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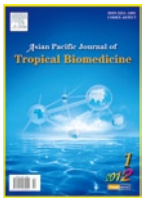

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# Ameliorative effects of *Spinacia oleracea* L. seeds on carbon tetrachloride ($CCl_4$) - induced hepatotoxicity: *In vitro* and in *vivo* studies

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ABSTRACT

**Objective:** To investigate the *in vitro* and *in vivo* protective effects of *Spinacia oleracea* L. (Chenopodiaceae) seeds on carbon tetrachloride ($CCl_4$)-induced hepatic toxicity.**Methods:** In the in vitro studies, different extracts (*i.e.* petroleum ether, ethanol and aqueous) and fractions derived from ethanol extract (*i.e.* chloroform, ethyl acetate and *n*-butanol) of *Spinacia oleracea* seeds were screened at a concentration of 100 μg/mL against carbon tetrachloride ($CCl_4$)-toxicity in rat hepatocyte culture. *In vivo* hepatoprotective activity was assessed in rats intoxicated with $CCl_4$. Level of biochemical markers along with histological changes were monitored to evaluate the extent of hepatoprotection. Silymarin was taken as reference drug.**Results:** In the *in vitro* screening, *n*-butanol fraction of *Spinacia oleracea* seeds was found to be more potent than other screened plant samples, hence selected further for phytochemical and *in vivo* studies. In the *in vivo* studies, the *n*-butanol fraction of *Spinacia oleracea* showed significant protection against $CCl_4$-induced hepatotoxicity as evident by restoration of biochemical and histological changes caused by $CCl_4$ intoxication. HPTLC fingerprinting of the *n*-butanol fraction of *Spinacia oleracea* confirmed the presence of 20-hydroxyecdysone (20-HE) besides other phytochemicals, which partially may explain the effects.**Conclusions:** The results of present study indicates the significant *in vitro* and *in vivo* hepatoprotective activity of *n*-butanol fraction of *Spinacia oleracea* on $CCl_4$-induced hepatotoxicity, and hence suggests its use as a potential therapeutic agent in liver diseases.

## 1. Introduction

Liver is an organ of paramount importance and its disorders are numerous with no effective remedies. Therefore, search for new medicines is still ongoing. In recent years, much interest has been developed in therapeutic evaluation of traditionally used herbals with that of the modern concept of evidence based evaluation[1].

*Spinacia oleracea* Linn. (Family-Chenopodiaceae), commonly known as "Paalak" in Hindi, is an erect herb with about 30-60 cm height. It is native to South-West Asia and cultivated throughout world as vegetables. Several parts of this plant are used in traditional Indian medicine for numerous therapeutic effects. The leaves are cooling and useful in febrile conditions, urinary calculi and lung inflammation. The seeds are cooling, laxative, and useful in difficult breathing, liver inflammation and jaundice[2,3]. Presence of 20-hydroxyecdysone, polypodine B and protein has been shown in *Spinacia oleracea* seeds[4, 5].

Literature review reports that very little work has been done on *Spinacia oleracea* seeds. Moreover, no scientific report is available regarding its hepatoprotective action, to the best of our knowledge. Therefore to validate the traditional claims, the present study was aimed to examine the potential hepatoprotective effects of *Spinacia oleracea* seeds against hepatotoxicity induced carbon tetrachloride ($CCl_4$), a most widely used hepatotoxin.

## 2. Material and methods

### 2.1. Chemicals and drugs

$CCl_4$, ethylene glycol tetraacetic acid (EGTA), hydroxyethyl piperazine ethane sulfonic acid, William's E medium, collagenase, thiobarbituric acid and 5, 5'-dithiobis-2-nitrobenzoic acid were procured from Sigma Chemical Co. (St. Louis, MO, USA). 20-hydroxyecdysone was purchased

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from Altavista Phytochemicals Pvt. Ltd, Hyderabad. All other chemicals and reagents used were of analytical grade and purchased from commercial sources.

### 2.2. Experimental animals

Wistar albino rats (200-250 g) of either sex were used for the studies. The animals were maintained under standard laboratory conditions of temperature [(25±2) ℃] and relative humidity [(55±5) %] with 12 h light-dark cycle. The animal studies were approved by the Institutional Animal Ethics Committee (379/01/ab/CPCSEA).

### 2.3. Plant material

The seeds of *Spinacia oleracea* were procured from the seed market, Sagar and authenticated by Dr. P. Tiwari, Botanist, Department of Botany, Dr. Hari Singh Gour Vishwavidyalaya, Sagar, India. The voucher specimen has been preserved (No. Bot/Her/889) for future reference.

### 2.4. Extraction and fractionation

The coarsely powdered seeds (800 g) were successively extracted with petroleum ether (60-80℃) and 95% ethanol using Soxhlet extractor. The marc left after the ethanol extraction was macerated with distilled water for 24 h. The solvents were removed by distillation under reduced pressure below 45℃ to afford petroleum ether extract of *Spinacia oleracea* seeds [yield 4.05% (w/w)], ethanol extract [yield 9.07% (w/w)] and aqueous extract, [yield 10.5% (w/w)], respectively.

The ethanol extract (30 g) was suspended in water (300 mL) and fractionated successively with chloroform (4 × 300 mL), ethyl acetate (4 × 300 mL) and *n*-butanol (4 × 300 mL) to afford chloroform fraction (3.7 g), ethyl acetate fraction (4.8 g) and *n*-butanol fraction (6.9 g), respectively.

### 2.5. Preliminary phytochemical screening

Preliminary phytochemical analysis was performed to identify the nature of phytoconstituents in different extracts and fractions[6].

### 2.6. In vitro hepatoprotective evaluation

#### 2.6.1. Isolation and culture of rat hepatocytes

The rat hepatocytes were isolated by the two step collagenase perfusion technique[7]. Briefly, the rats were anaesthetized by pentobarbital sodium (50 mg/kg, *i.p.*). After opening the abdomen, livers were perfused via the portal vein with $Ca^{2+}$- free phosphate buffer (pH 7.4), containing 135 mM NaCl, 15 mM $NaHCO_3$, 5 mM glucose, 5.9 mM KCl 0.74 mM $KH_2PO_4$ and 0.1 mM EGTA, at a flow rate of 25 mL/min to remove blood. After 10 min, the liver was reperfused for another 10 min with the same phosphate buffer containing 50 mg collagenase, 3 mM $CaCl_2$ and 22 mg pyruvate, at a flow rate of 35 mL/min. To produce a single cell suspension of hepatocytes, the collagenase-digested liver was removed, passed through a nylon mesh (mesh size, 0.3 nm), washed and centrifuged at 500 rpm for 5 min at 4℃. Hepatocytes were then resuspended and washed twice with washing medium. The cell viability was more than 85% as determined by trypan blue exclusion.

The freshly isolated hepatocytes (viability > 85%) were seeded at a density of 2 to $3\times10^3$ cells/60 nm tissue cultures plates in Williams medium E consisting 10% fetal calf serum, and 0.1 μM insulin at 37℃ in humidified atmosphere of 5% $CO_2$ in a $CO_2$ incubator. After 3 h of plating, the fresh Williams medium E containing 3% FCS, 0.1 μM dexamethasone, 5nM epidermal growth factor and 1 nM glucagons was added.

#### 2.6.2. Toxicity induction and drug treatment

The hepatocytes monolayer was exposed to $CCl_4$ (2.5 mM) [7] with or without plant samples (100 μg/mL) or silymarin (10 μM) and incubated for another 24 h at 37℃ in a humidified atmosphere of 5% $CO_2$ in a $CO_2$ incubator. After 24 h incubation, the leakage of alanine transaminase (ALT)[8] and lactate dehydrogenase (LDH)[7] in culture medium was determined.

### 2.7. Chromatographic studies

Precoated and preactivated TLC plates (E. Merck No. 5548) of silica gel 60 $F_{254}$ with the support of aluminium sheets 0.1 mm thick and 20 cm × 10 cm were used. The SOBF (10 mg) was weighed accurately and dissolved in 10 mL of methanol. The sample was applied in the form of a band using CAMAG LINOMAT V, an automatic sample applicator, maintaining a band width 6 mm, space 10 mm, 250 nL/s. The volume of sample applied was 10 μL. The mobile phase optimized and used was ethyl acetate: ethanol: water (16:2:1). 20-hydroxyecdysone (10 mg) was dissolved in methanol (10 mL). Vanilin-sulphuric reagent was used as detecting reagent.

### 2.8. Acute oral toxicity studies

The acute oral toxicity studies were performed on *n*-butanol fraction of *Spinacia oleracea* (SOBF) following OECD guideline[9]. On the basis of studies, the oral doses of 50, 100 and 200 mg/kg, b.w. were selected for the *in vivo* experiments.

### 2.9. In vivo hepatoprotective evaluation

#### 2.9.1. Experimental protocol

The experiment was conducted according to the method described previously[10]. Rats were randomly divided into seven groups, each consisting of six rats. Group I (normal control) rats received distilled water (1 mL/kg, *p.o.*) daily for 5 days and olive oil (1 mL/kg, *s.c.*) on days 2 and 3. Group II ($CCl_4$ control) rats received distilled water (1 mL/kg, *p.o.*) daily for 5 days and $CCl_4$: olive oil (1:1, 2 mL/kg, *s.c.*) on days 2 and 3. Group III (SOBF control) rats were treated with the SOBF (200 mg/kg, *p.o.*) daily for 5 days. Group IV rats were treated with silymarin (50 mg/kg, *p.o.*) daily for 5 days and received $CCl_4$: olive oil (1:1, 2 mL/kg, *s.c.*) on days 2 and 3,

30 min after administration of silymarin. Groups V-VII was treated with SOBF at a dose of 50, 100 and 200 (mg/kg, *p.o.*), respectively, for 5 days and received $CCl_4$: olive oil (1:1, 2 mL/kg, *s.c.*) on days 2 and 3, 30 min after administration of SOBF.

On the sixth day, under ether anesthesia, blood and liver samples were collected. The blood was allowed to clot for 30 min and serum was separated by centrifugation at 3 000 rpm at 4℃. The livers were immediately taken out and washed with ice-cold saline, stored at −80℃ and processed for determination of enzymatic and non-enzymatic antioxidants and histopathological studies.

#### 2.9.2. Estimation of functional and oxidative stress markers

Activities of the serum marker enzymes such as ALT and aspartate transaminase (AST)[8], alkaline phosphatase (ALP) [11] and LDH[8] were determined to assess the extent of hepatic toxicity and protection.

Lipid peroxidation was assayed by measuring the malondialdehyde (MDA) content[12]. The protein[12], GSH content[8] and activities of superoxide dismutase (SOD) [10], and catalase (CAT)[13], were determined in the liver homogenate accordingly to assess the extent of oxidative stress and antioxidant effect.

#### 2.9.3. Histopathological studies

The liver tissues were fixed with 10% formalin solution (pH 7.4) for 24 h and dehydrated with a sequence of ethanol solution (50%-100%), cleared in xylene and embedded in paraffin. The sections were cut 5 μm thick and stained with hematoxylin-eosin dye and then observed for the histological changes by photomicroscope.

### 2.10. Statistical analysis

The results of present study are presented as mean ± SEM. and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using Graph Pad Prism software. $P < 0.05$ was considered to be significant.

## 3. Results

### 3.1. Preliminary phytochemical analysis

In the preliminary phytochemical analysis, different extracts and fractions of *Spinacia oleracea* seeds showed the presence of sterols, triterpenoids, phenolics, tannins, flavonoids, alkaloids, glycosides and saponins (Table 1).

### 3.2. In vitro hepatoprotective evaluation

Incubation of hepatocytes with $CCl_4$ (2.5 mM) resulted in significant depletion (79%) in viability of hepatocytes. Similarly a significant ($P< 0.001$) increase of ALT and LDH (2.8 and 3 fold, respectively) was observed upon $CCl_4$ intoxication (Table 2). Treatment with different extracts and fractions of *Spinacia oleracea* (100 μg/mL) or silymarin

**Table 1.**
Preliminary phytochemical screening of various extracts and fractions of *Spinacea oleracea* L. (SO) seeds .

| Nature of phytoconstituents | Chemical test | SOPEE | SOEE | SOAE | SOCF | SOEAF | SOBF |
|---|---|---|---|---|---|---|---|
| Steroid and triterpenoids | Liebermann-Burchard Test | ++ | + | − | + | − | ++ |
| Phenolic and tannins | 5% Alco. $FeCl_3$ solution | − | + | ++ | − | ++ | +++ |
| Flavonoids | Shinoda test (Mg metal and HCl) | − | + | ++ | + | ++ | ++ |
| Saponin | Foam test | − | + | ++ | + | − | ++ |
| Alkaloid | Dragendorff and Mayer's reagent | − | + | − | + | − | − |
| Anthraquinone glycoside | Borntrager test | − | − | + | − | − | + |
| Carbohydrate | Molisch reagent and Fehling solution | − | + | ++ | − | + | + |
| Protein | Ninhydrin reagent | − | + | ++ | − | − | + |

*− , Absent; +, Trace; ++, Moderate; +++, Abundance.
SOPEE, petroleum ether extract; SOEE, ethanol extract; SOAE, aqueous extract; SOCF, chloroform fraction; SOEAF, ethyl acetate fraction; SOBF, *n*-butanol fraction.

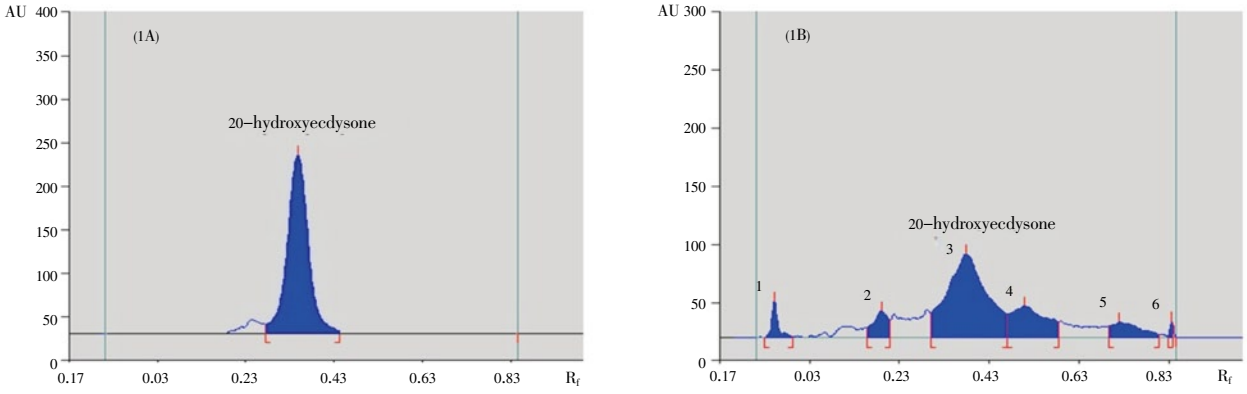


**Figure 1.** HPTLC fingerprinting of the *n*-butanol fraction of *Spinacia oleracea* revealing the presence of 20-hydroxyecdysone (20-HE, $\lambda_{max}$ 250 nm, $R_f$ 0.37).
1A: Standard of 20-HE; 1B: 20 HE in SOBF.

(10 μM) showed a varied protective effect against $CCl_4$ toxicity as evident from the restoration of ALT and LDH. The maximum restoration against enzyme leakage was observed with *n*-butanol fraction (69.13% and 82.60%, respectively for ALT and LDH), while the reference drug silymarin showed good protective effect (62.28% and 58.80% restoration, respectively for ALT and LDH). The most active *n*-butanol fraction (SOBF) was selected for further *in vivo* hepatoprotective studies.

**Table 2 .**
Protective effect of various extracts and fractions of *Spinacia oleracea* seeds and silymarin on $CCl_4$-induced toxicity in rat hepatocyte monolayer culture.

| Treatment | % Restoration | |
|---|---|---|
| | ALT | LDH (O.D./min/3 mL medium) |
| Normal | 100.00 | 100.00 |
| $CCl_4$ control | 0.00 | 0.00 |
| Silymarin (10 μM) | 62.28 | 58.80 |
| SOPEE (100 μg/mL) | 26.58 | 24.44 |
| SOEE (100 μg/mL) | 30.55 | 12.85 |
| SOAE (100 μg/mL) | 56.45 | 64.49 |
| SOCF (100 μg/mL) | 36.22 | 38.17 |
| SOEAF (100 μg/mL) | 30.00 | 23.25 |
| SOBF (100 μg/mL) | 69.13 | 82.60 |

The % restoration was calculated as 100 × (value of $CCl_4$ control − value of sample) / (value of $CCl_4$ control − value of normal control). SOPEE, petroleum ether extract; SOEE, ethanol extract; SOAE, aqueous extract; SOCF, chloroform fraction; SOEAF, ethyl acetate fraction; SOBF, *n*-butanol fraction.

### 3.3. Chromatographic studies

Chromatographic profile of SOBF in ethyl acetate: ethanol: water (16:2:1) revealed the presence of six spots. One of the spot showed an identical $R_f$ value with the standard compound 20-hydroxyecdysone at $R_f$ 0.37 when visualized under UV 250 nm Figure 1 (A − B).

### 3.4. Acute oral toxicity study

In acute oral toxicity studies, the SOBF did not show any sign and symptoms of toxicity and mortality up to 2 000 mg/kg dose, considered relatively safe.

### 3.5. In vivo hepatoprotective evaluation

The effect of SOBF on serum marker enzymes during $CCl_4$-induced hepatotoxicity is shown in Table 3. The elevated levels of AST, ALT, ALP and LDH due to $CCl_4$ intoxication were significantly ($P < 0.001$) prevented with SOBF treatment when compared with $CCl_4$ control rats. Maximum activity was found with higher dose. Silymarin also showed significant protective effect against $CCl_4$ induced alterations.

The elevated lipid peroxidation (measured in terms of MDA content) and reduced level of enzymatic and non-enzymatic antioxidants (SOD, CAT, and GSH, respectively) were observed in $CCl_4$ control rats (Table 4), whereas the SOBF treated groups showed marked ($P < 0.001$) rise in antioxidant

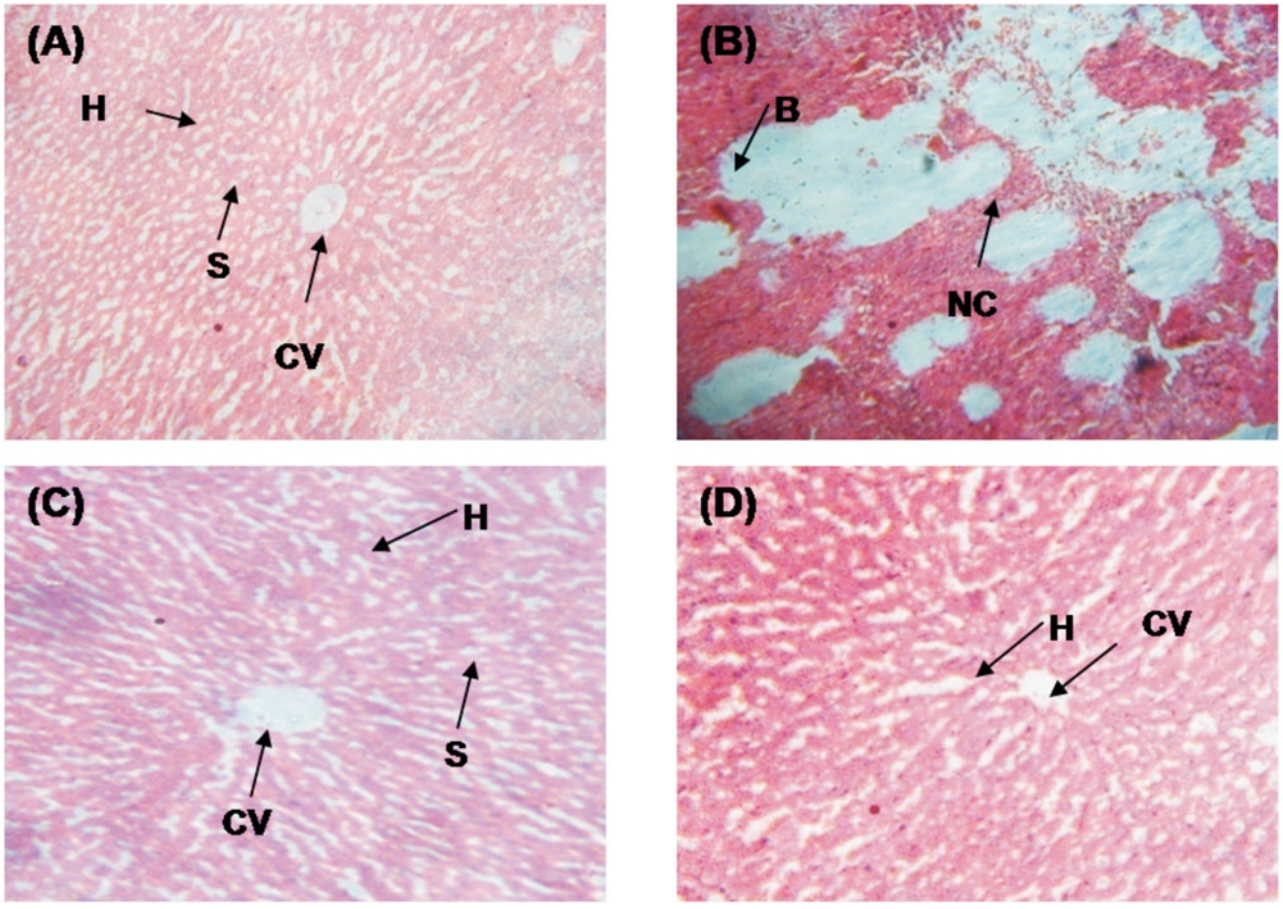


**Figure 2.** Histological changes in liver tissues by $CCl_4$ intoxication and prevention by *n*-butanol fraction of *Spinacia oleracea*.
A:Normal control, B: $CCl_4$ control, C: silymarin (50 mg/kg)+ $CCl_4$, D: SOBF (200 mg/kg)+ $CCl_4$.
H: hepatocytes, CV: central vein, S: sinusoids, NC: necrosis. B: balloning.

**Table 3.**
Effect of *n*-butanol fraction of *Spinacia oleracea* (L.) seeds and silymarin on AST, ALT, ALP and LDH in $CCl_4$- induced hepatotoxicity in rats.

| Biochemical parameters | Group I (Normal) | Group II[CCl4: olive oil (1:1, 2 mL/kg)] | Group III [SOBF (200 mg/kg)] | Group IV [Silymarin (50 mg/kg) + $CCl_4$] | Group V[SOBF (50 mg/kg) + $CCl_4$] | Group VI[SOBF (100mg/kg)+ $CCl_4$] | Group VII[SOBF (200 mg/kg) + $CCl_4$] |
|---|---|---|---|---|---|---|---|
| AST (IU/L) | 51.18 ± 5.47 | 185.38 ± 4.23$^{\#}$ | 52.09 ± 1.79 | 89.13 ± 8.11$^{*}$ | 131.47 ± 5.95$^{**}$ | 113.39 ± 3.27$^{*}$ | 96.89 ± 6.53$^{*}$ |
| ALT (IU/L) | 26.65 ± 2.23 | 158.03 ± 7.09$^{\#}$ | 28.99 ± 1.37 | 58.30 ± 3.20$^{*}$ | 114.95 ± 4.82$^{*}$ | 92.46 ± 5.88$^{*}$ | 62.88 ± 2.26$^{*}$ |
| ALP (IU/L) | 34.45 ± 1.89 | 123.43 ± 2.78$^{\#}$ | 31.58 ± 2.85 | 65.87 ± 1.73$^{*}$ | 104.38 ± 3.24$^{**}$ | 89.38 ± 2.37$^{*}$ | 81.41 ± 3.65$^{*}$ |
| LDH (IU/L) | 109.99 ± 3.77 | 243.10 ± 14.72$^{\#}$ | 105.23 ± 1.37 | 154.80 ± 9.05$_{*}$ | 203.92 ± 3.35$^{***}$ | 164.33 ± 3.38$^{*}$ | 185.99 ± 9.96* |

$^{\#}P < 0.001$ when compared with the normal control group (vehicle only). $^{*}P < 0.001$, $^{**}P < 0.01$, and $^{***}P < 0.05$, respectively when compared with the group treated with $CCl_4$ alone (one-way ANOVA followed by Tukey's multiple comparison test).

**Table 4.**
Effect of *n*-butanol fraction of *Spinacia oleracea* (L.) seeds and silymarin on hepatic MDA, GSH, SOD, and CAT in CCl4- induced hepatotoxicity in rats.

| Biochemical parameters | Group I (Normal) | Group II[CCl4: olive oil (1:1, 2 mL/kg)] | Group III[SOBF (200 mg/kg)] | Group IV [Silymarin (50 mg/kg) + $CCl_4$] | Group V[SOBF (50 mg/kg) + $CCl_4$] | Group VI[SOBF (100mg/kg)+ $CCl_4$] | Group VII [SOBF (200 mg/kg)+ $CCl_4$] |
|---|---|---|---|---|---|---|---|
| MDA(nM/mg protein) | 0.46 ± 0.04 | 1.36 ± 0.04$^{\#}$ | 0.48 ± 0.03 | 0.71 ± 0.03$^{*}$ | 1.09 ± 0.02$^{**}$ | 0.93 ± 0.03$^{*}$ | 0.79 ± 0.05$^{*}$ |
| GSH ($\mu$M/mg protein) | 7.80 ± 0.69 | 3.60 ± 0.38$^{\#}$ | 7.87 ± 0.23 | 6.79 ± 0.32$^{*}$ | 4.82 ± 0.17 | 5.86 ± 0.33$^{**}$ | 6.25 ± 0.25$^{*}$ |
| SOD (U/mg) | 38.90 ± 3.89 | 20.84 ± 1.23$^{\#}$ | 37.94 ± 0.79 | 33.31 ± 1.6$^{*}$ | 25.90 ± 2.28 | 31.62 ± 2.85$^{***}$ | 33.88 ± 1.98$^{**}$ |
| CAT (U/mg) | 55.35 ± 2.94 | 29.48 ± 0.65$^{\#}$ | 57.47 ± 2.56 | 46.08 ± 2.16$^{*}$ | 34.36 ± 1.88 | 39.97 ± 0.86$^{***}$ | 48.36 ± 1.71$^{*}$ |

$^{\#}P < 0.001$ when compared with the normal control group (vehicle only). $^{*}P < 0.001$, $^{**}P < 0.01$, and $^{***}P < 0.05$, respectively when compared with the group treated with $CCl_4$ alone (one-way ANOVA followed by Tukey's multiple comparison test).

levels with significant reduction in lipid peroxidation when compared with $CCl_4$-treated control group.

The hepatoprotective effect of SOBF was also confirmed by histopathological examination of the liver tissues of control and treated rats. The histological changes in liver architecture such as necrosis, ballooning degeneration, and loss of cellular boundaries due to $CCl_4$ intoxication were substantially prevented with SOBF and silymarin treatment Figure 2 (A-D).

## 4. Discussion

$CCl_4$-induced hepatic injury is frequently used as an *in vitro* and *in vivo* experimental model to evaluate the hepatoprotective activity of new drugs including plant extracts[14,15]. The present study represents the first attempt to assess the hepatoprotective effect of *Spinacia oleracea* seeds on $CCl_4$-induced hepatic toxicity and oxidative stress, *in vitro* and *in vivo*.

It is well established that $CCl_4$- is metabolized in the liver by cytochrome P450 system, especially by CYP-2E1. This metabolism produces highly reactive trichloromethyl radicals which convert into the trichloromethyl peroxy radicals. These free radicals bind covalently with cellular macromolecules, and leads to elevation of lipid peroxidation, disruption of $Ca^{2+}$ homeostasis and finally cell apoptosis and necrosis[16,17].

In the assessment of liver injury by $CCl_4$-, serum levels of a number of hepatic lysosomal enzymes are used as diagnostic indicators[8]. In our study, significantly increase in AST, ALT, ALP and LDH was observed upon $CCl_4$ intoxication, which indicated the increased permeability of hepatocytes and cellular leakage. However, pretreatment with SOBF significantly diminished the levels of serum enzymes indicating that SOBF could maintain the functional integrity of the hepatocyte membrane, thus protecting the hepatocytes against $CCl_4$-induced toxicity. In addition, the histopathological findings in rat livers are in agreement with biochemical results, which is supportive evidence for hepatoprotective activity.

It is well documented that lipid peroxidation is the principal cause of $CCl_4$ mediated hepatotoxicity[18]. MDA is one of the products of lipid peroxidation; its elevated level could reflect the extent of lipid peroxidative damage in hepatic cells. In the present study, elevated MDA level by $CCl_4$, was significantly suppressed with the treatment of SOBF indicating its antioxidant action.

Reduced GSH is one of the most abundant non-enzymatic biological antioxidant present in the liver. Together with SOD, and CAT, it efficiently scavenges reactive toxic metabolites of $CCl_4$[19]. In our study, a significant decline in GSH and SOD, CAT and GPx antioxidants was observed upon $CCl_4$ intoxication, which may be attributed to excessive free radicals by $CCl_4$. However, the SOBF could significantly increase the levels of antioxidants supporting its antioxidant effect against $CCl_4$ induced oxidative stress. These findings point out the involvement of potent antioxidant activity in the offered hepatoprotection.

Chemically, the *Spinacia oleracea* seeds have been reported as a good source of phytoecdysteroids *i.e.* 20-hydroxyecdysone and polypodine B. The potent antioxidant and hepatoprotective properties of 20-hydroxyecdysone has earlier been reported[20]. The presence of 20-hydroxyecdysone has been confirmed in the SOBF by chromatographic studies. Moreover, in our preliminary phytochemical studies, *n*-butanol fraction was also found to be positive for phenolic compounds, and flavonoids. Many such phytoconstituents are well known antioxidant and hepatoprotective agents[21,22]. Therefore, we can consider that these identified classes of compounds either as single or in combination might be responsible for the offered hepatoprotection against $CCl_4$ induced toxicity.

In conclusion, the results of present study demonstrate the potent hepatoprotective activity of *Spinacia oleracea* seeds against $CCl_4$ induced toxicity, which is supportive

evidence for its ethno pharmacological use for hepatic ailments. The most active (*in vitro* and *in vivo*) *n*-butanol fraction of *Spinacia oleracea* seeds, was found to be safe in acute toxicity studies up to 2 000 mg/kg, b.w. Hence due to its safety and potent activity, it is worth incorporating this fraction in herbal formulations for treating hepatotoxic conditions. Further studies to identify and characterize the active principle(s) and to elucidate the mechanism are in progress.

## Conflict of interest statement

We declare that we have no conflict of interest.

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## References

[1] Stickel F, Schuppan D. Herbal medicine in the treatment of liver diseases. *Dig Liver Dis* 2007; **39**: 293-304.

[2] Kirtikar KR, Basu, BD. *Indian medicinal plants*. Dehradun: Bishen Singh & Mahendra Paul Singh; 2005, p. 2078-2079.

[3] Chatterjee A. *The treatise of indian medicinal plants*. New Delhi, India: CSIR Publication; 1997, p. 69-70.

[4] Bathori M, Toth I, Szendrei K, Reisch J. Ecdysteroids in *Spinacea oleracea* and *Chenopodium bonus-Henericus*. *Phytochemistry* 1982; **21**: 236-238.

[5] Kang Z, Jiang JH, Wang D, Liu K, Du L. Kunitz-type trypsin inhibitor with high stability from *Spinacia oleracea* L. seeds. *Biochemistry* 2009; **74**: 102-109.

[6] Kokate CK, Purohit AP, Gokhale SB. *Pharmacognosy*. Pune, India: Nirali Prakashan Publication; 2005, p. 109-137.

[7] Reen RK, Karan M, Singh K, Karan V, Johri RK, Singh J. Screening of various *Swertia* species extracts in primary monolayer cultures of rat hepatocytes against carbon tetrachloride- and paracetamol-induced toxicity. *J Ethnopharmacol* 2001; **75**: 239-247.

[8] Raja S, Ahamed KFH, Kumar V, Mukherjee K, Bandyopadhyay A, Mukherjee PK. Antioxidant effect of *Cytisus scoparius* against carbon tetrachloride treated liver injury in rats. *J Ethnopharmacol* 2007; **109**: 41-47.

[9] OECD 423. *OECD Guideline for testing of chemicals acute oral toxicity-Acute Toxic Class Method adopted*. London: OECD publishing; 2001.

[10] Jain NK, Lodhi S, Jain A, Nahata A, Singhai AK. Effects of *Phyllanthus acidus* (L.) Skeels fruit on carbon tetrachloride-induced acute oxidative damage in liver of Wistar rats and mice. *J Chin Integra Med* 2011; **9**: 48-55.

[11] Jain NK, Singhai AK. Protective effects of *Phyllanthus acidus* (L.) Skeels leaf extracts on Acetaminophen and Thioacetamide induced hepatic injuries in Wistar rats. *Asian Pac J Trop Med* 2011; **4:** 470-474.

[12] Shrivastava A, Shivanandappa T. Hepatoprotective effect of the aqueous extract of the roots of *Decalepis hamiltonii* against ethanol-induced oxidative stress in rats. *Hepatol Res* 2006; **35**: 267-275.

[13] Jain NK, Lodhi S, Jain A, Nahata A, Singhai AK. Protective effects of *Phyllanthus acidus* (L.) Skeels extract on Acetaminophen mediated hepatic injury and oxidative stress in Wistar rats. *J Compl Integra Med* 2010; 7. DOI: 10.2202/1553-3840.1439.

[14] Jamshidzadeh A, Fereidooni F, Salehi Z, Niknahad H. Hepatoprotective activity of *Gundelia tourenfortii*. *J Ethnopharmacol* 2005; **101**: 233-237.

[15] Feng Y, Siu K, Ye X, Wang N, Yuen M, Leung C, et al. Hepatoprotective effects of berberine on carbon tetrachloride-induced acute hepatotoxicity in rats. *Chin Med* 2010; **5**: 1-6.

[16] Nada SA, Omara EA, Abdel-Salam OM, Zahran HG. Mushroom insoluble polysaccharides prevent carbon tetrachloride-induced hepatotoxicity in rat. *Food Chem Toxicol* 2010; **48**: 3184-3188.

[17] Ilavenil S, Kaleeswaran B, Ravikumar S. Protective effects of lycorine against carbon tetrachloride induced hepatotoxicity in Swiss albino mice. *Fund Clin Pharmacol* doi: 10.1111/j.1472-8206.2011.00942.x.

[18] Ozturk IC, Ozturk F, Gul M, Ates B, Cetin A. Protective effects of ascorbic acid on hepatotoxicity and oxidative stress caused by carbon tetrachloride in the liver of Wistar rats. *Cell Biochem Funct* 2009; **27**: 309-315.

[19] Xu J, Su Y, Cheng J, Li S, Liu R, Li W, et al. Protective effects of fullerenol on carbon tetrachloride-induced acute hepatotoxicity and nephrotoxicity in rats. *Carbon* 2010; **48**: 1388-1396.

[20] Bathori M, Pongracaz Z. Phytoecdysteroids-From isolation to their effects on Humans. *Current Med Chem* 2005; **12**: 153-172.

[21] Maheshwari DT, Yogendra Kumar MS, Verma SK, Singh VK, Singh SN. Antioxidant and hepatoprotective activities of phenolic rich fraction of Seabuckthorn (*Hippophae rhamnoides* L.) leaves. *Food Chem Toxicol* 2011; **49**: 2422-2428.

[22] Zeashan H, Amresh G, Singh S, Rao CV. Hepatoprotective and antioxidant activity of *Amaranthus spinosus* against $CCl_4$ induced toxicity. *J Ethnopharmacol* 2009; **125**: 364-366.