Hepatoprotective and antioxidant activity of standardized herbal extracts

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ABSTRACT

Background: *Phyllanthus emblica, Camellia sinensis, Mangifera indica, Punica granatum,* and *Acacia catechu* have been shown to possess widespread pharmacological application against multitude of diseases namely cancer, diabetes, liver disorders, and oxidative stress. **Objective:** We evaluated the hepatoprotective activity of the standardized herbal extracts against *tert*-butyl hydroperoxide (*t*-BH) induced toxicity and their mechanism of hepatoprotective action in human hepatocarcinoma cells (HepG2 cell line). **Materials and Methods:** The hepatoprotective activity was studied by observing the effect of these herbal extracts on *t*-BH induced reduction in cell viability of HepG2 cells. In addition, the reducing power of the extracts and their ability to scavenge free radicals were evaluated using two antioxidant assay systems: cell free [oxygen radical absorbance capacity (ORAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid)] (ABTS)] and cell based [cellular antioxidant activity (CAA)]. **Results and Discussion:** The results obtained showed that these extracts possess significant hepatoprotective activity. This may indicate that the plant extracts contain compounds, which can remove toxic metabolites following *t*-BH induced toxicity. The extracts exhibited significant antioxidant property as evident by the Trolox values and effective scavenging of DPPH and ABTS radicals. The extracts also demonstrated inhibition of AAPH-induced fluorescence in HepG2 cells. These results indicate the ability of the plant extracts to protect the liver cells from chemical-induced damage, which might be correlated to their radical scavenging potential. **Conclusion:** This study demonstrates that these extracts have potential hepatoprotective activity which is mainly attributed to the antioxidant potential, which might occur by reduction of lipid peroxidation and cellular damage.

Key words: *Acacia catechu, Camellia sinensis*, cellular antioxidant activity assay, hepatoprotection, *Mangifera indica, Phyllanthus emblica, Punica granatum*

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INTRODUCTION

Liver is a key organ that regulates metabolism, secretion, storage, and detoxifying functions in the body, and hepatic damage is often associated with distortion of these functions.[1] Liver cells possess a number of compensatory mechanisms to deal with reactive oxygen species (ROS) and its effects among these are the induction of antioxidant proteins such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GSHPx). Enzymatic antioxidant system [Cu–Zn, Mn–SOD, catalase, GSHPx, and GSH reductase (GR)] function by direct or sequential removal of ROS, thereby terminating their activities. An imbalance between the oxidative forces and antioxidant defense systems causes oxidative injury, which has been implicated in various diseases, such as atherosclerosis, diabetes, cancer, liver cirrhosis, etc.[2]

ROS is continuously generated in physiological conditions and effectively eliminated by several intracellular and extracellular antioxidant systems.[3] Uncontrolled production of ROS often leads to damage of cellular macromolecules (DNA, lipids, and protein) and other small antioxidant molecules. The most important ROS are the superoxide anion radical O_2^-, hydrogen peroxide (H_2O_2), alkoxyl (RO), peroxyl (ROO), hydroxyl radical (OH), and hypochlorous acid (HOCl). Other non-oxygen species existing as

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reactive nitrogen species (RNS), such as nitric oxide (NO) and peroxynitrite also have important bioactivity.[4] Free radical reaction is an important pathway in a wide range of unrelated biological systems. Among many ways of chemical-induced injury, the critical class of reaction is production of free radical intermediates which trigger a network of multifarious disturbances.[5]

Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages.[6-8] Liver possesses a unique metabolism and plays a pivotal role in the removal of substances from the portal circulation due to which it is susceptible to toxicity of drugs, xenobiotics, and oxidative stress.[9] The two distinct pathways in liver metabolism occur via cytochrome p-450 and GSH-peroxidase. The current treatment for hepatotoxicity includes drugs which influence the p-450 enzyme mechanism either by inhibiting (amiodarone, cimetidine, ciprofloxacin, etc.) or inducing (rifampicin, carbamazepine, phenobarbital, phenytoin) the metabolic activity of enzymes.

Recently, much attention has been focussed on investigating the hepatoprotective function of naturally occurring compounds and their mechanisms of action. Herein, we have examined the hepatoprotective activity of selected herbal extracts based on the measurement of cytotoxicity of *tert*-butyl hydroperoxide (*t*-BH) to HepG2 cells. Chemical-induced toxicity in HepG2 cells represents a suitable *in vitro* model for hepatotoxicological assessment of drugs, through analysis of different cytotoxic endpoints.[10] HepG2 cells have been used to investigate the metabolism and toxicity of drugs, since these cells retain many specialized functions that are characteristic to normal human hepatocytes, including synthesis and secretion of plasma proteins.[11]

In the absence of reliable modern hepatoprotective drugs, there are a number of traditional medicines recommended for treatment of liver diseases. Many herbs such as *Silybum marianum*,[12] *Tridax procumbens*,[13] and *Andrographis paniculata*[14] have been reported to possess hepatoprotective activity. Plants contain wide variety of bioactive molecules including terpenoids, steroids, phenols, and flavonoids. In addition to their nutritional value these phytoconstituents exhibit a wide array of pharmacological properties such as anti-inflammatory, antiviral, anti-proliferative, and anti-carcinogenic.[15] Plant derived phenolic, flavonoid, and polyphenolic compounds are considered to contribute to the prevention of diseases associated with oxidative stress.

In this study, we aimed to evaluate the hepatoprotective activity of five standardized herbal extracts namely *Phyllanthus emblica* Linn. (Euphorbiaceae), *Camellia sinensis* Linn. (Theaceae), *Punica granatum* Linn. (Punicaceae), *Mangifera indica* Linn. (Anacardiaceae), and *Acacia catechu* Linn. (Mimosaceae) on *t*-BH induced liver toxicity using HepG2 cells. Further, in order to elucidate the possible mechanism of action, we screened these extracts for their antioxidant activity using two assay systems: cell free [oxygen radical absorbance capacity (ORAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and {2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid)} (ABTS) radical scavenging tests] and cell based [cellular antioxidant activity (CAA) assay].

MATERIALS AND METHODS

Chemicals

2',7'-dichlorofluorescein diacetate (DCFH-DA), quercetin dihydrate, fluorescein sodium salt, Trolox (6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid), ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid)], AAPH (2,2'-azo-bis[2-methyl propionamidine] dihydrochloride), and *t*-BH were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,2-Diphenyl-1-picryl hydrazyl (DPPH) was purchased from Himedia (Mumbai, India). Gibco Life Technologies (Grand Island, NY) supplied Earle's minimum essential media (EMEM). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, USA).

Preparation of standardized herbal extracts

The herbal extracts were prepared in the industrial processing plant of our organization (Natural Remedies Pvt. Ltd., Bangalore). Dried fruits of *P. emblica* were refluxed with water, and the liquid extract was subjected to distillation under vacuum and spray dried. Dried leaves of *C. sinensis* were refluxed with 70% methanol in water, and the liquid extract was subjected to distillation under vacuum and partitioned with ethyl acetate. The ethyl acetate layer was concentrated under vacuum, dispersed in water, and spray dried. The dried peels of *P. granatum* were refluxed with methanol, and the liquid extract was subjected to distillation under vacuum to a thick paste and dried under vacuum. The dried bark of *M. indica* was refluxed with methanol, and the extract was subjected to distillation under vacuum to a thick paste and processed with methanol and acetone. The final paste was dried under vacuum. The dried heartwood of *A. catechu* was boiled with water. The extract was then concentrated to a thick paste and subjected for repeated processing with alcohol and water. The final paste was dried under vacuum. The extracts were quantified for the marker compounds using HPLC. The phytochemical constituents of each herbal extract are tabulated in Table 1.

Cell line and culture condition

HepG2 cell line (hepatocellular carcinoma) (# HB-8065™) was procured from American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were cultured

Table 1: Phytochemical composition of the herbal extracts

Herbal extracts	Plant part used	Phytochemical constituents	% w/w*
Phyllanthus emblica	Fruit	Gallic acid	5.7
		L-Ascorbic acid	0.8
		Methyl gallate	0.5
		Corilagin	0.03
Camellia sinensis	Leaves	EGCG	44.3
		ECG	17.4
		Epicatechin	5.7
Punica granatum	Fruit peel	Punicalagin	10.7
		Ellagic acid	2.6
Mangifera indica	Bark	Mangiferin	78
Acacia catechu	Heartwood	Catechin	66.9
		Epicatechin	23.1

*Determined by HPLC

in EMEM containing 10% FBS, 1 mM sodium pyruvate, and 2 g/l sodium bicarbonate under an atmosphere of 5% CO_2 at 37°C.

Cell viability assay

The viability of HepG2 cells was determined by a colorimetric MTT assay as described by Mosmann.[16] The assay is based upon the ability of mitochondria to catalyze the reduction of MTT bromide to insoluble formazan, the concentration of which is measured spectrophotometrically. HepG2 cells were first cultured in 96-well plates at a density of 4×10^4 cells/well for 24 h. After incubation the cells were washed with PBS and treated with different concentrations of *P. emblica* (15.6–125 μg/ml), *C. sinensis* (20–50 μg/ml), *M. indica* (31.25–250 μg/ml), *P. granatum* (12.5–75 μg/ml) and *A. catechu* (15.6–125 μg/ml), silymarin (10–50 μg/ml), and quercetin (0.68–3.38 μg/ml) for 24 h. Thereafter, the cells were washed and further incubated for 1 h with MTT (500 μg/ml). After 1 h, the formazan crystals were dissolved using DMSO (200 μl/well). The absorbance was measured colorimetrically at 570 nm. Consequently, the noncytotoxic concentrations were chosen for conducting the hepatoprotection and CAA studies.

Hepatoprotection activity

The hepatoprotective activity of the extracts was evaluated using well maintained HepG2 cells as per the method described in our previous publication.[17] *t*-BH was used as a hepatotoxicant and silymarin was used as a standard hepatoprotective drug. Confluent HepG2 cells were cultured in growth media (EMEM + 10% FBS) at a density of 5×10^4 cells/well in a 96-well tissue culture plate and incubated overnight. Postincubation, cells were treated with varying concentrations of extracts and incubated for 2 h, thereafter; *t*-BH (1 mM) was added and allowed for further 2 h incubation. Postincubation, the treated cells were washed with DPBS and incubated with MTT containing growth media. Finally, the medium was removed, and the formazan crystals were dissolved using DMSO. The optical density was measured at 570 nm.

Antioxidant activity studies

CAA assay

Cultured HepG2 cells were seeded at a density of 6×10^4 cells/well and incubated overnight. Postincubation cells were pretreated with the extracts at varying concentrations prior to addition of DCFH-DA (25 μM) and incubated for 1 h. Thereafter, AAPH (600 μM) was added, and the fluorescence kinetics was measured for 1 h using Fluostar Optima (BMG Labtech, Germany) at 37 °C, excitation 485 nm, emission 540 nm with a cycle time of 300 s. Quercetin was used as a reference standard. The median effective dose (EC_{50}) was calculated from the median effect plot of two independent trials. EC_{50} values were determined from the *X*-axis intersection value, where $\log (F_a/F_u) = 0$.[18]

ORAC assay

The assay was performed as per the method described by Alberto *et al.*[19] The reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction mixture was 200 μl. The extracts at different concentrations and sodium fluorescein (67 μM) solutions were added in the well of the microplate. The mixture was preincubated for 10 min at 37 °C. AAPH (12 mM) was added rapidly using a multichannel pipette. The microplate was immediately placed in the reader, and the fluorescence kinetics was measured at 485 nm excitation and 520 nm emission wavelengths for 90 min using Fluostar Optima (BMG Labtech, Germany). Trolox, a vitamin E analogue, was used as a reference standard. Sample curves (fluorescence versus time) were first normalized to the curve of the blank corresponding to the same assay. From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated. The net AUC corresponding to a sample and the regression equations between net AUC and sample concentration were calculated. ORAC values were expressed as μmoles of Trolox equivalent/g (TE/g).

ABTS radical scavenging assay

The ABTS radical scavenging activity was determined by the method of Auddy *et al.*[20] In brief, the total reaction mixture containing 10 mM PBS (pH 7.4), various concentrations of samples, and ABTS radical solution (0.238 mM) were mixed and immediately read at 734 nm using a VersaMax micro plate reader (Molecular Devices, USA). Gallic acid was used as a reference standard. The experiment was carried out in triplicates, half-maximal inhibitory concentration (IC_{50}) was determined using Finney software, and the values were expressed in μg/mL.

DPPH radical scavenging activity assay

The stable DPPH radical scavenging activity was determined by the method of Vani *et al.*[21] Reaction volume containing methanol, various concentrations of extracts, and DPPH (0.659 mM) were incubated at 25 °C for 20 min, following which the absorbance was read at 510 nm using a VersaMax micro-well plate reader (Molecular Devices, USA). Gallic acid was employed as the reference standard. Assay was performed in triplicates, and the IC_{50} values were calculated using Finney software, the values were expressed in µg/ml.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) of two independent experiments. Each experiment was performed in triplicates. Statistical differences between the treatments and the control were evaluated by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). The criterion for statistical significance was $^{*}P < 0.05$ and $^{**}P < 0.01$.

RESULTS

Cytotoxicity of the herbal extracts in HepG2 cells

All the herbal extracts were checked for MTT reducing potential. The results demonstrated that the extracts did not reduce MTT on their own and not cytotoxic up to the maximum tested concentration. Hereafter, we selected the noncytotoxic concentrations for further studies.

Effect of the herbal extracts on *t*-BH-induced hepatotoxicity

In this study, we examined the possible hepatoprotective effects of five standardized herbal extracts against *t*-BH-induced cytotoxicity, by preincubating the cells with or without the extracts or silymarin. A significant decrease in cell viability was observed upon treatment of HepG2 cells with *t*-BH (1 mM). Treatment with extracts demonstrated a significant dose-dependent protection toward cell toxicity resulting from *t*-BH exposure. As shown in Figure 1, *P. emblica* (12.5–75 µg/ml), *C. sinensis* (30–50 µg/ml), *M. indica* (62.5–250 µg/ml), *P. granatum* (12.5–75 µg/ml) and *A. catechu* (62.5–125 µg/ml) exhibited a significant dose-dependent increase in cell viability at the indicated concentration. Based on the IC_{50} values, the extracts showed their potency in the following order: *P. emblica* (IC_{50} = 32.4 µg/ml) ≥ *C. sinensis* (IC_{50} = 34.7 µg/ml) ≥ *P. granatum* (IC_{50} = 42.6 µg/ml) > *A. catechu* (IC_{50} = 114.8 µg/ml) > *M. indica* (IC_{50} = 190.5 µg/ml). The reference standard, silymarin demonstrated a dose-dependent increase in cell viability against *t*-BH-induced toxicity at concentrations ranging from 40 to 50 µg/ml [Figure 1], with 50% protection obtained at 49.0 µg/ml. Further, on comparing the IC_{50} values of the extracts with silymarin, *P. emblica*, *C. sinensis,* and *P. granatum* showed equipotent hepatoprotective activity against *t*-BH-induced cytotoxicity in HepG2 cells.

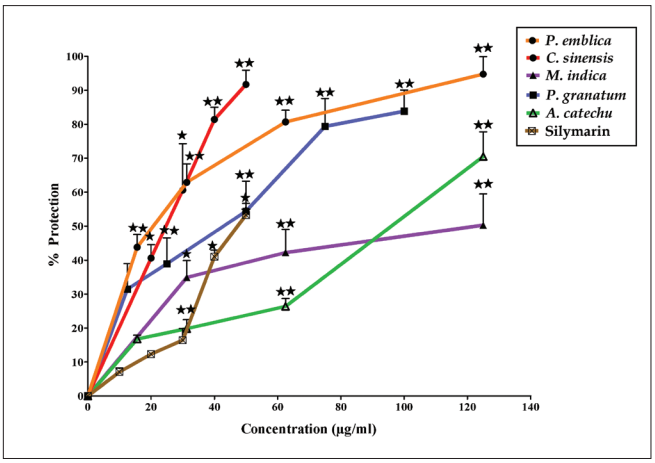

Figure 1: Effect of *P. emblica, C. sinensis, M. indica, P. granatum, A. catechu*, and silymarin on *t*-BH (1 mM) induced cytotoxicity in HepG2 cells. The values are expressed as mean ± SD. The criterion for statistical significance was $^{*}P < 0.05$ and $^{**}P < 0.01$

Determination of antioxidant potential of the herbal extracts

CAA assay

The antioxidant capacity of the extracts was estimated in terms of degree of inhibition on AAPH-induced fluorescence. At a concentration of 600 µM AAPH yielded a time-dependent increase of fluorescence. The reference standard quercetin exhibited dose-dependent inhibition of AAPH induced fluorescence at concentrations ranging from 0.68 to 3.38 µg/ml [Figure 2a], with an EC_{50} value of 4.65 µM (1.5 µg/ml). The increase in fluorescence from DCF formation was inhibited by the herbal extracts in a dose-dependent manner, as evident from the curves generated from cells treated with the extracts [Figure 2b–f]. Among all the extracts, *P. emblica* (7.81–125 µg/ml) exhibited maximum inhibition in fluorescence at the indicated concentrations with an EC_{50} value of 39.50 ± 0.70 µg/ml. The EC_{50} values of the extracts are listed in Table 2; based on which the inhibition of DCF formation was in the following order: *P. emblica* > *C. sinensis* ≥ *P. granatum* ≥ *M.indica* > *A. catechu.*

ORAC assay

The peroxyl radical scavenging activity of the extracts was determined by ORAC assay. This assay is based on the susceptibility of sodium fluorescein to AAPH, with concomitant loss of its fluorescence.[22] All the extracts showed significant antioxidant potential, and the values represent $ORAC_{ROO+}$ activities of the tested extracts

equivalent to Trolox. The $ORAC_{ROO+}$ values obtained for the extracts are shown in Table 2, based on which following degree of potency was obtained: *A. catechu* > *C. sinensis* ≥ *M. indica* > *P. emblica* ≥ *P. granatum*. The results showed that *A. catechu* with the $ORAC_{ROO+}$ value of 41589 ± 151.30 TE/g had the maximum antioxidant activity in comparison to other extracts [Table 2].

ABTS assay

All the extracts demonstrated ABTS free radical scavenging activity. Taking 0% inhibition in control with the absence of extract, regression equations were prepared from the concentrations of the extracts and the percentage inhibitions of free radical formation were calculated. IC_{50} values were calculated from these regression equations. Based on the IC_{50} values [Table 2], the following order of free radical scavenging activity was depicted: *A. catechu* > *C. sinensis* > *P. granatum* > *M. indica* > *P. emblica*. Further, in comparison to the reference standard gallic acid (IC_{50} = 1.32 ± 0.07 μg/ml), *A. catechu* (IC_{50} = 2.28 ± 0.14 μg/ml) showed lesser potency in scavenging ABTS free radicals [Table 2].

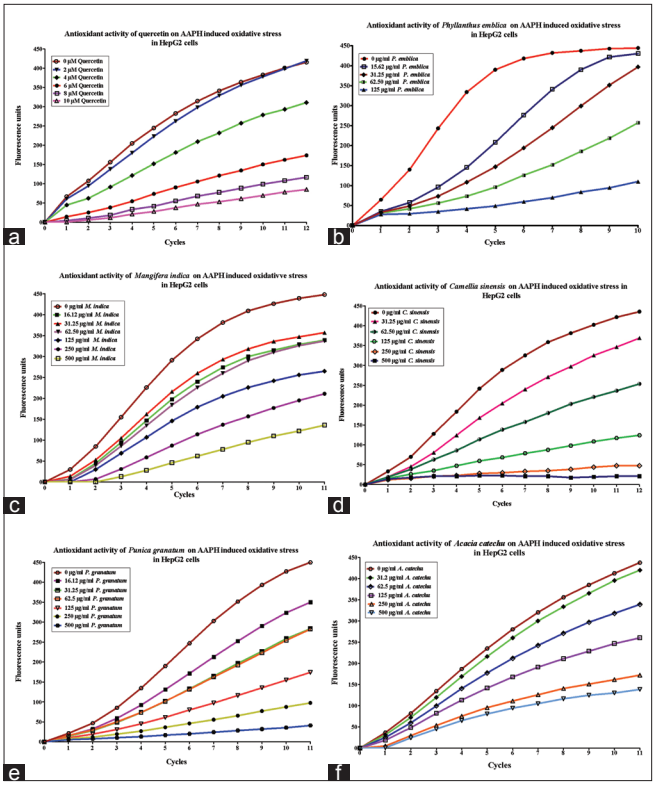

Figure 2: *In vitro* antioxidant activity of the (a) Quercetin, (b) *P. emblica*, (c) *M. indica*, (d) *C. sinensis*, (e) *P. granatum*, and (f) *A. catechu* at various concentrations as carried out by the CAA assay. The curves shown in the graph are from a single experiment which demonstrates the inhibition of increased fluorescence from DCF formation in a dose-dependent manner

DPPH assay

The herbal extracts were evaluated by their reactivity towards a stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH•). All the extracts examined were found to possess good DPPH radical scavenging activity. The IC_{50} values as depicted in Table 2 showed the following order of radical scavenging activity: *C. sinensis* > *P. granatum* > *A. catechu* > *P. emblica* ≥ *M. indica*. Further, on comparing the 50% scavenging activity of *C. sinensis* with the reference standard gallic acid, we observed that *C. sinensis* (IC_{50} = 5.00 μg/ml) was less potent than gallic acid (IC_{50} = 1.32 ± 0.07 μg/ml).

DISCUSSION

P. emblica is considered beneficial against various diseases namely cancer, diabetes, liver treatment, and various other diseases. Other useful properties of *P. emblica* include anti-tumor, anti-inflammatory, anti-bacterial, antioxidant, immunomodulatory, analgesic, etc.[23] In this study, we evaluated the protective effect of *P. emblica* against *t*-BH-induced cytotoxicity. Incubating HepG2 cells with 1 mM *t*-BH for 2 h caused a significant loss in the cell viability. Pretreatment with the extract resulted in a dose-dependent increase in cell viability at concentrations ranging from 15.6 to 125 μg/ml. Then, 50% protection was attained at a concentration of 32.36 μg/ml. Further, on comparing with silymarin, *P. emblica* was found equipotent in protecting the HepG2 cells against *t*-BH-induced oxidative damage. In order to elucidate the possible mechanism of hepatoprotection, we further evaluated the antioxidant potential of the extract. The results depicted that in the chemical-based assays, i.e. ORAC, ABTS, and DPPH, and the cell based assay, CAA the extract showed significant antioxidant and radical quenching potential [Table 2].

Table 2: ORAC, ABTS, DPPH, and CAA values of the herbal extracts

Extracts	ORAC (μMTE/g)*	ABTS [IC_{50} (μg/ml)]*	DPPH [IC_{50} (μg/ml)]*	CAA [EC_{50} (μg/ml)]*
Acacia catechu	41589 ± 151.30	2.28 ± 0.14	7.40 ± 1.16	203.50 ± 6.40
Phyllanthus emblica	3944 ± 128.70	5.08 ± 0.59	11.66 ± 1.05	39.50 ± 0.70
Mangifera indica	8260 ± 32.53	4.80 ± 0.06	12.81 ± 1.13	111.00 ± 19.80
Camellia sinensis	8777 ± 35.36	3.11 ± 0.13	5.00 ± 0.23	72.00 ± 4.20
Punica granatum	3481 ± 206.50	3.94 ± 1.30	7.11 ± 0.32	87.00 ± 11.30

*The values are represented as mean ± SD

However, *P. emblica* (EC_{50}= 39.50 μg/ml) displayed a greater degree of free radical scavenging ability in CAA assay, although with a lesser potency than quercetin (EC_{50}= 1.50 μg/ml). Previous studies have shown the hepatoprotective activity of the *P. emblica* fruit extract against a variety of toxins namely carbon tetrachloride (CCl_4), paracetamol, thioacetamide (TAA), alcohol, cyclophosphomide, and anti-TB drugs (rifampicin, isonizid, etc.).[24-30] Hence, the overall results indicate that the extract of *P. emblica* possesses a potent protective effect against *t*-BH-induced hepatic damage, and the main mechanism involved in the protection could be associated with its strong capability to reduce the intracellular level of ROS. The effective components which might act against oxidative damage are mainly phenolic compounds, flavonoids, tannins, phyllambic compounds, vitamin C, and others.[30]

C. sinensis commonly called 'Green tea' is rich in flavanol monomers known as catechins,[31] which have beneficial effects in cardiovascular diseases including LDL oxidative susceptibility, inflammation, abdominal disorders, etc.[32,33] In this study, we examined the *in vitro* hepatoprotective activity of leaves extract of *C. sinensis* on t-BH-induced toxicity in HepG2 cells. The extract demonstrated a significant protective effect against t-BH elicited cell death at a concentration range of 30–50 μg/ml, as evident by an increase in cell viability. As apparent from the IC_{50} values, *C. sinensis* displayed equipotent activity in comparison to the reference standard, silymarin. Further, to assess the mechanism of hepatoprotection, the extract was tested for its antioxidant activity. The ORAC, ABTS, and DPPH values indicated that *C. sinensis* possesses significant radical quenching property [Table 2]. However, among all the assays it demonstrated highest scavenging activity towards DPPH free radicals. In CAA assay, the extract exhibited 50% decrease in fluorescence at a concentration of 72 μg/ml; however, the radical quenching ability was less potent than the reference standard quercetin (EC_{50} = 1.50 μg/ml). Previous reports have stated the hepatoprotective activity of *C. sinensis* against acute liver injury induced by tamoxifen,[34] CCl_4,[35] 2-nitropropane,[36] lipopolysaccharide (LPS), and D-galactosamine.[37] Hence, it is possible that the mechanism of hepatoprotection of *C. sinensis* is due to its antioxidant effect, which is mainly influenced by polyphenols, epigallocatechin gallate, and epicatechin gallate.

In this study, the fruit peel extract of *P. granatum* exhibited significant hepatoprotective activity. The extract demonstrated a dose-dependent increase in cell viability at concentrations ranging from 12.5 to 75 μg/ml, with an IC_{50} value of 42.6 μg/ml. In comparison to silymarin (IC_{50} = 49.0 μg/ml), it showed nearly equipotent protective activity. Moreover, *P. granatum* showed good antioxidant property as evident from the Trolox values (3481 ± 206.50 μMTE/g) and scavenging of free radicals ABTS and DPPH. In CAA assay, AAPH-induced fluorescence was significantly reduced by *P. granatum*, with an EC_{50} value of 87.00 μg/ml. However, in comparison to quercetin (EC_{50} = 1.50 μg/ml) it showed less potent activity against free radical scavenging. Previous *in vivo* studies have been reported similar hepatoprotective properties of fruit peel and flowers' extracts of *P. granatum* against CCl_4, ferric nitrilotriacetate (Fe-NTA), and TCA-induced hepatotoxicity.[38-40] The extract showed protection against hepatic lipid peroxidation and preserved GSH levels and activities of antioxidant enzymes namely, catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione-*S*-transferase (GST). In total, these results suggest that hepatoprotection shown by the *P. granatum* extract may be due to its antioxidant properties.

M. indica and its components are commonly used in folk medicine for many curative effects. It possesses antioxidant,[41] immunomodulatory,[42] anti-mutagenic,[43] and anticancer[44] properties. In this study, we examined the possible hepatoprotective property of the bark extract of *M. indica* against *t*-BH-induced cytotoxicity. According to the results, *M. indica* at concentration ranging from 62.5 to 250 μg/ml resulted in concentration-dependent protection of HepG2 cells, thereby indicating the opposing actions against toxic stimuli [Figure 1]. However, on comparing with silymarin (IC_{50} = 49.0 μg/ml), it showed lesser efficiency, with 50% protection achieved at a concentration of 190.5 μg/ml. Further, we examined the antioxidant property of the extract in two different assay systems. In ORAC assay, the extract displayed a good activity with 8260 ± 32.53 μMTE/g. It showed efficient scavenging of ABTS and DPPH radicals with IC_{50} values of 4.80 μg/ml and 12.81 μg/ml, respectively. In CAA assay, *M. indica* showed moderate inhibition of AAPH-induced fluorescence, with an EC_{50} value of 111.00 μg/ml. Further, on comparing with quercetin (EC_{50} = 1.50 μg/ml) it showed less potent activity against free radical scavenging. These results are in good agreement with previous studies showing antioxidant and hepatoprotective activities of *M. indica*.[45-47] Rodeiro *et al.*[48] reported the hepatoprotective and antioxidant activity of the stem bark extract of *M. indica* against *t*-BH, ethanol, CCl_4, and LPS-induced cytotoxicity in rat primary hepatocytes. The extract was reported to prevent lipid peroxidation and GSH depletion produced by *t*-BH, apparently, mangiferin was identified as the main bioactive component responsible for scavenging ROS and free radicals involved in initiation of lipid peroxidation and in inhibiting the activity of CYP2E1.[49] Previous studies have also reported the hepatoprotective activity of *M. indica* against cumene hydroperoxide-induced toxicity.[50]

A. catechu and its phytoconstituents possess widespread

pharmacological properties namely hypoglycaemic, hepatoprotective, antipyretic, digestive, etc. In this study, we evaluated the hepatoprotective property of heartwood extract of *A. catechu* against *t*-BH-induced cytotoxicity. The extract exhibited dose-dependent protection at concentrations ranging from 62.5 to 125 μg/ml, 50% inhibition in *t*-BH induced toxicity was observed at 114.8 μg/ml. However, in comparison to silymarin (IC_{50} = 49.0 μg/ml) the extract showed very less protection. Further, the extract was tested for its antioxidant property, where it showed maximum activity in ORAC assay with the highest Trolox value (41589 ± 151.30 μMTE/g). It also demonstrated a significant effect toward ABTS and DPPH free radicals. *A. catechu* at the tested concentrations exhibited a marked decrease in AAPH-induced fluorescence, with an EC_{50} value of 203.50 μg/ml; although, in comparison to quercetin it showed very less radical quenching property. Prior *in vivo* studies have stated the hepatoprotective activity of *A. catechu* extract against CCl_4-induced liver damage.[51-53] These studies indicated that the protective effect resulted due to increase in the serum level of GOT, GPT, alkaline phosphates, improved serum lipid profile, restoration of structural integrity of hepatocyte cell membrane, and regeneration of damaged liver cells. Additionally, the hepatoprotective role of *A. catechu* was thought to be due to the presence of tannins, cyanidanol, and quercetin.[54,55] Hence, the probable mechanism by which *A. catechu* exerts its protective action is by minimizing the effects of free radicals, its antioxidant activity in association with the inhibition of lipid peroxidation.

In summary, it is well established that *t*-BH is metabolized by two distinct pathways in hepatocytes; one *via* cytochrome p-450, and other by glutathione (GSH) peroxidase converting *t*-BH to *t*-butanol and oxidized GSH.[56] These metabolic pathways increase cellular reactive metabolites which attack the membrane phospholipids, proteins, and nucleic acids. Thus, antioxidants that can inhibit free radical generation are important in terms of protecting the liver from chemical-induced damage by stabilizing the antioxidant systems in the cell. Our study clearly demonstrates that *P. emblica*, *C. sinensis*, *M. indica*, *P. granatum,* and *A. catechu* possess a significant protective effect against *t*-BH-induced cytoxicity. In conclusion, the results of the present investigation infer that these plant extracts possess potent antioxidant and hepatoprotective property, the former being probably responsible for the latter. Thus, the extracts can be beneficial in treating liver damages caused due to chemical or xenobiotic exposure.

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