ORIGINAL ARTICLE

Hepatoprotective activity of *Picrorhiza kurroa* Royle Ex. Benth extract against alcohol cytotoxicity in mouse liver slice culture

Sangeeta Sinha, Jyoti Bhat[1], Manoj Joshi[1], Vilas Sinkar[1], Saroj Ghaskadbi
Departments of Zoology, University of Pune, Pune, Maharashtra, [1]Department of Nutrition and Health, Unilever Research Centre, Bangalore, Karnataka, India

Kutki or *Picrorhiza kurroa* is a herbal medicinal plant belonging to Scrophulariaceae family and is found in the Himalayan region in India. This herb has been traditionally used in treating liver disorders. The antioxidant properties of *P. kurroa* were evaluated *in vitro* using different radical scavenging assays. Furthermore, liver slice culture system was used to test the antioxidant activity of this extract and ethanol was used as a hepatotoxin to generate oxidative stress. Hepatotoxicity was quantified in terms of release of intracellular marker enzymes lactate dehydrogenase, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase. Oxidative stress induced by ethanol and its modulation in the presence of *P. kurroa* extract was tested by estimating the levels of antioxidant enzymes like catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione-*S*-transferase, and of antioxidant molecules like uric acid and reduced glutathione that were quantitated along with lipid peroxidation. Our results clearly demonstrate that aqueous extract of *P. kurroa* with high antioxidant activity, as demonstrated using different radical scavenging assays, was effective in suppressing the deleterious effects of ethanol. Addition of *P. kurroa* aqueous extract along with ethanol restored the activities of antioxidant enzymes and significantly reduced lipid peroxidation.

Key words: Ethanol, hepatoprotective activity, liver slice culture, marker enzymes, oxidative stress, *Picrorhiza kurroa*

INTRODUCTION

Many studies have shown that reactive oxygen species (ROS) including oxygen-free radicals are causative factors in the etiology of degenerative diseases, including some hepatopathies. According to *in vitro* and *in vivo* studies, several classical antioxidants have been shown to protect hepatocytes against lipid peroxidation or inflammation, thereby preventing the occurrence of hepatic necrosis.[1] In the pathogenesis of ethanol-induced liver injury, oxidative stress plays an important role.[2,3] Free radicals generated on ethanol consumption react with various cellular components and cause damage to the tissues.

Peroxidative damage in alcoholic patients and enhanced lipid peroxide production in animals has been correlated with high ethanol consumption.[4] Increased formation of lipoperoxides, conjugated dienes and malondialdehyde (MDA) and reduced levels of antioxidants like Vitamin E and glutathione in the tissues have been demonstrated in experimental animals administered with ethanol for longer duration as well as alcoholic human patients.[5]

Picrorhiza kurroa (*P. kurroa*; Family Scrophulariaceae), a well-known herb in the Indian traditional *Ayurveda* system of medicine, has been used to treat disorders of the liver and is an important ingredient of many herbal preparations used for treatment of liver ailments.[6] Picroliv is a standardised iridoid glucoside mixture isolated from the roots and rhizomes of *P. kurroa*. It contains at least 60% of 1:1.5 mixture of picroside I and kutkoside and has been used as a hepatoprotective agent in diseases such as jaundice. In addition, the nitric oxide scavenging activity,[7] cardioprotective effect,[8] anti-cancer effect,[9,10] anti-diabetic activity,[11] and anti-viral effect[12] of *P. kurroa* extract have been reported. Picroliv, an active constituent of *P. kurroa*, has been found to be hepatoprotective against ethanol-induced hepatic injury in rats.[13-15] In the present study, we investigated the effects of aqueous extract of *P. kurroa* extract against ethanol-induced oxidative stress in liver slice cultures.

Liver slices, in culture, retain tissue-specific micro-architecture and maintain cell diversity, heterogeneity and functional identity. The liver slices contain all the liver cell types including kupffer, endothelial and

Access this article online	
Quick Response Code:	Website: www.greenpharmacy.info
	DOI: 10.4103/0973-8258.91234

Address for correspondence: Ms. Sangeeta Sinha, Department of Zoology, University of Pune, Ganeshkhind, Pune - 411 007, Maharashtra, India.
E-mail: sksinha@unipune.ac.in
Received: 28-04-2011; **Accepted:** 09-07-2011

biliary epithelial cells and thus mimic liver tissue *in vivo* by preserving their physiological characteristics.[16] Liver slice culture system has advantages over culture of dispersed hepatocytes, and has been successfully used for assaying hepatotoxic effect of several cytotoxic compounds such as paracetamol,[17] paraquat[18] as well as hepatoprotective activity of compounds such as curcumin,[19] *Gmelina arborea* extract,[20] and *Pterocarpus marsupium* extract.[21] In the present study, we evaluated the antioxidant activity of *P. kurroa* against ethanol-induced oxidative stress in liver slice culture by using a range of different *in vitro* biochemical assays.

MATERIALS AND METHODS

Chemicals

Ascorbic acid, aluminum chloride, 2,2-azobis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) diammonium salt, 1,1′-diphenyl-2-picrylhydrazyl (DPPH), diosgenin, ethylenediaminetetraacetic acid (EDTA), ferric chloride, Folin-Ciocalteu reagent, hydrogen peroxide, myoglobin, potassium ferricyanide, potassium phosphate (monobasic and dibasic), sodium carbonate, 1,1,3,3-tetraethoxypropane, 2,4,6-tripyridyl-s-triazine (TPTZ), 2-thiobarbituric acid and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co., St. Louis (USA).

All the common chemicals used were from one of the following companies: SRL, Mumbai (India), BDH, Mumbai (India), Hi-media, Mumbai (India), Merck, Mumbai (India), Accurex, Mumbai (India) and Novagen, Madison, WI (USA). Ethanol was obtained from Fluka, Buchs (Switzerland).

Preparation of *P. kurroa* Extract

The extract of *P. kurroa* was obtained from Anju phytochemicals (Bangalore, India). Extract preparation involved a standardised process. Briefly, the dried rhizomes of *P. kurroa* were ground to a fine powder and passed through a 20 # particle size mesh. The powder was extracted in distilled water three times for 3 h each at a temperature of 60-70°C. The extract was concentrated in multiple effect evaporators at 60-70°C and 650 mg Hg vacuum to about 40% w/w total solids. Concentrated extract was dried in spray drier using an inlet temperature of 170-180°C and outlet temperature of 75-80°C while a contact time of 5 min with hot air was maintained. The extract preparation was normalised for the picroside I and picroside II content. The amounts of picroside I and picroside II contents in the extract were determined using high performance liquid chromatography (HPLC) and were kept at 4.9 and 5.2% (w/w), respectively.

For preparation of the herb stock solution, 100 mg of dried extract powder was mixed in 100 ml of water by gentle shaking and warming at 90°C for 10 min. The suspension was centrifuged at 1600 *g* for 10 min. The amount of solids in the supernatant was measured gravimetrically.

Biologicals

Adult Swiss albino mice (6-8 weeks old) of either sex, bred and nurtured in the animal house of the Department of Zoology, University of Pune, were used for the experiment. Prior approval for the protocols involving animal work was obtained from the Pune University Institutional Animal Ethical Committee. Animals were maintained with 12:12 h light: dark cycle and were given food and water *ad libitum*. Mice used in these studies were sacrificed for the separate project is done by another investigator for pancreatic islet culture. Subsequently discarded animals were used to harvest liver which was sliced and used for all further experiments.

Quantitative determination of total phenols and total flavonoids

The total phenolic content of aqueous extract was measured using a modified Folin-Ciocalteu method[22] and gallic acid as standard. Amount of phenolics is expressed as milligram of gallic acid equivalents per gram powder of *P. kurroa* extract. Flavonoid content of the extract was measured following the method of Luximon-Ramma.[23] The values obtained were compared with a standard curve of quercetin concentrations and expressed as milligram of quercetin equivalents per gram powder of *P. kurroa* extract. All the assays were replicated at least three times and values expressed are an average of these replicates.

In vitro antioxidant capacity assays

DPPH radical scavenging ability of the extract was tested following the method of Aquino.[24] The ability of the extract to reduce ferric complex was assayed according to the protocol developed by Pulido,[25] whereas the potential of extract to inhibit ABTS radical cation ($ABTS^{+}$) formation was evaluated by measuring the lag time in formation of the radical by $ABTS^{+}$/ferrymyoglobin assay.[26] In all these assays, antioxidant activity is expressed as equivalent to μg/ml of standard antioxidant L-ascorbic acid. All the assays were replicated at least three times and values expressed are an average of these replicates.

Liver slice culture in vitro

Liver slice cultures were prepared and maintained following the protocol developed by Wormser[27,28] and Invittox Protocol No. 42.[29] The liver lobes from the sacrificed mice were removed and transferred liver lobes were removed and transferred to prewarmed Krebs Ringer Hepes (KRH) buffer (2.5 mM Hepes, pH 7.4, 118 mM NaCl, 2.85 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM KH_2PO_4, 1.18 mM $MgSO_4$, 5 mM β-hydroxy butyrate and 4.0 mM glucose). Liver was then cut into thin slices using sharp scalpel blades and

the slices, weighing between 4 and 6 mg, were used for the experiment. Each experimental system contained 20-22 slices. These slices were washed with 10 ml KRH buffer every 10 min over a period of 1 h and then preincubated for 60 min in small plugged beakers containing 2 ml KRH on a shaker water bath at 37°C. At the end of preincubation, the medium was replaced by 2 ml of fresh KRH and incubated for 2 h at 37°C with alcohol (1732 mM) alone or alcohol with different concentrations of *P. kurroa* (0.5 mg/ml, 10 mg/ml and 20 mg/ml). At the end of incubation, each group of slices was homogenised in chilled potassium phosphate buffer (100 mM, pH 7.8) in an ice bath at 100 mg/ml concentration. The homogenates and spent medium were centrifuged at 10,000 rpm (9760 *g*) for 10 min at 4°C. The supernatants were collected and activities of cytotoxicity marker enzymes, namely, lactate dehydrogenase (LDH), glutamate oxaloacetate transaminase (GOT), and glutamate pyruvate transaminase (GPT) were checked. The homogenates were also assayed for the antioxidant enzymes, namely, catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-*S*-transferase (GST) and for the antioxidant molecules like reduced glutathione (GSH) and uric acid. Oxidative damage induced by ethanol was estimated by measuring lipid peroxidation.

Measurement of cytotoxic marker enzymes

Samples were assayed for cytotoxic marker enzymes, namely, LDH, GOT and GPT. LDH was measured using the standard protocol.[30] Enzyme units, both in the medium and in the tissue homogenate, were estimated and release of enzyme from liver slices was calculated as the ratio of enzyme activity found in the supernatant to the total enzyme (supernatant + homogenate) activity.[27,28] GOT and GPT activities were estimated using Accurex kits[31] (Accurex Biomedical Pvt. Ltd., India). Experiments were repeated at least four times and the values given are average of three replicates.

Measurement of lipid peroxidation

Lipid peroxidation was measured in terms of nanomoles of MDA equivalents formed[32] [briefly, samples were boiled with TBA reagent (20% TCA, 0.5% TBA, 2.5 N HCl and 6 mM EDTA] for 20 min in a boiling water bath. After cooling, the pink colour representative of thiobarbutiric acid reactive substances (TBARS) was measured at 532 nm. The amount of TBARS formed is expressed as nanomoles of TBARS formed per milligram protein.

Measurement of antioxidant enzymes

SOD,[33] CAT,[34] GR[35] and GPx[36] activities were determined in the tissue homogenate using the standard protocols. GST was estimated[37,38] using GST TagTM assay kit (Novagen, USA and Canada). All the assays were done at least four times and the values given are average of three replicates. Values are expressed as U/100 mg of tissue.

Measurement of antioxidant molecules

Uric acid

Levels of uric acid were determined using an accurex kit (Accurex Biomedical Pvt. Ltd., India). Uricase converts uric acid to allantoin and hydrogen peroxide. The generated hydrogen peroxide degrades a phenolic chromagen to form a red coloured compound, which is measured at 510 nm. Values are expressed as mg/100 mg of tissue and are average of four different experiments.

Glutathione

For the estimation of glutathione, the liver slices were homogenised in phosphate buffer saline (PBS) and were processed according to the protocol described by Teixeira and Meneghini.[39] Values are expressed as μmoles/100 mg of tissue and are average of four different experiments.

Measurement of protein

Protein was estimated following the method of Lowry *et al*.[40]

Statistical Analysis

All statistical tests were two-tailed, and the level of significance was set at 0.05. All data are presented as mean±standard deviation (SD) for all groups. Statistical analyses were performed with SPSS, version 18 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) with *post-hoc* test by Duncan's multiple-range test was used to examine differences among groups. The data were analysed by Student's *t*-test for two group comparison.

RESULTS

Determination of Total Phenolic and Flavonoid Contents of Extract

The aqueous *P. kurroa* extract was analysed for its total phenolic and flavonoid contents by biochemical methods. The total flavonoid contents of 20, 10 and 0.5 mg/ml of *P. kurroa* extract were 13.3±1.0, 9.2±1.1 and 5.6±1.4 mg

Table 1: Total phenolic and total flavonoid contents of *P. kurroa* extract

Extracts	Flavonoid contents (mg of quercetin equivalents/g *P. kurroa* extract)	Phenolic contents (mg of quercetin equivalents/g *P. kurroa* extract)
PE (20 mg/ml)	13.3±1.0	42±1.3
PE (10 mg/ml)	9.2±1.1	29.4±2.3
PE (0.5 mg/ml)	5.6±1.4	11±0.4

PE (20 mg/ml): 20 mg/ml *P. kurroa* extract, PE (10 mg/ml): 10 mg/ml *P. kurroa* extract, PE (0.5 mg/ml): 0.5 mg/ml *P. kurroa* extract. Data represent mean±SD of five independent experiments. Data were analysed using one-way ANOVA followed by Bonferroni's test

quercetin/*P. kurroa* extract, respectively [Table 1]. The total phenolic contents of 20, 10 and 0.5 mg/ml of *P. kurroa* extract were 42±1.3, 29.2±2.3 and 11±0.4 mg quercetin/*P. kurroa* extract, respectively [Table 1].

P. kurroa Extract Exhibits High *In Vitro* Antioxidant Activity

In both DPPH and ferrymyoglobin radical scavenging assays *P. kurroa* extract showed concentration-dependent activity [Table 2 and Figure 1]. In ferric reducing antioxidant power (FRAP) assay, the maximum ferric reducing ability of 1.1±0.03 µg/ml ascorbic acid equivalent (AEAC) was recorded, whereas in DPPH assay, the maximum radical scavenging activity was 0.8±0.03 µg/ml AEAC [Table 2].

Assessment of Ethanol-induced Hepatotoxicity

In liver slice cultures, the release of intracellular enzymes, namely, LDH, GOT and GPT, was used as cytotoxicity biomarkers. It was observed that in case of liver slices treated with ethanol, 51% LDH was released compared to 10.2% in the case of untreated liver slices. For GOT and GPT, release was 52.3% and 51.5%, respectively, for ethanol-treated liver slices compared to 9.9% and 9.2%, respectively, in the case of control tissue [Table 3].

Assessment of Hepatoprotection of *P. kurroa* Against Ethanol-induced Hepatotoxicity

P. kurroa by itself had no cytotoxic effects on the liver slices at a wide range of concentrations (0.5-0.1%; data not shown). Percent release of LDH from slices treated with the highest concentration (20 mg/ml) of *P. kurroa* extract alone (8.5%± 0.14) was found to be similar to that in the case of control untreated slices (10.2%±2.6) [Table 3]. In all the experiments, three concentrations of *P. kurroa*, viz., 20 mg/ml, 10 mg/ml and 0.5 mg/ml were used, while the time course for the release of cytotoxicity marker enzyme was followed only for the highest concentration of *P. kurroa* (20 mg/ml). *P. kurroa*, when added along with ethanol, inhibited the release of all three marker enzymes in a concentration-dependent manner. Ascorbic acid (10 mM) was used as a standard antioxidant and protected the liver slices from the cytotoxic effect of ethanol [Table 3].

Following the time course for release of LDH, GOT and GPT, it was found that these marker enzymes were released continuously over a period of 2 h in presence of ethanol, and when *P. kurroa* was added along with ethanol, this release was suppressed within 30 min [Figure 2a-c].

P. kurroa Extract Protects Liver Slices by Lowering Lipid Peroxide Formation Induced by Ethanol

Lipid peroxidation was measured in terms of TBARS and expressed as picomoles of MDA formed/100 mg tissue. In ethanol-treated liver slices, the amount of lipid peroxidation increased approximately threefold (3.3±0.05 pmol of MDA/100 mg tissue) compared [Figure 3] to control untreated liver slices (1.23±0.09 pmol of MDA/100 mg tissue). In the presence of *P. kurroa*, the ethanol-dependent

Table 2: DPPH (radical scavenging activity) and FRAP of various *P. kurroa* extract

	DPPH (µg/ml)	FRAP (µg/ml)
PE (20 mg/ml)	0.8±0.03	1.1±0.03
PE (10 mg/ml)	0.6±0.02	0.8±0.04
PE (0.5 mg/ml)	0.3±0.02	0.3±0.002

Radical scavenging activities are expressed as ascorbic acid equivalent antioxidant activity (AEAC). PE (20 mg/ml): 20 mg/ml *P. kurroa* extract, PE (10 mg/ml): 10 mg/ml *P. kurroa* extract, PE (0.5 mg/ml): 0.5 mg/ml *P. kurroa* extract. Data represent mean±SD of five independent experiments. Data were analysed using one-way ANOVA followed by Bonferroni's test; DPPH – 1,1'-diphenyl-2-picrylhydrazyl; FRAP – Ferric reducing antioxidant power

Table 3: Effects of *P. kurroa* extract on percent release of LDH, GOT and GPT used as cytotoxic markers from cultured liver slices in response to different treatments

	LDH	GOT	GPT
Control	10.2±2.6	9.9±3.2	9.2±4.1
OH	51±1.9^{ab}	52.3±1.3^{ab}	51.5±2.0^{ab}
10 mM AA	5.5±2.9	7.5±1.3	8.09±0.9
10 mM AA + OH	9.6±0.6	11.8±0.95	10.0±1.5
PE (20 mg/ml)	8.5±0.14	8.8±1.8	7.09±1.2
PE (20 mg/ml) + OH	14.3±4.5^{c}	13.8±4.0^{c}	15.2±3.9^{c}
PE (10 mg/ml) + OH	26.8±2.4^{c}	26.9±4.3^{c}	23.6±1.9^{c}
PE (0.5 mg/ml) + OH	38.1±1.6^{c}	36.4±1.1^{c}	37.3±7.2^{c}

^{abc}Data in the same row not sharing a common superscript indicate a significant difference (P<0.05) between groups after one-way ANOVA and Duncan's multiple-range test; mean±SD (n = 5). ^{ab}Indicates significant difference from control (P<0.001). ^{c}Indicates significant difference from ethanol-treated liver slices (P<0.05). Control: Untreated control, OH: Ethanol, AA (10 mM): 10 mM ascorbic acid, AA (10 mM) + OH: 10 mM ascorbic acid in the presence of ethanol, PE (20 mg/ml): 20 mg/ml *P. kurroa* extract, PE (20 mg/ml + OH): 20 mg/ml *P. kurroa* extract in the presence of ethanol, PE (10 mg/ml + OH): 10 mg/ml *P. kurroa* extract in the presence of ethanol, PE (0.5 mg/ml + OH): 0.5 mg/ml *P. kurroa* extract in the presence of ethanol. Data represent mean±SD of five independent experiments LDH – Lactate dehydrogenase; GOT – Glutamate oxaloacetate transaminase; GPT – Glutamate pyruvate transaminase;

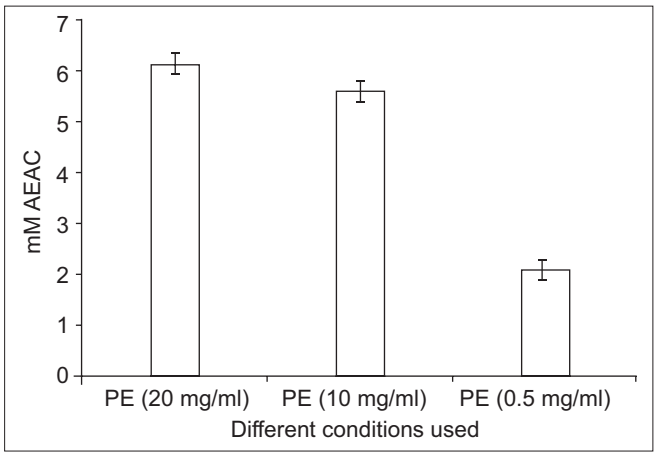

Figure 1: Inhibition of ferrylmyoglobin radical formation by various concentrations of *P. kurroa* extract. Inhibition was measured in terms of ascorbic acid equivalent antioxidant activity (AEAC). PE (20 mg/ml): 20 mg/ml *P. kurroa* extract, PE (10 mg/ml): 10 mg/ml *P. kurroa* extract, PE (0.5 mg/ml): 0.5 mg/ml *P. kurroa* extract. Data represent mean±SD of five independent experiments. Data were analysed using one-way ANOVA followed by Bonferroni's test

formation of TBARS was decreased in a concentration-dependent manner [Figure 3], and in the presence of *P. kurroa* alone (1.25 ± 0.02 pmol/100 mg tissue), the lipid peroxidation was almost similar to that of control untreated cells. Time course analysis of lipid peroxidation in ethanol-treated cells revealed gradual increase in the formation of TBARS which paralleled the increase in release of LDH, GOT and GPT in the liver slices. However, when the liver slices were treated with *P. kurroa* and ethanol together, lipid peroxidation reduced significantly ($P \leq 0.05$) in comparison to that of ethanol alone, within 2 h of treatment. Since lipid peroxidation is triggered by free radicals, *P. kurroa* extract appears to reduce the amount of free radicals generated [Figure 3].

P. kurroa Extract Modulates Antioxidant Status in Liver Cells in Response to Ethanol-induced Oxidative Stress

Antioxidant Enzymes

Intracellular antioxidant enzymes, SOD, CAT and GPx, were quantified. In the presence of ethanol, the activities of SOD and CAT were found to be significantly increased compared to control, whereas a small decrease was seen in GPx [Table 4]. In liver slices treated with *P. kurroa* extract alone, activities of SOD and CAT were similar to that of

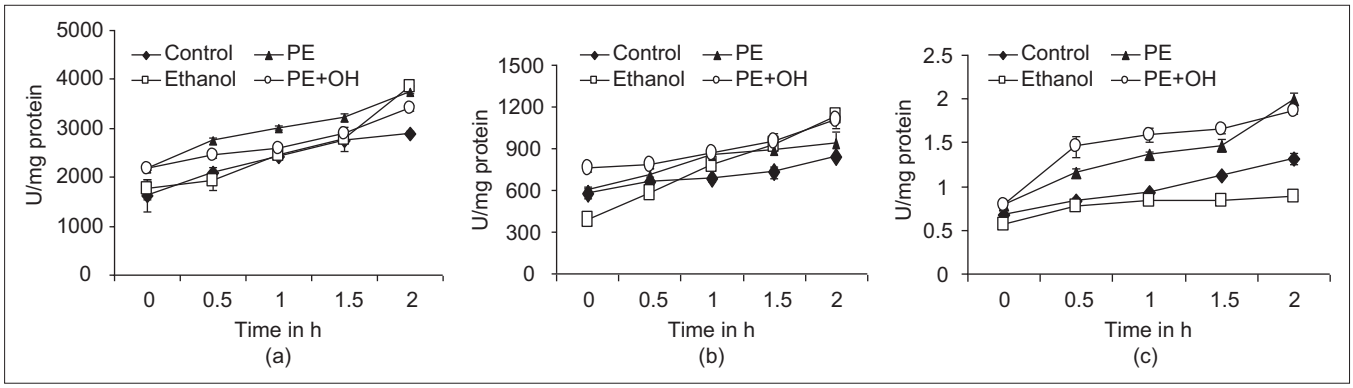

Figure 2: Time course for release of (a) LDH, (b) GOT, (c) GPT from cultured liver slices exposed to various treatments. Control: Untreated control, OH: Ethanol, PE (20 mg/ml): 20 mg/ml *P. kurroa* extract alone, PE + OH: *P. kurroa* and ethanol. Data represent mean±SD of five independent experiments. Treatment effects were analysed using one-way ANOVA followed by *post-hoc* test by Duncan's multiple-range test

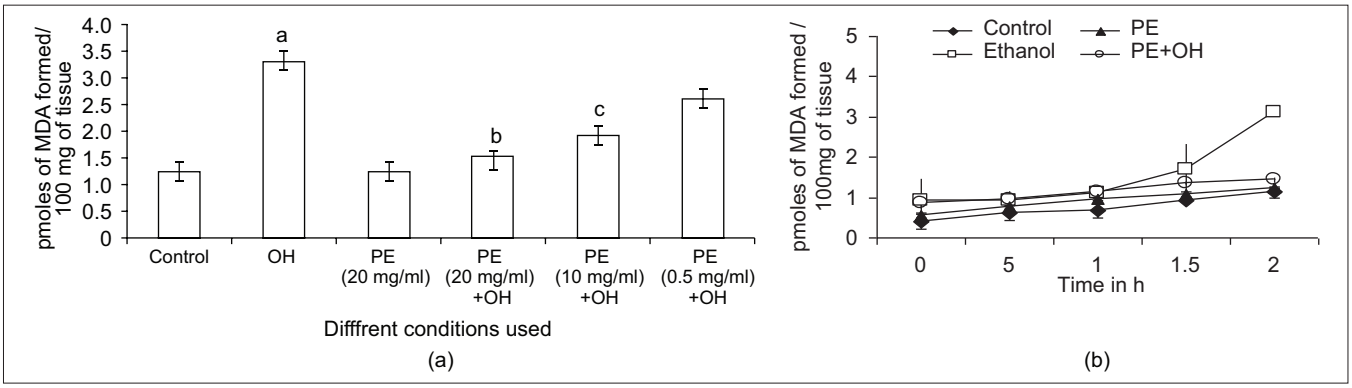

Figure 3: (a) Lipid peroxidation in liver slice culture. [a]Indicates significant difference from control ($P<0.001$). [b,c]Indicate significant difference from ethanol-treated liver slices ($P<0.05$). Control: Untreated control, OH: Ethanol, PE (20 mg/ml): 20 mg/ml *P. kurroa* extract, PE (20 mg/ml + OH): 20 mg/ml *P. kurroa* extract in the presence of ethanol, PE (10 mg/ml + OH): 10 mg/ml *P. kurroa* extract in the presence of ethanol, PE (0.5 mg/ml + OH): 0.5 mg/ml *P. kurroa* extract in the presence of ethanol. Treatment effects were analysed using one-way ANOVA by Duncan's multiple-range test. (b) Time course of lipid peroxidation (pmoles of MDA/100 mg of tissue) in liver slice culture measured at interval of 30 min. Control: Untreated control, OH: Ethanol, PE (20 mg/ml): 20 mg/ml *P. kurroa* extract alone, PE + OH: *P. kurroa* and ethanol. Data represent mean±SD of five independent experiments. Treatment effects were analysed using one-way ANOVA followed by *post-hoc* test by Duncan's multiple-range test

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

	Control	PE (20 mg/ml)	PE (20 g/ml+OH)	PE (10 mg/ml+OH)	PE (0.5 mg/ml+OH)	OH
CAT	2959±298	3279±734.4	3450±292[b]	3567±594[c]	3638±361	4375±525[a]
SOD	845±41	931±46	1060±122[b]	1131±131[c]	1207.2±143	1223.4±0.8[a]
GPx	1.4±0.3	1.9±0.7	1.9±0.3[b]	1.9±0.2[c]	2.08±0.2	0.72±0.1[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). Control: Untreated control, OH: Ethanol, PE (20 mg/ml): 20 mg/ml *P. kurroa* extract, PE (20 mg/ml + OH): 20 mg/ml *P. kurroa* extract in the presence of ethanol, PE (10 mg/ml + OH): 10 mg/ml *P. kurroa* extract in the presence of ethanol, PE (0.5 mg/ml + OH): 0.5 mg/ml *P. kurroa* extract in the presence of ethanol CAT - Catalase, SOD - Superoxide dismutase, GPx - Glutathione peroxidase

the control untreated slices. When various concentrations of *P. kurroa* were administered with ethanol, the liver slices showed dose-dependent protection against ethanol-induced rise in the activities of SOD and CAT. Following the time course, SOD and CAT showed a gradual increase, while GPx activity gradually dropped in ethanol-treated slices compared to controls within 30 min. Such change in the activity of all the enzymes was inhibited in the presence of extract [Figure 4a-c].

GR is involved in synthesis of GSH, which is a major antioxidant in the cell, and GST catalyses the conjugation of GSH to electrophilic centres via the sulphydryl group, on a wide variety of substrates. Liver slices treated with ethanol showed a decrease in the activity of these enzymes. Protection against such decrease was restored by *P. kurroa* extract in a concentration-dependent manner [Figures 5a and 6a]. Moreover, time course revealed that within 30 min, activities of GR and GST were restored [Figures 5b and 6b].

Antioxidant molecules

Glutathione and uric acid are small antioxidant molecules which directly scavenge the free radicals. Levels of both these molecules were decreased in liver slices treated with ethanol as compared to the untreated slices [Figures 7a and 8a]. Ethanol-induced decrease in glutathione and uric acid levels was normalised in a concentration-dependent manner by the addition of *P. kurroa* extract. Glutathione and uric acid levels decreased gradually over a period of 2 h in response to ethanol treatment, but such decline was suppressed when *P. kurroa* extract was included with ethanol. In case of control untreated liver slices and those treated with *P. kurroa* extract alone, glutathione and uric acid levels remained unchanged over a period of 2 h [Figures 7b and 8b].

DISCUSSION

P. kurroa is a well-known herb in the *Ayurvedic* system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract. Kutkin is the active principle of *P. kurroa* and comprises kutkoside and the iridoid glycosides, picrosides I, II, and III. Other identified active constituents are apocynin,

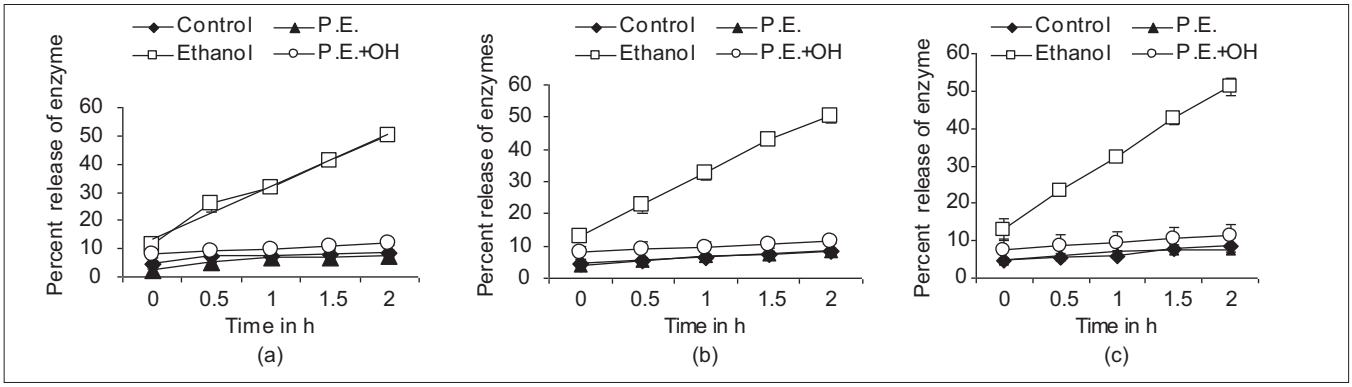

Figure 4: Time course for various antioxidant enzymes in the presence of ethanol and ethanol with *P. kurroa* extract: (a) catalase, (b) superoxide dismutase (SOD), and (c) glutathione peroxidase (GPx). Control: Untreated control, OH: Ethanol, PE (20 mg/ml): 20 mg/ml *P. kurroa* extract alone, PE + OH: *P. kurroa* and ethanol. Data represent mean±SD of five independent experiments. Treatment effects were analysed using one-way ANOVA followed by *post-hoc* test by Duncan's multiple-range test

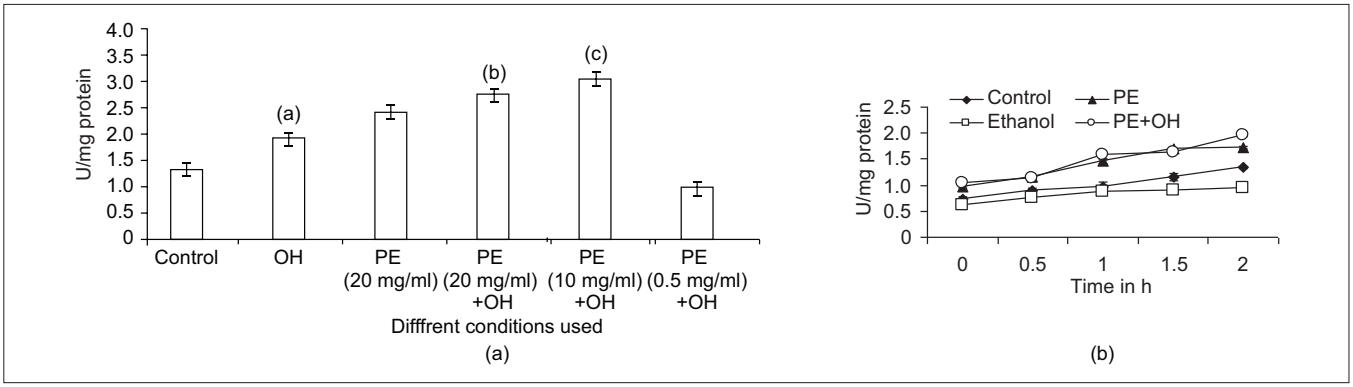

Figure 5: (a) Effect of *P. kurroa* extract on glutathione reductase in liver slice culture. [a]Indicates significant difference from control (P<0.001). [b,c]Indicate significant difference from ethanol-treated liver slices (P<0.05). Control: Untreated control, OH: Ethanol, PE (20 mg/ml): 20 mg/ml *P. kurroa* extract, PE (20 mg/ml + OH): 20 mg/ml *P. kurroa* extract in the presence of ethanol, PE (10 mg/ml + OH): 10 mg/ml *P. kurroa* extract in the presence of ethanol, PE (0.5 mg/ml + OH): 0.5 mg/ml *P. kurroa* extract in the presence of ethanol. (b) Time course for glutathione measured at an interval of 30 min under various conditions. Control: Untreated control, OH: Ethanol, PE (20 mg/ml): 20 mg/ml *P. kurroa* extract alone, PE + OH: *P. kurroa* and ethanol. Data represent mean±SD of five independent experiments. Treatment effects were analysed using one-way ANOVA followed by *post-hoc* test by Duncan's multiple-range test

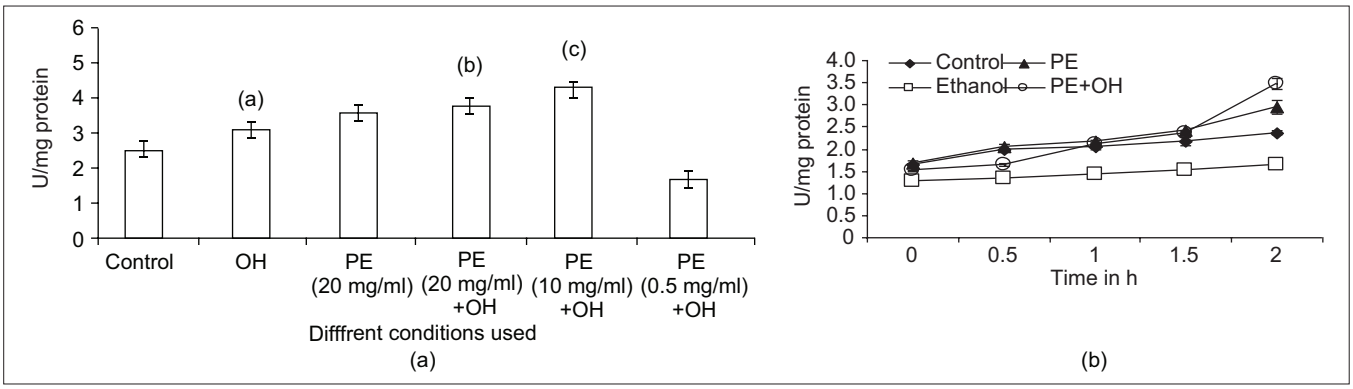

Figure 6: (a) Effect of *P. kurroa* extract on glutathione-*S*-transferase (GST) in liver slice culture. [a]Indicates significant difference from control (P<0.001). [b,c]Indicate significant difference from ethanol-treated liver slices (P<0.05). Control: Untreated control, OH: Ethanol, PE (20 mg/ml): 20 mg/ml *P. kurroa* extract, PE (20 mg/ml + OH): 20 mg/ml *P. kurroa* extract in the presence of ethanol, PE (10 mg/ml + OH): 10 mg/ml *P. kurroa* extract in the presence of ethanol, PE (0.5 mg/ml + OH): 0.5 mg/ml *P. kurroa* extract in the presence of ethanol. (b) Time course for GST measured at an interval of 30 min under various conditions. Control: Untreated control, OH: Ethanol, PE (20 mg/ml): 20 mg/ml *P. kurroa* extract alone, PE + OH: *P. kurroa* and ethanol. Data represent mean±SD of five independent experiments. Treatment effects were analysed using one-way ANOVA followed by *post-hoc* test by Duncan's multiple-range test

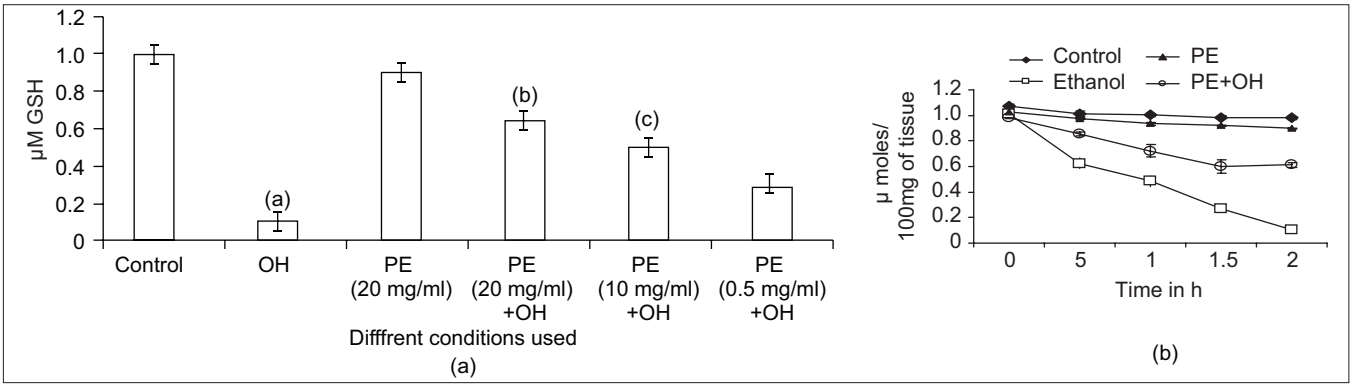

Figure 7: (a) Effect of *P. kurroa* extract on glutathione in liver slice culture. [a]Indicates significant difference from control (P<0.001). [b,c]Indicate significant difference from ethanol-treated liver slices (P<0.05). Control: Untreated control, OH: Ethanol, PE (20 mg/ml): 20 mg/ml *P. kurroa* extract, PE (20 mg/ml + OH): 20 mg/ml *P. kurroa* extract in the presence of ethanol, PE (10 mg/ml + OH): 10 mg/ml *P. kurroa* extract in the presence of ethanol, PE (0.5 mg/ml + OH): 0.5 mg/ml *P. kurroa* extract in the presence of ethanol. (b) Time course for glutathione measured at an interval of 30 min under various conditions. Control: Untreated control, OH: Ethanol, PE (20 mg/ml): 20 mg/ml *P. kurroa* extract alone, PE + OH: *P. kurroa* and ethanol. Data represent mean±SD of five independent experiments. Treatment effects were analysed using one-way ANOVA followed by *post-hoc* test by Duncan's multiple-range test

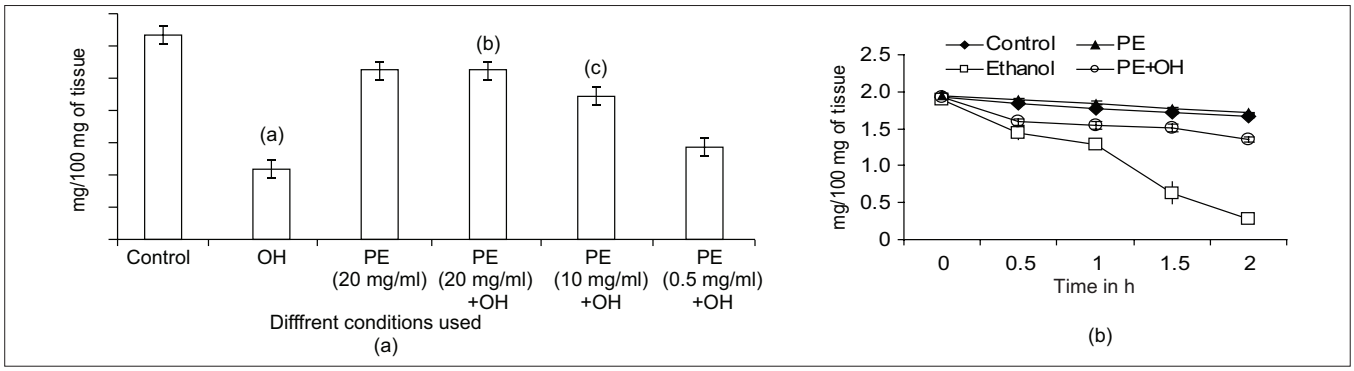

Figure 8: (a) Effect of *P. kurroa* extract on uric acid in liver slice culture. [a]Indicates significant difference from control (P<0.001). [b,c]Indicate significant difference from ethanol-treated liver slices (P<0.05). Control: Untreated control, OH: Ethanol, PE (20 mg/ml): 20 mg/ml *P. kurroa* extract, PE (20 mg/ml + OH): 20 mg/ml *P. kurroa* extract in the presence of ethanol, PE (10 mg/ml + OH): 10 mg/ml *P. kurroa* extract in the presence of ethanol, PE (0.5 mg/ml + OH): 0.5 mg/ml *P. kurroa* extract in the presence of ethanol. (b) Time course for uric acid measured at an interval of 30 min under various conditions. Control: Untreated control, OH: Ethanol, PE (20 mg/ml): 20 mg/ml *P. kurroa* extract alone, PE + OH: *P. kurroa* and ethanol. Data represent mean±SD of five independent experiments. Treatment effects were analysed using one-way ANOVA followed by *post-hoc* test by Duncan's multiple-range test

drosin, and nine cucurbitacin glycosides.[41,42] Apocynin is a catechol that has been shown to inhibit neutrophil oxidative burst in addition to being a powerful anti-inflammatory agent,[43] while the curcubitacins have been shown to be highly cytotoxic and they possess antitumour effects.[41]

The hepatoprotective action of *P. kurroa* is not fully understood but may be attributed to its ability to inhibit the generation of ROS and to scavenge free radicals.[44]

Antioxidative potential in terms of scavenging of DPPH and ferrylmyoglobin, and reducing ability by FRAP along with lipid peroxidative inhibitory activities of the aqueous *P. kurroa* extract was assayed in an *in vitro* system. In all the assays performed, *P. kurroa* extract revealed strong antioxidant activity and also inhibited lipid peroxidation significantly.

Furthermore, hepatoprotective effects of the *P. kurroa* extract in ethanol-induced toxicity were determined using liver slice culture model. The liver slice is a highly organised cellular community in which the different cell types are maintained. Liver slice culture is therefore a useful *in vitro* technique that offers the advantages of an *in vivo* situation and hence is a more suitable model for the experimental analysis of hepatotoxic events.[45]

We have quantified ethanol toxicity in liver slice culture in terms of release of LDH, GOT and GPT into the medium. These three cytosolic enzymes are mainly present in the periportal hepatocytes and are released when the cells are damaged by a hepatotoxin. It was observed that in case of liver slices treated with ethanol, approximately 51%, 52.3% and 51.3% cytotoxicity marker enzymes (LDH, GOT and GPT, respectively) were released as compared to 10% in the case of control untreated liver slices [Table 3]. *P. kurroa* (20 mg/ml) extract showed a high hepatoprotective effect against ethanol-induced cytotoxicity [Figure 2].

Ethanol administration is known to induce an increase in lipid peroxidation either by enhancing the production of ROS and/or by decreasing the level of endogenous antioxidants.[46] Amount of MDA equivalents is estimated by measuring the levels of TBARS and has been widely used in studies of anti-lipid peroxidation activity of natural phytochemicals in cultured cells[47] [Figure 3]. The TBARS formation, enhanced in the presence of ethanol, was significantly reduced in the presence of *P. kurroa* extract.

In the present study, we found that the activities of antioxidant enzymes, SOD and CAT, linearly increased gradually up to 2.0 h in the liver tissue treated with ethanol. Increase in CAT and SOD activities in plasma and liver[48] and in hepatic tissue[49,50] has been reported during ethanol toxicity. Significant increase in CAT activity was found[51-53] in the hepatic tissue of rats treated with ethanol for a period of 4 weeks. In our liver slice culture system, even a short exposure to ethanol led to increase in the activities of CAT and SOD and decrease in the activity of GSH-Px [Table 3].

GSH-Px mediated H_2O_2 degradation is considered the preferential pathway of elimination of a low concentration of H_2O_2. GSH-Px requires GSH for degradation of H_2O_2. In the present study, as enough GSH was not present, reduced activity of GSH-Px was recorded. This result correlated with the levels of glutathione (GSH) which were found to be significantly decreased ($P<0.05$) in the presence of ethanol and was restored in the presence of the *P. kurroa* extract [Figure 7]. The levels of uric acid, another antioxidant molecule, were also decreased with ethanol treatment and were normalised within 30 min in the presence of *P. kurroa* extract [Figure 8].

GR indirectly contributes in combating oxidative stress by regenerating GSH. The activity of GR was significantly decreased in ethanol-treated liver cells [Figure 5]. Earlier reports have also revealed decrease in GR activity[54] in hepatic tissues of rats treated with ethanol, which leads to impaired conversion of oxidised glutathione to reduced form, thereby altering the GSH/GSSG ratio.[53] GST catalyses the conjugation of GSH to electrophilic centres on a wide variety of substrates, via the sulphydryl group. This activity is useful in the detoxification of endogenous compounds such as peroxidised lipids. Hepatic GST activity was found to be decreased in ethanol-treated liver cells and such decrease was prevented in the presence of *P. kurroa* extract [Figure 5]. The enhanced activity of GST, a phase II enzyme, in case of cells treated with *P. kurroa* extract alone would also lead to increased detoxification of the reactive metabolites generated from the ethanol metabolism in the liver of *P. kurroa* treated liver tissue.

Thus, all these data together strongly point out high antioxidant and hepatoprotective activity of the extract of *P. kurroa* and justify its inclusion in many *Ayurvedic* preparations used as hepatoprotectants.

CONCLUSION

Thus, the present data provide a rationale for use of *P. kurroa* as a suitable herbal treatment from Indian medicinal plants for the management of alcohol-induced liver damage.

ACKNOWLEDGMENT

VPS, MKJ, SSG designed the study. SS and JB prepared the aqueous extract from *P. kurroa*. SS conducted all *in vitro* experiments. All authors read and approved the final version of the manuscript.

REFERENCES

1. Hsiao G, Lin YH, Lin CH, Chou DS, Lin WC, Sheu JR. Biol Pharm Bull 2001;24:1271-6
2. Christina AJ, Saraswathy GR, Robert SJ, Kothai R, Chidambaranathan N, Nalini G, *et al*. Inhibition of CCl4-induced liver fibrosis by *Piper longum* Linn? Phytomedicine 2006;13:196-8.

3. Zima T, Fialová L, Mestek O, Janebová M, Crkovská J, Malbohan I, *et al*. Oxidative stress, metabolism of ethanol and alcohol-related diseases. J Biomed Sci 2001;8:59-70.
4. Schlorff EC, Husain K, Soman SM. Dose- and time-dependent effects of ethanol on plasma antioxidant system in rat. Alcohol 1999;17:97-105.
5. Pal R, Nath R, Gill KD. Lipid peroxidation and antioxidant defense enzymes in various regions of adult rat brain after coexposure to cadmium and ethanol. Pharmacol Toxicol 1993;73:209-14.
6. Ansari RA, Aswal BS, Chander R, Dhawan BN, Garg NK, Kapoor NK, *et al*. Hepatoprotective activity of Kutkin- the iridoid glycoside mixture of Picrorhiza kurroa. Indian J Med Res 1988;87:401-7.
7. Jagetia GC, Baliga MS. The evaluation of nitric oxide scavenging activity of certain Indian medicinal plants *in vitro*: A preliminary study. J Med Food 2004;7:343-8.
8. Senthil KSH, Anandan R, Devaki T, Santhosh Kumar M. Cardioprotective effects of *Picrorhiza kurroa* against isoproterenol-induced myocardial stress in rats. Fitoterapia 2001;72:402-5.
9. Jeena KJ, Joy KL, Kuttan R. Effect of *Emblica officinalis, Phyllanthus amarus* and *Picrorhiza kurroa* on N-nitrosodiethylamine induced hepatocarcinogenesis. Cancer Lett 1999;136:11-6.
10. Rajaprabhu D, Rajesh R, Jeyakumar R, Buddhan S, Ganesan B, Anandan R. Protective effect of *Picrorhiza kurroa* on antioxidant defense status in adriamycin-induced cardiomyopathyin rats. J Med Plant Res 2007;1:80-5.
11. Joy KL, Kuttan R. Anti-diabetic activity of *Picrorhiza kurrooa* extract. J Ethnopharmacol 1999;67:143-8.
12. Saraswat B, Visen PK, Patnaik GK, Dhawan BN. *Ex vivo* and *in vivo* investigations of picroliv from *Picrorhiza kurroa* in an alcohol intoxication model in rats. J Ethnopharmacol 1999;66:263-9.
13. Rastogi R, Saksena S, Garg NK, Kapoor NK, Agarwal DP, Dhawan BN. Picroliv protects against alcohol-induced chronic hepatotoxicity in rats. Planta Med 1996;62:283-5.
14. Shukla B, Visen PK, Patnaik GK, Dhawan BN. Choleretic effect of picroliv, the hepatoprotective principle of *Picrorhiza kurroa*. Planta Med 1991;57:29-33.
15. Visen PK, Shukla B, Patnaik JK. Hepatoprotective activity of Picroliv. The active principle of *Picrorhiza kurroa* on rat hepatocytes against paracetamol toxicity. Drug Dev Res 1991;22:209-10.
16. Negi AS, Kumar JK, Luqman S, Shanker K, Gupta MM, Khanuja SP. Recent advances in plant hepatoprotectives: A chemical and biological profile of some important leads. Med Res Rev 2007;28:746-72.
17. Ramachandra SS, Quereshi AA, Viswanath SAH, Patil T, Prakash T, Prabhu K, *et al*. Hepatoprotective activity of *Calotropis procera* flowers against paracetamol-induced hepatic injury in rats. Fitoterapia 2007;78:451-4.
18. Togashi H, Shinzawa H, Wakabayashi H, Nakamura T, Yong H, Yamada N, *et al*. Superoxide is involved in the pathogenesis of paraquat-induced injury in cultured rat liver slices. Hepatology 1991;14:707-14.
19. Naik RS, Mujumdar AM, Ghaskadbi S. Protection of liver cells from ethanol cytotoxicity by curcumin in liver slice culture *in vitro*. J Ethnopharmacol 2004;95:31-7.
20. Sinha S, Priyajali D, Sujata B, Devasagayam TP, Saorj G. Bark and fruit extracts of *Gmelina arborea* protect liver cells from oxidative stress. Pharm Biol 2006;44:237-43.
21. Mahnaaz M, Swati K, Devasagayam TP, Saroj G. *Pterocarpus marsupium* extract reveals strong *in vitro* antioxidant activity. Drug Discov Ther 2009;3:151-61.
22. Wolfe K, Wu X, Liu RH. Antioxidant activity of apple peels. J Agric Food Chem 2003;51:609-914.
23. Luximon-Ramma A, Bahorun T, Soobrattee MA, Aruoma OI. Antioxidant activities of phenolic, proantocyanidin and flavonoid components in extracts of Cassia fistula. J Agric Food Chem 2002;50:5042-7.
24. Aquino R, Morelli S, Lauro MR, Abdo S, Saija A, Tomaino A. Phenolic constituents and antioxidant activity of an extract of Anthuriumversicolor leaves. J Nat Prod 2001;64:1019-23.
25. Pulido R, Bravo L, Saura-Calixto F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing antioxidant power assay. J Agric Food Chem 2000;48:3396-402.
26. Alzorkey N, Nakahara K. Antioxidant activity of some edible Yemeni plants evaluated by ferrylmyoglobin/$ABTS^{+}$ assay. Food Sci Tehnol Res 2001;7:141-4.
27. Wormser U, Ben-Zakine S. The Liver slice system: An *in vitro* acute toxicity test for assessment of hepatotoxins and their antitodes. Toxicol *in vitro* 1990;4:449-51.
28. Wormser U, Ben Z S, Stivelband E, Eizen O, Nyska A. The Liver slice system: A rapid *in vitro* acute toxicity test for primary screening of hepatotoxic agents. Toxicol *in vitro* 1990;4:783-9.
29. Invittox protocol no.42. The ERGATT/FRAME Data Bank of *in vitro* techniques in toxicology: Liver slice hepatotoxicity screening system. INVITTOX, England. 1992.
30. Whalefeld AW. UV-method with L-lactate and NAD. In: Bergmeyer HU, editor. Methods of Enzymatic Analysis. Vol. 3. Weinheim: Verlag, GmbH; 1983. p. 126-133.
31. Bergmeyer HU, Hørder M, Rej R. International Federation of Clinical Chemistry (IFCC) Scientific Committee, Analytical Section: Approved recommendation (1985) on IFCC methods for the measurement of catalytic concentration of enzymes. Part 3. IFCC method for alanine aminotransferase (L-alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2). J Clin Chem Clin Biochem 1986;24:481-95.
32. Devasagayam TP. Lipid peroxidation in rat uterus. Biochim Biophys Acta 1986;876:507-14.
33. Beauchamp C, Fridovich I. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Anal Biochem 1971;44:276-87.
34. Aebi, HE. Catalase In: Bermeyer HU, editor. Methods of Enzymatic Analysis. 1983. Vol. 3. Weinhein: Verlagchemie GmbH; 1983. p. 277-82.
35. Goldberg DM, Spooner RJ. Glutathione reductase. In: Bermeyer HU, editor. Methods of Enzymatic Analysis. Vol. 3. 1983. Verlagchemie GmbH; 1983. p. 258-65.
36. Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium-deficient rat liver. Biochem Biophys Res Commun 1976;71:952-8.
37. Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 1974;249:7130-9.
38. Smith DB, Johnson KS. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene 1988;67:31-40.
39. Teixeira HD, Meneghini R. Chinese hamster fibroblasts overexpressing CuZn-superoxide dismutase undergo a global reduction in antioxidants and an increasing sensitivity of DNA to oxidative damage. Biochem J 1996;315:821-5.
40. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with folin phenol reagent. J Biol Chem 1951;193;265-75.
41. Weinges K, Kloss P, Henkels WD. Natural products from medicinal plants. XVII. Picroside-II, a new 6-vanilloyl-catapol from Picrorhiza kuroa Royle and Benth. Justus Liebigs Ann Chem 1972;759:173-82.
42. Stuppner H, Wagner H. New cucurbitacin glycosides from Picrorhiza kurroa. Planta Med 1989;55:559-63.
43. Hirnwich HE, Nahum LH, Pakieten N, Fazekas JF, DU Bots, H. Effects of alcohol on metabolism. Am J Physiol 1982;101:57-68.
44. Freinkel N, Arky RA, Singer DL, Cohen AK, Bleicher SJ, Anderson JB, *et al*. Alcohol hypoglycemia IV. Current concepts of its pathogenesis. Diabetes 1965;14:350-61.
45. Gandolfi AJ, Wijeweera J, Brendel K. Use of precision-cut liver slices as an *in vitro* tool for evaluating liver function. Toxicol Pathol

1996;24:58-61.
46. Rouach H, Fataccioli V, Gentil M, French SW, Morimoto M, Nordmann R. Effect of chronic ethanol feeding on lipid peroxidation and protein oxidation in relation to liver pathology. Hepatology 1997;25:351-5.
47. Mahendran P, Shyamala CS. The modulating effect of *garcinia cambogia* extract on ethanol induced peroxidative damage in rats. Indian J Pharmacol 2001;33:87-91.
48. Ishii H, Kurose I, Kato S. Pathogenesis of alcoholic liver disease with particular emphasis on oxidative stress. J Gastroenterol Hepatol 1997;12: S272-82.
49. Wu SJ, Ng LT. Antioxidant and free radical scavenging activities of wild bitter melon (*Momordica charantia* Linn. var. *abbreviata* Ser.) in Taiwan, LWT-Food Sci. Technol 2007;41:323-30.
50. Bindu MP, Sreekant KS, Annamali PT, Augusti KT. Effect of S-allyl cysteine sulphoxide on lipid metabolism and free radical scavengers in alcohol fed rats. Curr Sci 2002;82:628-31.
51. Devi BG, Schenker S, Mazloum B, Handerson, GI. Ethanol-induced oxidative stress and enzymatic defenses in cultured fetal rat hepatocytes. Alcohol 1996;13:327-32.
52. Pigeolet E, Corbisier P, Houbion A, Lambert D, Michiels C, Raes M, *et al*. Glutathione peroxidase, superoxide dismutase, and catalase inactivation by peroxides and oxygen derived free radicals. Mech Ageing Dev 1990;51:283-97.
53. Halliwell B, Gutteridge JM. Free Radicals in Biology and Medicine. Oxford: Clarendon Press; 1993. p. 22-81.
54. Dinu V, Zamfir O. Oxidative stress in ethanol intoxicated rats. Rev Roum Physiol 1991;28:63-7.

How to cite this article: Sinha S, Bhat J, Joshi M, Sinkar V, Ghaskadbi S. Hepatoprotective activity of Picrorhiza kurroa Royle Ex. Benth extract against alcohol cytotoxicity in mouse liver slice culture. Int J Green Pharm 2011;5:244-53.

Source of Support: Nil, **Conflict of Interest:** None declared.