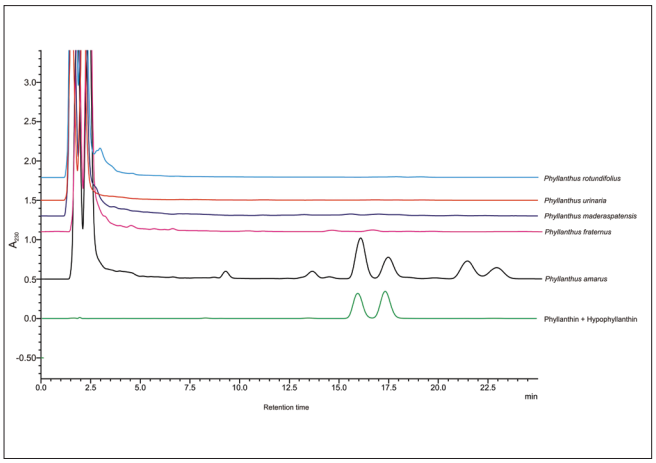

Figure 1: High performance liquid chromatography chromatogram of the *Phyllanthus species*

The MTT assay is based on the ability of viable cells to reduce MTT to an insoluble formazan product. In brief, HepG2 cells were cultured in 96-well plates at a seeding density of 4 × 10^4 cells/well. After 24 h incubation, the cells were treated with the extracts of *P. amarus*, *P. fraternus*, *P. rotundifolius*, *P. urinaria,* and *P. maderaspatensis* at concentrations ranging from 6.25 to 100 µg/ml in separate wells for 24 h. Thereafter, the cells were washed and incubated for 1 h with MTT. MTT was dissolved in phosphate-buffered saline (PBS) at a concentration of 5 mg/ml and added to the cells to the final concentration of 500 µg/ml. After 1 h, the medium was removed and the remaining formazan crystals were dissolved in 200 µl of DMSO. The optical density was measured using a microplate reader at a wavelength of 570 nm. Consequently, noncytotoxic concentrations were selected for hepatoprotective studies.

In vitro hepatoprotection activity study

The hepatoprotective activity of the extracts was evaluated against *t*-BH-induced toxicity using well-maintained HepG2 cells. Cells were seeded at a density of 5 × 10^4 cells/well and incubated overnight. Postincubation, the cells were treated with varying concentrations of extracts (*P. amarus*, *P. fraternus*, *P. rotundifolius*, *P. urinaria,* and *P. maderaspatensis*) in separate wells of a 96-well plate and incubated for 2 h. Silymarin (10 to 50 µg/ml) was used as a reference standard.[11] After incubation, the cells were treated with *t*-BH at a concentration of 1 mM and allowed to incubate for 2 h. Thereafter, the supernatant was discarded and the cells were washed with DPBS, and the fresh growth medium along with MTT was added and incubated for 1 h to allow the formation of formazan crystals. Finally, the medium was removed, and the formazan crystals were dissolved using DMSO; the absorbance was measured at 570 nm.[11]

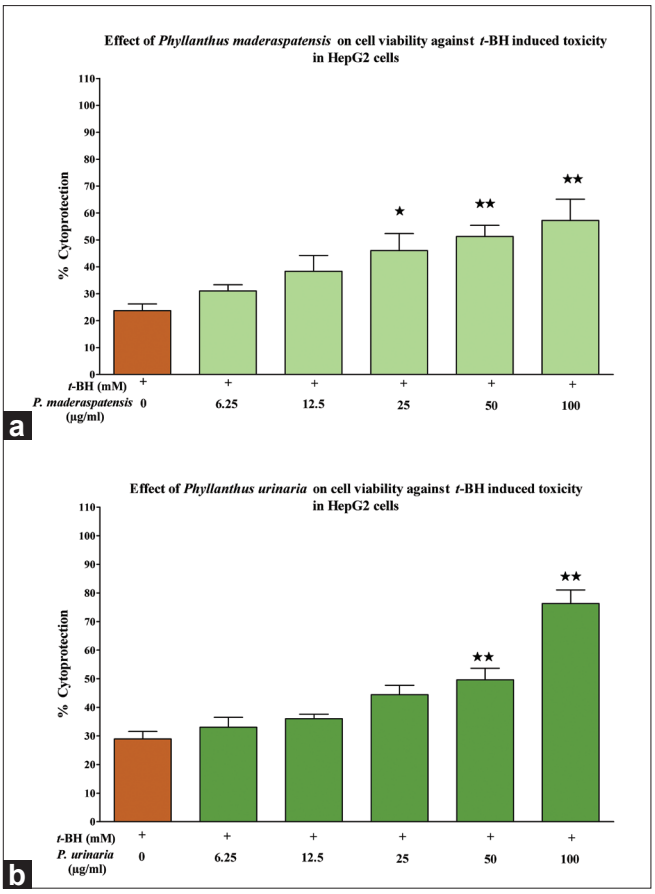

Figure 2: Effect of the *Phyllanthus species* on *t*-BH-induced toxicity in HepG2 cells. HepG2 cells were incubated in the presence/absence of (a) *P. maderaspatensis* and (b) *P. urinaria* for 2 h, prior to treatment with *t*-BH (1 mM) for 2 h. Thereafter, the cells were processed for the MTT assay. The results are expressed as mean ± SD. **P*<0.05 and ***P*<0.001 compared to the *t*-BH (1 mM) control

Statistical analysis

Results are expressed as mean ± standard deviation (SD). A sample concentration providing 50% cytoprotection was calculated from graph plotting, percentage protection against sample concentration [Figure 2a and b]. The experiment was performed in triplicates. Statistical analysis was performed using Dunnett's multiple comparison tests and one-way analysis of variance (ANOVA) using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Differences between the groups were considered statistically significant at $P<0.05$ and $P < 0.01$. Results are expressed as percentage protection, i.e., the percentage increase in cell viability compared to the viability of cells treated with *t*-BH alone. The percent protection is calculated as below:[12]

% Protection = (Mean of sample treated − Mean of *t*-BH treated)/(Mean of untreated − Mean of *t*-BH treated) × 100.

RESULT AND DISCUSSION

Cytotoxicity of the extracts

Based on the MTT assay, the noncytotoxic concentrations of the extracts were determined. The cytotoxicity experiment showed that all tested extracts were noncytotoxic to HepG2 cells up to a concentration of 100 μg/ml.

Chemical profile of high performance liquid chromatography

The HPLC chromatograms of extracts *P. amarus*, *P. fraternus*, *P. rotundifolius*, *P. urinaria*, and *P. maderaspatensis* are shown in Figure 1. Only *P. amarus* was found to contain phyllanthin and hypophyllanthin.

Effect of the extracts on *t*-BH-induced hepatoxicity

We examined the hepatoprotective activity of the five species of *Phyllanthus* against *t*-BH-induced toxicity in HepG2 cells. The extracts of *P. amarus*, *P. fraternus*, *P. rotundifolius*, *P. urinaria,* and *P. maderaspatensis* were tested at concentrations ranging from 6.25 to 100 μg/ml in the presence of *t*-BH (1 mM). Among all the extracts, only *P. maderaspatensis* (25to100 μg/ml) and *P. urinaria* (50to100 μg/ml) exhibited dose-dependent cytoprotection against *t*-BH-induced cell toxicity at the indicated concentrations [Figure 2a, b]. *P. maderaspatensis* and *P. urinaria* showed significant cytoprotection of 44% and 66%, respectively. Based on the % cytoprotection and IC_{50} values, *P. urinaria* (IC_{50} = 72 μg/ml) was found to have a more potent hepatoprotective activity than *P. maderaspatensis*. However, the extracts showed lesser potency when compared with the reference standard, silymarin (IC_{50} = 49.0 μg/ml).

t-BH exerts toxicity by forming covalent bonds with cellular molecules, resulting in cell injury. It causes leakage of lactate dehydrogenase (LDH) and formation of malonydialdehyde in hepatocytes.[13] Furthermore, *t*-BH causes the depletion of cellular glutathione levels and can also induce DNA damage.[13,14] Since all these phenomenons resemble the conditions that occur during oxidative stress in the cells and tissues; plants with antioxidant properties can be used to treat *t*-BH induced cytotoxicity and the resultant liver disorders.[3,15,16]

A large number of medicines have been developed for health problems pertaining to the liver. Hepatoprotectants, therapeutic agents that prevent damage to the liver, currently include both synthetic as well as natural products. However, to search for newer and better hepatoprotective agents, natural resources such as traditional medicinal plants have always been considered as an important source for new molecules to be used as medicines.[2,17]

In this context, HepG2 cells were was selected as the model to investigate the direct effects of *P. amarus*, *P. fraternus*, *P. maderaspatensis*, *P. urinaria*, and *P. rotundifolius* on *t*-BH-induced cytotoxicity. After treatment with *t*-BH, cell viability was decreased with the impairment of membrane integrity as demonstrated in Figure 2a and b. Treatment with the extracts showed that the protective activity of *P. urinaria* (IC_{50} = 72 μg/ml) was higher than *P. maderaspatensis*. However, both the extracts showed lesser potency in comparison to the reference drug, silymarin. Interestingly, the extracts demonstrated a significant protective activity despite the absence of key active phytoconstituents, phyllanthin and hypophyllanthin, which apparently suggests the involvement of other phenolic compounds present in the extracts.

In a similar study, the ethanolic extract of *P. urinaria* was reported to protect against an acetaminophen overdose by down-regulating hepatic cytochrome P450 CYP2E1 protein.[18] The chemical composition analysis indicated that the protective effect of the extract was majorly due to the presence of corilagin and gallic acid.[18] The methanolic extract of *P. urinaria* has also been reported to protect against CCl_4-induced liver toxicity by attenuating the increase in serum glutamate-oxalate transaminase (GOT), elevating the activity of reduced glutathione peroxidase (GSH-Px)[19] and increasing intracellular free Ca^{2+} concentrations in liver cells.[20]

In line with our results, *P. maderaspatensis* has been reported to possess a significant hepatoprotective activity against acetaminophen-induced hepatotoxicity, despite the absence of phyllanthin and hypophyllanthin.[7,21]

To summarize, this study illustrates for the first time the hepatoprotective potential of *P. urinaria* and *P. maderaspatensis* against *t*-BH induced cytotoxicity in HepG2 cells. Besides, an interesting conclusion can also be drawn that phyllanthin and hypophyllanthin may not be exclusively responsible for the hepatoprotective activity as these are not found in *P. urinaria* and *P. maderaspatensis*. This inference opens the possibility of considering these plants an adjunctive medicine for the treatment of liver disease associated with chemical toxicities.

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