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Original Article

PHYTOCHEMICAL EVALUATION, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF *HOPEA PONGA* (DENNST) MABBERLY EXTRACTS

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ABSTRACT

Objective: The present study was aimed at evaluating the *in vitro* antioxidant and antibacterial activities of methanol and aqueous extracts of *Hopea ponga.*

Methods: *Hopea ponga* leaf and stem sample were powdered and extracted with methanol and water. The extracts were screened for various phytochemical by HPLC and TLC studies. The total phenolic and flavonoid contents were assessed by spectrophotometric method and the antioxidant activity was estimated using 2,2 diphenyl-1-picrylhydrazyl (DPPH) free radical scavening activity and inhibition of lipid peroxidation assay. Antibacterial activity was evaluated by disc diffusion method & MIC by Broth dilution method.

Results: The methanol extracts exhibited significant ($p < 0.05$) higher antioxidant activity with an IC50 value of 108µg/ml and 122 µg/ml in leaf and stem extracts respectively in the DPPH radical scavenging method, 147.2 µg/ml 136 µg/ml in the hydroxyl radical scavenging assay compared to aqueous extracts. The amount of total phenolics in methanol leaf and stem was around 286mg and 213mg gallic acid equivalent (GAE) respectively. The total flavonoid contents of methanol extracts of stem and leaf were around 108.3 mg and 86 mg catechin equivalents per gram respectively. Methanol leaf and stem extracts were found to possess maximum antibacterial activity with MIC ranging around167-648ug/ml against tested strains. High Performance Liquid Chromatography (HPLC) analysis indicated the presence of phenolic compounds such as quercetin, gallic acid, *para* hyroxybenzoic acid.

Conclusion: The results indicate that the extracts had significant free radical scavenging and antibacterial activity. The results suggest that *Hopea ponga,* may be a good source of natural antioxidants and antibacterial source.

Keywords: *Hopea ponga,* Antioxidant activity, Lipid peroxidation, Total phenolic content, Minimum Inhibitory concentration (MIC).

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide anion, hydroxyl radicals and hydrogen peroxide are chemically reactive molecules derived from oxygen. They are generated in living organisms as by product through the normal metabolic pathways. ROS can readily react and oxidize most of the biomolecules including carbohydrates,proteins, lipids and DNA [1]. The accumulation of ROS has been proved to cause oxidative damage to tissue affecting cellular metabolism particularly in arthritis, cancer, inflammation and heart diseases. Consequently, antioxidants that can neutralize direct ROS attacks and terminate free radical mediated oxidative reaction would have beneficial activities in protecting the human body from such diseases. The commonly used synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxylanisole (BHA) are restricted by legislative rules because of their toxic effects and possible carcinogenic properties[2]. Nowadays antibiotic resistance among bacterial pathogen is major public health problem worldwide [3]. Among the wide array of antibiotics β lactam are the most widely used accounting about 50% of all systemic antibiotics in use. The most common cause of bacterial resistance to β lactam antibiotics is the production of β lactamase. Hence, there is a need for searching alternative source of antibiotics. Plant based medicines have been part of traditional healthcare in most parts of the world for thousands of years [4]. The medicinal value of these plants lies in some chemical substances known as phytochemicals [5]. Most of the antioxidant potential in herbs and spices are due to the redox properties of phenolic compounds that allow them to act as reducing agent, hydrogen donator and free radical quencher [6].

India is the largest producer of medicinal herbs and appropriately called the botanical garden of the world [7]. Over the past few decades, there has been much interest in natural material as a source of new anti bacterial agents. Different extracts from traditional medicinal plants have been tested. Many reports show the effectiveness of traditional herbs against micro organisms, as a result, plant have become one of the bases of modern medicine. [8]. Approximately 20% of the plants found in the world have been submitted to pharmacological or biological tests [9]. *Hopea ponga* is an endemic tree belonging to family Dipterocarpaceae, found in the tropical evergreen forests of south western India distributed all along the Western Ghats of Karnataka [10]. It is a large trees with oblong, leaves, Flowers are pink color with glabrous racemose panicles. Fruit is green, turns red when matures. *Hopea ponga* has been categorized as an endangered tree species under the International Union for Conservation of Nature red list of threatened species. Traditionally the plant is used in piles and snake bite[11]. The tree is economically important as timber, the bark is also a good tanning material and astringent with the slow speed of diffusion [12] Sukesh et al [13] evaluated seed wings of *Hopea ponga* for its antioxidant and antibacterial activity and observed higher activity in methanol extract. Rose et al [14] evaluated the leaf for its antioxidant activity and there is no information about comparative study of antioxidant and antimicrobial activity of *Hopea ponga* leaf and stem extracts hence in this context a present study was designed to evaluate the antioxidant and antibacterial activity.

MATERIALS AND METHODS

Diphenyl picryhydrazyl (DPPH), Trichloroacetic acid (TCA), Deoxyribose Potassium ferricyanide and ferric chloride & Dimethyl sulphoxide (DMSO) were purchased from Sisco Research Lab. All other chemicals and solvents used were of analytical grade available commercially. Bacterial cultures such as Gram positive *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, and Gram negative bacteria *Escherichia Coli* ATCC 8739, *Pseudomonas aerugenosa* ATCC 27853 were procured from National Chemical

Laboratory Pune, India. The bacterial cultures were grown on the nutrient agar medium and stored at 4ºC. To prepare bacterial strain for test, initially a loopfull of culture from the slant was transferred into nutrient broth solution (10 ml) and stored at 37ºC for 24h.

The plant parts such as leaves and stem were collected from the Andar reserve forest from the Western Ghats, shade dried at room temperature, powdered and stored in polythene bags until use.

Extraction of plant material

Fifty grams of dried and powdered plant material were extracted at room temperature for 24 hrs with a mass to volume ratio of 1: 10 g/ml using methanol as solvents in soxhlet apparatus following the method of Razali [15]. Aqueous extract was also prepared in the similar way at 90̊C for 2 -4 hours. The extracted samples were filtered using No 1whatmannfilter paper and muslin cloth respectively. All the extracts were concentrated under vaccum and stored at 4ºCprior to use.

HPLC – fingerprint analysis

High performance liquid chromatography was used to analyze the presence of phenolics in the extract. Chromatography was performed using C-18 column (250 mm x 4.6 x 5 µm). Detection was in the range of 210-500 nm. The extract was dissolved in corresponding solvent followed by filtration of the solution using Millipore filter. The elution system was acetonitrile ammonium dihydrogen phosphate and orthophosphoric acid. The flow rate was employed at 0.8 ml/min. The injected volume of the sample was 20 µl. The standard phenolics used were gallic acid, catechin, epicatechin, quercetin, *para* coumaric acid, caffeic acid, chlorogenic acid, vanillin dihydrobenzoic acid and sinapic acid.

Total Phenolic content

The concentration of polyphenol was quantified following the method of [16]. Fifty micro liters of test sample was mixed with 2 ml of 2% sodium carbonate and allowed to stand at room temperature for 2 min. At this time 100 µl of 50% Folin Ciocalteau reagent was added and the reaction mixture was allowed to stand at room temperature for 30 min and readings were taken at 720 nm. Gallic acid was used as standard for calibration curve. The polyphenol content of the extract was expressed as gallic acid equivalent.

Total flavonoids

The amount of flavonoid was quantified following the method of [17]. Two hundred microliters of test sample was mixed with 5 ml of cinnmaaldehyde and allowed to stand at room temperature for 30 min. The absorbance was measured against the reagent blank at 640 nm. Catechin was used as the standard for the calibration curve. The concentration of flavonoid content was calculated from the standard calibration curve and was expressed as catechin equivalent.

DPPH radical scavenging activity

The antioxidant activity of the extract was measured based on the scavenging activity of the stable DPPH free radical. The activity was determined by the method described by [18]. Plant extracts in different concentrations ranging from 50-250µg/ml were added to 3 ml of DPPH solution (0.004%). One ml of water in place of plant extract was used for control. Absorbance was determined at 520 nm after 30 min and percent of inhibition was calculated by following equation 1.

$$Antioxidant\ activity\ \left((\%) = \frac{A_0 - A_t}{A_0}\ x\ 100\right)$$

where A_0 is the absorbance of control and A_t is the absorbance of extract. All of the experiments were performed in triplicates and the mean values are presented in the results section of this paper.

Reducing power

The reducing power of the extract was determined following Oyaizu method [19]. Different amounts of extracts ranging from 2-10µg/ml were mixed with 2.5 ml of 0.2M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide and the mixture was incubated at 50ºC for 20 min. Afterwards, 2.5 ml of 10% Trichloro acetic acid was added and centrifuged for 10 min. The upper layer of the solution was mixed with 2.5 ml of distilled water and absorbance was measured at 700 nm after adding 0.5 ml of 0.1% ferric chloride. The increased absorbance of the reaction mixture indicated the reducing power. Ascorbic acid was used as standard

Hydroxyl radicals scavenging activity

The ability of the extract to inhibit non site specific hydroxyl radical mediated peroxidation was carried out according to the method described by Halliwell [20]. The reaction mixture contained 100µl of extract at various concentrations, 500 µl (5.6 mM) of deoxy ribose in KH_2PO_4/ NaOH Buffer (50 mM, pH 7.5), 200 µl of premixed 100 mM $FeCl_3$ and 104 mM EDTA (1:1v/v) solution, 100 µl of H_2O_2 (1.0 mM) and 100 µl of ascorbic acid. The tubes were vortexed and incubated at 50ºC for 30 min, thereafter 1 ml of 2.8% TCA and 1 ml of 1% TBA were added and kept in a water bath for 30 min and cooled. The absorbance of the solution was taken at 540 nm. The extent of oxidation was calculated by the absorbance. The percentage of inhibition values were calculated from the formula.

$$Antioxidant\ activity\ \left((\%) = \frac{A_0 - A_t}{A_0}\ x\ 100\right)$$

where A_0 is the absorbance of control and A_t is the absorbance of extract. All of the experiments were performed in triplicates and the mean values are presented in the results section of this paper.

Lipid peroxidation assay

A modified thiobarbituric acid reactive species (TBARS) assay described by Ohkowa, [21] was used in this study to measure lipid peroxides formed using egg yolk homogenate as lipid rich media [22] Malonaldehyde (MDA), a secondary product of oxidation of polyunsaturated fatty acid reacts with two molecules of thiobarbutric acid (TBA) yielding pinkish red chromogen with absorbance at 540 nm. In this method 0.5 ml of egg homogenate, (10% in distilled water) and various extracts of concentrations ranging from 5-20µg/ml were a mixed in test tube and the volume was made upto one ml by distilled water. Finally, 0.05 ml of $FeSO_4$ (0.07M) was added to the above mixture and incubated for 30 min to induce lipid peroxidation. Thereafter, 1.5 ml of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 ml of 0.8% TBA and 0.05 ml 20% TCA were added, vortexed and heated in boiling water bath for 1 hour. After cooling 5 ml of butanol was added to each tube and centrifuged at 3000 rpm for 10 min, The absorbance of the organic upper layer was measured at 540 nm. Water used in place of extract served as control. Percentage of inhibition was calculated according to formula

$$\left((\%) = \frac{1 - E}{C}\ x\ 100\right)$$

Where C is the absorbance of control and E is the absorbance of extract. All of the experiments were performed in triplicates and the mean values are presented in the results section of this paper.

Antibacterial activity

The disc diffusion method was employed for screening of the antimicrobial activities of the extracts. A suspension of the test microorganism (0.1 m1 of 10^8 CFU/ml) was spread on the Muller Hinton Agar medium. Sterile filter paper discs (6 mm in diameter) were soaked with 10 µl of the extract and placed on the inoculated plates [23]. After being kept at 4ºCfor half an hour the plates were incubated at 37ºC for 24 hrs. The diameters of zone of inhibition were measured in millimeters. All the experiments were performed in triplicate.

The Minimum Inhibitory Concentration (MIC) of methanol extract was determined by broth dilution method of NCCLS [24]. The lowest concentration of the plant extract inhibiting the visible growth of the organism was considered as MIC.

Statistical analysis

All the analyses were performed in triplicate and data was statistically analyzed using ANOVA, P<0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

Methanol recorded a higher yield compared to water in both leaf and stem sample. The yield of methanol was around 10.2% and 8.9% in leaf and stem respectively. The water extract showed 9.3% and 7.8% in leaf and stem respectively.

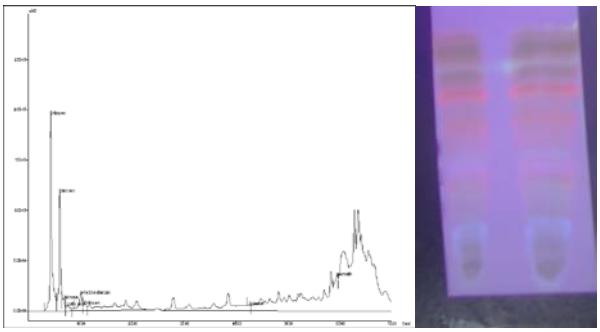

Fig. 2: HPLC and TLC analysis of Hopea ponga extract

The result of HPLC analysis of *Hopea ponga* extracts showed the presence of quercetin, *para* coumaric acid *para benzoic acid* and epicatechin. TLC analysis of *Hopea ponga* extracts were separated into ten bands with R_f value ranging between 0.17- 1.2 cm [Fig.1].

Total phenolic content

The leaf extracts exhibited higher phenolic compounds compared to stem extracts [Fig.2]. Methanolic leaf extract recorded a significant (p <0.05) phenolic content compared to water extracts and methanol stem extract. The methanolic leaf extract showed 286mgGAE/g of phenolic content followed by water leaf extract 213mg GAE/g. The present study indicates methanol as the ideal solvent for the extraction of the phenolic compounds. The methanolic stem extract had higher flavonoid content compared to other extracts. The methanolic stem extract recorded 108mgcatechin equivalent /gram compared to other extracts. Phenolic compounds in plant constitute a major class of secondary plant metabolites with bioactive potential attributed to antioxidant and antibacterial activities. Free phenolic acids or derivatives present in the ester or ether form are found in varying quantities throughout plant tissues in responses to characteristic synthesis pattern resulting from encounters of different forms of environmental stress. Therefore, different herbal parts will offer distinct qualities of material used for cuisine, food preservation and herbal medicine [25].

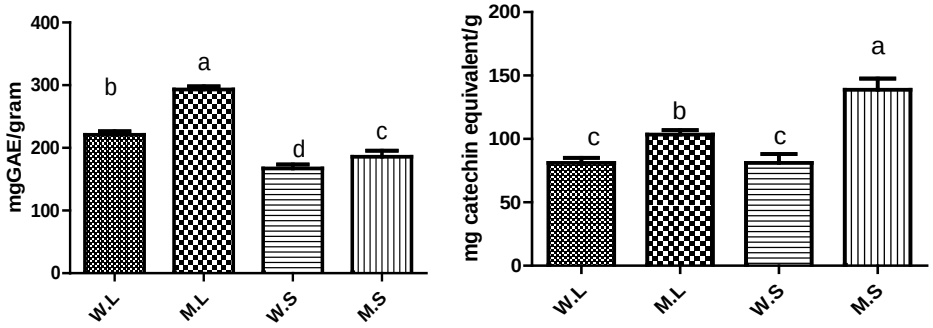

Fig. 2: Total phenolic and flavonoid content of the crude extracts of *Hopea ponga*

DPPH scavenging activity

Both the leaf and stem extracts of methanol showed statistically significant (p<0.05) DPPH scavenging activity compared to water extracts [Fig.3]. The DPPH scavenging activity increased with increase in the concentration of the extracts.

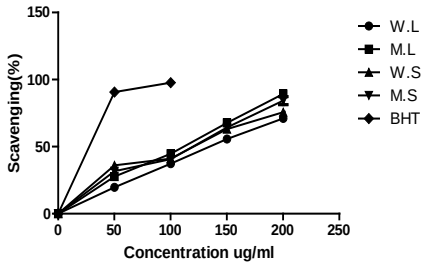

Fig. 3a: DPPH scavenging activity of water and methanol extracts of *Hopea ponga* and BHT: Values are means of three replicates

The IC_{50} value of the methanol leaf and stem extract was around 108µg/ml and 122 µg/ml which is comparably lower to the water extracts of leaf and stem (134 µg/ml and 146 µg/ml) respectively. DPPH is a stable nitrogen centered free radical, the color of which changes from violet to yellow, upon reduction by either process of hydrogen or electron donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers [26]. A positive correlation was observed between DPPH scavenging activity and total phenolic content of extracts [Fig.3b].

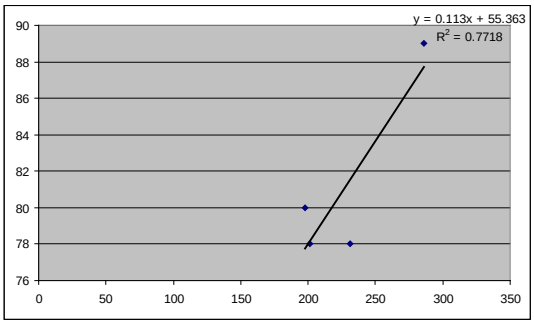

Fig. 3b: Correlation between DPPH scavenging activity and Total phenol content

Reducing Power assay

The reductive ability of extracts is represented in [Fig. 4] The reduction potential was lesser compared to the standard (Ascorbic acid). An increase in the reducing power was observed with an increase in the concentration of the extracts. The reducing properties are generally associated with the presence of reductones which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom[27]. Yen have observed a direct correlation between antioxidant activities and reducing power of certain plant extracts[28].

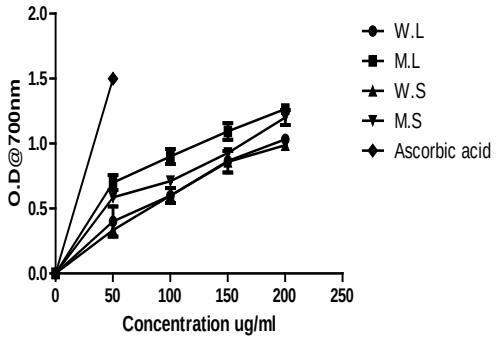

Fig. 4: Reducing power of water and methanol extracts of *Hopea ponga* and Ascorbic acid: Values are means of three replicates

Hydroxyl radical scavenging activity

The scavenging activities towards the hydroxyl radical are shown in [Fig 5] Both water and methanol extracts were able to scavenge the hydroxyl radical. The scavenging activity increased with increase in the concentration of the extracts. Methanol extract of stem had the significantly ($p<0.05$) higher scavenging activity upto 82% with IC_{50} values 114.85µg/ml compared to other extracts.

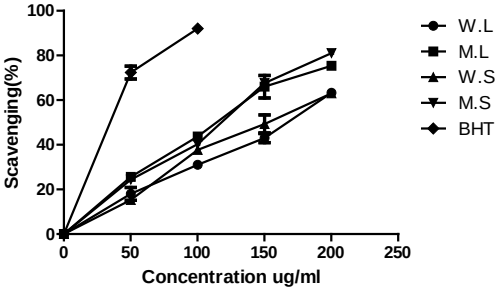

Fig. 5: Hydroxyl radical scavenging activity of water and methanol extracts of *Hopea ponga* and BHT: Values are means of three replicates

Lipid Peroxidation assay

The methanol & water extracts of stem and leaves inhibited lipid peroxidation induced by ferrous sulphate in egg homogenate. A concentration dependent manner inhibition was observed. The Methanol extract of leaf and stem inhibited upto 74% and 72% for 200 µg/ml and water extract of leaf inhibited 58% and 54% for 200µg/ml respectively [Fig.6]. The IC_{50} values of these two extracts where around 144 µg/ml and 136 µg/ml which were statistically significant ($p<0.05$) compared to water extracts of stem and leaf. In biological systems, degradation of poly unsaturated fatty acids in the cell membrane generates a large number of degradation products such as Malonoaldehyde (MDA) which are found to be an important cause for cell membrane destruction and cell damage. MDA, one of the major products of lipid peroxidation has been extensively used as index for lipid peroxidation and as a marker for oxidative stress. The reaction of MDA with TBA has been widely adopted as a sensitive assay method for lipid peroxidation [29].

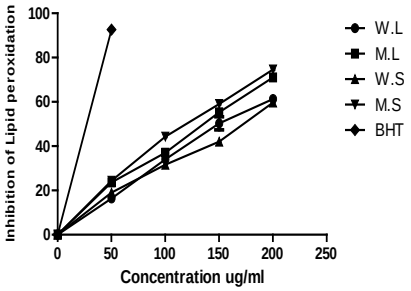

Fig. 6: Inhibition of Lipid peroxidation of water and methanol extracts of *Hopea ponga* and BHT: Values are means of three replicates

Antibacterial activity

Both stem and leaves extracts of methanol showed significant ($P<0.05$) inhibition to microbes tested. Gram +ve strains (*Staphylococcus areus* and *Bacillus subtilis*) were significantly sensitive compared to Gram –ve strains (*Escherichia coli and Pseudomonasaeruginosa*). A possible explanation for these observations may be attributed to the significant differences in the outer layers of Gram negative and Gram positive bacteria. Gram negative bacteria possess an outer membrane and a unique periplasmic space not found in Gram positive bacteria The resistance of Gram negative bacteria towards antibacterial substances is related to the hydrophilic surface of their outer membrane which is rich in lipopolysaccharide molecules, presenting a barrier to the penetration of numerous antibiotic molecules. The membrane is also associated with the enzymes in the periplasmic space which are capable of breaking down the molecules introduced from outside. However, the Gram positive bacteria do not possess such outer membrane and Cell wall structures [30]. Phytochemical constituents such as alkaloids, flavonoids, tannins, phenols, saponins, and several other aromatic compounds are secondary metabolites of plants that serve a defense mechanism against any microorganisms, insects and other herbivores.

Table 1: Antibacterial activity of methanol and water extracts of *Hopea ponga* extracts

Extracts/Antibiotic	Antibacterial activity (DIZ) in mm			
	S. aureus	*B. subtilis*	*P. aeuroginosa*	*E. coli*
Water Leaf	15.1 ± 1.8^{c}	18.2 ±3.5^{b}	10.1 ±2.2^{c}	13.0±.2.0^{b}
Stem	12.3 ± 0.9^{d}	11.6 ±1.8^{c}	5.7 ±1.6^{d}	4.5 ±.1.9^{d}
Methanol Leaf	18.3 ±2.4^{b}	20.1 ±4.3^{b}	15.3 ±3.1^{b}	16.3 ±2.6^{b}
Stem	14.7 ± 1.7^{c}	16.5 ±3.6^{b}	9.8 ±2.2^{c}	10.3 ±2.9^{c}
Streptomycin (10 µl/ml)	25.4 ± 0.5^{a}	30.1 ±0.4^{a}	26.0 ±0.2^{a}	25.0 ±0.1^{a}

Values presented are means of six replicates, ± Standard error. The values followed by different superscript differ significantly ($P<0.05$).

The presence of phenols, tannins, saponins and steroids in the extracts could be responsible for the observed antimicrobial property. These bioactive compounds are known to act by different mechanisms. Tannins bind to proline rich proteins and interfere with the protein synthesis. The antimicrobial activities of phenolic compounds may involve multiple modes of action for eg, oils degrade the cell wall, interact with the composition and disrupt cytoplasmic membrane, damage membrane protein, interfere with membrane integrated enzymes,cause leakage of cellular components, coagulate cytoplasm, deplete the proton motive force, change fatty acid and phospholipid constituents, impair enzymatic mechanism for energy production and metabolism, alter nutrient uptake and electron transport. Antimicrobial property of saponin is due to its ability to cause leakage of proteins and certain enzymes from the cell.

Steroids have been reported to have antibacterial properties, the correlation between membrane lipids and sensitivity for steroidal compound indicates the mechanism in which steroids specifically associate with membrane lipid and exert its action by causing leakages from liposomes [31].

Table 2: MIC of Methanol extract against four Microorganisms by Broth dilution method

Microorganisms	Minimum inhibitory concentration(µg/ ml)
S. aureus	185.32 ± 32.13
B. subtilis	167.75 ± 26.52
P. aeuroginosa	648.21 ± 26.22
E. coli	642.45 ±42.76

CONCLUSIONS

The antioxidant and antibacterial activities of *Hopea ponga* extracts were evaluated for the first time. Methanol extracts of leaf and stem showed a considerable antioxidant activity in various methods such as DPPH Scavenging activity, reducing power, and inhibition of lipid peroxidation. Methanol extracts exhibited a significant antibacterial activity. The present study indicates methanol as an excellent solvent for the extraction of antioxidants and antibacterial compounds. The observed antioxidant and antibacterial activity might be due to the presence of high level of phenolic compounds. On the basis of the present results, it may be suggested that extracts may be used as a natural antioxidant and antibacterial source. Further purification and characterization of the extracts may yield the novel compound which can be used in food & pharmaceutical industries.

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