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Original Article

PHYTOCHEMICAL, ANTIBACTERIAL AND ANTIOXIDANT STUDIES ON LEAF EXTRACTS OF *PIPER BETLE* L.

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ABSTRACT

Objective: In the present research, a clear systematic investigation of antibacterial and antioxidant potential of ambadi variety of *P. betle* of Mysore region has been carried out.

Methods: Antibacterial activity of aqueous and solvent extracts of *Piper betle* L. was evaluated by agar cup diffusion and disc diffusion methods against some common pathogenic bacteria. The antioxidant property of all the solvent extracts were evaluated by diphenyl picryl hydrazyl (DPPH), nitric oxide (NO) and hydrogen peroxide scavenging assay. The IC_{50} values were calculated and compared with standard BHT and AA.

Results: The results indicated that methanol and chloroform extracts of this variety recorded significant antibacterial activity and all the extracts recorded good radical scavenging capacity. Phytochemical analysis revealed that phenols, tannins, flavanoids, glycosides and proteins are present in aqueous, methanol and petroleum ether extracts.

Conclusion: The results obtained in this study clearly indicate that *P. betle* extracts has a significant potential to use as antibacterial agent. The free radical scavenging capability by DPPH was better and IC_{50} value found was lesser than the standard used for all the extracts, which suggest that *P. betle* extracts have potent antioxidant activity. The solvent extracts are effective against free radical mediated diseases

Keywords: *P. betle*, Antibacterial, Antioxidant, Minimum inhibitory concentration.

INTRODUCTION

Infectious diseases are a significant cause of morbidity and mortality worldwide accounting for approximately 50 % of all deaths in the world especially in developing countries [1]. Despite the progress made in understanding the biology and control of pathogenic microorganisms sporadic incidents of epidemics have emerged due to drug resistant microbes posing an enormous threat to public health.

The alarming increase in the rate of infectious diseases with antibiotic resistant microorganisms and side effects of some synthetic antibiotics has led to an increasing interest towards medicinal plants as a natural alternate to synthetic drugs [2]. Although most antibiotics are still active, the rapid progression of resistance suggests that many of these drugs may not be effective for much longer. Research and development of new therapies for treating bacterial infections are of immediate need.

The natural products such as probiotics, enzymes and secondary metabolites of plants are in great demand owing to their extensive biological properties and bioactive components which have proved to be useful against large number of diseases. Raskin *et al.* [3] reported that rediscovery of the connection between plants and health is responsible for launching the new generation of botanical therapeutics, multi component botanical drugs, dietary supplements and functional foods. WHO is encouraging, promoting and facilitating the effective use of herbal medicines in developing countries for health programmes. There are literally thousands of published scientific papers around the globe on herbal drugs. A search of Pubmed database produce approximately 1360 reports in journals and also a search of Napralt database largest database of world in herbal drugs 6550 species have been published experimental antimicrobial activity of which 4000 species had ethno medical data [4]. Plant derived natural products have provided many novel prototype bioactive molecules some of which have led to production of important drugs that are available in the market today.

The free radicals are chemical agents such as O_2, OH, RO, ROO, H_2O_2 produced in the living body during the chemical reaction that contribute to the development and cellular life [5]. Production of free radicals and other reactive species in cells and body tissues has been linked to ageing and several diseases in human being [6]. Antioxidants play an important role in scavenging free radicals and/or chain breaking of the oxidation reaction chemically. Based on growing interest in free radical biology and lack of effective therapies for most chronic diseases, the expediency of antioxidants in protection against these diseases is defensible.

Several synthetic compounds such as butylated hydroxyanisole (BHA) and butylated hydroxyl toluene (BHT) are used as antioxidants, but the use of BHA and BHT is proved to be toxic and carcinogenic [7]. Hence, naturally occurring antioxidants are capable of protecting the human body from diseases by preventing damage of critical bio-molecules and food spoilage and retarding oxidative rancidity of lipids [8]. Several plant extracts, natural foods, vitamin C and E, β-carotene and tocopherol and phytochemicals such as flavanoids, isoflavones, phenolic flavones, anthocyanins, coumarins and lignans are known to possess antioxidant property [9].

The antimicrobial potential of botanicals and benefits of natural antioxidants has encouraged the authors to scientifically validate the antibacterial and antioxidant potential of a local Ambadi variety of P. betle belonging to family Piperaceae. P. betle is a perennial dioceious, semi woody climber, nodes swollen, papillose when young, glabrous at maturity, leaves alternate, simple, bright green in color, berries are rarely produced, cocresent into a fleshy spadix. The leaves have a strong pungent aromatic flavor and are widely used as masticators in Asia [10]. It is popularly known as "Pan" or "Veelayadhele" in Kannada and "betle vine" or betle pepper in English.

Ethno pharmacological data records that it is a stimulant, expectorant, relives gastrointestinal disorders, stomachic, used for rheumatic bone pains and bronchial asthma. In Philippines, fresh crushed leaves are used as antiseptic for cuts and wounds. Its oil is used in China as the counter irritant in swellings, bruises, painful sores and enlarged glands. Its juice along with honey or a liquid extract is useful to treat coughs, dyspnoea and indigestion among

children. In India, leaves are smeared with oil and applied on the breast to promote milk secretion in lactating women, eliminates bad breath, prevents tooth decay, and used to treats vaginal discharge and stop bleeding in the nose [11].

Various pharmacological activities of *P. betle* such as antifungal [12], gastro protective [13], wound healing [14], hepato protective [15], antioxidant [16, 17], anti inflammatory [18], anti-malarial [19], antiallergic [20], antidiabetic activity and antifertility on male rats [21] have been reported.

Reports are available on the phytochemistry, antimicrobial and antioxidant potential of *P. betle* cultivated in Tamilnadu, West Bengal and others parts of India [22-24]. The Agro climatic conditions influence the production of secondary metabolites or phyto constituents considerably in the plants Mysore is well known for the cultivation and consumption of four varieties of *P. betle* of which Ambadi is one of the most popular varieties. The ambadi variety of *P. betle* in Mysore region has not been evaluated for antibacterial and antioxidant potentiality. The characteristic feature of ambadi var. of *P. betle* leaves are deep green, medium sized, coarse in texture, pungent with good keeping quality [10]. In the present study, authors have evaluated *in-vitro* antibacterial and antioxidant potential of this variety of *P. betle*.

MATERIALS AND METHODS

Plant material

Healthy leaves of *P. betle* var. ambadi was collected from Mysore, Karnataka and used for the preparation of aqueous and different solvent extracts. The plant and variety was identified by the taxonomists of the Department of Studies in Botany, University of Mysore and local cultivars. A voucher specimen of the plant has been deposited in the Herbarium, Department of Studies in Botany, University of Mysore, Mysore. Karnataka, India.

Test pathogens

Authentic cultures of human pathogenic bacteria viz., *E. coli* (MTCC 7410), *Kleb. pneumoniae* (MTCC 7407), *B. subtilis* (MTCC 121), *B. cereus* (MTCC 1272), *Salm. typhi* (MTCC 733), *Ent. aerogenes* (MTCC 7325) and *Staph. aureus* (MTCC 7443) obtained from Microbial Type Culture Collection, Chandigarh, India-which served as test bacteria. All the bacterial cultures were maintained in nutrient agar slants with periodic sub-culture.

Preparation of extracts

Aqueous extract

A fresh mature leaf of *P. betle* (50g) was macerated with 50 ml sterile distilled water in a Waring blender (Waring International, New Hartford, CT, USA) for 10 min. The macerate was first filtered through double layered muslin cloth and then centrifuged at 4000 rpm for 10 min. The supernatant was filtered through Whatman No. 1 filter paper and sterilized at 120 °C for 10 min. The extract was deep freezed and lyophilized. The concentrated extract was preserved at 5 °C in an airtight bottle until use.

Solvent extract

Mature leaves were washed thoroughly with water, shade dried and powdered with the help of waring blender. 50 g of the powder was filled in a thimble and extracted successively with increasing order of polarity i.e., with petroleum ether, chloroform, ethyl acetate and methanol in a Soxhlet extractor for 48 h. The extracts were concentrated using rotary flash evaporator and preserved at 5 °C in an airtight bottle until further use. All the extracts were tested for antibacterial and antioxidant activity.

Antibacterial activity

Agar cup diffusion assay

Antibacterial activity of aqueous extract and solvent extracts was determined by agar cup diffusion [25] and disc diffusion methods [26]. Cups were made in plates containing 20 ml of nutrient agar media using sterile cork borer (6 mm) and inoculum containing 10^6 CFU/ml of bacteria were spread uniformly over the solid media with a sterile swab moistened with the bacterial suspension. The dried aqueous and solvent extracts were reconstituted respectively in sterile distilled water and methanol to a concentration of 100 mg/ml. Aqueous and solvent extracts of 100 µl were placed in the cups made in the inoculated plates. Also, 100 µl of sterilized distilled water and methanol were placed in the cups separately which served as negative control and 100 µl of antibiotic containing 100 µg streptomycin (streptomycin sulfate IP; 1 mg/ml) served as positive control. The plates were incubated for 24 h at 37 ° C and zone of inhibition if any around the cups were measured in mm. For each treatment four replicates were maintained and repeated twice.

Disc diffusion assay

The disc diffusion method was carried out according to NCCLS protocol. One hundred (100 µl) aqueous and solvent extracts were loaded to sterile discs of 6 mm and placed on inoculated nutrient agar in the petri plates, also sterile discs loaded with 100 µl of sterilized distilled water and methanol served as negative control. Antibiotic disc of streptomycin (20 µg) (streptomycin sulfate IP) served as positive control. The plates were incubated for 24 h at 37 °C and zones of inhibition if any around the discs were measured in mm. For each treatment four replicates were maintained and repeated twice.

Minimum inhibitory concentration (MIC)

MIC was determined in 96-well flat bottom microtiter plates based on micro dilution assay which is an automated turbidometric and colorimetric method as described by Das *et al.* [27]. Inoculum of the test bacteria was prepared from 24 h old bacterial cultures in sterile/saline water and turbidity of the suspension was adjusted to 0.5 McFarland. The crude extracts of methanol, ethyl acetate and chloroform were diluted to the concentration of 100 mg/ml which served as the stock solution. The 96-well plates were filled with 200 µl of nutrient broth and 100 µl of the extract to the first well. A two-fold serial dilution was made along the rows and final concentrations were 5 to 0.019 mg/ml. A 10 µl inoculum of each test bacteria was added to each well.

The wells containing nutrient broth with inoculum and solvent served as negative control. The plates were incubated at 37 °C for 24 h and the optical density was measured at 620 nm using micro plate reader (LT4000, LABTECH Instruments, UK). The lowest concentration that inhibited visible growth of the bacteria was recorded as the MIC based on the optical density.

The MIC was also confirmed by adding 10 µl of TTC (2, 3, 5-triphenyl tetrazolium chloride, Sigma) dissolved in sterile distilled water (TTC 2 mg/ml) to each well and incubated at 37 °C for 30 min in the dark [28]. Viable organisms reduced the dye to pink color compound. The lowest concentration at which the color change occurred was taken as the MIC and tests were repeated in triplicate.

Antioxidant assay

Antioxidant capacity of different extracts of *P. betle* was estimated by scavenging diphenyl picryl hydrazyl (DPPH), nitric oxide (NO) and hydrogen peroxide.

DPPH radical scavenging assay

The free radical-scavenging assay of different extracts was measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH described by Blois method [29]. Stock solutions of extracts (0.001 g/ml) were prepared in DMSO. Different concentrations (20, 40, 60, 80 and 100 µg) of test solutions were prepared from stock and made up to 2 ml with methanol. Solution of DPPH (0.1 mmol) in methanol was prepared and 1 ml of this solution was added to each of the above test solutions. The mixture was shaken vigorously and incubated for 30 min and the absorbance of each test solution was measured at 517 nm. All the tests were run in triplicate and expressed as the mean±standard deviation (SD). Ascorbic acid (AA) was used as a standard or positive control and DMSO was used as negative control. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH}^{\cdot} \text{ scavenging effect (\%)} = \left[\left(\text{Ac} - \text{Ab} \middle| \text{Ac}\right) \times 100\right]$$

Where, A_c is absorbance of the negative control, A_b is the absorbance of the test solution.

Hydroxyl radical scavenging assay

The ability of the compounds to effectively scavenge hydrogen peroxide was determined according to the method of Jayabharathi *et al.* [30] and it was compared with that of butylated hydroxyanisole (BHA) as the standard. The hydroxyl radicals ($OH^{\cdot}$) in aqueous media were generated through the Fenton system. The test solutions of the different extracts were prepared with DMSO (0.001g ml^{-1}). A volume of 5 ml assay mixture containing the following reagents: safranin (11.4 µ mol), EDTA–Fe (II) (40 µmol), H_2O_2 (1.76 µmol), the test solution [4, 8, 12, 16 and 20 µl (5 mg/ml)] and a phosphate buffer (0.067 mol, pH 7.4) was prepared and incubated at 37 °C for 30 min in a water bath and the absorbance was measured at 520 nm. BHA which suppressed hydroxyl radical was used as positive control. All the tests were done in triplicate and expressed as the mean±standard deviation (SD). The suppression ratio for $OH^{\cdot}$ was calculated by the following equation:

$$\text{Suppression ratio (\%)} = \left[\left(\text{Ao} - \text{Ai} \middle| \text{Ao}\right) \times 100\right]$$

Where A_0 = the absorbance of the control; A_i = absorbance of the test solution.

Nitric oxide scavenging assay

Nitric oxide radical scavenging assay was performed according to literature method [31]. The assay was based on the generation of nitric oxide (NO) from sodium nitroprusside (SNP) and it was measured by the Griess reagent. At physiological pH, aqueous solution of sodium nitroprusside spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions. The generated nitrite ion was quantified by the Griess reagent. A volume of 1.5 ml (10 mM) sodium nitroprusside in phosphate buffer saline (pH 7.4) was mixed with various concentrations (20, 40, 60, 80 and 100 µg) of 1 ml extract and the mixture was incubated at 25 °C for 150 min. After incubation, the formed nitric oxide reacts with 1.5 ml Griess reagent (1 % sulfanilamide, 2 % phosphoric acid and 0.1 % naphthyl ethylene diamine dihydrochloride). The reaction mixture was allowed for 30 min at room temperature. The test solution (4 ml) was made-up with the phosphate buffer (pH 7.4). The absorbance of the reaction mixture was measured at 546 nm against the appropriate blank. All the samples and controls were determined in triplicates. The scavenging percentage was determined using the following equation;

$$\text{Nitric oxide}^{\cdot} \text{ scavenging effect (\%)} = \left[\left(\text{Ac} - \text{At} \middle| \text{Ac}\right) \times 100\right]$$

Where, A_c= absorbance of control and A_t= absorbance of sample mixture with extract.

The antioxidant ability of the extracts is expressed as 50 % inhibitory concentration (IC_{50}) in µg/ml.

Phytochemical analysis

Phytochemical analyses of aqueous and solvent extracts were carried out for the detection of secondary metabolites such as tannins, alkaloids, flavanoids, terpenoids, steroids, carbohydrates, proteins and saponins. The dried extracts were reconstituted in methanol and 1 ml of each extract was subjected to standard phytochemical analysis [32].

Statistical analysis

Statistical calculations were carried out using one way ANOVA and the significance of the differences between means were calculated using Tukey's HSD multiple range test under the significance level of $P<0.05$.

RESULTS AND DISCUSSION

RESULTS

Antibacterial activity

The result of antibacterial activity of different extracts of *P. betle* by agar cup diffusion method is presented in table 1. Aqueous extract did not show activity against any of the test bacteria. All solvent extracts showed activity, but inhibition zone varied among the solvent extracts. Methanol extract recorded highest zone of inhibition. Petroleum ether extract recorded least inhibition zone against all the tested bacteria and no activity was recorded against *B. cereus* and *Kleb. pneumoniae*. Higher inhibition zone of 21-30 mm was recorded in methanol extract. *B. subtilis* was highly susceptible with maximum inhibition zone of 30 mm in methanol extract and least zone of inhibition of 21 mm was recorded against *Ent. aerogenes.* Chloroform and ethyl acetate extracts recorded a moderate inhibition zone ranging from 18-22 mm and 18-20 mm, respectively. *E. coli* was highly susceptible to chloroform and ethyl acetate extracts with the inhibition zone of 22.75 mm and 20.25 mm, respectively. *Kleb. pneumoniae* and *Staph. aureus* also recorded highest inhibition zone of 20.25 mm in ethyl acetate extract. *B. subtilis* and *Salm. typhi* recorded least inhibition zone of 18.25 mm in both chloroform and ethyl acetate extracts. Petroleum ether extract recorded a lower inhibition range of 10-14 mm against all the tested bacteria.

Table 1: Antibacterial activity of different extracts of *P. betle* against human pathogenic bacteria by agar cup diffusion method (in mm). Each value represents mean±SD of four independent replicates

Test bacteria	Aqueous	Pet. Ether	Chloroform	Ethyl acetate	Methanol	Streptomycin
B. cereus	0.00	0.00	19.75 ± 0.27^{b}	18.75 ± 0.62^{abc}	25.25 ± 2.25^{bc}	21.0 ± 0.40^{bc}
B. subtilis	0.00	10.0 ± 0.00^{c}	18.25 ± 0.62^{b}	18.0 ± 0.57^{c}	30.25 ± 0.25^{a}	20.75 ± 0.47^{bc}
E. coli	0.00	12.25 ± 1.03^{b}	22.75 ± 0.47^{a}	20.25 ± 0.25^{ab}	25.5 ± 0.28^{bc}	23.75 ± 0.47^{a}
Ent. Aerogenes	0.00	14.25 ± 0.25^{a}	19.75 ± 0.47^{b}	19.5 ± 0.28^{abc}	21.5 ± 0.28^{c}	21.25 ± 0.62^{bc}
Kleb. pneumoniae	0.00	0.00±0.0	20.25 ± 0.62^{b}	20.0 ± 0.40^{abc}	25.75 ± 0.25^{bc}	19.75 ± 0.62^{c}
Salm. Typhi	0.00	10.25 ± 0.25^{c}	18.25 ± 0.47^{b}	18.25 ± 0.62^{bc}	28.50 ± 0.86^{ab}	22.5 ± 0.28^{ab}
Staph. aureus	0.00	10.5 ± 0.28^{bc}	18.5 ± 0.28^{b}	20.5 ± 0.28^{a}	25.25 ± 0.47^{bc}	19.25±0.47c

Fig. followed by different letters in columns differ significantly when subjected to Tukey's HSD ($P<0.05$).

Table 2: Antibacterial activity of different extracts of *P. betle* against human pathogenic bacteria by disc diffusion method. Each value represents mean±SD of four independent replicates

Test bacteria	Aqueous	Pet. Ether	Chloroform	Ethyl acetate	Methanol	streptomycin
B. cereus	0.00	0.00	14.5 ± 0.28^{c}	13.25 ± 0.25^{bc}	23.75 ± 0.47^{cd}	21.0 ± 0.40^{bc}
B. subtilis	0.00	10.0 ± 0.00^{b}	15.00 ± 0.00^{c}	11.75 ± 0.25^{cd}	27.25 ± 0.94^{a}	20.75 ± 0.47^{bc}
E. coli	0.00	13.25 ± 0.28^{a}	18.25 ± 0.25^{a}	18.75 ± 0.47^{a}	23.5 ± 0.28^{abc}	23.75 ± 0.47^{a}
Ent. aerogenes	0.00	14.25 ± 0.25^{a}	16.75 ± 0.25^{b}	14.25 ± 0.25^{b}	18.75±0.47a	21.25 ± 0.62^{bc}
Kleb. pneumoniae	0.00	10.00±0.00	18.5 ± 0.28^{a}	17.5 ± 0.28^{a}	25.0 ± 0.40^{abc}	19.75 ± 0.62^{c}
Salm. typhi	0.00	10.0 ± 0.25^{b}	14.25 ± 0.25^{c}	11.5 ± 0.28^{de}	26.25 ± 0.47^{ab}	22.5 ± 0.28^{ab}
Staph. aureus	0.00	10.0±0.00	14.25 ± 0.25^{c}	17.5 ± 0.28^{a}	24.75 ± 0.40^{bc}	19.25 ± 0.47^{c}

Fig. followed by different letters in columns differ significantly when subjected to Tukey ($P<0.05$).

The results of antibacterial activity of different extracts of *P. betle* by disc diffusion method are presented in table 2. All the solvent extracts recorded inhibitory activity against the tested bacteria, but the zone of inhibition was slightly lesser than the zones produced in agar cup diffusion method. The highest inhibition zone was recorded in methanol extract that ranged between 18 to 27 mm and the least zone of inhibition was recorded by petroleum ether extract that ranged between 10 to14.25 mm. Chloroform and ethyl acetate recorded moderate activity with the inhibition zone of 14.25 to18.25 mm and 11.50-18.75 mm, respectively. The standard antibiotic streptomycin recorded inhibition in the range of 19.25 and 23.75 mm which was lesser than that recorded by methanol extract.

The minimum inhibitory concentration (MIC) was determined for methanol, chloroform and ethyl acetate extract as they recorded good antibacterial activity against the test bacteria and the results are presented in table 3. The MIC of methanol extract against the test bacteria was in the range of 0.208-0.312 mg/ml and that of chloroform extract was 0.130-0.416 mg/ml. The MIC results indicate that minute quantity (0.13 mg/ml) of the extracts is sufficient to inhibit some bacteria. Lowest MIC value of ethyl acetate and chloroform extracts was 0.130 mg/ml against *B. subtilis* and *Ent. aerogenes,* respectively. Highest MIC value of 0.312 mg/ml of methanol extract and 0.416 mg/ml of ethyl acetate and chloroform extracts was against *B. cereus*.

Table 3: **MIC of Methanol, Chloroform and E. Acetate extract of *P. betle* against Human pathogenic bacteria (mg/ml).**

Extracts (mg/ml)	Test bacteria						
	B. cereus	***B. subtilis***	***E. coli***	***Ent. aerogenes***	***Kleb. pneumoniae***	***Salm. typhi***	***Staph. aureus***
Methanol	0.312	0.208	0.232	0.208	0.260	0.260	0.312
Ethyl acetate	0.416	0.182	0.242	0.130	0.364	0.208	0.234
Chloroform	0.416	0.169	0.199	0.130	0.312	0.150	0.208

Antioxidant activity

The results of antioxidant activity of different extracts of *P. betle* in different assay are presented in table 4.

DPPH radical scavenging assay

The free radical scavenging ability of extracts was evaluated by the change in absorbance by reduced DPPH. The ability of the extracts to neutralize hydroxyl radical was expressed as 50% inhibitory concentration (IC_{50}) in µg/ml. The results of DPPH showed that the methanol extract of *P. betle* was most active with an IC_{50} of 17±0.24 µg/ml, followed by ethyl acetate, chloroform and petroleum ether with IC_{50} of 18±0.11, 20±0.55, 21±0.36 µg/ml, respectively. All the tested extracts showed higher radical scavenging activity compared to standard ascorbic acid which recorded antioxidant potential at an IC_{50} of 25±0.22 µg/ml.

Hydroxyl radical scavenging assay

The IC_{50} value of the tested extracts was in following order methanol (35±0.60 µg), ethyl acetate (42±0.81 µg), petroleum ether (49±0.07 µg), chloroform (55±0.15 µg), and BHT (24±0.48 µg) indicating moderate activity methanol and ethyl acetate of the extracts compared with synthetic antioxidant BHT.

Nitric oxide scavenging assay

The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extract inhibits nitrite formation by directly competing with oxygen in reaction with NO. The tested extracts of *P. betle* have less potent nitric oxide scavenging activity compared with that of the standard BHT. The extract showed a moderate dose dependent inhibition of nitric oxide with an IC_{50} value in the order of methanol<ethyl acetate<chloroform<petroleum ether. The IC_{50} value of methanol (47±0.26 µg) and ethyl acetate (53±0.05 µg), was slightly higher than the standard BHT (44±0.29 µg).

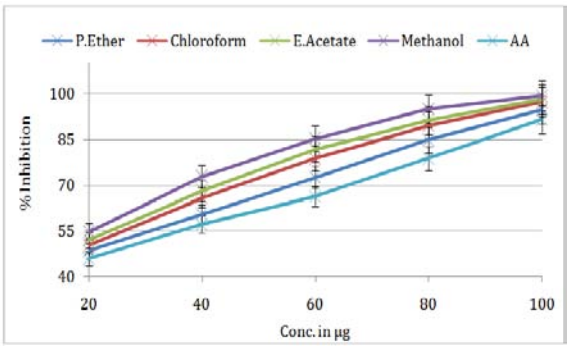

Fig. 1: DPPH free radical scavenging activity of different extracts of *P. betle* and standard Ascorbic Acid at different concentrations

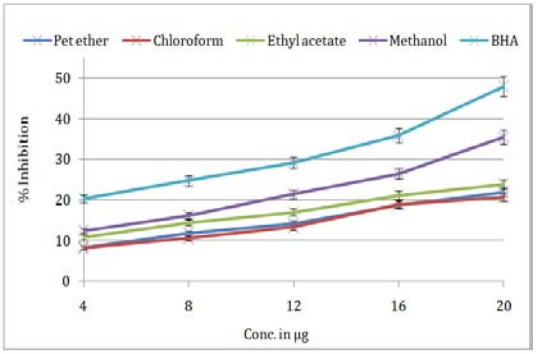

Fig. 2: Hydroxyl radical scavenging activity of different extracts of *P. betle* and standard BHA at different concentrations

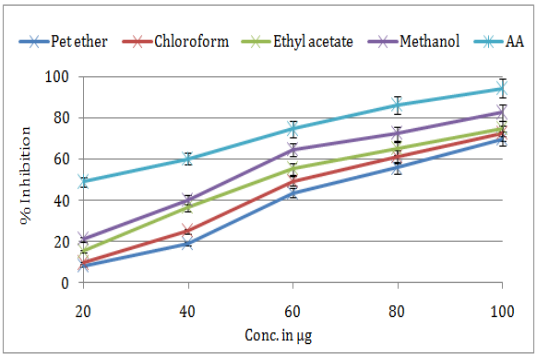

Fig. 3: Scavenging effect of nitric oxide radical of different extracts of *P. betle* and standard AA at different concentrations

The scavenging effect of the tested extracts on different free radicals is concentration related and the suppression ratio increases with the increasing concentration of the sample in all the antioxidant assays (fig. 1-3).

Phytochemical analysis

Phytochemical analysis of aqueous and solvent extracts is presented in table 5. Phenols, tannins, flavanoids, glycosides and proteins were present in aqueous, methanol and petroleum ether extracts. Methanol and petroleum ether extracts also contained steroids. Alkaloids and glycosides were present in chloroform extract. Terpenoids are present only in petroleum ether extract. Saponins were present in aqueous extract only.

Table 4: The IC_{50} values of DPPH radical, hydrogen peroxide and nitric oxide radical scavenging by different extracts of *P. betle*

Solvent extract	IC_{50} (µg/ml)		
	DPPH	H_2O_2	nitric oxide
Petroleum Ether	21±0.23	49±0.10	69±0.17
Chloroform	20±0.40	55±0.30	56±0.05
Ethyl Acetate	18±0.61	42±0.52	53±0.26
Methanol	17±0.13	35±0.22	47±0.44
AA	25±0.34	---	23±0.21
BHA	---	22±0.08	---

Values are mean of three independent replicates±Standard deviation

Table 5: Phytochemical analysis of aqueous and solvent extracts of *P. betle*

Phytochemical groups	Extracts				
	Aqueous	Petroleum ether	Chloroform	Ethyl acetate	Methanol
Alkaloids	-	+	+	-	-
Flavanoids	+	+	-	+	+
Terpenoids	-	-	-	-	-
Tannins	+	+	-	+	+
Steroids	-	+	-	-	+
Glycosides	+	+	+	+	+
Carbohydrates	-	-	-	+	+
Proteins	+	+	+	+	+
Saponins	+	-	-	-	-

+= Present; -= Absent

DISCUSSION

Natural products are both a fundamental source of new chemical diversity and an integral part of today's pharmaceutical compendium. Many currently available antibacterial agents have undesirable toxicity and these drugs have led to rapid development of drug-resistant strains. About 75-80% of the world's population in developing countries mainly depends on herbal medicine for primary health care because of better compatibility with the human body and less side effects [33].

Considering these facts, in the present investigation aqueous and solvent extracts of ambadi variety of *P. betle*, a high yielding and popular variety of Mysore was evaluated for antibacterial activity against a band of pathogenic bacteria know to cause infectious diseases in human beings. The activity of all the extracts was tested by employing both agar cup and disc diffusion method. Among the various extracts, aqueous extract did not showed activity against any of the test bacteria, while all other solvent extracts showed activity with varied zones of inhibition. Among the solvent extracts which showed activity, methanol extract was most potent with maximum inhibition zone ranging between 21 to 30 mm.

The inhibition zone was higher in cup diffusion method which may be due to the maximum diffusion of the extracts into the media, leading to the spread of the phytochemical to a wider diameter recording higher inhibition zone [34]. Methanol extract was the most promising in inhibiting the bacteria, followed by extracts of chloroform and ethyl acetate in both the assays. The inhibition zone recorded by methanol extract (21-30 mm) is higher than that of standard antibiotic streptomycin (19.25-23.75 mm) at tested concentration which speaks of the significance of methanol extract. The lowest MIC of ethyl acetate and chloroform extract was 0.130 mg/ml and highest value was 0.416 mg/ml which indicates that minute quantity of 0.13 mg/ml of the extracts are sufficient to inhibit some important bacteria.

The tested bacteria are associated with the different type of infections such as urinary tract infections, wound infections, gastro enteritis and pneumonia [35]. The significant inhibition of the bacteria associated with these infections by of *P. betle* extracts indicated the presence of the antibacterial compounds.

The literature showed that various extracts of *P. betle* has recorded good antibacterial activity against various human pathogens. Aqueous extract of *P. betle* has shown good activity against dental plaque forming bacteria *Streptococcus sanguins* and *Streptococcucs mitis*, but in the present investigation aqueous extract did not showed any activity against the tested bacteria. Several other workers have reported that the solvent extracts of *P. betle* has good antibacterial potential of which methanol is highly potent followed by ethanol, chloroform and ethyl acetate against many human pathogenic bacteria [23, 36, 37]. The results in the literature and present investigation confirm that methanol extract of ambadi variety of *P. betle* is also a potent extract against pathogenic bacteria.

The antioxidant activity includes free radical scavenging capacity, inhibition of lipid peroxidation, metal ion chelating ability and reducing ability. DPPH was used as substrate to evaluate the free radical scavenging activity of *P. betle* extracts. DPPH radical contains an odd electron which is responsible for the formation of blue color [38]. When DPPH accepts an electron from the extract it gets converted into a colorless diphenyl-picryl hydrazine. The reduced DPPH could be quantified spectrophotometrically at 517 nm. The various extracts of *P. betle* has good scavenging activity with IC_{50} values at a lower concentration of methanol, ethyl acetate, chloroform and petroleum ether extracts, which is lesser than that of standard, this indicates that *P. betle* extracts have better scavenging ability than standard ascorbic acid.

Hydrogen peroxide is a weak oxidizing agent, but sometimes can be toxic to the cell membrane generating hydroxyl radical while entering into the cell and react with iron (II) and copper (II) which is toxic to the cell. By oxidation of this thiol groups (-SH) it can inactivate a few enzymes directly. The oxidation, of (-SH) groups by oxidants may thus lead to disruption of various cellular functions and even lead to cell death [39]. *P. betle* extracts have shown H_2O_2 decomposing activity at a lower concentration of methanol, E. acetate, petroleum ether, chloroform extracts, which was slightly lesser than the standard BHA.

Nitric oxide is a potent plaotropic mediator of physiological process such as the smooth muscle relaxant, neuro signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. The NO reacts with superoxide and gives rise to various other reactive nitrogen species (RNS) such as NO_2, N_2O_4 and peroxy nitrite which attack and damage various cellular molecules [40]. E. acetate and methanol extracts showed scavenging activity at a concentration slightly higher than that of the standard by decreasing the amount of nitrite which is toxic to the cell.

The results of present investigation suggested that the *P. betle* extracts especially ethyl acetate and methanol extracts have moderate to high free radical scavenging activity in all the methods due to the presence of phenols, flavanoids in the extracts which has also been reported by some authors in literature [17, 23, 41]. The majority of antioxidant compounds are phenols, flavanoids, catechins and isocatechins which are evidently present in *P. betle* extracts resulting in antioxidant activity [42]. The free radical scavenging activity of *P. betle* extracts against DPPH was very effective, while slightly effective against hydroxyl and nitric oxide compared to the synthetic antioxidants like BHA and AA.

Phytochemical analysis revealed the presence of phenols, tannins and steroids in most of the extracts which indicated that *P. betle* extracts are phenol rich compounds. The literature showed that *P. betle* leaves are rich in a wide variety of secondary metabolites such as phenols (chavicol, hydroxychavicol), volatile oils (saforle, eugenol, isoeugenol, methyl ester), fatty acids (steraic acid and palmatic) acid which may be responsible for the disruption of cell membrane. cell wall and degradation of cell components leading to the death of bacteria as reported by many researchers [35, 43, 44]. Nalina and Rahim [45] have reported that the sterols present in the *P. betle* extracts interact with the bacterial cell wall and cell membrane leading to the alteration in their primary structure leading to pore formation and degradation of bacterial components. Saleem *et al.* [46] have reported that terpenoids present in the plant extracts may be involved in bacterial membrane disruption and flavanoids of plant extracts form a complex with the extracellular proteins of the bacterial cell wall.

CONCLUSION

In conclusion, the present study a potent antibacterial activity against a band of pathogenic bacteria by *P. betle* extracts especially methanol extract and good scavenging ability of all the extracts leads to scientific validation of the plant. A natural substance which is a part of daily diet nutritional supplement with antimicrobial and antioxidant property constitutes a new source of herbal drug. The antibacterial and antioxidant potential of this plant is a great relevance as *P. betle* is an edible plant and generally consumed and no extra processing is needed for its administration. Further exploration on the isolated element on antimicrobial and antioxidant activity may lead to chemical entities for clinical use.

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CONFLICT OF INTERESTS

Declared None

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