

# Antimicrobial Property of *Piper betel* Leaf against Clinical Isolates of Bacteria

Arani Datta, Shreya Ghoshdastidar and Mukesh Singh\*

Molecular Biology Laboratory,  
School of Biotechnology & Life Sciences  
Haldia Institute of Technology, HIT Campus  
P.O. HIT, Haldia, Dist. Purba Medinipur, W.Bengal  
\*Email: singhmukesh8@rediffmail.com

## Abstract

The leaves of *Piper betel* (locally known as Paan) have long been in use in the Indian local system of medicine for its antioxidant and antimicrobial properties. In the present work, the antimicrobial activity of ethanol extract of *Piper betel* leaves was evaluated against human pathogenic bacteria (both gram-positive and gram-negative). The leaf powder was subjected to phytochemical screening and was found to contain carbohydrate, protein, polyphenolic compounds, flavonoid, alkaloids and total antioxidant. The ethanol extract showed strong free radical scavenging activity as seen by DPPH model. The extract confirmed significant antimicrobial activity against all bacterial strains tested. The effect of the extract was almost proportional to the concentration of the extract tested. The MIC for the bacterial strains was in the range of 25 µg to 40 µg. Concurring with the disc diffusion results, the MIC of the *Proteus vulgaris* was found to be least 25 µg while for *Staphylococcus aureus* it was approximately 40 µg. Time-kill kinetics of the ethanol extract treated bacterial strains demonstrated similar results, showing decline in the growth curve after six hour in most of the strains. Crude ethanol extract of *Piper betel* showed strong antimicrobial activity against the tested pathogenic bacterial strains. The results also indicate that scientific studies carried out commonly use herbs having traditional claims of effectiveness might warrant fruitful results.

**Keywords:** antimicrobial activity, *Piper betel*, phytochemical, pathogenic isolates

## Introduction

*Piper betel* Linn (Piperaceae) leaves is widely used as a post meal mouth freshener and the crop is extensively grown in India, Sri Lanka, Malaysia, Thailand, Taiwan and other Southeast Asian countries. Due to strong pungent aromatic flavour betel leaves are used as masticatory by the Asian people. Its common names are betel (in English), paan (in Indian), phlu (in Thai) and sirih (in Bahasa Indonesian). Grown abundantly in many parts of India, betel is an evergreen dioecious herb that needs warm and moist growth conditions for its growth. Leaves of betel vine are used with various condiments such as areca nut (kattha), cloves, cardamom, arecanut, candied rose and fennel for chewing purposes (Verma et al., 2004). Indian system of medicine and health has adopted the use of betel leaves in various ways. In Indian folkloric medicine, betel leaf is popular as an antiseptic and is commonly applied on wounds and lesions for its healing effects. This particular property has paved way for further experimental studies, which have established paan extract to have antimicrobial and antileishmanian properties (Sarker et al., 2008). Fresh juice of betel leaves is also used in many ayurvedic preparations. Betel leaves have long been studied for their diverse pharmacological actions.

Traditional healers from different remote communities in India claim that their medicine obtained from these betel leaves is cheaper and more effective than modern medicine. Patients belonging to these communities have a reduced risk of acquiring infectious diseases from resistant pathogens than the people from urban areas who may be treated with regular antibiotics. A novel approach to the prevention of antibiotic resistance of pathogenic species is the use of new compounds that are not based on existing synthetic antimicrobial agents (Shah et al., 2005). It is imperative that evaluation of the potential use of folkloric medicine for the treatment of infectious diseases produced by common pathogens be performed on a scientific base. Many plants are thus becoming probable sources of important drugs and pharmaceutical industries, nowadays, have come to consider this traditional medicine as a source of bioactive agents which can be used in the preparation of synthetic medicine. Furthermore, they are possible source for new as well as potent antibiotics to which the pathogenic

strains are not resistant. Reports of various researches show that betel extract and betel oil exhibit antimicrobial and antioxidant activities in model systems (Salleh *et al.*, 2002; Lei *et al.*, 2003; Bhattacharya *et al.*, 2006).

The objective of this study includes the evaluation of the phytochemical constituents of the ethanol extract of the betel leaf and investigating the efficacy of the same as an antimicrobial agent on the four pathogenic bacterial species.

## **Materials and methods**

### ***Plant Materials***

Fresh betel vine leaves were collected from the nearby village in Haldia of West Bengal, India. The leaves were shade dried and crushed into fine powder with electric blender. The powdered sample was sealed in polythene bags and was stored in desiccators until further uses.

### ***Preparation of ethanol extract***

Dried and powdered betel vine leaves (50 g) were Soxhlet extracted with 300 ml of ethanol for about 16 h. The crude plant extract extracted in solvent was removed from the Soxhlet and was concentrated to dryness in rotary vacuum evaporator below 50 °C and stored until needed for the bioassays at -4 °C.

### ***Phytochemical screening***

Biochemical assays were carried out on the powdered betel vine ethanol extract for screening presence of bioactive components such as carbohydrate, protein, polyphenolic compounds, flavonoid, alkaloids and total antioxidant (Devmurari *et al.*, 2010; Bratati *et al.*, 2006). Similarly, antioxidant potential of the said extract was evaluated by DPPH model.

### ***Microbial strains and inoculum preparation***

The microorganisms used in this study were clinical isolates of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Staphylococcus aureus*.

Bacterial strains stock cultures were maintained at 4°C on nutrient agar medium. Active cultures were prepared by inoculating fresh nutrient broth medium with a loopful of cells from the stock cultures at 37°C for overnight. To get desirable cell counts for bioassays, overnight grown bacterial cells were subcultured in fresh nutrient broth at 37°C.

### ***In-vitro antimicrobial screening***

*In vitro* antimicrobial activity of the ethanol extracts of betel vine was screened against a total of four above mentioned bacterial strains.

### ***Disc diffusion method***

The antimicrobial activity of ethanol extract of betel vine was screened using disc diffusion technique. The agar plates were prepared by pouring 15 ml of molten nutrient agar media into sterile petriplates. The plates were allowed to solidify and 0.1 % inoculum suspension was swabbed uniformly with sterile cotton and was allowed to stand for 15 minutes. The different dilutions of extracts (0, 20, 40, 60 and 80 %) from initial concentration of 1 mg/ml were loaded on 6 mm autoclaved filter paper discs. The loaded disc was placed on the surface of medium and the compound was allowed to diffuse for 5 minutes and the plates were incubated at 37°C for 24 hrs. At the end of incubation, inhibition zones formed around the disc were measured with ruler in millimeter. These studies were performed in triplicate.

### ***Determination of MIC by visual analysis***

The ethanol extract was later tested to determine the Minimal Inhibitory Concentration (MIC) for each bacterial strain. Freshly, grown bacterial strains 100 µL (10<sup>6</sup> cells/mL) in nutrient broth was inoculated in tubes with nutrient broth supplemented with different concentrations (10 – 500 µL) from the stock extract (1 mg/ml) and antibiotic, respectively and incubated for 24 h at 37 °C. Presence of turbidity denoted presence of micro organism in the test tube after the period of incubation where as the complete absence of any turbidity indicates complete inhibition of microbial growth. The test tube with the lowest dilution with no detectable growth by visual inspection was considered the MIC. The MIC was calculated for the individual bacterial species.

### ***Time-kill Kinetics of the ethanol extract***

The ethanol extract was later tested to determine the time-kill kinetics for each bacterial strain. Freshly grown bacterial strains 100  $\mu$ L (about  $10^6$  cells/ml) in nutrient broth was inoculated in tubes with nutrient broth supplemented with different concentrations (0, 25  $\mu$ g, 50  $\mu$ g and 100  $\mu$ g) of the extract at 37 °C and optical density was recorded at 1h intervals up to 15 h. Graphs were plotted on the basis of the turbidity varying over a period of time. The growth rate thus obtained was studied for any signs of bactericidal effects of the plant extract.

Ceftriaxone (commercial name Monocef) (1 mg/ml) was used as positive control. A solution of the solvent in which dried extract was dissolved served as negative control.

### **Statistical analysis**

All experiments were carried out in triplicate. Data points were represented by the mean of the measured values. Statistical analysis was carried out using MS-Excel software.

### **Result and discussion**

The relative efficiency of bactericidal activity by betel leaf ethanol extract to that of a broad-spectrum antibiotic such as ceftriaxone on the above mentioned microorganisms suggest the possibility of a more cost-effective and potentially harmless antibacterial agent. The results obtained support the fact that more work needs to be done on the purification, identification and quantification of the active components and the toxicity of active components, their side effects and pharmacokinetic properties with the view of their use for *in vivo* studies.

The phytochemical analysis of the betel leaf extract revealed the presence of important bioactive components (Table 1). From other scientific studies and researches it was observed that the presence of antioxidants in other medicinal plants imparted antimicrobial properties to those plants. These studies prompted us to study the antimicrobial properties of betel leaf extract. The antimicrobial effects were studied for four bacteria to start with viz *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Proteus vulgaris* and *Staphylococcus aureus*. To study the antimicrobial properties disc diffusion test, determination of MIC by visual testing and time-kill kinetics was studied on the growth tested bacteria.

Table 1 Phytochemicals, and antioxidant screening of ethanol extract of Piper betel leaves

<b>Phytochemical</b>	<b>Presence/Absence</b>
Carbohydrate	++
Protein	++
Phenolic components	+++
Flavanoids	++
Total antioxidant	+++

From the experiment on disc diffusion method, all the micro organisms demonstrated maximum zone of inhibition at 0% dilution of the ethanol extract, i.e., when the betel leaf extract was 100% concentrated it showed maximum inhibitory effect on the microorganisms. It can also be added that the microorganism *Proteus vulgaris* exhibited maximum zone of inhibition at 0% dilution of plant extract at 13mm followed by *Klebsiella* at 11.5mm. From the disc diffusion method it could be concluded that *Proteus vulgaris* and *Klebsiella* were

inhibited to a great extent by the betel leaf extract, followed by *Pseudomonas* and *Staphylococcus aureus* (Table 2)

Table 2. Zones of inhibition of different dilution of ethanolic extract of *Piper betel* (1 mg/ml) and antibiotic ceftriaxone (1mg/ml) against four clinical bacterial isolates under study (values are mean  $\pm$  SD of three replicas).

Bacterial Strain	Zone of inhibition (mm)					
	Dilution Percentage					
	0	20	40	60	80	Ceftriaxone
<i>Pseudomonas aeruginosa</i>	16 $\pm$ 0.24	14 $\pm$ 0.15	13 $\pm$ 41	11 $\pm$ 0.02	9 $\pm$ 0.75	12 $\pm$ 0.9
<i>Proteus vulgaris</i>	10 $\pm$ 0.5	9.5 $\pm$ 0.25	8 $\pm$ 0.5	8 $\pm$ 0.25	7 $\pm$ 0.5	8 $\pm$ 0.5
<i>Staphylococcus aureus</i>	13 $\pm$ 0.43	12 $\pm$ 0.25	10 $\pm$ 0.6	10 $\pm$ 0.15	8 $\pm$ 0.25	20 $\pm$ 0.5
<i>Klebsiella pneumonia</i>	14 $\pm$ 0.15	14 $\pm$ 0.1	12 $\pm$ 0.25	11 $\pm$ 0.47	10 $\pm$ 0.15	17 $\pm$ 0.55

Previous report also confirmed antimicrobial property of Piper betel leaf on pathogenic fungi and bacteria (Shitut et al., 1999).

The minimum inhibitory concentration of the leaf extract was determined by visual observations and it was observed that the MIC for the bacterial strains was in the range of 25  $\mu$ g to 100  $\mu$ g. Concurring with the disc diffusion results, the MIC of the *Proteus vulgaris* was found to be least (25  $\mu$ g) while for *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* were approximately 40, 35 and 25  $\mu$ g. To further bolster the antimicrobial properties of the betel leaf extract, experiments were conducted to study the effects of the leaf extract on the life cycle of the four bacterial strains, using a time-kill kinetics. The life cycle was determined by plotting a graph between the turbidity of the cell culture (as measured by the spectrophotometer) and the time duration.

The growth inhibitory action (time-kill kinetics) of betel leaf ethanol extract was tested on bacterial strains over a period of 15 hours, for three different concentrations of the extract (25  $\mu$ g/ml, 50  $\mu$ g/ml and 100  $\mu$ g/ml) and was compared with the life cycle of the bacteria without the extract (Figure 1).

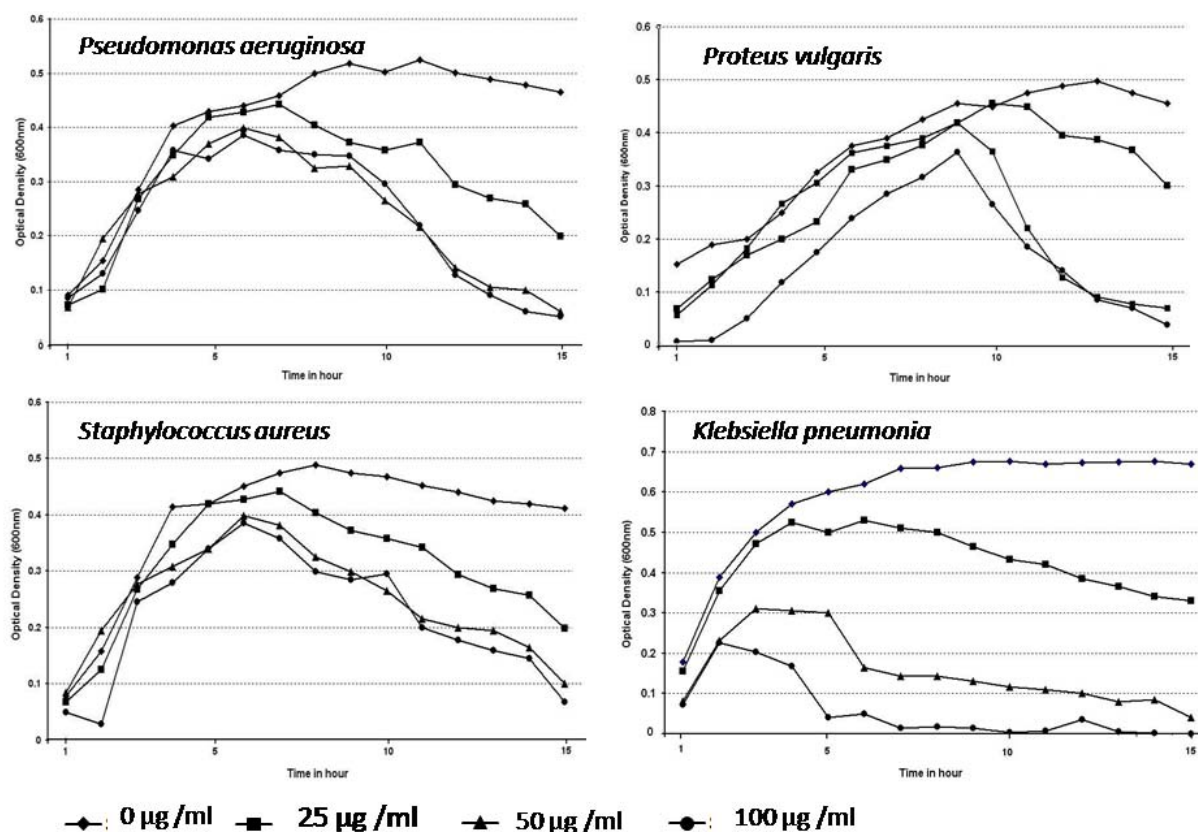


Figure 1

Time-kill curves of the tested clinical isolates of bacteria. Isolates were grown in LB medium supplemented with different concentrations (0, 25, 50 100 µg/ml) of ethanolic extract of *Piper betel* (values are mean of three replicas).

For *Pseudomonas aeruginosa* presence of 25 µg/ml of betel leaf extract the growth cycle curve shows a consistent rise in growth for a period of 3 ½ hours before forcing it into a ‘stationary’ state. It shows steep decline after the 6<sup>th</sup> hour which continues for about 4 more hours after which it stays minimal. There were not much significant differences seen with the other concentrations of the extract.

The growth inhibitory action of test extract on *Klebsiella pneumonia* was tested in a similar way and it was observed that in presence of extract the bacterial life cycle was forced into the death phase at the end of 4<sup>th</sup> hours ( with 50 µg/ml and 100 µg/ml ). Similar results were also seen for other two bacterial strains.

## Conclusion

The relative efficiency of bactericidal activity by betel leaf ethanol extract to that of a broad-spectrum antibiotic such as ceftriaxone on the above mentioned microorganisms suggest the possibility of a more cost-effective and potentially harmless antibacterial agent. The results obtained support the fact that more work needs to be done on the purification, identification and quantification of the active components and the toxicity of active components, their side effects and pharmacokinetic properties with the view of their use for *in vivo* studies.

## ACKNOWLEDGMENTS

This study was carried out in the research laboratory of School of Biotechnology and Life sciences, Haldia Institute of Technology. We gratefully acknowledge this institute for financial support of this research from the institute.

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