Amelioration of CCl_4-induced hepatosuppression by *Spinacia oleracea* L. leaves in wistar albino rats

R.S. Gupta* and Dharmendra Singh
Reproductive Physiology Section, Centre for Advanced Studies,
Department of Zoology, University of Rajasthan, Jaipur – 302 004, India

Summary

The present study deals with the amelioration by *Spinacia oleracea* L. leaves extract against hepatosuppression induced by carbon tetrachloride (CCl_4), 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 ($P \leq 0.001$) altered by the single dose of CCl_4 (1.0 ml/kg b wt, i.p, with olive oil, 1:1). Pretreatment with *S. oleracea* L. prior to the administration of CCl_4, 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 CCl_4 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 CCl_4 bioactivation through selective inhibitors of ROS (reactive oxygen species) like antioxidants brought about significant inhibition of TBARS suggesting possible involvement of $O_2^{\bullet -}$, $HO_2^{\bullet}$, HO_2^-, H_2O_2 and $^{\bullet}OH$. 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 CCl_4-induced hepatosuppression by *S. oleracea* L.

***Correspondence to:** Dr. R.S. Gupta, Assistant Professor,
TR – 03, Teacher's Hostel, University of Rajasthan, Jaipur – 302 004, INDIA
Email : gupta_rs@hotmail.com; **Fax :** +91-141-2701137; **Phone No.** +91-141-2711228

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 hepatotoxicants like drugs, chemicals and alcohol daily routine life, it has become imparative 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 hepatotoxicant including CCl_4, 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 CCl_4 are largely due to its active metabolite, trichloromethyl free radical (CCl_3^- and/or CCl_3OO^-) 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 provides 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 CCl_4– 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 CCl_4, at the single dose of 1.0 ml/kg.b.wt., i.p., with olive oil, 1 : 1, on 7^{th} day.
Group III	:	Animals received *S. oleracea* extract (100 mg/kg.b.wt./day, P.O.) for 7 days and CCl_4 as group II.
Group IV	:	Animals received *S. oleracea* extract (200 mg/kg.b.wt/day, P.O.) for 7days and CCl_4 as group II.
Group V	:	Animals received silymarin (100 mg/kg.b.wt./day, P.O.) for 7 days and CCl_4 as group II.

Assessment of liver functions

After 36 hours of CCl_4 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 alongwith 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 CCl_4 to rats caused significant ($P \leq 0.001$) 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 ($P \leq 0.001$) upon single dose of CCl_4 administration alone to rats in group II. Pretreatment of *Spinacia oleracea* leaves extract to rats for 7 days prior to the CCl_4 administration, exhibited dose dependent, marked protection against hepatic damage through serum-marker enzyme levels alongwith total bilirubin and total protein contents in group III & IV. The significant ($P \leq 0.001$) protection against CCl_4-induced hepatic aberrations through serum-marker enzymes alongwith total bilirubin and total protein levels were achieved with the pretreatment of silymarin to rats for 7 days prior to the CCl_4 administration in group V.

Single dose of carbon tetrachloride (CCl_4) 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 CCl_4 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 CCl_4 administration in group V (Table II).

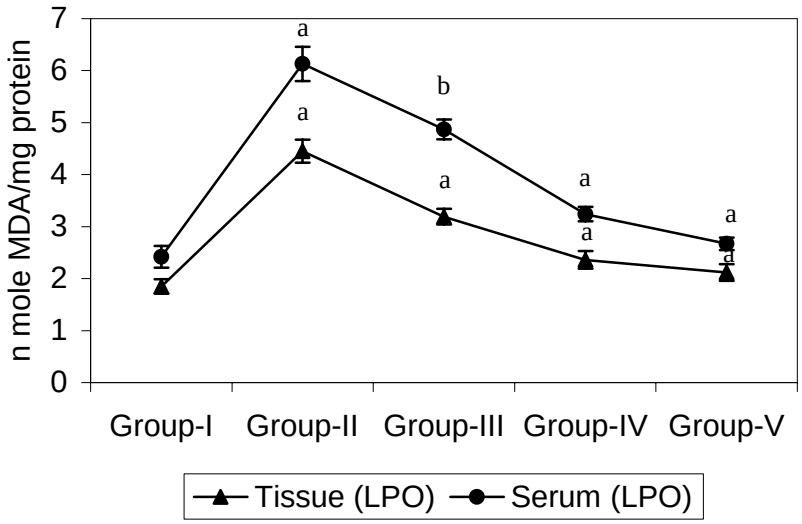

Fig. 1 : Showing amelioration by *S. oleracea* leaves and silymarin in male wistar albino rats through serum and tissue lipid peroxidation. Data points with different letter notations (a,b) are significantly different at a = $p \leq 0.001$; b = $p \leq 0.01$.

Rats treated with a single intraperitoneal dose of CCl_4 to rats, developed a significant ($P \leq 0.001$) 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 CCl_4 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 CCl_4, was achieved significantly ($P \leq 0.001$) in group V against CCl_4-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.

Treatment design	GGT (IU/L)	AST (IU/L)	ALT (IU/L)	LDH (IU/L)	SDH (IU/L)	GDH (IU/L)	ALP (KAU)	Total Bilirubin (mg/100ml)	Total protein (gm/dL)
Normal (vehicle treated) (Gp. I)	8.65 ± 0.97	120.08 ± 2.16	102.17 ± 2.09	85.16 ± 1.66	42.34 ± 1.84	7.89 ± 0.03	20.79 ± 1.29	0.72 ± 0.08	6.56 ± 0.31
CCl_4 (1.0 ml/kg.b.wt./on 7th day, i.p.) (Gp. II)	28.72 ± 1.24[a]	298.42 ± 4.32[a]	245.22 ± 3.73[a]	133.77 ± 2.21[a]	108.21 ± 2.29[a]	15.44 ± 1.22[a]	48.52 ± 2.38[a]	1.68 ± 0.12[a]	3.95 ± 0.17[a]
CCl_4 + *S. oleracea* extract (100 mg/kg.b.wt./day, P.O.) (Gp. III)	20.32 ± 1.12[a]	221.14 ± 3.44[a]	192.33 ± 2.94[a]	118.10 ± 1.93[a]	85.10 ± 1.92[a]	11.87 ± 0.93[c]	40.10 ± 1.45[c]	1.20 ± 0.06[b]	4.58 ± 0.22[c]
CCl_4 + *S. oleracea* extract (200 mg/kg.b.wt./day, P.O.) (Gp. IV)	13.21 ± 1.03[a]	168.10 ± 2.45[a]	144.21 ± 2.24[a]	105.08 ± 1.31[a]	72.08 ± 1.09[a]	9.22 ± 0.26[a]	32.10 ± 1.29[a]	1.02 ± 0.08[a]	5.05 ± 0.28[b]
CCl_4 + Silymarin (100 mg/kg.b.wt./day, P.O.) (Gp. V)	10.87 ± 0.98[a]	138.17 ± 2.38[a]	118.42 ± 2.14[a]	90.15 ± 1.37[a]	61.39 ± 1.43[a]	8.88 ± 0.38[a]	25.30 ± 1.32[a]	0.93 ± 0.04[a]	5.76 ± 0.45[b]

Levels of significance : Data are mean ± SEM (n=6)

a = P≤ 0.001 Gp. II compared with control (Gp. I).

a = P ≤ 0.001 ; b = P ≤ 0.01; c = P≤ 0.05 Gp. III, IV and V compared with Gp. II

Table II : Amelioration by *Spinacia oleracea* L. leaves and silymarin through hepatic antioxidant parameters in the control and experimental rats.

Treatment design	SOD μ mole/mg protein	CAT μ mole H_2O_2 consumed/ min/mg protein	GSH n mole/g tissue	GR n mole NADPH consumed/ min/mg protein	GPx n mole NADPH consumed/ min/mg protein	GST μ mole CDNB-GSH conjugate formed/min/mg proteins	Ascorbic acid mg/g tissue	β-Carotene mg/g tissue
Normal (vehicle treated) (Gp. I)	9.25 ± 0.46	58.21 ± 2.10	3.32 ± 0.18	16.45 ± 0.56	11.18 ± 0.48	6.58 ± 0.33	2.95 ± 0.26	2.24 ± 0.12
CCl_4 (1.0 ml/kg.b.wt./on 7th day, i.p.) (Gp. II)	4.85 ± 0.12[a]	30.14 ± 1.58[a]	1.92 ± 0.08[a]	8.32 ± 0.16[a]	5.76 ± 0.27[a]	3.21 ± 0.14[a]	1.02 ± 0.16[a]	0.93 ± 0.04[a]
CCl_4 + *S. oleracea* extract (100 mg/kg.b.wt./day, P.O.) (Gp. III)	6.12 ± 0.22[a]	37.28 ± 1.76[c]	2.28 ± 0.10[c]	10.15 ± 0.15[a]	7.10 ± 0.19[b]	3.86 ± 0.12[b]	1.72 ± 0.14[b]	1.34 ± 0.10[b]
CCl_4 + *S. oleracea* extract (200 mg/kg.b.wt./day, P.O.) (Gp. IV)	7.35 ± 0.25[a]	46.12 ± 1.68[a]	2.82 ± 0.09[a]	12.46 ± 0.24[a]	9.16 ± 0.20[a]	5.05 ± 0.09[a]	2.06 ± 0.12[a]	1.97 ± 0.13[a]
CCl_4 + Silymarin (100 mg/kg.b.wt./day, P.O.) (Gp. V)	8.82 ± 0.42[a]	53.24 ± 1.72[a]	3.10 ± 0.07[a]	14.29 ± 0.19[a]	10.66 ± 0.18[a]	5.76 ± 0.08[a]	2.82 ± 0.09[a]	2.06 ± 0.11[a]

Levels of significance :
a = P≤ 0.001
a = P ≤ 0.001 ; b = P ≤ 0.01; c = P≤ 0.05

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

Data are mean ± SEM (n=6)

Discussion

Hepatic cells appear to participate in a variety of enzymic metabolic activities. In case of CCl_4 intake by experimental animals or human beings, the toxic species (CCl_3^- or $CCl_3OO^{-)}$ 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 CCl_4 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 CCl_4 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 CCl_4 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 CCl_4 is the result of reductive dehalogenation, which is catalyzed by *P*-450 and forms the highly reactive trichloromethyl free radical (CCl_3^-). This then readily interact with molecular oxygen to form the trichloromethyl peroxy radical (CCl_3OO^-) [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 CCl_4 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 CCl_4-induced hepatic damage in rats.

The lipid peroxidative degradation of biomembranes is one of the principle cause of hepatotoxicity induced by CCl_4 [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 CCl_4 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 CCl_4 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 detoxificaion pathway involves GSH conjugation of the trichloromethyl radical, a P 450 2E1-mediated CCl_4 metabolite. Previous studies on the mechanism of CCl_4-induced hepatotoxicity have shown that GSH plays a key role in detoxifying the reactive toxic metabolites of CCl_4 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 CCl_4. Moreover, GSH contribute to the detoxification of CCl_4 and it has been suggested that one of the principle cause of CCl_4-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 CCl_4 treatment, were significantly restored by the pretreatment of *S. oleracea* leaves extract, dose depently and silymarin, prior to the CCl_4 administration. These findings were further confirmed by the inhibitory effect of CCl_4 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* leeaves extract in the protection of CCl_4-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 autooxidation of lipids in solution, but is about 50% more effective in retarding hydroperoxide formation in phosphatidylcholine liposomes [38]. Lutein is effective at inhibiting autooxidation 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 $O_2^{\bullet -}$ 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 CCl_4-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 CCl_4 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 peroxy 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.

Acknowledgement

The authors are grateful to Head, Department of Zoology and Prof. N.K. Lohiya, Coordinator, CAS, Department of Zoology and Head, Department of Chemistry, University of Rajasthan, Jaipur for providing necessary facilities. U.G.C. (regional office) Bhopal (India) is also duly acknowledged for the financial support.

10

References

1. Gonzalez F J. The molecular biology of cytochrome P 450s. Pharmacological Reviews 1988; 40 : 243-288.

2. Eaton D L, Gallagher E P, Bammler TK, Kunze K L. Role of cytochrome P 450 1A2 in chemical carcinogenesis : Implications for human variability in expression and enzyme activity. Pharmacogenetics 1995; 5 : 259-274.

3. Williams A T, Raymond F B. Carbon tetrachloride hepatotoxicity : An example of free radical-mediated injury. Seminars in Liver Disease 1990; 10 : 279-284.

4. Senthilkumr K T M, Rajkapoor B, Kavimani S. Protective effect of *Enicostemma littorale* against CCl_4-induced hepatic damage in rats. Pharmaceutical Biology 2005; 43 : 485-487.

5. Lee K J, Woo ER, Choi C Y, et al. Protective effect of acteoside on carbon tetrachloride induced hepatotoxicity. Life Sciences 2004 ; 74 : 1051-1054.

6. Flora K, Hahn M, Rosen H, et al. Milk thistle (*Silybum marianum*) for the therapy of liver disease. Am J Gastroenterol 1998; 93 : 139-143.

7. Blumenthal M. Herb sales down in mainstream market, up in natural food stores. Herbalgram 2002; 55 : 60-62.

8. Karen M P H, Kris M D. A review of *Silybum marianum* (Milk Thistle) as a treatment for alcoholic liver disease. J Clin Gastroenterol 2005; 39 : 520-528.

9. Kirtikar K R, Basu B D. Indian Medicinal Plants. Blatter E, Caius J F, Mhaskar K S, eds. IInd eds., Vol. 9, Oriental enterprises, Dehradun, India, 2001 : 2864-2865.

10. Bergan M, Varshavsky L, Gottlieb H E, Grossman S. The antioxidant activity of aqueous spinach extract: Chemical identification of active fractions. Phytochemistry 2001; 58 : 143-152.

11. Jain S K, De Fillipps R A. Medicinal Plants of India. Reference Publication, Algoriac, MI, 1991.

12. Ohkawa H, Ohishi N, yagi K. Assay for lipid peroxidation in animal tissue by thiobarbituric acid reaction. Anal biochem 1979; 95: 351-358.

13. Marklund S, Marklund G. Involvement of superoxide anion radical in auto-oxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974 ; 47 : 469-474.

14. Aebi H. Catalase in vitro. In: Colowick, SP, Kaplan, NO, editors. Methods in enzymology. Vol. 105. Academic Press : New York, 1984 : 121-126.

15. Moron MA, Dipierre JW, Mannervick B. Levels of glutathion, glutathione reductase and glutathione-s-transferase activity in rat lung and liver. Biochem Biophys Acta 1979 ; 582 : 67-78.

16. Paglia DE, Velentine WM. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967; 70 : 158-169.

17. Carlberg I, Mennervick B. Glutathione reductase. In: Methods in Enzymalogy. Vol. 113. Academic Press : New York, 1985 : 484-490.

18. Habig WH, Pabst MJ, Jacoby, WB. Glutathione-S-transferase - the first step in mercapturic acid formation. J Biol Chem 1974 ; 249 : 7130-7139.

19. Omaye ST, Turnbull JD, Sauberlich HE. Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. Meth Enzymol 1971 ; 62 : 1-6.

20. Bradley DW, Hombeck CL. Clinical evaluation of an improved TFA micro method for plasma and serum vitamin A. Biochem Med 1973 ; 7 : 78-83.

21. Schneider WC, Hogeboom GH. Intracellular distribution of enzymes. V. Further studies on the distribution of cytochrome *c* in rat liver homogenates. J Biol Chem 1950; 183: 123-128.

22. Omura T, Sato R. The carbon monoxide binding pigment of liver microsomes. J Biol Chem 1964; 239: 2370-2378.

23. Bishayee A, Sarkar A, Chatterjee M. Hepatoprotective activity of carrot (*Daucus carota* L.) against carbon tetrachloride intoxication in mouse liver. J Ethnopharmacol 1995; 47: 69-74.

24. Drotman R B, Lowhorn G T. Serum enzymes as indicators of chemical induced liver damage. Drug Chem Toxicol 1978; 1 : 163-167.

25. Pradeep K, Rajmohan C V, Anand K G, Karthikayan S. Effect of pretreatment of *Cassia fistula* Linn. leaf extract against subacute CCl_4 induced hepatotoxicity in rats. Indian J Exp Biol 2005; 43 : 526-530.

26. Achliya GS, wadodkar SG, Dorle AK. Evaluation of hepatoprotective effect of Amalkadi Ghrita against carbon tetrachloride induced hepatic damage in rats. J Ethnopharmacol 2004; 90: 229-232.

27. Brent JA, Rumack BH. Role of free radicals in toxic hepatic injury II. Clinical Toxicol 1993; 31 : 173-196.

28. Williams AT, Burk RF. Carbon tetrachloride hepatotoxicity : An example of free radical mediated injury. Seminars in Liver Disease 1990; 10 : 279-284.

29. Zangar RC, Benson JM, Burnett VL, Springer DL. Cytochrome P 450 2E1 is the primary enzyme responsible for low dose carbon tetrachloride metabolism in human liver microsomes. Chemico-Biological Interactions 2000; 125 : 233-243.

30. Kaplowitz N, Aw TY, Simon FR, Stolz A. Drug induced hepatotoxicity. Ann Int Med 1986; 104: 826-839.

31. Floyd RA. Role of oxygen free radicals in ischemia and hart diseases. FASEB J 1990; 4 : 2587-2592.

32. Singh RP, Padmanathi B, Rao AR. Modulatory influence of *Adhatoda vesica* (*Justicia adhatoda*) leaf extract on the enzymes of xenobiotic metabolism antioxidant status and lipid peroxidation in mice. Mol Cell Biochem 2000 ; 213 : 99-109.

33. Boyer TD, Vessey DA, Holcomb C, Saley N. Studies of the relationship between the catalytic activity and binding of non-substrate ligands by the glutathione-s-transferases. Biochemical J 1984 ; 217 : 179-185.

34. Recknagel RO, Glende Jr EA, Britton RS. Free radical damage and lipid peroxidation. In : Meeks RG, editor. Hepatotoxicity. CRC Press, Florida, 1991: 401-436.

35. Stocker R, Frei B. Endogenous antioxidant defense in human blood plasma. In: Sies H, edr. Oxidative Stress: Oxidants and Antioxidants. Academic Press, London, 1991; 213-243.

36. Blot W J, Li J Y, Taylor P R, et al. The initial trials: Mortality rates by vitamin-mineral intervention group. Am J Clin Nutri 1995; 62: 45-65.

37. Mandal PK, Bishayee A, Chatterjee M. Stimulation of tissue repair by *Mikania cordata* root extract in carbon tetrachloride-induced liver injury in mice. Phytother Res 1993; 7: 103-105.

38. Lim B P, Nagaro A, Terao J, et al. Antioxidant activity of xanthophylls in peroxyl radical – mediated phospholipid peroxidation. Biochem Biophys Acta 1992; 1126: 178-184.

39. Zhang L-X, Cooney R V, Bertram J S. Carotenoids entrance gap junctional communication and inhibit lipid peroxidation in C3H/10T1/2 Cells: Relationship to their cancer chemopreventive action. Carcinogenesis 1991; 2: 2109-2114.

40. Baumann J, Wurm J, Von Bruchhausen F. Prostaglandin synthetase inhibition by flavonoids and phenolic compounds in relation to their O_2^- scavenging properties. Arch Pharm (Weinheim) 1980; 313: 330-337.

41. Sorata Y, Takahama U, Kimura M. Protective effect of quercetin and rutin on Photosensitised lysis of human erythrocytes in the presence of hematoporphyrin. Biochem Biophys Acta 1984; 799: 313-317.

42. Singh G, Kawatra A, Sehgal S. Nutritional composition of selected green leafy vegetables, herbs and carrots. Plant Foods Hum Nutri 2001; 56: 359-364.