Phyllanthin of Standardized *Phyllanthus amarus* Extract Attenuates Liver Oxidative Stress in Mice and Exerts Cytoprotective Activity on Human Hepatoma Cell Line

Rajesh Krithika*, Ramtej J Verma*,**, Pranav S Shrivastav†, Lonchin Suguna*

*Department of Biochemistry, Central Leather Research Institute, Council of Scientific and Industrial Research (CSIR), Chennai, India, **Zoology Department, †Chemistry Department, School of Sciences, Gujarat University, Ahmedabad, India

Background: ***Phyllanthus amarus*****, a traditional herbal liver-protecting medicine, is known to contain an active ingredient phyllanthin. Many research studies and clinical trials performed in the past using this plant have given contentious results which clearly accentuates the need for the standardization of the extracts.** ***Aim:*** **In this study,** ***P. amarus*** **extract was standardized for phyllanthin content by high performance thin layer chromatography (HPTLC) and high performance liquid chromatography (HPLC) analysis. The preventive role of a standardized extract of** ***P. amarus*** **against CCl_4-induced hepatotoxicity in vivo and in vitro using mice model and human hepatoma HepG2 cell line, respectively, was investigated.** ***Methods:*** **Phyllanthin was used as a marker phytochemical for the standardization of** ***P. amarus*** **extract. The extracts were verified for phyllanthin content by HPTLC and HPLC. Female mice were orally administered with CCl_4 either with or without standardized** ***P. amarus*** **extract in three different doses. Similarly, the cytoprotective role of the standardized extract in vitro was studied in HepG2 cell line.** ***Results:*** **Oral administration of CCl_4 resulted in increased oxidative stress, decreased antioxidative defense, and liver injury. Treatment with** ***P. amarus*** **along with CCl_4 significantly mitigated the increase in activities of liver marker enzymes, lipid peroxidation, and bilirubin content. It also increased the antioxidant enzymatic and non-enzymatic defense parameter levels. The results of the in vitro study conducted in HepG2 cells indicated that the hepatotoxin lowered 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolism and increased the release of transaminases which were corrected with co-incubation with** ***P. amarus*****.** ***Conclusion:*** **The study established a significant liver-protecting role of standardized** ***P. amarus*** **extract due to the presence of active ingredient phyllanthin. (J Clin Exp Hepatol 2011;1:57–67)**

The realization that nature provides the largest supply of plants blessed with medicinal properties is fascinating and worth exploring. As the world's potential plant resource has been underutilized till date, limitless opportunity exists in developing phytomedicines. The Indian subcontinent through ancient ages had followed this rich tradition of employing medicinal plants. However, these herbal medicaments suffer from major drawbacks such as lack of standardization of crude extracts and insufficient data on randomized placebo-controlled clinical studies. Consequently, skepticism regarding their efficacy cannot be ruled out. A systematic approach toward validation of such therapeutic claims has to be carried out, which will pave the way for phytotherapeutic compounds to attain scientific credibility and acceptance. Standardization of extracts which ensures a minimal level of active ingredient pertaining to a plant species is, thus, essential and of utmost importance.

Liver disorders are a major cause of morbidity and a global search is on for agents which can alleviate conditions of hepatic damage. The scarcity of trustworthy contemporary liver-protecting drugs had increased the market potential of their traditional counterparts.[1] Many Indian medicinal plants have been reported to have an excellent potential for the treatment of various infections and are considered as best alternatives for clinical allopathic drugs. *Phyllanthus* is one of the largest genus falling under kingdom Plantae, represented by around 1200 species. Until 1985, many species of *Phyllanthus* in India were collectively called as *Phyllanthus niruri* L. Surprisingly, *P. niruri* has been proven to be endemic to West Indies and America and has not been found to occur in India.[2,3] Many phytochemical and pharmacological studies reported from Indian subcontinent using *P. niruri*

Keywords: Hepatitis B virus, hepatoprotection, HepG2, high performance liquid chromatography, high performance thin layer chromatography, lipid peroxidation
Received: 07.07.2011; *Accepted:* 12.08.2011
Address for correspondence: Ramtej J Verma, Professor, Zoology Department, School of Sciences, Gujarat University, Navrangpura, Ahmedabad – 380009, India
E-mail: ramtejverma@gmail.com
Abbreviations: ALT: alanine transaminase; ANOVA: analysis of variance; AST: aspartate transaminase; CAM: complementary and alternative medicines; CAT: catalase; DMSO: dimethylsulfoxide; GSH: glutathione; HBV: hepatitis B virus; HETP: height equivalent of theoretical plates; HPLC: high performance liquid chromatography; HPTLC: high performance thin layer chromatography; HQC: high quality control; LDH: lactate dehydrogenase; LPO: lipid peroxidation; LQC: low quality control; MDA: malondialdehyde; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TAA: total ascorbic acid
doi: 10.1016/S0973-6883(11)60123-0

were apparently done on one of these three species namely *P. amarus*, *P. fraternus*, and *P. debilis*.[2] Unfortunately, even today these are sold under the same vernacular name and are advocated by traditional practitioners.[4] The case is even worse in research scenario as many previously published reports indicate that *P. amarus* is synonymous to *P. niruri*[5,6] which significantly lowers the reliability on the data obtained from such research outcomes.

Phyllanthus amarus is one among the most studied plant for its hepatoprotective property. It is called Bhuiamlki in Sanskrit. Presently, it is included as a major ingredient in various Ayurvedic preparations prescribed for jaundice. Interestingly, the use of this plant for liver ailments has been mentioned in Caraka Samhita which is a most ancient book on holistic Ayurvedic medicine. Even today, *P. amarus* is prescribed for the treatment of acute liver infections by many Indian practitioners. Various studies performed in the past have suggested a beneficial role of *P. amarus* for the treatment of hepatitis B virus (HBV) infection. The in vitro inhibitory potential of *P. amarus* on hepatitis B surface antigens was demonstrated first by Thyagarajan et al.[7] The suppression of viral release, inhibition of polymerase activity of HBV in culture medium, has been reported by Lee et al.[8] A similar antihepatitis property of *P. amarus* at cellular level has been reported by Jayaram and Thyagarajan.[9] Studies revealed that the antiviral property of *P. amarus* is mediated via its inhibitory effect on HBV polymerase activity and mRNA transcription and interaction with CCAAT-enhancer-binding proteins (C/EBP) and HBV enhancer I.[10] Very recently, Ravikumar et al[11] have shown that the methanolic extract of *P. amarus* significantly inhibited hepatitis C viral enzymes NS3 protease and NS5B RNA-dependent RNA polymerase and prevented viral replication as determined by TaqMan Real time RT-PCR. However, other reports have suggested a nil to very minimal role of *P. amarus* on HBV replication[12,13] which may be due to the difference in the plant source material used. In addition to this, previously conducted experimental studies and clinical trials using *P. amarus* have also produced controversial results.[14,15] This clearly emphasizes the need for the standardized preparations of *P. amarus* to exterminate such disparities.

Phyllanthin and hypophyllanthin are reported to be the active principles accountable for liver-protecting property of many *Phyllanthus* species.[16] High performance thin layer chromatography (HPTLC)[17] and high performance liquid chromatography (HPLC)[18,19] studies have been reported for the standardization of the extract for major lignins present in these species. Murugiayah et al[20] have analyzed *P. niruri* and determined four lignins in plasma by HPLC fluorescence and applied the method for bioavailability studies in rats. Recently, Yadav et al[21] have studied the synergistic effects of silymarin and standardized the ethanolic extract of *P. amarus* in rats against carbon tetrachloride (CCl_4)-induced acute liver toxicity and have concluded it to be the most effective among others owing to the higher percentage of phyllanthin in the *P. amarus* extract. The protective effect of phyllanthin against ethanol-induced on primary cultured rat hepatocytes has been by Chirdchupunseree and Pramyothin.[22]

Carbon tetrachloride is used as an experimental model to induce hepatotoxicity in many in vivo and in vitro systems. This model mimics viral hepatitis and is constantly employed to study the liver-protecting property of many compounds. Lipid peroxidation mechanism has emerged as a possible key event in pathogenesis of liver injury after CCl_4 administration. It undergoes metabolic activation to free radical products which have the ability to initiate lipid peroxidation.[23] This study was undertaken to explore the hepatoprotective and antioxidative property of the standardized extract of *P. amarus* against CCl_4-induced hepatotoxicity in vivo and in vitro. The inhibitory effect of *P. amarus* has been ascribed to the presence of a bioactive lignin marker phyllanthin, as determined by HPTLC and HPLC analysis.

MATERIALS AND METHODS

Chemicals

The chemicals used in the entire study were procured from Sigma-Aldrich (St. Louis, MO, USA), unless specified. HPLC grade ethyl acetate, hexane, acetone, and CCl_4 were obtained from Merck Specialties Pvt. Ltd. (Mumbai, India). Phyllanthin standard was procured from Southern Petrochemical Industries Corporation (SPIC), Chennai, India. Water used in the entire analysis was prepared from Milli-Q water purification system procured from Millipore (Bangalore, India).

Plant Material

The aerial parts of *P. amarus* were obtained through the courtesy of the Botany Department, Gujarat University, Ahmedabad, India, between the months of August and September 2007. The identity of the plant material was confirmed by Dr AKS Rawat, Scientist and Head, Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute (Ref: NBRI/PH/1-6-1/8), Lucknow, India. The aerial parts of *P. amarus* were shade dried, powdered, and soaked with ethanol:water (3×1000 mL, 50:50, v/v) at room temperature. The solvent was evaporated under reduced pressure in a rotary evaporator at 40°C. The yield of dry hydroalcoholic extract was 8.5% (w/w). The detailed procedure for extraction, purification, and characterization is reported in our previous report.[24]

High Performance Thin Layer Chromatography and High Performance Liquid Chromatography Analysis

The *P. amarus* extracts were evaluated for phyllanthin content by HPTLC and HPLC. Thin layer chromatography was

performed on pre-coated silica gel 60 F_{254} plates from E. Merck (Darmstadt, Germany) with a semi-automatic Linomat IV band spotter from CAMAG (Switzerland). A mobile phase consisting of hexane:acetone:ethyl acetate (7:2:1, v/v/v) was used for developing the plates. The plates were dried in a stream of air and then immersed in a freshly prepared solution of vanillin in concentrated sulfuric acid:ethanol (5:95 v/v) mixture.[17] The dried plates were then heated at 110°C to develop the color of the spots. Scanning was done with CAMAG TLC scanner 3 using win-CATS software at 540 nm. For reverse-phase separation of phyllanthin from other constituents, a Shimadzu LC-VP HPLC-UV system (Kyoto, Japan) consisting of LC-10AD prominence pump, SIL-HTc autosampler, CTO 10 ASvp column oven, and a DGU-14A degasser was used. Chromatographic separation was achieved on a Phenomenex C18 (250 mm × 4.6 mm, length × inner diameter with 5 µm particle size) column (Grace, USA), maintained at 40°C in a column oven. The mobile phase consisted of acetonitrile:water (60:40, v/v). For isocratic elution, the flow rate of the mobile phase was kept at 0.5 mL/min. The optimum injection volume was 20 µL and the wavelength of the detector was set at 230 nm. The auto sampler temperature was maintained at 10°C and the pressure of the system was 1000 psi.

Standard Stock, Calibration Standards and Quality Control Sample Preparation

The standard stock solution of phyllanthin (1 mg/mL) was prepared by dissolving requisite amount in methanol. Calibration standards were made at 20.0, 40.0, 80.0, 160, 320, 640, 1000, 1500, and 2000 ng/mL concentrations while the quality control samples were prepared at three levels, viz. 1750 ng/mL (HQC, high quality control), 500 ng/mL (MQC, medium quality control), and 55.0 ng/mL (LQC, low quality control).

Extraction Protocol

The procedure followed for the preparation and extraction of phyllanthin from gelatin capsules was the same as reported by Sharma et al.[18] The macerated aerial parts of the plant material were refluxed for 1 h in methanol containing 3% KOH. The refluxed material was filtered and the residue was washed with methanol and the total volume of the solution was made to 100 mL; 20 µL of this solution was used for HPLC analysis.

Validation Procedures for High Performance Liquid Chromatography Analysis

System suitability experiment was performed by injecting six consecutive injections using MQC (500 ng/mL) sample at the start of each batch during method validation. System performance was studied by injecting one CS-1 sample at the beginning of each analytical batch and before re-injecting sample during method validation. The carry-over effect of the autosampler was evaluated by sequentially injecting solutions of standard phyllanthin equivalent to highest standard (CS-9) in the calibration range and the mobile phase. The linearity of the method was determined by analysis of five linearity curves containing nine non-zero concentrations. Standard plots were drawn by plotting mean peak area against the amount injected. Each calibration curve was analyzed individually by using least-square linear regression. Precision (% coefficient of variation) was examined by performing the intra- and inter-day analysis of three replicates at LQC, MQC, and HQC levels. Intra-day assay was performed with an interval of 3 h in 1 day, while the inter-day precision was evaluated over 5 consecutive days. Accuracy of the method was checked from the recovery studies by the standard addition method. Known amounts of standard phyllanthin (80%, 100%, and 120%) were added to pre-analyzed capsule material and the amounts were calculated from the standard calibration curve.

In vivo Study

Animals

Female mice (*Mus musculus*) of Swiss strain between the weight range of 32 and 35 g (6–8 weeks old) were maintained under controlled conditions (temperature 25 ± 2°C; relative humidity 50–55°C; 12 h light/dark cycle) in the Animal house of the Zoology Department, Gujarat University, Ahmedabad. They were maintained on certified pelleted rodent food supplied by Amrut Feeds, Pranav Agro Industries Limited, Pune, India, and water ad libitum. The experimental procedure was assessed and approved by "The committee for the purpose of control and supervision of Experiment on Animals" (Reg – 167/1999/CPCSEA), New Delhi, India. All animal care guidelines imposed by the Prevention of Cruelty to Animals Act, 1960 (59 of 1960), Government of India, were strictly followed during the entire course of the study.

Acute Oral Toxicity Study

The toxicity study was performed in female mice by following the guidelines imposed by the Organization for Economic Co-operation and Development (OECD) No – 425 [main dose test of Up-and-Down Procedure (UPD)]. The LD_{50} of the standardized extract was calculated using "AOT 425" software (Environment Protection Agency, Washington, DC, USA).

Experimental Protocol

The mice were divided into seven groups consisting of 10 animals each. Group I mice were marked as control and were given free access to food and drinking water, Group II received 0.2 mL of olive oil which was used as the vehicle to dissolve CCl_4, Group III were given *P. amarus* extract

(300 mg/Kg body weight, p.o.) which served as plant control group, Group IV were administered CCl_4 (826 mg, 0.2 mL olive oil/Kg body weight, p.o.), and Groups V–VII were orally treated with CCl_4 along with 100, 200, and 300 mg/Kg body weight/day of *P. amarus* extract. Treatment was continued for 30 days in all groups. After the last oral dose in each group, blood was collected by cardiac puncture and serum separated by centrifugation at 1000× *g* for 10 min. Liver was blotted free of blood and used for biochemical analysis.

Assessment of Liver Function

The lipid peroxidation was estimated in the liver by the method of Ohkawa et al.[25] The glutathione peroxidase (GPx) (EC 1.11.1.9) activity was assayed by the modified method of Pagila and Valentine.[26] The glutathione reductase (GR) (EC 1.11.1.9) activity was estimated by the method of Mavis and Stellwagen.[27] The activity of glutathione-S-transferase (GST) (EC 2.5.1.18) was measured by the method of Habig et al.[28] Superoxide dismutase (SOD) (EC 1.15.1.1) was assayed by using the method of Kakkar et al.[29] The liver catalase (CAT) (EC 1.11.1.6) activity was assayed by the method of Luck.[30] The glutathione (GSH) content was estimated by the method of Grunert and Philips.[31] Total ascorbic acid (TAA) content in the liver was estimated by the method of Roe and Kuether.[32] Protein content was determined by the method of Lowry et al[33] using bovine serum albumin as a standard.

Serum Parameters

Lactate dehydrogenase (LDH) was determined by using the method of King.[34] The γ-glutamyl transferase (γ-GT) activity in the serum was estimated following the method of Orlowski and Meister.[35] The serum bilirubin content was determined by using the method of Malloy and Evelyn.[36]

In vitro Study

Cell Culture and Treatment

HepG2 were obtained from ATCC, USA. These were grown on Eagle's minimum essential medium (EMEM, GIBCO, USA) supplemented with 10% (v/v) heat inactivated fetal calf serum, 2 mM L-glutamine, MEM non-essential amino acids, gentamycin (50 µg/mL), and penicillin (100 µg/mL). Cells were plated in a 24-well plate (3×10^4 cells/well in 1 mL of medium) for 48 h at 37°C and 5% CO_2. They were then treated with different agents for 24 h. Group I – Serum-free MEM Control; Group II – DMSO control (0.25% (v/v) in serum-free MEM); Group III – *P. amarus* control extract (600 µg/mL); Group IV – 0.4% (v/v) CCl_4 in 0.25% DMSO; Group V – CCl_4 (0.4% v/v) + *P. amarus* extract (200 µg/mL); Group VI – CCl_4 (0.4% v/v) + *P. amarus* extract (400 µg/mL); Group VII – CCl_4 (0.4% v/v) + *P. amarus* extract (600 µg/mL).

Parameters Studied

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Assay

After the treatment as above with various agents, 100 µL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (5 mg/mL) was added to each well and the cells were incubated at 37°C in the dark. After 4 h, the medium was removed and formazan crystals were dissolved in 100 µL of DMSO. Optical density was assessed using a microplate reader at 570/630 nm.[37] The percentage viability of the cells was calculated by the formula:

$$\frac{(A_{570}-A_{630})_{\text{sample}}}{(A_{570}-A_{630})_{\text{control}}}\times 100\%$$

Alanine Transaminase (ALT) and Aspartate Transaminase (AST) Leakage

After 24 h treatment with various additives, the transaminases activities (ALT and AST) were measured in the medium by International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) method as the kinetic reaction at 340 nm using kits procured from Agappe Diagnostics, Ernakulam, Kerala, India.

Hepatoprotective Index

The liver-protecting activity of the standardized *P. amarus* extract in vivo and in vitro was expressed as the hepatoprotective percentage (H)[38] which was calculated using the formula:

$$H=\left(1-\frac{T-V}{C-V}\right)\times 100$$

where *T* is the mean value of plant extract along with the toxin CCl_4 (groups 5, 6, or 7), *C* is the mean value of CCl_4 alone (group 4), and *V* is the mean value of vehicle control animals (group 2).

Statistical Analysis

The in vivo and in vitro data were expressed as mean ± SEM. Results were statistically analyzed by the one-way analysis of variance (ANOVA) followed by LSD multiple comparison tests using SPSS software version 13.0. Differences were considered significant when the *P* values were <0.001.

RESULTS

Standardization of *Phyllanthus amarus* Extract

Phyllanthin was used as a marker for standardizing the *P. amarus* extract. The higher inhibitory effect of *P. amarus* extract can be related to the presence of phyllanthin as evident from HPTLC and HPLC studies for the plant extracts and standard phyllanthin sample. The plant extract in ethanol:water (50:50, v/v) showed seven-well resolved spots on TLC plates with a retention factor of 0.32

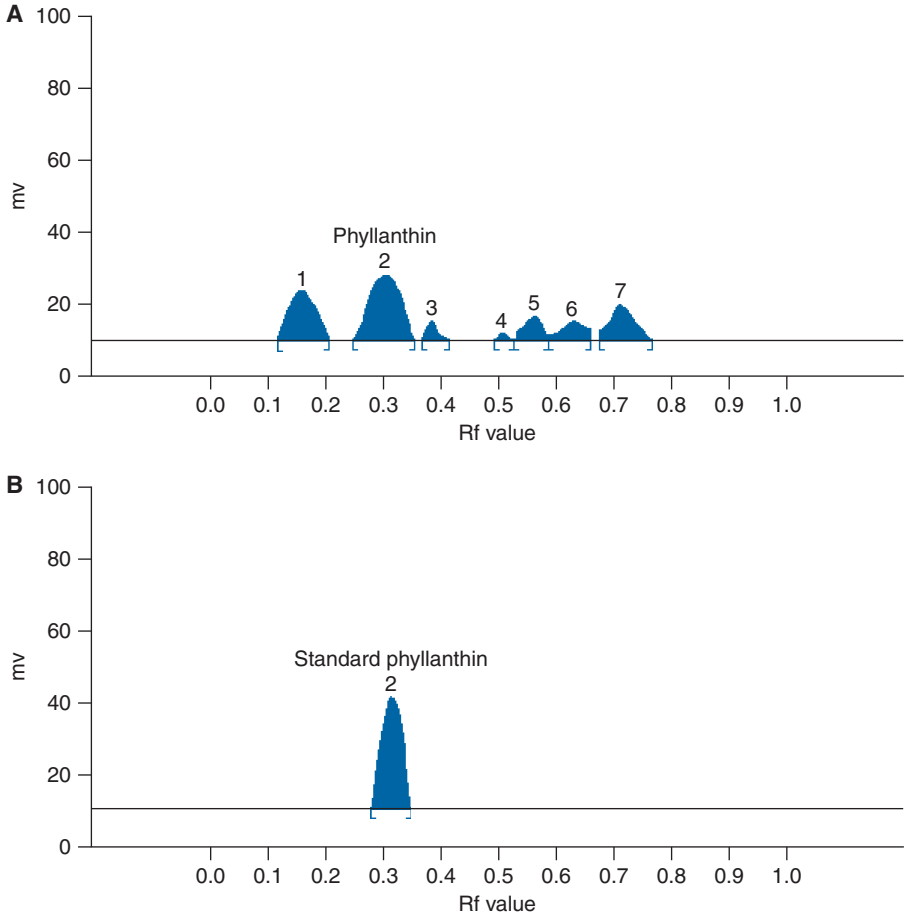

Figure 1 High performance thin layer chromatography analysis of (A) *Phyllanthus amarus* extract and (B) standard phyllanthin on pre-coated silica gel 60 F_{254} plates with hexane:acetone:ethyl acetate (7:2:1, v/v/v) as the mobile phase.

corresponding to phyllanthin (Figure 1). The retention time for phyllanthin on the reversed-phase HPLC column was 22.3 min in a total run time of 40 min (Figure 2). The capacity factor, which describes the rate at which the analyte migrates through the column, was 3.95 based on the dead time of 4.5 min. The number of theoretical plates and the height equivalent of theoretical plates (HETP) were 1989 and 0.125 mm, respectively. Throughout the method validation, the precision (% CV) of system suitability test was observed in the range of 0.07–0.14% for the retention time and 1.01–1.5% for the area response of phyllanthin. The signal to noise ratio for the system performance was ≥ 100 for phyllanthin. Carry-over evaluation was performed in each analytical run to ensure that it does not affect the accuracy and the precision of the proposed method. There was a negligible carry-over observed during autosampler carry-over experiment. All five calibration curves were linear over the concentration range of 20.0–2000 ng/mL (Table 1A). A straight-line fit was made through the data points by the least-square regression analysis to give the mean linear equation $y = 30.02x - 72.7$ with a correlation coefficient (r^2) of 0.9987. The standard deviation value for slope, intercept, and correlation coefficient observed were 0.013, 0.167, and 0.0008, respectively. The accuracy and the precision (% CV) observed for the calibration curve standards ranged from 98.2% to 100.2% and from 0.4% to 1.7%, respectively. The intra-day and inter-day precision and accuracy were established from validation runs performed at HQC, MQC, and LQC levels (Table 1B). The intra-day precision (% CV) ranged from 1.1% to 1.6% and the accuracy was within 98.8–100.5%. For the inter-day experiments, the precision varied from 1.3% to 1.8% and the accuracy was within 98.4 to 100.8%. The recovery of extracted samples varied from 98.4% to 99.1% for all the three samples with % CV values < 1.0 as shown in Table 1C.

Toxicity Study and Biochemical Analysis

The standardized extract of *P. amarus* was found to be non-toxic up to a dosage of 2000 mg/Kg body weight. Table 2 represents the results of lipid peroxidation levels, enzymatic, and non-enzymatic antioxidative parameters in the liver of various control and experimentally treated mice. No significant differences were observed between

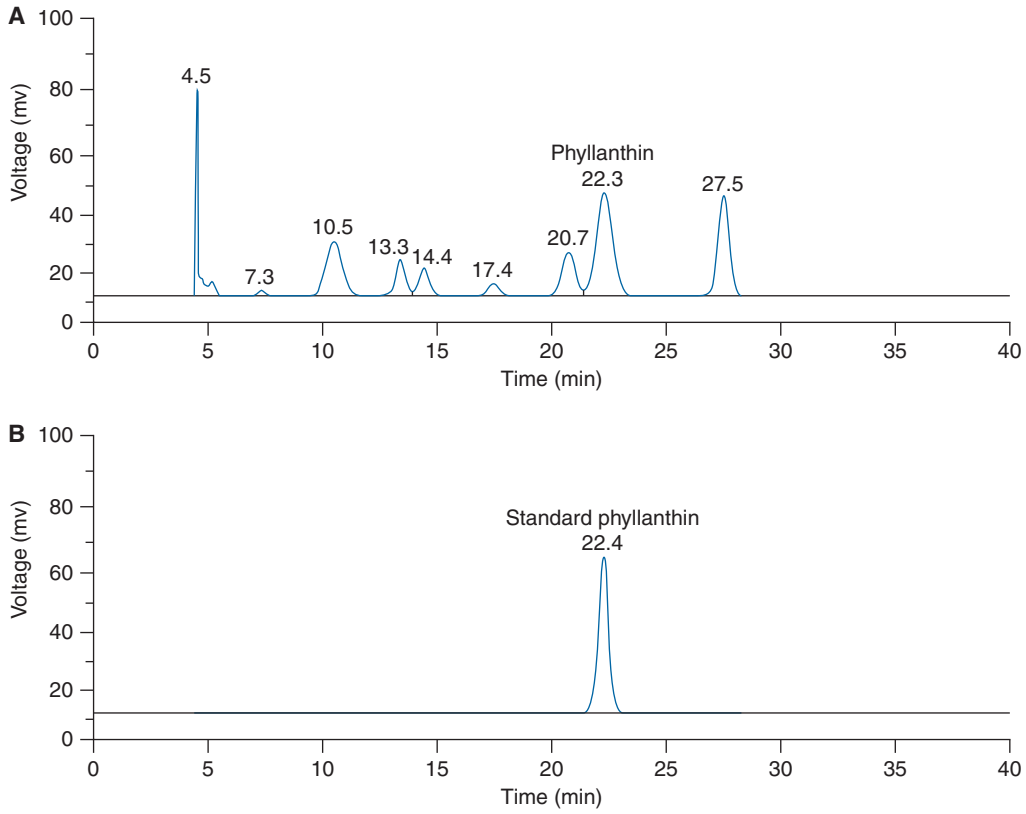

Figure 2 Reversed-phase high performance liquid chromatography chromatograms of (A) *Phyllanthus amarus* extract and (B) standard phyllanthin on phenomenex C18 column using acetonitrile:water (60:40, v/v) as the mobile phase.

Table 1A Mean peak areas obtained from five calibration curves containing nine calibration standards (CS-1 to CS-9).

Concentration (ng/mL)	Mean area (μV×sec)±SD
CS-1 (20.0)	592.5±10.1
CS-2 (40.0)	1175.2±18.2
CS-3 (80.0)	2395.3±39.3
CS-4 (160)	4778.2±61.4
CS-5 (320)	9505.1±83.3
CS-6 (640)	19,020.5±105.0
CS-7 (1000)	29,832.4±113.7
CS-8 (1500)	44,735.2±278.6
CS-9 (2000)	60,225.3±341.3

the animals of untreated control and vehicle control. *P. amarus* alone administered mice also resembled control mice. Oral administration of CCl_4 significantly ($P<0.001$) increased the LPO levels, while the antioxidative indices, TAA, GSH, GPx, GR, GST, SOD, and CAT, were found to be reduced ($P<0.001$) drastically. However, the co-treatment of CCl_4 along with *P. amarus* significantly decreased ($P<0.001$) the lipid peroxidation levels with a concurrent, dose-dependent recovery in the antioxidative defense mechanisms. Treatment with the highest dose of *P. amarus* exhibited a maximum protective effect as compared with the vehicle control.

Table 3 shows the results of clinical marker enzymes, viz. LDH and γ-GT and bilirubin in the serum of control and experimental mice. Results indicated that in CCl_4-induced mice, the levels of LDH, γ-GT, and bilirubin ($P<0.001$) were significantly increased when compared with the vehicle control group. Oral administration of *P. amarus* extract along with CCl_4 resulted in the restoration of these marker enzymes and bilirubin to near normal levels. On the other hand, *P. amarus* alone treated mice did not show any significant differences in these parameters as compared with the vehicle control.

Figures 3A–C depicts the results of MTT assay and leakage enzymes—ALT and AST in vitro. Treatment with CCl_4 caused a significant decrease ($P<0.001$) in the metabolism of MTT. Similarly, CCl_4 treatment for an exposure time of 24 h caused an increased release of intracellular enzymes ALT and AST. The toxin-induced changes were significantly reduced by *P. amarus* extract.

The calculated hepatoprotective percentage (H) has been tabulated (Table 4). Results indicated that the protection percentage was dose-dependent and maximum for *P. amarus* at the dosage regimen of 300 mg/Kg b.wt (Group VII) in vivo and 600 μg/mL (Group VII) in vitro.

Table 1B Intra-day and inter-day precision and accuracy for phyllanthin.

QC-ID	Nominal conc. (ng/mL)	Intra-day				Inter-day			
		n	Mean conc. found (ng/mL)[a]	% CV	% Accuracy	*n*	Mean conc. (found ng/mL)[b]	% CV	% Accuracy
HQC	1750	3	1759	1.1	100.5	15	1764	1.8	100.8
MQC	500	3	497	1.4	99.4	15	492	1.7	98.4
LQC	55.0	3	54.3	1.6	98.8	15	54.2	1.3	98.5

CV: coefficient of variation; *n*: total number of observations; HQC: high quality control; MQC: medium quality control; LQC: low quality control.
[a]Mean of three replicates at each concentration.
[b]Mean of three replicates for five precision and accuracy batches.

Table 1C Recovery results of phyllanthin added to pre-analyzed samples ($n=3$).

Original (μg/mg)	Amount added (μg/mg)	Amount found (μg/mg)	% Mean recovery (% CV)
12.26	9.84	21.74	98.4 (0.44)
12.29	12.30	24.34	99.1 (0.51)
12.25	14.76	26.66	98.7 (0.93)

CV: coefficient of variation.

Table 2 Effect of *Phyllanthus amarus* against CCl_4-induced changes in liver of mice.

Biochemical parameters	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
LPO (*n* moles MDA/mg protein/60 min)	1.74±0.45	1.71±0.33	1.70±0.67	3.93±0.99[a]	3.23±0.82[b]	2.45±0.55[b]	1.72±0.49[b]
GPx (*n* moles NADPH consumed/mg protein/min)	2.94±0.71	2.95±0.88	2.93±0.48	1.09±0.92[a]	1.60±0.45[b]	2.18±0.71[b]	2.95±0.71[b]
GR (*n* moles NADPH consumed/mg protein/min)	2.08±0.56	2.09±0.39	2.09±0.77	1.00±0.72[a]	1.34±0.93[b]	1.69±0.81[b]	2.07±0.91[b]
GST (μ moles of CDNB-GSH conjugate formed/ mg protein/min)	2.17±0.49	2.18±0.28	2.16±0.29	1.13±1.09[a]	1.50±0.62[b]	1.71±0.91[b]	2.16±0.38[b]
SOD (units/mg protein)	3.40±0.48	3.42±0.87	3.40±0.60	1.14±1.01[a]	1.89±0.51[b]	2.59±1.01[b]	3.41±0.99[b]
CAT (μ moles H_2O_2 consumed/mg protein/min)	10.29±1.19	10.32±1.41	10.28±1.37	4.32±1.09[a]	6.02±1.38[b]	7.98±1.52[b]	10.30±1.12[b]
GSH (μg/100 mg tissue weight)	40.40±1.21	40.35±1.67	40.52±0.99	17.33±1.21[a]	24.26±1.43[b]	32.30±0.69[b]	40.32±1.32[b]
TAA (mg/g tissue weight)	5.14±0.96	5.17±0.78	5.15±0.99	3.03±0.87[a]	4.16±1.12[b]	4.49±0.96[b]	5.16±0.92[b]

LPO: lipid peroxidation; MDA: malondialdehyde; GPx: glutathione peroxidase; NADPH: nicotinamide adenine dinucleotide phosphate; GR: glutathione reductase; GST: glutathione-S-transferase; CDNB: chloro-dinitro benzene; GSH: glutathione; SOD: superoxide dismutase; CAT: liver catalase; TAA: total ascorbic acid.
Results are expressed as mean±SEM, $n=10$. No significance difference was noted between groups 1, 2, and 3.
[a]As compared between vehicle control (group 2) and toxin treated (group 4).
[b]As compared between toxin treated (group 4) and toxin+antidote treated (groups 5, 6, and 7). Level of significance $P<0.001$.

Table 3 Effect of *Phyllanthus amarus* against CCl_4-induced changes in serum of mice.

Biochemical parameters	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
LDH (μmol pyruvate liberated/mg protein/min)	0.84±0.45	0.85±0.78	0.84±1.12	1.75±0.87[a]	1.54±0.67[b]	1.28±0.92[b]	0.87±0.87[b]
γ-GT (μmol *p*-nitroaniline liberated/mg protein/min)	0.77±0.66	0.77±0.56	0.76±0.95	1.51±0.39[a]	1.33±0.76[b]	1.02±0.92[b]	0.78±0.99[b]
Bilirubin (mg/L)	1.22±0.99	1.20±0.94	1.19±0.86	3.03±0.78[a]	2.49±1.12[b]	1.92±0.93[b]	1.22±0.76[b]

LDH: lactate dehydrogenase; γ-GT: γ-glutamyl transferase.
Results are expressed as mean±SEM, $n=10$. No significance difference was noted between groups 1, 2, and 3.
[a]As compared between vehicle control (group 2) and toxin treated (group 4).
[b]As compared between toxin treated (group 4) and toxin+antidote treated (groups 5, 6, and 7). Level of significance $P<0.001$.

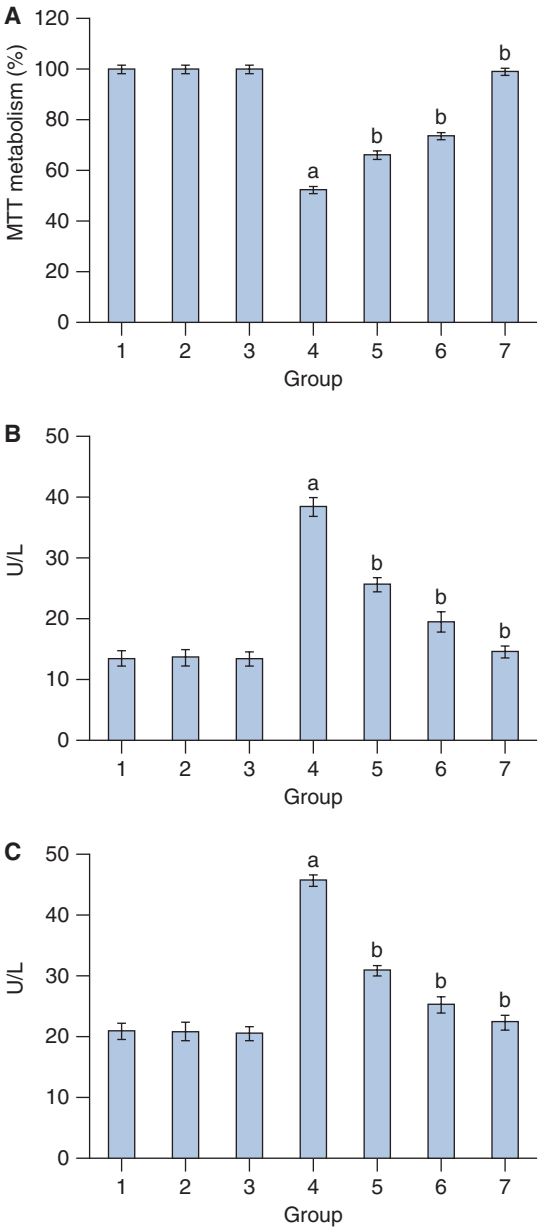

Figure 3 Protective effect of *Phyllanthus amarus* against CCl_4-induced toxicity in HepG2 as measured by (A) MTT assay (B) alanine transaminase leakage and (C) aspartate transaminase. Results are expressed as mean±SEM ($n=5$). No significance difference was noted between groups 1, 2, and 3. [a]Compared with vehicle control (group 2) and CCl_4 treated (group 4); [b]compared with CCl_4 treated (group 4) and CCl_4+ antidote treated (groups 5, 6, and 7). Level of significance $P<0.001$. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Table 4 Hepatoprotective index (%).

In vivo parameters	Group V (CCl_4+ *P. amarus* 100 mg/ Kg b.wt)	Group VI (CCl_4+ *P. amarus* 200 mg/ Kg b.wt	Group VII (CCl_4+ *P. amarus* 300 mg/ Kg b.wt
LPO	32.0	67.0	99.5
GPx	27.4	59.0	100.0
GR	31.0	63.0	98.2
GST	32.0	55.0	98.1
SOD	33.0	64.0	99.6
CAT	29.0	61.0	99.7
GSH	30.0	65.0	99.8
TAA	53.0	68.0	99.5
LDH	23.0	52.0	98.0
γ-GT	24.0	66.0	98.6
Bilirubin	30.0	60.7	98.9
In vitro parameters	**Group V (CCl_4+ *P. amarus* 200 μg/mL)**	**Group VI (CCl_4+ *P. amarus* 400 μg/mL)**	**Group VII (CCl_4+ *P. amarus* 600 μg/mL)**
MTT	29.0	44.8	98.0
ALT	51.8	76.2	96.0
AST	60.0	82.6	94.1

LPO: lipid peroxidation; GPx: glutathione peroxidase; GR: glutathione reductase; GST: glutathione-S-transferase; SOD: superoxide dismutase; CAT: liver catalase; GSH: glutathione; TAA: total ascorbic acid; LDH: lactate dehydrogenase; γ-GT: γ-glutamyl transferase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ALT: alanine transaminase; AST: aspartate transaminase.

DISCUSSION

The demand for complementary and alternative medicines (CAM) is increasing among patients with liver injury. Undoubtedly, medicinal plants containing well-defined ingredients can be considered as supreme liver-protecting agents. Currently, strict rules are imposed on herbal preparations by many regulatory agencies worldwide, and the presence of sufficient quantity of any known marker compound in them is considered mandatory. Chromatographic fingerprinting of plant extracts is a holistic method for detection and quality control of active ingredients. The phenomenon of 'standardization' has now been accepted as an essential part of medicinal plant research.

Phyllanthus amarus belongs to the family Euphorbiaceae and is widely distributed in India, China, and other tropical countries. This plant has been used extensively as a folklore medicine and has been reported to be antiviral, antibacterial, antidiabetic, antiplasmodial, and antioxidative[39] in nature. *P. amarus* has also been proved in providing protection against ethanol[1] and paracetamol.[40] The antihepatoxic potential of phyllanthin against galactosamine-induced cytotoxicity in primary cultured rat hepatocytes has been reported by Syamasundar et al.[16] The hepatoprotective potential of the methanolic extract of *P. amarus* on ethanol-induced oxidative stress was studied by Faremi et al.[41] Similarly, the liver-protecting role of *P. amarus* against aflatoxin B1-induced hepatic damage and its effect on the fibrotic markers as against alcohol and poly-unsaturated fatty acid-induced toxicity has been studied by Naaz et al[42] and Surya Narayanan et al,[43] respectively.

However, earlier attempts made to demonstrate the hepatoprotective property of this plant against CCl_4-induced liver damage had produced debatable results. For example, Sane et al[44] have compared the liver-protecting properties of two species of *Phyllanthus* namely *P. amarus* and *P. debilis* and have concluded the latter to be a better hepatotonic. Likewise, a study conducted in Taiwan which compared the antioxidative and hepatoprotective ability of 11 species of *Phyllanthus* has also reported *P. amarus* to be effective in decreasing only the elevated ALT levels.[45]

Oxidative stress plays a significant role in the pathogenesis of many diseases, and antioxidants have emerged as the key therapeutic agents in the management of various associated disorders. The enzymatic and non-enzymatic antioxidative scavengers are considered as natural protectors against oxidative assaults frequently encountered within living systems. CCl_4 toxicity has been generally accepted as an excellent tool for the induction of oxidative injury in various experimental models. This investigation was thus carried out to explore the possible liver protective mechanism of the standardized extract of *P. amarus* in vivo and in vitro against CCl_4-induced toxicity.

The significant reduction seen in the oxidative defense parameters (Table 2) with a concomitant increase in lipid peroxidation after a 30-day treatment with CCl_4 suggested oxidative stress. Malondialdehyde (MDA), a secondary product of lipid peroxidation, was utilized as an indicator of tissue damage. Oral administration of CCl_4 caused significant reduction in the activities of antioxidative enzymes—GPx, GR, GST, SOD, and CAT. A similar decrease was observed in the hepatic GSH and TAA contents. These changes were effectively mitigated by co-treatment of CCl_4 along with the standardized *P. amarus* extract in a dose-dependent manner. The maintenance of near normal levels of hepatic MDA and revival of both enzymatic and non-enzymatic antioxidative status in mice administered with *P. amarus* extract along with CCl_4 offers strong supportive evidence to confirm the antioxidative potential of the plant extract.

Carbon tetrachloride treatment for 30 days caused a significant rise in LDH, γ-GT activities, and bilirubin content in the serum of mice (Table 3). Any increase seen in the activities of these intracellular liver enzymes indicates leakage due to membrane damage. Bilirubin assay is also considered as a sensitive indicator of liver cell damage as it substantiates the functional integrity of the liver with the severity of necrosis.[46] A significant reduction seen in activities of LDH and γ-GT after extract treatment suggests recovery toward normalization, which might be due to the recoupment of the cell membrane, while the suppression of increased bilirubin content indicates the ability of the extract to stabilize biliary dysfunction which is prone to occur after CCl_4 liver injury. The overall results of the in vivo study indicated that the standardized *P. amarus* extract reversed the pathological changes of CCl_4-induced liver injury in mice.

The in vitro hepatoprotective study was carried out in HepG2 cells which are considered to be one of the best alternatives to hepatocytes.[47] This cell line is known to resemble normal liver cells both morphologically and biochemically. A significant reduction was observed in MTT metabolism in CCl_4-treated cells, which indirectly measured the survival rate and the extent of mitochondrial damage.[48] The increased leakage of enzymes, ALT and AST, after a 24 h incubation period with the hepatotoxin envisaged membrane damage. Co-incubation of the standardized extract of *P. amarus* along with CCl_4 improved MTT metabolism and curtailed the leakage of ALT and AST in a dose-dependent manner (Figures 3A–C).

Kiemer et al[49] have studied the antiinflammatory activity of *P. amarus* extract in a model of acute hepatitis and had suggested that this particular of the plant may be responsible for the antihepatitis activity. The role of *P. amarus* extract in combating the oxidative stress and hepatic cell damage in clinically diagnosed hepatitis B patients by virtue of its antioxidative property and reduction of lipid peroxidation has been recently reported by Nikam et al.[50] Our study confirmed the hepatoprotective role of *P. amarus* both in vivo and in vitro. In conclusion, the standardized extract of *P. amarus* provided significant protection against CCl_4-induced hepatic damage by decreasing the levels of leakage enzymes and by enhancing the antioxidative defense mechanisms. Collectively, the data presented established that the protective effect offered by the standardized extract of *P. amarus* is mediated by its antioxidative ability which is due to the presence of higher percentage of phyllanthin as evident by the HPTLC and HPLC studies.

ACKNOWLEDGMENTS

The fellowship assistance to Dr Krithika Rajesh (Research Associate) from the Indian Council of Medical Research (ICMR 45/37/2009BMS-TRM) is gratefully acknowledged. The authors also gratefully acknowledge the instrumentation facility provided by Zydus Cadila, Ahmedabad. The authors are thankful to Ms. Aishwariya Bhaskaran for her valuable support in literature collection.

CONFLICTS OF INTEREST

All authors have none to declare.

REFERENCES

1. Pramyothin P, Ngamtin C, Poungshompoo S, Chaichantipyuth C. Hepatoprotective activity of *Phyllanthus amarus* Schum. et. Thonn. extract in ethanol treated rats: *in vitro* and *in vivo* studies. *J Ethnopharmacol* 2007;114:169–73.
2. Bagchi GD, Srivastava GN, Singh SC. Distinguishing features of medicinal herbaceous species of *Phyllanthus* occurring in Lucknow district (UP), India. *Int J Pharmacog* 1992;30:161–8.
3. De B, Datta PC. Pharmacognostic evaluation of *Phyllanthus amarus*. *Pharm Biol* 1990;28:81–8.

4. Khatoon S, Rai V, Rawat AK, Mehrotra S. Comparative pharmacognostic studies of three *Phyllanthus* species. *J Ethnopharmacol* 2006;104:79–86.
5. Manjrekar AP, Jisha V, Bag PP, et al. Effect of *P. niruri.* Linn. treatment on liver, kidney and testes in CCl_4 induced hepatotoxic mice. *Ind J Exp Biol* 2008;46:514–20.
6. Islam A, Selvan T, Mazumder UK, Gupta M, Ghosal S. Antitumour effect of phyllanthin and hypophyllanthin from *Phyllanthus amarus* against Ehrlich ascites carcinoma in mice. *Pharmacologyonline* 2008;2:796–807.
7. Thyagarajan SP, Thiruneelakantan K, Subramanian S, Sundaravelu T. *In vitro* inactivation of HBsAg by *Eclipta alba Hassk* and *Phyllanthus niruri* Linn. *Ind J Med Res* 1982;76(Suppl):124–30.
8. Lee CD, Ott M, Thyagarajan SP, Shafritz DA, Burk RD, Gupta S. *Phyllanthus amarus* down-regulates hepatitis B virus mRNA transcription and replication. *Eur J Clin Invest* 1996;26:1069–76.
9. Jayaram S, Thyagarajan SP. Inhibition of HBsAg secretion from Alexander cell line by *Phyllanthus amarus*. *Ind J Pathol Microbiol* 1996;39:211–5.
10. Ott M, Thyagarajan SP, Gupta S. *Phyllanthus amarus* suppresses hepatitis B virus by interrupting interactions between HBV enhancer I and cellular transcription factors. *Eur J Clin Invest* 1997; 27:908–15.
11. Ravikumara YS, Raya U, Nandhithaa M, et al. Inhibition of hepatitis C virus replication by herbal extract: *Phyllanthus amarus* as potent natural source. *Vir Res* 2011;158:89–97.
12. Niu JZ, Wang YY, Qiao M, et al. Effect of *Phyllanthus amarus* on duck hepatitis B virus replication in vivo. *J Med Virol* 1990;32:212–8.
13. Munshi A, Mehrotra R, Panda SK. Evaluation of *Phyllanthus amarus* and *Phyllanthus maderaspatensis* as agents for postexposure prophylaxis in neonatal duck hepatitis B virus infection. *J Med Virol* 1993;40:53–8.
14. Subramoniam A. Too many hepatoprotective drugs and a little hepatoprotection. *Curr Sci* 1995;69:898–9.
15. Leelarasamee A, Trakulsomboom S, Maunwongyathi P, et al. Failure of *Phyllanthus amarus* to eradicate hepatitis B surface antigen from symptomless carriers. *Lancet* 1990;335:1600–1.
16. Syamasundar KV, Singh B, Thakur RS, Hussain A, Kiso Y, Hikino H. Antihepatoprotective principles of *Phyllanthus niruri* herbs. *J Ethnopharmacol* 1985;14:41–4.
17. Tripathi AK, Verma RK, Gupta AK, Gupta MM, Khanuja SP. Quantitative determination of phyllanthin and hypophyllanthin in Phyllanthus species by high-performance thin layer chromatography. *Phytochem Anal* 2006;17:394–7.
18. Sharma A, Singh RT, Handa SS. Estimation of phyllanthin and hypophyllanthin by high performance liquid chromatography in *Phyllanthus amarus*. *Phytochem Anal* 1993;4:226–9.
19. Murugaiyah V, Chan KL. Determination of four lignans in *Phyllanthus niruri* L. by a simple high-performance liquid chromatography method with fluorescence detection. *J Chromatogr A* 2007a;1154:198–204.
20. Murugaiyah V, Chan KL. Analysis of lignans from *Phyllanthus niruri* L. in plasma using a simple HPLC method with fluorescence detection and its application in a pharmacokinetic study. *J Chromatogr B* 2007b;852:138–44.
21. Yadav NP, Pal A, Shanker K, et al. Synergistic effect of silymarin and standardized extract of *Phyllanthus amarus* against CCl_4-induced hepatotoxicity in *Rattus norvegicus*. *Phytomedicine* 2008; 15:1053–61.
22. Chirdchupunseree H, Pramyothin P. Protective activity of phyllanthin in ethanol-treated primary culture of rat hepatocytes. *J Ethnopharmacol* 2010;128:172–6.
23. Boll M, Weber LW, Becker E, Stampfl A. Mechanism of carbon tetrachloride-induced hepatotoxicity. Hepatocellular damage by reactive carbon tetrachloride metabolites. *Z Naturforsch [C]* 2001; 56:649–59.
24. Krithika R, Mohankumar R, Verma RJ, et al. Isolation, characterization and antioxidative effect of phyllanthin against CCl_4-induced toxicity in HepG2 cell line. *Chem Biol Interact* 2009; 181:351–8.
25. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979;95: 351–8.
26. Pagila DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte peroxidise. *J Lab Clin Med* 1967;70:158–69.
27. Mavis RD, Stellwagen E. Purification and subunit structure of glutathione reductase from Baker's yeast. *J Biol Chem* 1968;243: 809–14.
28. Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases – the first enzymatic step in mercapturic acid formation. *J Biol Chem* 1974;249:7130–9.
29. Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. *Ind J Biochem Biophys* 1984; 21:130–2.
30. Luck H. A spectrophotometric method for the estimation of catalase. In: *Methods of Enzymatic Analysis*, Bergmeyer HU, ed. Academic Press: New York 1963:886–7.
31. Grunert RR, Philips PH. A modification of the nitroprusside method of analysis for glutathione. *Arch Biochem* 1951;30:217–25.
32. Roe JH, Kuether CA. The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. *J Bio Chem* 1943;147:399–407.
33. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with Folin–phenol reagent. *J Biol Chem* 1951;193:265–75.
34. King J. The dehydrogenases or oxido reductase – lactate dehydrogenase. In: *Practical in Clinical Enzymology*, Van D, eds. Nostrand Co. Ltd: London 1965:83–93.
35. Orlowski M, Meister A. Isolation of γ-glutamyl transpeptidase from hog kidney. *J Biol Chem* 1965;240:338–47.
36. Malloy HT, Evelyn KA. The determination of bilirubin with photoelectric colorimeter. *J Biol Chem* 1937;119:481–90.
37. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. *J Immunol Meth* 1983;65:55–63.
38. Chandan BK, Saxena AK, Shukla S, et al. Hepatoprotective potential of *Aloe barbadensis* Mill. Against carbon tetrachloride induced hepatotoxicity. *J Ethnopharmacol* 2007;111:560–6.
39. Karuna R, Reddy SS, Baskar R, Saralakumari D. Antioxidant potential of aqueous extract of *Phyllanthus amarus* in rats. *Ind J Pharmacol* 2009;41:64–7.
40. Wongnawa M, Thaina P, Bumrungwong N, Nitiruangjarat A, Muso A, Prasartthong V. Effect of *Phyllanthus amarus* Schum & Thonn. and its protective mechanism on paracetamol hepatotoxicity in rats. *Acta Hort* 2005;680:195–201.
41. Faremi TY, Suru SM, Fafunso MA, Obioha UE. Hepatoprotective potentials of *Phyllanthus amarus* against ethanol-induced oxidative stress in rats. *Food Chem Toxicol* 2008;46:2658–64.
42. Naaz F, Javed S, Abdin MZ. Hepatoprotective effect of ethanolic extract of *Phyllanthus amarus* Schum. et Thonn. on aflatoxin B1-induced liver damage in mice. *J Ethnopharmacol* 2007;113: 503–9.
43. Surya Narayanan B, Latha P, Rukkumani R. Protective effects of *Phyllanthus amarus* on fibrotic markers during alcohol and polyunsaturated fatty acid-induced toxicity. *Toxicol Mech Methods* 2011; 21:48–52.
44. Sane RT, Kuber VV, Chalissery MS, Menon S. Hepatoprotection by *Phyllanthus amarus* and *Phyllanthus debilis* in CCl_4-induced liver dysfunction. *Curr Sci* 1995;68:1243–6.
45. Lee CY, Peng WH, Cheng HY, Chen FN, Lai MT, Chiu TH. Hepatoprotective effect of *Phyllanthus* in Taiwan on acute liver damage induced by carbon tetrachloride. *Am J Chin Med* 2006;34:471–82.

46. Fan G, Tang J, Bhadauria M, et al. Resveratrol ameliorates carbon tetrachloride induced acute liver injury in mice. *Env Toxicol Pharmacol* 2009;28:350–6.
47. Sassa S, Sugita O, Galbraith RA, Kappas A. Drug metabolism by the human hepatoma cell, HepG2. *Biochem Biophys Res Commun* 1987;143:52–7.
48. Kaviarasan S, Ramamurthy N, Gunasekaran P, Varalakshmi E, Anuradha CV. Fenugreek (*Trigonella foenum graecum*) seed extract prevents ethanol-induced toxicity and apoptosis in change liver cells. *Alcohol Alcoholism* 2006;41:267–73.
49. Kiemer AK, Hartung T, Huber C, Vollmar AM. Phyllanthus amarus has anti-inflammatory potential by inhibition of iNOS, COX-2, and cytokines via the NF-kB pathway. *J Hepatol* 2003;38:289–97.
50. Nikam PS, Nikam SV, Sontakke AV. Lipid peroxidation and anti-oxidants during treatment of hepatitis-B with *Phyllanthus amarus*. *Int J Biol Med Res* 2010;1:41–3.