Amelioration of CCl₄-induced hepatosuppression by *Spinacia oleracea* L. leaves in wistar albino rats

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Summary

The present study deals with the amelioration by *Spinacia oleracea* L. leaves extract against hepatosuppression induced by carbon tetrachloride (CCl₄), which was evaluated in terms of serum-marker enzymes like GGT (gamma glutamyl transferase), AST, ALT (aspartate and alanine transaminases), LDH (lactate dehydrogenase), SDH (sorbitol dehydrogenase), GDH (glutamate dehydrogenase), ALP (alkaline phosphatase) and serum-total bilirubin, total protein levels alongwith concomitant hepatic-antioxidants like SOD (superoxide dismutase), CAT (catalase), GSH (reduced glutathione), GPx (glutathione peroxidase) GR (glutathione reductase), GST (glutathione-s-transferase), ascorbic acid (vitamin-c), β-carotene and cytochrome P-450 enzyme whereas LPO (lipid peroxidation) was monitored in both serum and liver contents. These biochemical parameters were significantly (P< 0.001) altered by the single dose of CCl₄ (1.0 ml/kg b wt, i.p, with olive oil, 1:1). Pretreatment with *S. oleracea* L. prior to the administration of CCl₄, at the doses of 100 and 200 mg/kg.b.wt./day, P.O. for 7 days, significantly restored to all the serum and liver parameters near to the normal levels, respectively. However, silymarin was used as a reference standard, prior to the administration of CCl₄ to rats. These findings indicate the hepatoprotective potential of *S. oleracea* L. against hepatosuppression possibly involve mechanism related to its ability to block the P-450 mediated CCl₄ bioactivation through selective inhibitors of ROS (reactive oxygen species) like antioxidants brought about significant inhibition of TBARS suggesting possible involvement of O₂•−, HO₂•, HO₂, H₂O₂ and •OH. In conclusion, the amelioration may be attributed to the combined synergistic effects of its constituents rather than to any single factor as the leaves are rich in carotenoid contents (β-carotene, lutein, zeaxanthine), ascorbic acid, flavonoids and p-caumaric acid. Thus *S. oleracea* L., showing protection in liver, may prove promising as a rich source of antioxidants because its use is cost effective, especially for peoples in adverse and hazardous circumstances, who are living in poverty.

Key words : Antioxidants, free radicals, marker enzymes, hepatoprotection.

Running title : Amelioration of CCl₄-induced hepatosuppression by *S. oleracea* L.
Introduction

The mammalian liver disease remains a serious health problem because liver has been reported as a highly toxicity sensitive organ. With the extensive use of hepatotoxins like drugs, chemicals and alcohol daily routine life, it has become imperative to safeguard human populations inhabiting poverty against liver cirrhosis. Liver cirrhosis can be life threatening when the entirely or most of the liver is exposed with any hepatotoxin including CCl4, which is required metabolic activation, particularly by the liver cytochrome P-450 enzymes, to form reactive toxic metabolites, which in turn cause liver cirrhosis in experimental animals and humans [1,2].

Carbon tetrachloride is one of the most commonly used hepatosuppressing agent in the experimental study of liver disease because it has been administered to humans in vehicles ranging from shampoo to a drug against hook worm. The hepatotoxic effect of CCl4 are largely due to its active metabolite, trichloromethyl free radical (CCl3- and/or CCl3OO-) by chronic or acute vehicles [3,4].

In a serious liver injury through oxidative stress, currently available drugs have little effect and, to creates a demand to develop new drugs. Herbs have attracted a great deal of interest as physiologically functional foods and as a source for the development of drugs because herbal constituents may have stimulating or regenerating effect on hepatocytes and restored the activities of hepatic system through their anti-hepatotoxic, antioxidant and anti-hyperlipidemic activities [5].

Silybum marianum has been commonly standardized for 70% to 80% silymarin content. A hepatology clinic patient survey found that 31% were using over-the-counter "alternative agents" for the therapy for their liver diseases, the most common being milk thistle (Silymarin) [6]. In 2001, milk thistle ranked # 12 of the 20 top selling herbs in the mainstream U.S. market [7]. Therefore, familiarity with this herb, its constituents, current usage and potential drug interactions is increasingly important for health care providers as growing numbers of those with liver conditions elect to use this supplement [8].

Spinacia oleracea L. (Chenopodiaceae), commonly known as 'Spinach' in English and 'palak' in Hindi, found throughout India, and used in inflammation of liver and in jaundice [9]. The spinach is well reported to be a good source of minerals, vitamin B-complex, ascorbic acid, carotenoids, flavonoids, apocyanin and p-coumaric acid [10]. The leaves are used for bowel and lung inflammation, febrile affliction and cooling [11].

The current study has therefore, been undertaken to determine the possible amelioration by S. oleracea on CCl4– induced hepatosuppression in wistar albino rats.

Materials and Methods

Plant material and extraction

The leaves of Spinacia oleracea L were collected from locally cultivated fields near Jaipur and identified from the Deptt. of Botany, University of Rajasthan, Jaipur and placed in the herbarium for future reference (Voucher No. RUBL-19867). The leaves were shade dried and pulverized. The powder was treated with petroleum ether for defatting as well as to remove chlorophyll. The powder was packed into a soxhlet apparatus and subjected to hot continuous percolation using alcohol (95% v/v) as solvent. The extract was concentrated under vacuum and dried in a vacuum desiccator (yield 7.4% w/w) and then suspended in olive oil and used for the hepatoprotective studies.

Silymarin was obtained from German Remedies Ltd., Mumbai, for using as a standard drug for experimentation.
Experimental protocol

After aclimatisation, the animals were divided into the following groups of 6 rats each:

Group I : Animals served as control and received vehicle only.

Group II : Animals received CCl₄, at the single dose of 1.0 ml/kg.b.wt., i.p., with olive oil, 1 : 1, on 7th day.

Group III : Animals received *S. oleracea* extract (100 mg/kg.b.wt./day, P.O.) for 7 days and CCl₄ as group II.

Group IV : Animals received *S. oleracea* extract (200 mg/kg.b.wt/day, P.O.) for 7 days and CCl₄ as group II.

Group V : Animals received silymarin (100 mg/kg.b.wt./day, P.O.) for 7 days and CCl₄ as group II.

Assessment of liver functions

After 36 hours of CCl₄ administration, rats of each group were anesthesized with ether and blood was collected by cardiac puncture in heparinized vials. Serum – GGT, AST, ALT, LDH, SDH, GDH, ALP, total bilirubin and total protein were determined using diagnostic kits along with serum LPO [12], respectively. GGT (batch no. 34004), AST (batch no. 61105), ALT (batch no. 60805) kits were purchased from Accurex Biomedical Pvt. Ltd., Mumbai, India. LDH (lot no. 6854), SDH (lot no. 6810), GDH (lot no 6988), ALP (lot no. 7093), total bilirubin (lot no. 6801), and total protein (lot no. 6808) kits were purchased from Span Diagnostic Ltd., Surat, India.

After the collection of blood, the liver was immediately excised, washed with cold saline, blotted, weighed and a part of it was minced and homogenized in ice cold 1.15% w/v KCL in a potter Elvehjem Teflon glass homogenizer for 1 min. to make a 10% w/v liver homogenate. Lipid peroxidation (TBARS) [12] was measured in the liver homogenate. The activities of hepatic antioxidants like SOD [13], CAT [14], GSH [15], GPx [16], GR [17], GST [18], ascorbic acid [19] and β-carotene [20] were also determined. A liver microsomal fraction was prepared [21] and the cytochrome P-450 content in this fraction was measured from a reduced carbon monoxide difference spectrum [22], respectively.

Ethical aspects

The study was approved by the ethical committee of the University Department of Zoology, Jaipur, India. Indian National Science Academy, New Delhi (INSA, 2000) guidelines were followed for maintenance and use of the experimental animals.

Statistical process

The results obtained in the present study were expressed as the mean ± SEM for each parameter and statistically processed by applying Student ‘t’ test.

Results

The results of biochemical parameters revealed that the administration of CCl₄ to rats caused significant (P< 0.001) liver damage as evidenced by marker enzymes and antioxidant defense system through liver and serum contents (Table I & II).
Table I depicts that the activities of GGT, AST, ALT, LDH, SDH, ALP, total bilirubin and total protein levels in serum were altered significantly (P < 0.001) upon single dose of CCl₄ administration alone to rats in group II. Pretreatment of *Spinacia oleracea* leaves extract to rats for 7 days prior to the CCl₄ administration, exhibited dose dependent, marked protection against hepatic damage through serum-marker enzyme levels along with total bilirubin and total protein contents in group III & IV. The significant (P ≤ 0.001) protection against CCl₄-induced hepatic aberrations through serum-marker enzymes along with total bilirubin and total protein levels were achieved with the pretreatment of silymarin to rats for 7 days prior to the CCl₄ administration in group V.

Single dose of carbon tetrachloride (CCl₄) to rats caused a significant (P < 0.001) decline in hepatic antioxidants level such as SOD, CAT, GSH, GPx, GR, GST, ascorbic acid and β-carotene with a concurrent decline in hepatic cytochrome P-450 level in comparison to control in group II (Table II). In contrast, pretreatment of *Spinacia oleracea* leaves extract to rats for 7 days prior to the CCl₄ administration, afforded a significant dose dependent elevation in the depleted levels of hepatic antioxidants and cytochrome P-450 enzyme level in group III & IV (Table II).

The remarkable protection in the depleted levels of hepatic antioxidants and cytochrome P-450 enzyme level have been monitored with the pretreatment of silymarin to rats for 7 days prior to the CCl₄ administration in group V (Table II).

Rats treated with a single intraperitoneal dose of CCl₄ to rats, developed a significant (P ≤ 0.001) elevation of lipid peroxidation (LPO) in both serum and liver contents in comparison to control in group II (Fig. 1). In contrast, the pretreatment with *Spinacia oleracea* leaves extract to rats, prior to the CCl₄ administration, showed a significant dose dependent restoring effect on elevated lipid peroxidation in both serum and liver contents in group III & IV (Fig. 1). Inhibition of lipid peroxidation in both serum and liver contents with the pretreatment of silymarin to rats, prior to the administration of CCl₄, was achieved significantly (P ≤ 0.001) in group V against CCl₄-induced elevation of lipid peroxidation in group II (Fig. 1).
Table I: Amelioration by *Spinacia oleracea* L. leaves and silymarin through serum biochemical parameters in the control and experimental rats.

<table>
<thead>
<tr>
<th>Treatment design</th>
<th>GGT (IU/L)</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>LDH (IU/L)</th>
<th>SDH (IU/L)</th>
<th>GDH (IU/L)</th>
<th>ALP (KAU)</th>
<th>Total Bilirubin (mg/100ml)</th>
<th>Total protein (gm/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (vehicle treated)</td>
<td>8.65 ± 0.97</td>
<td>120.08 ± 2.16</td>
<td>102.17 ± 2.09</td>
<td>85.16 ± 1.66</td>
<td>42.34 ± 1.84</td>
<td>7.89 ± 0.03</td>
<td>20.79 ± 1.29</td>
<td>0.72 ± 0.08</td>
<td>6.56 ± 0.31</td>
</tr>
<tr>
<td>CCl₄ (1.0 ml/kg.b.wt./on 7th day, i.p.)</td>
<td>28.72 ± 1.24ᵃ</td>
<td>298.42 ± 4.32ᵃ</td>
<td>245.22 ± 3.73ᵃ</td>
<td>133.77 ± 2.21ᵃ</td>
<td>108.21 ± 2.29ᵃ</td>
<td>15.44 ± 1.22ᵃ</td>
<td>48.52 ± 2.38ᵃ</td>
<td>± 0.12ᵃ</td>
<td>± 0.17ᵃ</td>
</tr>
<tr>
<td>CCl₄ + S. oleracea extract (100 mg/kg.b.wt./day, P.O.)</td>
<td>20.32 ± 1.12ᵃ</td>
<td>221.14 ± 3.44ᵃ</td>
<td>192.33 ± 2.94ᵃ</td>
<td>118.10 ± 1.93ᵃ</td>
<td>85.10 ± 1.92ᵃ</td>
<td>11.87 ± 0.93ᶜ</td>
<td>40.10 ± 1.45ᶜ</td>
<td>± 0.06ᵇ</td>
<td>± 0.22ᶜ</td>
</tr>
<tr>
<td>CCl₄ + S. oleracea extract (200 mg/kg.b.wt./day, P.O.)</td>
<td>13.21 ± 1.03ᵃ</td>
<td>168.10 ± 2.45ᵃ</td>
<td>144.21 ± 2.24ᵃ</td>
<td>105.08 ± 1.31ᵃ</td>
<td>72.08 ± 1.09ᵃ</td>
<td>9.22 ± 0.26ᵃ</td>
<td>32.10 ± 1.29ᵃ</td>
<td>± 0.08ᵃ</td>
<td>± 0.28ᵇ</td>
</tr>
<tr>
<td>CCl₄ + Silymarin (100 mg/kg.b.wt./day, P.O.)</td>
<td>10.87 ± 0.98ᵃ</td>
<td>138.17 ± 2.38ᵃ</td>
<td>118.42 ± 2.14ᵃ</td>
<td>90.15 ± 1.37ᵃ</td>
<td>61.39 ± 1.43ᵃ</td>
<td>8.88 ± 0.38ᵃ</td>
<td>25.30 ± 1.32ᵃ</td>
<td>± 0.04ᵃ</td>
<td>± 0.45ᵇ</td>
</tr>
</tbody>
</table>

Levels of significance:
- a = P < 0.001
- b = P < 0.01
- c = P < 0.05

Data are mean ± SEM (n=6)
Table II: Amelioration by *Spinacia oleracea* L. leaves and silymarin through hepatic antioxidant parameters in the control and experimental rats.

<table>
<thead>
<tr>
<th>Treatment design</th>
<th>SOD (µ mole/mg protein)</th>
<th>CAT (µ mole H₂O₂ consumed/min/mg protein)</th>
<th>GSH (n mole/g tissue)</th>
<th>GR (n mole NADPH consumed/min/mg protein)</th>
<th>GPx (n mole NADPH consumed/min/mg protein)</th>
<th>GST (µ mole CDNB-GSH conjugate formed/min/mg proteins)</th>
<th>Ascorbic acid (mg/g tissue)</th>
<th>β-Carotene (mg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (vehicle treated) (Gp. I)</td>
<td>9.25 ± 0.46</td>
<td>58.21 ± 2.10</td>
<td>3.32 ± 0.18</td>
<td>16.45 ± 0.56</td>
<td>11.18 ± 0.48</td>
<td>6.58 ± 0.33</td>
<td>2.95 ± 0.26</td>
<td>2.24 ± 0.12</td>
</tr>
<tr>
<td>CCl₄ (1.0 ml/kg.b.wt./on 7th day, i.p.) (Gp. II)</td>
<td>4.85 ± 0.12 a</td>
<td>30.14 ± 1.58 a</td>
<td>1.92 ± 0.08 a</td>
<td>8.32 ± 0.16 a</td>
<td>5.76 ± 0.27 a</td>
<td>3.21 ± 0.14 a</td>
<td>1.02 ± 0.16 a</td>
<td>0.93 ± 0.04 a</td>
</tr>
<tr>
<td>CCl₄ + <em>S. oleracea</em> extract (100 mg/kg.b.wt./day, P.O.) (Gp. III)</td>
<td>6.12 ± 0.22 a</td>
<td>37.28 ± 1.76 c</td>
<td>2.28 ± 0.10 c</td>
<td>10.15 ± 0.15 a</td>
<td>7.10 ± 0.19 b</td>
<td>3.86 ± 0.12 b</td>
<td>1.72 ± 0.14 b</td>
<td>1.34 ± 0.10 b</td>
</tr>
<tr>
<td>CCl₄ + <em>S. oleracea</em> extract (200 mg/kg.b.wt./day, P.O.) (Gp. IV)</td>
<td>7.35 ± 0.25 a</td>
<td>46.12 ± 1.68 a</td>
<td>2.82 ± 0.09 a</td>
<td>12.46 ± 0.24 a</td>
<td>9.16 ± 0.20 a</td>
<td>5.05 ± 0.09 a</td>
<td>2.06 ± 0.12 a</td>
<td>1.97 ± 0.13 a</td>
</tr>
<tr>
<td>CCl₄ + Silymarin (100 mg/kg.b.wt./day, P.O.) (Gp. V)</td>
<td>8.82 ± 0.42 a</td>
<td>53.24 ± 1.72 a</td>
<td>3.10 ± 0.07 a</td>
<td>14.29 ± 0.19 a</td>
<td>10.66 ± 0.18 a</td>
<td>5.76 ± 0.08 a</td>
<td>2.82 ± 0.09 a</td>
<td>2.06 ± 0.11 a</td>
</tr>
</tbody>
</table>

Levels of significance:

- a = P < 0.001
- b = P < 0.01
- c = P < 0.05

Data are mean ± SEM (n=6)

- Gp. II compared with control (Gp. I).
- Gp. III, IV and V compared with Gp. II.
Discussion

Hepatic cells appear to participate in a variety of enzymic metabolic activities. In case of CCl₄ intake by experimental animals or human beings, the toxic species (CCl₃⁻ or CCl₃OO⁻) are altered the hepatic metabolism through hepatic damage by oxidative stress, which are similar to that of acute viral hepatitis [23].

It is postulated that administration of CCl₄ could cause cell lysis, resulting in the release of cytoplasmic enzymes of the liver into the blood circulation, leading to their increase in levels in serum and this property is often implicated to assess the extent of CCl₄ induced hepatocellular damage [24,25].

The efficacy of any hepatoprotective drug is dependent on its capacity of either reducing the harmful effect or restoring the normal hepatic physiology through the production of structural integrity of hepatocytic cell membrane or regeneration of damaged liver cells that has been disturbed by a hepatotoxin [4].

The observations of the present study are in accordance with these reports. Pretreatment to rats with S. oleracea leaves extract, dose dependently and silymarin inhibited the increase level of all hepatic marker enzymes in serum, indicating the liver protective activity of S. oleracea leaves. Stabilization of serum-total bilirubin and total protein level by the pre-administration of S. oleracea leaves extract to rats, dose dependently for 7 days prior to the CCl₄ administration is further a clear indication of the improvement of functional status of the hepatic cells [26].

It is now generally accepted that the hepatotoxicity by CCl₄ is the result of reductive dehalogenation, which is catalyzed by P-450 and forms the highly reactive trichloromethyl free radical (CCl₃⁻). This then readily interact with molecular oxygen to form the trichloromethyl peroxo radical (CCl₃OO⁻) [27]. Both radicals are capable of binding to proteins or lipids or of abstracting a hydrogen atom from an unsaturated lipid, which initiate lipid peroxidation and liver damage and by play a significant role in the pathogenesis of diseases [28]. Therefore, the suppression of P-450 can result in a reduction in the level of the reactive metabolites, and correspondingly, less tissue injury. The metabolic activation of CCl₄ is believed to be mediated through P 450 2E1 [29]. These results support this hypothesis, in that there is a good correlation between the lower P 450 2E1 enzyme activities in the S. oleracea leaves extract and silymarin treated hepatic microsomes in vivo and the level of protection against CCl₄-induced hepatic damage in rats.

The lipid peroxidative degradation of biomembranes is one of the principle cause of hepatotoxicity induced by CCl₄ [30]. Because lipid peroxidation is viewed as a complicated biochemical reaction involving free radicals, oxygen, metal ions and a host of other factors in the biological system. Since lipids constitute nearly 60% of the compounds in biomembranes, only major perturbation is bound to affect structure and function of the cell. In recent year, lipids and their derivatives have been recognized as important molecules in signal transduction. Lipid peroxidation is the focus of intense activity in relation to its possible involvement in health and disease [31].

In our present investigation, the measurement of lipid peroxidation in the liver and serum, is a convenient method to monitor oxidative cell damage. Inhibition of elevated LPO has been observed in S. oleracea leaves extract, dose-dependently and silymarin treated groups due to its antioxidant and free radical scavenging activities through reestablishment of biomembranes of the hepatic parenchymal cells.

The antioxidant enzymes have been suggestive to playing an important role in maintaining physiological levels of oxygen and hydrogen peroxide and eliminating peroxides generated from inadvertent exposure to xenobiotics and drugs. Any natural compound with antioxidant properties may help in maintaining health when continuously taken as components of dietary food, spices or
drugs [32]. The increase in the levels of antioxidant profiles i.e. SOD, CAT, GR and GPx by S. oleracea leaves extract dose-dependently and silymarin may be attributed to have biological significance in eliminating reactive free radicals that may affect the normal functioning of cells. GST is a soluble protein located in the cytosol, and plays an important role in the detoxification and excretion of xenobiotics [33]. Moreover, the GST functionally binds GSH and the endogenous or exogenous substances. Since it increase the solubility of hydrophobic substances, it also plays an important role in the storage and excretion of xenobiotics. S. oleracea leaves extract and silymarin that increase the GST activity and metabolize toxic compounds to non-toxic compound, protect the liver.

Our study further revealed that the exposure with CCl₄ significantly, decreased the activities of non-enzymic antioxidants namely-GSH, β-carotene and vitamin C in liver might be responsible for hepatocellular injury. The pretreatment of S. oleracea leaves extract dose-dependently and silymarin for 7 days prior to the CCl₄ administration were enhanced significantly non-enzymic antioxidants. GSH and vitamin C exist in their inter convertible forms and participate in the detoxification of the toxic reactive oxygen species. Regeneration to their reduced forms is brought about by reduced glutathione because the detoxification pathway involves GSH conjugation of the trichloromethyl radical, a P 450 2E1-mediated CCl₄ metabolite. Previous studies on the mechanism of CCl₄-induced hepatotoxicity have shown that GSH plays a key role in detoxifying the reactive toxic metabolites of CCl₄ and that liver necrosis begins when the GSH stores are markedly depleted [34]. GSH is largely mediated through the activity of GST and forms adducts with the toxic metabolites of CCl₄. Moreover, GSH contribute to the detoxification of CCl₄ and it has been suggested that one of the principle cause of CCl₄-induced liver injury is lipid peroxidation caused by its free radical derivatives [34]. Vitamin C is considered to be the most important antioxidant in extracellular fluids [35] and also acts to protect membranes against peroxidation by enhancing the activity of α-tocopherol, the chief lipid soluble and chain breaking antioxidant. β-carotene is a potent free radical quencher, singlet oxygen scavenger and lipid peroxidation [36].

Thus the results of the present investigation are clearly demonstrate that various biochemical changes, produced in the serum and liver of rats by CCl₄ treatment, were significantly restored by the pretreatment of S. oleracea leaves extract, dose dependently and silymarin, prior to the CCl₄ administration. These findings were further confirmed by the inhibitory effect of CCl₄ on cytochrome P-450 level was also compensated by pretreatment of S. oleracea, dose dependently and silymarin through maintenance of its normal level. The role of Spinacia oleracea leaves extract in the protection of CCl₄-induced loss in cytochrome P-450 content may be considered as an indication of improved protein synthesis in the hepatic cells [37].

Mechanism of action by constituents of S. oleracea

The amelioration by S. oleracea against hepatosuppression, appears to be offered by its constituents, including β-carotene, lutein, zeaxanthine, flavonoids, vitamin C, p-coumaric acid and micronutrients. Free radical scavenging compounds such as β-carotene and vitamin C can protect DNA from oxidizing radical reactions. β-Carotene is a potent free radical quencher, singlet oxygen scavenger, and lipid antioxidant [36]. β-Carotene has already been reported to quench not only singlet oxygen, but also to scavenge a variety of free radical species. Zeaxanthine is only as effective as β-carotene in inhibiting autoxidation of lipids in solution, but is about 50% more effective in retarding hydroperoxide formation in phosphatidylcholine liposomes [38]. Lutein is effective at inhibiting autoxidation of cellular lipids [39]. Vitamin C is considered to be the most important antioxidant in extracellular fluids [35] and also acts to protect membranes against peroxidation by enhancing the activity of α-tocopherol, the chief lipid-soluble and chain breaking antioxidant. Flavonoids are typical phenolic compounds and reported that LPO can be inhibited by flavonoids, possibly through their activity as strong O²⁻ scavengers [40] and singlet oxygen quenchers [41]. p-Coumaric acid derivatives are very strong antioxidants and have a ability of scavenge free radicals.
Copper, iron, manganese and zinc contents are present in maximum amount in Spinacia oleracea [42]. Adequate zinc supplementation inhibits LPO and has been described as an antioxidant. Increased availability of Zn, Cu, and Mn results in proper activity of Cu Zn SOD and Mn/Fe SOD.

Therefore, the amelioration by S. oleracea leaves against hepatosuppression may be attributed to the combined synergistic effects of its constituents rather than to any single factor.

**Conclusion**

Based on the present findings, it can be concluded that the probable mechanism by which the S. oleracea leaves exerts its protective action against CCl₄-induced hepatocellular metabolic alterations could be by the stimulation of hepatic regeneration through an improved synthesis of proteins, or due to its ability to block the bioactivation of CCl₄ by inhibiting the P 450 2E1 activity and/or its accelerated detoxification and the potential to minimise the deleterious effects of free radicals including the peroxyl radicals and its antioxidant activity in combination with the inhibition of lipid peroxidation, thereby the S. oleracea leaves can be ranked as hepatoprotective agent by the combined synergistic effect of its constituents and micronutrients rather than to any single factor through free radicals scavenging activity.

Further, fractionation and isolation of its active constituents are in progress in our laboratory and then will be pharmacologically evaluated for hepatoprotection and will be reported else where because Spinacia oleracea leaves are used as a dietary vegetable and are freely available in all over the world, it is worthwhile to conduct detailed studies in order to explore the full potential of this plant in human hepatoprotection, from the point of view of cost and availability for people at all socioeconomic levels.

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