ORIGINAL ARTICLE

In vitro evaluation and comparison of antioxidant and antibacterial activities of leaf extracts of *Hopea ponga* (Dennst.) Mabberly

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Background: *Hopea ponga* has been categorised as an endangered tree species under International Union for Conservation of Nature red list of threatened species. **Objective:** To study the antibacterial, antioxidant activities, total phenolic and flavonoid contents of *Hopea ponga* leaf, extracted from different solvents *viz*., petroleum ether, chloroform and methanol. **Materials and Methods:** Antibacterial activity of leaf extract of *Hopea ponga* was determined by disc diffusion method against *Escherichia coli*, *Proteus vulgaris, Bacillus cereus*, *Streptococcus faecalis and Pseudomonas aeruginosa.* The antioxidant activities of the extracts were assayed through *in-vitro* models such as 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), ferric reducing antioxidant power, hydrogen peroxide and hydroxyl scavenging activities. Total flavonoid content was determined using aluminium chloride colorimetric method and total phenolic content determined using Folin ciocaulteu reagent method. **Results**: Methanol extract showed higher scavenging activity against 2,2-diphenyl-1-picrylhydrazyl and reducing power activity against ferric ion (97.24 ± 0.11 and 88.01 ± 0.03) compared to other extracts. Methanol extract showed significant antibacterial activity against *P. vulgaris* and *B. cereus*. Among the tested extracts, methanol extract exhibited higher content of phenols (1318.749 mg of ferulic acid equivalent/100 gram dry weight of extract) and flavonoids (457.832 mg of quercetin equivalent/100 gram dry weight of extract). **Conclusions**: The present study suggests that the methanol extract may contain active compounds, which have effective antioxidant and antimicrobial activity. Hence, this endangered species have to be explored and conserved to serve the mankind.

Key words: Antioxidant, antimicrobial, dipterocarpaceae, *Hopea ponga*, total phenolic, total flavonoid

INTRODUCTION

Plants have been a valuable source of natural products for maintaining human health. According to World Health Organization, medicinal plants would be the source to obtain a variety of drugs.[1] Plants produce numerous secondary metabolites and have been historically used as pharmaceuticals, fragrances, flavour compounds, dyes and agrochemicals. Even today, metabolites are major source of new drugs.[2] Over three-quarters of the world population rely mainly on plant extracts for healthcare.[3]

Dipterocarpaceae is the most typical family of tropical forest trees in the Malesian region with a geographical distribution that extends to South America and Africa.

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The family comprises approximately 500 species in 17 genera.[4] *Hopea* is one of these genera, containing over 100 species. *Hopea ponga* has been categorised as an endangered tree species under International Union for Conservation of Nature (IUCN) red list of threatened species.[5] *H. ponga* is a common subcanopy tree in wet evergreen forest that grows up to 900 m and the species is endemic to the Western Ghats[6] and exploited for timber in the past.[7] Dipterocarpaceous plants are reported to be an abundant source of stilbenoids[8,9] and possess effective biological properties.[10-13] The aim of the present study is to evaluate antioxidant and antimicrobial activities of *H. ponga* leaves.

MATERIALS AND METHODS

Collection of Leaves and Extraction

Fresh leaves of *H. ponga* were collected from 8 years old tree at Community Agrobiodiversity Centre (CAbC) conservation garden, Puthoorvayal, Kalpetta, Wayanad District, in the state of Kerala, India. The leaves were shade dried at room temperature until moisture disappears. Using a table top mixer, 1,500 g of dried leaves were grounded and each 500 g of powder soaked with petroleum ether, chloroform and methanol

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and kept at room temperature for 12 hrs with 6-8 shakes. Extraction was carried out using Soxhlet[14] apparatus for 4 hours. Solvent collected and evaporated using rotary evaporator at respective temperature (60°C, 68°C and 65°C). Colloidal stage of extract from solvents were collected and dried under laminar air flow and dried extract transferred to amber screw cap bottles and kept at room temperature until use.

Hydroxyl Scavenging Activity

The scavenging activity of extracts on hydroxyl radical was measured.[15] The reaction mixture contained 1,750 µl phosphate buffer solution (pH 7.4), 50 µl 2-deoxy-2-ribose of 80 mM, 50 µl Ethylenediaminetetraacetic acid (EDTA) of 4 mM, 50 µl $FeCl_3$ of 4 mM, 50 µl H_2O_2 of 20 mM, 50 µl ascorbic acid of 4 mM and 200 µl of extract of various concentration was vortexed and allowed to incubate for one hour at 37°C. One ml of 2% trichloroacetic acid (TCA) and 1 ml of 1% thiobarbituric acid (TBA) were added and kept in a boiling bath for 15 min. After cooling, the pink chromogen revealed the formation of thiobarbituric reactive substances (TBARS). The absorbance of the mixture at 532 nm was measured using a spectrophotometer. The hydroxyl radical scavenging activity was calculated as,

Hydroxyl scavenging activity (%) = [(Ac-At)/Ac] ×100

Ac: Absorbance of the control
At: Absorbance of the extracts/standard.

Hydrogen Peroxide Assay

The ability of the extracts to scavenge hydrogen peroxide was determined.[15] A solution of 2 mM hydrogen peroxide was prepared using distilled water. Extracts of 62.5, 125, 250, 500, 1,000 µg/ml concentrations were added to 600 µl hydrogen peroxide and incubated for 30 min at 37°C. Absorbance of hydrogen peroxide at 230 nm was determined against a blank solution and compared with ascorbic acid, the reference compound measured at 230 nm using spectrophotometer and tabulated.

Hydrogen peroxide activity (%) = [(Ac-At)/Ac] ×100

Ac: Absorbance of the control
At: Absorbance of the extracts/standard.

Ferric Reducing Antioxidant Power Assay

Ferric reducing antioxidant power (FRAP) of leaf extract determined.[16] The FRAP stock solutions included 300 mM acetate buffer (3.1 g sodium acetate trihydrate and 16 ml glacial acetic acid) pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. Fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3.6H_2O$ solution and then warmed at 37°C before using. Extracts (150 ml) were allowed to react with 2850 µl of the FRAP solution for 30 min under dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm.

The Reducing power of the extract was calculated according to the following equation,

Reducing power (%) = [(Ac-At)/Ac] ×100

Ac: Absorbance of the control
At: Absorbance of the extracts/standard.

Each antioxidant activity assay was done three times from the same extract in order to determine their reproducibility. Analysis of variance was used to test any difference in antioxidant activities resulting from these methods.

2, 2-diphenyl-1-picrylhydrazyl Assay

Free radical scavenging activity was evaluated by measuring the scavenging activity of the extracts on 2, 2-diphenyl-1-picrylhydrazyl (DPPH).[17] The DPPH radical scavenging activity (%) of the sample was calculated as [1-(absorbance of sample/absorbance of control)] ×100**.** 0.016% butylated hydroxyl toluene in methanol was taken as standard. Methanol was taken as solvent for preparation of samples. Pure methanol was taken as blank. One mg/ml of DPPH was used as assay reagent (oxidant). The following reagents were added serially 2.7 ml of methanol, 100 µl of sample (100 µl methanol in case of blank and Butylated hydroxytoluene (BHT) in case of standard), and 200 µl of DPPH reagent. The mixture was kept in dark incubation at room temperature (RT) for 30 min. Samples were visualised in ultraviolet-visual (UV-VIS) spectrophotometer at wavelength 517 nm.

2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) Assay

2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay of leaf extract was determined.[15] The ABTS stock solution included 7.4 mM ABTS + solution and 2.6 mM potassium persulfate solution. Working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 hrs at RT in the dark. The solution was then diluted by mixing 1 ml ABTS + solution with 60 ml methanol. (Fresh ABTS + solution was prepared for each assay). Leaf extracts (150 µl) were allowed to react with 2,850 µl of the ABTS + solution for 2 hrs in a dark condition. Then the absorbance was taken at 734 nm using spectrophotometer.

Determination of Total Phenolic Content

Four concentrations of the extracts (25, 50, 75, 100 mg) taken in separate test tubes. Added 600 µl of 10 × Folin ciocalteu reagent (FCR) to samples. After 2 minutes of incubation at RT, 960 µl of sodium carbonate was added. Absorbance was measured at 760 nm. The concentration of total phenolic

Table 1: Antioxidant activities of *Hopea ponga* leaf extract

Concentration µg/ml	FRAP (%)			Hydrogen peroxide (%)			Hydroxyl scavenging (%)			ABTS (%)			DPPH (%)		
	PE	CHL	ME	PE	CHL	ME	PE	CHL	ME	PE	CHL	ME	PE	CHL	ME
1000	94.58± 0.23	93.33± 0.26	97.24± 0.11	99.56± 0.13	91.20± 0.17	70.25± 0.16	95.60± 0.29	93.20± 0.37	91.20± 0.22	85.98± 0.12	73.86± 0.34	99.03± 0.16	79.46± 0.12	74.20± 0.17	88.01± 0.23
500	80.85± 0.19	88.00± 0.34	57.15± 0.42	64.57± 0.45	81.55± 0.44	61.50± 0.12	64.57± 0.12	81.55± 0.18	64.80± 0.34	68.43± 0.23	44.73± 0.69	98.76± 0.27	54.17± 0.14	68.95± 0.28	85.51± 0.16
250	78.96± 0.12	47.76± 0.18	27.29± 0.25	54.81± 0.33	60.54± 0.68	47.25± 0.16	54.80± 0.14	65.24± 0.19	49.20± 0.12	61.74± 0.17	30.03± 0.14	98.48± 0.45	52.86± 0.15	66.68± 0.10	67.16± 0.07
125	36.52± 0.14	33.95± 0.17	10.36± 0.17	48.54± 0.21	31.00± 0.47	35.00± 0.18	48.54± 0.31	31.00± 0.25	24.40± 0.43	57.70± 0.38	15.73± 0.18	98.34± 0.32	49.64± 0.18	63.95± 0.14	42.27± 0.11
62.5	35.26± 0.16	33.90± 0.25	3.82± 0.31	10.20± 0.14	13.00± 0.53	21.50± 0.29	10.20± 0.16	13.00± 0.13	9.20± 0.18	57.19± 0.16	10.79± 0.41	98.21± 0.15	43.46± 0.26	63.19± 0.09	20.29± 0.13

Values calculated from three replicate data were expressed as mean±standard deviation; PE – Petroleum ether; CHL – Chloroform; ME – Methanol; FRAP- Ferric reducing antioxidant power

Table 2: Total phenolic and flavonoid content of *Hopea ponga* leaf extract

Extract	Total phenolic content (mg of ferulic acid equivalent/100 g dry weight of extract)	Total flavonoid content (mg of quercitin equivalent/100g dry weight of extract)
Petroleum ether	204.53±2.6	414.87±5.2
Chloroform	128.28±4.5	398.41±6.8
Methanol	1318.74±6.8	457.83±1.6

Values calculated from three replicate data were expressed as mean±standard deviation

compounds in extract was expressed as mg of ferulic acid equivalent per 100 gram dry weight of extract.[18]

Determination of Total Flavonoid Content

The total flavonoid content was determined using aluminium chloride colorimetric method.[19] Plant extracts (0.5 ml) separately mixed with 1.5 ml of 95% methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After mixing, the solution was incubated for 30 min at room temperature. Absorbance of the reaction mixtures measured at 415 nm. The concentration of total flavonoids in all extracts was expressed as mg of quercetin equivalent per 100 gram dry weight of extract.

Antibacterial Activity

Six bacterial strains (*Pseudomonas aeroginosa, Escherishia coli, Staphylococcus faecalis, Proteus vulgaris* and *Bacillus cereus*) were used in this study. Stock cultures were maintained at 4°C on slant of nutrient agar. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of nutrient broth for bacteria that were incubated at 24 hrs at 37°C. The assay was performed by disc diffusion method. Antibacterial activity of the given sample was determined by disc diffusion method on Muller Hinton agar (MHA) medium. The test solutions of the dried extracts at the concentrations of 1,000 µg, 500 µg, 250 µg, 100 µg/ml were impregnated on sterile discs. Ampicillin was used as positive control. The disc impregnated with Dimethyl sulfoxide (DMSO) was used as negative control. The discs were placed on the surface of the MHA agar for bacteria and incubated at 37°C for 24 hrs. Inhibition zones were calculated as the difference between disc diameter (6 mm) and the diameters of inhibition.[20] The antibacterial activities were evaluated by the determination of minimum inhibitory concentration (MIC) by micro broth dilution assay.[21]

RESULTS AND DISCUSSION

Antioxidant activities measured in petroleum ether, chloroform and methanol extracts using ABTS, DPPH, FRAP, hydroxyl scavenging and hydrogen peroxide assays. FRAP, DPPH and ABTS assays are commonly used for the assessment of free radical-scavenging abilities of herbal medicines and health foods.[22,23] ABTS is a synthetic radical that can be used to estimate scavenging activities for both polar and non-polar samples.[24] The antioxidant activities of different radicals were presented in Table 1.

The methanolic extract showed effective results in scavenging DPPH and ABTS radicals and also effectively reduces ferric ion compared to chloroform and petroleum ether extract. Moreover, the hydroxyl radical and hydrogen peroxide are ineffectual against petroleum ether followed by chloroform extract. The scavenging activity increases with increasing concentration of the extract and the result is comparable with the antioxidant activity of seed wing from *H. ponga*.[25] The scavenging ability of leaf extracts of *H. ponga* is also found to be remarkably higher compared to other genus of Dipterocarpaceae family.[26,27]

Phenolic compounds are very important plant constituents because of their scavenging ability due to their hydroxyl group; they are also powerful chain breaking antioxidants and have been associated with antioxidant activity[28] The concentration of phenols and flavonoids present in the leaf extracts are given in Table 2. The highest concentration of phenols (1,318.749 mg of ferulic acid equivalent/100 g dry weight of extract) and flavonoids (457.832 mg of QE/100g dry weight of extract) were observed in methanolic extract

Table 3: Antibacterial activity of *Hopea ponga* leaf extract

Bacterial strains	ATCC no.	Petroleum ether (mm)				Chloroform (mm)				Methanol (mm)				Ampicillin (mm)
		1000 µg/ml	500 µg/ml	250 µg/ml	100 µg/ml	1000 µg/ml	500 µg/ml	250 µg/ml	100 µg/ml	1000 µg/ml	500 µg/ml	250 µg/ml	100 µg/ml	
Escherichia coli	25922	12	09	06	06	13	11	10	07	11	08	05	05	40
Pseudomonas aeruginosa	27853	08	08	05	NA	10	09	08	06	06	06	04	NA	38
Proteus vulgaris	13315	11	09	06	05	13	12	10	8.5	19	16	15	10	40
Bacillus cereus	11778	14	11	08	06	10	07	05	05	18	13	07	06	40
Streptococcus faecalis	29212	16	12	09	03	09	07	06	04	14	11	08	05	32

NA – No activity; ATCC – American type culture collection

Table 4: Minimum Inhibitory Concentration of *Hopea ponga* leaf extract

Bacterial strains	Minimum inhibitory concentration (µg/ml)		
	Petroleum ether	Chloroform	Methanol
Escherichia coli	250	100	250
Pseudomonas aeruginosa	500	250	500
Proteus vulgaris	250	125	100
Bacillus cereus	250	250	100
Streptococcus faecalis	125	250	125

followed by petroleum ether and chloroform extracts. The phenolic content and flavonoid content of *H. ponga* leaf is higher compared to other genus of Dipterocarpaceae family.[26,27,29]

The antibacterial activity was performed by disc diffusion method and the results are presented in Table 3. Chloroform extract showed moderate activity against *E. coli* whereas *P. vulgaris* and *B. cereus* were sensitive to methanolic extract. *S. faecalis* exhibited maximum zone of inhibition against petroleum ether. The MIC values of the plant extract against the tested bacterial isolates ranged from 100 to 500 µg/ml [Table 4]. The methanol extract at the least concentration of 100 µg/ml inhibits the growth of *P. vulgaris* and *B. cereus*. *E. coli* have been inhibited by chloroform extract at the same concentration. *S. faecalis* is susceptible to the methanolic and petroleum ether extract at 125 µg/ml concentration.

The present study revealed that the methanol leaf extract of *H. ponga* possess significant biological properties against *P. vulgaris* and may be used to cure *Proteus* infections. The methanol extract also exhibits high scavenging ability against different free radicals, which in turn implies that the extract may contain active compounds which have effective antioxidant and antimicrobial activities. Hence, this endangered species have to be explored and conserved to serve the mankind.

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