
*Original Research Article***IN-VITRO ANTIBACTERIAL ACTIVITY OF *PIPER BETEL* LEAF EXTRACTS**

**Fawad Ali Bangash, Hashmi A.N., Mahboob A., Zahid M., Hamid B., Muhammad S.A.,
Shah Z.U., Afzaal H.**

Faculty of Pharmacy, Riphah International University, Islamabad Pakistan.

ABSTRACT:

Ethanol, petroleum ether and chloroform extracts of *Piper betel* leaf were tested against Gram-positive (*Bacillus subtilis* ATCC 6633), *Staphylococcus aureus* ATCC 29213, *Micrococcus luteus* ATCC 9341) and Gram-negative (*Escherichia coli* ATCC 25922, *Pseudomonas putida*, *Salmonella typhi* ATCC 19430, *Shigella flexneri* ATCC 25929, *Pseudomonas aeruginosa* ATCC 33347, *Vibrio cholerae*, *Klebsiella pneumoniae*, *Proteus mirabilis* ATCC 49565) bacterial strains by Agar-well Diffusion Method. All the crude extracts showed a broad spectrum of antibacterial activity inhibiting both the gram-positive and gram-negative bacteria. Petroleum ether extracts of *Piper betel* were found to be least effective against most of the tested organisms. Moderate antibacterial activity has been studied with chloroform extracts while ethanolic fractions have been investigated to show optimum activity against nearly all chosen strains. Levofloxacin, the semi-synthetic broad spectrum antibiotic was used as standard Compare to levofloxacin, bio active fractions of *Piper betel* showed maximum activity against the *Klebsiella pneumonia*. This study reports the possible activity of *Piper betel* leaf in arresting the growth of selected bacterial strains. It has been expected that the present work on antimicrobial isolation of the plant bio active parts will lead to the researchers who continue work that may bring clinical success concerning the fatal diseases.

Keywords: *Piper betel*, antimicrobial fraction, pathogenic species

Address for Correspondence : Syed Aun Muhammad, Riphah institute of pharmaceutical sciences, 7th avenue, G7/4, Islamabad Pakistan. Tel.: +92301-4413995, Email: aunmuhammad78@yahoo.com

INTRODUCTION:

Plants are rich source of active metabolites that affects against variety of microbial species. New drugs are needed to eradicate the resistant microorganisms because existing antibacterial compounds are going to be resistant against a number of microbial strains and are becoming superseded (Ekpendu *et al.*, 1994). Antibacterial compounds extracted from plants are not related with side effects as the laboratory prepared compounds and can be successfully used against many infectious diseases (Iwu *et al.*, 1999). The utilization of bio active plant extracts and medicinal drugs have massive impact in curative approaches (Sousa *et al.*, 1991; Kubo *et al.*, 1993; Shapoval *et al.*, 1994; Artizzu *et al.*, 1995; Izzo *et al.*, 1995).

The *Piper betel* plant investigated in this study is a climbing plant, silky heart shaped leaves and white catkin. The Betel (*Piper betel*) is the leaf of a vine belonging to the Piperaceae family. It

originated from South and South East Asia including India, Bangladesh, Sri Lanka, and is typically too sensitive to grow outside the tropics geographic.

Piper betel plant leaves are rich in a wide variety of secondary metabolites such as phenolic compounds (chavicol, hydroxyl chavicol), volatile oils (safrole, eugenol, isoeugenol, eugenol methyl ester), fatty acids (stearic and palmitic) and hydroxyl fatty acids (stearic, palmitic, myristic) which in vitro illustrate the antibacterial properties and might be used as an choice, useful, cheap and safe antibacterial for the treatment of microbial infections.

Piper betel has been conventionally used as compost, carminative, antiseptic agents, antifungal and antibacterial (Jenie, 2001). It has also been reported for the cure of stomach problems, worms and as a general tonic. It is often chewed in combination with the betel nut (*Areca catechu*), as a stimulatory. Some evidence suggests that betel leaves have immune boosting properties as well as anti-cancer properties (Fathilah *et al.*, 2010).

In recent years, multiple drug resistance has developed due to indiscriminate use of existing antimicrobial drugs in the treatment of infectious diseases. These problems stress a transformed attempt to find the antimicrobial agents effective against the pathogenic microorganisms resistant to current antibiotics. Therefore there is an extensive requirement to establish alternative antibacterial molecules for the treatment of infectious diseases from other sources. One of the possible strategies towards this objective is the rational localization of bioactive photochemical (Chattopadhyay *et al.*, 2009).

It has been reported that *Piper betel* plant is effective against various bacterial strains including *Bacillus cereus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Micrococcus luteus*, *Staphylococcus aureus*, *Aeromonas hydrophila*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella Enteritidis* (Suppakul *et al.*, 2006), *Streptococcus mutans* (Sharma and Khan, 2010), *Streptococcus pyogenes* (Caburian and Marina, 2010), *Enterococcus faecium*, *Actinomyces viscosus*, *Streptococcus sanguis*, *Fusobacterium nucleatum*, *Prevotella intermedia*. The reported isolation and extraction techniques were employed using solvent-ethanol and water (Sharma and Khan, 2010), ethanol (Suppakul *et al.*, 2006), acetone and methanol (Sharma and Khan, 2010). For antimicrobial assay, nutrient agar well diffusion (Caburian and Marina, 2010; Suppakul *et al.*, 2006) and broth dilution methods (Caburian and Marina, 2010; Sharma and Khan, 2010) have been used. The trend of results with respect to solvents reported was in relation that the antimicrobial activity is more in organic solvent extract than in aqueous solvent extract.

In the present investigation, an attempt was made to elucidate the possible antibacterial activity of different extracts of *Piper betel* plant (Ethanol, Chloroform, Petroleum ether) against the common Gram-positive and Gram-negative bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Pseudomonas putida*, *Salmonella typhi*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Klebsiella pneumoniae*, *Proteus mirabilis*) and also to compare their antibacterial potential against the test strains with respect to standard antimicrobial drug, levofloxacin.

MATERIALS AND METHODS:

Materials:

All chemicals used were of analytical-reagent grade and obtained from Riphah Institute of Pharmaceutical Sciences (Islamabad, Pakistan) lab supplies. *Piper betel* leaves were imported from Sirilanka and evaluated for specie confirmation.

Bacterialstrains:

Eleven bacterial strains (3 Gram-positive and 8 Gram-negative) were used for the study. The Gram-positive strains were *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 29213), *Micrococcus luteus* (ATCC 9341), and the Gram-negative strains included *Escherichia coli* (ATCC 25922), *Pseudomonas putida*, *Salmonella typhi* (ATCC 19430), *Shigella flexneri* (ATCC 25929), *Pseudomonas aeruginosa* (ATCC 33347), *Vibrio cholerae*, *Klebsiella pneumoniae*, *Proteus mirabilis* (ATCC 49565). The bacterial stock cultures were obtained from the culture collection unit of Department of Pharmaceutical Biotechnology, Riphah Institute of Pharmaceutical Sciences (Islamabad, Pakistan). The viability tests for each isolate were carried out by resuscitating the organism in nutrient agar medium. The stock on nutrient agar medium was incubated for 24h at 37°C following refrigeration storage at 4°C until required for sensitivity testing.

Preparation of sample:

The leaves of *Piper betel* were air-dried for 1 week, followed by crushing in pestle and mortar. To achieve fine dry powder, crushed leaves were further grinded with the help of Grinder Machine (Model No.SF-1012). The powder was weighed in a single pan electronic weighing balance and divided into three equal portions. All the three portions of dried powdered leaves were extracted with three different solvents i.e. Chloroform, Ethanol and Petroleum ether. The herbal extracts were prepared at the rate of 1g/50ml in soxhlet extractor. All the three mixtures were continuously extracted for about 4 hours at 100°C. The extract was then collected and concentrated under reduced pressure in a Rotary Vacuum Evaporator (Lot No. M10019040) until semisolid substance was obtained. The temperature of the rotary evaporator was adjusted according to the type of solvent under process. The semisolid mass was then air-dried to obtain solid mass. The powder was weighed and percentage yield for all the extracts was calculated. Powdered substances were reconstituted with same solvent as used for extraction to make 1% stock solutions (0.1g of dried extract in 10ml) and then stored under refrigeration at 4°C for further testing antibacterial activity.

Percentage yield of chloroform extract was 5% (1g powder was used, residue weight after drying of the extract was 0.16g), petroleum ether extract was 15% (1g powder was used, residue weight after drying of was 0.15g) and of Ethanolic extract was 39.5% (1g powder was used, residue weight after drying was 0.395g). This shows that ethanolic extraction has higher percentage yield as compared to, in other two solvents.

For positive control 1% levofloxacin solution was used and for the negative control all the three solvents (in which *Piper betel* leaf extract was prepared) were used i-e, ethanol, chloroform, petroleum ether. No zone of inhibition has been observed in negative control.

Antibacterial Assay:

The antibacterial activity of *Piper betel* leaf extract was determined by Agar Well Diffusion method against selected bacteria strains, as mentioned previously. In this method, pure isolate of each bacterium was sub-cultured in nutrient broth at 37°C for 24 hours. Turbidity of inoculated nutrient broth was checked and standardized by McFarland solution (0.5ml). About 100 micro liter of each test bacterium was spread with the help of sterile spreader on to a sterile nutrient agar plate so as to achieve a confluent growth. The plates were allowed to dry and then a sterile metal borer of diameter 8mm was used to form three wells in each of the agar plates. Each of the three wells was labeled for three different extract solutions. Subsequently, 50 micro liter volume of each extract was poured in relatively labeled well on nutrient agar plates. Levofloxacin, used as a positive control, in the centre of all the nutrient agar plates. The plates were allowed to stand for 30 minutes for diffusion to take place and then incubated at 37°C for 24 hours. After completion of incubation period, zones of inhibition were measured and recorded to the nearest size in millimeters.

RESULTS AND DISCUSSION

Following the extraction of dried leaves of *Piper betel* plant using Ethanol, Chloroform and Petroleum ether by reflux condensation method, the antimicrobial activity of the extracts was determined by agar-well diffusion method. Table shows the antimicrobial activity of the *Piper betel* leaf extracts on the selected Gram-positive and Gram-negative bacterial strains. The extracts were effective against both Gram-positive and Gram-negative bacteria.

Table 1: Zones of Inhibition (mm) of Ethanolic, Chloroform and Petroleum ether extracts on Bacterial Species.

No.	Bacterial Strain	Positive Control Levofloxacin	Ethanol Extract	Chloroform Extract	Petroleum ether Extract
1	<i>Staphylococcus aureus</i>	16	18	10	4
2	<i>Bacillus subtilis</i>	25	25	20	25
3	<i>Micrococcus luteus</i>	21	13	15	12
4	<i>Pseudomonas putida</i>	8	26	15	20
5	<i>Klebsiella pneumoniae</i>	8	33	23	33
6	<i>Escherichia coli</i>	7	12	8	4
7	<i>Shigella flexneri</i>	19	18	9	8
8	<i>Pseudomonas aeruginosa</i>	29	20	20	18
9	<i>Salmonella typhi</i>	20	23	13	17
10	<i>Proteus mirabilis</i>	33	20	15	16
11	<i>Vibrio cholerae</i>	65	58	44	45

The **ethanolic extract** turned out to be most effective for its antibacterial activity against all eleven selected bacterial strains. Widest zone of inhibition with a diameter of 58mm was observed in case of *Vibrio cholerae*, followed by *Klebsiella pneumoniae* with zone of inhibition extending up to 33mm(fig; 1). Amongst the Gram-negative bacteria, the extract showed higher activity against *Pseudomonas putida*, *Escherichia coli* and *Salmonella* with a diameter of zone of inhibition 26mm, 12mm and 23mm respectively.

The **Chloroform extract** was less effective for antibacterial activity as compared to Ethanolic extract. Similarly, widest zone of inhibition with a diameter of 44mm was observed in case of *Vibrio cholerae*, followed by *Klebsiella pneumoniae* with zone of inhibition extending up to 23mm. Zones of inhibition produced by chloroform extract for Gram-negative *Pseudomonas putida* and *Escherichia coli* were also observed to be wider than the positive control levofloxacin (Fig 1).

The **Petroleum ether extract** was least effective among all three extracts for its antibacterial activity. Like other two extracts, petroleum ether extract came up with widest zone of inhibition with a diameter of 45mm was observed in case of *Vibrio cholerae*, followed by *Klebsiella pneumoniae* with zone of inhibition extending up to 33mm. Only one Gram-positive bacteria *Bacillus subtilis* showed comparable results with levofloxacin (Fig 2).

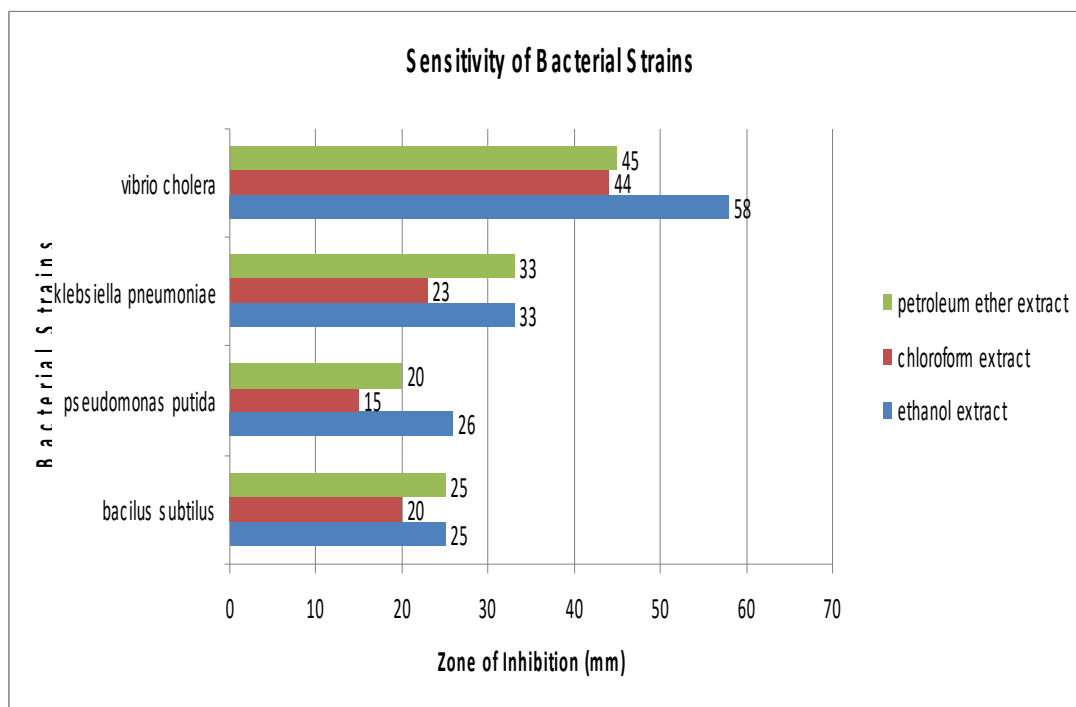


Fig 1: Comparative activities of *Piper betel* extracted with ethanol (blue), chloroform (red) and petroleum ether (green)

From this investigation, it was observed that *Piper betel* leaf extracts successfully inhibited growth of both groups of bacterial strains. It has been reported that the major constituents of *Piper betel* leaf are phenolic in nature that may be responsible for inhibiting bacterial growth. *Piper betel* leaf also possesses some volatile oils. As phenolic groups are slightly acidic in nature

and also possess slight polarity, maximum yield and maximum activity was observed for ethanolic extracts. Ethanol is slightly polar organic solvent that sufficiently dissolve slightly polar organic compounds. Whereas other two solvents are non-polar in nature, thus their percentage yield and activity was relatively less.

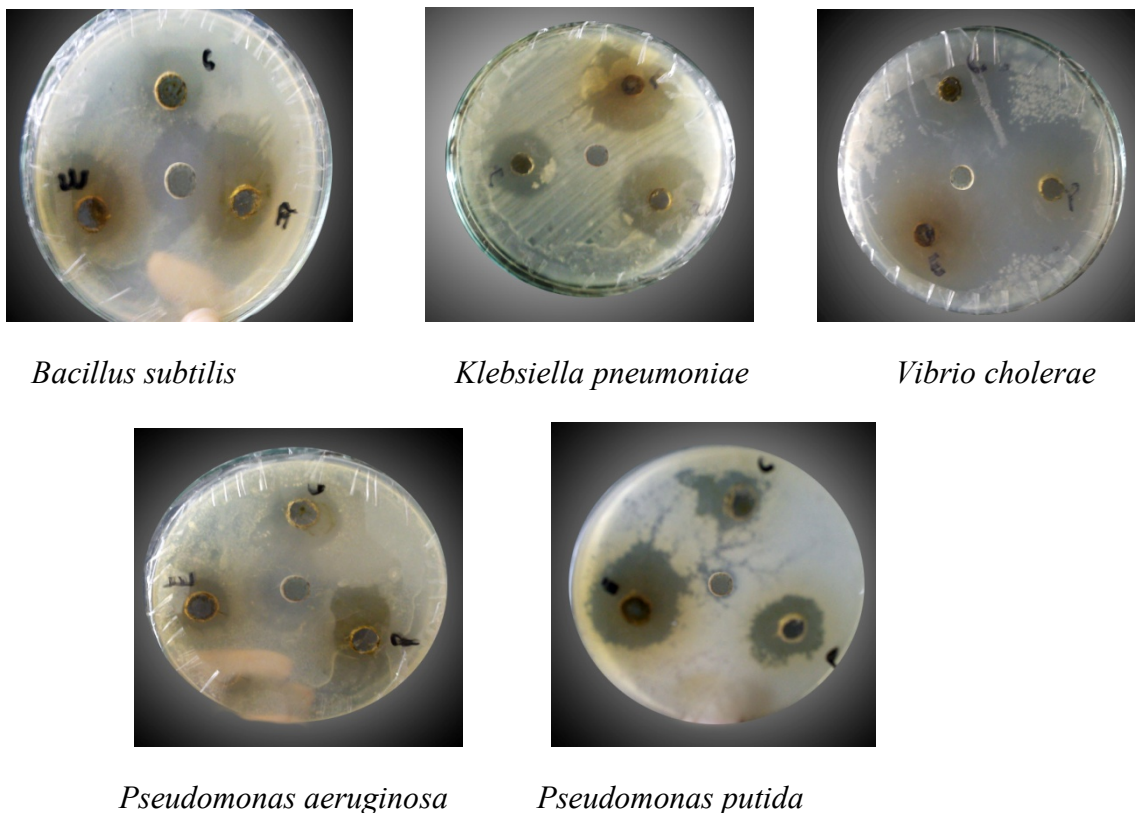


Fig 2: Bacterial strains demonstrating susceptibility to *Piper betel* extracts

CONCLUSION

From the results of this present study, it has been concluded that the extract of *Piper betel* leaf obtained through reflux condensation method was more effective against tested bacterial strains. The extracts were most effective against *Vibrio cholerae*, followed by *Klebsiella pneumoniae*, whereas *Escherichia coli* were found to be the least sensitive strain, all these belong to Gram-negative species. Among Gram-positive bacteria only the *Bacillus subtilis* proved to be the most sensitive strain.

The maximum yield was observed in case of ethanolic extract i.e. 39.5%, whereas yield for petroleum ether and chloroform extract was equivalent to 15% and 5% respectively. This result necessitated the investigation of the specific constituent that was responsible for antibacterial activity and such characterization studies are underway.

This study confirms the efficacy of *Piper betel* leaf extracts and their potential to be a good choice for the development of new strategies to treat gram-negative infections specially those of *Vibrio cholerae* and *Klebsiella pneumoniae*.

ACKNOWLEDGEMENT

The Authors are grateful to the Riphah Institute of Pharmaceutical Sciences (Islamabad, Pakistan) for supporting this research and bearing all expenses. We also express our gratitude to Dr. M. Zaman Ashraf for their guidance and help in this research study.

REFERENCES:

- 1 T. O. Ekpendu, A. A. Akshomeju, J. I. Ologun, *letters in applied microbiology*, **30**, 379-384 (1994).
- 2 M.W. Iwu, A. R. Duncan, C. O. Okunji, New antimicrobials of plant origin, In Janick J (Ed.) Perspectives on New crops and New Uses. ASHS Press, Alexandria., VA, p. 457-462 (1999).
- 3 M. Sousa, C. Pinheiro, M. E. O. Matos, F. J. Matos, M. I. Lacerda, A. A. Craveiro, Universidade Federal do Ceara, Fortaleza, p. 385-388 (1991).
- 4 L. Kubo, H. Muroi, M. Himejima, *Journal of Agricultural and Food Chemistry*, **41**, 1016-1019 (1993).
- 5 E. E. S. Shapoval, S. M. Silveira, M. L. Miranda, C. B. Alice, A. T. Henriques, *Journal of Ethnopharmacology*, **44**, 136-142 (1994).
- 6 N. Artizzu, L. Bonsignore, F. Cottiglia, G. Loy, *Fitoterapia*, **66**, 174-175 (1995).
7. A. A. Izzo, D. I. Carlo, G. Biscardi, D. Fusco, R. Mascolo, N. Borrelli, F. Capasso, F. Fasulo, M. P. Autore, *Phytotherapy research* **9**, 281-286 (1995).
8. Lucy Sun Hwang, Chin-Kun Wang, Ming-Jen Sheu and, Lung-Sen Kao. 1992. Phenolic Compounds in Food and Their Effects on Health. American Chemical Society
9. T. Nalina, Z.H.A. Rahim. The Crude Aqueous Extract of *Piper betel* L. and its Antibacterial Effect towards *Streptococcus mutans*. American Journal of Biotechnology and Biochemistry **3** (1): 10-15, 2007.
10. A.B. Caburian and O.O. Marina. Characterization and Evaluation of Essential Oil from the leaves of *Piper betel* L. E-International Scientific Research Journal **2** (1), 2010.
11. A. R. Fathilah, R. Sujata, A. W. Norhanom and M. I. Adenan, Antiproliferative activity of aqueous extract of *Piper betel* L. and *Psidium guajava* L. on KB and HeLa cell lines. Journal of Medicinal Plants Research **4** (11):987-990, 2010.
- 12 R.R Chattopadhyay, S. K. Bhattacharyya, C. Medda, S. Chanda and A.Bag. A comparative evaluation of antibacterial potential of some plants used in Indian traditional medicine for the treatment of microbial infections. Braz. arch. biol. technol. **52**(5), 2009.
13. L.S.Hwang, C.K. Wang, M.J. Sheu and, L.S. Kao. Phenolic Compounds in Food and Their Effects on Health. American Chemical Society, 1992.

-
14. T. Nalina and Z.H.A. Rahim. The Crude Aqueous Extract of *Piper betel* L. and its Antibacterial Effect towards *Streptococcus mutans*. American Journal of Biotechnology and Biochemistry 3 (1): 10-15, 2007.
 15. P.Suppakul, N. Sanla-ead and P.Phoopuritham. Antimicrobial and Antioxidant Activities of Essential Oil of Betel (*Piper betel* Linn.) Kasetsart J. Nat. Sci 40:91-100, 2006.
 16. S.Sharma and I.A. Khan. Evaluation of Antimicrobial, Antioxidant and Anti-inflammatory Activities of Hydroxychavicol for its Potential Use as oral care Agent. Antimicrobial Agents Chemother 53(1):216-222, 2009.