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Research Article

ANTIBACTERIAL ACTIVITY OF FRESH LEAF JUICE OF *PIPER BETLE* AGAINST *ESCHERICHIA COLI, BACILLUS SUBTILIS* AND *STAPHYLOCOCCUS AUREUS*

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ABSTRACT
Bacteria acquire novel characters by horizontal gene transfer. Acquisition of antibiotic resistance is a serious consequence that makes bacteria robust to fight against antibiotics and thus, survive, grow and multiply even with the treatment of antibiotic. This is due to overuse and misuse of antibiotics. Therefore, it is the need of the hour to find out solutions and develop new strategies. In herbal medicine, many plant products are used in the treatment of various ailments. Various laboratories are engaged in the study of medicinal plants for their antibacterial efficacy. In the present investigations, we have studied the antibacterial activity of fresh leaf of *Piper betle* (the edible Paan/leaf used for making betel quid) against *Escherichia coli, Bacillus subtilis and Staphylococcus aureus*. The fresh leaf juice exhibited bacteriostatic action against *Escherichia coli* and *Bacillus subtilis*. The zone of inhibition for *E.coli* was 16.00 ± 3.46 mm by disc diffusion method and 12.13 ± 0.64 mm by agar well diffusion method and MIC 2.5%. For *B. subtilis* it was 13.86 ± 3.53mm by disc diffusion method and 11.33 ± 1.86 mm by agar well diffusion method and MIC 0.31%. There was no any effect on *S. aureus.*

Keywords: *Antibiotic Resistance, Plant Derived Antimicrobials, Piper Betle, Antibacterial Susceptibility Assays*

INTRODUCTION
Due to increasing use and misuse of antibiotics, bacteria and fungi are acquiring resistance against the existing antibiotics (D'Agata *et al.,* 2008; Rosenblatt-Farrell, 2009; Davies and Davies, 2010). Therefore, there is need of developing novel antimicrobials. However, there is a paradox in the speed with which the pathogens are acquiring resistance and the discovery of new antibiotics (Conly and Johnston, 2005). Hence, screening of antimicrobial activity of traditional medicinal plants is very important (Harvey, 2008; Saklani and Kutty, 2008). In the present investigation antimicrobial activity of fresh leaf juice of *Piper betle* was studied against *Escherichia coli*, *Bacillus subtitis* and *staphylococcus aureus. E. Coli* is a normal inhabitant of the human intestine and is an opportunistic pathogen involved in bacillary dysentery. *Bacillus subtilis* is a Gram positive and most studied soil bacteria. *S.aureus* is a Gram positive coccus and a serious pathogen involved in most of the wound and hospital infections. Methicillin resistant *S. aureus* is resistant to most of the currently available antibiotics. Pan (betel leaf) is a tropical creeper, belonging to the family Piperaceae. In India, the betel leaf and Areca nut together have a ceremonial value. People chew it to sweeten the breath. Betel leaf is in use from ancient times as a digestive and a mouth freshener. Betel leaf is considered as aphrodisiac (Guha, 2006). It has anthelmintic property (Ali and Mehta, 1970). Besides, it also possesses antioxidant potential (Choudhury and Kale, 2002). It is also used as an external application for boils.

MATERIALS AND METHODS
Preparation of Fresh Leaf Juice of Piper Betle: Fresh and healthy leaves of *Piper betle* were purchased from local vegetable market (Itwara Bazzar, Amravati). It was authenticated at the Department of Botany, Govt. Vidarbha Institute of Science and Humanities, Amravati. The leaves were washed with sterile water, blotted with sterile muslin cloth, crushed and squeezed to get the leaf juice. The leaf juice was

filtered through sixteen layered sterile muslin cloth, collected in a chilled sterile glass vial and used immediately for testing the antibacterial activity. All the operations mentioned above were performed in an aseptic condition.

Culture of test organisms: *E. coli* (MTCC 739), *B. subtilis* (MTCC 736) and *S. aureus* (MTCC 3160) were purchased from Institute of Microbial Technology (IMTECH), Chandigarh. *E. coli, B. subtilis and S. aureus* were suspended in nutrient broth and incubated at 37^{o}C for 24 hours. Thereafter, a loopful suspension from this broth was inoculated on the slant of nutrient agar for further use. After 24 hours of incubation the bacterial strains were maintained at 4^{o}C. Every time two slants were inoculated, one for `in-use' and the other for archiving.

Preparation of Inoculum

Optical Density of inoculum is directly proportional to the number of cells present in it. The inoculum was prepared by the direct colony suspension method in which colonies from overnight-incubated culture from the slant were suspended into pre-chilled 0.9 % saline. Cells from overnight-incubated cultures are in logarithmic phase of growth. So, as to have uniformity throughout the course of experimentation, the optical density of the inoculum was set to 0.2. This turbidity gave semi confluent growth, when the lawn was prepared on the petri dish.

Antimicrobial Study

Antimicrobial activity was studied by disc diffusion method and agar well diffusion method. All steps described below were carried out aseptically.

Disc Diffusion Method (Bauer et al., 1966)

The Whatman filter paper No.1 discs of the diameter 5mm were used. The discs were sterilized in dry air oven. Each disc was soaked in 5µl of fresh leaf juice. 0.1ml of the inoculum of test organism was spread using sterile glass spreader on the surface of nutrient agar. A disc soaked in leaf juice was placed on the medium at the center of the plate. The plates were initially transferred to refrigerator for 30 min. to allow the diffusion of the contents from disc to the surface of the agar and then incubated at 37°C for 12 hrs. The diameter of the zone of inhibition was measured. Discs soaked in Ciprofloxacin (10µg/ml) and sterile water, were used as a positive and negative control respectively.

Agar Well Diffusion Method (Perez et al., 1990)

Wells of the diameter 3 mm were punched in the agar using sterile gel puncher and filled with 10 µl of the fresh leaf juice of *Piper betle.* The antibiotic Ciprofloxacin (10µg/ml) was used as the positive control while sterile water as the negative control. The plates were incubated at 4°C for 30 min to allow diffusion of the contents from the well into the medium and then incubated at 37°C for 12 hours. The diameter of the zone of inhibition was measured.

Determination of Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial, which inhibits the visible growth of a microorganism after overnight incubation. The MICs were determined by the broth microdilution method (Andrews, 2001). In the first tube 400µl of the leaf juice was added. From tube No.2 to 11, in each tube, 200 µl nutrient broth was dispensed. In the 2nd tube, 200 µl fresh leaf juice from tube no. 1 was added and mixed well. From tube No.3, 200 µl was transferred to 3rd tube and mixed well. From 3rd tube 200 µl was transferred to 4th tube and likewise serial dilution was carried up to 11th tube. Finally, from 11th tube 200 µl solution was discarded. Thus, all tubes from tube No.1 to 11 contained 200 µl of serially diluted leaf juice.

Then, 800 µl of sterile nutrient broth was added in each tube. The 12th tube was treated as blank, which neither contained antibiotic nor leaf juice. A loopful suspension of the test organism was inoculated in each tube. All tubes were incubated at 37^{o}C with constant agitation to prevent formation of a gradient of leaf juice. Since leaf juice was having colour, another set of 12 tubes was prepared as described earlier but not inoculated with test organisms. This group was served as a negative control. In these tubes, suspension of test organism was inoculated after completion of incubation period and just before taking the O.D. Ciprofloxacin was used as a positive control where, the highest concentration was 10µg/ml in the first tube, which was serially diluted up to 11th tube so as to have 10 µg/ml in the first tube to 0.00975

µg/ml in the 11th tube. Rest of the procedure of addition of culture medium and inoculation was carried as per the experimental group.

Statistical Analysis

Each experiment was carried out for six times. Arithmetic mean and standard deviation were calculated using six observations.

RESULTS AND DISCUSSION

The results of the disc diffusion method and agar well diffusion method are displayed in plate No.1 and table No.1. There was a zone of inhibition of 16.00 ± 3.46mm for *E. coli*, for *B. subtilis* 13.86 ± 3.53 mm and for *S. aureus* there was no zone of inhibition surrounding the disc soaked in fresh leaf juice of *Piper betle.* In control, a zone of inhibition of 38.75 ± 3.06mm for *E. coli*, for *B. subtilis* 33.71±1.70 mm and for *S. aureus* 33.5 ± 0.68 was observed surrounding the disc soaked in ciprofloxacin.

There was a zone of inhibition of 12.13 ± 0.64 for *E. coli*, for *B. subtilis* 11.33 ± 1.86 mm and for *S. aureus* there was no zone of inhibition surrounding the well containing fresh leaf juice of *Piper betle.* In control, a zone of inhibition of 35.62 ± 1.92 mm for *E. coli*, for *B. subtilis* 35.00 ± 0.89 mm and for *S. aureus* 36.20±1.60 mm was observed surrounding the disc soaked in ciprofloxacin. After 24hrs of incubation, the zone of inhibitions surrounding the leaf juice soaked discs and well containing leaf juice were disappeared due to growth of bacteria.

Table 1: Diameter of Zone of Inhibition in mm Observed in Disc Diffusion Method and Agar Well Diffusion Method (Incubation Period 12 Hours, Temperature 37°C)

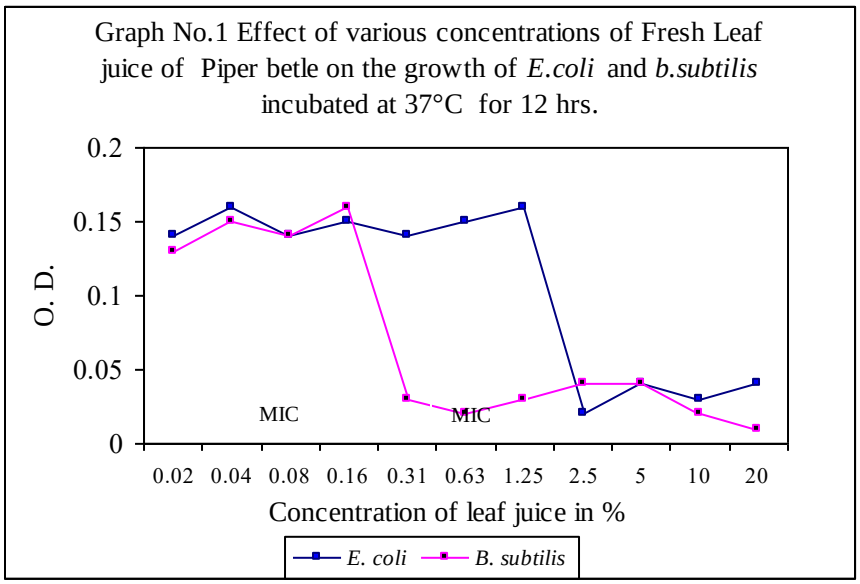
Microorganism	Diameter of Zone of Inhibition in mm in Disc Diffusion Method		Diameter of Zone of Inhibition in mm in Agar Well Diffusion Method	
	Ciprofloxacin	*P.Betle* Leaf Juice	Ciprofloxacin	*P.Betle* Leaf Juice
E. coli	38.75 ± 3.06	16.00 ± 3.46	35.62 ±1.92	12.13 ± 0.64
B. subtilis	33.71 ± 1.70	13.86 ± 3.53	35.00 ± 0.89	11.33 ± 1.86
S. aureus	33.5 ± 0.68	Nil	36.2 ± 1.6	Nil

The results of Minimum inhibitory concentration (MIC) are displayed in table No.2 and graph No.1. Minimum inhibitory concentration of fresh leaf juice of *Piper betle* was 2.5% in the case of *E. coli* and for *B. subtilis* it was 0.31%. There was no any inhibition for *S. aureus*.

Table 2: MIC of Fresh Leaf Juice of *Piper Betle* for the Test Organisms

Microorganism	Concentration of Fresh Leaf Juice of *Piper betle*											Blank	Result
	20 %	10 %	05 %	2.5 %	1.25 %	0.63 %	0.31 %	0.156 %	0.078 %	0.039 %	0.0195 %	0.00 %	
E.Coli	_	_	_	_	+	+	+	+	+	+	+	+	MIC 2.5 %
B.subtilis	–	–	–	–	–	–	–	+	+	+	+	+	MIC 0.31 %
S.aureus	+	+	+	+	+	+	+	+	+	+	+	+	No MIC

-indicates no bacterial growth and + indicates bacterial growth



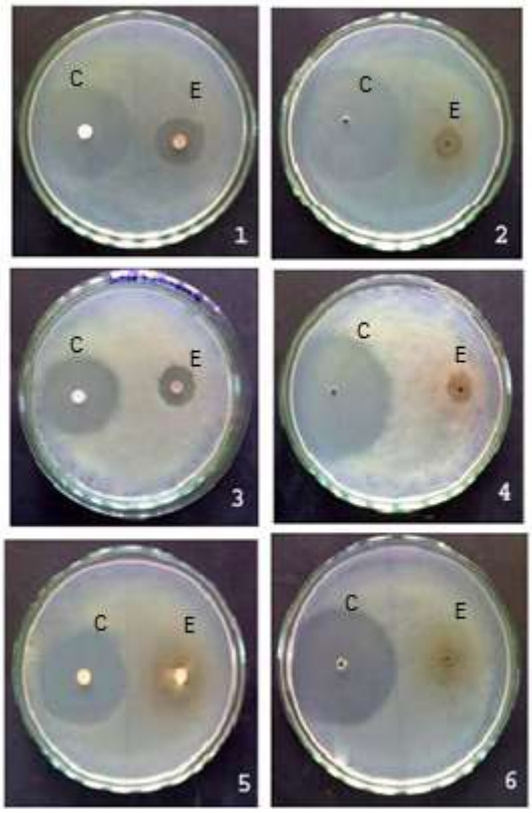


Plate I Showing the Zone of Inhibitions Observed by Disc Diffusion Method Figure 1, 3, 5 and Agar Well Diffusion Method Figure 2, 4, 6 C: Control (Ciprofloxacin), E: Experimental (Leaf Juice of *Piper Betle*) Figure 1 & 2 *E. Coli*, Figure 3 & 4 *B. Subtilis*, Figure 5 & 6 *S. Aureus*

The zone of inhibition for *E. coli* and *B. subtilis* around the disc soaked in the fresh leaf juice of *Piper betle* suggest the growth inhibitory effect of *Piper betle*. The results of the agar well diffusion method are also depicting the inhibitory effect of leaf juice against these bacteria. There was no zone of inhibition for *S. aureus.* There was no zone of inhibition in *S. aureus.* This indicates that *Piper betle* has no inhibitory

effect on *S. aureus*. It is a methicillin resistant strain, which is resistant to multiple antibiotics (Chambers and DeLeo, 2009). Since, it has developed defense mechanisms to adapt to various antibiotics, one of these mechanisms might have protected it from the action of leaf juice.

It was found that the extract of *Piper betle* leaves contains fatty acids, hydroxy fatty acid esters and hydroxychavicol (Nalina and Rahim, 2007). Fatty acids can act as anionic surfactants and have antibacterial and antifungal properties at low pH (Hayes and Berkovitz, 1979). These components might be the contributing factors for developing the zone of inhibition of *E. coli* and *B. subtilis* around the disc and well containing the fresh leaf juice of *Piper betle*. Moreover, B. *subtilis* is a Gram-positive bacterium. Fatty acids are selective against Gram-positive organisms by targeting the structure and function of bacterial cell walls and membranes (Kabara *et al.,* 1972).

In *E. coli* and *B. subtilis* 12 hours incubation exhibited zone of inhibition while a further continuation of the incubation up to 24 hours resulted in growth of test organism in the zone of inhibition, suggesting the bacteriostatic action of leaf juice against these test organisms. This might be due to time dependant inactivation and loss of inhibitory molecules from the juice. Even, these microorganisms might have developed a defense to tolerate the unfavorable conditions in the early hours of incubation. The susceptibility of *B. subtilis* (MIC 0.31%) was more than *E.coli* (MIC 2.5%).

REFERENCES

Ali SM and Mehta RK (1970). Preliminary pharmacological and anthelmintic studies of the essential oil of *Piper betle. Indian Journal of Pharmacy* **32** 132-133.

Andrews JM (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy* **48**(Suppl.1) 5-16.

Bauer AW, Kirby WM, Sherris JC and Turk M (1966). Antibiotic susceptibility testing by a standardized disk diffusion method. *American Journal of Clinical Pathology* **45**(4) 493-496.

Bhattacharya S, Mula S, Gamre S, Kamat JP, Bandyopadhyay SK and Chattopadhyay S (2007). Inhibitory property of *Piper betle* extract against photosensitization-induced damages to lipids and proteins. *Food Chemistry* **100** 1474–1480.

Chambers HF and DeLeo FR (2009). Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nature Reviews Microbiology* **7**(9) 629-641.

Choudhury D and Kale RK (2002). Antioxidant and non-toxic properties of *Piper betle* leaf extract: *in vitro* and *in vivo* studies. *Phytotherapy Research* **16** 461-466.

Conly J and Johnston B (2005). Where are all the new antibiotics? The new antibiotic paradox. *The Canadian Journal of Infectious Diseases and Medical Microbiology* **16**(3) 159-160.

D'agata EMC, Dupont-Rouzeyrol M, Magal P and Ruan S (2008). The impact of different antibiotic regimens on the emergence of Antimicrobial resistance bacteria. *PLoS One* **3**(12) e4036.

Davies J and Davies D (2010). Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews* **74**(3) 417-433.

Guha P (2006). Betel leaf: The neglected green gold of India. *Journal of Human Ecology* **19**(2) 87-93.

Harvey AL (2008). Natural products in drug discovery. *Drug Discovery Today* **13**(19-20) 894-901.

Hayes ML and Berkovitz BK (1979). The reduction of fissure caries in Wistar rats by a soluble salt of non anionic acid. *Archives of Oral Biology* **24** 663-666.

Kabara JJ, Swieczkowski DM, Conley AJ and Truant JP (1972). Fatty acids and derivatives as antimicrobial agents. *Antimicrobial Agents and Chemotherapy* **2** 23-28.

Liao YL, Chiang YC, Tsai TF, Lee RF, Chan YC and Hsiao CH (1999). Contact leukomelanosis induced by the leaves of *Piper betle* L. (Piperaceae): A clinical and histopathologic survey. *Journal of American Academy of Dermatology* **40** 583-589.

Nalina T and Rahim ZHA (2007). The Crude Aqueous Extract of *Piper betle* L. and its antibacterial effect towards *Streptococcus mutans. American Journal of Biotechnology and Biochemistry* **3**(1) 10-15.

Perez C, Paul M and Bazerque P (1990). Antibiotic assay by agar well diffusion method. *Acta Biologiae et Medicinae Experimentalis* **15** 113-115.

Ramji N, Iyer R and Chandrasekharan S (2002). Phenolic antibacterials from *Piper betle* in the prevention of halitosis. *Journal of Ethnopharmacology* **83** 149-152.
Rosenblatt-Farrell N (2009). Landscape of antibiotic resistance. *Environmental Health Perspectives* **117**(6) A240-A250.
Saklani A and Kutty SK (2008). Plant derived compounds in clinical trials. *Drug Discovery Today* **13**(3-4) 161-171.