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To cite this article: R. Srirama, H. B. Deepak, U. Senthilkumar, G. Ravikanth, B. R. Gurumurthy, M. B. Shivanna, C. V. Chandrasekaran, Amit Agarwal & R. Uma Shaanker (2012) Hepatoprotective activity of Indian *Phyllanthus*, *Pharmaceutical Biology*, 50:8, 948-953, DOI: [10.3109/13880209.2011.649858](https://doi.org/10.3109/13880209.2011.649858)

To link to this article: <https://doi.org/10.3109/13880209.2011.649858>



Published online: 06 Apr 2012.



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RESEARCH ARTICLE

# Hepatoprotective activity of Indian *Phyllanthus*

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## Abstract

**Context:** *Phyllanthus* (Euphorbiaceae) species are traditionally well-known for their medicinal properties including hepatoprotective activity.

**Objective:** The study assessed the hepatoprotective and antioxidant activities of 11 *Phyllanthus* species, *P. amarus* Schumach., *P. urinaria* L., *P. debilis* Klein ex Willd., *P. tenellus* Roxb., *P. virgatus* G. Forst., *P. maderaspatensis* L., *P. reticulatus* Poir., *P. polyphyllus* Willd., *P. emblica* L., *P. indofischerii* Bennet. and *P. acidus* (L.) Skeels.

**Materials and methods:** The dried leaves and stems of each plant species were extracted in methanol and successively in water. The extracts were screened for hepatoprotective activity at a concentration of 50 µg/mL against *tert*-butyl hydroperoxide (*t*-BH) induced toxicity in HepG2 cells. Seven extracts from five species that showed hepatoprotective activity were assessed for their 50% effective concentration (EC<sub>50</sub>) values and their antioxidant activity using a DPPH assay. Phyllanthin and hypophyllanthin contents were also determined in these *Phyllanthus* species.

**Results:** The methanol extracts of *P. polyphyllus*, *P. emblica* and *P. indofischerii* showed high levels of hepatoprotective activity with EC<sub>50</sub> values of 12, 19 and 28 µg/mL and IC<sub>50</sub> of 3.77, 3.38 and 5.8 µg/mL for DPPH scavenging activity respectively against an IC<sub>50</sub> of 3.69 µg/mL for ascorbic acid. None of these activities could be attributed to phyllanthin and hypophyllanthin.

**Discussion and conclusion:** The hepatoprotective and antioxidant activities of *P. indofischerii* are demonstrated for the first time in literature. The study also confirms the hepatoprotective and antioxidant activities of leaves of *P. emblica* and *P. polyphyllus*. The molecule(s) responsible for the activities is being investigated.

**Keywords:** HepG2 cells, DPPH, phyllanthin, hypophyllanthin, *Phyllanthus*, hepatoprotective activity

## Introduction

The genus *Phyllanthus* (Euphorbiaceae) constitutes one of the most important groups of plants with high medicinal value (Ved & Goraya, 2008). In India, *Phyllanthus* species traded as raw drugs (Srirama et al., 2010) have been traditionally used for treating liver disorders, intestinal infections and diabetes (Sharma et al., 2000; Mahishi et al., 2005; Rajakumar & Shivanna, 2009). Recently, Shivanna and Rajkumar (2011) reported that *P. amarus* is extensively recommended by local communities in Hosanagara taluk of Shimoga district in Karnataka,

India for treating jaundice in humans. These plants are also used as an astringent, diuretic, antipyretic, laxative, tonic, antibacterial, antioxidative, immunomodulatory, antiviral, antiatherosclerotic and antineoplastic as well as to treat dropsy (Thyagarajan & Jayaram, 1992; Prakash et al., 1995; Calixto et al., 1998).

Fifty-three species of *Phyllanthus* are distributed in India of which 23 are endemic (Balakrishnan & Chakrabarty, 2007). Only a few of these *Phyllanthus* species have been investigated for their medicinal value. For example, *P. amarus*, *P. urinaria*, *P. emblica*, *P. acidus*, *P. tenellus*,

*P. polyphyllus*, *P. fraternus*, *P. simplex*, *P. maderaspatensis*, and *P. reticulatus* have been reported for a variety of medicinal activities including antioxidant, anticancer, nephro-protective, hepatoprotective, antibacterial, anti-inflammatory, antitumor, anti-HIV, anti-herpes Simplex Virus activities (Ravikanth et al., 2011; Eldeen et al., 2011; Faremi et al., 2008; Lin et al., 2008; Luo et al., 2009, 2011; Sharma et al., 2009; Sultana et al., 2008; Kundu et al., 2009; Rao et al., 2006; Asha et al., 2007; Shabeer et al., 2009). Although a number of molecules have been isolated and chemically characterized from *Phyllanthus* species, the bioactivity of only few have been determined (Calixto et al., 1998; Kinjo et al., 2003; Rao et al., 2006). With particular reference to their hepatoprotective activity, only few species (*P. amarus*, *P. urinaria*) have been screened in HepG2 cells against *tert*-butyl hydroperoxide.

The present study aims to investigate the hepatoprotective activity of 11 *Phyllanthus* species against liver damage mediated by toxin (*t*-BH) in HepG2 cells and determine its antioxidant activity.

## Materials and methods

### Plant material

*Phyllanthus* species (Euphorbiaceae) – *P. amarus* Schumach., *P. urinaria* L., *P. debilis* Klein ex Willd, *P. tenellus* Roxb., *P. virgatus* G.Forst., *P. maderaspatensis* L., *P. reticulatus* Poir., *P. polyphyllus* Willd., *P. emblica* L., *P. indofischerii* Bennet. and *P. acidus* (L.) Skeels were selected for the study. Individual plant specimens of the above species were collected in 2009–2010 from southern India and Andaman and Nicobar Islands. The plant species were identified and authenticated by the Botanical Survey of India, Kolkata. The specimens of these plant species were deposited at the School of Ecology and Conservation, University of Agricultural Sciences, Bangalore. The details of voucher and place of deposition are given in Table 1. These species comprised of 6 herbs, 2 shrubs and 3 trees.

### Preparation of the plant extract

The leaves and stems of each plant species were dried and ground into a fine powder. The tissue powder (10g)

was extracted with methanol under reflux three-times for 30–40 min each and filtered using a Whatman qualitative filter paper (Grade 1–11  $\mu$ m particle size) to obtain methanol extract. The remaining residue after methanol extraction was successively refluxed in water three-times for 30–40 min and the pooled aqueous extract was filtered using a Whatman Qualitative filter paper (Grade 1–11  $\mu$ m particle size). The solvents were then dried in a rotary evaporator under reduced pressure at a temperature of 60°C to obtain a dark brown to black paste in case of methanol and a dark brown powdered form in case of aqueous extracts. The methanol and successive water extract yield of all species was about 10% except *P. maderaspatensis* and *P. virgatus* which yielded about 6–8%.

### Determination of phyllanthin and hypophyllanthin

Phyllanthin and hypophyllanthin are the two major lignans of the *Phyllanthus* genus. These two compounds present in *P. amarus* have been shown to have hepatoprotective effect against carbon tetrachloride and galactosamine and ethanol induced hepatotoxicity in primary cultured rat hepatocytes and HepG2 cells (Syamsunder et al., 1985; Krithika et al., 2009; Chirdchupunseree & Pramyothin, 2010). Here, we attempted to examine if these lignans were also responsible in the hepatoprotective activity exhibited by other *Phyllanthus* species.

Estimation of phyllanthin and hypophyllanthin was done based on Sharma et al. (1993). The methanol extracts (100 mg) and 300 mg of the aqueous extracts of *Phyllanthus* species were dissolved in 10 mL of HPLC grade methanol. The samples were sonicated for 3 min. These samples were kept in boiling water bath for 5 min and then allowed to stand until they reached the room temperature. The samples were made up to 10 mL with HPLC grade methanol. They were sonicated again for 6 min and filtered with 0.22  $\mu$  filters (Rankem). The filtrate was used for estimation of phyllanthin and hypophyllanthin using HPLC. The standard phyllanthin and hypophyllanthin were dissolved separately at a concentration of 0.16 mg/mL in methanol were used as the standards.

The phyllanthin and hypophyllanthin was detected using a C18 column at 230 nm in HPLC (Agilent). The mobile phase consisted of acetonitrile and phosphate

Table 1. List of *Phyllanthus* species with the voucher number and location details.

Sl. No.	Species name	Habit	Voucher no.	Location
1	<i>P. acidus</i> (L.) Skeels	Tree	SK 225	Gandhi Krishi Vigyan Kendra, Karnataka
2	<i>Phyllanthus amarus</i> Schumach. & Thonn.	Herb	SK 108, SK717	Kolhapur, Maharashtra; Carbynchowk, South Andaman
3	<i>P. debilis</i> Klein ex Willd	Herb	SK 113, SK784	Chennai, Tamil Nadu; North-South road, Great Nicobar
4	<i>P. emblica</i> L.	Tree	SK 102, SK622	Sholapur, Maharashtra; Harmutty, Assam
5	<i>P. indofischeri</i> Bennet.	Tree	SK 541	Biligiri Rangaswamy Temple Hills, Karnataka
6	<i>P. maderaspatensis</i> L.	Herb	SK 112	Chennai, Tamil Nadu
7	<i>P. polyphyllus</i> Willd.	Shrub	SK 115	Gandhi Krishi Vigyan Kendra, Karnataka
8	<i>P. reticulatus</i> Poir.	Shrub	SK226, SK621	Gandhi Krishi Vigyan Kendra, Karnataka; Harmutty, Assam
9	<i>P. tenellus</i> Roxb.	Herb	SK 116	Gandhi Krishi Vigyan Kendra, Karnataka
10	<i>P. urinaria</i> L.	Herb	SK114, SK 764	Gandhi Krishi Vigyan Kendra, Karnataka; East-west road Great Nicobar
11	<i>P. virgatus</i> G.Forst.	Herb	SK111, SK785A	Chennai, Tamil Nadu; North-South road, Great Nicobar

buffer in the ratio of 83:17. The phyllanthin and hypophyllanthin standards (Natural Remedies Private Limited, Bangalore) were used for obtaining the standard curves. The flow rate was adjusted to 1.9 mL/min. The presence of phyllanthin and hypophyllanthin in the extracts were determined depending on the retention time, and the concentrations corresponding to their peak area was estimated using the standard curves obtained from reference phyllanthin and hypophyllanthin.

### Assessment of hepatoprotective activity of extracts against *t*-BH induced cytotoxicity in HepG2 cells

The hepatoprotective activity of the extracts of *Phyllanthus* species were assessed on HepG2 cells treated in the presence or absence of *tert*-butyl hydroperoxide toxin. HepG2 (ATCC No. HB-8065 HepG2) is a cultured immortalized hepatoma cell line from humans widely used for cytotoxicity, xenobiotic metabolism and carcinogenesis studies (Thabrew et al., 1997). The HepG2 cells have many of the morphological and biochemical characteristics of normal hepatocytes including the synthesis and secretion of plasma proteins (Knowles et al., 1980; Thabrew et al., 1997; Sohn et al., 2005).

The methanol extracts were prepared in plain DMSO at a stock concentration of 20 mg/mL whereas the successive water extract was prepared in Dulbecco's phosphate buffer saline (DPBS) at a stock concentration of 2 mg/mL, filtered through 0.22  $\mu$ m sterilized filter and further diluted in Dulbecco's phosphate buffer saline (DPBS).

The HepG2 cells were seeded at  $5 \times 10^4$  cells/well in a 96 well plate containing Earle's minimum essential media (EMEM) amended with 10% fetal bovine serum (FBS) and incubated overnight at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The used media was discarded and the wells were washed with (DPBS). Fresh EMEM with 10% FBS media along with different concentrations (3.125, 6.25, 12.5, 25, 50, 100 and 200  $\mu$ g/mL) of the extracts and silymarin as the positive control were added to the plate and incubated for 2 h.

The plate with the HepG2 cells and the extracts was divided into two halves. The *t*-BH toxin (1 mM) was added to one half of the plate and the other half was devoid of *t*-BH to assess the cytotoxicity of the extracts. The plate was incubated at 37°C for 2 h. The used media with the extracts and with silymarin in the positive control was discarded, washed with DPBS twice and fresh EMEM with 10% FBS media and [3-(4,5-D-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)] (500  $\mu$ g/mL) were added and incubated for 1 h. MTT, a yellow formazone is reduced to a purple formazan in living cells as these indicate the viability of cells. Post incubation, the supernatant was removed, and 100% DMSO was added to dissolve the formazan crystals. The absorbance of each well was measured at 570 nm using a VERSAmx tunable Micro plate reader (Molecular Devices) (Chandrasekaran et al., 2010). From the assay, we arrived at the non-cytotoxic

concentration of the extract to further assess their hepatoprotective activity.

The absorbance data was converted to number of cells following the standard relation between cell number and optical density. For each concentration of the extract, the mean  $\pm$  standard deviation (SD) of cell number was computed based on the five replicates.

These results were compared using one-way ANOVA and Dunnett's multiple comparison tests. The differences between the groups were considered statistically significant at  $p < 0.05$  and  $p < 0.01$ . The results are expressed in percentage hepatoprotection. The percentage protection was calculated according to Kinjo et al. (2003).

$$\% \text{protection} = \frac{\text{Sample value} - \text{Mean of } t\text{-BH control}}{\text{Mean of Cell control} - \text{Mean of } t\text{-BH control}} \times 100$$

where, sample value = viable cell number treated with the concentrations of extracts and *t*-BH

*t*-BH control = cell number treated with the toxin  
*t*-BH only

Cell control = cell number of the negative control

The EC<sub>50</sub> was calculated by using median effective plot. All the statistical analysis was performed using GraphPad Prism program (GraphPad Software, Inc., San Diego, CA, U.S.A.).

### DPPH assay

The antioxidant activity of the *Phyllanthus* species was assessed based on the free radical scavenging activity against 2, 2-diphenyl-1-picrylhydrazil (DPPH) free radical. The assay was performed according to Sharma and Bhat (2009) with slight modification. A total reaction mixture of 200  $\mu$ l was prepared in a 96 micro well plate. The reaction mixture consisted of 20  $\mu$ l of the methanol and aqueous extracts of *Phyllanthus* species or positive control (ascorbic acid) dissolved in methanol or negative control (methanol), 80  $\mu$ l of 50 mM Tris HCl buffer and 100  $\mu$ l of 50  $\mu$ M DPPH solutions. The extracts and positive control were diluted to a final concentration of 100  $\mu$ g/mL (3.125, 6.25, 12.5, 25, 50, 100  $\mu$ g/mL) and 10  $\mu$ g/mL (0.3125, 0.625, 1.25, 2.5, 5 and 10  $\mu$ g/mL), respectively. The reaction mixture was incubated at 37°C for 30 min. The decrease in the absorbance value was measured at 517 nm using ELISA reader (TECAN). The radical scavenging activity was computed and 50% Inhibitory concentration (IC<sub>50</sub>) was calculated for the antioxidant activity by linear regression.

## Results and discussion

Among the species of *Phyllanthus* studied, the methanol extracts of *P. debilis*, *P. virgatus*, *P. polyphyllus*, *P. emblica* and *P. indofischeri* and successive water extracts of *P. virgatus* and *P. polyphyllus* showed hepatoprotective

Table 2. Hepatoprotective and antioxidant activities along with phyllanthin and hypophyllanthin content of eleven *Phyllanthus* species.

<i>Phyllanthus</i> species	Extract	Hepatoprotective activity (50 µg/mL)	EC <sub>50</sub> value of the hepatoprotective activity (µg/mL)	IC <sub>50</sub> value of the antioxidant activity (µg/mL)	Phyllanthin (%)	Hypophyllanthin (%)
<i>P. polyphyllus</i> Willd.	Methanolic	Active	12	3.77	-	-
	Aqueous	Active	24	14.85	-	-
<i>P. emblica</i> L.	Methanolic	Active	19	3.38	-	-
	Aqueous	Not active	-	-	-	-
<i>P. indofischeri</i> Bennet.	Methanolic	Active	28	5.8	-	-
	Aqueous	Not active	-	-	-	-
<i>P. debilis</i> Klein ex Willd	Methanolic	Active	74	19.1	-	-
	Aqueous	Not active	-	-	-	-
<i>P. virgatus</i> G.Forst.	Methanolic	Active	>100	8.46	-	-
	Aqueous	Active	>100	53.89	-	-
<i>P. amarus</i> Schumach.	Methanolic	Not active	-	-	2.3	1.1
	Aqueous	Not active	-	-	0.027	0.013
<i>P. urinaria</i> L.	Methanolic	Not active	-	-	-	-
	Aqueous	Not active	-	-	-	-
<i>P. tenellus</i> Roxb.	Methanolic	Not active	-	-	-	-
	Aqueous	Not active	-	-	-	-
<i>P. maderaspatensis</i> L.	Methanolic	Not active	-	-	-	-
	Aqueous	Not active	-	-	-	-
<i>P. reticulatus</i> Poir.	Methanolic	Not active	-	-	-	-
	Aqueous	Not active	-	-	-	-
<i>P. acidus</i> (L.) Skeels	Methanolic	Not active	-	-	-	-
	Aqueous	Not active	-	-	-	-

<sup>1</sup>Positive control: Silymarin (EC<sub>50</sub> = 32 µg/mL) for hepatoprotective activity and Ascorbic acid (IC<sub>50</sub> = 3.69 µg/mL) for antioxidant activity.

activity at 50 µg/mL against *t*-BH induced cytotoxicity in HepG2 cells (Table 2). The EC<sub>50</sub> of the positive control silymarin was 32 µg/mL, while that of the methanol extracts of *P. polyphyllus*, *P. emblica* and *P. indofischeri* were 12, 19 and 28 µg/mL, respectively (Figure 1). The antioxidant activity of the methanol extracts of these three species was determined by the DPPH assay. The IC<sub>50</sub> for *P. polyphyllus*, *P. emblica* and *P. indofischeri* were 3.77, 3.38 and 5.8 µg/mL respectively compared to an IC<sub>50</sub> of 3.69 µg/mL from the positive control ascorbic acid (Table 2).

The results of the study indicated a significant hepatoprotective activity of *P. polyphyllus*, *P. emblica*, and *P. indofischeri*. The former two species have been studied previously for their hepatoprotective and antioxidant activities (Pramyothin et al., 2006; Liu et al., 2008; Raj Kapoor et al., 2008; Luo et al., 2009). Raj Kapoor et al. (2008) reported the hepatoprotective and antioxidant activity of *P. polyphyllus* in acetaminophen-induced hepatotoxicity in rat model. The hepatoprotective activity of *P. polyphyllus* was attributed to the inhibition of lipid peroxidation, enhancement of the antioxidant enzyme levels and by free radical scavenging (Raj Kapoor et al., 2008). In addition, preliminary phytochemical studies suggest that the flavonoids present in the plant could be responsible for the hepatoprotective activity (Raj Kapoor et al., 2008). However, a strong hepatoprotective activity and antioxidant activity of *P. indofischeri* is reported for the first time in literature. *P. indofischeri* is an endemic tree found only in

the Deccan plateau of India and shares morphological similarities with *P. emblica* (amla, Indian gooseberry) (Ganesan, 2003; Balakrishnan & Chakrabarty, 2007). The fruits of *P. indofischeri* are sold in the trade name of Amla (Ganesan, 2003; Ravikanth et al., 2011) but this species has not been studied for its bioactivity. The results of the experiment also indicated that the traditionally known hepatoprotective activity of admixtures of *Phyllanthus* raw drug could be due to the invariable presence of *P. emblica*, *P. indofischeri* and *P. polyphyllus*, all possessing hepatoprotective activity. The presence of *P. amarus* in the admixture might also contribute to the above activity (Khatoun et al., 2006).

Traditionally, *P. amarus* has been predominantly used in treating liver disorders (Sharma et al., 2000). Two phytochemicals namely phyllanthin and hypophyllanthin that are present in *P. amarus* have been argued to be responsible for the hepatoprotective activity. However, none of the species examined other than *P. amarus* was found to have phyllanthin and hypophyllanthin in the extracts studied. Clearly the hepatoprotective activity of the species reported here could be due to the activity of other chemical constituents.

## Conclusion

In conclusion, the methanol extracts of *P. polyphyllus*, *P. emblica* and *P. indofischeri* show promising hepatoprotective activity. Characterization of the active principles,

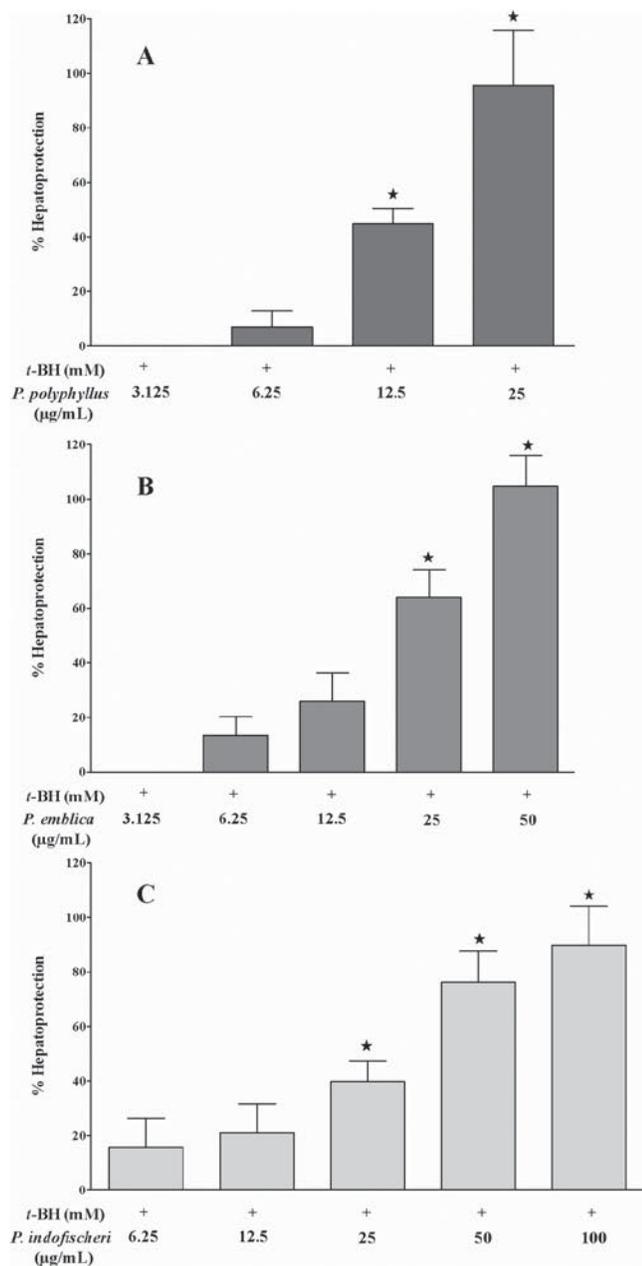


Figure 1. Hepatoprotective activity of methanol extracts of (A) *P. polyphyllus*, (B) *P. emblica* and (C) *P. indofischeri* against *t*-BH induced cell damage in HepG2 cells. \* $p < 0.001$  as compared to *t*-BH (1 mM). Error bars indicate standard deviation.

their mode of action and other *in vivo* experiments are needed to evaluate their therapeutic value as natural hepatoprotective agents. The biomolecule(s) with activity is currently being investigated.

## Acknowledgements

The work was supported by grants from the Department of Biotechnology, Government of India. We acknowledge the cooperation of the Forest Departments of Karnataka, Kerala and Andaman and Nicobar Islands for providing the necessary permission to visit the various forest divisions in the respective regions.

## Declaration of interest

The work was supported by grants from the Department of Biotechnology, Government of India (BT/PR 8359/NDB/51/145/2006).

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