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

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ABSTRACT

Present study was aimed to investigate antioxidant and hepatoprotective activities of phenolic rich fraction (PRF) of Seabuckthorn leaves on CCl_4 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 CCl_4 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 CCl_4 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 CCl_4 induced oxidative damage in the liver.

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 (CCl_4) 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 CCl_4 is believed to be induced by the trichloromethyl radical ($^{\cdot}CCl_3$). 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 antioxidative 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

Abbreviations: ABTS, 2,2′-Azino-di-[3-ethylbenzthiazoline sulfonate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCl_4, Carbon tetrachloride; CAT, catalase; GGT, γ-glutamyl transpeptidase; GSH, glutathione; GPX, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-*S*-transferase; PRF, phenolic rich fraction; ROS, reactive oxygen species; RP-HPLC, reverse phase high performance liquid chromatography; SOD, superoxide dismutase; TAS, Total Antioxidant Status.

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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, a-tocopherol, c-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; Kallio 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 CCl_4 induced oxidative damage in rats.

2. Materials and methods

2.1. Chemicals and reagents

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), dithibisnitrobenzoic acid (DTNB), glutathione and nicotinamide adenine dinucleotide reduced (NADH) were purchased from Sigma chemical company, USA. Tris(hydroxymethyl)aminomethane, sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), 2-thiobarbituric acid, potassium ferricyanide [$K_3Fe(CN)_6$], sorbitol, trichloroacetic acid, xylenol orange, and sucrose were procured from Merck, India.

2.2. Apparatus

High performance liquid chromatography (HPLC) Waters, Bio-Rad Spectrum 3000 spectrophotometer, Buchi Rotavapor R-124, Allied Frost Lyophilizer FD-5, Afcoset Electronic balance FX-300, REMI Cooling centrifuge CPR-24.

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 of 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 w/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 5000*g* for 10 min at 4 °C. Ethanol 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 (HITOSICC, 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 the 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 Na_2CO_3 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; Acetonitrile:Methanol 75:25) and peaks were detected at 370 nm. Identification of compounds was performed on the basis of the retention time, coinjections, 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-Dawly 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 canula 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 CCl_4, 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 CCl_4 in olive oil (1:1) at 2.0 g/kg of body weight 6 h after the last dose of PRF/saline. After 24 h of CCl_4 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)

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 D, 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)

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