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Water-extractable phytochemicals from *Phyllanthus niruri* exhibit distinct *in vitro* antioxidant and *in vivo* hepatoprotective activity against paracetamol-induced liver damage in mice

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ABSTRACT

The antioxidative and hepatoprotective potential of *Phyllanthus niruri*, a widely used medicinal plant in Brazil, were investigated. The aqueous extracts of leaves showed inhibition against thiobarbituric acid-reactive species (TBARS), induced by different pro-oxidants (10 μM $FeSO_4$ and 5 μM sodium nitroprusside) in rat liver, brain and kidney homogenates. The extracts also lowered the formation of TBARS in phospholipids extracted from egg yolk. The plant exhibited strong antioxidant activity in the 2,2-diphenyl-1-picrylhydrazyl free radical (IC_{50}, 43.4 ± 1.45 μg/ml) and iron chelation assay. The hepatoprotective activity of the extracts were also demonstrated *in vivo* against paracetamol-induced liver damage, as evidenced by the decrease in serum glutamate oxaloacetate transaminase (GOT), and glutamate pyruvate transaminase (GPT), and increased catalase activity in the liver in treatment groups, compared to the control. The results of the present study suggest the potential use of *P. niruri* in the treatment of various diseases, among which liver disease is the most important, due to its ability to act as an antioxidant. Furthermore, since the treatment of human intoxications with paracetamol is always limited to the administration of *N*-acetyl-cysteine, additional studies would be important to determine whether aqueous extracts of *P. niruri* could increase the efficacy of *N*-acetyl-cysteine against paracetamol acute hepatotoxicity.

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1. Introduction

Reactive oxygen species (ROS) are generated spontaneously in cells during metabolism and are implicated in the aeitology of different degenerative diseases, such as heart diseases, stroke, rheumatoid arthritis, diabetes and cancer (Halliwell, Gutteridge, & Cross, 1992). In addition to chronic diseases, oxidative stress can also play a fundamental role in the acute hepatoxicity of several drugs, including the world-widely used analgesic and antipyretic paracetamol (Angela, Richard, Sandra, Robert, & Jack, 2005). Studies have shown that the use of polyphenolic compounds found in tea, fruits and vegetables is associated with low risk of these diseases (Hertog, Hollman, & Van, 1993). Consequently, there is a great deal of interest in edible plants that contain antioxidants and health-promoting phytochemicals as potential therapeutic agents. One such plant is *Phyllanthus niruri* L. (Euphorbiaceae), which grows to the rainforests of amazon and other tropical areas throughout the world, including the Bahamas, southern India and China (Vieira, 1999). In Brazil, the plant is known as "Quebra Pedra" which literally can be translated as "stone breaker". The plant is traditionally used in Brazilian folk medicine for the treatment of several pathological conditions, including urolithiasis. Uriston®, a *P. niruri* extract has shown high efficacy after extracorporeal shock wave lithotripsy for renal stones (Micali et al., 2006). *P. niruri* has gained world-wide attention due to its effects against Hepatitis B (Yeh et al., 1993). Recent research on this plant reveals that its antiviral activity extends to the human immunodeficiency virus (HIV). The HIV-1 reverse transcriptase inhibition properties of *P. niruri* can be obtained with a simple water extract of the plant (Qian-Cutrone, 1996). Several bioactive molecules, such as lignans, phyllanthin, hypophyllanthin, flavonoids, glycosides and tannins, have been reported in the extracts of *P. niruri* (Rajeshkumar et al., 2002).

In this study, we evaluated the relationship between pharmacological and antioxidant effects of aqueous extracts of *P. niruri* leaves, which are used as a herbal tea by local communities in Brazil. The hepatoprotective effects of crude methanolic and aqueous extracts against carbon tetrachloride (CCl_4)-induced liver damage

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in rats have been investigated (Harish & Shivanandappa, 2005). A perusal of the literature revealed that the hepatoprotective effects of water extracts on paracetamol-induced liver damage remains to be studied. Herein, we report the antioxidant and hepatoprotective effect of the aqueous extract of *P. niruri* on paracetamol-induced liver damage in Wistar mice. This is particularly important in view of the fact that the treatments of acute human intoxications with paracetamol are limited and frequently not effective and rely, basically, on the use of *N*-acetyl-cysteine (Groote & Steenbergen, 1995). Liver damage was assessed by serum enzymes (GOT and GPT), liver catalase actvitiy and thiobarbituric acid (TBARS)-reactive species. The *in vitro* antioxidant activity of the drug was evaluated by studying the lipid peroxidation product, malondialdehyde (MDA), using different pro-oxidants in rat liver, brain and kidney homogenates and also phospholipid extracted from egg yolk. Antiradical activitiy of the extract was further confirmed by scavenging of the DPPH radical. The aim of this study was to confirm the antioxidative and hepatoprotective activity of water extracts, these being non-toxic and environmentally friendly and suitable for their use in tea and other formulations.

2. Materials and methods

2.1. Materials

Thiobarbituric acid (TBA), malonaldehyde- bis-dimethyl acetal (MDA) 2,2-diphenyl-1-picrylhydrazyl (DPPH·), quercetin, rutin and phenanthroline were purchased from Sigma (St. Louis, MO, USA). Sodium nitroprusside (SNP) was obtained from Merck (Darmstadt, Germany) and iron (II) sulphate from Reagen (Rio de Janeiro, RJ, Brazil).

2.2. Preparation of plant extract

The plant was purchased from the local market and authenticated by a botanist at University Federal de Santa Maria. Two different batches of the *P. niruri*, which were available in the market as Chileno (PN1) and Prenda (PN2), were used in the study. Dried plant material (25 g) was soaked in boiling water (250 ml) for 15 min, allowed to cool and filtered using Whatman filter paper. The obtained residues were further extracted, twice, and then concentrated using a rotary evaporator. Filtrates were dried to a powder in an oven at 40–50 °C, giving a percentage yield of 21–23%. Serial dilutions of these were made to obtain the desired concentration of plant for the experiment.

2.3. Test animals

All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals. Two to three month old Wistar rats (200–250 g), from our own breeding colony, were used for *vitro* studies. For *in vivo* studies, male albino mice weighing 24–30 g were used. The animals were kept in separate cages with access to water and food *ad libitum*, in a room with controlled temperature (22 °C ± 3) and in a 12 h light/ dark cycle with lights on at 7:00 a.m.

2.4. In vitro assays

2.4.1. Production of TBARS from animal tissues

Production of TBARS was determined using a modified method of Ohkawa, Ohishi, and Yagi (1979). The rats were killed by anaesthetizing them mildly in ether and the tissues (liver, kidney and brain) were quickly removed and placed on ice. One gramme quantities of tissues were homogenised in cold 100 mM Tris-buffer pH 7.4 (1:10 w/v) with ten up and down strokes at approximately 1200 rev/min in a Teflon glass homogenizer. The homogenates were centrifuged for 10 min at 1400*g* to yield a pellet that was discarded and a low-speed supernatant (S1) used for the assay. The homogenates (100 μl) were incubated with or without 50 μl of the various freshly prepared oxidants (iron and nitroprusside) and different concentrations of the plant extracts, together with an appropriate volume of deionized water, to give a total volume of 300 μl at 37 °C for 1 h. The colour reaction was carried out by adding 200, 500 and 500 μl each of the 8.1% sodium dodecyl sulphate (SDS), acetic acid (pH 3.4) and 0.6% TBA, respectively. The reaction mixtures, including those of serial dilutions of 0.03 mM standard MDA, were incubated at 97 °C for 1 h. The absorbance was read after cooling the tubes at a wavelength of 532 nm in a spectrophotometer.

2.4.2. Production of TBARS from phospholipid

Production of TBARS from phospholipid was determined using the method of Ohkawa et al. (1979) but with modifications. The egg yolk was weighed to 1 g and mixed with a solution of hexane-isoprapanol (3:2) and filtered. The solution was dried in a rotary evaporator at 60 °C. Then 0.2 g of the phospholipid was diluted to 10 ml with water, centrifuged and used as a homogenate. The remaining procedure was the same as that mentioned for the tissue except that the colour reaction was carried out without SDS by adding 600 μl of TBA and 600 μl of acetic acid (pH 3.4) for 1 hour. The tubes were cooled with tap water and 2 ml of n-butanol was finally added and the mixture centrifuged. The supernatant was taken and the absorbance was read at 532 nm in a spectrophotometer.

2.4.3. DPPH radical-scavenging

Scavenging of the stable radical, DPPH·, was assayed *in vitro* (Hatano, Kagawa, Yasuhara, & Okuda, 1988). The extract (10–100 μg) was added to a 0.5 ml solution of DPPH (0.25 mM in 95% ethanol). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm in a spectrophotometer. Percent inhibition was calculated from the control. Vitamin C was used as a standard compound in the DPPH· assay.

2.4.4. Iron chelation assay

The ability of the aqueous extract to chelate Fe(II) was determined using a modified method of Puntel, Nogueira, and Rocha (2005). Briefly, 150 μl of freshly prepared 2 mM FeSO4 were added to a reaction mixture containing 168 μl of 0.1 M Tris–HCl (pH 7.4), 218 μl saline and the aqueous extract of the plant (10–100 μg/μl). The reaction mixture was incubated for 5 min, before the addition of 13 μl of 0.25% 1,10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm in the spectrophotometer.

2.5. In vivo hepatoprotective activity

2.5.1. General

Animals were divided into three groups comprising five mice in each group. Group 1 (control) received only distilled water. Group 2 (Paracetamol-control) received 250 mg/kg acetaminophen only. Group 3 (Paracetamol + Plant extract) received a single dose of acetaminophen (250 mg/kg) + (100 mg/kg) of plant extract. Plant extract was administered three hours after the administration of acetaminophen. All the treatments were done by means of a gavage or gastric tube. The treatments were continued for seven days and on the 8th day of the experiment all animals were anaesthized and killed by heart puncture and blood samples were obtained. Livers were quickly removed, placed on ice and homogenised in seven volumes of NaCl (0.9%). The homogenates were centrifuged at

4000g for 10 min to yield a low-speed supernatant fraction (S_1) that was used for the catalase and TBARS assays.

2.5.2. Enzyme assays

Serum was obtained from clotted blood samples by centrifugation at 1500g for 10 min. The activities of enzymes, glutamate pyruvate transaminase (alanine aminotransferase) and gulatamate oxaloacetate transaminase (aspartate aminotransferase) were determined using assay kits according to the supplier (Lab Test, MG, Brazil) specifications.

Catalase (CAT) activity was measured by the method of Aebi & Bergmeyer, 1983. An aliquot of liver supernatant (10 μl) was added to a quartz cuvette and the reaction was started by the addition of freshly prepared H_2O_2 (30 mM) in phosphate buffer (50 mM, pH 7.0). The rate of H_2O_2 decomposition was measured spectrophotometrically at 240 nm during 120 s. The activity of CAT was expressed as μmol H_2O_2/mg tissue/min.

2.5.3. Lipid peroxidation assay

After the end of centrifugation, an aliquot of 200 μl of liver S_1 was incubated for 1 h at 37 °C and then used for lipid peroxidation determination. Thiobarbituric acid-reactive species (TBARS) production was determined as described by Ohkawa et al. (1979).

2.6. Phenolics content

The total phenol content was determined by adding 0.5 ml of the aqueous extract to 2.5 ml, 10% Folin–Ciocalteau's reagent (v/v) and 2.0 ml of 7.5% sodium carbonate. The reaction mixture was incubated at 45 °C for 40 min, and the absorbance was measured at 765 nm in the spectrophotometer. Gallic acid was used as a standard phenol (Singleton, Orthofer R, & Lamuela-Raventos, 1999). The mean of three readings was used and the total phenol content was expressed as milligrammes of gallic acid equivalents/g extract.

2.7. Determination of total flavonoids

Flavonoids were expressed as quercetin equivalents. Quercetin was used to make the calibration curve [0.04, 0.02, 0.0025 and 0.00125 mg/ml in 80% ethanol (v/v)]. The standard solutions or extracts (0.5 ml) were mixed with 1.5 ml of 95% ethanol (v/v), 0.1 ml of 10% aluminium chloride (w/v), 0.1 ml of 1 mol/l sodium acetate and 2.8 ml of water. The volume of 10% aluminium chloride was substituted by the same volume of distilled water in the blank. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. The mean of three readings was used and the total flavonoid content was expressed as milligrams of quercetin equivalents/g of extract (Kosalec, Bakmaz, Pepeliniak, & Vladimir-Knezevic, 2004).

2.8. Thin-layer chromatography (TLC) of plant extracts

Methanolic extract of leaves was submitted to characterisation by thin-layer chromatography (Silica gel coated TLC plates, Merck; mobile phase, *n*-butanol:ethyl acetate:water (2:4:4). Chromatograms were evaluated under UV light at 254 and 365 nm to detect the presence of flavonoids. The presence of flavonoids was further confirmed by spraying the plates with 5% $AlCl_3$ in ethanol. Rutin and quercetin were used as standard flavonoids.

2.9. Statistical analysis

The results were expressed as means ± standard deviation. The data were analyzed statistically by one way ANOVA and different group means were compared by Duncan's multiple range test; $P < 0.05$ was considered significant in all cases. The software package Statistica was used for analysis of data.

3. Results

3.1. In vitro assays

Rat liver homogenate was induced with iron and sodium nitroprusside to cause lipid peroxidation and the effect of *P. niruri* aqueous extracts was determined. There was a statistically ($P < 0.05$) significant increase (about 75% and 85.4%) in the formation of TBARS in iron(II) sulphate (10 μM)- and SNP (5 μM)-induced liver homogenates when compared to the basal or normal (Table 1). *P. niruri* significantly reduced the accumulation of lipid peroxides in a dose-dependent manner, from 2 to 10 μg/ml for iron and 10–80 μg/ml for SNP. However, the plant afforded greater protection against iron-induced lipid peroxidation than SNP, as it was active at low concentration. Table 2 shows the interaction (inhibition) of the plant extracts with Fe(II)- and SNP-induced lipid peroxidation in rat's brain. The result revealed that incubation of the brain tissue in the presence of 10 μM Fe(II) and 5 μM SNP caused 80.5% and 85.2% increases in the MDA content of the brain homogenates when compared to the basal brain homogenates. However, aqueous extracts of *P. niruri* caused a significant inhibition ($P < 0.05$) in Fe(II)-induced lipid peroxidation, in the brain in a dose-dependent (10–80 μg/ml). For SNP-induced lipid peroxidation, the aqueous extract significantly ($P < 0.05$) reduced the MDA content in a dose-dependent manner up to 40 μg/ml, beyond which there was less inhibition of lipid peroxidation (Table 2).

Table 3 shows the inhibition of the extracts with Fe(II)- and SNP-induced lipid peroxidation in kidney of rat. Incubation of tissue in the presence of 10 μM Fe(II) and 5 μM SNP caused 73.3% and 85.3% increases in the MDA content of the kidney homogenates when compared to the normal kidney homogenates. *P. niruri* extracts were found to be effective against iron (2–10 μg/ml) and SNP (5–60 μg/ml)-induced inhibition in a dose-dependent manner (Table 3). The phospholipid from egg yolk was also induced with iron and SNP to cause lipid peroxidation, to further confirm the antioxidative potential of *P. niruri* extracts. There were statistically

Table 1
The inhibitory effect of aqueous extracts between different batches of *P. niruri* (PN) on iron sulphate-and sodium nitroprusside (SNP)-induced lipid peroxidation in a rat liver homogenate *in vitro*

Treatments	PN concentration (μg/ml)	MDA (nmole/g.tissue) in PN1	MDA (nmole/g.tissue) in PN2
Normal	–	0.82 ± 0.06	0.82 ± 0.11
Control	–	3.31 ± 1.20	3.40 ± 1.10
Iron + PN	2	2.83 ± 0.09	3.26 ± 0.12
Iron + PN	4	2.20 ± 0.122	2.54 ± 0.04
Iron + PN	6	1.70 ± 0.275	1.80 ± 0.21
Iron + PN	8	0.76 ± 0.084[c]	1.39 ± 0.13[a]
Iron + PN	10	0.49 ± 0.109[c]	1.07 ± 0.05[a]
Normal	–	0.82 ± 0.06	0.82 ± 0.06
Control	–	5.63 ± 0.085	5.55 ± 0.095
SNP + PN	10	5.06 ± 0.054	5.13 ± 0.10
SNP + PN	20	4.45 ± 0.26	4.64 ± 0.60
SNP + PN	40	2.37 ± 0.1	4.02 ± 0.09
SNP + PN	60	1.17 ± 0.05[b]	3.62 ± 0.05
SNP + PN	80	1.16 ± .084[b]	2.55 ± 0.12

Values represent the means ± SE ($n = 6$).
All the values in columns are significantly ($P < 0.05$) different by DMR test compared to the control group.
Values in columns followed by same letters are non-significantly different from each other.
Differences between batches of the plant were analyzed by one way ANOVA and found to be non-significant ($P = 0.63$) for iron- and sodium nitroprusside- ($P = 0.33$) induced inhibition.

Table 2
The inhibitory effect of aqueous extracts between different batches *P. niruri* (PN) on iron sulphate-and sodium nitroprusside (SNP)-induced lipid peroxidation in a rat brain homogenate *in vitro*

Treatments	PN concentration (μg/ml)	MDA (nmol/g.tissue) in PN1	MDA (nmol/g.tissue) in PN2
Normal	–	0.91 ± 0.10	0.91 ± 0.10
Control	–	4.69 ± 0.09	4.81 ± 0.07
Iron + PN	10	3.43 ± 0.052	4.05 ± 0.12
Iron + PN	20	1.84 ± 0.091	3.1 ± 0.13
Iron + PN	40	0.86 ± 0.098	2.44 ± 0.06[b]
Iron + PN	60	0.144 ± 0.018[a]	2.21 ± 0.02[b]
Iron + PN	80	0.097 ± 0.02[a]	1.90 ± 0.05
Normal	–	0.91 ± 0.10	0.91 ± 0.10
Control	–	6.20 ± 0.07	6.24 ± 0.08
SNP + PN	5	4.7 ± 0.052	5.21 ± 0.11
SNP + PN	10	3.26 ± 0.097[b]	3.59 ± 0.32
SNP + PN	20	2.75 ± 0.042	2.91 ± 0.045
SNP + PN	40	1.50 ± 0.087	2.59 ± 0.032[c]
SNP + PN	60	3.40 ± 0.10[b]	2.71 ± 0.117[c]

Values represent the means ± SE ($n = 6$).
All the values in columns are significantly ($P < 0.05$) by DMR test compared to control group. Values in columns followed by same letters are non-significantly different from each other.
Difference between batches of the plant were analyzed by one way ANOVA and found to be non-significant ($P = 0.195$) for iron- and sodium nitroprusside- ($P = 0.79$) induced inhibition.

Table 3
The inhibitory effect of aqueous extracts between different batches of *P. niruri* (PN) on iron sulphate- and sodium nitroprusside (SNP)-induced lipid peroxidation in a rat kidney homogenate *in vitro*

Treatments	PN concentration (μg/ml)	MDA (nmol/g.tissue) in PN1	MDA (nmol/g.tissue) in PN2
Normal	–	0.82 ± 0.093	0.82 ± 0.093
Control	–	3.12 ± 0.22	3.26 ± 0.12
Iron + PN	2	2.54 ± 0.160	2.64 ± 0.04[a]
Iron + PN	4	1.80 ± 0.131	2.39 ± 0.03[a]
Iron + PN	6	1.23 ± 0.153[a]	2.19 ± 0.10
Iron + PN	8	0.85 ± 0.073[a]	1.97 ± 0.66
Iron + PN	10	0.59 ± 0.095	1.65 ± 0.07
Normal	–	0.82 ± 0.07	0.82 ± 0.07
Control	–	5.60 ± 0.23	5.52 ± 0.29
SNP + PN	5	5.19 ± 0.130[b]	5.19 ± 0.05
SNP + PN	10	5.01 ± 0.06[b]	4.83 ± 0.23
SNP + PN	20	4.80 ± 0.09	3.77 ± 0.32
SNP + PN	40	3.53 ± 0.11	3.22 ± 0.03
SNP + PN	60	1.92 ± 0.05	2.38 ± 0.21

Values represent the means ± SE ($n = 6$).
All the values in columns are significantly ($P < 0.05$) by DMR test compared to control group. Values in columns followed by same letters are non-significantly different from each other.
Difference between batches of the plant were analyzed by one way ANOVA and found to be non-significant for iron- ($P = 0.80$) and for sodium nitroprusside ($P = 0.80$)-induced inhibition.

($P < 0.05$) significant increases (about 70% and 76.8%) in the formation of TBARS in iron(II) sulphate (10 μM) and SNP (5 μM), respectively, when compared to the basal (Table 4). The extracts of PN2 & PN1 afforded higher protection against iron-induced lipid peroxidation (84.7–98.8%) than did SNP (61%) at the concentration 100 μg/ml, showing the greater potential of the extract against iron overload. Both the batches of the plant statistically ($P < 0.05$) reduced the production of TBARS in a concentration-dependent manner in all the tested pro-oxidant-induced oxidative stresses (Tables 1–4). However, batch 1 showed a higher antioxidant potential. The results of statistical analysis (ANOVA) revealed a non-significant difference ($P > 0.05$) between the batches of the plant (Tables 1–4) showing the similar natures of extracts. The extracts also exhibited strong antioxidant activity in the DPPH· and iron chelation assay (Fig. 1). The IC_{50} values obtained for DPPH·-scavenging of PN1 & PN2 were 43.4 ± 1.45 μg/ml and 47.9 ± 1.5 μg/ml, respectively, which were comparable for the reference standard, vitamin C 28.4 ± 0.25 (data not shown). For iron chelation the extract showed maximum activity at a concentration of 50 μg/ml, beyond which there was less inhibition (Fig. 1).

Table 4
The inhibitory effect of aqueous extracts between different batches of *P. niruri* (PN) on iron sulphate- and sodium nitroprusside (SNP)-induced lipid peroxidation in phospholipid homogenate *in vitro*

Treatments	PN concentration (μg/ml)	MDA (nmol/g.tissue) in PN1	MDA (nmol/g.tissue) in PN2
Normal	–	1.96 ± 0.34	1.96 ± 0.34
Control	–	6.63 ± 0.095	6.43 ± 0.05
Iron + PN	20	4.89 ± 0.03	4.92 ± 0.12
Iron + PN	40	3.26 ± 0.14	3.52 ± 0.13
Iron + PN	60	2.57 ± 0.09	2.51 ± 0.04
Iron + PN	80	1.19 ± 0.1[a]	1.53 ± 0.23
Iron + PN	100	0.093 ± 0.01[a]	0.98 ± 0.01
Normal	–	1.96 ± 0.34	1.96 ± 0.34
Control	–	8.43 ± 0.082	8.40 ± 0.09
SNP + PN	20	5.97 ± 0.12	6.49 ± 0.05
SNP + PN	40	5.25 ± 0.03	5.46 ± 0.14
SNP + PN	60	4.48 ± 0.07	4.88 ± 0.04
SNP + PN	80	3.50 ± 0.036[b]	3.69 ± 0.18
SNP + PN	100	3.30 ± 0.035[b]	3.21 ± 0.06

Values represent the means ± SE ($n = 6$).
All the values in columns are significantly ($P < 0.05$) by DMR test compared to control group. Values in columns followed by same letters are non-significantly different from each other.
Difference between batches of the plant were analyzed by one way ANOVA and found to be non-significant ($P = 0.87$) for iron- and sodium nitroprusside ($P = 0.86$)-induced inhibition.

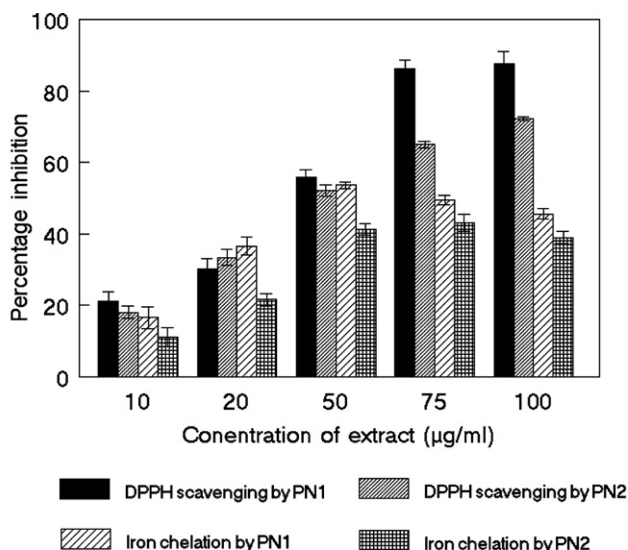

Fig. 1. DPPH radical-scavenging and iron chelation by the extracts of *P. niruri*. *Values are mean ± SE ($n = 3$).

3.2. In vivo assays

Treatment of paracetamol (250 mg/kg) induced a marked increase in the serum hepatic enzyme levels of GPT and GOT of mice as compared to the normal controls (Figs. 2 and 3) indicating liver damage. However, the administration of an aqueous extract of *P. niruri* (100 mg/kg) after the paracetamol treatment caused a significant reduction of GPT and GOT enzymes, almost comparable to

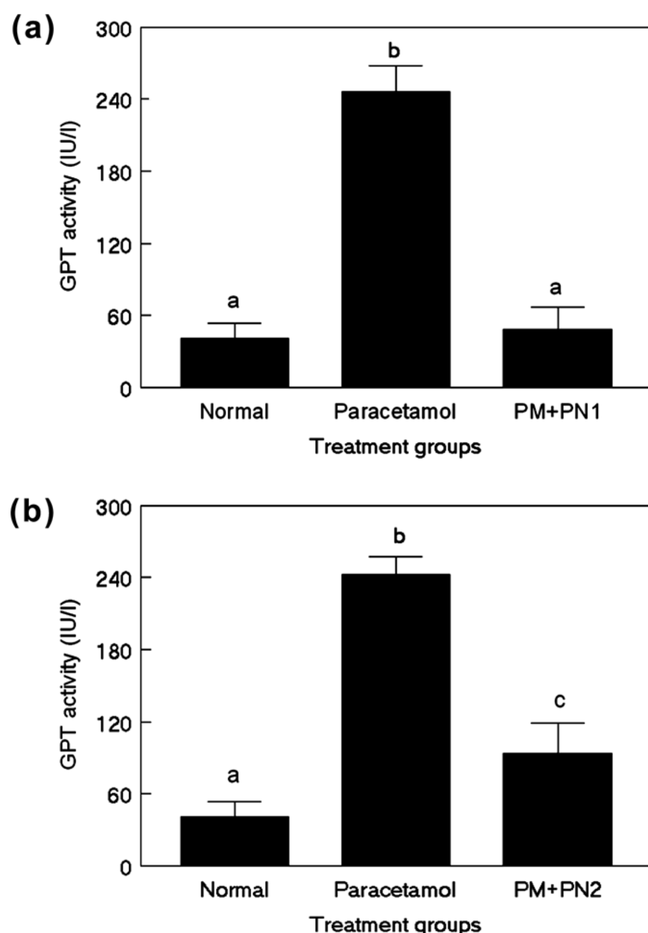

Fig. 2. (a). Hepatoprotective action of *P. niruri* in mice: (a) serum enzyme activity of GPT in control and treated groups for batch 1 (PN1). (b) serum enzyme activity of GPT in control and treated groups for batch 2 (PN2).*Values are means ± SE ($n = 5$); bars with different letters differ significantly at $P < 0.05$ by DMRT.

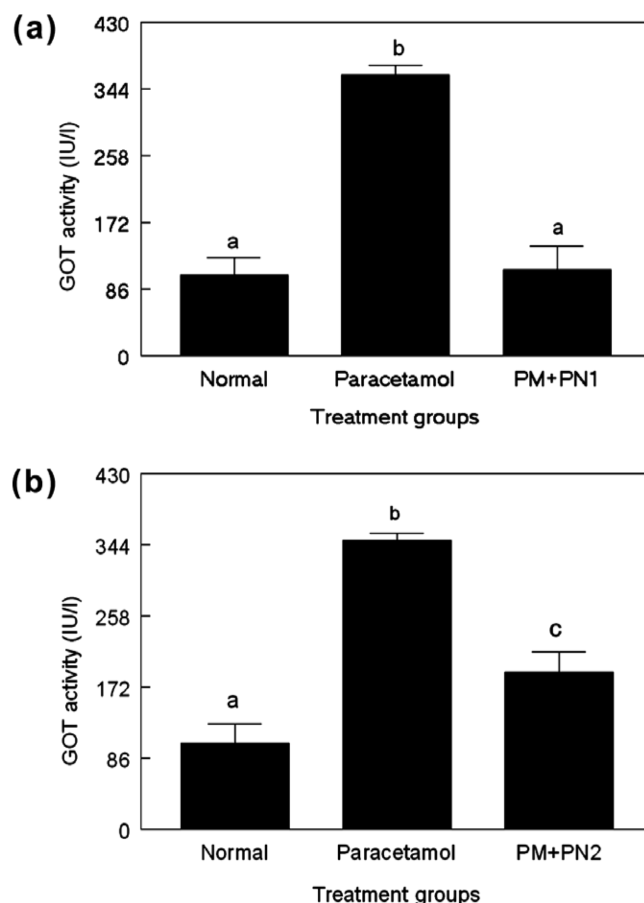

Fig. 3. Hepatoprotective action of *P. niruri* in mice: (a) serum enzyme activity of GOT in control and treated groups for batch 1 (PN1). (b) serum enzyme activity of GOT in control and treated groups for batch 2 (PN2). *Values are means ± SE ($n = 5$); bars with different letters differ significantly at $P < 0.05$ by DMRT.

the normal control group. There was a significant difference ($P < 0.05$) in the activities of GPT and GOT enzymes between the batches (PN1 and PN2) of the plant. However, batch 1 caused a greater reduction of these enzymes in the sera of mice than did batch 2 (Figs. 2 and 3). The hepatoprotective activity of extracts was further confirmed by studying the activity of catalase and TBARS in mice liver. Treatment with paracetamol caused a significant decrease ($P < 0.05$) in the hepatic catalase activity. This activity was abolished in the group treated with 100 mg/kg of *P. niruri* extract, showing the hepatoprotective activity of the crude extract (Fig. 4). There was a significant increase in TBARS formation in the liver of the group treated with a single oral dose of paracetamol (250 mg/kg), when compared to the control ($P < 0.05$). The increase was about 73%. The treatment with plant extracts significantly decreased ($P < 0.05$) the lipid peroxidation and showed about 44.8–49.3% inhibition in MDA compared to the paracetamol group (Fig. 5). The statistical analysis (ANOVA) showed a non-significant ($P > 0.05$) difference in the catalase activity and lipid peroxidation between the batches, which showed that both the batches of the plant were equally effective against liver damage.

The leaf extract of PN1 had high contents of phenolics (111 ± 0.072 mg/g) and flavonoids (2.11 ± 0.03 mg/g) which may be responsible for antioxidant and hepatoprotective activity of the plant. Similar levels of phenolics (108 ± 0.02) and flavonoids (1.93 ± 0.02) were also detected in water extracts of PN2 (Table 5). The TLC analysis for flavonoids showed a yellow spot of rutin at R_f 0.41 which indicates the presence of rutin in the leaves.

4. Discussion

Oxidative stress is now recognised to be associated with more than 100 diseases, as well as with the normal aging process (Ghasanfari, Minaie, Yasa, Leilu, & Azadeh, 2006). There is a strong correlation between thiobarbituric acid-reactive substances (TBARS) as a marker of lipid peroxidation and products that reflect oxidative damage to DNA (Chen, Wu, & Huang, 2005). Increases in the formation of TBARS in iron(II) sulphate (10 μM)-induced oxidative stress, as compared to the normal, suggest possible damage of tissues with an overload of iron. Free iron in the cytosol and mitochondria can cause considerable oxidative damage by increasing superoxide production, which can react with Fe(III) to regenerate Fe(II) that participates in the Fenton reaction (Fraga & Oteiza, 2002). Iron overload results in the formation of lipid peroxidation products, which have been demonstrated in a number of tissues, including the liver and kidneys (Houglum, Filip, Witztum, & Chojkier, 1990). Storage of iron in the liver leads to liver cirrhosis. Rats overloaded with iron showed toxic effects, such as hepatocellular hypertrophy, cardiomyopathy, pancreatic atrophy, splenic white pulp atrophy and hemosiderosis in the liver, heart, pancreas and endocrine glands, respectively (Whittaker, Berlett, & Stadtman, 1997). The possible mechanisms of iron toxicity include free radical-mediated peroxidative reactions, which are readily catalysed by iron. The protections offered by the aqueous extracts of *P. niruri* suggest that they may be useful in the treatment of liver, brain and kidney diseases resulting from iron overload.

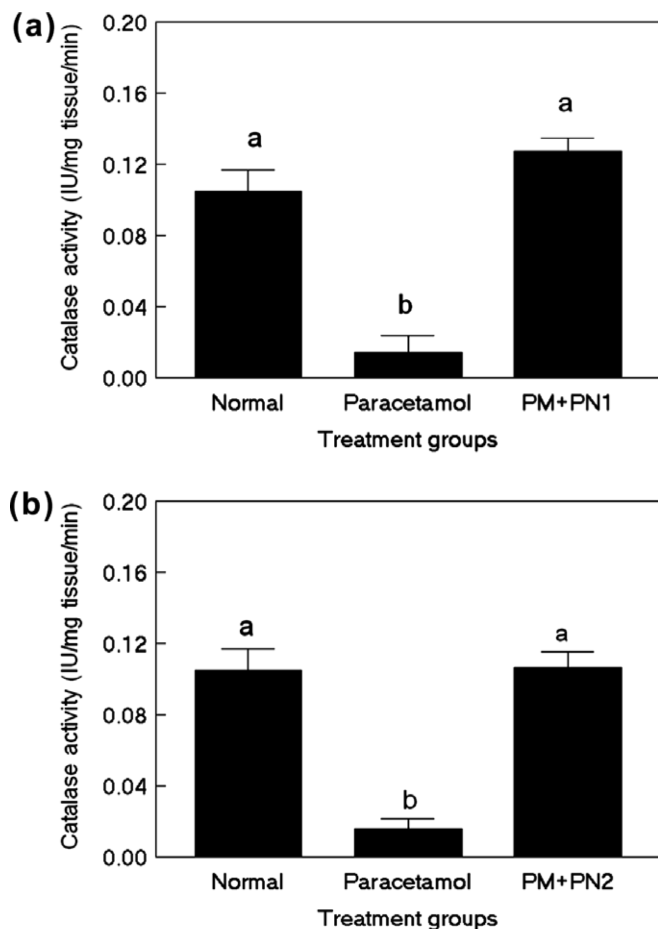

Fig. 4. Hepatoprotective action of *P. niruri* in mice: (a) catalase activity in control and treated groups for batch 1 (PN1). (b) catalase activity in control and treated groups for batch 2 (PN2). *Values are means ± SE ($n = 5$); bars with different letters differ significantly at $P < 0.05$ by DMRT.

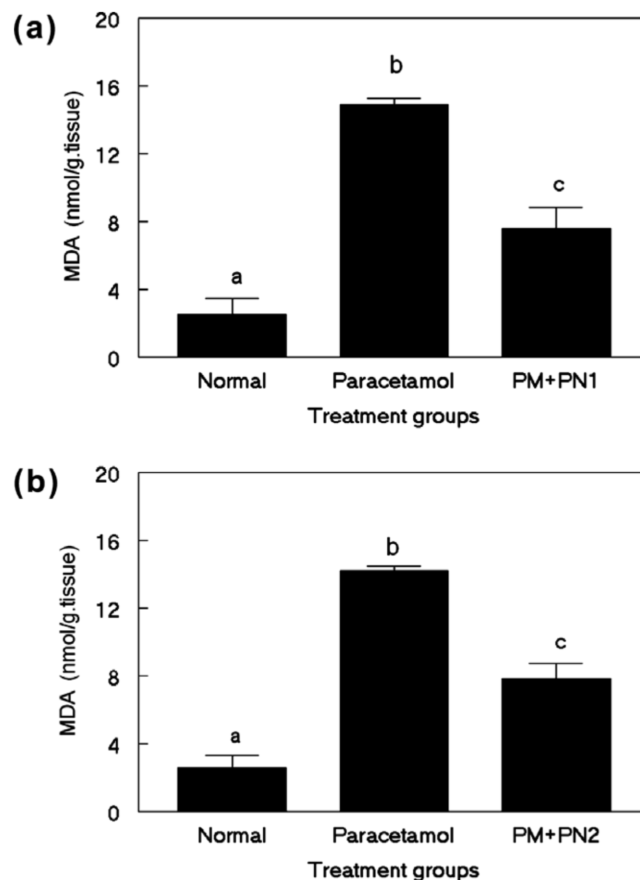

Fig. 5. Hepatoprotective action of *P. niruri* in mice: (a) lipid peroxide content (MDA) in control and treated groups for batch 1 (PN1). (b) lipid peroxide content (MDA) in control and treated groups for batch 2 (PN2). *Values are means ± SE ($n = 5$); bars with different letters differ significantly at $P < 0.05$ by DMRT.

Sodium nitroprusside is an anti-hypertensive drug which acts by relaxation of vascular smooth muscle; consequently it dilates peripheral arteries and veins. However, SNP has been reported to cause cytotoxicity through the release of cyanide and/or nitric oxide (Bates, Baker, Guerra, & Harrison, 1990). Although nitric oxide acts independently, it may also cause neuronal damage in cooperation with other reactive oxygen species (ROS). Rat liver methionine synthase has also been inactivated by SNP. The inactivation has been attributed to nitric oxide released by SNP. The nitric-oxide-induced inactivation of methionine synthase could offer a rational explanation for the cellular and cytotoxic effects of this highly reactive molecule (Nicolaou, Waterfield, Kenyon, & Gibbons, 1997). The protection offered by *P. niruri* extracts on tissues and phospholipids proves the antioxidant activity of crude extracts of plant and suggest that they may be useful for diseases resulting from overload of SNP. The results on the tissues suggest that the *P. niruri* elicits hepatoprotective, neuroprotective and renal protection through antioxidant activity at a low concentration (less than 100 μg/ml) and, in most of the cases, has the ability to reduce the TBARS production to less than the basal level. DPPH radical-scavenging activity of the extracts also revealed very high potency, considering the fact that free radical quenching properties were only from the crude extracts with the IC_{50} value at 43.4 ± 1.45 μg/ml. Such high free radical-scavenging properties of the crude extracts are shown by few other plants (Gulcum, Oktay, Krreccr, & Kufrevioglu, 2003). The use of iron chelation is a popular therapy for the management of Fe(II)-associated oxidative stress in brain. The iron

Table 5
Contents of phenolics and flavonoids in different extracts of *P. niruri*

Extracts	Total phenolics (GE[A] mg/g)	Total flavonoids (Quer[B] mg/g)
PN1 (aqueous)	111 ± 0.07	2.11 ± 0.03
PN1 (methanol)	115 ± 0.05	2.81 ± 0.043
PN2 (aqueous)	108 ± 0.02	1.93 ± 0.02
PN2 (methanol)	118 ± 0.1	2.16 ± 0.05

GE[A] is gallic acid equivalents.
Quer[B] is quercetin equivalents.

chelating ability of the *P. niruri* is again an indicator of the neuroprotective property of the plant because iron is involved in the pathogenesis of Alzeimer's and others diseases by multiple mechanisms (Elise & James, 2002).

Hepatoprotective activity of *P. niruri* was shown by its ability to inhibit paracetamol-induced liver damage in mice. Paracetamol is a well-known antipyretic and analgesic agent, which is safe in therapeutic doses, but can produce fatal hepatic necrosis in experimental animals and humans (Amar & Schiff, 2007; Schidt, Rochling, Cassey, & Lee, 1997) and is employed as an experimental hepatotoxic agent. An obvious sign of hepatic injury is the leaking of cellular enzymes into the plasma due to the disturbance caused in the transport functions of hepatocytes (Zimmerman & Seeff, 1970). The estimation of enzymes in the serum is a useful quantitative marker of the extent and type of hepatocellular damage. The mice treated with an overdose of paracetamol developed significant hepatic damage, which was observed through substantial

increases in the concentrations of serum parameters. Treatment of the rats with *P. niruri* extracts at 100 mg/kg, orally, for 7 days after paracetamol administration, resulted in a significant reduction of paracetamol-induced elevation of serum marker enzymes and appears to be protective in reducing the injurious effect of paracetamol observed in the study. This is an indication of the stabilization of plasma membrane, as well as repair of hepatic tissue damage caused by paracetamol toxicity. Our results for hepatoprotection are in line with previous studies where *P. niruri* showed significant hepatoprotection *in vivo* and *vitro* (Harish & Shivanandappa, 2005; Shamasundar et al., 1985). We also observed significant increases in the liver levels of TBARS, a marker of lipid peroxidation which was decreased by the treatment with plant extracts. Paracetamol severely decreased the level of catalase in liver which was abolished by the treatment with the plant. In fact, *P. nuiri* treatment restored the catalase activity to levels similar to that found in control mice, which reinforces the hepatoprotective potential of the plant.

Chronic viral hepatitis B and C, alcoholic liver disease, non-alcoholic fatty liver disease, and hepatocellular carcinoma are major problems which still remain unresolved. Therapies developed along the principles of modern medicine are often limited in their efficacy, carry the risk of adverse effects, and are often too costly, especially for the developing world. Therefore, treating liver diseases with plant-derived compounds, such as *P. niruri*, which are easily available and do not require laborious pharmaceutical synthesis seems highly attractive. Phytochemical analysis of the plant showed the presence of high contents of phenolics and flavonoids which may be responsible for the activity of the plant, beside other phytochemicals. Herbals and herbal extracts, which contain different classes of polyphenols, are very attractive, not only in modern phytotherapy, but also for the food industry, due to their use as preservatives. It has been reported (Calliste, Trouillas, Allais, Simon, & Duroux, 2001) that phenolic acids and their glycosides, aglycones, and monoglycosyl or diglycosyl flavonoids are distributed in the different solvents as a function of polarity and water extracts contain the most polar compounds. These facts might explain the strong scavenging and antioxidant activity of water extracts of *P. niruri.* The TLC analysis also confirmed the presence of rutin in leaves which is a known hepatoprotective agent against liver damage (Khalid, Sheikh, & Gilani, 2002).

In conclusion, the results of this study demonstrated the high efficacy of the crude aqueous extracts of *P. niruri* in free radical-scavenging, inhibition of reactive oxygen species and lipid peroxidation, which may be associated with its high medicinal use as a functional food and effectiveness in treatment of different diseases, among which liver disease is the most important. Although the use of *P. niuri* extracts is safe for humans, detailed clinical trials have not yet been done to assess the potential hepatotoxicity of this plant after administration of high doses to experimental animals and healthy human volunteers.

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