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Phytochemical analysis, antimicrobial, insecticidal and antiradical activity of *Hydnocarpus pentandra* (Buch.-Ham.) Oken

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Abstract

The present study was conducted to evaluate antimicrobial, insecticidal and radical scavenging activity of leaf extract of *Hydnocarpus pentandra* (Buch.-Ham.) Oken belonging to the family Achariaceae.

Extraction process of shade dried and powdered leaf was carried out by maceration technique. Extract was screened for phytochemicals by standard tests. Antibacterial and antifungal activity of leaf extract was determined by Agar well diffusion and Poisoned food technique respectively. Antiradical activity of leaf extract was evaluated by two in vitro assays namely 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azinobis 3-ethylbenzothiazoline 6-sulfonate (ABTS) free radical scavenging assays. Insecticidal activity of leaf extract was determined against II instar and IV instar larvae of *Aedes aegypti*.

Preliminary phytochemical analysis showed the presence of alkaloids, flavonoids, tannins, saponins, glycosides, triterpenes and steroids in the leaf extract. Leaf extract exhibited marked inhibitory activity against Gram positive bacteria when compared to Gram negative bacteria. *Bacillus cereus* (zone of inhibition 1.86±0.05cm) and *Escherichia coli* (zone of inhibition 1.06±0.05cm) were inhibited to highest and least extent respectively. Extract was effective in inhibiting mycelial growth of seed-borne fungi. Among fungi, the susceptibility to extract was in the order: *Curvularia* sp. (53.64% inhibition) > *Fusarium* sp. (45.81% inhibition) > *Alternaria* sp. (35.08% inhibition). The extract exhibited concentration dependent larvicidal activity with marked activity being observed against II instar larvae (LC_{50} value 0.79mg/ml) when compared to IV instar larvae (LC_{50} value 1.37mg/ml). Leaf extract scavenged DPPH and ABTS radicals dose dependently with an IC_{50} value of 13.91µg/ml and 6.03µg/ml respectively.

The plant is shown to be an important source of bioactive agents. The observed bioactivities could be attributed to the phytochemicals present in the leaf extract. Further studies on characterization and bioactivity determination of isolated components from leaf extract are to be carried out.

Keywords: *Hydnocarpus pentandra*, Phytochemical, Agar well diffusion, Poisoned food technique, DPPH, ABTS, *Aedes aegypti*

Introduction

Discoveries and developments made in the field of chemotherapy, in particular antibiotics, resulted in a dramatic change in the treatment of infectious diseases which once caused devastating situation in history. Antibiotics have revolutionized the field of medicine as they have prevented huge number of deaths due to infectious diseases. Despite of advances in chemotherapy, emerging and re-emerging infectious diseases caused serious threat to therapy. Infections caused by drug resistant microorganisms remain an important problem in clinical practice. Antibiotic resistant pathogens are of serious concern in both hospital and community settings. Moreover, the tendency of microbes to transmit resistance genes to susceptible strains is another serious problem. Besides, many antibiotics are costly and exhibit certain side effects. Natural products including plants and their metabolites are considered to be a promising alternative for disease therapy. Plants have been widely used in the traditional treatment of several diseases by people all over the world. Use of botanicals is safer, cheaper and is not associated with resistance development problem [1][2][3][4][5][6][7][8].

Pathogens such as bacteria, viruses and fungi are known to cause several diseases in crops. Among these, fungi represent the dominant group of phytopathogens responsible for causing a number of diseases in crops (both in field and storage conditions)

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leading to decreased productivity and huge economic loss in severe cases. Crop losses of >50% can occur in severe cases. Seed-borne fungi are known to affect the germination and vigor of seeds. The use of chemical fungicides has been considered promising to control fungal diseases of plants. However, their unrestrained use results in environmental pollution, toxic effects on non-target organisms and emergence of resistant strains of pathogens. Diseases caused by fungicide resistant strains are difficult to control. Plants and plant based formulations are one among the promising alternatives for chemical agents. Studies have shown the potential of plants to inhibit a wide range of phytopathogenic fungi. Fungicides from botanical origin are promising as they are cheaper, eco-friendly and do not cause adverse effects on non-target organisms [9][10][11][12][13][14].

The role of mosquitoes as important vectors transmitting dreadful human diseases like malaria, filariasis, Japanese encephalitis, and dengue is well reported. *Aedes aegypti* is one of the important mosquito vectors that transmit diseases viz. dengue and chikungunya. Management of mosquito-borne diseases involves several strategies, among which, killing larvae of mosquitoes is one of the routinely used methods. Synthetic chemicals such as organochlorines and organophosphates are widely used as larvicidal agents. However, the indiscriminate use of these chemical agents is associated with drawbacks such as high cost, deleterious effects on environment, adverse effects on non-target organisms including humans and emergence of resistant forms. Natural products such as plant based formulations are shown to be one of the best alternatives for chemicals and the use of botanicals is eco-friendly and do not cause harm to non-target organisms [15][16][17][18][19][20][21].

Reactive oxygen species such as free radicals (superoxide radical and hydroxyl radical), hydrogen peroxide and singlet oxygen are formed during normal metabolism. Free radicals are chemical species having an unpaired electron in outer orbit. Free radicals are highly reactive and are known to cause damage to biomolecules such as proteins, nucleic acids and lipids. Excessive production of free radicals results in oxidative damage which is implicated in several diseases or disorders such as ageing, cancer, cardiovascular diseases and neurodegenerative diseases. Cells have enzymatic and non-enzymatic antioxidant defense system. A balance exists between free radical generation and antioxidant defense in normal conditions. However, in pathophysiological conditions, there is an extra need for antioxidants in the form of food. Interest in botanicals with antioxidant activity has triggered nowadays due to suspected ill effects associated with the use of synthetic antioxidants. Several plants are shown to exhibit marked antioxidant potential which is mainly due to phenolic compounds and flavonoids [22][23][24][25][26][27][28].

The genus *Hydnocarpus* Gaertn. Belonging to the family Achariaceae include 40 species that are distributed worldwide. *H. pentandra* (Buch.-Ham.) Oken [*H. laurifolia* (Dennst.) Sleumer; *H. wightiana* Blume] is a large evergreen tree. Leaves are alternate, ovate or oblong-lanceolate, acuminate, more or less serrate. Stipules are linear and pubescent. Petiole is around 1.2cm in length. Flowers are dioecious, in axillary cymes. Sepals and petals are 5 in number. Petals are free, white and imbricate. Stamens are 5 in number, ovary is densely pubescent and stigma is 5-lobed. Fruit is an indehiscent berry with a hard rind, globose, hard, brown tomentose and contain many seeds having an oily endosperm. The plant is common in forests. Seeds yield an oil (hydnocarpus oil, chaulmoogra oil) which is used in the treatment of skin diseases such as leprosy [29][30][31][32].

The plant *H. pentandra* is a medicinal plant and is traditionally used for treatment of various ailments or diseases. In certain areas of Aurangabad district of Maharashtra state of India, the paste of seeds is used for treatment of eczema, white patches, itching and infection. The oil is used in skin problems and mixture of oil and lemon juice is applied on burned skin and leprosy [33]. The seed and seed oil of *H. pentandra* are used traditionally to treat ailments such as leprosy, skin diseases, eczema, dermatitis, tubercular laryngitis, chronic ulcers, dyspepsia, flatulence and verminosis by local people of sacred groves of Trissur district, Kerala, India [34]. The plant is reported to exhibit bioactivities such as larvicidal [19], antioxidant [35], antibacterial [36] and anticancer [28][37] activity. The present study was performed to investigate antimicrobial, insecticidal and antiradical potential of methanol extract of leaves of *H. pentandra*.

Materials and methods

Chemicals and media

Culture media viz. Nutrient agar, Nutrient broth and Potato dextrose agar were purchased from HiMedia, Mumbai. Chemicals viz. DPPH and ABTS were obtained from Sigma Chemical Co., USA. Other chemicals namely methanol, ascorbic acid, potassium persulfate, dimethyl sulfoxide (DMSO) and chloramphenicol were purchased from Hi Media, Mumbai.

Plant material

The plant was collected near Shiralakoppa, Shivamogga district, Karnataka during February 2017. The plant was authenticated by Dr. Vinayaka K.S, Assistant Professor, KFGC, Shikaripura, and Karnataka on the basis of its floral and other characteristics.

Extraction and phytochemical analysis

Maceration process was employed for extraction. Leaves were separated, washed using clean water, dried under shade and powdered in a blender. 10g of leaf powder was transferred into a stopper container and 100ml of methanol (extraction solvent) was added. The container was left for 48 hours at room temperature and stirred occasionally. The content was filtered through muslin cloth and Whatman filter paper No. 1, re-extraction was done twice

and the pooled filtrates were evaporated to dryness [2],[35],[38]. The crude methanol extract was subjected to standard phytochemical tests to detect phytochemicals namely alkaloids, flavonoids, tannins, saponins, triterpenes, glycosides and sterols [39][40].

Antibacterial activity of leaf extract

Agar well diffusion assay was carried out to investigate antibacterial potential of leaf extract. 24 hours old Nutrient broth cultures of test bacteria (Gram positive bacteria - *Staphylococcus aureus* NCIM 5345, *Staphylococcus epidermidis* NCIM 2493, *Bacillus subtilis* NCIM 2063 and *Bacillus cereus* NCIM 2016; Gram negative bacteria - *Escherichia coli* NCIM 2065, *Pseudomonas aeruginosa* NCIM 2200 and *Salmonella typhimurium* NCIM 2501. The bacteria were procured from NCL, Pune) were inoculated on the surface of sterile Nutrient agar plates using sterile cotton swabs. With the help of a sterile cork borer, wells of 8mm diameter were punched in the inoculated` plates and labeled. To respective wells, leaf extract (20mg/ml of DMSO), chloramphenicol (1mg/ml of sterile distilled water) and DMSO were transferred. The plates were incubated in upright position for 24 hours at 37ºC and the zones of inhibition formed were measured [38][41].

Antifungal activity of leaf extract

Poisoned food method was carried out to evaluate antifungal potential of leaf extract against three seed-borne fungi viz. *Curvularia* sp., *Alternaria* sp. and *Fusarium* sp. the test fungi were inoculated at the centre of control (without extract) and poisoned potato dextrose agar (0.5mg extract/ml of medium) plates aseptically followed by incubating plates in upright position for 5 days at room temperature. The diameter of fungal colonies was measured in mutual perpendicular directions using a ruler. The antifungal potential of leaf extract (in terms of inhibition of mycelial growth) was determined using the formula:
Inhibition of mycelial growth (%) = (Dc – Dt / Dc) x 100, where 'Dc' refers to colony diameter in control plate and 'Dt' refers to colony diameter in poisoned plates [42][43].

Insecticidal activity of leaf extract

The insecticidal potential of leaf extract was assessed, in terms of larvicidal effect, against II and IV instar larvae of *A. aegypti*. In brief, 25 larvae were transferred into 50ml of sterile dechlorinated water with different concentrations of leaf extract (0.0, 0.25, 0.50, 1.0 and 2.0mg/ml). The flaks were kept undisturbed for 24 hours. The number of dead larvae (the larvae which failed to move even after probing with a needle) was counted and the mortality of larvae (%) was calculated using the formula:

Mortality (%) = [number of dead larvae / total number of larvae] x 100 [15][18]. LC_{50} value was calculated. LC_{50} value indicates the concentration of extract required to cause 50% mortality of larvae.

Antiradical activity of leaf extract

DPPH assay

In clean and labeled tubes, 3ml of DPPH radical solution was mixed with 1ml of various concentrations viz. 3.125 to 100µg/ml of leaf extract and ascorbic acid (reference antioxidant). The tubes were incubated at room temperature in dark for 30 minutes followed by measuring absorbance of content of each tube at 517nm spectrophotometrically. A mixture of 1ml methanol and 3ml DPPH radical solution was taken as control. Radical scavenging potential of each concentration of extract and ascorbic acid was calculated using the formula:
Radical scavenging activity (%) = (Ac – At / Ac) x 100, where 'Ac' and 'At' refers to absorbance of DPPH control and absorbance of DPPH in presence of extract/ascorbic acid. IC_{50} value was calculated which indicates the concentration required to scavenge 50% of free radicals [13][44].

ABTS assay

In clean and labeled tubes, 3ml of ABTS radical solution (generated by mixing and incubating ABTS stock and potassium persulfate) was mixed with 1ml of various concentrations viz. 3.125 to 100µg/ml of leaf extract and ascorbic acid (reference antioxidant). The tubes were incubated at room temperature in dark for 30 minutes followed by measuring absorbance of content of each tube at 730nm spectrophotometrically. A mixture of 1ml methanol and 3ml DPPH radical solution was taken as control. Radical scavenging potential of each concentration of extract and ascorbic acid was calculated using the formula:
Radical scavenging activity (%) = (Ac – At / Ac) x 100, where 'Ac' and 'At' refers to absorbance of ABTS control and absorbance of ABTS in presence of extract/ascorbic acid. IC_{50} value was calculated which indicates the concentration required to scavenge 50% of free radicals [13].

Statistical analysis

All experiments were performed in triplicates and the results are presented as Mean±Standard deviation (S.D). The IC_{50} value (for antiradical activity) and LC_{50} value (for larvicidal activity) were calculated by linear regression analysis.

Results and discussion

The therapeutic potential of plants is due to the presence of a variety of secondary metabolites distributed in various parts of the

plants. Phytochemicals such as alkaloids, polyphenolic compounds and terpenes exhibit various bioactivities such as antimicrobial, antioxidant and anticancer activity. It is very important to screen plant materials for phytochemicals so as to relate the phytochemical and the bioactivity observed [1],[23],[38],[39],[40],[45]. Many methods are employed in the extraction of plant materials. Maceration is a simple and one of the widely used methods for extraction and is applied to obtain essential oils and other bioactive compounds in plants. In maceration, the plant material is dried and powdered to improve the surface area. The dried powder is then added to a suitable solvent and left for several days at ambient temperature with occasional stirrings. The content is pressed and the crude solvent extract is obtained by filtration or decantation.

In the present study, we subjected the powdered leaf material of *H. pentandra* to maceration process for extraction. Methanol was selected as extraction solvent as methanol can dissolve many phytochemicals. Methanol has been used by various researchers and is found to be the best solvent for extraction of many bioactive plant metabolites [1],[8],[47]. Preliminary phytochemical analysis of the leaf extract of *H. pentandra* showed that the extract possess phytochemicals namely alkaloids, tannins, saponins, flavonoids, triterpenes, glycosides and saponins (Table 1). In an earlier study, phytochemicals namely glycosides, flavonoids, tannins, saponins, triterpenoids and steroids were detected in the root extract of *H. pentandra* [30]. The study of David and George [48] which involved HPTLC protocol revealed that the leaf extract of *H. pentandra* is rich in secondary metabolites like alkaloids, essential oils, steroids, triterpenes, flavonoids, flavonoid glycosides, flavonolignans, phenolics, tannins and saponins.

Table 1: Phytoconstituents detected in leaf extract of *H. pentandra*

Phytoconstituents	Leaf extract
Alkaloids	+
Tannins	+
Saponins	+
Flavonoids	+
Triterpenes	+
Glycosides	+
Sterols	+

'+' Present

Antibacterial activity of *H. pentandra*

The result of antibacterial activity of *H. pentandrus* is shown in Table 2. Leaf extract was found to inhibit all test bacteria. Among Gram positive bacteria, highest and least inhibitory activity was observed against *B. cereus* and *B. subtilis* respectively. Inhibition of *S. aureus* was more or less similar to *B. cereus*. *P. aeruginosa* was inhibited to higher extent while *E. coli* exhibited least susceptibility among Gram negative bacteria. Reference antibiotic exhibited stronger antibacterial activity with high activity against Gram positive bacteria. No inhibitory activity was displayed by DMSO. The inhibitory activity of leaf extract was maximum against Gram positive bacteria when compared to Gram negative bacteria. Similar observations were made in an earlier study by Shirona et al. [36] where the methanol extract of leaf *H. pentadra* inhibited *B. subtilis* to higher extent when compared to *E. coli*. The lower susceptibility of Gram negative bacteria to leaf extract could be due to the presence of an outer membrane (exterior to peptidoglycan layer) which acts as an additional barrier to the entry of extract.

Table 2: Antibacterial activity of leaf extract of *H. pentandra*

Test bacteria	Zone of inhibition in cm (Mean±S.D; n=3)		
	Leaf extract	Antibiotic	DMSO
S. aureus	1.80±0.00	3.30±0.10	0.00±0.00
S. epidermidis	1.56±0.05	3.73±0.050	0.00±0.00
B. subtilis	1.40±0.00	3.30±0.10	0.00±0.00
B. cereus	1.86±0.05	3.60±0.00	0.00±0.00
S. typhimurium	1.30±0.10	2.10±0.00	0.00±0.00
P. aeruginosa	1.40±0.00	2.76±0.05	0.00±0.00
E. coli	1.06±0.05	2.53±0.05	0.00±0.00

Antifungal activity of *H. pentandra*

Table 3 shows the result of antifungal potential of leaf extract against seed-borne fungi. The extract was effective in causing inhibitory activity against all test fungi as revealed by considerable reduction in the mycelial growth of fungi in poisoned plates when compared to control plates. Among fungi, highest and least susceptibility to extract was observed in case of *Curvularia* sp. (53.64% inhibition) and *Alternaria* sp. (35.08% inhibition) respectively. Extent of inhibition of *Fusarium* sp. by extract was 45.81%. In an earlier study, the leaf extract of *H. pentandra* was shown to exhibit inhibitory activity against *Candida tropicalis* [49].

Table 3: Antifungal activity of leaf extract of *H. pentandra*

Test fungi	Colony diameter in cm (Mean±S.D; n=3)	
	Control	Leaf extract
Curvularia sp.	4.53±0.05	2.10±0.00
Alternaria sp.	5.13±0.11	3.33±0.05
Fusarium sp.	4.30±0.00	2.33±0.11

Insecticidal activity of *H. pentandra*

The result of larvicidal potential of leaf extract is shown in Table 4. The extract was found to exhibit dose dependent mortality of II and IV instar larvae of *A. aegypti*. II instar larvae were more susceptible to leaf extract when compared to IV instar larvae. All concentrations of extract (0.25-2.0mg/ml) were effective in causing mortality of II instar larvae while IV instar larvae were not affect by extract concentration 0.25mg/ml. A mortality of >50% of II and IV instar larvae was observed at extract concentration 1mg/ml and 2mg/ml respectively. The LC_{50} value of extract was 0.79mg/ml and 1.37mg/ml respectively for II instar and IV instar larvae. In an

earlier study, Sivaraman et al. [19] observed larvicidal potential of various solvent extracts of seeds of *H. pentandra* against *A. aegypti* and *Culex quinquefasciatus*. Chloroform extract was found to exhibit marked larvicidal activity. In another study, a fraction consisting of hydnocarpic acid and chaulmoogric acid from hexane extract of *H. pentandra* seed was shown to exhibit antifeedant activity against *Helicoverpa armigera* [50].

Table 4: Larvicidal activity of leaf extract against II and IV instar larvae

Concentration (mg/ml)	Mortality of larvae (%)	
	II instar larvae	IV instar larvae
0.00	00.00±00.00	00.00±00.00
0.25	10.00±00.00	00.00±00.00
0.50	36.66±05.77	20.00±00.00
1.00	60.00±00.00	36.66±05.77
2.00	96.66±05.77	70.00±10.00

DPPH radical scavenging activity of *H. pentandra*

One of the most widely used in vitro antiradical assays is DPPH assay. This method uses an organic nitrogen centred free radical called DPPH. In alcoholic solution, DPPH exhibits absorption maxima at 517nm and is purple in color. Substances (antioxidants) having the potential to donate hydrogen will convert purple colored DPPH radical into yellow colored non-radical form DPPHH (diphenylpicryl hydrazine). The assay is simple, rapid and the results obtained are reproducible. The assay has been widely used to evaluate antiradical activity of plants and their metabolites [13],[38],[51][52][53][54][55]. In the present study, the leaf extract was shown to exhibit concentration dependent scavenging of DPPH radicals with an IC_{50} value of 13.91µg/ml. A scavenging of >50% was observed at extract concentration 25µg/ml and higher (Figure 1). Based on the IC_{50} values, the DPPH radical scavenging potential of leaf extract (13.91µg/ml) in this study was higher than that of the result of DPPH radical scavenging potential by leaf extract (>20µg/ml) of the study carried out by George et al. [28]. The study of Krishnan et al. [35] showed marked scavenging of DPPH radicals by ethyl acetate extract when compared to methanol extract of *H. pentandra*. In another study, Shirona et al. [36] showed dose dependent scavenging of DPPH radicals by methanolic extract of leaves. In the present study, the scavenging potential of leaf extract of *H. pentandra* observed was lower than that of ascorbic acid (IC_{50} value 3.06µg/ml). It is evident from the study that the leaf extract possess hydrogen donating ability and therefore it can act as a free radical scavenger.

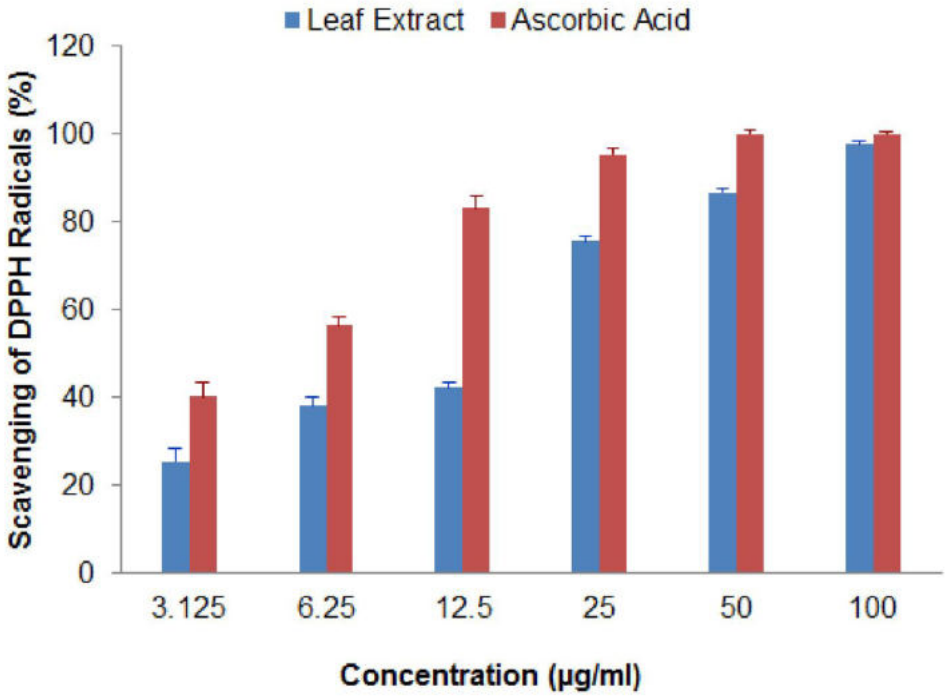

Figure 1: Scavenging of DPPH radicals by leaf extract and ascorbic acid

ABTS radical scavenging activity of *H. pentandra*

Unlike DPPH assay, the assay involving scavenging of ABTS radicals requires generation of ABTS radicals. It is done by reacting

ABTS stock (7mM) and an oxidizing agent such as potassium persulfate or potassium permanganate (2.45mM). The resulting blue-green ABTS radical solution is used for assay. Substances capable of donating electrons reduce the colored radical solution to colorless neutral form which is evidenced by suppression of characteristic long wavelength absorption spectrum. The assay is widely used to evaluate antiradical activity of various kinds of specimens including plant extracts [13],[46],[52],[54],[56],[57]. In the present study, the leaf extract exhibited dose dependent scavenging of ABTS radicals with an IC_{50} value of 6.03µg/ml. A scavenging of >50% was observed at extract concentration 6.25µg/ml and higher (Figure 2). Based on the IC_{50} values, the ABTS radical scavenging potential of leaf extract (6.03µg/ml) in this study was higher than that of the result of ABTS radical scavenging potential by leaf extract (>30µg/ml) of the study carried out by George et al. [28]. In the present study, the scavenging potential of leaf extract of *H. pentandra* observed was lower than that of ascorbic acid (IC_{50} value 2.48µg/ml). It is clear from the result of the study that the leaf extract possess electron donating potential and therefore the extract can act as a free radical scavenger.

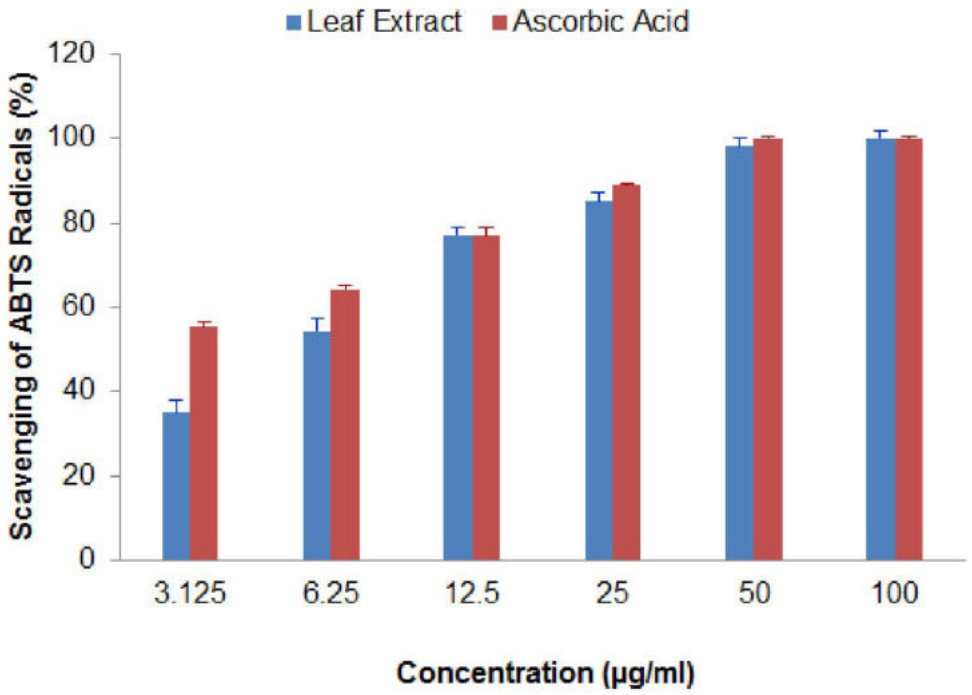

Figure 2: Scavenging of ABTS radicals by leaf extract and ascorbic acid

Conclusions

Plants have been extensively used as resources for developing therapeutic agents. In the present study, the leaf extract of *H. pentandra* is shown to exhibit antimicrobial, antiradical and insecticidal activity. The plant can be used in the treatment of microbial infections, oxidative damage and to control mosquito vectors. The observed bioactivities could be attributed to the phytochemicals such as alkaloids, flavonoids, saponins and others that are detected in the leaf extract. Further studies on purification and characterization of components and their bioactivity determination are to be carried out.

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Conflicts of Interest

Authors declared no conflicts of interest.

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