

Original Article***Pterocarpus marsupium* extract reveals strong *in vitro* antioxidant activity**Mahnaaz Mohammadi¹, Swati Khole¹, Thomas Paul Asir Devasagayam², Saroj S. Ghaskadbi^{1,*}¹ Department of Zoology, University of Pune, Pune, India;² Radiation Biology and Health Sciences Division, Bhabha Atomic Research Centre, Mumbai, India.

ABSTRACT: Diabetes mellitus is a complex chronic disease characterized by hyperglycemia, which via several mechanism leads to an increase in production of reactive oxygen species (ROS) leading to various secondary complications. Thus, a drug having both antidiabetic and antioxidant properties would have great therapeutic value for overcoming the oxidative load in diabetes. The present study was aimed at extensively evaluating the antioxidant properties of an anti-diabetic plant extract of stem bark of *Pterocarpus marsupium* using various *in vitro* radical scavenging assays as well as by using liver slice cultures as a model system. Our results demonstrate that the whole aqueous extract showed high antioxidant activity in all different assays used and also protected mitochondria against oxidative damage. Ethanol was used as an inducer of oxidative stress in liver slice culture and cytotoxicity was estimated by quantitating release of cytotoxicity marker enzymes such as lactate dehydrogenase (LDH). Additionally, levels of antioxidant enzymes (AOEs) namely superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase were also estimated. The whole aqueous extract significantly reduced LDH release along with reduction of lipid peroxidation compared to ethanol treated slices. These results indicate that the *P. marsupium* extract may serve as a potential source of natural antioxidant for treatment of diabetes.

Keywords: Antioxidant activity, *P. marsupium*, liver slice culture, *in vitro* antioxidant assays

1. Introduction

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and insufficiency of secretion or

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action of endogenous insulin. It is a common disease affecting over 124 million individuals worldwide.

Persistent hyperglycemia during diabetic conditions leads to production of free radicals or impaired antioxidant defenses via several mechanisms (1). Also there is strong evidence that diabetes induces changes in the activity of antioxidant enzymes in various tissues (2). Many studies reveal that antioxidants capable of neutralizing free radicals are effective in preventing experimentally induced diabetes in animal models (3,4) as well as reducing the severity of diabetic complications (5). Additionally, supplementation with antioxidants in diabetic patients provided greater protection against free radical induced damage (6). Recent reviews suggest that certain herbal plants possess both antidiabetic and antioxidative activities and that their proper use in the diet may help decrease the oxidative load in diabetes mellitus. Therefore, various plant or herb extracts having both antidiabetic and antioxidant activity would have a better therapeutic value than other treatments for overcoming the oxidative load in diabetes.

Pterocarpus marsupium Roxb (from the family Leguminosae) known in the vernacular as "vijaysar" or "Bijasar" is a large tree that commonly grows in the central, western, and southern parts of India and in Sri Lanka. Various portions of the bark are used as astringent, anti-diarrheal, antacid, for treatment of toothache and for the management of diabetes (7). The heartwood of *P. marsupium* is known to be useful in arthritis, gout, bronchitis, skin infections asthma, diabetes, etc. The flavonoids marsupium, pterosupin, and liquiritigenin are reported to possess antihyperglycemic (8) and antihyperlipidemic (9) activities. The aqueous extract of stem bark was found to reduce the blood glucose level in alloxan-induced diabetic rats (10). In one laboratory study, pancreatic beta-cell regeneration was observed in alloxan-induced diabetic rats that received the flavonoid fraction from the bark of *P. marsupium* (11).

In view of this, the present study evaluated the antioxidant activity of the stem bark of *Pterocarpus marsupium* against ethanol induced oxidative stress

in liver slice cultures and by using different *in vitro* biochemical assays.

2. Materials and Methods

2.1. Chemicals

Chemicals were from one of the following companies: SRL (Mumbai, India), BDH (Mumbai, India), Hi-media (Mumbai, India), Sigma-Aldrich (St Louis, MO, USA), Merck (Mumbai, India), Accurex (Mumbai, India), and EMD (Madison, WI, USA) assay kits. Ethanol was obtained from Fluka, Buchs, Switzerland. Stem bark of *P. marsupium* was obtained and authenticated from the pharmacy.

2.2. Biologicals

Adult Swiss albino mice (6-8 weeks old) of either sex, bred in the animal house of Department of Zoology, University of Pune, were used for preparations of liver slices. Prior approval for the protocols involving animals during this work was obtained from Pune University Institutional Animal Ethical Committee. Animals were maintained at 12:12 h light/dark cycle and were given food *ad libitum*.

2.3. Preparation of sample

Stem bark of *P. marsupium* was used for preparing 10% aqueous extract. Briefly, 10 g of powder was dissolved in 100 mL of distilled water and stirred for 1 h. The suspension obtained was centrifuged and the supernatant was collected and used as the whole aqueous extract. Sequential extraction of *P. marsupium* bark powder was done using petroleum ether, chloroform, and methanol using Soxhlet apparatus. The powder was extracted for 16 h with each solvent to remove the soluble matter. The extract was evaporated to dryness in vacuum desiccators. Finally, the powder was extracted with distilled water, centrifuged, dried, and used as the soxhlet aqueous extract.

2.4. *In vitro* antioxidant capacity assays

Whole aqueous extract (Aq), soxhlet methanolic extract (Met) and soxhlet aqueous extract (Aqs) of *P. marsupium* were used for these assays. All the assays were replicated at least three times and values expressed are an average of these replicates.

DPPH (1,1'-diphenyl-1-picrylhydrazyl) radical scavenging ability of the extracts was checked according to Aquino, *et al.* (12). The ability of the extracts to reduce ferric complex was assayed according to the protocol developed by Alzorkey, *et al.* (13), whereas the potential of the extracts to inhibit 2,2'-azobis-3-ethylbenzthiazoline-6-sulfonic acid

radical cation (ABTS^{•+}) formation was evaluated by measuring the lag time in formation of the radical by the ABTS^{•+}/ferrylmyoglobin assay (14). In all these assays, antioxidant activity is expressed as equivalent of $\mu\text{g/mL}$ of standard antioxidant L-ascorbic acid.

2.5. ABTS^{•-} and CO₃^{•-} radical anions scavenging assay by pulse radiolysis

The pulse radiolysis experiments were carried out at National Centre for Free Radical Research, University of Pune. The ability of the extracts to scavenge ABTS^{•-} and CO₃^{•-} radicals was determined by pulse radiolysis. ABTS^{•-} radical was produced by the reaction of radiolytically generated azide radicals with ABTS^{•-}. CO₃^{•-} radicals were generated using a reaction mixture containing 0.05 M NaHCO₃ and 0.05 M Na₂CO₃ saturated with N₂O. In the presence of the extract, the decay of ABTS^{•-} and CO₃^{•-} were correlated with the concentration of ascorbic acid equivalents (15).

2.6. Isolation of rat liver mitochondria and exposure to oxidative stress

Three month old female Wistar rats were used for the isolation of mitochondria from liver (16). Oxidative stress to mitochondria was generated by 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), which generates peroxy radical and ascorbate-Fe²⁺ which generates [•]OH like radicals (16).

2.7. TBARs assay

The ability of the extracts to protect lipids from oxidative damage was assessed by measuring inhibition of lipid peroxide and hydroperoxide formation. Briefly, mitochondria were treated with AAPH and or ascorbate-Fe²⁺ in the absence or presence of extract and were incubated at 37°C in a shaker water-bath. These were then reacted with TBA reagent, boiled for 30 min and the pink color developed due to thiobarbituric acid reactive substance (TBARs) formed was estimated at 532 nm spectrophotometrically and expressed as malonaldehyde (MDA) equivalents after accounting for appropriate blanks. Malonaldehyde was prepared by acid hydrolysis of tetramethoxypropane (16).

2.8. Lipid hydroperoxide assay

Oxidative damage to mitochondria by AAPH and ascorbic acid-Fe²⁺ was measured in terms of lipid hydroperoxides formed using FOXII reagent (17). The working reagent was routinely calibrated against solutions of cumene hydroperoxide of known concentration. Samples were incubated at 37°C for 30 min, and then reacted with FOXII reagent, spun and

absorbance was read at 560 nm.

2.9. Estimation of protein carbonyl

Protein carbonyls were measured using 2,4-dinitrophenyl hydrazine (DNPH) (18) and are expressed as nmoles of protein formed per mg protein.

2.10. Measurement of protein sulphhydryl

Protein sulphhydryls were quantitated using Ellman's reagent (5,5-dithiobis-2-nitrobenzoic acid) and are expressed as nmoles protein sulphhydryls per mg protein (19).

2.11. Liver slice culture

Liver slice cultures were as described earlier (20,21). Liver slices were incubated with or without whole aqueous extract and damage was induced by ethanol. At the end of incubation, the culture medium was collected and used for estimation of lactate dehydrogenase (LDH), glutamate pyruvate transaminase (GPT), and glutamate oxaloacetate transaminase (GOT), which were used as cytotoxicity markers. The slices were homogenized and the homogenates were centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was assayed for LDH, GPT, GOT and antioxidant enzymes catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx), and antioxidant molecules glutathione and uric acid. Oxidative damage induced by ethanol was estimated by measuring lipid peroxidation. Cholesterol, triglyceride, and bilirubin were assayed as lipid metabolic markers.

2.12. Measurement of cytotoxicity markers

Samples were assayed for cytotoxic marker enzymes namely LDH, GPT, and GOT. LDH activity was assayed according to the protocol of Whaley (22). GPT and GOT were estimated using Accurex kits (Accurex Biomedical Pvt. Ltd., Mumbai, India) (23). Enzyme units in both the supernatant and tissue homogenate were measured. Release of enzymes from liver slices was calculated as the ratio of enzyme activity found in the supernatant to the total enzyme (supernatant + homogenate) activity.

2.13. Measurement of antioxidant enzymes

Superoxide dismutase (SOD) activity was measured spectrophotometrically using the method of Beauchamp and Fridovich (24). The activity of catalase was measured spectrophotometrically at 240 nm as described by Aebi (25). Glutathione peroxidase activity was determined according to the protocol described by Lawrence and Burk (26). Glutathione reductase (GR) was measured by the method of Goldberg (27). Assays

were repeated three times and the values given are an average of these replicates. Values are expressed as μ moles/100 mg tissue.

2.14. Measurement of antioxidant molecules

Levels of uric acid were determined using an Accurex kit (Accurex Biomedical Pvt. Ltd.) (28). Uricase converts uric acid to allantoin and hydrogen peroxide. The generated hydrogen peroxidase degrades a phenolic chromagen in the presence of peroxidase to form a red colored compound, which is measured at 510 nm. The values are expressed as mg/100 mg tissue and the values given are replicates of three experiments.

Total glutathione levels were estimated in liver slice homogenates following the protocol of Teixeria and Meineghini (29). For the determination of GSSG, the cell extract was treated with 4-vinyl pyridine to a final concentration of 0.1% (v/v) and then incubated for 1 h. The glutathione content was determined as above. The amount of GSH could be determined by subtracting the GSSG content from total glutathione. Values are expressed as micromoles/100 mg of tissue and the values are an average of three different experiments.

2.15. Measurement of lipid peroxidation

Lipid peroxidation in liver slices was estimated in terms of thiobarbituric reactive substances formed (30). Tissue homogenate was prepared in 5% TCA. To 1 mL of homogenate, 4 mL of 0.5% TBA in 20% TCA was added and this was incubated at 95°C for 30 min. This was immediately cooled on ice, during which the color changed from orange to pink. After centrifugation at 4,000 rpm for 10 min, the difference in the absorbance of the supernatant at 532 nm (specific) and 600 nm (non-specific) was measured to estimate pmol of malonaldehydes (MDA).

2.16. Measurement of lipid metabolic markers

Cholesterol was estimated using an Accurex kit (Accurex Biomedical Pvt. Ltd.) using a three-step reaction (31). Cholesterol esterase hydrolyses cholesterol esters into free cholesterol and fatty acids. The free cholesterol is acted upon by cholesterol oxidase to form cholest-4-en-3-one and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide oxidatively couples with 4-amino-antipyrene and phenol to produce red quinoneimine dye which has an absorbance maximum at 510 nm. Triglyceride levels were estimated by an Accurex kit (32). Hydrolysis of triglycerides by lipoprotein lipase releases glycerol, which is converted to glycerol-3-phosphate by glycerol kinase. This glycerol-3-phosphate is oxidized to dihydroxyacetone phosphate and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide oxidizes

the phenolic chromogen to a red colored compound, which is measured at 510 nm. Bilirubin levels were measured by an Accurex kit (33). In the presence of dimethyl sulphoxide, bilirubin reacts with diazotized sulphanic acid to produce a violet colored compound azobilirubin, which is measured at 546 nm. The values given are an average of three replicates and are expressed as mg/100 mg tissue.

3. Results

3.1. *P. marsupium* extracts exhibit high in vitro antioxidant activity

Antioxidant activities of whole aqueous, soxhlet aqueous and soxhlet methanolic extracts was evaluated by their radical scavenging ability, ferric reducing ability, and radical formation inhibition activity.

In DPPH and ferrylmyoglobin/ABTS^{•+} assays, the whole aqueous extract exhibited the highest radical scavenging as well as inhibition of ABTS^{•+} radical formation, with a value of 1.22 ± 0.01 and 3.74 ± 0.05 $\mu\text{g}/\text{mL}$, respectively, of ascorbic acid equivalent antioxidant capacity (AEAC) (Figures 1 and 2). In Ferric reducing antioxidant power (FRAP) assay, the maximum ferric reducing ability of 0.803 ± 0.03 $\mu\text{g}/\text{mL}$ ascorbic acid equivalent was shown with the 0.05% soxhlet methanolic extract (Figure 3).

Additionally, pulse radiolysis was used to quantitate radical scavenging activity of these extracts. Decay of ABTS^{•-} (Figure 4a) and CO₃^{•-} (Figure 5a) radicals was monitored in the presence of ascorbic acid and the linear plot of pseudo-first order rate constant (K_{abs}) versus ascorbic acid concentration was used to calibrate the standard curve for estimation of ascorbate equivalents in the extracts. The pseudo-first order rate constants for the decay of ABTS^{•-} and CO₃^{•-} were determined for the known concentrations of extracts and from the calibration curve, the ascorbate equivalents, present in different extracts were determined. Figures 4b-d and Figures 5b-d show ABTS^{•-} and CO₃^{•-} radical scavenging activities by whole aqueous, aqueous soxhlet, and methanolic soxhlet extracts, respectively. Maximum ABTS^{•-} radical scavenging activity was exhibited by the 1% soxhlet methanolic extract with a value of 2.3 $\mu\text{g}/\text{mL}$ of AEAC and maximum CO₃^{•-} radical scavenging activity was exhibited by 0.1% methanolic soxhlet extract with a value of 3.9 $\mu\text{g}/\text{mL}$ of AEAC.

3.2. *P. marsupium* extracts protect biomolecules from oxidative damage

These extracts were checked for their ability to protect rat liver mitochondria against oxidative damage induced by AAPH and ascorbate-Fe²⁺. Lipid peroxides and hydroperoxides were used as a measure of damage to lipids, while protein sulphhydryl and protein

carbonyl were quantitated as a measure of damage to protein.

The whole aqueous extract showed the highest ability to inhibit formation of lipid peroxides followed by soxhlet aqueous against ascorbate-Fe²⁺ and AAPH induced oxidative damage respectively (Table 1). Similar results were obtained for lipid hydroperoxide formed in response to damage by ascorbate-Fe²⁺ and AAPH. The nmoles of lipid hydroperoxide formed was significantly ($p < 0.05$) reduced against ascorbate-Fe²⁺ and AAPH as compared to oxidatively damaged mitochondria (Table 2).

Protein carbonyl and protein sulphhydryl were quantitated as a measure of oxidative damage to proteins induced by AAPH. Protein carbonyl formation was reduced substantially ($p < 0.05$) by 1% whole

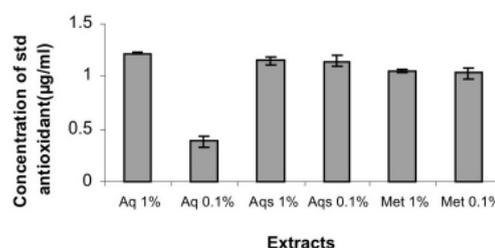


Figure 1. DPPH radical scavenging activity of various *P. marsupium* extracts. Aq, whole aqueous extract; Aqs, soxhlet aqueous extract; Met, soxhlet methanolic extract. Radical scavenging activity is expressed as ascorbic acid equivalent antioxidant activity (AEAC). Values are mean \pm SD of three different experiments.

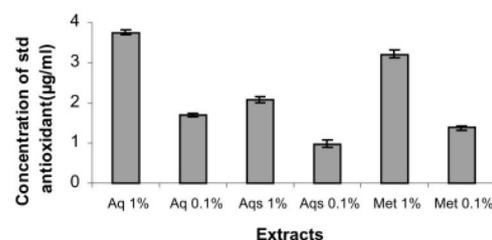


Figure 2. Inhibition of ABTS^{•-} radical formation by various *P. marsupium* extracts. Aq, whole aqueous extract; Aqs, soxhlet aqueous extract; Met, soxhlet methanolic extract. Inhibition is measured in terms of ascorbic acid equivalent antioxidant activity (AEAC). Values are mean \pm SD of three different experiments.

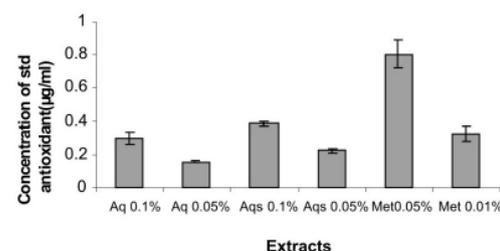


Figure 3. Ferric reducing ability of various *P. marsupium* extracts. Aq, whole aqueous extract; Aqs, soxhlet aqueous extract; Met, soxhlet methanolic extract. Radical scavenging activity is expressed as ascorbic acid equivalent antioxidant activity (AEAC). Values are mean \pm SD of three different experiments.

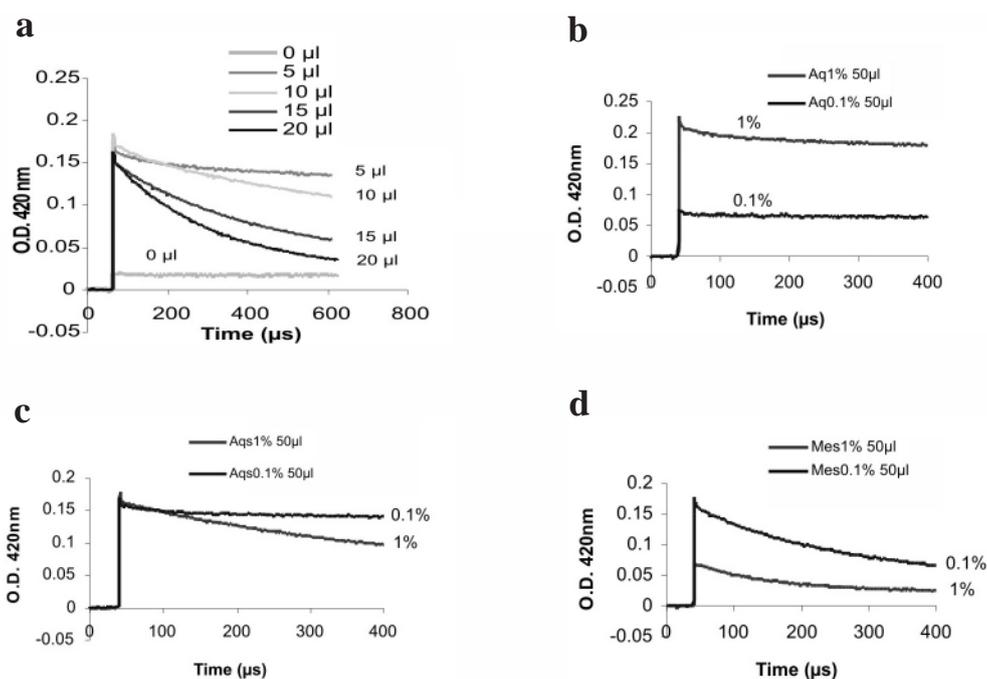


Figure 4. Kinetic decay of $ABTS^{\bullet-}$ radical generated by pulse radiolysis. $ABTS^{\bullet-}$ radical scavenging was compared in the presence of (a) different concentrations of ascorbic acid, (b) 1% and 0.1% of whole aqueous extract, (c) 1% and 0.1% of soxhlet aqueous extract, and (d) 1% and 0.1% of soxhlet methanolic extract of *P. marsupium*.

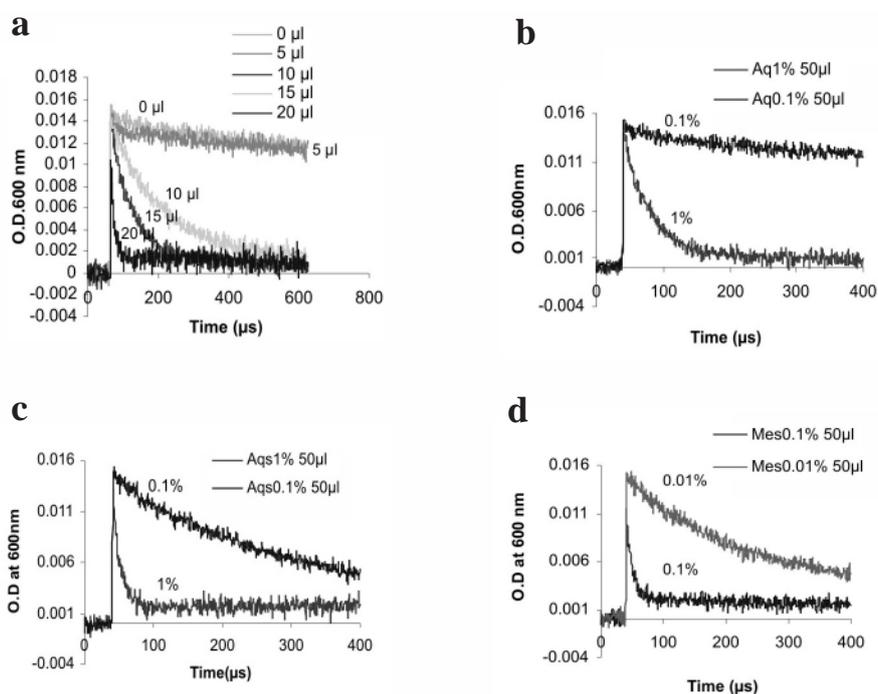


Figure 5. Kinetic decay of $CO_3^{\bullet-}$ radical generated by pulse radiolysis. $CO_3^{\bullet-}$ radical scavenging was compared in the presence of (a) different concentrations of ascorbic acid, (b) 1% and 0.1% of whole aqueous extract, (c) 1% and 0.1% of soxhlet aqueous extract, and (d) 0.1% and 0.01% of soxhlet methanolic extract of *P. marsupium*.

aqueous extract as compared to damaged mitochondria. Similarly, the 1% whole aqueous extract was able to protect mitochondria significantly ($p < 0.05$) by inhibiting depletion of the protein sulphhydryls against damaged mitochondria (Table 3).

3.3. Protection of liver slices from ethanol induced oxidative stress

The ability of whole aqueous extract of *P. marsupium* to protect liver cells against ethanol induced oxidative

Table 1. Inhibition of lipid peroxidation by *P. marsupium* measured in terms of nmoles of TBARs formed/mg protein in rat liver mitochondria damaged by ascorbate-Fe²⁺ and AAPH. Lipid peroxidation in controls and oxidatively damaged mitochondria by ascorbate-Fe²⁺ and AAPH was 0.5 ± 0.1 and 1.93 ± 0.37 nmoles of TBARs/mg protein, respectively. Lipid peroxidation in controls and oxidatively damaged mitochondria by APPH were 0.44 ± 0.04 and 1.01 ± 0.08 nmoles of TBARs, respectively. Values are represented as mean ± SD of three different experiments.

Extract	Nmoles of TBARs formed/mg protein by	
	Ascorbate-Fe ²⁺	AAPH
Aqueous extract 1%	0.32 ± 0.07 ^a	0.27 ± 0.04 ^a
Aqueous extract 0.1%	0.46 ± 0.05 ^a	0.34 ± 0.02 ^a
Aqueous soxhlet extract 1%	0.41 ± 0.07 ^a	0.29 ± 0.04 ^a
Aqueous soxhlet extract 0.1%	0.55 ± 0.09 ^a	0.32 ± 0.05 ^a
Methanol soxhlet extract 1%	0.42 ± 0.06 ^a	0.31 ± 0.03 ^a
Methanol soxhlet extract 0.1%	0.49 ± 0.1 ^a	0.34 ± 0.07 ^a

^a These values differ significantly ($p < 0.05$) from the AAPH and ascorbate-Fe²⁺ induced damage to rat liver mitochondria (Student's *t*-test).

Table 2. Inhibition of lipid peroxidation measured in terms of nmoles of lipid hydroperoxides formed/mg protein in rat liver mitochondria damaged by ascorbate-Fe²⁺ and AAPH by different extracts of *P. marsupium*. Lipid hydroperoxides formed in controls and oxidatively damaged mitochondria by ascorbate-Fe²⁺ were 152.32 ± 3.8 and 455.05 ± 5.71 nmoles of lipid hydroperoxides/mg protein. Lipid hydroperoxides formed in controls and oxidatively damaged mitochondria by APPH were 132.64 ± 3.99 and 405.55 ± 11.86 nmoles of lipid hydroperoxides/mg protein. Values are represented as mean ± SD of three different experiments.

Extract	Nmoles of lipid hydroperoxides formed/mg protein by	
	Ascorbate-Fe ²⁺	AAPH
Aqueous extract 1%	292.89 ± 17.58 ^a	254.18 ± 10.72 ^a
Aqueous extract 0.1%	326.85 ± 15.11 ^a	300.51 ± 11.9 ^a
Aqueous soxhlet extract 1%	296.07 ± 17.37 ^a	258.94 ± 10.81 ^a
Aqueous soxhlet extract 0.1%	300.51 ± 19.65 ^a	270.04 ± 15.3 ^a
Methanol soxhlet extract 1%	303.05 ± 16.82 ^a	261.79 ± 11.58 ^a
Methanol soxhlet extract 0.1%	309.71 ± 18.56 ^a	270.68 ± 14.25 ^a

^a These values differ significantly ($p < 0.05$) from the AAPH and ascorbate-Fe²⁺ induced damage to rat liver mitochondria (Tukey's *t*-test).

Table 3. Protein sulphhydryl formed in rat liver mitochondria damaged by AAPH by different extracts of *P. marsupium*. Protein sulphhydryl in controls and oxidatively damaged mitochondria were 36.65 ± 1.99 and 26.67 ± 0.18 nmoles protein sulphhydryl/mg protein, respectively. Protein oxidation measured in terms of nmoles of protein carbonyl formed/mg protein in oxidatively damaged rat liver mitochondria by AAPH by different extracts of *P. marsupium*. Protein carbonyl in controls and oxidatively damaged mitochondria were 7.59 ± 0.54 and 23.13 ± 1.09 nmoles protein carbonyl/mg protein, respectively. Values are represented as mean ± SD of three different experiments

Extract	nmoles Protein sulphhydryl/mg protein	nmoles Protein carbonyl/mg protein
Aqueous extract 1%	31.6 ± 0.5 ^a	12.84 ± 0.28 ^a
Aqueous extract 0.1%	27.97 ± 0.59 ^a	17.25 ± 1.33 ^a
Aqueous soxhlet extract 1%	30.97 ± 0.68 ^a	14.56 ± 0.25 ^a
Aqueous soxhlet extract 0.1%	30.17 ± 0.85 ^a	14.78 ± 0.29 ^a
Methanol soxhlet extract 1%	30.72 ± 0.62 ^a	14.43 ± 0.65 ^a
Methanol soxhlet extract 0.01%	27.54 ± 0.58 ^a	16.30 ± 0.31 ^a

^a These values differ significantly ($p < 0.01$) from the AAPH and ascorbate-Fe²⁺ induced damage to rat liver mitochondria (Student's *t*-test).

stress was checked using liver slice culture and assaying the release of intracellular enzymes namely LDH, GOT, and GPT. As shown in Figure 6a, the % release for LDH, GOT, and GPT was significantly decreased to 12.6 ± 1.06, 15.3 ± 1.1, and 16.4 ± 0.79, respectively, in slices treated with ethanol and 1% whole aqueous extract as compared to ethanol treated slices which had a percent release of 53 ± 1.83, 41 ± 1.4, and 41 ± 0.7 for LDH, GOT, and GPT, respectively. The time course

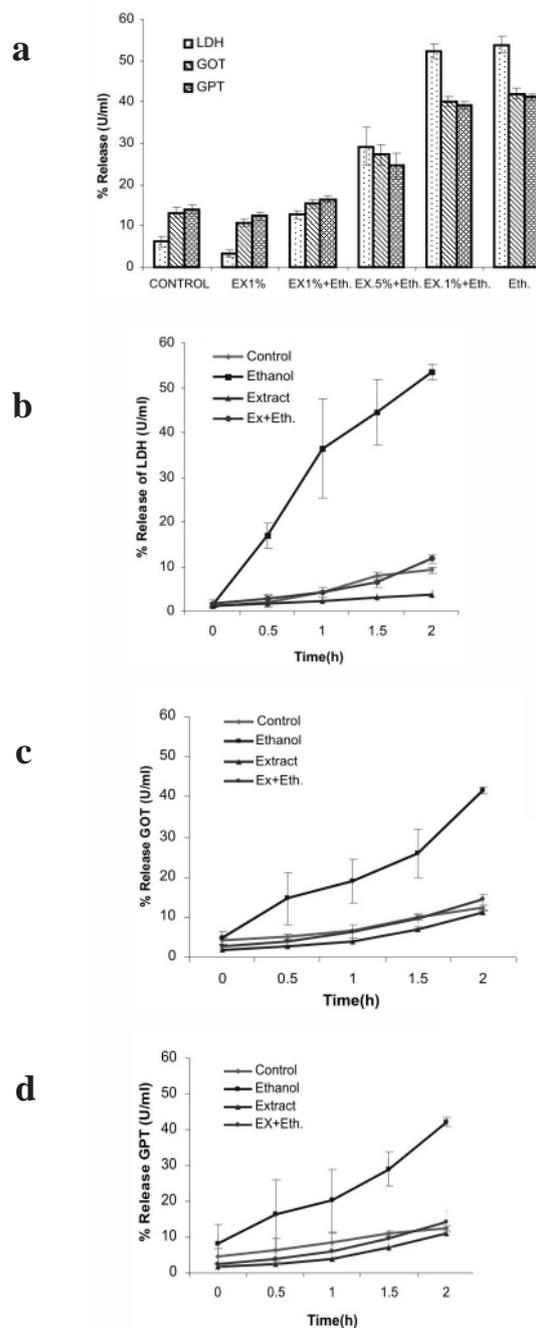


Figure 6. Effect of whole aqueous extract of *P. marsupium* on release of various cytotoxicity markers from liver slice culture. (a) Measurement of cytotoxicity markers in the presence or absence of ethanol (Eth.) alone or with different concentrations of whole aqueous extract (EX). (b), (c) and (d) are time course for LDH, GOT and GPT, respectively. Values are mean ± SD of three different experiments.

of release of these enzymes showed that these enzymes were released over the period of 2 h and this release was inhibited within 30 min in the presence of 1% *P. marsupium* extract (Figures 6b-d).

3.4. *P. marsupium* extract protects liver slices by lowering lipid peroxide formation due to ethanol

It was observed that in ethanol-treated liver cells the amount of MDA formed was substantially higher (1.29 pmol/100 mg tissue) compared to the control (0.27 pmol/100 mg tissue) (Figure 7a). Following the time course for lipid peroxidation, a gradual increase in lipid peroxidation over a period of 2 h was observed, which was inhibited within 30 min in the presence of 1% *P. marsupium* extract (Figure 7b).

3.5. *P. marsupium* extract modulates antioxidant status in liver cells in response to ethanol induced oxidative stress

3.5.1. Modulation of antioxidant enzymes

Intracellular antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase

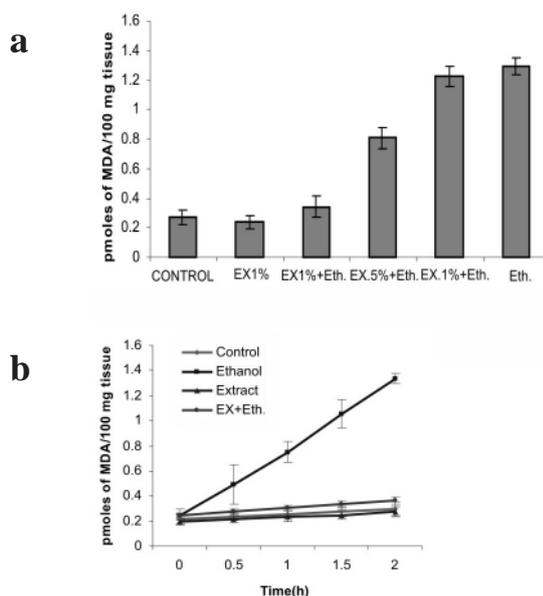


Figure 7. Effect of whole aqueous extract of *P. marsupium* on lipid peroxidation of liver slices. (a) Protection conferred by different concentrations of whole aqueous extract in the presence of ethanol, as measured as picomoles of malonaldehyde formed per mg of tissue. (b) Time course for lipid peroxidation measured at an interval of 30 min. Values are mean \pm SD of three different experiments.

(GPx) were quantitated. In the presence of ethanol, the activities of SOD and catalase were found to be significantly increased as compared to controls whereas a small decrease was seen in GPx as compared to control (Table 4). Activities of all these enzymes were normalized in the presence of *P. marsupium* extract in a concentration-dependent manner. Following the time course for all three enzymes, SOD and catalase showed a gradual increase, while GPx activity gradually dropped in ethanol treated slices compared to control. The activity of all the enzymes was restored in the presence of extract (Figure 8).

GR is involved in synthesis of reduced glutathione, which is a major antioxidant in the cell. Liver slices treated with ethanol showed a decrease in the activity of this enzyme, which was restored by whole aqueous extract in a concentration dependent manner (Figure 9a). Moreover time course revealed that within 30 min, activity of GR was restored (Figure 9b).

3.5.2. Modulation of antioxidant molecules

Glutathione and uric acid are small antioxidant molecules, which directly scavenge free radicals. Activity of both these molecules was decreased in liver slices treated with ethanol as compared to untreated slices (Figures 10a and 11a). The whole aqueous extract of *P. marsupium* normalized the levels of both these molecules within 30 min in a concentration dependent manner (Figures 10b and 11b).

3.5.3. *P. marsupium* regulates lipid metabolic markers

Cholesterol, triglyceride, and bilirubin levels were checked as major lipid metabolic markers. In liver slices treated with ethanol both cholesterol (0.9 mg/100 mg tissue) and triglycerides (3.09 mg/100 mg tissue) were found to be markedly decreased as compared to untreated slices (12.18 and 13.72 mg/100 mg tissue, respectively). 1% *P. marsupium* extract was able to restore these levels. Bilirubin levels in liver slices treated with ethanol showed a small increase (0.39 ± 0.01 mg/100 mg tissue), as compared to control (0.2 ± 0.02 mg/100 mg tissue). Slices treated with 1% *P. marsupium* extract did not show any change compared to control untreated slices (Table 5). Cholesterol and triglyceride levels decreased gradually over a period of 2 h during ethanol treatment and were normalized

Table 4. Specific activity of catalase, SOD and GPx (U/mg protein) under different conditions

	Control	Extract 1%	EX 1% + OH	EX 0.5% + OH	EX 0.1% + OH	OH
CAT	1,014.1 \pm 64.13	908 \pm 57.52	1,198.2 \pm 126.59 ^b	1,366.4 \pm 90.72 ^b	1,724.5 \pm 48.83 ^c	2,040.7 \pm 96.23 ^a
SOD	338.12 \pm 17.82	303.38 \pm 19.80	551.36 \pm 17.93 ^b	636.88 \pm 17.28 ^b	707.61 \pm 5.28 ^c	762.6 \pm 18.90 ^a
GPx	1.37 \pm 0.0344	1.38 \pm 0.0459	1.33 \pm 0.0571 ^b	1.32 \pm 0.058 ^b	1.32 \pm 0.204 ^c	1.31 \pm 0.197 ^a

^a These values differ significantly ($p \leq 0.001$) from the control group (student's *t*-test); ^b These values differ significantly ($p \leq 0.001$) from ethanol treated group (student's *t*-test); ^c These values differ significantly ($p \leq 0.1$) from ethanol treated group (student's *t*-test).

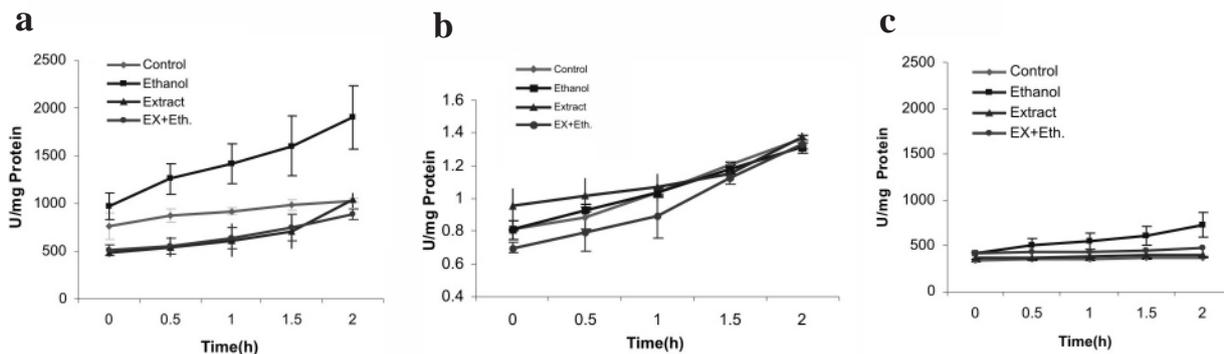


Figure 8. Time course for various antioxidant enzymes in the presence of ethanol and ethanol with whole aqueous extract. (a) Catalase, (b) superoxide dismutase (SOD), and (c) glutathione peroxidase (GPx). Values are mean \pm SD of three different experiments.

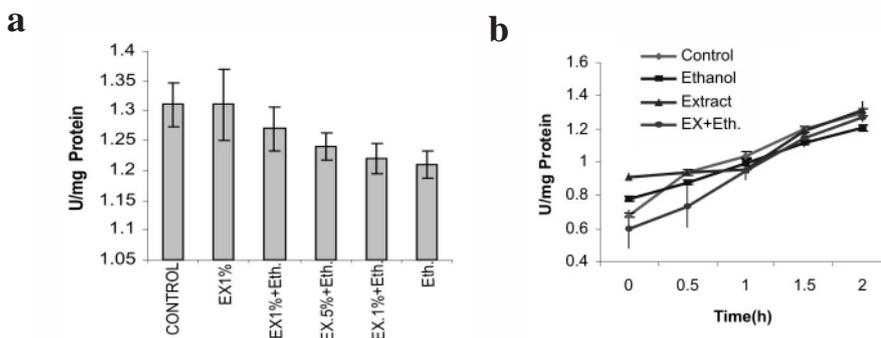


Figure 9. Effect of *P. marsupium* extract on glutathione reductase in liver slice culture. (a) Level of glutathione reductase in the presence of ethanol and ethanol along with different concentrations of whole aqueous extract. (b) Time course for glutathione reductase in the presence of ethanol and ethanol with 1% whole aqueous extract. Values are mean \pm SD of three different experiments.

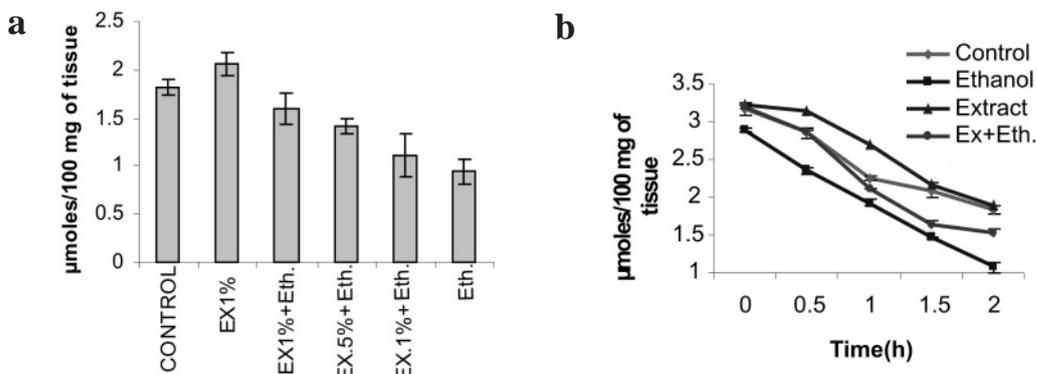


Figure 10. Effect of *P. marsupium* extract on glutathione amount in liver slice culture. (a) Amount of glutathione under different conditions. (b) Time course for glutathione measured at an interval of 30 min under various conditions. Values are mean \pm SD of three different experiments.

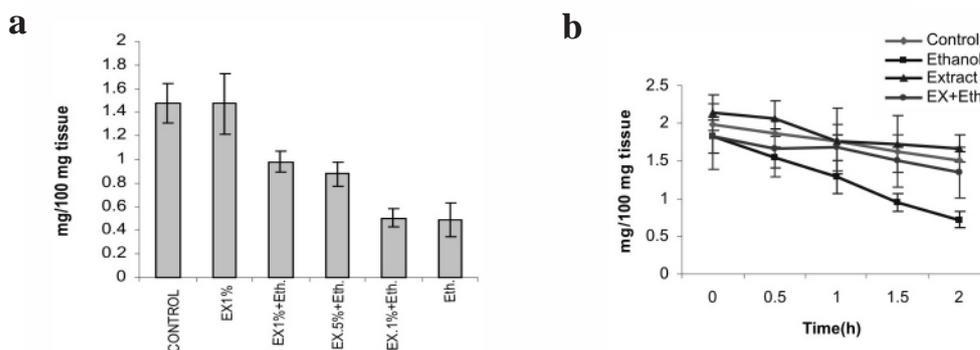


Figure 11. Effect of *P. marsupium* extract on uric acid amount in liver slice culture. (a) Amount of uric acid under various conditions. (b) Time course for uric acid measured at an interval of 30 min under various conditions. Values are mean \pm SD of three different experiments.

Table 5. Amount of cholesterol, triglyceride, and bilirubin (mg/100 mg tissue) in liver slice culture under different conditions

	Control	Extract 1%	EX 1% + OH	EX 0.5% + OH	EX 0.1% + OH	OH
Cholesterol	12.18 ± 2.18	11.02 ± 2.15	4.35 ± 0.95 ^b	3.12 ± 1.0 ^b	5.1 ± 0.1 ^b	0.905 ± 0.54 ^a
Triglyceride	13.72 ± 2.22	11.7 ± 3.16	8.07 ± 1.59 ^b	6.78 ± 1.37 ^b	3.99 ± 0.53 ^b	3.09 ± 0.33 ^a
Bilirubin	0.20 ± 0.02	0.21 ± 0.03	0.22 ± 0.02 ^b	0.22 ± 0.05 ^b	0.35 ± 0.03 ^b	0.39 ± 0.01 ^a

^a These values differ significantly ($p \leq 0.001$) from the control group (Student's *t*-test); ^b These values differ significantly ($p \leq 0.001$) from ethanol treated group (Student's *t*-test).

within 30 min in the presence of 1% *P. marsupium* extract along with ethanol. Bilirubin levels increased gradually over a period of 2 h during ethanol treatment and was restored within 30 min in the presence of 1% *P. marsupium* extract (Figure 12).

4. Discussion

Reactive oxygen species have been implicated in the etiology of many human diseases such as cardiovascular ailments, cancer, diabetes, Alzheimer's disease, arthritis, and neurodegenerative, and in the process of aging. Antioxidants confer protection either by inhibiting formation of radicals or by scavenging free radicals (34). Recently, a large number of plant extracts have been explored for their ability as strong antioxidants.

Pterocarpus marsupium has been used in ayurvedic medicine for the treatment of diabetes over a long period of time. Two major phenolic constituents of *P. marsupium* namely pterostilbene and marsupium were able to significantly decrease plasma glucose levels by 42 and 33%, respectively, in streptozotocin induced diabetic rats. Though the exact mechanism of the antidiabetic effect of this extract is yet to be understood, some diabetics use it regularly as an antidiabetic drug. Demonstration of the strong antioxidant activity of this extract would certainly increase its potential as an anti-diabetic drug. Therefore, the aim of the present study was to evaluate antioxidant activity of *P. marsupium* using *in vitro* assays and liver slice cultures as a model system.

The total antioxidant activity of *P. marsupium* extracts was evaluated using different *in vitro* antioxidant activity assays. These assays correspond to an action of antioxidants at different levels. The DPPH[•] radical scavenging assay corresponds to the primary radical scavenging activity of an antioxidant, whereas ferrylmyoglobin/ABTS^{•+} corresponds to the ability of an antioxidant to inhibit radical formation. The FRAP assay, on the other hand, evaluates the reducing ability of the antioxidant. The whole aqueous extract of *P. marsupium* exhibited high antioxidant potential in all of the above mentioned assays except the FRAP assay, wherein the soxhlet methanolic extract showed high reducing power (Figures 1-3). Pulse radiolysis reveals the ability of antioxidant to scavenge secondary radicals such as ABTS^{•-} and CO₃^{•-}. The soxhlet methanolic extract showed high ABTS^{•-} as well as CO₃^{•-} radical

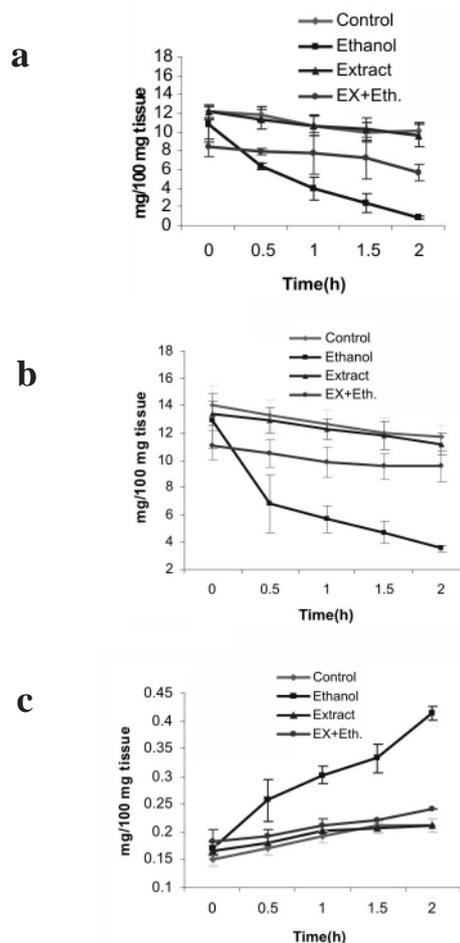


Figure 12. Effect of *P. marsupium* extract on various lipid metabolic markers in liver slice culture. Time course for cholesterol (a), triglyceride (b), and bilirubin (c) were measured at an interval of 30 min under various conditions. Values are mean ± SD of three different experiments.

scavenging activity. The whole aqueous extract on the other hand was best in inhibiting formation of lipid peroxides and lipid hydroperoxide induced by AAPH and ascorbate-Fe²⁺ in rat liver mitochondria (Tables 1 and 2). Similarly, the whole aqueous extract inhibited formation of protein carbonyls as well as inhibited depletion of protein sulphhydryls induced by AAPH in rat liver mitochondria (Table 3). This data clearly shows that the whole aqueous extract and soxhlet methanolic extract behave differentially in different assays. This differential activity of the extracts could be attributed to different components present in the extract.

The antioxidant activity of the extract was

additionally evaluated using liver slice cultures as a model system. Oxidative stress was induced by addition of ethanol to the liver slice culture. These cultures offer a very good test system because they provide a model of the considerable complexity of structurally and functionally intact cells. These cultures retain intact tissue architecture and more closely mimic the *in vivo* situation as compared to isolated hepatocytes.

Ethanol used to induce oxidative stress was cytotoxic to the cells as measured by the increase in LDH, GOT, and GPT release in treated cells. This cytotoxic effect was substantially lowered in a concentration dependent manner on addition of whole aqueous extract (Figure 6) probably through reduction of oxidative stress.

Increased lipid peroxidation has already been associated with liver injury in animals fed ethanol (35) or in hepatocytes treated with ethanol *in vitro* (36). In our studies when cultures were treated with *P. marsupium* extract along with ethanol, lipid peroxidation was substantially reduced as compared with control cells (Figure 7). The fact that lipid peroxidation was significantly reduced in *P. marsupium*-treated cultures indicates that this extract has strong antioxidant activity. Activity of four antioxidant enzymes namely catalase, SOD, GR, and GPx was measured. In the presence of ethanol the activities of SOD and catalase substantially increased, probably in response to oxidative stress induced by ethanol. Addition of whole aqueous extracts along with ethanol lead to a decrease in these enzyme levels, which was comparable to those seen in untreated cultures (Table 4). The levels of GPx (Table 4) and GR (Figure 9), on the other hand, decreased in the presence of ethanol and were normalized on addition of whole aqueous extracts in a concentration dependent manner. This result correlated to the levels of glutathione, which also decreased in the presence of ethanol and was restored in the presence of the extract (Figure 10). The levels of uric acid, another antioxidant molecule, were also decreased with ethanol treatment and were normalized within thirty minutes in the presence of *P. marsupium* extract (Figure 11).

Cholesterol, bilirubin, and triglyceride levels were estimated as markers of liver specific lipid metabolism. Both cholesterol and triglycerides decreased substantially in the presence of ethanol and in the presence of extract this decrease was prevented (Figures 12a and b). We also checked for bilirubin, the product of cholesterol degradation and it was found to be increased with ethanol treatment and was restored in the presence of the extract (Table 5). However, this increase was not as prominent as the decrease in cholesterol. This was a bit surprising because the majority of reports have shown association of fat accumulation with alcohol intake. However, all those observations were made on chronic treatment of tissues whereas in our system cells are given acute exposure to ethanol (37). Our data

demonstrates that during acute exposure to alcohol *P. marsupium* extract helps to maintain cholesterol as well as triglyceride levels.

Both the reduction in lipid peroxidation and restoration of the activity of AOE's clearly demonstrate that in the presence of *P. marsupium* extracts free radical generation is significantly reduced. The antioxidant activity exhibited by *P. marsupium* extract in this system seems to be due to its interaction with free radicals leading to their inactivation rather than altering the activity of antioxidant enzymes, which scavenge them.

Antioxidant activity of the plant extracts is often attributed to the presence of flavonoids. *P. marsupium* is known to contain pterostilbene, an analogue of resveratrol, which is shown to have peroxyl radical scavenging activity (38). Other purified flavonoids, marsupin, pterostilbene, and epicatechin have also been shown to possess antidiabetic activity (39). Our data clearly show that *P. marsupium* extract has strong antioxidant activity in all *in vitro* assays and protects liver cells from ethanol induced oxidative stress. Thus the strong antioxidant activity shown in *P. marsupium* extracts substantially increases its therapeutic value.

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