hepatoprotective, antifungal, and antioxidant effects [11]. However, the methanolic and *n*-hexane fractions have not been evaluated for their hepatoprotective effects. The aqueous ethanol extract of *Z. spinosa* reduces gamma glutamyl transferase (GGT), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels, indicating the stabilization of plasma membranes of carbon tetrachloride (CCl_4)-exposed hepatocytes in animals [11, 12]. The chloroform extract of *Z. spinosa* is effective against human colon (HCT116) and liver (HEPG2) cancer cells. It induces significant cytotoxicity in HCT116 cells. It also holds significant analgesic and anti-inflammatory potential [8]. *Hammada elegans* is a shrub or undershrub belonging to the family Amaranthaceae that exerts antidiabetic, antiseptic, and anti-inflammatory effects [13, 14]. Triterpenoids, flavonoids, tannins, alkaloids, glycosides, and saponins are reported to be responsible for its analgesic, anti-inflammatory, hepatoprotective, and antioxidant effects [15–17]. The ethanolic extract shows moderate antimicrobial activities against numerous bacterial species, such as *Enterococcus faecalis*, *Escherichia coli*, *Moraxella lacunata*, *Proteus mirabilis*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Bacillus subtilis*, *Micrococcus luteus*, *Sarcina ventriculi*, and *Staphylococcus aureus*, and fungi, such as *Candida albicans*, *Candida tropicalis*, *Aspergillus flavus*, *Aspergillus fumigatus*, and *Penicillium chrysogenum* [15].

The current investigation focuses on the hepatoprotective effectiveness of methanolic and *n*-hexane fractions of *Z. spinosa* and *H. elegans* against CCl_4-induced liver injury in rats and their antioxidant potentials.

2 Experimental Procedure

2.1 Plant material

Z. spinosa and *H. elegans* were collected from Wadi Hafr Al-Batin and Umm oshr Al-Butaihan, Saudi Arabia on 24 February 2016. The specimen vouchers were deposited in the herbarium of the Medicinal Aromatic and Poisonous Plants Research Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia with voucher numbers SY282 and SY 270, respectively.

2.2 Extraction and fractionation

Whole plants were air-dried, ground, and soaked in methanol for one week. After filtering, the methanol was evaporated using a Buchi rotary evaporator. The process was repeated three times using methanol. Further, the greenish methanolic crude extract was mixed with distilled water, and partitioned using a separating funnel with solvents of increasing polarity (*n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water). Crude methanolic and *n*-hexane fractions were used for biological evaluations.

2.3 Animals and chemicals

Albino rats (aged two months and weighing 190–202 g) of both sexes were obtained from the College of Pharmacy, Experimental Animal Care Center, King Saud University. The rats were housed in standard polypropylene animal cages at a controlled temperature (22±2°C) and a 12-h light-dark cycle with unrestricted access to water and food (Purina chow). Rats were maintained in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, and the experimental protocol was approved by the Institutional Animal Ethics Committee of the College of Pharmacy, King Saud University (clearance number, 05763-891; 6 January, 2014). All solvents used were of analytical grade. Silymarin was purchased from Sigma Aldrich Company (St. Louis, MO, USA).

2.4 Acute toxicity test

The acute oral toxicity of *H. elegans* methanolic (HEM) and *n*-hexane (HEH) extracts, and *Z. spinosa* methanolic (ZSM) and *n*-hexane extracts (ZSH) was evaluated on rats at several doses (0.1–0.5 mL/kg). The rats were monitored continuously to identify symptoms of toxicity and mortality [18].

2.5 CCl_4-induced liver toxicity

Rats of both sexes were randomly divided into eleven groups (n=6)—group 1 (control), group 2 (CCl_4 only), group 3 (CCl_4+silymarin), group 4 (CCl_4+HEM 250 mg/kg), group 5 (CCl_4+HEM 500 mg/kg), group 6 (CCl_4+HEH 250 mg/kg), group 7 (CCl_4+HEH 500 mg/kg), group 8 (CCl_4+ZSM 250 mg/kg), group 9 (CCl_4+ZSM 500 mg/kg), group 10 (CCl_4+ZSH 250 mg/kg), and group 11 (CCl_4+ZSH 500 mg/kg). The test extracts were administered intraperitoneally. Pretreatment with the test extracts and silymarin was initiated three weeks before CCl_4 administration, and was continued until the end of the experiment. Blood samples were withdrawn directly into tubes containing disodium EDTA from the retro orbital plexus of all rats under anesthesia 24 h after CCl_4 treatment. After centrifuging the clotted blood samples at 2,500 ×*g* for 10 min, serum samples were separated. The rats were sacrificed after blood withdrawal. Liver tissues were isolated for biochemical and histopathological analyses. They were cleaned with refrigerated normal saline, and 10% (w/v) liver homogenates were prepared in ice cold 0.15 M KCl solution using a motorized Teflon pestle.

2.6 Estimation of hepatotoxicity marker enzymes and bilirubin

Commercially available kits (Roche) were used with a Reflotron plus Analyzer to analyze serum biochemical parameters, such as alkaline phosphatase (ALP), bilirubin, GGT, AST, and ALT [19-22].

2.7 Estimation of lipid profile

To evaluate lipid profile, levels of total cholesterol, triglycerides, very low-density lipoprotein (VLDL), LDL, and high-density lipoprotein (HDL) were measured using commercial diagnostic kits [23].

2.8 Determination of malondialdehyde (MDA)

Liver tissue homogenates (10% w/v) were prepared in KCl (0.15 M) at 4°C in a Potter–Elvehjem type C homogenizer. After incubating the tissue homogenates (1 mL) at 37°C for 3 h in a metabolic shaker, they were mixed with 10% aqueous trichloroacetic acid (1:1), centrifuged at 800 ×*g* for 10 min, and the supernatants were separated. Equal volumes (1 mL) of supernatants and thiobarbituric acid solution (0.67% in water) were mixed, and placed in a water bath for 10 min at 100°C. After cooling, the samples were diluted with 1 mL of distilled water, and the optical densities were measured using a spectrophotometer at 535 nm. Sample MDA levels were estimated by comparing with a standard curve of MDA solution [23].

2.9 Estimation of non-protein sulfhydryls (NP-SH) and total protein

Ice-cold 0.02 mM EDTA was used for the homogenization of liver tissues. Tissue homogenates (5 mL) were mixed with distilled water (4 mL) and 50% trichloroacetic acid (1 mL) in test tubes. After shaking to mix for 10 min, the mixtures were centrifuged at 3,000 rpm, and the supernatants (2 mL) were mixed with Tris buffer (0.4 M, pH 8.9, 4 mL) and 5,5′-dithio-bis (2-nitrobenzoic acid) (0.1 mL) for 5 min; the optical densities were immediately measured at 412 nm against the blank reagent.

A colorimetric method was used to determine total serum protein (g/L); the optical density of the titrated blue/violet product was measured at 546 nm, and sample protein concentrations were calculated based on the following equation [23].

$$Total\ serum\ protein\ (g/L) = \frac{Absorbance\ of\ sample}{Absorbance\ of\ standard} \times Concentration\ of\ standard$$

2.10 Histopathological assessment

An American-made optical rotary microtome was used for producing 5-μm sections of liver tissues. The sections were stained with hematoxylin and eosin, and pathomorphological changes were studied under a light microscope [24].

2.11 Antioxidant activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to evaluate the antioxidant potential of all four extracts at

different concentrations (100, 150, 200, and 2,500 μg/mL). Freshly prepared purple colored DPPH solution turned yellowish after incubating with test samples for 30 min at room temperature, and color changes were measured using a spectrophotometer (UVmini-1240, Shimadzu, Japan) at 514 nm. The following equation was used for the calculation of antioxidant activity [25]:

$$Antioxidant\ activity\ (\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

2.12 Statistical analyses

Results are expressed as mean±standard error (SE) of four samples. The data were statistically analyzed by ANOVA, followed by Dunnett's multiple comparisons test.

3 Results and Discussion

3.1 Acute toxicity

No mortality or clinical pathologies were observed after treatment with the four extracts even at the highest dose (0.5 mL/kg, p.o.).

3.2 Effects of the extracts on hepatotoxicity marker enzymes and bilirubin in the serum

Results of biochemical parameters indicative of liver function are summarized in Table 1. CCl_4 administration caused severe hepatotoxicity in rats as evidenced by significantly elevated serum levels of ALT, AST, ALP, GGT, and bilirubin compared to control. The elevation in these levels was significantly reduced by silymarin pretreatment (10 mg/kg). Pretreatment with HEH and HEM significantly reduced these levels at both doses (250 and 500 mg/kg) compared to control; however, HEH was more effective than HEM. Pretreatment with ZSM and ZSH yielded non-significant results at both doses (250 and 500 mg/kg).

3.3 Effect of the extracts on lipid profile

CCl_4-exposed rats had significantly elevated levels of triglycerides, cholesterol, VLDL, and LDL compared to normal rats (Table 2). These levels were significantly lower in CCl_4-exposed rats pretreated with silymarin than in rats exposed to CCl_4 only. HEH and HEM pretreatment at both doses (250 and 500 mg/kg) significantly ($p<0.001$) lowered these levels compared to CCl_4 exposure alone. However, ZSM and ZSH pretreatment at both doses (250 and 500 mg/kg) demonstrated insignificant ($p>0.05$ and 0.01) improvement in the lipid profile.

3.4 Effect of the extracts on MDA, NP-SH, and total protein in liver tissue homogenates

CCl_4 exposure without any treatment significantly ($p<0.001$) increased MDA, while decreased total protein and NP-SH levels compared to control (Table 3). CCl_4-exposed rats pretreated with HEH or HEM at both doses (250 and 500 mg/kg) had significantly ($p<0.001$) decreased MDA, total protein, and NP-SH levels compared to rats exposed to CCl_4 alone. However, ZSM and ZSH pretreatment at both doses (250 and 500 mg/kg) caused insignificant changes ($p>0.05$ and 0.01).

3.5 Effect of the extracts on liver histopathology

As shown in Figure 1, histopathological findings were consistent with results of biochemical analyses. Severe necrosis and focal hepatic cellular decay of the lobules were observed in the CCl_4 only group compared to the control group. Hepatocytes and the central vein were normal in rats pretreated with the plant extracts. In addition, liver tissues of rats treated with silymarin exhibited a normal central vein and sinusoidal hepatocytes. HEH pretreatment at 500 mg/kg was found to be as effective as silymarin with negligible liver toxicity.

3.6 Antioxidant activities of the extracts

The plant extracts exerted antioxidant effects in a dose-dependent manner (Table 4). HEM, HEH and ZSM at 250 μg/mL showed significant antioxidant activities of 38.16, 35.65 and 31.18%, respectively. However, the antioxidant activities of ZSM and ZSH were insignificant. These results further reinforced the significant hepatoprotective potentials of HEM and HEH.

Hepatic diseases remain a worldwide health concern, despite remarkable progress in the field of medicine. Numerous hepatoprotective herbal formulations exist [23]. Plant extracts rich in phenolic compounds are associated with hepatoprotective effects [26–29]. To our

knowledge, this is the first study on the methanolic and *n*-hexane extracts of the selected plant species. CCl_4 becomes toxic after biotransformation by cytochrome P450 to trichloromethyl (CCl_3•) and trichloromethylperoxy (CCl_3OO•) free radicals, which further initiate lipid peroxidation and disturb the levels of bilirubin and other biomarker enzymes [30,31]. The current study showed that CCl_4 damages the structure and functions of the liver, thereby severely disturbing the levels of serum marker enzymes and bilirubin. The results indicated that the tested extracts protect the liver from damage and restore enzyme levels.

Both the extracts showed significant antioxidant as well as hepatoprotective activities; accordingly, a previous study showed that antioxidant effects translate to anti-inflammatory activities [32]. Histopathology results (Figure 1) indicated that HEH at 500 mg/kg shows the best hepatoprotective effects. CCl_4 damages hepatocytes as observed by the increased release of various cytosolic enzymes into the blood; therefore, the levels of these enzymes in the blood act as an indicator of the extent of hepatotoxicity [33]. The identification and characterization of pharmacologically active phytoconstituents is crucial for new drug development. Although HEH showed significant therapeutic potential, further studies are required to explore the exact phytoconstituent responsible for its hepatoprotective activity.

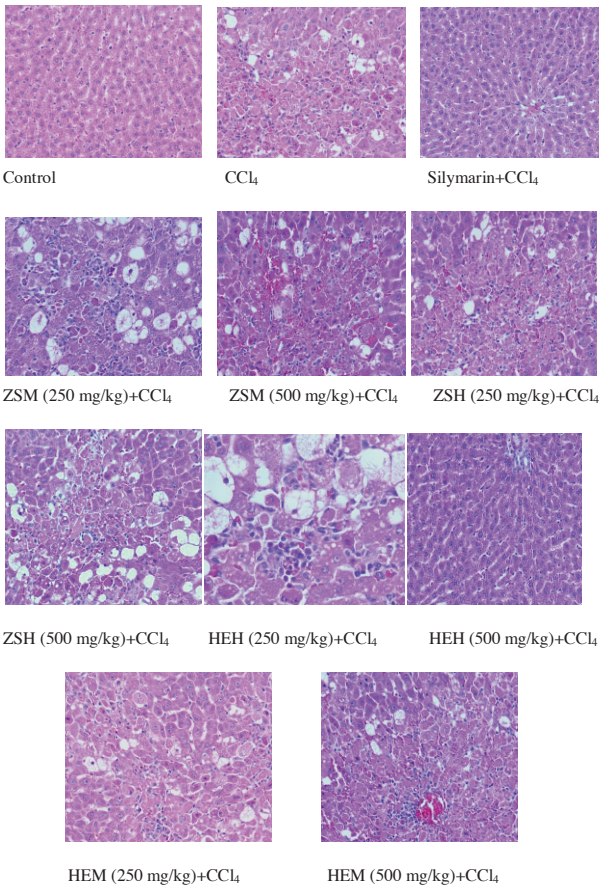

Figure 1: Liver histopathology in CCl_4-exposed rats after treatment with different plant extracts at the indicated doses.

Table 1: Effect of Extracts on serum marker enzymes of control and treated rats.

TREATMENTS	Dose mg/kg	SGOT(AST) U/l Mean±S.E	SGPT(ALT) U/l Mean±S.E	ALP U/l Mean±S.E	GGT(U/l) Mean±S.E	BILIRUBIN mg/dL Mean±S.E
Normal		99.66±13.20	22.50±1.53	373.16±9.29	5.11±0.15	0.58±0.01
CCl_4		341.00±8.16***[a]	300.00±6.87***[a]	616.33±10.11***[a]	19.05±0.78***[a]	3.04±0.06***[a]
Silymarin+CCl_4	**10**	167.16±10.38***[b]	97.96±5.23***[a]	414.16±6.63***[b]	6.70±0.30***[b]	0.96±0.04***[b]
HEM+CCl_4	**250**	268.33±8.33***[b]	247.83±4.39***[b]	572.00±8.73*[b]	16.63±0.57*[b]	2.36±0.06***[b]
HEM+CCl_4	**500**	198.50±4.20***[b]	152.83±7.20***[b]	455.83±9.71***[b]	10.58±0.41***[b]	1.63±0.07***[b]
ZSM+CCl_4	**250**	351.66±6.78[b]	302.50±7.17[b]	614.33±8.05[b]	18.61±0.33[b]	3.06±0.05[b]
ZSM+CCl_4	**500**	312.33±6.39*[b]	284.83±6.76[b]	589.50±11.38[b]	17.30±0.33[b]	2.91±0.04[b]
ZSH+CCl_4	**250**	336.66±6.40[b]	285.16±6.53[b]	611.33±5.37[b]	19.33±0.25[b]	2.98±0.05[b]
ZSH+CCl_4	**500**	300.33±4.39**[b]	278.83±7.85[b]	607.50±6.65[b]	16.13±0.29**[b]	2.81±0.05*[b]
HEH+CCl_4	**250**	242.83±4.89***[b]	235.66±12.91**[b]	545.83±9.63***[b]	14.13±0.26***[b]	1.99±0.06***[b]
HEH+CCl_4	**500**	193.50±6.50***[b]	149.16±8.11***[b]	466.50±10.63***[b]	9.80±0.32***[b]	1.53±0.04***[b]

All values represent mean ± SEM. *p<0.05; **p<0.01; ***p<0.001; ANOVA, followed by Dunnett's multiple comparision test. [a] As compared with Control group. [b] As compared with CCl_4 only group.

Table 2: Effect of Extracts on metabolism and serum lipoproteins of control and treated rats.

TREATMENTS	Dose mg/kg	Cholesterol (mg/dl) Mean±S.E	Triglycerides (mg/dl) Mean±S.E	HDL (mg/dl) Mean±S.E	LDL (mg/dl) Mean±S.E	VLDL (mg/dl) Mean±S.E
Normal		112.50±3.54	87.36±2.53	47.88±1.68	47.14±4.10	17.47±0.50
CCl_4		247.66±3.01*** [a]	192.00±7.30*** [a]	25.56±0.90*** [a]	183.70±4.66*** [a]	38.40±1.46*** [a]
Silymarin+ CCl_4	**10**	132.00±3.42*** [b]	110.26±3.85*** [b]	44.58±1.43*** [b]	65.36±2.38*** [b]	22.05±0.77*** [b]
HEM+ CCl4	**250**	194.83±4.23*** [b]	171.00±3.29* [b]	29.91±1.68* [b]	130.71±5.49*** [b]	34.20±0.65* [b]
HEM+ CCl4	**500**	152.16±2.99*** [b]	132.83±2.91*** [b]	42.26±3.00*** [b]	83.33±3.98*** [b]	26.56±0.58*** [b]
ZSM+ CCl4	**250**	241.50±5.81 [b]	200.50±4.95 [b]	26.53±0.80 [b]	174.86±6.19	40.10±0.99 [b]
ZSM+ CCl4	**500**	230.50±3.54** [b]	177.16±5.29 [b]	27.20±0.74 [b]	167.86±4.28* [b]	35.431.05 [b]
ZSH+ CCl4	**250**	255.33±3.33 [b]	191.33±6.65 [b]	25.91±0.68 [b]	191.15±3.98 [b]	38.26±.133 [b]
ZSH+ CCl4	**500**	236.66±3.67* [b]	170.00±4.88* [b]	28.65±0.68* [b]	174.01±4.76 [b]	34.00±0.97* [b]
HEH+ CCl4	**250**	199.50±3.75*** [b]	157.66±3.42** [b]	32.30±1.18*** [b]	135.66±4.19*** [b]	31.53±0.68** [b]
HEH+ CCl4	**500**	158.66±6.42*** [b]	139.16±3.36*** [b]	40.30±141*** [b]	90.53±5.97*** [b]	27.83±0.67*** [b]

All values represent mean ± SEM. *p<0.05; **p<0.01; ***p<0.001; ANOVA, followed by Dunnett's multiple comparision test.
[a] As compared with Control group. [b] As compared with CCl_4 only group.

Table 3: Effect of Extracts on MDA , NP-SH and Total Protein in liver tissue.

Treatments	Dose mg/kg	MDA (nmol/g)	NP-SH (nmol/g)	Total Protein (g/l)
Normal		1.08±0.05	7.29±0.17	119.36±2.99
CCl_4		8.34±0.24*** [a]	1.51±0.17*** [a]	55.83±1.71*** [a]
Silymarin+ CCl_4	**10**	1.20±0.05*** [b]	7.17±0.38*** [b]	107.38±2.99*** [b]
HEM+ CCl_4	**250**	2.61±0.15*** [b]	5.28±0.37*** [b]	73.05±2.67*** [b]
HEM+ CCl_4	**500**	2.33±.10*** [b]	5.07±0.19*** [b]	96.60±2.67*** [b]
ZSM+ CCl_4	**250**	7.38±0.52 [b]	1.72±0.22 [b]	60.67±2.44 [b]
ZSM+ CCl_4	**500**	7.50±0.33 [b]	1.65±0.14 [b]	65.86±2.20** [b]
ZSH+ CCl_4	**250**	8.60±0.38 [b]	1.63±0.06 [b]	57.88±1.56 [b]
ZSH+ CCl_4	**500**	6.72±0.34** [b]	1.80±0.04 [b]	65.46±1.00*** [b]
HEH+ CCl_4	**250**	2.78±0.09*** [b]	4.16±0.21*** [b]	74.25±1.63*** [b]
HEH+ CCl_4	**500**	2.69±0.11*** [b]	4.51±0.25*** [b]	91.41±1.99*** [b]

All values represent mean ± SEM. *p<0.05; **p<0.01; ***p<0.001; ANOVA, followed by Dunnett's multiple comparision test.
[a] As compared with Control group. [b] As compared with CCl_4 only group.

Table 4: Antioxidant Activities of Samples.

Samples Code	100 (g/mL)%	150(g/mL)%	200(g/mL)%	250(g/mL)%
ZSM	9.85	13.72	16.47	31.18
ZSH	5.63	7.80	12.35	18.27
HEM	15.28	27.62	31.21	38.16
HEH	11.72	17.24	22.83	35.65

HEM, *Hammada elegans* methanolic extract; HEH, *H.Elegans n*-hexane fraction; ZSM, *Zilla Spinosa* methanolic extract; ZSH, *Z. Spinosa n*-hexene fraction

4 Conclusions

It can be concluded that HEM and HEH of *H. elegans* significantly protect against CCl_4-induced hepatotoxicity, and exert antioxidant effects in a dose-dependent manner. However, ZSM and ZSH crude extracts showed poor hepatoprotective and antioxidant effects. This study identifies the association between antioxidant activity and hepatoprotective efficacy. HEH at 500 mg/kg exhibits potent pharmacological activity, and holds significant therapeutic potential.

Ethics approval: Animals were maintained in accordance with the recommendations of the 'Guide for the Care and Use of Laboratory Animals' approved by institutional animal ethics committee of College of Pharmacy, King Saud University, Riyadh, Saudi Arabia (Clearance No. 05763–891; January 6, 2014).

Competing interests: The authors declare that they have no competing interests.

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