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Original Article

Antioxidant and Hepatoprotective Activities of Hydroalcohol Extract of *Homalium zeylanicum* on Carbon tetrachloride Induced Liver Damage in Wistar Rats

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ABSTRACT

Objectives: The ethyl acetate fraction of bark and leaf part of *Homalium zelanicum* showed potential antioxidant and hepatoprotective activities. So, this study is aimed to evaluate antioxidant and hepatoprotective activities of hydroalcohol extract of *Homalium zelanicum* in carbon tetrachloride induced hepatotoxicity. **Methods:** To evaluate antioxidant potential, *in vitro* antioxidant assays e.g. DPPH, superoxide, hydroxyl and nitric oxide and *ex vivo* antioxidant assay e.g. cell based antioxidant protection in erythrocytes (CAP-e) were performed. Hepatoprotective activities were evaluated by estimating biochemical parameters such as serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), total bilirubin (TB) and total protein (TP). Hepatic markers such as thiobarbituric acid reactive species (TBARS), superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) were evaluated. **Results:** Both bark and leaf of *Homalium zeylanicum* showed improvement in antioxidant protection of hepatocytes. As well as the extracts exhibited significant (P<0.001) hepatoprotection against CCl_4-induced liver injury. **Conclusion:** Among all the extracts, hydroalcohol extract of bark of *Homalium zeylanicum* (HZB, 400 mg/kg) showed significant improvement in restoring serum and hepatic markers and the results were supported with histopathological observations.

Key words: Antioxidant, CCl_4, Hepatic marker, CAP-e, *Homalium zeylaicum*.

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INTRODUCTION

Liver is a pivotal organ in human for metabolism and detoxification of xenobiotics, environmental pollutants, and chemotherapeutic agents and regulates cellular homeostasis.[1] It is more susceptible to get damaged by various hepatotoxicants. In recent years, liver diseases greatly impact global health care system. The plant based drugs like *Silybum marianum*, *Phyllanthus emblica*, *Picrorhiza kurroa* etc. are widely and successfully used in the treatment of liver disorders. But still, there is need of less toxic and high efficacy plant based drugs.[2-4]

Homalium zeylanicum Benth. is predominantly found in evergreen forest and native to Eastern ghats of Andhra Pradesh of India. Previously, the plant was placed in the family Flacourtiaceae but in recent Angiosperm Phylogeny Group (APG)-IV system of classification, it is placed in Salicaceae. Traditional tribes of Rayalaseema region of Andhra Pradesh use leaves and bark of *H. zeylanicum* in the treatment of malaria, diabetes, rheumatism, wound healing, and inflammation.[5-7] In our previous studies, we have reported the presence of phenolics, flavonoids, steroids and terpenes in the species.[8] Experimental evidences reflect antioxidant, anti-inflammatory, antidiabetic, hepatoprotective, antidyslipidemic, and anthelmintic properties.[9-14] On basis of our earlier studies on hepatoprotective activities of ethyl acetate extract of *H. zeylanicum*, we aimed to establish antioxidant and hepatoprotective effect of hydroalcohol extract of *H. zeylanicum* on carbon tetrachloride induced liver cirrhosis in wistar rats.

MATERIALS AND METHODS

Chemicals, reagents and assay kits

All chemicals, reagents and solvents were of analytical grade and purchased from HiMedia Laboratories Pvt. Ltd., India, Central Drug House (P) Ltd. India, and SISCO Research Laboratories Pvt. Ltd. (SRL), Mumbai, India. The standard drug silymarin was purchased from Cadila Pharma Ltd., India. Assay kits were purchased from HiMedia Laboratories Pvt. Ltd., and Tulip Group, Coral Clinical System, India.

Plant material

H. zeylanicum (bark and leaf) was collected from Tirumala hills, Chittoor district, Andhra Pradesh, India. The species were identified by Dr. K. Madhava Chetty, Taxonomist, Sri Venkateswara University, Tirupati, Andhra Pradesh, India. The voucher specimen was deposited in Regional Plant Resource Centre for future reference (7545/RPRC).

Preparation of plant extract

The bark and leaves of *H. zeylanicum* were shade dried. The plant material (each of 700 g) was defatted with petroleum ether and then extracted with hydroalcohol (70%) by hot maceration at 60-70°C for 72 h. The extracts were dried to semisolid mass in a rotatory evaporator (R-100, Buchi, Switzerland).[15] The percentage yields of hydroalcohol extract of bark and leaf of *H. zeylanicum* were recorded to be 16.14% w/w (HZB) and 14% w/w (HZL) respectively.

In vitro antioxidant assays

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

Serial diluted hydroalcohol extracts of *H. zeylanicum* (HZB and HZL, 50-200 µg/ml) were mixed with DPPH solution (0.15%). After five min incubation at room temp, absorbance of the reaction mixture was recorded at 515 nm by using microplate Reader (Bio-Tek Synergy H1MF, USA).[13] Ascorbic acid was used as reference drug.

$$\% \text{ inhibition} = (A_{control} - A_{sample})/A_{control} \times 100$$

Superoxide (SOD) radical scavenging activity

Hydroalcohol extracts of *H. zeylanicum* (HZB and HZL, 50-200 µg/ml) was mixed with NBT (1 ml, 156 µM), and NADH (1 ml, 468 µM). PMS (100 µl, 60 µM) was added to initiate the reaction and incubated at 25°C for 5 min. Then, absorbance was read at 560 nm by using microplate Reader (Bio-Tek Synergy H1MF, USA).[13] Quercetin was used as reference drug.

$$\% \text{ inhibition} = (A_{control} - A_{sample})/A_{control} \times 100$$

Hydroxyl radical scavenging activity

The reaction mixture was formed by hydroalcohol extracts of *H. zeylanicum* (HZB and HZL, 50-200 µg/ml), 2-deoxy-2-ribose (100 µl, 28 mM), ferric chloride (200 µl, 200 µM), EDTA (1 mM), hydrogen peroxide (100 µl, 1 mM)), and ascorbic acid (100 µl). After incubation of the reaction mixture at 37°C for 1 h, each 1 ml of TBA and TCA were added and heated at 100°C for 20 min. the, the reaction mixture was allowed to cool to room temp and absorbance was recorded at 532 nm by using microplate Reader (Bio-Tek Synergy H1MF, USA).[13] Ascorbic acid was used as reference drug.

$$\% \text{ inhibition} = (A_{control} - A_{sample})/A_{control} \times 100$$

Nitric oxide radical scavenging activity

Hydroalcohol extracts of *H. zeylanicum* (HZB and HZL, 50-200 µg/ml) was mixed with sodium nitroprusside (10 mM) and incubated at 25 °C for 1.5 h. Then the reaction mixture was added to Griess reagent (1 ml) and absorbance was recorded at 546 nm by using microplate Reader (Bio-Tek Synergy H1MF, USA).[13] Quercetin was used as reference drug.

$$\% \text{ inhibition} = (A_{control} - A_{sample})/A_{control} \times 100$$

Ex vivo antioxidant studies

Cell based antioxidant protection in erythrocytes (CAP-e) assay

The sample solutions were prepared by dissolving HZB and HZL (each 0.5 g) in normal saline (0.9%, 5 ml) incubated on a rocker for 20 min. Then, sample solution was centrifuged at 2400 r/min for 10 min and filtered. RBCs of albino rats (0.1 ml) were treated with different dilutions of HZB and HZL. RBCs were treated with standard oxidising agent and considered as positive control and untreated RBCs were considered as negative control. The radical generator 2,2´-azobis-2-amidinopropane hydrochloride (AAPH) was added to initiate oxidative damage in cells and extent of damage was indicated by using 2,7´-dichlorofluorescein diacetate (DCF-DA). Fluorescence intensity of reaction mixture was measured by using microplate reader (Synergy H1M, BioTek, USA). Trolox was used as standard drug and IC_{50} of HZB and HZL were calculated (mg/ml).[14]

$$\text{Fluorescence Intensity (FI)} = (FI_{max}\text{-}FI_{sample}) / (FI_{sample}\text{-}FI_{untreated})$$

Experimental animals

Swiss albino rats (120-150 g) of both sexes were purchased from Imgenex India Pvt. Ltd., Bhubaneswar, Odisha. All the rats were acclimatized for a week in standard laboratory conditions (25-30°C and 12 h light-dark cycle). Animals were fed with standard rat diet and water *ad libitum*.[14] Experiments were conducted as specified guidelines of CPCSEA and approval of of Institutional Animal Ethical Committee (IAEC) of Regional Plant Resource Centre vide Regd. No. 1807/GO/R/S/15/CPCSEA.

Acute toxicity study

Acute toxicity of HZB and HZL were performed according to OECD guidelines. Swiss albino rats (150-200 g) of either sex were divided into 10 groups (n=6). Animals were fasted overnight before the experiments. Animals were administered with five different doses (500, 1000, 2000, 3000 and 4000 mg/kg b.w.) of HZB and HZL and kept under observation for a period of 72 h. The behavioral changes in sign and symptoms such as irritability, restlessness, fearfulness, touch response, gait, tremor, excess urination and defecation were observed.[16,17]

Experimental design

Animals of either sex were randomly divided into 7 groups (n=6).

Group I (Normal control): Single daily dose of Tween 80 (1 ml/kg b. w. of distilled water) for 14 days.

Group II (Toxic control): Single daily dose of Tween 80 (1 ml/kg b. w. in distilled water, p. o.) + CCl_4/olive oil (1:1 v/v, 1 ml/kg b. w., i. p.) on every 72 h for 14 days.

Group III (Standard control): Single daily dose of silymarin (0.7 ml/kg b. w.) + CCl_4/olive oil on every 72 h for 14 days

Group IV-V (HZB, low and high dose): Single daily dose of HZB 300 and 400 mg/kg b. w., respectively + CCl_4/olive oil on every 72 h for 14 days.

Group VI-VII (HZL, low and high dose): Single daily dose of HZL 300 and 400 mg/kg b. w., respectively + CCl_4/olive oil on every 72 h for 14 days

All the animals were anesthetized with diethyl ether after 24 h of the last treatment and sacrificed by cervical dislocation. Blood was collected by cardiac puncture and allowed to coagulate. After 30 min, blood was centrifuged at 2500 r/min for 10 min at 4°C (5424 R, Eppendorf, Germany). Serum was collected and stored at 4°C for biochemical analysis. Liver was removed and rinsed with cold normal saline. Liver was dissected into two sections. One section was kept in formalin (10%) for histopathological examination and other section was stored at -86°C deep freezer (U410-86, Eppendorf, Germany) for analysis of hepatic markers.[14,15]

Biochemical estimation of serum markers

Biochemical estimation of serum markers such as serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), total bilirubin (TB) and total protein (TP) were performed by following the standard protocols of diagnostics kits (Coral Clinical System, India).

Biochemical estimation of hepatic markers

Estimation of thiobarbituric acid reactive substance (TBARS)

Thiobarbituric acid reactive substance was estimated by using assay kit. The blank, standard and sample solutions were prepared by mixing each of 100 µl of water, MDA and liver homogenate to 2 ml of colour development solution respectively. The reaction mixture was warmed for 1 h and then cooled for 10 min. Then the mixture was centrifuged at 3000 r/min for 10 min at 4°C and absorbance was recorded at 540 nm by using multimode microplate reader (Synergy H1MF, BioTek, USA).

Estimation of superoxide dismutase (SOD)

Liver homogenate (0.5 ml) was mixed with sodium carbonate (1 ml, 50 mM), NBT (0.4 ml, 24 µM), EDTA (0.2 ml, 0.1 mM) and hydroxylamine hydrochloride (0.4 m, 1 mM). Then, absorbance of the reaction

mixture was read at 560 nm at every 30 s for 5 min by using multimode microplate reader (Synergy H1MF, BioTek, USA).[18]

Estimation of catalase (CAT)

The reaction mixture was prepared by mixing liver homogenate (1 ml), H_2O_2 (1 ml, 30 mM) and phosphate buffer (1 ml, 50 mM, pH 7.4). Then, absorbance of the reaction mixture was recorded at 240 nm by using by multimode microplate reader (Synergy H1MF, BioTek, USA).[19]

Estimation of reduced glutathione (GSH)

The reaction mixture was prepared by mixing liver homogenate (0.01 ml), phosphate buffer (2 ml, pH 8.4), 5,5′-dithiobis (2-nitrobenzoic acid) (0.5 ml) and distilled H_2O (0.4 ml). Then, absorbance was read at 412 nm within 15 min by using multimode microplate reader (Synergy H1MF, BioTek, USA).[20]

Histopathological studies

Liver tissues were embedded in paraffin and tissue sections (5 µM) were prepared by using microtome (MRM-ST, Medimeas, India). Tissue sections were stained with haematoxylin-eosin (H & E) for the microscopic observations (Olympus BX51).[14]

Statistical analysis

All the experimental datas were expressed as mean ± SEM. Statistical analysis was performed by using ANOVA followed by Tukey's multiple comparison tests using GraphPad Prism software (version 7.0, GraphPad, USA).

RESULTS

In vitro antioxidant assays

Antioxidant activities of hydroalcohol extracts of *H. zeylanicum* (HZB and HZL) were assessed by different *in vitro* antioxidant assays (DPPH, SOD, NO and OH) and the results were expressed as IC_{50}. In DPPH assay, the IC_{50} value of HZB and HZL was found to be 62.51 ± 0.28 and 89.24 ± 0.36 µg/ml respectively whereas IC_{50} value of standard drug ascorbic acid was 25.7 ± 0.13 µg/ml. In SOD radical scavenging assay, the IC_{50} value of HZB and HZL was found to be 76.44 ± 0.15 and 93.13 ± 0.21 µg/ml respectively whereas IC_{50} value of standard drug quercetin was 28.44 ± 0.26 µg/ml. In hydroxyl radical scavenging assay, the IC_{50} value of HZB and HZL was found to be 87.69 ± 0.33 and 109.25 ± 0.62 µg/ml respectively whereas IC_{50} value of standard drug ascorbic acid was 30.2 ± 0.37 µg/ml. In nitric oxide radical scavenging assay, the IC_{50} value of HZB and HZL was found to be 71.58 ± 0.47 and 98.73 ± 0.66 µg/ml respectively whereas IC_{50} value of standard drug quercetin was 28.6 ± 0.1 µg/ml (Table 1).

Table 1: *In vitro* antioxidant assays of hydroalcohol extract of leaf and bark of *H. zeylanicum*.

Assays	HZB (µg/ml)	HZL (µg/ml)	Standard (µg/ml)
DPPH	62.51 ± 0.28	89.24 ± 0.36	Ascorbic acid (25.7 ± 0.13)
SOD	76.44 ± 0.15	93.13 ± 0.21	Quercetin (28.44 ± 0.26)
OH	87.69 ± 0.33	109.25 ± 0.62	Ascorbic acid (30.2 ± 0.37)
NO	71.58 ± 0.47	98.73 ± 0.66	Quercetin (28.6 ± 0.1)

DPPH-2,2-diphenyl-1-picrylhydrazyl; SOD-superoxide; OH-hydroxyl; NO-nitric oxide; HZB-hydroalcohol extract of bark of *H. zeylanicum*; HZL-hydroalcohol extract of leaf of *H. zeylanicum*

Table 2: IC_{50} values of hydroalochol extract of bark and leaf of *H. zeylanicum* (HZB and HZL) in CAP-e assay.

Plant part	IC_{50} (mg/ml)
HZB	48.23
HZEL	71.46
Standard drug (Trolox)	40.75

Ex vivo antioxidant studies

Cell based antioxidant protection in erythrocytes (CAP-e) assay

CAP-e assay of ethyl acetate extract of bark and leaf of *H. zeylanicum* (HZB and HZL) were performed. IC_{50} values of HZB and HZL were found to be 48.23 and 71.46 mg/ml, respectively whereas the IC_{50} value of standard drug trolox was found to be 40.75 mg/ml (Table 2).

Acute toxicity studies

In acute toxicity studies of hydroalcohol extract of bark and leaf of *H. zeylanicum*, both HZB and HZL treated groups showed 50% death at dose of 4000 mg/kg b.w. so, LD_{50} was recorded as 4000 mg/kg. Thus, effective dose were determined to be 400 mg/kg b.w. (high dose) and 300 mg/kg b.w. (low dose).

Biochemical estimation of serum markers

The administration of hepatotoxicant (CCl_4) caused liver damage and indicated by the level of serum markers e.g. SGOT, SGPT, ALP, TP and TB. The level of SGOT, SGPT, ALP and TB were significantly ($^c p< 0.001$) elevated in the toxic control group (SGOT, 88.16 ± 1.27 U/l; SGPT, 146.32 ± 1.16 U/l; ALP, 190.62 ±0.9 U/l; TB, 3.1 ± 0.16 mg/dl) as compared to normal group (SGOT, 41.33 ± 0.19 U/l; SGPT, 55.29 ± 1.44 U/l; ALP, 70.37 ± 0.88 U/l; TB, 0.98 ± 0.04 mg/dl). But, pretreatment of HZB (400 mg/kg) was significantly ($^c p< 0.001$) lowered the level of SGOT (56.13 ± 1.54 U/l), SGPT (72.63 ± 1.50 U/l), ALP (97.51 ± 0.98 U/l), and TB (1.43 ± 0.01 mg/dl) which were comparable to positive control group (SGOT, 49.77 ± 1.13 U/l; SGPT, 63.48 ± 1.23 U/l; ALP, 77.48 ± 1.22 U/l; TB, 1.1 ± 0.03 mg/dl). In toxic control group, TP level was significantly ($^c p< 0.001$) reduced (2.16 ± 0.49 mg/dl) than the normal control group (5.33 ± 0.25 mg/dl). But, HZB (400 mg/kg) treated groups exhibited significant ($^a p< 0.05$) increase in the level of total protein (4.71 ± 0.14 mg/dl) in comparison to the positive control group (4.89 ± 0.31 mg/dl) (Table 3).

Biochemical estimation of hepatic markers

The level of MDA was estimated by TBARS assay kit. MDA level was significantly ($^c p< 0.001$) increased in toxic control group (7.4 ± 0.28 nM/mg protein) as compared to normal control group (3.6 ± 0.44 nM/mg protein). However, administration of HZB (400 mg/kg) was significantly ($^c p< 0.001$) reduced the MDA level (4.5 ± 0.36 nM/mg protein) and was comparable to positive control group (3.89 ± 0.12 nM/mg protein) (Table 4). The level of SOD was found to be 7.98 ± 0.35 U/mg protein in normal control group whereas CCl_4 administration significantly ($^c p< 0.001$) depleted the level of SOD in toxic control group (4.1 ± 0.74 U/mg protein). The reduced level of SOD was significantly ($^a p< 0.05$) restored in HZB (400 mg/kg) treated group (6.32 ± 0.12 U/mg protein) and comparable to positive control group (7.22 ± 0.55 U/mg protein). Catalase enzyme activity was significantly ($^c p< 0.001$) decreased in toxic control group (20.76 ± 1.19 U/mg protein) in comparison to normal control group (40.51 ± 1.32 U/mg protein). However, the administration of HZB (400 mg/kg) group showed significant ($^a p< 0.05$) rise in the level of

Table 3: Biochemical estimation of serum markers.

Groups	SGOT (U/l)	SGPT (U/l)	ALP (U/l)	TP (mg/dl)	TB (mg/dl)
Normal control	41.33 ± 0.19	55.29 ± 1.44	70.37 ± 0.88	5.33 ± 0.25	0.98 ± 0.04
Toxic control	88.16 ± 1.27^{c}	146.32 ± 1.16^{c}	190.62 ±0.9^{c}	2.16 ± 0.49^{c}	3.1 ± 0.16^{c}
Positive control	49.77 ± 1.13^{c}	63.48 ± 1.23^{c}	77.48 ± 1.22^{c}	4.89 ± 0.31^{c}	1.1 ± 0.03^{c}
HZB 300	58.36 ± 1.46^{c}	79.50 ± 1.70^{c}	113.08 ± 1.45^{c}	3.57 ± 0.18^{c}	1.64 ± 0.01^{b}
HZB 400	56.13 ± 1.54^{c}	72.63 ± 1.50^{b}	97.51 ± 0.98^{c}	4.71 ± 0.14^{a}	1.43 ± 0.01^{a}
HZL 300	71.24 ± 1.59^{c}	93.42 ± 1.67^{c}	139.08 ± 0.96^{c}	4.4 ± 0.17^{b}	1.77 ± 0.04^{b}
HZL 400	66.35 ± 1.06^{c}	88.50 ± 1.81^{c}	127.38 ± 0.94^{c}	4.57 ± 0.24^{b}	1.50 ± 0.09^{b}

Values are given in mean ± SEM (n=6). Statistical significance differences were represented as $^{a}p< 0.05$, $^{b}p< 0.01$, and $^{c}p< 0.001$ in comparing the normal, negative, positive and test control groups as evaluated by one way ANOVA and Tukey's multiple range test.

SGPT-serum glutamate pyruvate transaminase, SGPT-serum glutamate oxaloacetate transaminase, ALP-alkaline phosphatase, TP-total protein, TB-total bilirubin, HZB-hydroalcohol extract of bark of *H. zeylanicum*, HZL-hydroalcohol extract of leaf of *H. zeylanicum*

Table 4: Biochemical estimation of hepatic markers.

Groups	MDA (nM/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	GSH (nM/mg protein)
Normal control	3.6 ± 0.44	7.98 ± 0.35	40.51 ± 1.32	40.57 ± 1.69
Toxic control	7.4 ± 0.28^{c}	4.1 ± 0.74^{c}	20.76 ± 1.19^{c}	23.7 ± 1.34^{c}
Positive control	3.89 ± 0.12^{c}	7.22 ± 0.55^{c}	36.95 ± 1.27^{c}	36.92 ± 1.58^{b}
HZB 300	5.33 ± 0.02^{a}	5.16 ± 0.02^{c}	27.57 ± 1.62^{b}	28.69 ± 1.22^{b}
HZB 400	4.5 ± 0.36^{c}	6.32 ± 0.12^{a}	30.16 ± 1.33^{a}	30.97 ± 0.36^{a}
HZL 300	5.11 ± 0.36^{a}	6.23 ± 0.22^{a}	25.31 ± 1.58^{c}	29.54 ± 1.14^{b}
HZL 400	5.10 ± 0.35^{ns}	6.14 ± 0.28^{a}	28.32 ± 1.33^{c}	29.63 ± 1.50^{b}

Values are given in mean ± SEM (n=6). Statistical significance differences were represented as $^{a}p< 0.05$, $^{b}p< 0.01$, and $^{c}p< 0.001$ in comparing the normal, negative, positive and test control groups as evaluated by one way ANOVA and Tukey's multiple range test.

MDA-malondialdehyde, SOD-superoxide dismutase, CAT-catalase, GSH-reduced glutathione, HZB-hydroalcohol extract of bark of *H. zeylanicum*, HZL-hydroalcohol extract of leaf of *H. zeylanicum*.

catalase (30.16 ± 1.33 U/mg protein) and comparable to positive control group (36.95 ± 1.27 U/mg protein) (Table 4). On CCl_4 administration, the level of GSH was significantly ($^{c}p< 0.001$) reduced in toxic control group (23.7 ± 1.34 nM/mg protein) in comparison to normal control group (40.57 ± 1.69 nM/mg protein). However, the treatment of HZB (400 mg/kg) was significantly ($^{a}p< 0.05$) elevated the GSH level (30.97 ± 0.36 nM/mg protein) and was comparable to toxic control group (36.92 ± 1.58 nM/mg protein) (Table 4).

DISCUSSION

In human body, free radicals are continuously generated in metabolism and pathological conditions. These radicals severely affect biological macromolecules and leads to degenerative diseases. To counteract free radicals endogenous antioxidants and antioxidant enzymes are present in our body system which may fail in case of excess increase in free radicals and oxidative stress. Till date, many synthetic drugs are available to treat but these have adverse side effects. In the present study, *in vitro*, *ex vivo* and *in vivo* antioxidant potential and hepatoprotection ability of hydroalcohol extract of bark and leaf of *H. zeylanicum* were evaluated.

DPPH assay is simple and rapid antioxidant assay to measure the radical scavenging potential of any substance. It is a stable free radical and has ability to accept electron or hydrogen atom to become stable reduced form of DPPH molecule. DPPH solution shows absorbance at 517 nm and appears as violet coloured solution and upon reduction it becomes colourless. From the disappearance of its colour, the antioxidant activity of substance can be measured. In the present study, HZB exhibited less IC_{50} value (62.51 ± 0.28 µg/ml) as compared to HZL which demonstrated better antioxidant activity of HZB. In normal physiological processes, superoxide radicals are generated in our body which directly or indirectly damage essential biomolecules by formation of hydrogen peroxide, peroxy radicals or peroxy nitrite radicals. Also, it is associated with initiation mechanism of lipid peroxidation. In the present study, superoxide radicals are generated by PMS-NADH system. HZB extract showed higher inhibitory action towards scavenging superoxide radicals and exhibited lower IC_{50} value (76.44 ± 0.15 µg/ml) as compared to HZL. Hydroxyl radicals are highly reactive oxygen species that reacts with polyunsaturated fatty acids (PUFA), membrane proteins, DNA and other biomolecules. Also, it initiates lipid peroxidation in cells. In this study, HZB showed promising free radical scavenging activity inhibition towards hydroxyl radicals. As shown in Table 1, HZB possessed less IC_{50} value (87.69 ± 0.33 µg/ml) than HZL which demonstrated its antioxidant potential. Nitric oxide is highly reactive free radical generated from L-arginine. It is produced in endothelial cell, and brain. Primarily it reacts with proteins and other free radicals such as superoxide radicals to form toxic peroxynitrite radicals. In *in vitro* experiment, it is produced from sodium nitroprusside and resulted in increase in the absorbance at 546 nm. However, different concentrations of HZB reduced its absorbance and exhibited IC_{50} value of 71.58 ± 0.47 µg/ml. Thus it concluded that HZB possess strong antioxidant potential than HZL. Among both the extracts, HZB showed highest radical scavenging activity which may be attributed to the presence of phenolic and flavonoids that neutralize free radicals by donating hydrogen or electron to it.[21-23]

CAP-e assay was performed to evaluate antioxidant potential of the extracts in *ex vivo* (RBC) model. The oxidation of fluorescent indicator DCF-DA to DCF occurs in the presence of free radicals and developed green solution. However, the addition of HZB exhibited highest cellular antioxidant potential which was attributed to the presence of phenolic, flavonoids and other antioxidant principles which have ability to penetrate cell membrane of RBC and thus protected from oxidative damage.[14]

In experimental *in vivo* animal model, the administration of hepatotoxicant CCl_4 induces hepatotoxicity by altering metabolic functions and also causes morphological change in hepatic cells. Hepatic cells consist of high concentration of SGOT, SGPT and ALP and due to damage in cell membrane, these enzymes leak into the blood and mark hepatic injury. Hepatoprotective effect of the extracts can be evaluated by the extent of decrease in the level of these enzymes in blood serum. Among all the extracts, HZB (400 mg/kg) was significantly lowered the level of SGOT, SGPT and ALP in CCl_4 intoxicated rats and the results were comparable to positive control group.[24-26] The administration of CCl_4 caused disruption of polyribosome in endoplasmic reticulum and thus reduces biosynthesis of proteins. The level of total protein was significantly restored in HZB (400 mg/kg) treated groups than other groups. The results were found to be comparable to positive control group.[27,28] Hepatotoxin induced liver injury cause alteration in the secretion of bile by the liver which resulted in increase in the level of TB in serum. But, oral administration of HZB (400 mg/kg) was significantly lowered the level of TB as compared to other extracts and the results were comparable to positive control group.[29]

MDA is an oxidative stress marker in hepatic injury. In CCl_4-induced hepatotoxicity, the trichloromethyl or trichloroperoxy free radicals initiate lipid peroxidation and damage the cell membrane. In experimental rats, the administration of CCl_4 caused increase in lipid peroxidation and the level of MDA increased in toxic control group. But, administration of HZB (400 mg/kg) showed significant reduction in MDA level towards normal than other groups.[30] Superoxide radicals are produced in respiratory electron transport reaction. It reacts with nitrous oxide to form peroxynitrite and cause damage to biological molecules. It can be removed by SOD an antioxidant enzyme which helps in conversion of superoxide free radicals into hydrogen peroxide and molecular oxygen. Hydrogen peroxide, a reactive oxygen species produced as a result of superoxide formation and aerobic metabolism. It plays critical role in apoptosis, inflammation and immune cell activation. Catalase, an antioxidant enzyme involved detoxification of hydrogen peroxide and form oxygen and water. On exposure to hepatotoxicant, superoxide and hydroxyl radicals are generated in the cells and its level increase and inactivate antioxidative enzymes such as SOD and CAT. But, pretreatment of HZB (400 mg/kg) showed significant increase in the level of these enzymes than other groups and the results were comparable to positive control group.[31] Glutathione is an oxidant present in cells. Intracellular GSH is responsible for detoxification of xenobiotics such as free radicals. Oxidative stress induced pathological conditions caused reduction in the level of GSH. In the present study, CCl_4-induced hepatic injury wass marked by decrease in the level of GSH in toxic control group as compared to normal control group. However, pretreatment of HZB (400 mg/kg) showed significant improvement in the level of GSH than other groups and the results were found to be similar to positive control group.[31]

The histopathological studies directly support the results of biochemical estimations of serum and hepatic markers. The groups pretreated with hydroalcohol extract of bark and leaf of *H. zeylanicum* showed significant improvement in hepatotoxicity as compared to the toxic control group. The histopathological results of HZB 400 group were found to be similar with standard drug treated groups (Figure 1).

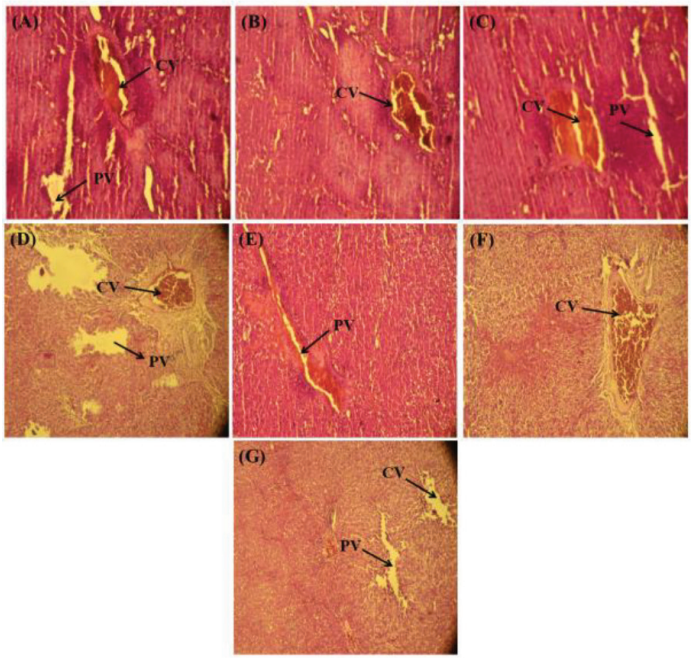

Figure 1: Photomicrographs of liver sections in experimental rats.
A: Normal group showed the presence of CV, PV and normal hepatocytes. B: Toxic (CCl_4) group revealed severe damage in normal cellular architecture and disappearance of PV and narrowed CV. C: Standard (silymarin) treated group showed significant recovery of hepatocytes, CV and PV. D and E: HZB-400 and HZB-300 treated group showed moderate recovery of hepatocytes, CV and PV. F and G: HZL-400 and HZL-300 treated group recovered with appearance of CV, PV and hepatocytes.
CV-central vein, PV-portal vein

CONCLUSION

Hydroalcohol extract of bark and leaf of *H. zeylanicum* possess antioxidant and hepatoprotective activities. The possible bio-efficacy of the extract may be attributed to high content of antioxidants such as phenolic and flavonoids which are responsible for management of oxidative stress in liver cirrhosis. Also, histopathological studies also corroborate the activity of the extracts. Thus, the present study validates the usage of this plant in traditional medicine for treatment of liver disorder and further research is needed to establish bioactive molecules of this species.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

Angiosperm: Phylogeny Group (APG); **HZB:** Bark of *H. zeylanicum*; **HZL:** Leaf of *H. zeylanicum*; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **SOD:** Superoxide; **CAP-e:** Cell based antioxidant protection in erythrocytes; **SGOT:** Serum glutamate oxaloacetate transaminase; **SGPT:** Serum glutamate pyruvate transaminase; **ALP:** Alkaline phosphatase; **TB:** Total bilirubin; **TP:** Total protein; **TBARS:** Thiobarbituric acid reactive

substance; **SOD:** Superoxide dismutase; **CAT:** Catalase; **GSH:** Reduced glutathione.

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