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Antioxidant activity and hepatoprotective potential of *Phyllanthus niruri*

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Abstract

Antioxidant activity and hepatoprotective potential of *Phyllanthus niruri*, a widely used medicinal plant, were investigated. Methanolic and aqueous extract of leaves and fruits of *P. niruri* showed inhibition of membrane lipid peroxidation (LPO), scavenging of 1,1-diphenyl-2picrylhydrazyl (DPPH) radical and inhibition of reactive oxygen species (ROS) in vitro. Antioxidant activity of the extracts were also demonstrable in vivo by the inhibition of the carbon tetrachloride (CCl_4) – induced formation of lipid peroxides in the liver of rats by pretreatment with the extracts. CCl_4 – induced hepatotoxicity in rats, as judged by the raised serum enzymes, glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT), was prevented by pretreatment with the extracts, demonstrating the hepatoprotective action of *P. niruri*.

Keywords: *Phyllanthus niruri*; Antioxidant activity; Lipid peroxidation; Hepatoprotective

1. Introduction

Free radicals, from both endogenous and exogenous sources, are implicated in the etiology of several degenerative diseases, such as coronary artery diseases, stroke, rheumatoid arthritis, diabetes and cancer (Halliwell, Gutteridge, & Cross, 1992). High consumption of fruits and vegetables is associated with low risk for these diseases, which is attributed to the antioxidant vitamins and other phytochemicals (Ames, Shigenaga, & Hagen, 1993; Prior, 2003; Weisburger, 1999). There is a great deal of interest in edible plants that contain antioxidants and health – promoting phytochemicals, in view of their health implications.

Phyllanthus niruri (family: Euphorbiaceae) is a perennial herb distributed throughout India. Whole plant, fresh leaves and fruits are used to treat various ailments, particularly hepatitis (Chopra, Nayar, & Chopra, 1986). Antitumor and anticarcinogenic activities of *Phyllanthus amarus* have also been reported (Rajeshkumar et al., 2002). Other medicinal properties, such as hypolipidaemic (Khanna, Rizvi, & Chander, 2002) and antiviral (Venkateswaran, Millman, & Blumberg, 1987; Wang, Cheng, Li, Meng, & Malik, 1994) activities of *P. niruri* have also been shown. Several bioactive molecules, such as lignans, phyllanthin, hypophyllanthin, flavonoids, glycosides and tannins, have been shown to be present in the extracts of *P. niruri* (Rajeshkumar et al., 2002). Using a rat hepatocyte primary culture Shamasundar et al. (1985) have shown that phyllanthin and hypophyllanthin protected cells against carbon tetrachloride cytotoxicity whereas triacontanal was protective against galactosamine toxicity. *P. niruri* is used as one of the components of a multiherbal preparation for treating liver ailments (Kapur, Pillai, Hussain, & Balani, 1994). However, a hepatoprotective effect of *P. niruri* has not been demonstrated in vivo. Several studies have shown that the hepatoprotective effect is associated with

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antioxidant rich plant extracts, (De, Shukla, Ravishankar, & Bhavasar, 1996; Dwivedi et al., 1990; Emmanuel, Amalaraj, & Ignacimuthu, 2001). In this study, we report the potent antioxidant activity of the fruits and leaves of *P. niruri* in several in vitro systems, and the hepatoprotective property in vivo.

2. Materials and methods

2.1. Materials

Nicotinamide adenine dinucleotide (reduced) (NADH), nitroblue tetrazolium chloride (NBT) and phenazine methosulphate (PMS) were procured from Sisco Research Laboratories, India. Thiobarbituric acid (TBA), and 1,1-diphenyl-2picrylhydrazyl (DPPH) were purchased from Sigma chemical Co., USA. All other chemicals were purchased from Ranbaxy and Qualigens, India.

2.2. Preparation of plant extract

The plants were collected from the fields near Mysore. The taxonomic identification was confirmed by the Department of Botany, University of Mysore. Leaves and fruits were separated and dried at room temperature, powdered, sieved and stored prior to further use. The powder (100 g) was homogenized with water (800 ml) and the homogenate was kept in a shaker at 40 °C for 24 h and then filtered using Whatman No. 1 paper. The filtrate was concentrated in a lyophilizer. The yield of the aqueous extract was 56 g from 100 g of leaf powder and 8 g from 25 g of fruit powder. The remaining residue, after aqueous extraction, was extracted with methanol (similarly to the aqueous extraction) and filtered on Whatman filter paper. The filtrate was evaporated to dryness under reduced pressure. The yields of the methanolic extract were 20 and 4.6 g for leaves and fruits, respectively. The extracts were resuspended in a known volume of the respective solvent and used for in vitro antioxidant activity assays.

2.3. Phenolics content

Total phenolics content in the extracts was determined by using Folin–phenol reagent as described by Yildirim, Mavi, and Kara (2001) with slight modification. One millilitre of the extract was added to 10 ml deionized water and 2.0 ml of Folin–phenol reagent. The mixture was then allowed to stand for 5 min and 2.0 ml sodium carbonate were added to the mixture. The absorbance was measured at 765 nm in a spectrophotometer. Phenolic content was calculated with guaiacol as the standard and expressed as milligrammes of guaiacol equivalent per g dry weight.

2.4. Microsomal membrane lipid peroxidation

Rat liver (1 g) was homogenized in 5 ml (0.02 M) tris buffer (pH 7.4) and microsomes were isolated by the calcium aggregation method (Vijayalakshmi & Anandatheerthavarada, 1990). The pellet was resuspended in 0.1 M phosphate buffer. Microsomal lipid peroxidation was assayed by the thiobarbituric acid method (Buege & Aust, 1978). To 100 μl of microsomes were added ferrous sulphate (100 μM) and ascorbate (100 μM) with or without *P. niruri* extract (20–500 μg/ml) in 0.1 M phosphate buffer (pH 7.4) and incubated at 37 °C for 1 h. This was followed by the addition of 20% trichloroacetic acid (2 ml) and 1% thiobarbituric acid (2 ml). The mixture was heated in a boiling water bath for 10 min, cooled, centrifuged and the colour in the supernatant was read at 535 nm in a spectrophotometer. Percent inhibition was calculated against a control without the extract.

2.5. DPPH radical scavenging

Scavenging of the stable radical DPPH was assayed in vitro (Hatano, Kagawa, Yasuhara, & Okuda, 1988). The extract (5–100 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.

2.6. Superoxide anion scavenging activity

Superoxide anion was generated by the reaction of NADH and phenazine methosulphate (PMS) coupled to the reduction of nitro blue tetrazolium chloride (NBT) (Nishikimi, Rao, & Yagi, 1972) with slight modification. The reaction mixture contained NBT (100 μM), NADH (300 μM) with or without plant extract (0.05–2 mg/ml for aqueous extract and 0.1–6 mg/ml for methanolic extract) in a total volume of 1 ml Tris buffer (0.02 M, pH 8.3). The reaction was started by adding PMS (30 μM) to the mixture and the absorbance change was recorded at 560 nm every 30 s for 1 min. Percent inhibition was calculated against a control without the extract.

2.7. Hepatoprotective action

Male adult rats of the Wistar strain (200–225 g), bred in the animal house of the Institute, were maintained in individual cages and divided into six groups of four animals each. Group one (control) was administered orally with the vehicle sunflower oil only (1 ml/kg body weight). Group two was administered a single dose of CCl_4 (dissolved in sunflower oil) at 1 ml/kg body weight. Groups three and four, were pretreated with aqueous and methanolic extracts (whole plant) of *P. niruri* (100 mg/kg body weight), followed by the administration of CCl_4 (1 ml/kg body weight). Groups five and six received the plant extracts (aqueous and methanolic) alone. The animals had free access to standard pellet diet and water. Rats were sacrificed by ether anaesthesia 24 h after the treatment.

2.8. Enzyme assay

Blood was collected by cardiac puncture, allowed to clot and centrifuged at 1000*g* to obtain the serum. The enzymes, glutamate-puruvate transaminase (alanine aminotransferase) and glutamate oxaloacetate transaminase (aspartate aminotransferase), were assayed by the DNPH method of Reitman and Frankel as described by Bergmeyer (1974). Briefly, enzyme assay was started by adding 50 µl serum to the reaction mixture containing the substrates in 0.1 M phosphate buffer and DNPH reagent as the chromogen. The enzyme activity was expressed as units/litre, computed directly from the absorbance values.

The liver was removed, washed with 0.9% saline and 10% w/v homogenate was prepared in cold 0.1 M phosphate buffer (pH 7.2). Total lipid peroxide content in the homogenate was assayed by the TBA method (Buege & Aust, 1978). Briefly, to 1 ml of the homogenate were added 2 ml TCA–TBA solution (0.67% TBA in 20% TCA) mixed thoroughly and heated for 15 min in a boiling water bath. After cooling and centrifuging at 4 °C at 2000 rpm for 10 min, the absorbance of the supernatant was read at 535 nm in a spectrophotometer.

2.9. Statistical analysis

IC_{50} values, from the in vitro data, were calculated by regression analysis. Results from in vivo experiments were analyzed by Duncan's multiple range test (Snedecor & Cochran, 1980) to detect inter group differences where *P*-values < 0.05 were considered statistically significant.

3. Results and discussion

Aqueous and methanolic extracts of *P. niruri* were potent inhibitors of microsomal lipid peroxidation induced by Fe^{2+} and ascorbate in vitro. Both leaf and fruit extracts showed antioxidant activity (Fig. 1). Results on inhibition of the superoxide (ROS) in vitro showed that the aqueous extracts of leaf and fruit were more potent than methanolic extracts (Fig. 2). DPPH radical-scavenging activity of all the extracts of *P. niruri* revealed very high potency (Fig. 3) considering the fact that the free radical quenching properties were only from the crude extracts with the IC_{50} values at 10–30 µg/ml. Such high free radical scavenging properties of the crude extracts are shared by few other plants (Gulcum, Oktay,

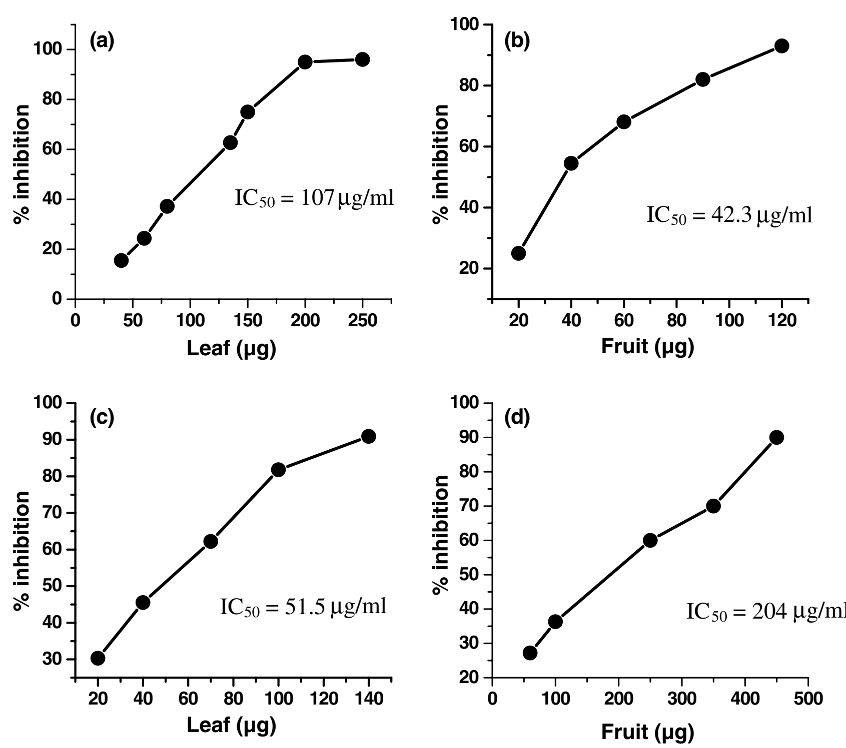

Fig. 1. Inhibition of microsomal lipid peroxidation in vitro by the extracts of *P. niruri* ((a) and (b) aqueous extract; (c) and (d) methanolic extract). *Values are means ± SE of three replicates.

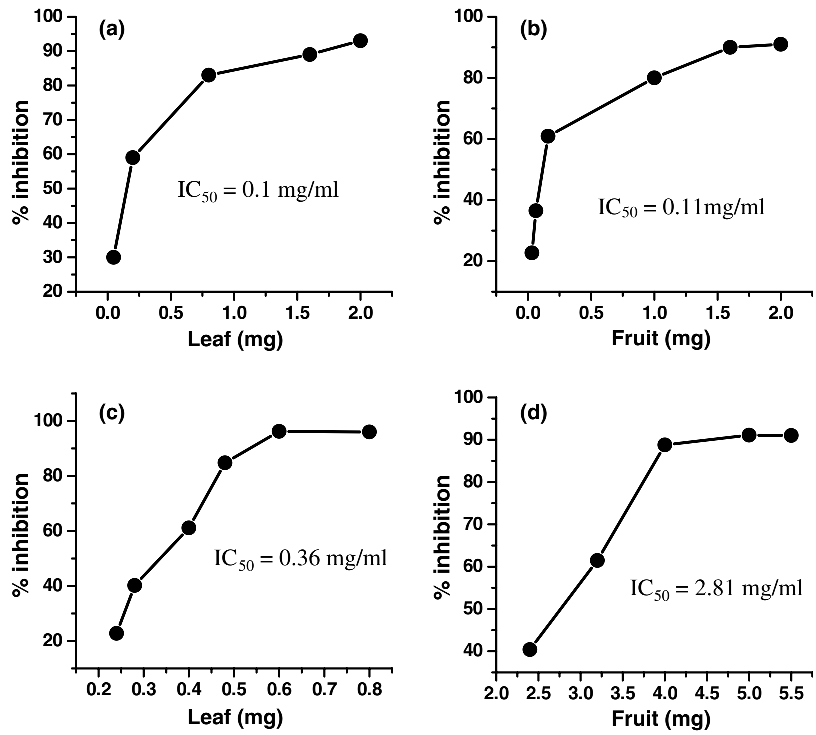

Fig. 2. Inhibition of superoxide production in vitro by the extracts of *P. niruri* ((a) and (b) aqueous extract; (c) and (d) methanolic extract). *Values are means ± SE of three replicates.

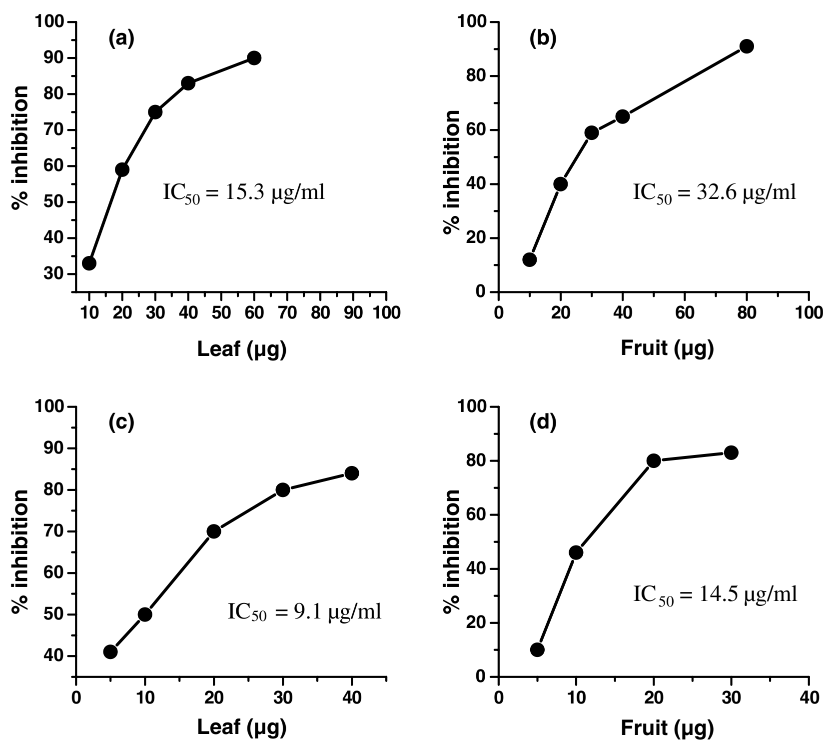

Fig. 3. DPPH radical scavenging by the extracts of *P. niruri* ((a) and (b), aqueous extract; (c) and (d) methanolic extract). *Values are means ± SE of three replicates.

Krreccr, & Kufrevioglu, 2003). There appears to be no correlation between antioxidant activity and phenolics content of the *P. niruri* extracts (Table 1), which suggests that, besides phenolics, other chemical constituents may contribute to the antioxidant activity. Phenolic chemical constituents such as flavonoids and tannins have been

Table 1
Total phenolics content of *P. niruri*

Extract	Phenolic content (mg/g), mean ± SE	
	Aqueous	Methanolic
Leaves	97.4 ± 3.0	105 ± 4.2
Fruits	360 ± 33.0	31.8 ± 0.2

reported from *P. niruri* (Rajeshkumar et al., 2002). However, their distributions in the leaf and fruits are not known. Non-phenolic antioxidant molecules of *P. niruri* remain to be identified.

Antioxidant potential of the *P. niruri* extracts in vivo was shown by their ability to inhibit CCl_4 – induced lipid peroxidation in the liver of rats. Results show that both aqueous and methanolic extracts of *P. niruri* were effective antioxidants in vivo (Fig. 4) and the extracts per se did not induce LPO in the liver of rats.

Experiments were done to demonstrate the hepatoprotective potential of *P. niruri* extracts in rats by inducing liver damage by CCl_4 with or without pretreatment. Results showed that CCl_4 induced a rise of the serum enzymes, GOT and GPT, well known markers for hepatic injury (Achliya, Kotagale, Wadodkar, & Dorle, 2003). Pretreatment of rats with the extracts markedly reduced CCl_4 – induced changes in the serum enzymes. The extracts per se did not affect the serum enzymes (Figs. 5 and 6).

Earlier studies (Shamasundar et al., 1985) have shown that chemical constituents of *P. niruri* such as phyllanthin and hypophyllanthin, could protect against

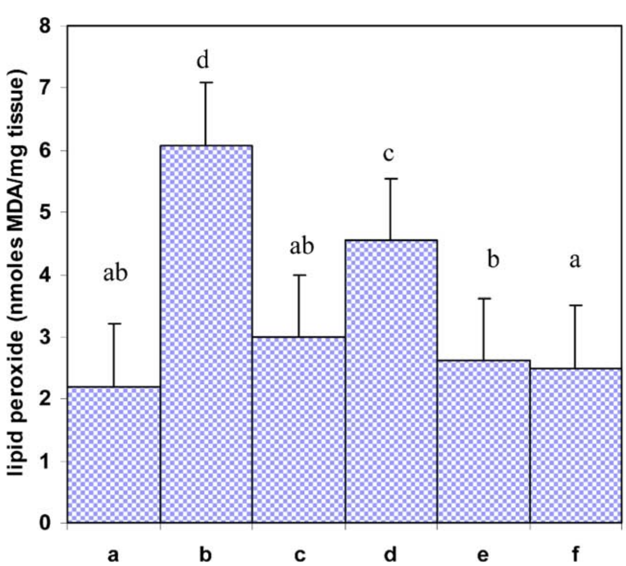

Fig. 4. Antioxidant activity of the *P. niruri* extract in vivo: lipid peroxide content in the liver of normal and CCl_4 treated rats with or without pretreatment: (a) Control; (b) CCl_4-treated; (c) aqueous extract pretreated followed by CCl_4-administration; (d) methanolic extract pretreated followed by CCl_4 administration; (e) and (f) treated with aqueous and methanolic extracts, respectively. The values are means ± SEM ($n = 4$), bars with different letters differ significantly at $P < 0.05$ by DMRT.

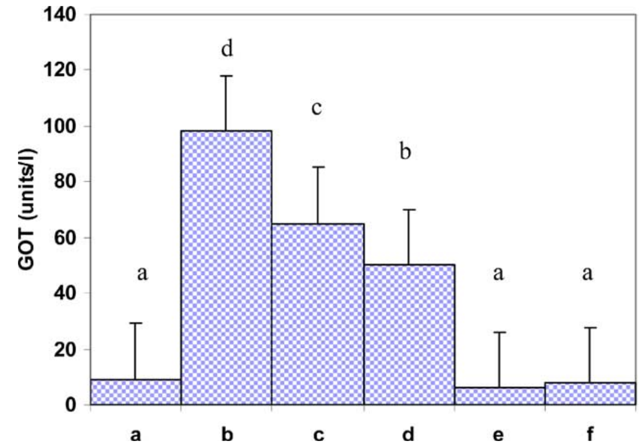

Fig. 5. Hepatoprotective action of *P. niruri* in rats: serum enzyme profile of GOT in control and treated rats: treatments (a)–(f) are same as in Fig. 4. The values are means ± SEM ($n = 4$), bars with different letters differ significantly at $P < 0.05$ by DMRT.

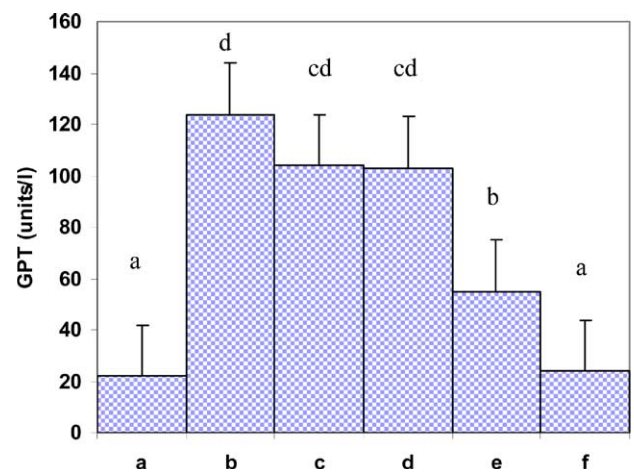

Fig. 6. Hepatoprotective action of *P. niruri* in rats: serum enzyme profile of GPT in control and treated rats: treatments (a)–(f) are same as in Fig. 4. The values are means ± SEM ($n = 4$), bars with different letters differ significantly at $P < 0.05$ by DMRT.

cytotoxicity of CCl_4 in an isolated hepatocyte primary culture. Our studies have provided in vivo evidence for the hepatoprotective action of *P. niruri*.

Several studies have reported the medicinal properties of *P. niruri* (Chopra et al., 1986). *P. niruri* is used in folk medicine to treat hepatitis and other viral infections (Venkateswaran et al., 1987; Wang, 2000; Wang et al., 1994). Many bioactive molecules have been reported in *P. niruri*, showing various activities, such as antiviral, antinociceptive, and antispasmodic activities and inhibition of calcium oxalate formation in the kidney (Freitas, Schor, & Boim, 2002; Qian-Cutrone, 1996; Santos, Filho, Yunes, & Calixto, 1995). The unusually high potency of the crude extracts of *P. niruri* in free radical scavenging, inhibition of ROS and lipid peroxidation is reported for the first time. This could be associated with its high medicinal value.

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