



Hepatoprotective Potential of *Mesembryanthemum forsskalii* Fruits Extract Against Carbon Tetrachloride-Induced Liver Toxicity in Mice

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ABSTRACT

The present study aimed to investigate the ameliorative effect of the methanolic extract of *Mesembryanthemum forsskalii* fruits extract against the hepatotoxicity of carbon tetrachloride (CCl₄) in mice at two different doses (low; 100 mg/kg BW and high; 500 mg/kg BW). The obtained results showed that CCl₄ injection significantly reduced the body weights of mice and increased the relative liver weight. Treatment with *M. forsskalii* extract after CCl₄ injection restores the weights of mice to the normal range and reduced the relative weight of the liver. Mice that were injected with CCl₄ exhibited elevation in the liver enzymes; aspartate aminotransferase (AST) and alanine aminotransferase (ALT), reduction in the cholesterol content, and altered the architecture of the hepatic cells. Animals treated with *M. forsskalii* extract at a low dose after CCl₄ administration enhanced the liver functions as evidenced by histological examination and liver enzymes analysis. Immunohistochemical investigation showed that the administration of *M. forsskalii* extract at a low dose downregulated the expression pattern of P53 and upregulated the expression of Bcl-2 in liver tissue. In conclusion, our findings indicated that the methanolic extract of *M. forsskalii* at a low dose (100 mg/kg BW) attenuated the hepatotoxicity induced by CCl₄ through upregulation of Bcl-2 expression and restoring liver enzymes to their normal range.

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Key words

Mesembryanthemum forsskalii, Hepatotoxicity, CCl₄, Liver enzymes, immunohistochemistry

INTRODUCTION

Toxins, infectious agents, and serum inflammatory mediators are the main reasons leading to the loss of functional liver capacity (Edwards and Wanless, 2013). Experimental animals that expose to CCl₄ exhibit different morphological alterations in livers (Li *et al.*, 2019; Owujuyigbe *et al.*, 2020; Begum *et al.*, 2022). Fibrosis, focal inflammation, cell infiltration, hepatic cell degeneration

and regeneration, early portal cirrhosis, and alteration of the lobular structure are the most prominent changes that appear after exposure to CCl₄ (Izzularab *et al.*, 2021; Ammar *et al.*, 2022). CCl₄-induced hepatotoxicity was proved by the excess serum contents of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (GGT) (Baradaran *et al.*, 2019). A slight increase in triglycerides (TG) was reported in rats exposed to 10-100 ppm of CCl₄ (Ikatsu and Nakajima, 1992). 1 ml/kg BW of CCl₄ induced higher induction of oxidative stress in the brain compared to that of liver indicating brain vulnerability for CCl₄ neurotoxicity (Ritesh *et al.*, 2015). The same dose induced severe hepatotoxicity in mice where, CCl₄ increased the liver enzymes level and hepatic oxidative stress parameters and significantly decreased all hematological parameters except neutrophils and toxic neutrophils counts. Moreover, CCl₄ significantly suppressed the antioxidants, respiratory burst, phagocytic, serum lysozyme, and bactericidal activities. Also, CCl₄

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raised serum levels of tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and diminished IL-10, IL-12 (Elshopakey and Elazab 2021). 1.25 ml/kg BW of CCl₄ induced hepatotoxicity related parameters in rats (Al-Yahya *et al.*, 2013). CCl₄ in 2 ml/kg BW induced oxidative stress and hepatotoxicity in mice, where a marked alteration in histology and marked inflammation was induced in CCl₄ treated mice (Ullah *et al.*, 2020).

Many drugs are now available for the treatment of liver failure disease; however, the majority showed many serious side effects besides their cost-effectiveness. Therefore, several studies have been focused on finding alternative natural sources including plants to replace conventional drugs in the last two decades (Katiyar *et al.*, 2012). Many desert medicinal plants showed therapeutic and hepatoprotective effects against CCl₄-induced hepatotoxicity in mice and rats (Wahid *et al.*, 2016; Ammar *et al.*, 2022; Ayoka *et al.*, 2022; Doudach *et al.*, 2022; Nwaechefu *et al.*, 2022; Ouassou *et al.*, 2021). The methanolic extract of *Corrigiola telephiifolia* roots showed hepatoprotective potentiality through improving the blood biochemical parameters as well as enhancement of the hepatic tissues in CCl₄ induced hepatotoxicity in mice (Doudach *et al.*, 2022). Liver enzymes in rats treated with the methanol extract of *Cajanus cajan* at 100 and 200 mg/kg BW showed lower levels of liver enzymes compared to CCl₄ treated group (Nwaechefu *et al.*, 2022). The extract of *C. cajan* also reduced the inflammatory cell infiltration, hepatic fibrosis and necrosis and severe hepatocytes apoptosis induced by CCl₄ (Nwaechefu *et al.*, 2022). The aqueous extract of *Caralluma europaea* stem showed hepatoprotective potential in CCl₄ induced hepatotoxicity rats (Ouassou *et al.*, 2021). The ethanolic flower extract of *Salix subserrata* reduced the liver enzymes in CCl₄ treated rats and also ameliorated the negative impact of CCl₄ on liver tissues (Wahid *et al.*, 2016). Naringenin isolated from *Citrus sinensis* ameliorated the increased levels of liver enzymes induced by CCl₄ as well as attenuated the pathological changes in liver tissues. Naringenin also enhanced the expression of Bcl-2 (Ammar *et al.*, 2022). CCl₄ induced hepatotoxicity in rats through destruction of the liver tissues. The administration with the alkaloid fraction of the *Vitex doniana* showed protective effect in rats through improving various blood parameters and also restoration of the normal appearance of the destructive liver tissues induced by CCl₄ (Ayoka *et al.*, 2022).

Desert plants that are growing wildly in the kingdom of Saudi Arabia (KSA); have remarkable medicinal and commercial value to local communities (Aati *et al.*, 2019). Among these plants, *Mesembryanthemum forsskalii* (family Aizoaceae) which has multiuse and is considered a rich source of natural products and bioactive secondary

metabolites that have not been well exploited until now (Moawad *et al.*, 2016; El-Amier *et al.*, 2021). It was reported that *M. forsskalii* has more than 14 unsaturated fatty acids representing about 5.6% dominated by linoleic and oleic acids and also saturated fatty acids dominated by palmitic acid (Bilel *et al.*, 2020). In addition to the fatty acids, significant amounts of carbohydrates, protein, and amino acids were also detected in seeds of *M. forsskalii* (Al-Jassir *et al.*, 1995). In the northern part of KSA, the seeds of *M. forsskalii* are used as a traditional food for the public due to its high content of carbohydrates, protein, and fats (Najib *et al.*, 2004; Abdel-Hamid *et al.*, 2021).

Seeds oil extract of *M. forsskalii* showed antifungal activity against different fungal strains such as *Penicillium chrysogenum*, *P. stii*, *P. oxalicum*, *Fusarium oxysporum*, and *Aspergillus fumigatus*, *A. niger*, *A. flavus*, *A. carneus*, *Alternaria alternata*, *Rhizopus oryzae*, *Cladosporium cladosporioides* and *Paecilomyces lilacinus* (Bilel *et al.*, 2020). The effect of seeds oil extract of *M. forsskalii* was more prominent on *P. chrysogenum*, *A. fumigatus*, *A. flavus* and *A. carneus* (Bilel *et al.*, 2020). Samh seeds diets decreased the lipid peroxidation in streptozotocin (STZ)-induced diabetic of Wistar Albino rats (Al-Faris *et al.*, 2010). The nanoparticle prepared using *M. forsskalii* seeds showed antimicrobial activity against Gram positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) as well as antifungal activity against *Candida albicans*. The nanoparticles also showed anticancer activity against LoVo cancer cell lines with low IC₅₀ (28.3 μ g/ml) (Abed and Mohammed, 2021). The biological activity of one species of *Mesembryanthemum* (*M. nodiflorum*) showed an analgesic effect and significant inhibitory activity against colon, cervix, liver and melanocyte carcinoma. Moreover, the ethanolic extract of the aerial parts of *M. nodiflorum* had a significant hypoglycemic effect, anti-oxidant, anti-inflammatory and hepatoprotective activity (El-Hawary *et al.*, 2020). Some of the secondary metabolites of *M. forsskalii* such as flavonoids, alkaloids, saponins, and phenolics are proven to act as potent antioxidant agents (Bilel *et al.*, 2020; El-Amier *et al.*, 2021). Hence, it may contribute individually or in combination (synergistic effect) to protective capability against liver toxicity. Many researchers demonstrated that drugs of herbal origin may have potential antioxidant activities which may contribute to the protective action of liver toxicity if added as supplementary medications (Hamzawy *et al.*, 2015; Singh *et al.*, 2016). So far, there is no previous study had evaluated the phytochemical and biological activity of *M. forsskalii*. Accordingly, the main objective of our study is to evaluate the hepatoprotective potency of the aqueous methanol extract of *M. forsskalii*

fruits against hepatotoxicity induced by CCl₄.

MATERIALS AND METHODS

Plant collection and extract preparation

M. forsskalii was collected from Al-Adare region in the Aljouf district in the Northern part of KSA. Fruits were separated from the rest of the plant, dried at room temperature, and grounded into powder using an electrical grinder. Powdered materials were macerated in aqueous methanol until exhaustion for 24h. The solvent was evaporated under vacuum at 45°C using a rotary evaporator to obtain the aqueous methanolic crude extract for further study (Mahalel, 2015).

Determination of the LD₅₀ of M. forsskalii extract

A total number of 30 adult male mice of eight weeks old (25–30g) were divided into 5 groups of 6 mice each. These groups were injected with a single dose of plant extract at 3, 3.5, 4, 4.5, and 5g/kg of plant extract, respectively (Dhanarasu *et al.*, 2016). Mice were monitored for 24h to assess the acute toxicity of *M. forsskalii* extract. LD₅₀ of the extract was calculated using the arithmetic method of Finney (Finney, 1985). According to the following formula:

$$LD_{50} = Dm - \sum (z*d)/n$$

Where Dm is the highest dose that kills all animals. z is mean of dead animals between two successive groups. d is the constant factor between two successive groups and n is number of animals per each group.

Carbon tetrachloride (CCl₄) preparation

CCl₄ of 100 % concentration and olive oil were obtained from Algomhoria Company, Egypt. CCl₄ dissolved in olive oil was given by intraperitoneal (i.p) injection in a dose of 0.8 ml/Kg body weight of mice twice weekly for three weeks.

Experimental design

Based on the LD₅₀ value, low and high doses of *M. forsskalii* extract were used in the study as a 100 and 500 mg/kg BW, respectively. Six experimental groups of mice (6 mice/group) were divided as follows: Group 1 (Gp1): Mice were administered with 200 µl of olive oil and served as vehicle control; Gp2: mice were injected intraperitoneal (i.p) with CCl₄ as 0.8 ml/kg BW twice a week for three weeks (3 days intervals); Gp3: mice had administered orally with *M. forsskalii* extract as 100 mg/kg BW, 7 times/ 15 day (every other day); Gp4: mice had injected with CCl₄ as in Gp2, then followed by administration with *M. forsskalii* extract as in Gp3 (100 mg/kg BW); Gp5: mice had administered with *M. forsskalii* extract as 500

mg/kg BW (7 times/15 day, day after day) and Gp6: mice had injected i.p with CCl₄ as in Gp2, then followed by administration with *M. forsskalii* extract as in Gp5.

Determination of the total body weights and liver relative weight

The body weight of mice was assessed at the beginning of the experiment and at the end of the experiment after treatment with CCl₄ and/or *M. forsskalii* extract. The significant difference between the body weight before and after treatment was assessed. After decapitation, livers were removed, dried by blotting paper, and weighed on a digital balance. To calculate the relative liver weight, the liver weight was divided by the total body weight x 100.

Biochemical analysis

Twenty-four-hours after the last treatment, mice were sacrificed. The blood was collected from the orbital plexus in heparinized glass tubes from each mouse, allowed to stand for 30 min at room temperature, and centrifuged at 3000 rpm for 15 min to separate the blood serum samples. The separated serum was used for the estimation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using the protocol described by Ahmadi *et al.* (2011) and Habibi *et al.* (2015). Total serum protein was determined by the method of Biuret (Perez Gutierrez *et al.*, 2011). Albumin was determined using the method of Webster (Webster, 1977). The contents of cholesterol and triglycerides were also determined using commercial kits. All the above estimations were performed using standard autopak kits using a spectrophotometer.

Histological examinations

Liver tissues were sliced into small pieces and then immersed in neutral formalin buffered (10%) for 24h. The fixed tissues were processed routinely, embedded in paraffin (to get paraffin sections 4–5 µm), sectioned, deparaffinized, and dehydrated using the standard techniques (Suvarna *et al.*, 2018). Sections were then stained with hematoxylin-eosin and studied for histopathological changes. During the microscopic examination representative photos were captured (Nikon TE 2000-U microscope (NIKON, Tokyo, Japan).

Immunohistochemical studies

Representative sections of the formalin-fixed, paraffin-embed liver tissue from different groups were used for P53 and Bcl-2 expression investigation. Tissues were deparaffinized in xylene and rehydrated in descending ethanol series. Antigen retrieval was accomplished through microwave irradiation of the sections in 10 mM sodium citrate buffer. After microwave antigen repair, the sections

were incubated at 4°C overnight with rabbit anti-P53 and anti-Bcl-2 antibody (1:100, dilution), followed by incubation at 37°C in PV6001 for 30 min. The bound immune complexes were developed by the addition of 3, 3'-diaminobenzidine tetrahydrochloride (DAB) substrate and the nuclei were stained with hematoxylin (Sigma-Aldrich). The sections were incubated with a normal goat serum as a negative control. Samples were viewed by using Nikon TE 2000-U microscope (NIKON, Tokyo, Japan). Immunohistochemical positivity was evaluated by proportion and staining intensity for each protein.

Statistical analysis

Analysis of variance (ANOVA) using Minitab (ver. 12.21) was used to evaluate the significant difference in blood parameters in control and treated groups of mice. ANOVA was also used to assess the difference between the relative body weight before and after treatment and also the relative liver weight in the control and treated groups.

RESULTS

LD₅₀ of M. forsskalii fruit extract

The estimation of the median lethal dose (LD₅₀) in the experimental animals was carried out according to Finney, 1985. The result showed that the LD₅₀ of *M. forsskalii* extract was 3.625 g/kg BW (Table I).

Table I. Determination of LD₅₀ of *Mesembryanthemum forsskalii* on albino Swiss mice.

Group	N	Dose	No. of dead mice	Z	d	Z*d	% of mortality
1	6	5 g/kg wt.	6				100 %
2	6	4.5 g/kg wt.	4	5	0.5	2.5	66.7 %
3	6	4 g/kg wt.	4	4	0.5	2	66.7 %
4	6	3.5 g/kg wt.	4	4	0.5	2	66.7 %
5	6	3 g/kg wt.	3	3.5	0.5	1.75	50 %
Total						8.25	

Effect of M. forsskalii extract on the body weight and relative liver weight

The total body weight of mice was reduced significantly after CCl₄ treatment. The weight was not significantly changed after treatment with either *M. forsskalii* alone or after CCl₄ followed by 100 mg/kg BW from *M. forsskalii* extract (Gp5) and increased significantly after CCl₄ followed by 500 mg/kg BW from *M. forsskalii* extract (Gp6) (Table II).

The relative liver weight of mice was increased significantly after CCl₄ treatment (Gp2) compared to

vehicle control (Gp1). The reduction of relative liver weight was observed after treatment with CCl₄ followed by *M. forsskalii* extract (either 100 or 500 mg/kg BW) (Gp5 and Gp6). Low or high doses of *M. forsskalii* after CCl₄ treatment (Gp5 and Gp6) reduced liver weight observed in treated mice (Gp2). There was no significant change between the relative weights of vehicle control (Gp1) and that of CCl₄ followed by 100 or 500 mg/kg BW (Gp5 and Gp6) (Table II).

Biochemical changes

Treatment of the animals with CCl₄ (Gp2) resulted in a significant increase in ALT compared to the vehicle control (Gp1). Both low dose (100 mg/kg BW) (Gp5) and high dose (500 mg/kg BW) (Gp6) significantly reduced the levels of ALT in the treated mice compared to the CCl₄ treated mice (Gp2) (p<0.05). The reduction of the ALT content was more prominent after treatment with the low dose (100 mg/kg BW). No significant difference was observed in the animal group which received 100 mg/kg BW alone (Gp3) and the vehicle control group (Gp1). But the treatment of mice with 500 mg/kg BW alone (Gp4) significantly increased the content of ALT compared to the vehicle group (Gp1) (Table III).

CCl₄ administration to the mice increased AST levels (Gp2) when compared to the vehicle group (Gp1). Treatment of mice with a high dose (500 mg/kg BW) after CCl₄ treatment (Gp6), enhanced the AST content, whereas treatment with a low dose (100 mg/kg BW) (Gp5) reduced the AST content in the serum comparing to the CCl₄ treated group (Gp2) to a level almost normal as indicated by that of the vehicle control group (Gp1) indicating that low dose of *M. forsskalii* has improved the liver enzymes.

Although *M. forsskalii* extract at any dose has not significantly changed the albumin and the total protein levels, protein content was elevated after treatment with low or high doses (Gp5 and Gp6) compared to that of CCl₄ treated group (Gp2). Albumin content also was elevated after the administration of 500 mg/kg BW from *M. forsskalii* (Gp6) but the elevation of the content was not significant (Table III).

Even the CCl₄ treated mice (Gp2) showed lower cholesterol content than vehicle control (Gp1), the content of cholesterol in blood serum showed a significant increase after treatment with *M. forsskalii* either with low or high doses (Gp5 and Gp6), respectively comparing to CCl₄ treated group (Gp2) (Table III).

A high dose of *M. forsskalii* (Gp6) had significantly increased the content of triglycerides compared to CCl₄ treated group (Gp2), whereas a low dose (Gp5) elevated the content of triglycerides compared to the CCl₄ treated group (Gp2) but the increase was not significant (Table III).

Table II. Effect of *M. forsskalii* fruits extract on the total body weight and relative liver weight of mice.

Groups	Gp-1	Gp-2	Gp-3	Gp-4	Gp-5	Gp-6
Total body weight (g)						
Before treatment	33.4 ± 0.09	34.9 ± 2.3	30.7 ± 1.0	31.8 ± 1.0	34.5 ± 6.7	30.9 ± 4.9
After treatment	34.3 ± 1.7	33.7 ± 1.4*	29.0 ± 1.6	33.6 ± 0.9	35.8 ± 7.1	32.0 ± 5.2*
Relative weight (%)						
Liver	4.8 ± 0.7	6.4 ± 1.1*	6.0 ± 0.2*	5.6 ± 0.4	6.0 ± 0.3	5.1 ± 2.5

Data are expressed as means ± SD. Gp-1, control mice (negative control); Gp-2, mice that were injected with CCl₄ (positive control); Gp-3, mice were injected with 100 mg/kg BW of *O. forsskalii*; Gp-4, mice were injected with 500 mg/kg BW of *O. forsskalii*; Gp-5, mice were injected with 100 mg/kg BW after CCl₄ administration, and Gp-6, mice were injected with 500 mg/kg BW of *M. forsskalii* after CCl₄ administration. * Denotes significant difference at p < 0.05. Difference was assessed between Gp1 vs. Gp2, Gp2 vs. Gp5, Gp2 vs. Gp6, Gp1 vs. Gp3 and Gp1 vs. Gp4.

Table III. Effect *M. forsskalii* fruits extract on biochemical parameters and liver function in CCl₄ induced hepatotoxicity in mice.

Groups	Treatment	ALT U/L	AST U/L	Protein	Albumin	Cholest.	Triglyc.
Gp-1	Vehicle (negative control)	32.5 ± 3.5	24.0 ± 5.5	4.12 ± 0.4	3.14 ± 0.26	61.2 ± 6.5	106 ± 20
Gp-2	CCl ₄ (positive control)	60.0 ± 2.8*	39.5 ± 17.6	4.15 ± 0.06	3.14 ± 0.21	47.9 ± 6.4*	111 ± 24
Gp-3	Extract (100 mg/kg)	37.0 ± 4.2	33.5 ± 8.5	4.18 ± 0.19	3.09 ± 0.04	60.3 ± 7.7	123 ± 32
Gp-4	Extract (500 mg/kg)	47.0 ± 5.2*	46.0 ± 0.0*	4.76 ± 0.09	3.46 ± 0.09*	62.7 ± 14.4	171 ± 13*
Gp-5	CCl ₄ + Extract (100 mg/kg)	40.0 ± 5.5*	26.5 ± 5.5	4.18 ± 0.16	3.05 ± 0.08	56.9 ± 5.0*	139 ± 24
Gp-6	CCl ₄ + Extract (500 mg/kg)	41.5 ± 3.5*	40.5 ± 5.3	4.67 ± 0.41	3.41 ± 0.18	84.6 ± 9.1*	179 ± 21*

ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cholest., cholesterol; Triglyc., triglycerides. Protein and albumin were expressed as g/dl, cholesterol and triglycerides were expressed as mg/100 ml. For other abbreviations, details of groups and statistical details see Table II.

From the previous results, it is clear that treating mice with high doses of *M. forsskalii* (500 mg/kg BW) had a negative effect on liver enzyme function either in control mice or the mice treated with CCl₄. Treated mice with low doses either alone or post CCl₄ treatment had significantly improved the liver enzyme's function and other blood parameters.

Histopathological changes

Liver tissue sections were either stained with H and E (Fig. 1) or immune stained for the detection of the expression levels of the cell cycle regulator and tumor suppressor protein, P53 (Fig. 2), and the expression levels of the anti-apoptotic Bcl-2 (Fig. 3). The histological observations of liver tissues support the results obtained from serum enzyme assays. Negative control tissue sections exhibited no apparent pathological alterations (Fig. 1A). No cavitation, necrosis, or fibrosis was found in control sections. In contrast, sections from CCl₄-only treated mice displayed apparent cavitation in broad areas, necrosis with inflammation, and loss of cellular boundaries (Fig. 1B). On the other hand, liver sections treated with low and high dose of *M. forsskalii* extract (100mg and 500mg/kg BW, respectively) separately showed no marked variation in the histological architecture of the liver tissue (Fig. 1C,

D). Interestingly, the broad cavitation in the liver was attenuated in mice treated with *M. forsskalii* extract during the experimental periods (Fig. 1E, F). The administration of *M. forsskalii* extract resulted in less cavitation in the liver. Importantly, the remaining cavitation level and the range of the necrotic cells in the 100 mg/kg BW-treated group (Fig. 1E) were lower than that in the 500 mg/kg BW-treated group (Fig. 1F).

Liver sections prepared from *M. forsskalii* -treated groups displayed less fibrosis as compared to the CCl₄-only control group. This group's liver sections showed regeneration of hepatocytes, almost toward near-normal liver architecture and possessing higher hepatoprotective action. The improvement in the 100 mg/kg group (Fig. 1E) was more obvious in comparison to that of the 500 mg/kg group (Fig. 1F). We also examined the distribution of fibrosis in different liver regions (from the central vein region to hepatic portal veins). Both doses of the extract reduced apparent liver injury caused by CCl₄, compared to the group of mice that were treated with CCl₄ only.

The immunostaining study revealed that CCl₄ had enhanced the expression of the cell cycle regulator and necro-apoptotic driver, P53 in the liver tissues (Fig. 2B). However, this expression was relatively reduced after treatment with the extract from *M. forsskalii* at both doses

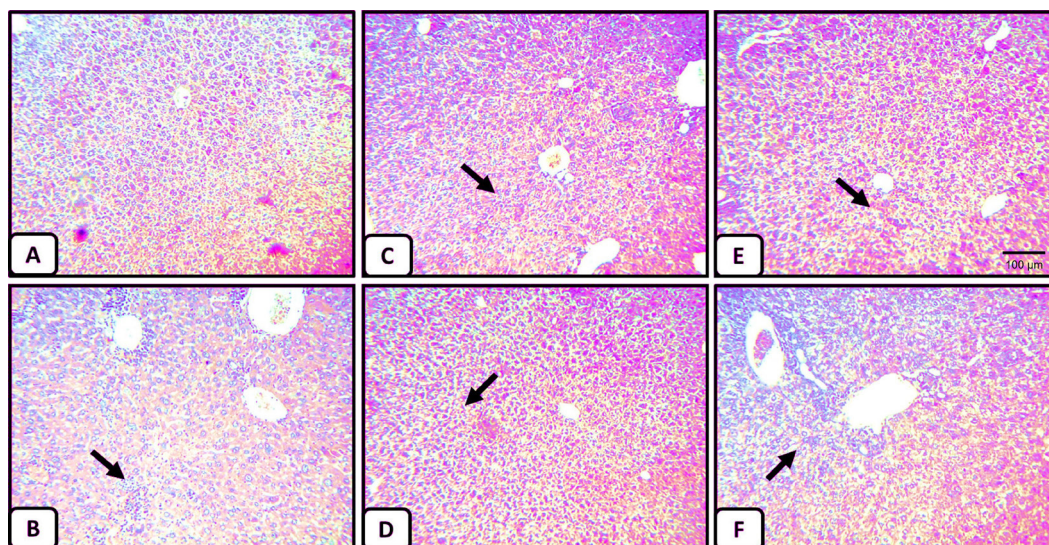


Fig. 1. Histopathology of liver tissues. (A) Liver section of normal negative control mice (vehicle) shows central vein surrounded by hepatic cord of cells (normal architecture), (B) liver section of CCl_4 -treated mice showing massive fatty changes, focal central vein congestion, a variety of cavitations and necrosis in hepatocytes with inflammation, and loss of cellular boundaries (indicated by arrow), (C) liver section of mice treated with 100mg/kg showing normal liver architecture (magnification 5X), (D) liver section of mice treated with the 500mg/kg showing normal liver architecture, (E) liver section of mice treated with CCl_4 and 100mg/kg of *M. forsskalii* showing absence of cavitations, necrosis, and inflammatory cells, and regeneration of hepatocytes around central vein toward near normal liver architecture but slight congestion in central vein (indicated by arrow), and (F) liver section of mice treated with CCl_4 and 500 mg/kg of *M. forsskalii* showing mild central vein congestion (indicated by arrow), ballooning, and necrosis with sinusoidal dilatation H and E x 50.

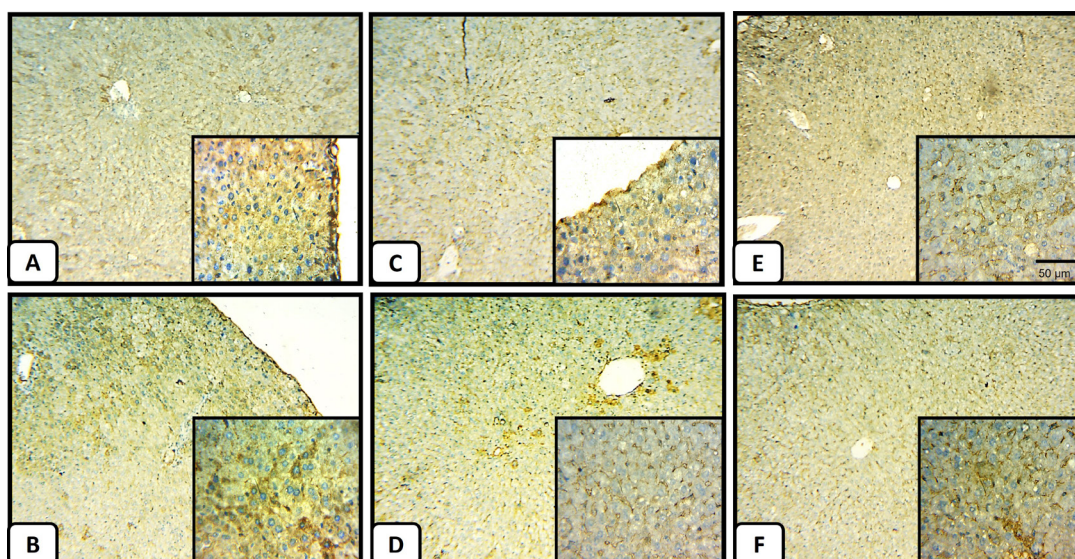


Fig. 2. Immunohistochemistry of P53 in the liver tissues. (A) Section from a negative control mice liver shows the normal pattern of P53 with mild staining. (B) The liver section obtained from CCl_4 -intoxicated mice shows extensive staining (indicated by brown color) for P53. (C) and (D) a liver section from normal mice treated at low and high doses, respectively, showing a non-appreciated difference in P53 staining pattern (magnification, x 50 for the main photo and x 100 for the hyper-focused region in the small box at the left bottom of each panel). (E) and (F) Liver tissue sections prepared from the 100 mg/kg and 500mg/kg of *M. forsskalii* after CCl_4 treatment, respectively, show less P53 staining compared to (B), however (E) shows a closer staining pattern to the normal liver (A).

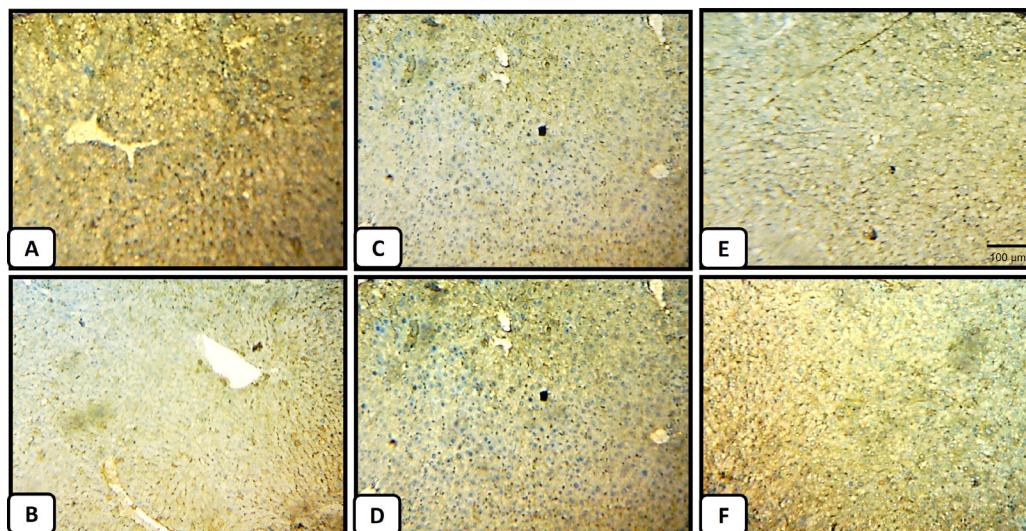


Fig. 3. Immunohistochemistry of the cytoprotective molecule, Bcl-2, in the liver tissues. (A) A representative section from a negative control mice liver shows a normal pattern of Bcl-2 with mild staining. (B) The liver section obtained from CCl_4 -intoxicated mice shows faint staining (indicated by brown color) for Bcl-2. (C) Liver section from normal mice treated with *M. forsskalii* extract at a low dose shows a detectable reduction in the expression of Bcl-2 in the normal liver when introduced at a low dose (Magnification, x 50), (D) A representative liver section from normal mice treated at high dose shows mild staining ability by Bcl-2, indicating a very limited effect of *M. forsskalii* extract on Bcl-2 expression when administered at high dose, (E) and (F) Liver tissue sections prepared from the 100 mg/kg and 500 mg/kg of *M. forsskalii* after CCl_4 treatment groups, respectively, showing the relative restoration of the Bcl-2 staining compared to (B), however (F) shows closer staining pattern to the normal liver (A).

(Fig. 2E, F) but the effect was more prominent when the extract was administered at a low dose (Fig. 2E). There was no appreciated change in the expression level in P53 under the extract treatment when administered to normal animals as represented in (Fig. 2C, D) if compared to the negative control (Fig. 2A). On the other side, the expression level of the tumor-promoting and cytoprotective molecule, Bcl-2, was significantly enhanced in the liver section of animals challenged by CCl_4 (Fig. 3B). Interestingly, this elevated expression had been reverted after treatment with the plant extract at both doses (Fig. 3E, F). Similarly, the expression of Bcl-2 in the liver from normal animals did not show detectable changes before and after the administration of the extract neither at a low dose nor a higher one (Fig. 3C, D) when compared to the negative control (Fig. 3A).

DISCUSSION

The change in dietary habits as well as the chemoprevention show considerable effective strategies against oxidative stress and are the main focus of the area of research these days (Lee and Park, 2003). CCl_4 is a known, reliable, and commonly used chemical to induce liver damage through oxidative degradation in the adipose tissue which resulted in fatty infiltration of the hepatocytes (Pal *et al.*, 2014; Wang *et al.*, 2019; Begum *et al.*, 2022)

and increased permeability, acute toxicity and may be hepatic necrosis (Naji *et al.*, 2017). Increasing relative liver weight and the level of liver enzymes such as AST and ALT in the serum is the first clue to liver toxicity (Tsai *et al.*, 2009; Bahashwan *et al.*, 2015). The current investigation was undertaken to evaluate the possible protective effect of *M. forsskalii* extract against carbon tetrachloride-induced hepatotoxicity and oxidative stress in mice. CCl_4 causes acute hepatocyte injuries and altered membrane integrity and as a result enzymes in hepatocytes leak out (Hu *et al.*, 2000). However, after treatment with *M. forsskalii*, the pathological increases in AST and ALT were significantly restored at least after treatment with a low dose. These results indicate that *M. forsskalii* can protect against CCl_4 -induced hepatocyte injuries. Extract from *M. forsskalii* fruits showed the protective capability against treatment through the restoration of the liver enzymes level to their normal values and restoration of the normal relative weights. The protective potentiality of *M. forsskalii* against CCl_4 -induced hepatotoxicity may be attributed to the presence of some secondary metabolites well known as an antioxidant and radical scavenging agents such as flavonoids, saponins, and phenolics.

Many plants are used as hepatoprotective natural sources against CCl_4 -induced hepatotoxicity. For instance, the aqueous ethanol extract of the pods of *Acacia senegal*

was used efficiently as a hepatoprotective agent against hepatotoxicity induced by CCl₄ in rats through the restoration of the blood serum enzymes and bilirubin and also restoration of the architecture of the liver through the presence of normal hepatic cords, absence of necrosis and fatty infiltration through histology of liver sections (Pal *et al.* 2014; Azubuiké *et al.* 2018). It was reported that leaves extract of *Pyrenacantha staudtii* has a protective effect against CCl₄ induced liver toxicity and damage, as well as CCl₄ induced elevations in the liver enzymes were significantly reduced by 750 mg/kg and 1500 mg/kg BW of the plant extract (Anosike *et al.*, 2008). As well as (Ouassou *et al.*, 2021) found that *Caralluma europaea* stem extract (250 mg/kg BW) had a significant hepatoprotective effect by ameliorating CCl₄-induced alterations of some biochemical parameters.

In line with the results of the current study, the blood parameters were improved in CCl₄ induced animals after administration of different plant extracts such the flowers extract of *S. subserrata* (Wahid *et al.*, 2016), roots extract of *C. telephiifolia* (Doudach *et al.*, 2022) and the extract of *C. cajan* (Nwaechefu *et al.*, 2022). In accordance with our results, the inflammatory cell infiltration, hepatic fibrosis, necrosis and severe hepatocytes apoptosis induced by CCl₄ were ameliorated by the extract of *C. cajan* (Nwaechefu *et al.*, 2022). Administration of *S. subserrata* extract ameliorated the negative impact of CCl₄ on liver tissues, where the appearance of the liver tissues was restored to the normal appearance post-administration of *S. subserrata* extract (Wahid *et al.*, 2016). Naringenin from *C. sinensis* ameliorated the negative impact of CCl₄ on liver tissue appearance. Moreover, it enhanced the expression of Bcl-2 (Ammar *et al.*, 2022). The alkaloids extract of *V. doniana* restored the normal appearance of the destructive liver tissues induced by CCl₄ (Ayoka *et al.*, 2022).

The lower level of cholesterol in CCl₄ treated mice than that of normal (vehicle control) may be attributed to loosing of an animal's appetite after CCl₄ treatment compared to other groups which could be concluded from the amounts of food remaining in the CCl₄ treatment group comparing to other groups. It seems that animals hardly eat after treatment with CCl₄. It was reported that feed intake was significantly reduced after CCl₄ administration compared with the constant feed intake by the control group (Uemitsu and Nakayoshi, 1984). The effect of decreasing cholesterol and the nonsignificant changes in the content of triglycerides, protein, and albumin in CCl₄ treated mice may also be attributed to the decrease in the number of hepatocytes due to liver damage by CCl₄ and consequently decrease in the liver capacity to synthesize these metabolites (Bhandarkar and Khan, 2003). The significant increase in cholesterol and triglycerides after

treatment with either high or low doses from *M. forsskalii* indicates the preliminary evidence for the protective role of *M. forsskalii*. This may refer to the restoring of the liver ability to synthesize these metabolites after its retardation by the CCl₄ effect.

It is well established that CCl₄ induces liver damage through necrosis. Immunostaining results from our study showed that CCl₄ alone induced relative up-regulation of P53 and down-regulation in the expression of Bcl-2 in the liver tissues. Importantly, unlike high dose, administration of the *M. forsskalii* at a low dose significantly restored this effect by the downregulation of P53 and upregulation of Bcl-2 expression. CCl₄ is reported to induce hepatic damage as a result of metabolic conversion of the radicals through lipid peroxidation and disturbance of the activities of the antioxidant enzymes (Adesanoye and Farombi, 2010), induce oxidative stress, and cause liver injury by the formation of free radicals (Manna *et al.*, 2006). On the other hand, carbon tetrachloride causes noticeable toxicity by enhancing liver lipid peroxidation as found by increased concentrations of hepatic malondialdehyde (Poli, 1993; Dalton *et al.*, 2009). Results from the current study have not included any evidences for the antioxidant activity of the *M. forsskalii* extract, the antioxidant activity of each fraction from the same fruits should be evaluated.

In conclusion, the administration of a low dose of *M. forsskalii* (100 mg/kg BW) improved the liver function through restoration of liver enzymes, reducing P53 expression, and increasing the Bcl-2 expression to the normal levels after CCl₄ treatment. However, 500 mg/kg BW prominently showed no beneficial effect after CCl₄ induced toxicity except enhancing Bcl-2 expression; even it exerts some toxic effect on the liver enzymes in mice.

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IRB approval

Jouf University Institutional Animal Ethics Committee approved the protocol under the number: JU - 49 / 2014.

Ethical statement

All procedures were conducted under ethical guidelines of animal care and approved by the animal care and use committee of at Jouf University, Saudi Arabia. We

took all the possible procedures to reduce mice sufferance.

Statement of conflict of interest

The authors declare no competing interests.

REFERENCES

- Aabed, K., and Mohammed, A.E., 2021. Phytoproduct, Arabic gum and *Opophytum forsskalii* seeds for bio-fabrication of silver nanoparticles: Antimicrobial and cytotoxic capabilities. *Nanomaterials*, **11**: 2573. <https://doi.org/10.3390/nano11102573>
- Aati, H., El-Gamal, A., Shaheen, H., and Kayser, O., 2019. Traditional use of ethnomedicinal native plants in the Kingdom of Saudi Arabia. *J. Ethnobiol. Ethnomed.*, **15**: 1–9. <https://doi.org/10.1186/s13002-018-0263-2>
- Abdel-Hamid, A.M.E., Ibrahim, M., and Alnusairi, G.S.H., 2021. Comparison of Egyptian and Saudi *Mesembryanthemum forsskalii* Hochst. ex Boiss as an unconventional alternative protein of wheat and barley. *Indian J. exp. Biol.*, **59**: 194–201.
- Adesanoye, O.A. and Farombi, E.O., 2010. Hepatoprotective effects of *Vernonia amygdalina* (Asteraceae) in rats treated with carbon tetrachloride. *Exp. Toxicol. Pathol.*, **62**: 197–206. <https://doi.org/10.1016/j.etp.2009.05.008>
- Ahmadi, A., Ebrahimzadeh, M.A., Ahmad-Ashrafi, S., Karami, M., Mahdavi, M.R., and Saravi, S.S.S., 2011. Hepatoprotective, antinociceptive and antioxidant activities of cimetidine, ranitidine and famotidine as histamine H2 receptor antagonists. *Fundam. clin. Pharmacol.*, **25**: 72–79. <https://doi.org/10.1111/j.1472-8206.2009.00810.x>
- Al-Faris, N.A., Al-Sawadi, A.D. and Alokail, M.S., 2010. Effect of samh seeds supplementation (*Mesembryanthemum forsskalii* Hochst) on liver enzymes and lipid profiles of streptozotocin (STZ)-induced diabetic Wistar rats. *Saudi J. Biol. Sci.*, **17**: 23–28. <https://doi.org/10.1016/j.sjbs.2009.12.004>
- Al-Jassir, M.S., Mustafa, A.I., and Nawawy, M.A., 1995. Studies on samh seeds (*Mesembryanthemum forsskalii* Hochst) growing in Saudi Arabia 2: Chemical composition and microflora of samh seeds. *Pl. Fd. Hum. Nutr.*, **48**: 185–192. <https://doi.org/10.1007/BF01088439>
- Al-Yahya, M., Mothana, R., Al-Said, M., Al-Dosari, M., Al-Musayeib, N., Al-Sohaibani, M., Parvez, M.K., and Rafatullah, S., 2013. Attenuation of CCl₄-induced oxidative stress and hepatonephrotoxicity by Saudi Sidr honey in rats. *Evid.-based Complement. Altern. Med.*, Article ID 569037. <https://doi.org/10.1155/2013/569037>
- Ammar, N.M., Hassan, H.A., Abdallah, H.M.I., Affi, S.M., Elgamal, A.M., Farrag, A.R.H., El-Gendy, A.E.-N.G., Farag, M.A., and Elshamy, A.I., 2022. Protective effects of naringenin from *Citrus sinensis* (var. Valencia) peels against CCl₄-Induced hepatic and renal injuries in rats assessed by metabolomics, histological and biochemical analyses. *Nutrients*, **14**: 841. <https://doi.org/10.3390/nu14040841>
- Anosike, C.A., Ugwu, U.B., and Nwakanma, O., 2008. Effect of ethanol extract of *Pyrenacantha staudtii* leaves on carbontetrachloride induced hepatotoxicity in rats. *Biokemistri*, **20**. <https://doi.org/10.4314/biokem.v20i1.56433>
- Ayoka, T.O., Ezema, B.O., Eze, C.N. and Nnadi, C.O., 2022. Antioxidants for the prevention and treatment of non-communicable diseases. *J. Explor. Res. Pharmacol.*, **7**: 178–188. <https://dx.doi.org/10.14218/JERP.2022.00028>
- Azubuikwe, N.C., Onyemelukwe, A.O., and Maduakor, U., 2018. Hepatoprotective effects of the leaf extracts of *Cassia occidentalis* against carbon tetrachloride-induced hepatotoxicity in albino rats. *Liver*, **1**: 4–5.
- Bahashwan, S., Hassan, M.H., Aly, H., Ghobara, M.M., El-Beshbishy, H.A., and Busati, I., 2015. Crocin mitigates carbon tetrachloride-induced liver toxicity in rats. *J. Taibah Univ. Med. Sci.*, **10**: 140–149. <https://doi.org/10.1016/j.jtumed.2014.09.003>
- Baradaran, A., Samadi, F., Ramezani, S.S., and Yousefdoost, S., 2019. Hepatoprotective effects of silymarin on CCl₄-induced hepatic damage in broiler chickens model. *Toxicol. Rep.*, **6**: 788–794. <https://doi.org/10.1016/j.toxrep.2019.07.011>
- Begum, R., Papia, S.A., Begum, M.M., Wang, H., Karim, R., Sultana, R., Das, P.R., Begum, T., Islam, M., and Manwar, N., 2022. Evaluation of hepatoprotective potential of polyherbal preparations in CCl₄-induced hepatotoxicity in mice. *Adv. Pharmacol. Pharm. Sci.*, **2022**. <https://doi.org/10.1155/2022/3169500>
- Bhandarkar, M. and Khan, A., 2003. Protective effect of *Lawsonia alba* Lam., against CCl₄ induced hepatic damage in albino rats. *India. J. Exp. Biol.*, **41**: 85–87.
- Bilel, H., Elsherif, M.A., and Moustafa, S.M.N., 2020. Seeds oil extract of *Mesembryanthemum forsskalii* from Aljouf, Saudi Arabia: Chemical composition, DPPH radical scavenging and antifungal activities. *Oilseeds, Fats, Crops Lipids*, **27**: 10. <https://doi.org/10.1051/ocl/2020005>
- Dalton, S.R., Lee, S.M.L., King, R.N., Nanji, A.A.,

- Kharbanda, K.K., Casey, C.A., and McVicker, B.L., 2009. Carbon tetrachloride-induced liver damage in asialoglycoprotein receptor-deficient mice. *Biochem. Pharmacol.*, **77**: 1283–1290. <https://doi.org/10.1016/j.bcp.2008.12.023>
- Dhanarasu, S., Selvam, M. and Al-Shammari, N.K.A., 2016. Evaluating the pharmacological dose (oral LD50) and antibacterial activity of leaf extracts of *Mentha piperita* Linn. grown in Kingdom of Saudi Arabia: A pilot study for nephrotoxicity. *Int. J. Pharmacol.*, **12**: 195200. <https://dx.doi.org/10.3923/ijp.2016.195.200>
- Doudach, L., Omari, N. El, Mrabti, H.N., Touhami, F., Mrabti, N.N., Benrahou, K., Bouyahya, A., Cherrah, Y., Meddah, B. and Faouzi, M.E.A., 2022. Hepatoprotective effect of corrigiola telephiifolia pourr root methanolic extracts against CCl₄-induced hepatic damage in mice. *Biointerf. Res. appl. Chem.*, **12**: 2489-2502. <https://doi.org/10.33263/BRIAC122.24892502>
- Edwards, L. and Wanless, I.R., 2013. Mechanisms of liver involvement in systemic disease. *Best Pract. Res. clin. Gastroenterol.*, **27**: 471–483. <https://doi.org/10.1016/j.bpg.2013.08.002>
- El-Amier, Y.A., Al-hadithy, O.N., Fahmy, A.A., and El-Zayat, M.M., 2021. Phytochemical analysis and biological activities of three wild *Mesembryanthemum* species growing in heterogeneous habitats. *J. Phytol.*, **13**: 1–8. <https://doi.org/10.25081/jp.2021.v13.6403>
- El Hawary, S.S.E., Hassan, M., Mostafa, D., AbouZid, S., Sleem, A. and Mohammed, R., 2020. Comparative phytochemical and biological study for *Mesembryanthemum nodiflorum* and *Aptenia cordifolia* plants growing in Egypt. *Egypt. J. Chem.*, **63**: 2509-2522. <https://doi.org/10.21608/ejchem.2020.20877.2248>
- Elshopakey, G.E. and Elazab, S.T., 2021. Cinnamon aqueous extract attenuates diclofenac sodium and oxytetracycline mediated hepato-renal toxicity and modulates oxidative stress, cell apoptosis, and inflammation in male albino rats. *Vet. Sci.*, **8**: 9. <https://doi.org/10.3390/vetsci8010009>
- Finney, D.J., 1985. The median lethal dose and its estimation. *Arch. Toxicol.*, **56**: 215–218. <https://doi.org/10.1007/BF00295156>
- Habibi, E., Shokrzadeh, M., Chabra, A., Naghshvar, F., Keshavarz-Maleki, R., and Ahmadi, A., 2015. Protective effects of *Origanum vulgare* ethanol extract against cyclophosphamide-induced liver toxicity in mice. *Pharm. Biol.*, **53**: 10–15. <https://doi.org/10.3109/13880209.2014.908399>
- Hamzawy, M.A., El-Denshary, E.S.M., and Abdel-Wahhab, M.A., 2015. Effects of natural compounds in treatment and prevention of hepatotoxicity and hepatocellular carcinoma. *Hepatoma Res.*, **1**: 111–118. <https://doi.org/10.4103/2394-5079.167378>
- Hu, Y.Y., Liu, C.H., Wang, R.P., Liu, C., Zhu, D.Y., and Liu, P., 2000. Protective actions of salvianolic acid A on hepatocyte injured by peroxidation *in vitro*. *World J. Gastroenterol.*, **6**: 402. <https://doi.org/10.3748/wjg.v6.i3.402>
- Ikatsu, H. and Nakajima, T., 1992. Hepatotoxic interaction between carbon tetrachloride and chloroform in ethanol treated rats. *Arch. Toxicol.*, **66**: 580–586. <https://doi.org/10.1007/BF01973389>
- Izzularab, B.M., Megeed, M., and Yehia, M., 2021. Propolis nanoparticles modulate the inflammatory and apoptotic pathways in carbon tetrachloride-induced liver fibrosis and nephropathy in rats. *Environ. Toxicol.*, **36**: 55–66. <https://doi.org/10.1002/tox.23010>
- Katiyar, C., Gupta, A., Kanjilal, S., and Katiyar, S., 2012. Drug discovery from plant sources: An integrated approach. *Ayu J.*, **33**: 10-19. <https://doi.org/10.4103/0974-8520.100295>
- Lee, B.M. and Park, K.K., 2003. Beneficial and adverse effects of chemopreventive agents. *Mutat. Res. Fundam. Mol. Mech. Mutagenesis*, **523**: 265–278. [https://doi.org/10.1016/S0027-5107\(02\)00342-1](https://doi.org/10.1016/S0027-5107(02)00342-1)
- Li, J., Niu, R., Dong, L., Gao, L., Zhang, J., Zheng, Y., Shi, M., Liu, Z., and Li, K., 2019. Nanoencapsulation of curcumin and its protective effects against CCl₄-induced hepatotoxicity in mice. *J. Nanomater.*, **2019**: 7140132. <https://doi.org/10.1155/2019/7140132>
- Mahalel, U., 2015. Allelopathic effect of saponins isolated from *Trigonella hamosa* L. and *Solanum lycopersicum* L. on germination and growth of *Allium cepa* L. *Catrina*, **12**: 95–99.
- Manna, P., Sinha, M., and Sil, P.C., 2006. Aqueous extract of *Terminalia arjuna* prevents carbon tetrachloride induced hepatic and renal disorders. *BMC Complement. Altern. Med.*, **6**: 1–10. <https://doi.org/10.1186/1472-6882-6-33>
- Moawad, A., Amin, E., and Mohammed, R., 2016. Diffusionordered spectroscopy of flavonol mixture from *Mesembryanthemum forsskaolii* (Aizoaceae). *Eur. J. Med. Pl.*, **16**: 1–8. <https://doi.org/10.9734/EJMP/2016/27794>
- Naji, K.M., Al-Shaibani, E.S., Alhadi, F.A., Al-Soudi, S.A., and D'souza, M.R., 2017. Hepatoprotective and antioxidant effects of single clove garlic against CCl₄-induced hepatic damage in rabbits.

- BMC Complement. Altern. Med.*, **17**: 1–12. <https://doi.org/10.1186/s12906-017-1916-8>
- Najib, H., Al-Dosari, M.N., and Al-Wesali, M.S., 2004. Use of samh seeds (*Mesembryanthemum forsskalii* Hochst) in the laying hen diets. *Int. J. Poult. Sci.*, **3**: 287–294. <https://doi.org/10.3923/ijps.2004.287.294>
- Nwaechefu, O.O., Olaolu, T.D., Akinwunmi, I.R., Ojezele, O.O. and Olorunsogo, O.O., 2022. *Cajanus cajan* ameliorated CCl₄-induced oxidative stress in Wistar rats via the combined mechanisms of anti-inflammation and mitochondrial-membrane transition pore inhibition. *J. Ethnopharmacol.*, **289**: 114920. <https://doi.org/10.1016/j.jep.2021.114920>
- Ouassou, H., Bouhrim, M., Daoudi, N.E., Mekhfi, H., Ziyat, A., Legssyer, A., Aziz, M., and Bnouham, M., 2021. Evaluation of hepatoprotective activity of *Caralluma europaea* stem extract against CCl₄-induced hepatic damage in Wistar rats. *Adv. Pharmacol. Pharm. Sci.*, **2021**. <https://doi.org/10.1155/2021/8883040>
- Owojuyigbe, O.S., Larbie, C., Firempong, C.K., Komlaga, G., Emikpe, B.O., and Otuechere, C.A., 2020. Extracts of *Hura crepitans* L. stem bark attenuate liver injury and inflammation induced by CCl₄ in rats. *Comp. clin. Pathol.*, **29**: 1199–1208. <https://doi.org/10.1007/s00580-020-03172-2>
- Pal, R., Hooda, M.S., Bias, C.S., and Singh, J., 2014. Hepatoprotective activity of *Acacia senegal* pod against carbon tetrachloride-induced hepatotoxicity in rats. *Int. J. Pharm. Sci.*, **26**: 165.
- Perez-Gutierrez, R.M., Anaya-Sosa, I., Hoyo, V.C., and Victoria, T.C., 2011. Effect of flavonoids from *Prosthechea michuacana* on carbon tetrachloride induced acute hepatotoxicity in mice. *Pharm. Biol.*, **49**: 1121–1127. <https://doi.org/10.3109/13880209.2011.570766>
- Poli, G., 1993. Liver damage due to free radicals. *Br. med. Bull.*, **49**: 604–620. <https://doi.org/10.1093/oxfordjournals.bmb.a072634>
- Ritesh, K.R., Suganya, A., Dileepkumar, H.V, Rajashekar, Y. and Shivanandappa, T., 2015. A single acute hepatotoxic dose of CCl₄ causes oxidative stress in the rat brain. *Toxicol. Rep.*, **2**: 891–895. <https://doi.org/10.1016/j.toxrep.2015.05.012>
- Singh, D., Cho, W.C., and Upadhyay, G., 2016. Drug-induced liver toxicity and prevention by herbal antioxidants: An overview. *Front. Physiol.*, **6**: 363. <https://doi.org/10.3389/fphys.2015.00363>
- Suvarna, K.S., Layton, C., and Bancroft, J.D., 2018. *Bancroft's theory and practice of histological techniques*. E-Book. Elsevier Health Sciences.
- Tsai, C.F., Hsu, Y.W., Chen, W.K., Chang, W.H., Yen, C.C., Ho, Y.C., and Lu, F.J., 2009. Hepatoprotective effect of electrolyzed reduced water against carbon tetrachloride-induced liver damage in mice. *Fd. chem. Toxicol.*, **47**: 2031–2036. <https://doi.org/10.1016/j.fct.2009.05.021>
- Uemitsu, N. and Nakayoshi, H., 1984. Evaluation of liver weight changes following a single oral administration of carbon tetrachloride in rats. *Toxicol. appl. Pharmacol.*, **75**: 1–7. [https://doi.org/10.1016/0041-008X\(84\)90069-3](https://doi.org/10.1016/0041-008X(84)90069-3)
- Ullah, H., Khan, A., Baig, M.W., Ullah, N., Ahmed, N., Tipu, M.K., Ali, H. and Khan, S., 2020. Poncirin attenuates CCL4-induced liver injury through inhibition of oxidative stress and inflammatory cytokines in mice. *BMC Complement. Med. Therap.*, **20**: 114. <https://doi.org/10.1186/s12906-020-02906-7>
- Wahid, A., Hamed, A.N., Eltahir, H.M. and Abouzied, M.M., 2016. Hepatoprotective activity of ethanolic extract of *Salix subserata* against CCl₄-induced chronic hepatotoxicity in rats. *BMC Complement. Alternat. Med.*, **16**: 110. <https://doi.org/10.1186/s12906-016-1238-2>
- Wang, R., Yang, Z., Zhang, J., Mu, J., Zhou, X., and Zhao, X., 2019. Liver injury induced by carbon tetrachloride in mice is prevented by the antioxidant capacity of Anji white tea polyphenols. *Antioxidants*, **8**: 64. <https://doi.org/10.3390/antiox8030064>
- Webster, D., 1977. The immediate reaction between bromocresol green and serum as a measure of albumin content. *Clin. Chem.*, **23**: 663–665. <https://doi.org/10.1093/clinchem/23.4.663>