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Comparative Evaluation on the Antibacterial Activity of Karpoori Variety *Piper betle* Leaves against Certain Bacterial Pathogens

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ABSTRACT

In recent years, there is an increased thirst for the production of plant-derived drugs for the treatment of many diseases. Having known the wide applications of betel leaves in day-to-day life, this investigation was performed to assess the antibacterial potential of betel leaves. Different extracts *viz.* methanol, ethanol, acetone, chloroform, and water were used against ten pathogenic bacteria. Among the extracts tested, methanol extracts prepared from Karpoori variety of *Piper betle* collected from Tamil Nadu exerted good antibacterial activity against test organisms. The pathogens *viz. Salmonella typhi*, *Streptococcus mutans*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris*, and *Streptococcus pyogenes* were exhibited greater susceptibility towards methanol extract of betel leaves. The results also revealed that there was a marked difference among the same variety of *P. betle* cultivated under two different regions in their antimicrobial nature.

INTRODUCTION

Nowadays, multidrug resistance has been observed in pathogenic microorganisms due to wide spread use of antibiotics. An increased antibiotic resistance to the existing antibiotics should be balanced with the discovery of new medication. An alternative product, which is more potent, low cost with fewer side effects and continuous large number availability, needs to be discovered to overcome antibiotic resistance of microorganism (Kaveti and Tan, 2011). Much focus is being given to traditionally used medicinal plants for their antimicrobial properties due to negative impacts displayed by synthetic drugs, such as antibiotic resistance, side effects, high cost, and loss of public reliance (Livermore, 2004). It is estimated that about 25% of modern medicines are directly derived from higher plants. It is interesting to note that about 60% of the antitumor and antimicrobial medicines currently available on the market are derived from natural products, mainly from higher plants (Kritikar and Basu, 1999).

The most popular medicinal plant known by the people all over the world is betel plant. The antiseptic property of the betel plant has been known since 600 BC (Sudewo, 2010). Indian ancient culture witnessed the vital role played by *Piper betle*. Indian traditional books of Ayurveda, Charaka Samhitas, and Kashyapa Bhojanakalpa enlisted the practice of chewing *P. betle* leaf after meals (Kumar *et al*., 2010). The *P. betle* (Tamil: Vetrilai) is a perennial dioecious glabrous climbing vine belonging to the family Piperaceae. Leaves are simple alternate and yellow to bright green in color (Sharma *et al*., 2009; Jayaweera, 1982).

In Tamilnadu, three varieties of *P. betle* leaves *viz*. Sirugamani, Karpoori, and Vellaikodi, are mostly available (Nandkarnis, 2007). The plant grows well in warm, humid climates (Nanayakkara *et al*., 2014). The comparative study is essential because the phytochemical concentration of the plant may tend to differ according to habitation, soil, climate, and agronomic practices followed. Even though numerous researches have been done on various medicinal uses of *P. betle* leaves, the comparative study between leaf varieties grow under different climate is limited. Therefore, the objective of this study is to compare antimicrobial properties of betel leaf Karpoori variety collected from Tamil Nadu and Kerala state against selected bacterial pathogens.

MATERIALS AND METHODS

Collection of plants

The *P. betle* Karpoori variety leaves grown in Tamil Nadu and Kerala state were selected for this study. Healthy and well-grown, young greenish leaves were collected directly from the cultivated fields in sterile polythene bags and transported to the laboratory. The leaves were washed alternatively with tap water and distilled water, then surface sterilization was done with 10% concentrated sodium hypochlorite solution to prevent the growth of microbes, rinsed again with sterile distilled water and shade dried at room temperature.

Preparation of plant extracts

Dried leaves were homogenized into a fine powder using mixer grinder. Five different solvents *viz*. methanol, ethanol, acetone, chloroform, and water were used to extract bioactive compounds from the sample. About 50g of powdered samples were loaded in Soxhlet apparatus with 250ml of respective solvent separately and extracted for about 72 h. The extraction was continued until the extractive become colorless. Finally, all the successive extracts were evaporated in rotary vacuum evaporator at 40°C. The crude extract thus obtained were transferred into glass vials and stored at 4°C until it is required.

Test organisms used

The bacterial pathogens used in this study were procured from Institute of Microbial Technology (IMTech), Chandigarh. The lyophilized cultures were revived by inoculating the strains into the nutrient broth. The stock culture was maintained on Nutrient Agar slants and stored at 4°C in a refrigerator.

Preparation of inoculum

The active young cultures for the study were prepared by subculturing a loop full of cells to the Nutrient broth and incubated for 24 h at 37°C. The 24-h-old cultures were suspended into sterile Nutrient broth to match turbidity of 0.5 Mc Farland standard, which is approximately 1.0 x 10^6 CFU/ml.

In vitro antibacterial activity of the extracts

Antibacterial activity of the *P. betle* leaf extract was assessed by disc diffusion method (Bauer *et al*., 1966). About 15–20ml of sterilized Mueller-Hinton (MH) agar medium was transferred to sterile petriplates and allowed to solidify. After solidification, the test bacterial cell suspension (0.1%) was uniformly spread over the agar surface using sterile cotton swabs. The sterile discs impregnated with 300µg/ml of the extracts were placed at equal distance and then incubated at 37°C for 24 h. The standard antibiotic ciprofloxacin (5µg/disc) was also placed to compare the inhibition results contributed by the extracts. At the end of incubation, the zone of growth inhibition around the disc was measured in mm unit.

Minimum inhibitory concentration

For the determination of minimum inhibitory concentration (MIC), twofold serial dilution method was followed with Mueller Hinton Broth (Ericsson and Sherriel, 1971). The plant extracts were diluted to the concentration ranging from 3.9 to 1000µg/ml. The MH broth tube inoculated with respective culture was served as a positive control, whereas uninoculated broth served as negative control. The tubes were incubated at 37°C for 24 h, and the results were recorded. The MIC was the lowest concentration of the extract, which did not permit any visible growth on the inoculated tubes.

Minimum bactericidal concentration

The minimum bactericidal concentration (MBC) was determined by subculturing the above (MIC) serial dilution after 24 h in MH agar plates and incubating at 37°C for 24 h. MBC is defined as the lowest concentration that inhibits the growth of any bacterial colony on the solid media (NCCLS, 1997).

RESULTS AND DISCUSSION

Several researchers have assessed the antimicrobial potential of *P. betle* leaf varieties and came up with promising results (Chakraborty and Shah, 2011; Shukla *et al*., 2009; Datta *et al*., 2011). Majority of them have stated that solvents such as methanol, ethanol, chloroform, ethyl acetate, petroleum ether, and acetone recorded good results than the aqueous extracts, which has been confirmed in our present study.

According to Pelczar *et al*. (1993), the antibacterial activity of the *P. betle* extract is contributed by sterol, which is present in high concentrations. The mode of action of sterol is alteration and creation of pores in the cell wall and membrane consequently lead to death of bacteria. The alkaloids, phenolic compounds, glycosides, and tannins may have direct action on the microbial membranes and contribute antimicrobial effect (McDonald *et al*., 1996; Kaveti *et al*., 2011).

Among the five different extracts tested, methanolic extract of betel leaves recorded appreciable antibacterial activity against the pathogenic microorganisms, whereas the work of Sutrapu *et al*. (2013) showed positive results for antimicrobial activity in ethanolic extracts of *P. betle* leaves. In this study, methanolic extracts prepared from Karpoori variety of betel leaves grown in Tamil Nadu exhibited wide spectrum of inhibition against the tested bacterial pathogens even in the low concentration. The inhibitory action was increased with increase in the concentration of extracts used (Figure 1). Ethanolic extracts from *P. betle* leaves also recorded good results after methanolic extracts. On comparison, aqueous extracts exhibited less inhibitory activity than the other extracts used in this study. Our results are comparable with the study of Swapna *et al*. (2012). They investigated the antibacterial efficacy of solvent extracts of Mokkathotapapada leaves of *P. betle* Linn. Cv. Kapoori. Among the four different solvents used, ether extracts exhibited high activity against tested bacteria even at lowest concentrations. They also stated that the inhibitory activity of the various solvent extracts was dose dependent, enhanced with increase in concentration.

Among the bacteria tested, *S. mutans* was highly susceptible and showed 17.7 ± 0.4 mm zone of inhibition against methanolic extract of Karpoori variety of betel from Tamil Nadu. This was followed by *S. typhi* (17.5 ± 0.5 mm), *E. coli* (16.5 ± 0.5 mm), *S. aureus* (13.9 ± 0.4 mm), *P. vulgaris* (13.9 ± 0.4 mm), *P. aeruginosa* (13.7 ± 0.2 mm), and *S. pyogenes* (13.4 ± 0.3 mm) at 300µg/ml concentration of the methanolic extracts. These results are in agreement with results of Subash kumar *et al*. (2013). According to them, maximum bactericidal activity was observed towards clinical isolates of *E. coli, P. aeruginosa,* and *S. aureus* with greatest zone of inhibition to the ethanol extracts of *P. betle* crude extracts. The MIC and MBC values for the extracts from Karpoori variety of *P. betle* leaves were also analyzed in this study. The MIC values ranged between 15.6 and 500µg/ml, whereas the MBCs were twofold greater than the MICs (Tables 1 & 2).

A comparative study was made on antimicrobial activity of some selected Indian medicinal plants using well diffusion method (Pandey *et al*., 2014). Among the plant extracts tested, *P. betle* extracts showed maximum antimicrobial activity against all microbes. It is noteworthy that *E. coli* was resistant to all the extracts tested except *P. betle* extract. *S. aureus* was the most susceptible one, whereas *E. coli* was the most resistant of all the bacteria tested toward other plant extracts. These results are due to differences in cell wall structure between Gram positive and Gram negative bacteria, with the Gram negative bacteria outer membrane acts as a barrier to many environmental substances, including antibiotic. On the contrary, methanolic extracts of *P. betle* leaves recorded 16.5 ± 0.5 mm zone of inhibition against *E. coli* strains in the present investigation.

From this investigation, Karpoori variety of *P. betle* cultivated in Tamil Nadu recorded pronounced antibacterial activity than Karpoori variety grown in Kerala state. The constituent in the oil may vary qualitatively and quantitatively based on different factors such as plant variety, soil, climate, and the agronomic practices followed to raise the crop *etc.* (Ramalakshmi *et al*., 2002; Sankar *et al*., 1996).This study shows the variation in phytochemical nature of leaves may be due to different soil and climatic condition.

Based on the results of the present investigation, methanolic extract from Karpoori variety of *P. betle* leaves found to contain certain microbicidal compounds, which should be explored further to develop drugs against potential pathogenic microbes. The study obviously justified the traditional use of betel leaves as antiseptics since from the ancient time.

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Table-1: List of bacterial pathogens used in this study

S. No	Bacterial strains
1.	*Salmonella typhi* MTCC1168
2.	*Klebsiella pneumoniae* MTCC39
3.	*Streptococcus mutans* MTCC121
4.	*Bacillus cereus* MTCC430
5.	*Streptococcus pneumoniae* MTCC423
6.	*Pseudomonas fluorescens* MTCC671
7.	*Staphylococcus aureus* MTCC90
8.	*Escherichia coli* MTCC40
9.	*Streptococcus pyogenes* MTCC442
10.	*Proteus vulgaris* MTCC426

Table-2: Antibacterial activity of Karpoori variety of *Piper betle* leaves collected from Tamil Nadu

S. No	Extracts used µg/ml	Bacterial pathogens used Zone of inhibition (mm)									
		A	B	C	D	E	F	G	H	I	J
1.	Methanol										
	300	17.5 ± 0.5	12.2 ± 0.5	17.7 ± 0.4	13.2 ± 0.4	13.3 ± 0.4	13.7 ± 0.2	13.9 ± 0.4	16.5 ± 0.5	13.4 ± 0.3	12.9 ± 0.4
	MIC	15.6	62.5	15.6	62.5	15.6	62.5	15.6	62.5	62.5	62.5
	MBC	31.2	125	31.2	125	31.2	125	31.2	125	125	125
2.	Ethanol										
	300	15.6 ± 0.5	11.3 ± 0.4	17.2 ± 0.5	12.2 ± 0.5	10.3 ± 0.5	11.6 ± 0.5	11.8 ± 0.5	14.8 ± 0.2	10.8 ± 0.4	12.2 ± 0.4
	MIC	62.5	62.5	15.6	62.5	62.5	62.5	62.5	62.5	62.5	62.5
	MBC	125	125	31.2	125	125	125	125	125	125	125
3.	Acetone										
	300	15.2 ± 0.3	9.7 ± 0.5	14.9 ± 0.5	10.6 ± 0.4	9.5 ± 0.5	11.2 ± 0.5	10.9 ± 0.2	11.6 ± 0.3	12.5 ± 0.4	10.2 ± 0.5
	MIC	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
	MBC	125	125	125	125	125	125	125	125	125	125
4.	Chloroform										
	300	13.5 ± 0.5	8.3 ± 0.4	12.3 ± 0.5	10.0 ± 0.4	8.2 ± 0.4	11.0 ± 0.5	10.3 ± 0.5	11.5 ± 0.5	10.7 ± 0.3	10.3 ± 0.5
	MIC	15.6	250	15.6	250	62.5	62.5	250	15.6	250	62.5
	MBC	32.5	500	32.5	500	125	125	500	32.5	500	125
5.	Aqueous										
	300	12.4 ± 0.3	8.0 ± 0.2	12.0 ± 0.3	8.8 ± 0.3	8.0 ± 0.02	10.0 ± 0.5	8.9 ± 0.3	9.4 ± 0.4	9.0 ± 0.3	9.2 ± 0.3
	MIC	62.5		62.5	250	250	62.5	250	62.5	125	125
	MBC	125		125	500	500	125	500	125	250	250

A- *Salmonella typhi*, B- *Klebsiella pneumoniae*, C- *Streptococcus mutans*, D- *Bacillus cereus*, E- *Streptococcus pneumoniae*, F- *Pseudomonas aeruginosa*, G- *Staphylococcus aureus*, H- *Escherichia coli*, I- *Streptococcus pyogenes*, J- *Proteus vulgaris.*

Table-3: Antibacterial activity of Karpoori variety of *Piper betle* leaves collected from Kerala

S. No	Extracts Used µg/ml	Bacterial pathogens used									
		A	B	C	D	E	F	G	H	I	J
1.	**Methanolic**										
	300	14.5 ± 0.4	9.9 ± 0.3	16.6 ± 0.3	11.2 ± 0.3	11.3 ± 0.5	11.7 ± 0.2	10.9 ± 0.3	13.8 ± 0.6	11.5 ± 0.3	11.0 ± 0.3
	MIC	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
	MBC	125	125	125	125	125	125	125	125	125	125
2.	**Ethanolic**										
	300	13.8 ± 0.4	9.8 ± 0.4	15.5 ± 0.6	10.2 ± 0.6	10.9 ± 0.6	11.5 ± 0.5	10.9 ± 0.4	12.9±0.5	10.9 ± 0.2	10.2 ± 0.6
	MIC	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
	MBC	125	125	125	125	125	125	125	125	125	125
3.	**Acetone**										
	300	11.5 ± 0.4	8.8 ± 0.2	15.7 ± 0.6	9.5 ± 0.02	10.2 ± 0.6	10.9 ± 0.4	9.9 ± 0.6	10.8 ± 0.4	9.9 ± 0.6	9.8 ± 0.4
	MIC	62.5	125	62.5	62.5	62.5	62.5	62.5	62.5	62.5	125
	MBC	125	250	125	125	125	125	125	125	125	250
4.	**Chloroform**										
	300	9.5 ± 0.4	8.9 ± 0.4	13.5 ± 0.3	8.9 ± 0.4	9.9 ± 0.3	10.1 ± 0.3	9.9 ± 0.3	9.5 ± 0.3	9.6 ± 0.3	8.4 ± 0.2
	MIC	62.5	125	62.5	125	62.5	62.5	62.5	62.5	125	125
	MBC	125	250	125	250	125	125	125	125	250	250
5.	**Aqueous**										
	300	8.9 ± 0.6	8.8 ± 0.5	10.8 ± 0.3	8.4 ± 0.5	9.5 ± 0.5	9.2 ± 0.4	9.2 ± 0.2	9.0 ± 0.5	8.5 ± 0.4	8.6 ± 0.4
	MIC	125	125	62.5	250	125	125	125	125	250	125
	MBC	250	250	125	500	250	250	250	250	500	250
6.	**Standard Ciprofloxacin (5µg/disc)**	28.3 ± 0.05	29.5 ± 0.03	27.4 ± 0.04	29.7 ± 0.04	28.6 ± 0.05	29.4 ± 0.04	28.6 ± 0.05	29.6 ± 0.04	28.8 ±0.03	27.4 ± 0.05

A-*Salmonella typhi*, B- *Klebsiella pneumoniae*, C- *Streptococcus mutans*, D- *Bacillus cereus*, E- *Streptococcus pneumoniae*, F- *Pseudomonas aeruginosa*, G- *Staphylococcus aureus*, H- *Escherichia coli*, I- *Streptococcus pyogenes*, J- *Proteus vulgaris.*

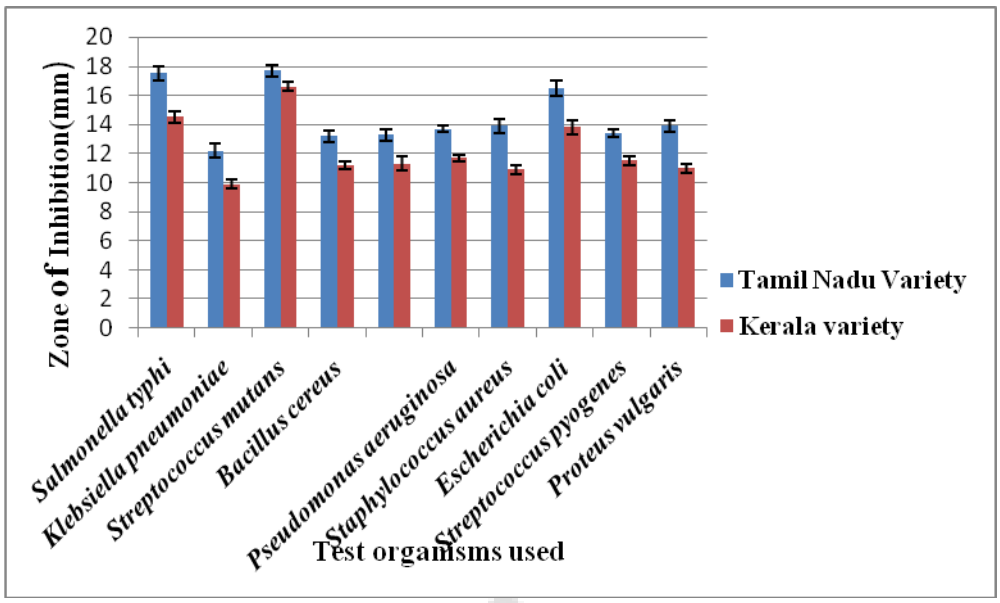

Figure-1: Antibacterial activity of methanolic extracts of Karpoori variety *betle* against selected pathogens.