Antioxidant and hepatoprotective activities of phenolic rich fraction of Seabuckthorn (*Hippophae rhamnoides* L.) leaves


**Defence Institute of Physiology and Allied Sciences (DIPAS), Lucknow Road, Timarpur, Delhi 110 054, India**

### Abstract

Present study was aimed to investigate antioxidant and hepatoprotective activities of phenolic rich fraction (PRF) of Seabuckthorn leaves on CCl4 induced oxidative stress in Sprague Dawley rats. Total phenolic content was found to be 319.33 mg gallic acid equivalent (GAE)/g PRF and some of its phenolic constituents, such as gallic acid, myricetin, quercetin, kaempferol and isorhamnetin were found to be in the range of 1.935–196.89 mg/g of PRF as determined by reverse-phase high-performance liquid chromatography (RP-HPLC).

Oral administration of PRF at dose of 25–75 mg/kg body weight significantly protected from CCl4 induced elevation in aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GGT) and bilirubin in serum, elevation in hepatic lipid peroxidation, hydroperoxides, protein carbonyls, depletion of hepatic reduced glutathione (GSH) and decrease in the activities of hepatic antioxidant enzymes; superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-S-transferase (GST). The PRF also protected against histopathological changes produced by CCl4 such as hepatocytic necrosis, fatty changes, vacuolation, etc. The data obtained in the present study suggests that PRF has potent antioxidant activity, prevent oxidative damage to major biomolecules and afford significant protection against CCl4 induced oxidative damage in the liver.

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### 1. Introduction

It is commonly recognized that reactive oxygen species (ROS) are involved in a variety of physiological and pathological processes, including cellular signal transduction, cell proliferation, differentiation and apoptosis, as well as ischemia – reperfusion, injuries, inflammation and many neurodegenerative disorders. In healthy individuals, ROS production is continuously balanced by natural antioxidant defense system. Oxidative stress is a process where the physiological balance between pro-oxidants and antioxidants is disrupted in favor of the former, ensuing in potential damage for the organism (Halliwell and Gutteridge, 1990). Carbon tetrachloride (CCl4) is frequently used as a chemical inducer of experimental tissue damages (Gurpreet et al., 2006; Upur et al., 2009; Mohamed et al., 2011). Transient tissue disorders after the administration of CCl4 is believed to be induced by the trichloromethyl radical (‘CCl3’). This free radical induces an adverse reaction by forming other free radicals after its administration in the early stage between intracellular uptake and transformation into storage types. Many biological substances such as membrane lipids, proteins, and nucleic acids are known to be injured by trichloromethyl radicals (Nomura and Yamaoka, 1999; Weber et al., 2003).

Dietary antioxidant intake may be an important strategy for inhibiting or delaying the oxidation of susceptible cellular substrates, and is thus relevant to disease prevention in many paradigms. Phenolic compounds such as flavonoids, phenolic acids, diterpenes and tannins have received attention for their high antioxidant activity (Rice-Evans et al., 1996). Converging evidence from both experimental and epidemiological studies have demonstrated that cereals, vegetables, and fruits contain a myriad of phenolic compounds.

Seabuckthorn (*Hippophae rhamnoides* L., Elaeagnaceae) has emerged as a versatile nutraceutical high altitude plant with diverse uses, from controlling soil erosion to being a source of...
horse fodder, nutritious foods, drugs, and skin-care products. Different parts of this plant are used in traditional medicine for the treatment of diseases, such as flu, cardiovascular diseases, mucosal injuries, skin disorders, hepatotoxicity and hypoglycemia (Beveridge et al., 1999; Upendra et al., 2008; Geetha et al., 2008; Zhang et al., 2010). All parts of this wonder plant are considered to be a good source of a large number of bioactive compounds, including carotenoids, tocopherols, sterols, flavonoids, lipids, vitamins, tannins, minerals, etc. (Upendra et al., 2008) which contribute to its wide usage as a natural antioxidant (Yogendra Kumar et al., 2011). Earlier studies have reported that SBT seed oil contain high amounts of unsaturated fatty acids, α-tocopherol, ω-tocopherol, b-tocotrienol, carotenoids, and flavonoids, which are known to have significant antioxidant, anti-bacterial, anti-atherogenic, cardioprotective and hepatoprotective activity (Negi et al., 2005; Basu et al., 2007; Yu et al., 2009). Furthermore, SBT berries has been reported to be a rich source of vitamins A, C, E, K, flavonoids, carotenoids, organic acids, and oils (Pintea et al., 2001; Kalio et al., 2002). In particular, Seabuckthorn (SBT) leaf extract has also hepatoprotective effects which might be due to its antioxidant activity (Geetha et al., 2008).

High performance liquid chromatography (HPLC) is an indispensable tool for the provisional identification of the main phenolic structures present in foods (Chirinos et al., 2009).

But so far to the best of our knowledge this is the first report on polyphenol content and antioxidant as well as hepatoprotective activities of the Seabuckthorn fraction. Hence, the aim of this study was to evaluate the antioxidant and hepatoprotective properties of phenolic rich fraction of Seabuckthorn leaves against CCl4 induced oxidative damage in rats.

2. Materials and methods

2.1. Chemicals and regents

3,4,5-trihydroxybenzoic acid [Gallic acid], phenazine methosulfate, nicotinamide adenine dinucleotide, nitroblue tetrazolium, myricetin, kaempferol,isorhamnetin (Sigma Aldrich Chemicals, USA). Bovine serum albumin, 1-chloro-2,4- dinitrobenzene (CDNB), 2,4-dinitrophenylhydrazine(2,4-DNPH), dithiobisnitrobenzen (DTNB), glutathione and nicotinamide adenine dinucleotide reduced (NADH) were purchased from Sigma chemical company, USA. Tris(hydroxymethyl)mammonemethane, sodium phosphate dibasic (Na2HPO4), sodium phosphate monobasic (NaH2PO4), dipotassium hydrogen phosphate (K2HPO4), potassium dichromate (K2Cr2O7), potassium ferricyanide (K3Fe(CN)6), sorbitol, trichloroacetic acid, xylenol orange, and sucrose were procured from Merck, India.

2.2. Apparatus


2.3. Plant material

Seabuckthorn (H. rhamnoides) fresh leaves were collected from hilly region of western Himalayas, India in the month of September, in which the plant grows widely under natural condition. Voucher specimen is preserved in Defence Institute for High Altitude Research, Leh after ethanobotanical identification of species.

2.4. Extraction procedure

Hundred grams of Seabuckthorn leaves powder was soaked in 70% ethanol (1:5 v/v) at room temperature (25 ± 1°C). After 24 h, the supernatant was decanted and the residue was re-soaked in respective fresh solvent. The process was repeated three times for complete extraction. Supernatants were pooled, filtered through muslin cloth, and centrifuged at 5000g for 10 min at 4°C. Ethanolic content was evaporated using Buchi Rotavapor R-124 (Buchi Labortechnik AG, Postfach, CH-9230, Flawil, Switzerland) at 4°C. Finally, solution was lyophilized in a Heto Lyophilizer (HTOSICC, Heto-Holten A/S, Denmark) and the dried extracts were stored in airtight dark bottles at 4°C (Nitin et al., 2010).

2.5. Preparation of phenolic rich fraction (PRF)

Five grams of obtained crude extract was dissolved in 100 ml water and sequentially extracted thrice using 100 ml hexane and ethyl acetate. Then solvent in each fractions were removed using rotary evaporator to obtain ethyl acetate fraction as phenolic rich fraction (PRF).

2.6. Determination of total phenol content

Total phenolics content was determined by the Folin–Ciocalteu method, which was adapted from Swain and Hillis (1959). One hundred and fifty microliters of extract/fraction, 2400 μl of nanopure water and 150 μl of 0.25 N Folin–Ciocalteu reagent were combined and then mixed well. The mixture was allowed to react for 3 min then 300 μl of 1 N Na2CO3 solution was added and mixed well. The solution was incubated at room temperature in the dark for 2 h. The absorbance was measured at 725 nm using a spectrophotometer and the results were expressed in milligram of gallic acid equivalents (GAE) per gram of extract/fraction.

2.7. HPLC analysis

The HPLC system consisted of a Waters HPLC system (Waters Corporation, USA) equipped with Waters 515 HPLC pump, Waters 717 plus auto sampler and Waters 2487 UV detector, interfaced with an IBM Pentium 4 personal computer. The separation was performed on a Symmetry C18 250 × 4.6 mm ID; 5 μm column (Waters, USA) by maintaining the gradient flow rate 1.0 ml/min of the mobile phase (Solution A: Water:O-Phosphoric acid 99.7:0.3 and Solution B; Aconitine:Methanol 75:25) and peaks were detected at 370 nm. Identification of compounds was performed on the basis of the retention time, conjugations, and spectral matching with standards. For the preparation of the calibration curve, standard stock solutions of gallic acid, myricetin, quercetin, kaempferol and isorhamnetin (1 mg/2 mL) were prepared in methanol, filtered through 0.22 μm filters (Millipore), and appropriately diluted (0.01–100 μg/mL) to obtain the desired concentrations in the quantification range. The calibration graphs were plotted after linear regression of the peak areas versus concentrations.

2.8. Animal treatment

Male albino Sprague-Dawley rats (n = 30), weighing 190–210 g were housed in cages (46 × 24 × 20 cm) with two animals per cage in a temperature (22 ± 1°C), humidity and light control room (lights on at 06:30 h, lights off at 18:30 h). Animals were provided with standard rat chow diet (Lipton India, Kolkata) and water ad libitum. All procedures and protocols used in the present study were approved by the Animal Care and Use Committee of the institute and followed the guidelines documented in the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. Rats were divided into five groups containing six rats each. PRF was dissolved in sterile saline containing 0.1% Tween-80, three different concentration of PRF was administered orally with the help of gastric cannula and the control group was maintained on saline containing 0.1% Tween-80.

Group 1 was administered saline, served as positive control. Group 2 was administered CCl4 served as negative control. Group 3 was administered 25 mg/kg of PRF orally for 7 days. Group 4 was administered 50 mg/kg of PRF orally for 7 days. Group 5 was administered 75 mg/kg of PRF orally for 7 days.

On the seventh day, the rats of the groups 2-5 were given a single oral dose of CCl4 in olive oil (1:1) at 2.0 kg/kg of body weight 6 h after the last dose of PRF/saline. After 24 h of CCl4 administration, rats were sacrificed. Blood was collected from heart for serum separation. Livers were isolated, portion of liver kept for histopathological evaluation and from remaining portion of liver, post mitochondrial suspension (PMS) was prepared.

2.9. Measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase(GGT) and bilirubin

2.9.1. Aspartate aminotransferase (AST)

The AST activity in serum was determined by the method of Reitman and Frankel (1957). An aliquot of 1 ml of substrate (2 mM α-ketoglutarate and 0.2 M L-aspartate) was incubated with 0.2 ml of serum sample for 1 h at 40°C. Then the reaction was clogged by the addition of 1 ml of dinitrophenyl hydrazine (1 mM). After 20 min, 10 ml of 0.4 N NaOH was added. The absorbance of the solution was measured at 505 nm after 30 min and distilled water served as a blank.

2.9.2. Alanine aminotransferase (ALT)

The ALT activity in serum was determined by the method of Reitman and Frankel (1957). An aliquot of 1 ml of substrate and 0.2 M L-alanine (For AST) was incubated with 0.2 ml of serum sample for 1 h at 40°C. Then the reaction was clogged by the addition of 1 ml of substrate (2 mM α-ketoglutarate and 0.2 M L-aspartate) was incubated with 0.2 ml of serum sample for 1 h at 40°C.
addition of 1 ml of dinitrophenyl hydrazine (1 mM). After 20 min, 10 ml of 0.4 N NaOH was added. The absorbance of the solution was measured at 505 nm after 30 min and distilled water served as a blank.

2.9.3. γ-glutamyl transpeptidase (GGT)

γ-glutamyl transpeptidase (GGT) activity was measured according to the reported method of Orlowsky and Meister (1963).

2.9.4. Bilirubin

Serum bilirubin level was determined by the diazo reagent method (Raghuramu et al., 1983). Briefly, 0.4 ml of serum diluted with 3.6 ml of distilled water in two separate tubes, one treated with 0.5% HCl (Blank) and other with Diazosal reagent (0.5% Sodium nitrite and 0.1% Sulfinic acid) and incubated for 30 min at room temperature. After the incubation read the absorbance of test at 540 nm against blank.

2.10. Measurement of Total Antioxidant Status (TAS) and glutathione (GSH)

2.10.1. Total Antioxidant Status (TAS)

Total Antioxidant Status (TAS) was measured in plasma as an 2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate (ABTS) radical cation decolorizing assay using a Randox kit (Randox Laboratories Ltd., UK).

2.10.2. Glutathione (GSH)

Glutathione content was assayed according to the method of Ellman (1959). An aliquot of 1.0 ml of hepatic PMS was precipitated with 1.0 ml of 10% meta phosphoric acid. The assay mixture contained 0.1 ml of aliquot, 2.7 phosphate buffer (0.1 M, pH 7.4) and 0.2 ml of DTNB (1 mg/ml in phosphate buffer 0.1 M, pH 7.4). The yellow color developed was read at 412 nm.

2.11. Measurement of SOD, GPX, CAT, GR, and GST

2.11.1. Superoxide dismutase (SOD)

SOD activity was measured spectrophotometrically using commercially available kit (Randox Laboratories, Crumlin, UK). One unit activity of SOD was determined by monitoring the 50% inhibition of the rate of reduction of 2-(4-Iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium under the conditions of the assay as mentioned in kits protocol.

2.11.2. Glutathione peroxidase activity (GPX)

GPX activity was measured according to the kit protocol (Randox Laboratories, Crumlin, UK). Enzyme activity was expressed as µmol NADPH oxidized/min/mg protein.

2.11.3. Catalase activity (CAT)

CAT activity was assayed according to the reported method of Aebi (1984). One unit of catalase activity was defined as the amount of enzyme required to decompose 1 µmol of hydrogen peroxide (H₂O₂) per minute. CAT activity was expressed as µmol of hydrogen peroxide reduced/min/mg protein.

2.11.4. Glutathione reductase (GR)

GR activity was measured using the method described by Racker (1955). PMS was incubated with GSSG and NADPH at pH 7.5 and changes in OD were measured at 340 nm for 3 min at an interval of 30 s.

2.11.5. Glutathione-S-transferase (GST)

Glutathione-S-transferase (GST) activity was measured by using the method of Habig and Jakoby (1981) following the formation of the conjugate between GSH and CDNB at 340 nm. The extinction coefficient value of 1 mM⁻¹ cm⁻¹ was used for calculations. The activity was expressed as µmol thioester formed/min/mg protein.

2.12. Protein assay

Protein concentration was measured by the conventional method of Lowry et al., 1951 using Bovine serum albumin was used as the standard.

2.13. Determination of lipid peroxidation (LPO) and protein carbonyls

2.13.1. Lipid peroxidation (LPO)

Lipid peroxidation levels were determined as malondialdehyde–thiobarbituric acid adducts according to the method (Ohkawa et al., 1979). Using a molar extinction coefficient of 1.56 × 10⁵ M⁻¹ cm⁻¹ and expressed as µmol TBARS/mg protein.

2.13.2. Protein carbonyls

The measurement of the rate of protein oxidation as reactive carbonyl derivative was determined using 2,4-dinitrophenhydrazine (DNPH) according to the method described by Reznick and Packer (1994). The absorbance was measured at 366 nm, and the carbonyl content was obtained as µmol carbons per milligram protein using a molar extinction coefficient of 22,000 M⁻¹ cm⁻¹.
2.14. Determination of lipid hydroperoxides by ferrous oxidation in xylene orange reagent assay (FOX-1)

Liver hydroperoxides were estimated according to the method of (Wolff, 2004). This method is extremely sensitive \( [\pm 500 (H_2O_2) = 2.2 \times 10^3 M^{-1} cm^{-1}] \) to measure low levels of hydroperoxide. Briefly, 50 μL of sample was added to 950 μL of ferrous oxidation in xylene orange reagent (FOX) containing 100 μM xylene orange, 250 μM ammonium ferrous sulfate, 100 mM sorbitol and 25 mM H_2SO_4. The reaction mixture was vortexed and incubated for 30 min at 27°C. The color developed was read at 560 nm. Hydroperoxides were expressed as μmol/mg tissue.

2.15. Histopathological studies

Pieces of liver tissues were excised, washed with normal saline and processed separately for histopathological observation. Initially the liver tissues were fixed in 10% buffered neutral formalin, dehydrated in gradual ethanol (50–100%), cleared in xylene, and embedded in paraffin. Sections were prepared and then stained with hematoxylin and eosin (H–E) dye. The sections were examined microscopically for histopathology changes, including cell necrosis, fatty change and vacuolation.

2.16. Statistical analysis

Values are expressed as means ± SEM, \( n = 6 \). Statistical analysis was performed with commercially available software (MINITAB). Data were evaluated for significance by one-way ANOVA. A \( P \) value of less than 0.05 was considered to be significant.

### 3. Results and discussion

#### 3.1. Preparation of phenolic rich fraction

In our previous study, relatively higher phenolic compounds and antioxidant activities were observed from 70% ethanolic extract in comparison with water extract (Nitin et al., 2010). We therefore used 70% ethanol as our extraction solvent and obtained a 23.27% yield of extracted material from SBT leaves. From this crude extract (SLE), phenolic-rich fraction (PRF) was prepared by sequential extraction using hexane and ethyl acetate. Total phenolic compounds of SLE HF, EAF and WF were determined using Folin–Ciocalteau method and expressed as gallic acid equivalent (GAE).

#### Table 1

Quantitative determination of marker compounds PRF by RP-HPLC analysis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gallic acid</th>
<th>Myricetin</th>
<th>Quercetin</th>
<th>Kaempferol</th>
<th>Isorhamnetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic rich fraction (PRF)</td>
<td>196.89 ± 9.30</td>
<td>1.935 ± 0.24</td>
<td>2.923 ± 0.25</td>
<td>2.842 ± 0.406</td>
<td>9.537 ± 0.75</td>
</tr>
</tbody>
</table>

Values expressed as mean ± standard deviation of three determinations.

#### Table 2

Protective effects of phenolic rich fraction (PRF) of Seabuckthorn leaves on Carbon tetrachloride (CCl₄) induced elevation in aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin and γ-glutamyl transpeptidase (GGT) depletion in hepatic reduced glutathione (GSH).

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein (mg/100 g tissue)</th>
<th>AST (U/I)</th>
<th>ALT (U/I)</th>
<th>Bilirubin (mg/dl)</th>
<th>GGT (µmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>78.2 ± 9.5</td>
<td>210.2 ± 10</td>
<td>102.6 ± 4.6</td>
<td>44.02 ± 10.2</td>
<td>1.06 ± 0.04</td>
</tr>
<tr>
<td>CCl₄</td>
<td>32.4 ± 4.6**</td>
<td>359.6 ± 7.7**</td>
<td>221.0 ± 7.8**</td>
<td>102.4 ± 9.4**</td>
<td>0.6 ± 0.06**</td>
</tr>
<tr>
<td>CCl₄ + PRF (25 mg/kg)</td>
<td>66.2 ± 5.5**</td>
<td>224 ± 14**</td>
<td>110.7 ± 7.2</td>
<td>46.2 ± 4.5**</td>
<td>1.0 ± 0.06**</td>
</tr>
<tr>
<td>CCl₄ + PRF (50 mg/kg)</td>
<td>72.6 ± 4.5**</td>
<td>216 ± 10.4**</td>
<td>108.4 ± 6.5**</td>
<td>45.11 ± 9.5**</td>
<td>1.02 ± 0.04**</td>
</tr>
<tr>
<td>CCl₄ + PRF (75 mg/kg)</td>
<td>76.8 ± 5.5**</td>
<td>1900 ± 3.3**</td>
<td>100.8 ± 7.6**</td>
<td>44.04 ± 4.5**</td>
<td>1.05 ± 0.07**</td>
</tr>
</tbody>
</table>

The data were expressed as mean ± SD, \( n = 6 \). Differences were considered to be statistically significant, if \( * P < 0.05, ** P < 0.001 \) compared with control and \( * P < 0.05, ** P < 0.001 \) compared with CCl₄ group.

#### Table 3

Protective effects of phenolic rich fraction (PRF) of Seabuckthorn leaves on Carbon tetrachloride (CCl₄) induced depletion in activities superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione-S-transferase (GST) and Total Antioxidant Status (TAS).

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD (U/mg protein)</th>
<th>CAT (U/mg protein)</th>
<th>GPX (U/mg protein)</th>
<th>GR (µmol/min/mg protein)</th>
<th>GST (µmol/min/mg protein)</th>
<th>GSH (µmol/g)</th>
<th>TAS (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>46.8 ± 3.5</td>
<td>18.1 ± 1.9</td>
<td>75.6 ± 5.2</td>
<td>21.6 ± 4.2</td>
<td>18.9 ± 0.7</td>
<td>35.4 ± 1.4</td>
<td>1.17 ± 0.2</td>
</tr>
<tr>
<td>CCl₄</td>
<td>19.3 ± 2.1**</td>
<td>9.7 ± 1.4**</td>
<td>36.2 ± 6.2**</td>
<td>11.7 ± 6.1*</td>
<td>8.4 ± 0.5**</td>
<td>18.4 ± 0.9**</td>
<td>0.52 ± 0.08**</td>
</tr>
<tr>
<td>CCl₄ + PRF (25 mg/kg)</td>
<td>31 ± 3.2*</td>
<td>13 ± 0.42*</td>
<td>55.8 ± 4.2**</td>
<td>16 ± 3.6</td>
<td>14.2 ± 0.7**</td>
<td>26.0 ± 2.1</td>
<td>0.8 ± 0.24*</td>
</tr>
<tr>
<td>CCl₄ + PRF (50 mg/kg)</td>
<td>40 ± 4.1*</td>
<td>15.9 ± 1.0</td>
<td>68.7 ± 5.4</td>
<td>19 ± 3.2</td>
<td>18 ± 0.4</td>
<td>31.6 ± 1.4</td>
<td>1.0 ± 0.2*</td>
</tr>
<tr>
<td>CCl₄ + PRF (75 mg/kg)</td>
<td>42 ± 3.6**</td>
<td>18.3 ± 0.82*</td>
<td>20.4 ± 4.6</td>
<td>18.2 ± 0.7</td>
<td>33.0 ± 2.1</td>
<td>1.1 ± 0.3*</td>
<td></td>
</tr>
</tbody>
</table>

The data were expressed as mean ± SD, \( n = 6 \). Differences were considered to be statistically significant, if \( * P < 0.05, ** P < 0.001 \) compared with control and \( * P < 0.05, ** P < 0.001 \) compared with CCl₄ group.
isorhamnetin, which further corroborates the observed trends in total phenol content and antioxidant activity.

3.3. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GGT) and bilirubin

In the present study, the effect of PRF of Seabuckthorn leaves was evaluated on rat model of acute oxidative stress: CCl₄ induced hepatotoxicity. CCl₄ afflicts acute oxidative injury to the liver causing oxidative damage and other changes around the central vein in the liver that leads to the leakage of the marker enzymes such as AST, ALT, GGT and bilirubin in serum (Gurpreet et al., 2006; Sreelatha et al., 2009). AST is an enzyme that is present in high quantities in the cytoplasm and mitochondria of liver, also present in the heart, skeletal muscle, kidney and brain. ALT is a hepatospecific enzyme that is principally found in the cytoplasm (Nyblom et al., 2006).

The activities of AST, ALT, GGT and bilirubin levels after 24 h of oral administration of CCl₄ at the dose of 2.0 g/kg of body weight are depicted in Table 2. As can be seen, CCl₄ administration resulted in a significant elevation in the levels of AST (171% in comparison of the saline treated control), ALT (215.3% in comparison of the saline treated control), GGT (332.6% in comparison of the saline treated control) and bilirubin (232.6% in comparison of the saline treated control). Administration of PRF at the dose range of 25–75 mg/kg body weight for 7 days prior to CCl₄ treatment dose dependently restored the activities of AST, ALT and GGT along with bilirubin levels (Table 2). These results indicate PRF of Seabuckthorn leaves possess potent hepatoprotective activity at lower concentration in comparison with the earlier reported hepatoprotective activity of Seabuckthorn leaves crude extract (Geetha et al., 2008).

3.4. Reduced glutathione (GSH) and Total Antioxidant Status (TAS)

The GSH system is a key component of the overall antioxidant defense system that protects the cell from the deleterious effects of reactive oxygen species (Meister and Anderson, 1983). Total Antioxidant Status (TAS) of a cell represents overall antioxidant capacity of the cell, i.e. the capacity to reduce oxidative stress.

CCl₄ treatment alone resulted in depletion of hepatic GSH to 51.8% and plasma TAS to 44.4% in comparison of the saline treated control. However, pretreatment of the rats with PRF at the dose range of 25–75 mg/kg body weight for 7 days prior to CCl₄ treatment dose dependently restored the GSH content (73.4%, 89.2% and 93.2%) as well as plasma TAS to 68.3%, 85.4% and 94% (Table 3).

3.5. Antioxidant enzyme activities

The body has an effective mechanism to prevent and neutralize the free radical induced damage. This is accomplished by a set of endogenous antioxidant enzymes, such as SOD, CAT, GPX, GR and GST. It is known that SOD converts superoxide anion into H₂O₂ and O₂, whereas CAT reduces H₂O₂ to H₂O, resulting in the detoxification of free radicals (Paolletti and Moccali, 1990). GPX reduces reactive peroxides to alcohols and water at the expense of GSH, which is oxidized to GSSG. GSSG is recycled to GSH by GSH reductase (GR). GR is the primary enzyme to maintain glutathione redox status. GST generally is viewed as a phase 2 enzyme, primarily involved in the detoxification of electrophilic compounds. Several recent studies also have demonstrated that GST plays a critical role in protecting cells against oxidant-mediated injury by catalyzing the decomposition of lipid hydroperoxides generated from oxidative damage of cellular lipid molecules (Xie et al., 2001; Yang et al., 2001).

CCl₄ treatment resulted in the depletion of the hepatic antioxidant enzymes (Yu et al., 2009). The activities of SOD, CAT, GPX, GR and GST were depleted to 41.2%, 53.5%, 47.8%, 54.1% and 44%, respectively of the saline treated control. The pretreatment of rats with PRF provided protection against the depletion in activities of these enzymes (Table 3). At the lower dose (25 mg/kg of body weight), the recovery of enzymes activities ranged from 66.2% (for SOD), 71.8% (for CAT), 73.8% (for GPX), 74% (GR) to 75.1% (for GST), while the higher doses of PRF, the recovery of enzymes ranged from 89.7% (SOD), 91.1% (for CAT), 92.5% (for GPX), 94% (GR) to 96.2% (for GST) of the saline treated control value (Table 3). This shows that PRF protects rats against oxidative injury by maintaining the levels of these enzymes even after CCl₄ treatment. This observation well correlated with the earlier reported antioxidant activity of Seabuckthorn leaves crude extract (Geetha et al., 2008) and Seabuckthorn seeds oil (Yu et al., 2009).

3.6. Lipid peroxidation (LPO), protein carbonyls and hydroperoxides

In biological systems, lipid peroxidation generates many aldehydes products, among which malondialdehyde is considered to be the most important derivative. Lipid peroxidation can reduce membrane fluidity, inactivate membrane-bound proteins, and
decompose into cytotoxic aldehydes such as malondialdehyde or hydroxynonenal (Richter, 1987). Water soluble hydroperoxides may react to metals to generate other kind of free radicals and causes membrane damage. The oxidative damage to proteins is reflected by increase in levels of protein carbonyls. Reaction of free radicals with the side chains of lysine, arginine, proline, threonine and glutamic acid residues of proteins leads to the formation of carbonyl derivatives (Stadtman and Berlett, 1997). Furthermore, aldehydes, such as 4-hydroxy-2-nonenal or malondialdehyde produced during lipid peroxidation can be incorporated into proteins by reaction with either the e-amino moiety of lysine or the sulfhydryl group of cysteine residues to form carbonyl derivatives (Uchida and Stadtman, 1993). The inhibition of protein oxidation and LPO is thus a crucial property of antioxidant compounds by virtue of which they can inhibit/impede the induction/progression of a number of diseases implicating oxidative stress.

In the present study, decline in the levels of antioxidant enzymes SOD, CAT, GPX, GST and GR observed in CCl₄ treated rats is a clear manifestation of excessive formation of hepatic LPO, hydroperoxides and protein carbonyls content. Previous studies also reported the decline in the levels of antioxidant enzymes and excessive formation of hepatic LPO in CCl₄ treated rats (Gurpreet et al., 2006; Yang et al., 2008).

Effect of PRF on CCl₄ induced oxidative stress was also assessed. CCl₄ treatment resulted in the elevation of hepatic LPO and Protein carbonyls to 160% and 179.3% in comparison to the saline treated control. Pretreatment of rats with PRF dose dependently reduces the hydroperoxides content (Fig. 2C).

3.7. Histopathological studies

The presence of injuries in livers of CCl₄ treated rats was revealed by histopathological examinations. Fig. 3A shows photomicrographs of hematoxylin–eosin stained liver tissues. In case of control, hepatocytes had normal architecture. Severe hepatocyte necrosis, fatty degeneration, vacuolation were found in rats 24 h after CCl₄ treatment Fig. 3B. Pretreatment of PRF at 25, 50 and 75 mg/kg body weight reduced the severity of CCl₄ induced liver intoxication Fig. 3C–E. These results clearly indicate the protection provided by PRF of Seabuckthorn leaves.

4. Conclusion

The data of the present study suggests that PRF of Seabuckthorn leaves has the potent antioxidant activity against free radicals, prevent oxidative damage to major biomolecules and afford significant protection against CCl₄ induced oxidative stress and liver damage in rats. Five individual phenolic compounds in the PRF sample identified by RP-HPLC may be responsible for antioxidant and hepatoprotective activities. Obtained results show that the PRF can be used as source of natural antioxidants and as a possible food supplement. Further studies in isolation of phenolic compounds and effects of these phenolics on antioxidant status in animal models are needed to evaluate their potential benefits.
Conflict of Interest

The authors declare that there are no conflicts of interest.

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