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Antioxidant and Hepatoprotective Activities of the Fractions of *Ficus microcarpa* using *in Vitro* and *ex Vivo* Models

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ABSTRACT

The aim of the present study was to investigate the antioxidant and hepatoprotective activities of the various fractions of the hydromethanolic extract of the leaves of *Ficus microcarpa* L.f (Moraceae). The antioxidant activities of the fractions were evaluated using H-donor activity, reducing power ability, nitric oxide, superoxide anion, hydroxyl radical, hydrogen peroxide scavenging activities, metal chelating ability, β-carotene bleaching and total peroxy radical assays, and total antioxidant activity by thiocyanate method. The pro-oxidant activity was measured using bleomycin-dependent DNA damage. *Ex vivo* models like lipid peroxidation, erythrocyte haemolysis and total antioxidant capacity in human plasma were also studied. Total phenolic and flavonoid content of the fractions were determined as gallic acid and quercetin equivalents, respectively. Increasing concentration of all the fractions showed increased antioxidant potential in a dose-dependent manner and the antioxidant activities were compared with standard drugs such as ascorbic acid, α-tocopherol, curcumin, quercetin and butylated hydroxy toluene (BHT) in different assays and the results were found to be statistically significant ($P<0.001$). None of the fractions exerted an obvious pro-oxidant activity when compared to ascorbic acid. The hepatotoxicity of carbon tetrachloride (CCl_4) on rat liver slice culture *in vitro* was also decreased by the leaf fraction which was evidenced by an increase of rat liver catalase, superoxide dismutase, glutathione peroxidase, reduced glutathione, and significant decrease of LDH and lipid peroxidation end products, and malondialdehyde levels. All the four fractions offered strong antioxidant and hepatoprotective activity justifying the claim of traditional use and serve as a potential source of natural antioxidants.

Keywords: carbon tetrachloride, Chinese banyan, DNA damage, lactate dehydrogenase, liver slice culture, pro-oxidant activity, reactive oxygen species

INTRODUCTION

There is extensive evidence to implicate free radicals in the development of degenerative diseases. The most important free radicals in the body are the derivatives of oxygen, which are known as reactive oxygen species (ROS). Oxygen is also required for the generation of reactive nitrogen species (RNS) and reactive chlorine species (RCS) (Nordberg and Arner 2001; Shao *et al*. 2008). Free radicals react with other compounds and capture electrons to gain stability, which can start a chain reaction initiating lipid peroxidation resulting in destabilization, disintegration and disruption of cell membranes. This results in excessive generation of ROS leading to the progression of a variety of pathophysiological processes such as ischemic-reperfusion injury, diabetes, inflammation, genotoxicity, emphysema, cirrhosis, arteriosclerosis, cancer, etc. (Halliwell and Gutteridge 1999; Gulcin *et al.* 2002). Antioxidants play a pivotal role in preventing or alleviating chronic diseases by reducing the oxidative damage to cellular components caused by ROS. A wide range of antioxidants from both natural and synthetic origin has been proposed for use in the treatment of various human diseases (Cuzzocrea *et al.* 2001). Liver is the major organ involved in the biotransformation of biological toxins and drugs. Hepatitis, cirrhosis and carcinoma are associated with the redox imbalance and oxidative stress. Carbon tetrachloride (CCl_4) is a potent hepatotoxin producing centrilobular hepatic necrosis. It produces a toxic intermediate which covalently binds to trichloromethyl free radical eventually initiating a chain of events ultimately leading to membrane lipid peroxidation and cell necrosis (Brattin *et al.* 1985; Recknagel *et al.* 1989).

Ficus microcarpa L.f (Moraceae), commonly known as Chinese banyan, is a large evergreen tree possessing few aerial roots. It is traditionally used in conditions such as diabetes, ulcers, burning sensations, hemorrhages, leprosy, itching, liver diseases and toothache (Warrier *et al.* 1995). This plant is reported to possess good anti-diabetic activity (Asokkumar *et al.* 2007). Cyclopropyl-triterpenes, ficusone, ficuspirolide and ficusolide were isolated from the heartwood of *F. microcarpa* (Kuo and Li 1999; Chiang *et al*. 2001). Based on its traditional use the present study was undertaken to investigate the antioxidant activity and hepatoprotective activities from the leaves of *F. microcarpa* using *in vitro* and *ex vivo* models.

MATERIALS AND METHODS

Plant materials

F. microcarpa was collected from Coimbatore district, Coimbatore and the plant was identified and authenticated by Dr. G. V. S Murthy, Director, Botanical survey of India, Tamil Nadu Agricultural University, Coimbatore, India and a voucher specimen (Ref No. BSI/SC/5/23/07-08/ Tech 297 dated 6th June 2007) was preserved in our department herbarium for further reference.

Chemicals and reagents

2,2 diphenyl-1-picryl hydrazyl hydrate (DPPH), linoleic acid, ammonium molybdate, β-carotene, curcumin, hydrogen peroxide, nitro blue tetrazolium salt (NBT), α-tocopherol, Tris HCl and HEPES buffer were purchased from HiMedia, Mumbai; 2-deoxy-2-ribose, xanthine oxidase, quercetin, hypoxanthine, Thiobarbitu-

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ric acid (TBA) and gallic acid were obtained from SRL, Mumbai; Folin Denis reagent was procured from SD Fine Ltd., Mumbai; calf thymus DNA from Genei Chemicals, Bangalore; ferrozine, 2'2-azobis (2-amidinopropane) dihydrochloride (AAPH) and Trolox obtained from Sigma Aldrich, USA; 2,7-dichloro flurescein diacetate from Fluka, Germany. 2'2-azobis (2-amidinopropane) was obtained from Acros organics, USA. Bleomycin sulphate was received from Biochem pharmaceutical industries Ltd., Mumbai. Lactate dehydrogenase kit was purchased form Agappe diagnostics, Kerala. All other chemicals used in the study were of analytical grade and was purchased from respective suppliers.

Preparation of the extract and fractionation

The shade-dried leaves were powdered mechanically and sieved through No. 22 mesh sieve and stored in an air-tight container until the time of use. About 750 g of leaf powder was soaked with 3.24 L of methanol: water (7: 3) for 12 h and then macerated at room temperature using a mechanical shaker for 4 h. The extract was filtered and the marc (residue) was again soaked with the same volume of methanol: water for 12 h and then further extracted for 4 h and filtered. The filtrates were then combined, concentrated under reduced pressure and evaporated at 40°C. Around 100 ml of the crude extract was mixed with 200 ml of petroleum ether, chloroform, ethyl acetate, chloroform: methanol (1:1) separately in the order of increasing polarity in a separating funnel and shaken for 30 min. The organic layer was separated and fresh solvent was added until the recovery of a clear organic layer. Each fraction was then concentrated under reduced pressure to obtain the petroleum ether fraction (PEF, 1.6%), ethyl acetate fraction (EAF, 1.18%) chloroform fraction (CF, 1.4%) and chloroform: methanol fraction (CMF, 1.22%), which were then stored at 4°C in amber colored jars until the time of use.

Experimental animals

Wistar albino rats of either sex (150-200 g) were procured from Kerala Agricultural University Veterinary College and Animal Science, Kerala, India and used for the *ex vivo* antioxidant and *in vitro* hepatoprotective studies. Rats were housed in standard polypropylene cages and kept under controlled room temperature (24 ± 2°C, relative humidity 45-55%) in a 12 h light-dark cycle and were given a standard laboratory diet with water *ad libitum*. The experiments were performed in accordance with CPCSEA guidelines.

Phytochemical screening

Phytochemical screening of the various fractions of *F. microcarpa* was performed for the presence of tannins, saponins, flavonoids, terpenoids, alkaloids, glycosides and steroids (Trease and Evans 2002).

1) *In vitro* antioxidant activity

DPPH radical scavenging assay

The hydrogen donating ability of fractions was examined in the presence of DPPH stable radical. One millilitre of 0.3 mM DPPH ethanol solution was added to 2.5 ml of sample solution of different concentrations of various fractions of *F. microcarpa* and allowed to react at room temperature. After 30 min the absorbance values were measured at 517 nm. Ethanol (1.0 ml) plus various fractions of *F. microcarpa* solution (2.5 ml) was used as a blank, DPPH solution (1.0 ml, 0.3 mM) plus ethanol (2.5 ml) served as negative control. The positive controls were those using the standard (ascorbic acid) solutions (Mensor *et al*. 2001).

Nitric oxide radical scavenging assay

Various concentrations of the fractions and sodium nitroprusside (10 mM) in phosphate buffer saline (0.025 M, pH 7.4) in a final volume of 3 ml were incubated at 25°C for 150 min. Control experiments without the test compounds but with an equivalent amount of buffer were prepared in the same manner as done for the test. Thereafter, 0.5 ml of incubation solution was removed and diluted with 0.5 ml Griess' reagent (1% sulphanilamide, 2% *o*-phosphoric acid and 0.1% naphthyethylene diamine dihydrochloride) and allowed to react for 30 min. The absorbance of the chromophore formed during diazotisation of nitrite with sulphanilamide and subsequent coupling with naphthyethylene diamine dihydrochloride was read at 546 nm. The percentage inhibition was calculated. The experiment was done in triplicate using curcumin (25-400 µg/ml) as positive control (Sreejayan and Rao 1997).

Deoxyribose degradation assay

The decomposing effect of fractions of *F. microcarpa* on hydroxyl radicals was determined by the assay of malondialdehyde chromogen formation due to 2-deoxy 2-ribose degradation. The assay mixture contained, in a final volume of 1 ml: 100 µl of 28 mM 2-deoxy 2-ribose dissolved in phosphate buffer, pH 7.4, 500 µl of the plant extract fractions of various concentrations in buffer, 200 µl of 200 mM ferric chloride (1:1 v/v) and 1.04 mM EDTA and 100 µl of 1.0 mM hydrogen peroxide and 100 µl of 1.0 µM ascorbic acid. After incubation of the test sample at 37°C for 1 h the extent of free radical damage imposed on the substrate deoxyribose was measured using thiobarbituric acid (TBA) test. Percentage inhibition of deoxyribose degradation was calculated. Quercetin was used as standard (Gomes *et al.* 2001).

NBT reduction assay

A reaction mixture with a final volume of 3 ml per tube was prepared with 1.4 ml of 50 mM KH_2PO_4-KOH, pH 7.4 containing 1 mM EDTA, 0.5 ml of 100 µm hypoxanthine, 0.5 ml of 100 µM NBT. The reaction was started by adding 0.066 units per tube of xanthine oxidase freshly diluted in 100 µl of phosphate buffer and 0.5 ml of test extract fractions in saline. The xanthine oxidase was added last. The subsequent rate of NBT reduction was determined on the basis of spectrophotometric determinations of absorbance at 560 nm. Ascorbic acid was used as standard (Guzman *et al.* 2001). The results are expressed as the percentage inhibition of NBT reduction with respect to the reaction mixture without test compound (saline only).

Reducing power ability

Reducing power ability was measured by mixing 1.0 ml fractions of various concentrations prepared with distilled water with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide and incubated at 50°C for 30 min. After that 2.5 ml of trichloroacetic acid (10%) were added to the mixture and centrifuged for 10 min at 3000 × *g*, 2.5 ml from the upper part were diluted with 2.5 ml water and shaken with 0.5 ml fresh 0.1%, ferric chloride. The absorbance was measured at 700 nm using a UV spectrophotometer. The reference solution was prepared as above, but contained water instead of the samples. Increased absorbance of the reaction mixture indicates increased reducing power. All experiments were done in triplicate using butylated hydroxyltoluene (BHT) as positive control (Yildirim *et al.* 2001).

Estimation of total phenolic component

Total soluble phenolics present in the fractions were determined with Folin-Denis reagent using gallic acid as a standard (Kaur *et al*. 2009) in a spectrophotometer at 760 nm. The fraction was mixed with 5 ml of Folin–Denis phenol reagent, 10 ml of Na_2CO_3 and diluted by a factor 100 with distilled water. The concentration of total phenolic component in the various fractions were determined as microgram of gallic acid equivalent.

Estimation of total flavonoid content

Total soluble flavonoid content of the different fractions were determined with aluminium nitrate using quercetin as a standard (Hsu 2006). The fractions (1000 µg) was added to 1 ml of 80% ethanol. An aliquot of 0.5 ml was added to test tubes containing 0.1 ml of 10% aluminium nitrate, 0.1 ml of 1 M potassium acetate and 4.3 ml of 80% ethanol. The absorbance of the supernatant was mea-

sured at 415 nm after 40 min at room temperature.

Bleomycin-dependent DNA damage

The reaction mixture contained 0.5 ml calf thymus DNA (10 µg/ml), 50 µg of 1.0 ml bleomycin sulfate, 1.0 ml of 5 mM magnesium chloride, 1.0 ml of 50 µM ferric chloride and 1.0 ml of different concentrations of fractions. The mixture was incubated at 37°C for 1 h. The reaction was terminated by addition of 0.05 ml EDTA (0.1 M). The colour was developed by adding 0.5 ml thiobarbituric acid (TBA) (1% w/v) and 0.5 ml hydrochloric acid (25% v/v) followed by heating at 37°C for 15 min. After centrifugation the extent of DNA damage was measured in a UV spectrophotometer at 532 nm employing ascorbic acid as positive control. Each determination was done in triplicate (Ng *et al.* 2003).

Thiocyanate method

The peroxy radical was determined by thiocyanate method using α-tocopherol as standard (Gutierrez *et al.* 2006) Increasing concentration of the various fractions (5-80 µg/ml) in 0.5 ml of distilled water were mixed with 2.5 ml of linoleic acid emulsion (0.02 M, in 0.04 M pH 7.0 phosphate buffer) and 2 ml phosphate buffer (0.04 M, pH 7) in a test tube and incubated in darkness at 37°C. At intervals during incubation, the amount of peroxide formed was determined by reading absorbance of red color developed at 500 nm by the addition of 0.1 ml of 30% ammonium thiocyanate solution and 0.1 ml of 20 mM ferrous chloride in 3.5% hydrochloric acid to the reaction mixture.

Hydrogen peroxide scavenging activity

Hydrogen peroxide (H_2O_2) solution (2 mmol/L was prepared with standard phosphate buffer (pH 7.4). Fraction samples (25-400 µg/ml) in distilled water were added to hydrogen peroxide solution (0.6 ml). Absorbance of hydrogen peroxide at 230 nm was determined spectrophotometrically after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging of hydrogen peroxide of both plant fractions and standard compound (α-tocopherol) were determined (Gulcin *et al.* 2003).

Metal chelating ability

The ferrous level was monitored by measuring the formation of the ferrous ion-ferrozine complex. The reaction mixture containing different concentrations of fractions (1.0 ml) were added to 2 mM ferrous chloride (0.1 ml) and 5 mM ferrozine (0.2 ml) to initiate the reaction and the mixture was shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. The positive control were those using ascorbic acid and all tests and analysis were run in triplicate. The percentage chelating effect of Ferrozine-Fe^{2+} complex formation was calculated (Huang and Kuo 2000).

β-carotene linoleic acid assay

A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml chloroform and 1.0 ml of this solution was then pipetted into a flask containing 20 mg of linoleic acid and 200 mg of Tween 40 emulsifier. Chloroform was completely evaporated using a vaccum evaporator. Aliquots of 5.0 ml of this emulsion were transferred into a series of tubes containing various concentration of fractions (25-400 µg/ml) or α-tocopherol for comparison. Optical density (OD) at 470 nm was taken for fractions and standard immediately (t=0) and at the intervals of 10, 20, 30 and 90 min (t=10, 20, 30 and 90). The tubes were incubated at 50°C in a water bath during the test. Measurement of OD was continued until the colour of β-carotene disappeared in the control (Jayaprakasha *et al.* 2002).

Total peroxy radical trapping potential (TRAP)

A water soluble azo initiator 2,2′ azo bis (2-amidino propane) dihydrochloride (AAPH) produced the peroxyl radicals while a spectrophotometric analysis of 2,7-dichlorofluresecin-diacetate (DCF-DA) monitored the scavenging activity of the plant extracts. A 350 µl of 1 mM stock of DCF in ethanol was mixed with 1.75 ml of 0.01 N sodium hydroxide and allowed to stand for 20 min before the addition of 17.9 ml of 25 mM sodium phosphate buffer (pH 7.2). The reaction mixture contained 0.5 ml of various concentration of the fractions of *F. microcarpa* in ethanol, 150 µl of activated DCF solution and 25 µl of AAPH (56 mM). The reaction was initiated with the addition of the AAPH. Absorbance was read at 490 nm. Trolox (6-hydroxy 2,5,7-8 tetra methyl chroman 2 carboxylic acid) was used as standard and the determination was done in triplicate (McCune and Johns 2002).

2) *Ex vivo* studies

Assay of lipid peroxidation

Lipid peroxidation induced by Fe^{2+}-ascorbate system in rat liver homogenate was estimated by TBA reaction method (Ajith and Janardhanan 2002). The reaction mixture consisted of rat liver homogenate 0.1 ml (25% w/v) in Tris-HCL buffer (20 mM, pH 7.0), potassium chloride (30 mM), ferrous ammonium sulphate (0.16 mM), ascorbate (0.06 mM), and various concentrations of the fractions in a final volume of 0.5 ml. The reaction mixture was incubated for 1 h at 37°C. After the incubation time, 0.4 ml was removed and treated with 0.2 ml sodium dodecyl sulphate (SDS) (8.1%), 1.5 ml TBA (0.8%), and 1.5 ml glacial acetic acid (20%, pH 3.5). The total volume was made up to 4 ml by distilled water and then kept in a water bath at 95-100°C for 1 h. After cooling, 1.0 ml of distilled water and 0.5 ml of *n*-butanol and pyridine mixture (15:1, v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 g for 10 min. The organic layer was removed and its absorbance at 532 nm was measured. Inhibition of lipid peroxidation was determined by comparing the OD of the treatments with that of control. Ascorbic acid was used as standard.

Assay of erythrocyte hemolysis

The blood was obtained from human volunteer's blood bank in heparinized tubes. Erythrocytes were separated from plasma and the buffy coat was washed three times with 10 volumes of 0.15 M sodium chloride. During the last wash, the erythrocytes were centrifuged at 3000 × *g* for 10 min to obtain a constantly packed cell preparation. Erythrocyte hemolysis was mediated by peroxyl radicals in this assay system. A 0.2 ml of 10% suspension of erythrocytes in phosphate buffered saline pH 7.4 (PBS) was added to the similar volume of 200 mM AAPH solution in PBS containing samples to be tested at different concentrations. The reaction mixture was shaken gently while being incubated at 37°C for 2 h. The reaction mixture was then removed, diluted with eight volumes of the PBS and centrifuged at 2000 × *g* for 10 min. The absorbance of the supernatant was read at 540 nm (A). Similarly, the reaction mixture was treated with 8 volumes of distilled water to achieve complete hemolysis, and the absorbance (B) of the supernatant obtained after centrifugation was measured at 540 nm using L-ascorbic acid as positive control (Ng *et al*. 2000).

Colorimetrtic method for the determination of the total antioxidant capacity of human plasma

To 100 µl of various fractions, 50 µl of the plasma sample diluted in phosphate buffer were added. The reaction was initiated by the addition of 100 µl of pre-warmed (37°C) 2'2-azobis (2-amidinopropane) ABAP (5 mg/ml), and tubes were incubated in a humidified thermostatic oven at 37°C for 60-75 min. Blanks consisting of different fractions, plasma samples, and phosphate buffer (50 and 100 µl, respectively) were run in parallel. The absorbance was measured at 450 nm. A standard curve of the water-soluble synthetic antioxidant Trolox, prepared prior to use, ranging from 0-10 µg/ml was equally assayed under the same condition (Kampa *et al*. 2002).

Calculation of 50% Inhibitory Concentration (IC_{50})

The concentration (mg/ml) of the various fractions required to sca-

venge 50% of the radicals was calculated by using the percentage scavenging activities at different concentrations of the extract. Percentage inhibition (I%) was calculated using the formula

$$I\ \% = \frac{(Ac-As) \times 100}{Ac}$$

where Ac is the absorbance of the control and As is the absorbance of the sample (Senevirathne *et al*. 2006).

3) *In vitro* hepatoprotective activity

Liver slice culture *in vitro*

Wistar albino rats weighing about 150-200 g were dissected after cervical dislocation and liver lobes were removed and transferred to pre-warmed Krebs's Ringer HEPES KRH medium (Wormser *et al.* 1990). Liver was then cut into thin slices using sharp scalpel blades. The liver slices weighing between 4 and 6 mg were used for the experiment. Each experimental system contained 20–22 slices weighing about 100–120 mg. The slices were washed with 10 ml KRH medium, every 10 min for a period of 1 h. They were then pre-incubated for 60 min in small plugged beakers containing 2 ml KRH medium on a shaker water bath at 37°C. The experiment was conducted by dividing into 4 groups as mentioned below:

Group I: control liver slices which were not treated either with $CC1_4$ or drug; Group II: CCl_4 (20 mM) alone; Group III: PEF (petroleum ether fraction), EAF (ethyl acetate fraction), CF (chloroform fraction) and CMF (chloroform methanol fraction) (50 and 100 µg/ml) were added and allowed to react for 2 h and maintained at 37°C with CCl_4; Group IV: silymarin (50 and 100 µg/ml) was added and allowed to react for 2 h and maintained at 37°C with CCl_4.

At the end of incubation, each group of slices was homogenized in an appropriate volume of chilled potassium phosphate buffer (100 mM, pH 7.8) in an ice bath to give a tissue concentration of 100 mg/ml. The homogenates were centrifuged at 10,000 rpm × *g* for 10 min at 4°C and the supernatant were used for the following estimation.

Estimation of lactate dehydrogenase (LDH)

To one ml supernatant, 2 ml of distilled water and 0.2 ml of NAD were added. The reaction mixture was mixed and then incubated at 37°C for 15 min. briefly, after incubation; 1.0 ml of dinitrophenyl hydrazine was added and again allowed to react for 15 min. To this add 10 ml of 0.4 N sodium hydroxide and intensity of colour developed was read at 440 nm using a blank without supernatant. LDH activity was expressed as µmoles of pyruvate liberated/minute (Naik *et al*. 2004).

Estimation of catalase (CAT)

The catalysis of H_2O_2 to H_2O in an incubation mixture adjusted to pH 7.0 was recorded at 254 nm. The reaction mixture contained 2.6 ml of 25 mM potassium phosphate buffer pH 7.0 and 0.1 ml of tissue homogenate and was incubated at 37°C for 15 min and the reaction was started with the addition of 0.1 ml of 10 mM H_2O_2. The time required for the decrease in absorbance from 0.45 to 0.4 representing the linear portion of the curve was used for the calculation of enzymic activity. One unit of catalase activity was defined as the amount of enzymes causing the decomposition of µM H_2O_2/mg protein/min at pH, 7.0 at 25°C (Aebi 1984).

Estimation of glutathione peroxidase (GPx)

The reduction of GSSG is coupled to the oxidation of NADPH through glutathione reductase. The reaction mixture contained 100 µl tissue homogenate solution and 800 µl 100 mM/l potassium phosphate buffer (pH 7.4), containing 1 mM/l EDTA, 1 mM/l sodium azide, 0.2 mM/l NADPH, 1 U/ml glutathione reductase and 1 mM/l GSH. After 5 min the reaction was started by the addition of 100 µl 2.6 mM hydrogen peroxide and the absorbance change at 340 nm in 3 min was recorded at 37°C. Various controls were carried out and suitably subtracted. Glutathione peroxidase activity was expressed as µM NADPH oxidized/min/mg protein at 37°C (Paglia and Valentine 1967).

Estimation of superoxide dismutase (SOD)

SOD activity was determined by the inhibition of autocatalyzed adrenochrome formation in the presence of tissue homogenate at 480 nm. The reaction mixture contained 150 µl of homogenate, 1.8 ml of 30 mM carbonate buffer (pH 10.2), and 0.7 ml of distilled water and 400 µl of epinephrine (45 mM). Auto oxidation of epinephrine to adrenochrome was performed in a control tube without the homogenate. The activity was expressed as units/mg tissue protein (Misra and Fridovich 1972).

Measurement of thiobarbituric acid reactive substances (TBARS)

For TBARS, 0.1 ml of tissue homogenate (Tris-HCl buffer, pH 7.5) was mixed with 2.0 ml of TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA mixed in 1:1:1 ratio). The resultant solution was placed in water bath for 15 min, cooled and centrifuged at 1000 rpm for 10 min. The absorbance of clear supernatant was measured against reference blank at 535 nm. The results were expressed as nM/min/mg tissue protein (Rukkumani *et al*. 2004).

Histopathology evaluation

A portion of liver tissue in each group after respective drug treatment was preserved in 10% formalin, embedded in paraffin, sectioned at 5 µm thickness and stained with hematoxylin-eosin and later the microscopic slides were photographed.

Statistical analysis

Results were expressed as mean ± S.E. of at least three independent experiments (n=3) for *in vitro* assays. Comparison of data between more than two groups were statistically evaluated using SPSS version 10.0 by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, $P<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Phytochemical screening

Preliminary phytochemical screening of the various fractions of *F. microcarpa* revealed the presence of tannins, saponins, flavonoids triterpenoids, alkaloids, glycosides and steroids.

DPPH radical scavenging assay

Various fractions obtained from the hydromethanolic fractions of *F. microcarpa* demonstrated H-donor activity. The highest DPPH radical scavenging activity was detected in chloroform fraction with an IC_{50} of 4.3 µg/ml followed by CMF (13 µg/ml), EA (15 µg/ml) and PEF (16 µg/ml). The DPPH-scavenging effect for various fractions was less than that of standard compound, ascorbic acid. DPPH was reduced with the addition of all fractions in concentration dependent manner and the results were statistically significant at $P<0.001$ (**Table 1A**). DPPH is a stable free radical which produces a violet solution in ethanol with strong absorption band at 517 nm (Sokmen *et al.* 2005). DPPH is widely used to evaluate the free radical scavenging effect of plant extracts. The bleaching of DPPH represents the capacity of the test drugs to scavenge free radicals independently. The mechanism of reduction of DPPH molecule is correlated with the presence of hydroxyl groups. The very good activity of hydromethanolic fractions of *F. microcarpa* is probably due to the presence of substances with available hydroxyl groups. According to the studies carried out by Mensor *et al*. (2001), IC_{50} values of *Vitex polygama* extract was 21.94 µg/ml,

Table 1A Antioxidant activities of the fractions of *F. microcarpa* by using different *in vitro* models.

Fractions	*In vitro* method (IC_{50} µg/ml)							
	DPPH	$O_2^{\cdot-}$	$OH^{\cdot}$	$NO_{\cdot}$	H_2O_2	Fe^{2+}chelating ability	Thiocyanate method	Trap method
PEF	16.0 ± 0.57	161.5 ± 0.99	36 ± 0.52	132.4 ± 1.51	141 ± 0.57	80.83 ± .044	39.3 ± 0.88	191.06 ± 0.52
EAF	15.0 ± 0.58	104 ± 3.0	41 ± 0.33	95.56 ± 1.59	160 ± 0.33	62.1 ± 0.60	45.6 ± 0.33	197.46 ± 3.31
CF	4.3 ± 0.88	151 ± 0.57	21 ± 0.32	83.93 ± 0.38	80.30 ± 0.43	41.6 ± 0.44	30.0 ± 0.57	131.36 ± 0.46
CMF	13.0 ± 0.57	107.7 ± 1.08	24 ± 0.35	123.5 ± 1.22	90.60 ± 0.33	71.03 ± 0.54	31.0 ± 0.42	102.16 ± 0.72
Ascorbic acid	3.2 ± 0.15	91.2 ± 0.56				31.13 ± 0.52		
Quercetin			18.33 ± 0.33					
Curcumin				76.5 ± 1.02				
α-tocopherol					72 ± 0.57		21.1 ± 0.57	
Trolox								91.83 ± 0.78

Values are expressed as the mean ± S.E.M, $n = 3$ with $P<0.001$ compared to positive control group
CF, chloroform fraction; CMF, chloroform-methanol fraction; EAF, ethyl acetate fraction; PEF, petroleum ether fraction

Table 1B Antioxidant activities of the fractions of *F. microcarpa* by using *ex vivo* models.

Fractions	*Ex vivo* method (IC_{50} µg/ml)	
	Lipid per oxidation	Erythrocyte hemolysis
PEF	72.03 ± 0.51	80.16 ± 0.72
EAF	45.06 ± 0.88	72.90 ± 72.09
CF	65.00 ± 0.88	53.16 ± 4.57
CMF	41.60 ± 0.53	62.07 ± 0.35
Ascorbic acid	40.00 ± 0.57	42.00 ± 0.57

Values are expressed as the mean ± S.E.M, $n = 3$ with $P<0.001$ compared to positive control group
CF, chloroform fraction, CMF, chloroform-methanol fraction, EAF, ethyl acetate fraction, PEF, petroleum ether fraction

Anadenanthera peregrina extract was 11.56 µg/ml and *Lantana trifolia* extract was 25.84 µg/ml. Our fractions also showed significant activity in DPPH scavenging when compared with other crude extracts.

Nitric oxide radical scavenging assay

Incubation of solutions of sodium nitroprusside in phosphate buffered saline at 25°C for 150 min resulted in the generation of nitric oxide. All the fractions of *F. microcarpa* effectively reduced the generation of nitric oxide (NO) radicals. The CF exhibited superior NO scavenging activity with an IC_{50} of 83.93 µg/ml (**Table 1A**). The scavenging activity of EAF, CMF and PEF was however lower than the standard curcumin (76.5 µg/ml). NO is also implicated in inflammation, cancer and other pathological conditions and the nitrite scavenging ability of all four fractions further expands the role of this plant as a potent antioxidant (Umamaheswari and Chatterjee 2008). Senevirathna *et al*. (2006) in their antioxidant studies on *Ecklonia cava* reported strong nitric oxide scavenging activities for organic ethyl acetate and aqueous chloroform fractions (IC_{50} values of 330 ± 0.05 µg/ml and 330 ± 0.03 µg/ml, respectively). According to the studies carried out by Ilavarasan *et al*. (2005), IC_{50} values of *Cassia fistula* aqueous extract was 22 µg/ml, and methanolic extract was 47 µg/ml in NO scavenging activity. Our fractions also showed significant activity in NO scavenging when compared with other plant extracts.

Deoxyribose degradation assay

The degradation of deoxyribose by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system was markedly decreased by the all fractions of *F. microcarpa* tested indicating a significant ($P<0.01$) hydroxyl radical scavenging activity. The IC_{50} 50% inhibitory concentrations were higher for CMF (24 µg/ml) than other fractions employed in the study but at a lower rate constant than quercetin (**Table 1A**). Hydroxyl radicals are the most reactive free radical which is produced via the Fenton reaction in living systems (Kunchandy and Rao 1990). The oxygen-derived hydroxyl radicals along with the added transition metal ion (Fe^{2+}) cause the degradation of deoxyribose into malondialdehyde which produces a pink chromogen with thiobarbituric acid (Halliwell *et al.* 1987). The IC_{50} value of methanol extract of *Bauhinia racemosa* in the hydroxyl radical scavenging assay (Kumar *et al*. 2005) was 1789.13 µg/ml and those reported by Xing *et al*. (2005) showed higher IC_{50} values (1250 µg/ml) compared to our fractions.

NBT reduction assay

Superoxide radicals generated *in vitro* by the system was determined by an NBT photo-reduction method. All fractions of *F. microcarpa* effectively scavenged the superoxide anion generated by the system at concentrations of 25-400 µg/ml in a dose dependent manner and the results were found to be significant ($P<0.001$) (**Table 1A**). The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion radical in the reaction mixture and thus of superoxide scavenging activity (Citoglu *et al.* 2004). Further, our values are more significant when compared with the IC_{50} values (109 µg/ml) of Kumar *et al*. (2005) and less significant when compared with values (IC_{50} 66 and 52 µg/ml) obtained from the studies of Rajapakshe *et al*. (2005).

Thiocyanate method

The total antioxidant activity by ammonium thiocyanate method measures the amount of peroxides produced at the initial stage of lipid peroxidation and the absorbance decreased with the increasing concentration of fractions indicating increased level of antioxidant activity by decreased formation of peroxides. The antioxidant activity might be due to hydroperoxide inhibition, inactivation of free radicals or complex formation with metal ions or combinations thereof. The total antioxidant activity of CF and CMF was found to be almost similar (IC_{50} 30 and IC_{50} 31.06 µg/ml, respectively). The other fractions, PEF and EAF, also showed good antioxidant activity (Table 1) which might be attributed to the presence of flavonoid-like phytochemicals (Gutierrez *et al.* 2006). Asokkumar *et al*. (2008) reported that the IC_{50} value (0.176 mg/ml) of the 70% ethanol extract of *Erythrina stricta* was higher compared to our fractions.

Hydrogen peroxide scavenging activity

The removal of H_2O_2 free radicals is very important for antioxidant defense in cell or food systems (Gulcin *et al.* 2004). The various fractions of *F. microcarpa* scavenged hydrogen peroxide in a concentration dependent manner

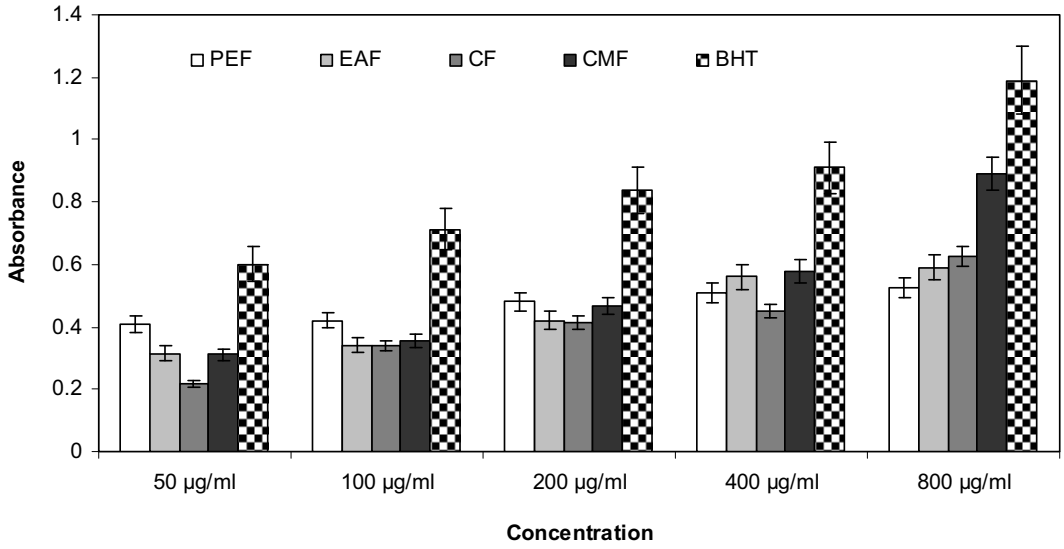

Fig. 1 Reductive ability of the fractions of *F. microcarpa*. CF, chloroform fraction; CMF, chloroform-methanol fraction; EAF, ethyl acetate fraction; PEF, petroleum ether fraction.

which could be seen by its graded increase in percentage of inhibition (**Table 1A**). The IC_{50} values of the crude extract of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour was 34 mg/ml and 16 mg/ml respectively as reported by Quettier-Deleu *et al*. (2000) and the H_2O_2 scavenging activity of these extracts are lower compared to our fractions.

Metal chelating ability

Ferrozine can quantitatively form complexes with Fe^{2+} but in the presence of ion chelating agents; the complex formation is disrupted resulting in a reduction in the red color of the complex (Senevirathne *et al.* 2006). Addition of the fractions of *F. microcarpa* interfere the ferrous-ferrozine complex linearly (from 10 to 160 µg/ml) in a dose-dependent manner. Among the fractions tested, the CF showed more pronounced ferrous chelating ability than other fractions tested (IC_{50} 41.6 µg/ml). Ascorbic acid exhibited the highest ferrous ion chelating ability with IC_{50} 35.13 µg/ml (**Table 1A**). All the fractions captured ferrous ion before ferrozine and thus have ferrous chelating ability. Metal chelating activity of chloroform and ethyl acetate fraction of *F. microcarpa* was higher than that of the chelating activity showed by 70% methanol fraction (IC_{50} 0.436 mg/ml) and *n*-hexane fraction (IC_{50} 0.66 mg/ml) of *Ecklonia cava* (Senivirathne *et al.* 2006).

Total peroxy radical trapping potential (TRAP)

TRAP is a spectrophotometric assay involving DCF-DA. This assay uses AAPH to generate peroxyl radicals and DCF-DA as the oxidizable substrate for the peroxyl radicals. The oxidation of DCF-DA by peroxyl radicals converts DCF-DA to dichlrofluorescein (DCF). Therefore the produced DCF can be monitored spectrophometrically. DCF-DA has been used to determine the antioxidant property of phytoconstituents (Dufour *et al*. 2007). Addition of increasing concentrations of fractions to the solution containing AAPH decreased the luminescence produced by DCF and the absorbance decreased in a linear fashion. CMF exhibited promising activity with an IC_{50} of 102.16 µg/ml when compared to other fractions employed in this study (**Table 1A**).

Reducing power ability

For the measurements of the reductive ability, we investigated the transformation of Fe^{3+} into Fe^{2+} in the presence of the various fractions (Singh and Rajini 2004). **Fig. 1** shows the reductive capabilities of various fractions of *F. microcarpa* when compared to the standard, BHT. Like the antioxidant activity, the reducing power increased significantly ($P<0.01$) with increasing amount of the fractions. CMF of *F. microcarpa* showed the highest reducing power ability than the other fractions tested. However, the activity of the vari-

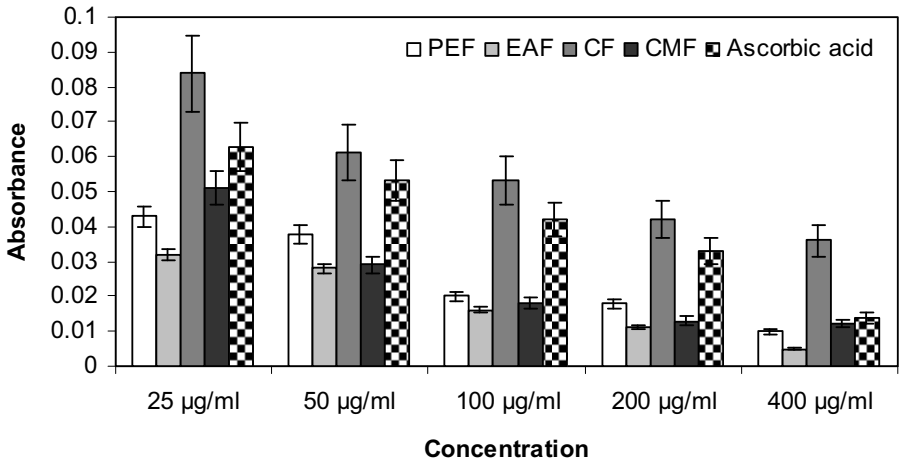

Fig. 2 Antioxidant activity of the fractions of *F. microcarpa* by using bleomycin-dependent DNA damage. CF, chloroform fraction; CMF, chloroform-methanol fraction; EAF, ethyl acetate fraction; PEF, petroleum ether fraction.

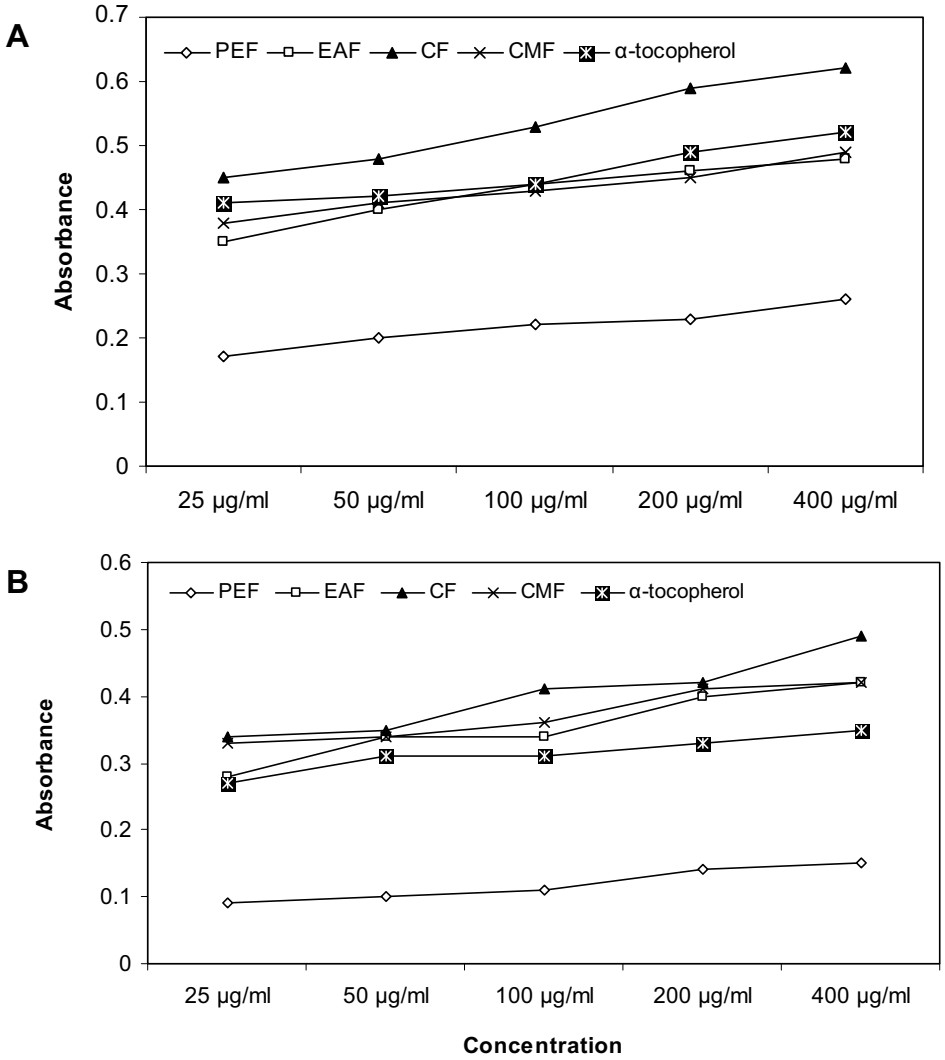

Fig. 3 Antioxidant activity of the fractions of *F. microcarpa* at 0 (**A**) and 90 (**B**) minutes by using β-carotene bleaching assay. CF, chloroform fraction; CMF, chloroform-methanol fraction; EAF, ethyl acetate fraction; PEF, petroleum ether fraction.

ous fractions was less than the standard. The order of reducing ability of the fractions are CMF > PEF > CF > EAF. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Kuda *et al*. (2005) have reported that fucoidan and crude alginate showed reducing abilities (absorbance less than 1.0) at a concentration of 10 mg/ml which were lower than that of our reducing abilities.

Bleomycin-dependant DNA damage

The DNA damage imposed by bleomycin is represented in **Fig. 2**. All the fractions decreased the absorption and bleomycin-Fe^{3+} is not converted into bleomycin-Fe^{2+}, thereby preventing the DNA degradation. Bleomycin binds iron ions and the bleomycin-iron complex will degrade DNA in the presence of O_2 and a reducing agent ascorbic acid. The reaction occurs by attack of ferric-bleomycin peroxide (BLM-Fe (III)-O_2H^-) on the DNA (Ng *et al.* 2000). These results confirm that none of the fractions exhibited pro-oxidant activity. When compared the fractions of the author's crude extract with the isolated compounds from *Aster tataricus*, our fractions were devoid of pro-oxidant activity whereas, quercetin and kaempferol exhibited some pro-oxidant activity as reported by Ng *et al*. (2003).

β-carotene linoleic acid assay

The antioxidant activity of the fractions of *F. microcarpa* and α-tocopherol increased gradually in a concentration dependent manner and this was due to the bleaching of the color of β-carotene. The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated β-carotene molecules. As β-carotene molecules lose their double bonds by oxidation, the compound loses its orange color (Elzaawely *et al.* 2005). Optical density was measured at time intervals of 0, 10, 20, 30 and 90 min and the bleaching action increased with increasing of time (**Fig. 3A, 3B**). The different fractions can hinder the extent of β-carotene-bleaching by neutralizing the free radical formed in the system.

Total phenolic content

Phenolics are ubiquitous secondary metabolites in plants and possess a wide range of therapeutic uses such as antioxidant, antimutagenic, anticarciongenic, free radical sca-

Table 2 Total phenolic and flavonoid contents and total antioxidant capacity of the fractions of *F. microcarpa*.

Fractions	Total phenolic content (µg gallic acid equivalent /mg)	Total flavonoid content (µg quercetin equivalent/mg)	Total antioxidant capacity (µg trolox equivalent/mg)
PEF	5.4	15.09	4.1
EAF	11.9	15.92	2.4
CF	7.4	20.16	3.8
CMF	6.5	16.41	2.3

CF, chloroform fraction, CMF, chloroform-methanol fraction, EAF, ethyl acetate fraction, PEF, petroleum ether fraction

venging activities and also decrease cardiovascular complications (Yen *et al.* 1993; Singh *et al*. 2008; Kundu *et al*. 2009). The total phenolics content in the fractions of PEF, EAF, CF and CMF was calculated from the calibration curve using regression equation and expressed as µg gallic acid equivalents (GAC). The amounts of total phenolic content in various fractions were correlated with gallic acid equivalent antioxidant activity. Among all fractions, significantly higher phenolic content was found in EAF (11.9 µg/GAC/1000 µg). The content of total phenolics in the fractions decreased in the order of PEF > EAF > CF > CMF (**Table 2**). Gulcin *et al*. (2002) reported that the aqueous extract of *Cetraria islandica* contain 0.0387 µg pyrocatechol equivalent of phenols.

Total flavonoid content

Flavonoids are a group of polyphenolic compounds which exhibit several biological effects such as anti-inflammatory, hepatoprotective, anti-ulcer, anti-allergic, anti-viral and anti-cancer effects (Cao *et al.* 1997). Many complex-forming reagents like alluminium nitrate and potassium acetate with alcohol, ionizes generally hydroxy groups of flavones, flavonols, flavanones and chalcones and produce a considerable absorption maximum. This is due to formation of Al^{3+} complexation by interaction of flavonoid with aluminum ions. Since all flavonoids have hydroxy group, it produces complexation with aluminium ions (Litvinenko and Maksyutina 1965). Hence we have used this method for estimation of total flavonoid content in our fractions. The total flavonoid content in the fractions was expressed as µg quercetin equivalent. Highest flavonoid content was detected in CF (20.16 µg/mg) while the other fractions showed lower flavonoid content (**Table 2**). The high flavonoid contents in the fractions may be responsible for its free radical scavenging activity. Hsu (2006) reported that the total flavonoid content of *Polygonum aviculare* L. extract was 112.7 mg/g quercetin equivalent of flavonoids. Comparison with other studies implies that our fractions contain a high quantity of flavonoids.

Ex vivo studies

Lipid peroxidation

Initiation of lipid peroxidation by ferrous sulphate takes place either through the ferryl-perferryl complex or through the $OH^{\cdot}$ radical by Fenton's reaction. Lipid peroxidation is initiated by active oxygen species attacking unsaturated fatty acids, and is propagated by a chain reaction cycle involving lipid peroxy radicals and lipid hydroperoxides. Lipid peroxidation has been identified as one of the basic reactions involved in oxygen free radical induced cellular damage and plays an important role in the pathogenesis of several diseases (Govindarajan *et al.* 2004). All the fractions of *F. microcarpa* were effective in inhibiting the lipid peroxidation induced by Fe^{2+}-ascorbate system in rat liver homogenate (**Table 1B**). The generation of malondialdehyde (MDA) and related substances that react with thiobarbituric acid (TBA) was found to be inhibited by the fractions in a concentration dependant manner with P<0.01. Citoglu *et al*. (2004) examined the lipid peroxidation activities of ethanol extract of *Ballota* species. Their IC_{50} values ranged from 12 to 50 mg/ml whereas all the fraction of *F. microcarpa* exhibited inhibition of lipid peroxidation at much lower concentrations with IC_{50} ranging from 41 to 72 µg/ml. According to the study carried out by Ajith and Janardhan (2002) the IC_{50} value of the ethyl acetate extract of *Plellinus rimosus* was also higher (162 µg/ml) than our plant fractions.

Erythrocyte hemolysis

The azo compound generates free radicals by its unimolecular thermal decomposition. The rate of generation of peroxyl radicals can be easily controlled and measured by adjusting the concentration of AAPH. Therefore, the hemolysis induced by AAPH clearly demonstrates the oxidative erythrocyte membrane damage by peroxyl radical (Govindarajan *et al.* 2004). The peroxyl radical generated by AAPH on addition to erythrocyte suspension and its subsequent scavenging action produced by graded higher concentration of the fraction are represented in **Table 1B**. The results were compared with ascorbic acid and an increase in inhibition was noticed for all the samples tested.

Total antioxidant capacity (TAC) assay

A great variety of methods have been proposed for the assay of total antioxidant capacity of serum or plasma. Antioxidant capacity is the measure of moles of a given free radical scavenged by a test solution, independently of the capacity of any one antioxidant present in the mixture. Therefore, plasma being a heterogenous solution of diverse antioxidants, the antioxidant status is better reflected by antioxidant capacity and expressed as trolox equivalent (Kampa *et al.* 2002). This method is based on the inhibition by antioxidants of the generation of peroxyl radicals by the radical cation ABAP. The relative ability of various fractions of *F. microcarpa* to scavenge free radicals was compared to standard amounts of synthetic antioxidant trolox (Desmarchelier *et al*. 1999). Among the fractions tested, PEF contains 4.1 µg/mg trolox equivalents/100 µg. The antioxidant activity decreased in the order of PEF > CF > EAF > CMF (**Table 2**).

In vitro hepatoprotective activity

Induction of liver damage by CCl_4 is a widely used method in the study of liver protective activity of many drugs. CCl_4 produces an experimental liver damage which histologically resembles that of viral hepatitis (James and Pickering 1976). CCl_4 appears to be metabolized to its trichloromethyl free radical or peroxytrichloromethyl free radical which then mediates subsequent damage producing lipid peroxidation via attack at unsaturated sites in fatty acids in membrane phospholipids. CCl_4 can induce centrilobular necrosis resulting in severe steatosis. This is mainly due to the translocation of fat from peripheral adipose tissue to liver. It is generally believed that inhibition of the generation of free radicals or antioxidant activity is important in the protection against CCl_4-induced liver lesion (Ha *et al.* 2005). In the

Table 3 Effect of *F. microcarpa* on LDH and lipid peroxidation in liver slice culture *in vitro*.

Treatment	Concentration of LDH (nmole pyruvate/min/mg protein)		Lipid peroxidation (nmole MDA/min/mg protein)	
Untreated control	6.71 ± 0.21		1.11 ± 0.01	
CCl_4 alone	16.48 ± 0.22 a		3.75 ± 0.03a	
Fractions	50 µg/ml	100 µg/ml	50 µg/ml	100 µg/ml
PEF+ CCl_4	10.58 ± 0.170 b	5.08 ± 0.395 b	2.06 ± 0.004 b	1.73 ± 0.019 b
EAF+ CCl_4	10.87 ± 0.304 b	5.42 ± 0.142 b	2.41 ± 0.001 b	1.17 ± 0.001 b
CF+ CCl_4	12.15 ± 0.200 b	8.42 ± 0.156 b	2.55 ± 0.002 b	1.86 ± 0.021 b
CMF CCl_4	13.09 ± 0.036 b	8.37 ± 0.196 b	3.07 ± 0.010 b	1.23 ± 0.002 b
Silymarin+CCl_4	9.50 ± 0.154 b	7.65 ± 0.068 b	2.13 ± 0.005 b	1.31 ± 0.022 b

Values are expressed as mean ± S.E.M of n=6 a P<0.01 Vs untreated control, b P<0.01 when compared with CCl_4 treated group.
CF, chloroform fraction, CMF, chloroform-methanol fraction, EAF, ethyl acetate fraction, PEF, petroleum ether fraction

Table 4 Effect of *F. microcarpa* fractions on antioxidant enzymes level in liver slice culture *in vitro*.

Treatment	CAT (µmol/min/mg protein)		SOD (µmol/min/mg protein)		GSH (µmol/min/mg protein)		GPx (µmol/min/mg protein)	
Untreated control	5.23 ± 0.06		48.36 ± 0.06		1.54 ± 0.01		1.01 ± 0.004	
CCl_4 alone	1.37 ± 0.14 a		14.81 ± 0.40a		0.337 ± 0.001a		0.396 ± 0.002a	
Fractions	50 µg/ml	100 µg/ml	50 µg/ml	100 µg/ml	50 µg/ml	100 µg/ml	50 µg/ml	100 µg/ml
PEF+CCl_4	1.60 ± 0.01c	2.46 ± 0.19b	20.39 ± 0.45b	24.3 ± 0.45b	0.50±0.03b	0.91±0.0.02b	0.49 ± 0.02c	0.77± 0.05b
EAF+CCl_4	1.51 ± 0.02c	2.21 ± 0.07b	20.04 ± 1.38b	28.80 ± 0.65b	0.43 ± 0.01b	0.57 ± 0.08b	0.41± 0.02c	0.67 ± 0.01b
CF+CCl_4	1.73 ± 0.04c	2.46 ± 0.20b	23.2 ± 0.96b	30.0± .45b	0.63 ± 0.08b	0.67 ± 0.302b	0.53 ± 0.02c	0.83 ± 0.01b
CMF+CCl_4	1.42 ± 0.02c	2.95 ± 0.41b	22.0 ± 0.51b	29.0 ± 0.09b	0.48 ± 0.05b	0.82 ± 0.01b	0.47 ± 0.03c	0.81 ± 0.03b
Silymarin+CCl_4	1.60 ± 0.07c	3.62 ± 0.06b	33.64 ± 0.59b	42.6 ± 0.52b	0.65 ± 0.05b	1.37 ± 0.02b	0.43 ± 0.08c	0.86 ±0.01b

Values are expressed as mean ± SEM; n=5 in each group. a $P < 0.01$ when compared to control. b $P < 0.01$ and c $P > 0.05$, when compared to CCl_4 control.
CF, chloroform fraction, CMF, chloroform-methanol fraction, EAF, ethyl acetate fraction, PEF, petroleum ether fraction

liver slice culture system, leakage of LDH was used as a marker to study the hepatotoxicity induced by CCl_4. LDH is a cytosolic enzyme mainly present in periportal hepatocytes and released when the cells are lysed by hepatotoxin. The amount of enzymes released is proportional to the extent of damage caused to the cells (Naik *et al.* 2004). In the slices treated with CCl_4 alone, almost 2.5 times more LDH was released after pre incubation for 2 h as compared to control liver slices (**Table 3**). All the fractions of *F. microcarpa* exhibited hepatoprotective activity by reducing the release of LDH on induction with CCl_4 and the results were significant. Liver slice culture is therefore an *in vitro* technique that offers the advantages of *in vivo* situation and hence can be used for the experimental analysis of hepatotoxic events.

CAT, SOD, GPx and GSH are some of the antioxidant enzymes that protect liver cells from oxidative stress induced by highly reactive free radicals. The defensive action of liver cells against oxidative stress involves the use of enzymatic and non-enzymatic antioxidants (Murugesh *et al*. 2005). In the presence of CCl_4, the activities of enzymatic and non-enzymatic antioxidant enzymes were found to be significantly ($P<0.01$) decreased compared to the control (**Table 4**). The antioxidant enzyme levels significantly ($P<0.01$) increased in all the fraction-treated groups and the results were comparable with that of standard hepatoprotective agent, silymarin.

The level of lipid peroxidation is a measure of membrane damage and alterations in structures and function of cellular membranes. The levels of thiobarbituric acid reactive substances (TBARS) are an indirect measurement of lipid peroxidation (Ilavarasan *et al.* 2003). In CCl_4-treated liver cells, the amount of lipid peroxidation end products increased significantly ($P<0.01$) by two-fold (3.75 ± 0.03) when compared to control slices (1.11 ± 0.01). All the fractions showed significant reduction in MDA content at a dose of 100 µg/ml and the reduction was less in the 50 µg/ml treated group (**Table 3**). The fractions inhibited the lipid peroxidation in a concentration-dependent manner and the results obtained were however less than that of silymarin-treated liver slices ($P < 0.01$).

Histological examination of liver slice culture

Histological examination of the untreated control liver slices showed normal hepatic parenchyma (HE staining, x 100) (**Fig. 4A**). The liver slices exposed to CCl_4 alone exhibited mild periportal inflammation and macrovesicular steatosis (HE staining, x 100) (**Fig. 4B**). In slices treated simultaneously with the chloroform fraction, normal hepatic parenchyma with mild periportal inflammation was observed (HE staining, x 100) (**Fig. 4C**). Histopathological studies revealed that concurrent administration of CCl_4 with the fraction afforded protection of the liver cells, which further confirmed the hepatoprotection offered by the fractions.

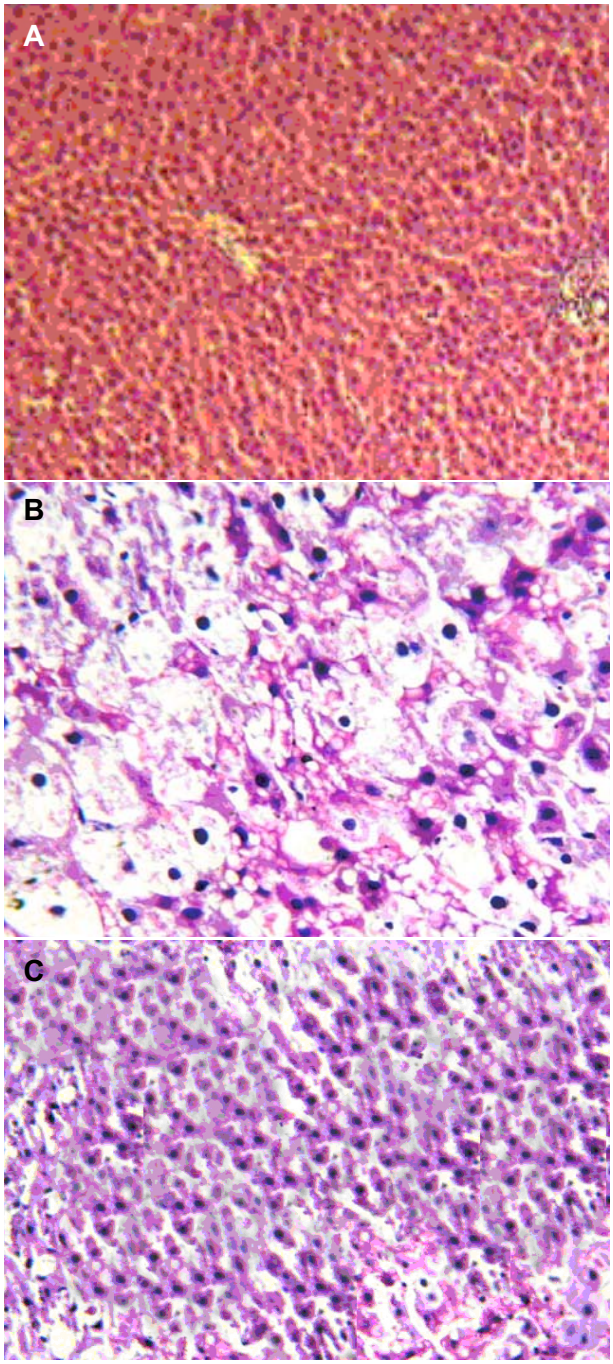

Fig. 4 Photomicrographs of the liver tissue of control group (**A**) showing normal hepatic parenchyma. Haemotoxylin and eosin staining, x 100; (**B**) CCl_4-treated group showing mild periportal inflammation and macrovesicular steatosis. Haemotoxylin and eosin staining, x 100; (**C**) CF+CCl_4-treated group showing almost normal hepatic parenchyma with mild periportal inflammation. Haemotoxylin and eosin staining, x 100.

CONCLUSIONS

In summary, plant-based medicine is beneficial to human health because it increases antioxidant defense against oxidative damage. Based on the active profile exposed through various *in vitro* and *ex vivo* assays, it can be concluded that the hydromethanolic fractions of *F. microcarpa* showed significant antioxidant and hepatoprotective activities. The isolation of bioactive components in the extracts could certainly help to ascertain the individual potency of the compounds. Exploitation of its antioxidant activity could be further explored using *in vivo* assay systems, to increase the overall antioxidant activity and to protect against various ailments that are induced by oxidative stress.

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