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Antimicrobial Activity of Betel Leaf Extracts

Aye Thida Htun[*]*, Saw Myat Thwe, Phyo Mon Oo, Wai Mi Aung, May Thandar Htun, Moh Moh Lwin, Rai Kit, Swe Swe & Yi Yi Myint*

Department of Medical Research (Upper Myanmar)

Piper betle L. is a well-known medicinal plant and widely distributed in Myanmar. In this study, antimicrobial activity of different extracts (95% ethanol and distilled water) and fresh juice of *Piper betle* L. leaf was studied on *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. Ceftriaxone was used as positive control. Antimicrobial activity of *Piper betle* L. leaf extracts was determined by agar disc diffusion method. Among the different extracts and fresh juice, 95% ethanolic extract showed more larger zone of inhibition, i.e., 7 mm to 12 mm for *Staphylococcus aureus* and *Pseudomonas aeruginosa* and 8 mm to 13 mm for *Escherichia coli*. Fresh juice (up to 30 µl) did not show the significant zone of inhibition on above microorganisms. Broth dilution method was used for determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ethanolic and aqueous extract of betel leaf. Although MIC and MBC of aqueous extracts did not show the concentration up to 11 mg/ml, MIC of ethanolic extract was observed in the range from 1 mg/ml to 8 mg/ml and MBC from 2 mg/ml to 9 mg/ml. Phytochemical analysis of *Piper betle* L. leaf was carried out according to the quality control method of World Health Organization. The presence of phenolic and tannin compounds seemed to exert antimicrobial activity. So, this study will provide referential information about the antimicrobial activity of different extracts and fresh juice of *Piper betle* L.

Key words: Piper betle L., Antimicrobial activity, Phenol, Tannin

INTRODUCTION

The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide, becoming an important cause of morbidity and mortality in immune compromised patients in developing countries.[1]

Although a large number of antimicrobial agents have been discovered, pathogenic microorganisms are constantly developing resistance to these agents. In addition to resistance problem, antibiotics are associated with adverse effects including hypersensitivity reactions, bone marrow depression, liver disease and kidney disease.[2] Resistance to antimicrobial agents has become an increasingly important and pressing global problem. A major cause in the UK is methicillin-resistant *Staphylococcus aureus* (MRSA).[3] Due to the emergence of the antibiotic resistant pathogens, plants are being looked upon as an excellent alternate to combat the spread of multidrug resistant microorganisms. There are some advantages of using antimicrobial compounds of medicinal plants, such as relatively less expensive, acceptance due to the long history of use and being renewable in nature.[4]

The medicinal value of plants lies in some chemical substances that produce a definite physiological action on human body. Therapeutic treatments can get great benefit from the use of plant extracts with

*To whom correspondence should be addressed.
Tel: +95-9796139133
E-mail: ayethidahtundmrum@gmail.com

known antimicrobial properties. Hence, more studies concerning with the use of plants in the treatment of infectious diseases should be emphasized.[5]

In the era of antimicrobial resistance to most anti-infective agents, newer, effective, safe and affordable agents should be explored scientifically. World Health Organization also encourages research programmes for traditional, complementary and alternative medicine by the use of national resources rather than chemical agents. Hence, more studies concerning with antimicrobial activity of natural product are required to be done.

Piper betle L. (Piperaceae) leaves are widely used as a medicinal plant and extensively grown in India, Sri Lanka, Malaysia, Thailand, Taiwan, Myanmar and other Southeast Asian countries. These leaves have antimicrobial activity towards bacteria in mouth i.e., *Streptococcus viridans, Staphylococcus aureus* and *Streptococcus mutans* and also heals many bacterial diseases. These compounds are assumed that they could inhibit food borne pathogens as well as food spoilage micro-organisms.[6] The ethanolic extracts of *Piper betle* L. (Kun) had significant antibacterial activity on both gram-positive and gram-negative bacteria.[7]

The present study was aimed to examine the *in vitro* antimicrobial activity of leaf extracts of *Piper betle* L. on some bacteria. The antimicrobial efficacy was assessed by agar disc diffusion technique.

MATERIALS AND METHODS

Piper betle L. leaves were collected from Kyaukse District, Mandalay Region. The test plant was identified by the taxonomist according to The Flora of Ceylon at Pharmacology Research Division, Department of Medical Research (Upper Myanmar).[8] Phytochemical analysis was done by the quality control method for WHO.[9]

Extraction of Piper betle L. leaves

Piper betle L. leaves were collected and thoroughly washed with water, and then air-dried for about two weeks. The dried leaves were powdered and extracted by percolation.[10] The powdered leaves of 100 g was percolated with 600 ml of 95% ethanol for a week in a percolator. The liquid extract was filtered and evaporated on the water bath until to get constant weight and stored in dessiccator. Aqueous extract of *Piper betle* L. leaves was obtained by using reflux apparatus. Fresh juice was prepared by using mortar and pestle and filtered the solution.

Determination of antimicrobial activity of different extracts of Piper betle L. leaves

The antimicrobial activity of different extracts of *Piper betle* L. leaves was determined by agar disc diffusion technique according to modified Kirby and Bauer method.[11]

Preparation of medium

Mueller-Hinton agar plate was prepared according to the procedure of the manufacturer's recommendation and sterilized by moist heat at 121°C for 15 minutes. After autoclaving, 25 ml of the media was poured into 9 cm diameter petridishes and allowed to set at room temperature. It was prepared freshly before use. When the agar had solidified, the plates were dried at 50°C by placing them in the upright position in the oven with the lids tilted. The plates were then labeled.

Preparation of bacterial suspension

A few colonies of organisms from the sub-culture to be tested were picked with a wire loop and introduced into test tube containing peptone solution. These tubes were incubated at 37°C for 3-4 hours to produce the growth turbidity.

Preparation of impregnated disc of different plant extracts

The discs, 6 mm in diameter, were spread out separately in petridishes, so that each

disc was not less than 2 mm from its neighbors. They were sterilized by dry heat at 160°C for 1 hour. Ethanolic extracts of betel leaf (30 mg, 60 mg, 90 mg, 120 mg, 150 mg, etc.,) were dissolved in 300 µl of 95% ethanol.

From the stock solution, 10 µl of solution was impregnated to discs resulting in the range of 1 mg/disc to 11 mg/disc, respectively and dried in the oven at 37°C to evaporate the solvent. Discs for aqueous extract of *Piper betle* L. leaf were done by the same procedure. Ceftriaxone (30 µg) was used as positive control reference standard. Disc as negative control was prepared using the same solvent employed to dissolve the plant extract.

Antimicrobial susceptibility test

Antimicrobial susceptibility test was determined by a standard disc diffusion technique using Mueller-Hinton agar according to the recommendations of Clinical and Laboratory Standards Institute (CLSI).

A sterile cotton swab was dipped into the bacterial suspension. Freshly grown liquid cultures of the test pathogens were seeded over the Mueller-Hinton agar (MHA) plates with a sterile cotton swab. The swab was streaked in at least three directions through the angle of 60° over the surface of the Mueller-Hinton agar to obtain uniform growth. A final sweep was made around the edge of the agar surface. After the inoculum has dried for a few minutes, the sterile filter paper discs impregnated with plant extracts were placed on the seeded MHA plates at equal distance with a sterile forceps and gently pressed down to ensure contact with the medium. The plates were incubated at 37°C for 24 hours. Following overnight incubation. The zone of inhibition around the discs occurred in the pate. The inhibition zones were recorded as millimeter.

Determination of minmum inhibitory concentration and minimum bactericidal concentration

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation, and minimum bactericidal concentrations (MBCs) as the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic-free media.

The determination of the MIC involves a semi-quantitative test procedure which gives an approximation to the least concentration of an antimicrobial needed to prevent microbial growth. MIC/MBC values can be determined by a number of standard test procedures. The most commonly employed methods are the tube dilution method and agar dilution method. Serial dilutions are made of the products in bacterial growth media. The test organisms are then added to the dilutions of the products, incubated, and scored for growth.

Determination of MIC and MBC of ethanolic and aqueous extracts of Piper betle L. leaf

The ethanolic and aqueous extracts of *Piper betle* L. leaf which showed zone of inhibition more than 7 mm were proceeded for MIC by broth dilution method.[12] Different concentrations of ethanolic and aqueous extract of *Piper betle* L. leaf ranging from 0.5 mg/ml to 11 mg/ml were tested against different test organisms. A series of nine tubes for each test organisms was prepared. Each tube contains 200 µl of test organisms in 2 ml of Mueller-Hinton broth. The different dilution of 0.5 ml of ethanolic extract of *Piper betle* L. leaf was added to the tubes. The ninth tube was used as control tube which contained Mueller-Hinton broth, 95% ethanol with test organisms. Then, the different dilution of aqueous extract was also done and incubated at 37°C for 24 hours. After incubation, minimum inhibitory concentration was recorded as tube with lowest concentration at which no visible turbidity was observed. For determination of MBC, one loopful from each tube of above dilutions was streaked on Mueller-Hinton agar plate and incubated at 37°C for 24 hours.

RESULTS

Plant identification

The plant was identified as *Piper betle* L. belonging to the family Piperaceae.

Yield percentage of plant extracts

The different weights of plant extracts of *Piper betle* L. leaves are shown in Table 1.

Table 1. Determination of yield (%) of different extracts of *Piper betle* L. leaves

Solvent	Weight (g)	Yield % (W/W)
95% ethanol	100	7.4
Aqueous	100	16

Phytochemical analysis

The results from qualitative tests of leaves are shown in Table 2.

Table 2. Results of phytochemical tests on *Piper betle* L. leaves

No.	Type of compound	Results
1.	Alkaloid	+
2.	Carbohydrates	+
3.	Glycosides	+
4.	Phenols	+
5.	Amino acids	-
6.	Saponin	+
7.	Starch	+
8.	Tannins	+
9.	Flavonoids	-
10.	Steroids	+
11.	Reducing sugars	+
12.	Terpenoids	-
13.	Cyanogenic glycosides	-

+=Detected, - =Not detected

Antibacterial activity of different extracts of Piper betle L. leaves

Antimicrobial activity of *Piper betle* L. leaf extracts was determined by agar disc diffusion method. Among the different extracts and fresh juice, 95% ethanolic extract showed larger zone of inhibition, i.e., 7 mm to 12 mm for *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and 8 mm to 13 mm for *Escherichia coli*. Fresh juice (up to 30 µl) did not show the significant zone of inhibition on above microorganisms. These results are shown in Table 3.

Table 3. Antibacterial activities of different extracts of *Piper betle* L. leaves

Test drug	Diameter of inhibition zone of different extracts of *Piper betle* L. leaves (mm)		
	Staph aureus	*P. aeruginosa*	*E. coli*
Ethanol 95% extract	12	12	13
Aqueous extract	9	9	10
Fresh juice (30 µl)	-	-	-

Paper disc 6 mm, Standard antibiotic Ceftriaxone 30 µg

Table 4. MIC and MBC of ethanolic and aqueous extracts of *Piper betle* L. leaf

Test organisms	Extract (mg/ml)		
	Ethanolic		Aqueous
	MIC	MBC	MIC
Staphylococcus aureus	1	2	>11
Escherichia coli	1	2	>11
Pseudomonas aeruginosa	8	9	>11

MIC=Minimum Inhibitory Concentration
MBC=Minimum Bactericidal Concentration

Determination of MIC and MBC of Piper betle L.

MIC and MBC of *Piper betle* L. extracts were determined by broth dilution method. MIC and MBC of ethanolic extract of *Piper betle* L. leaf were each of 8 mg/ml and 9 mg/ml for *Pseudomonas aeruginosa*, and 1 mg/ml and 2 mg/ml for *Staphylococcus aureus and Escherichia coli*, respectively. Minimum inhibitory concentration of aqueous extracts of *Piper betle* L. did not show the concentration up to 11 mg/ml on these bacteria. These results of ethanolic and aqueous extracts were shown in Table 4.

DISCUSSIONS

The yield of the aqueous extract of *Piper betle* L. leaf is higher than 95% ethanolic extract. This may be due to the constituents of leaves are more soluble in distilled water than alcohol. From phytochemical investigations, it was observed that alkaloids, glycosides, steroids, phenolic com-pounds, amino acids, tannin and carbohydrates were significantly present and flavonoid and cyanogenic glycosides were absent in the betel leaf. The phenolic compounds were among the most active

components against gram-positive and gram-negative bacteria.[13] Regarding the medicinal value of *Piper betle* L. leaves, antimicrobial properties may be due to the presence of phenolic compounds.

In this study, antimicrobial activity of the two extracts (95% ethanol and distilled water) and fresh juice of *Piper betle* L. leaf was studied on *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* by agar disc diffusion method. Among the different extracts and fresh juice, 95% ethanolic extract showed larger zone of inhibition than aqueous extract on *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. According to the results obtained from this study, ethanolic extract of betel leaf was more effective for antimicrobial activity than distilled water. This is due to the active constituents of *Piper betle* L. for anti-microbial activity may be more soluble in alcohol.

MIC and MBC of ethanolic extract of *Piper betle* L. leaf were each of 8 mg/ml and 9 mg/ml for *Pseudomonas aeruginosa*, and 1 mg/ml and 2 mg/ml for *Staphylococcus aureus and Escherichia coli*, respectively. MIC of aqueous extracts did not show the concentration up to 11 mg/ml on these bacteria. The previous study observed that MIC of ethanolic extract of *Piper betle* L. was 1 mg/ml for *Staphylococcus aureus* and *Escherichia coli*, and 2 mg/ml for *Pseudomonus aeruginosa*.[7]

Thus, the result of present study was agreed with the findings of this previous study for *Staphylococcus aureus* and *Escherichia coli* but different for *Pseudomonus aeruginosa*. This disparity may be due to different extraction methods, different species of strains and resistance of organisms.

Conclusion

The phytochemical analysis revealed the presence of various phytochemical constituents such as alkaloids, glycosides, steroids, phenolic compounds, amino acids, tannin and carbohydrates. Among the two extracts and fresh juice of *Piper betle* L. leaf, the antimicrobial activity of ethanolic extract was more effective against the test microorganisms such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*.

Recommendation

Conducting the study on the antimicrobial activity of *Piper betle* L. leaf on other microorganisms is needed.

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REFERENCES

1. Ara N, Nur MH, Amran MS, Wahid MI & Ahmed M. *In vitro* antimicrobial and cytotoxic activities of leaves and flower extracts from *Gliricidia sepium. Pakistan Journal of Biological Sciences* 2009; 12(1): 87-90.
2. Al-Barl MA, Sayeed MA, Rahman MS & Mossadik MA. Characterization and anti-microbial activities of a phenolic acid derivative produced by *Streptomyces bangladeshiensis* a novel species collected in Bangladesh. *Research Journal of Medicine and Medical Sciences* 2006; 1: 77-81.
3. Adcock H Pharmageddon: Is it too late to tackle growing resistance to anti-infectives? *Pharmaceutical Journal* 2002; 269: 599-600.
4. Reddy JL & Jose B. Evaluation of antibacterial activity of the bark, flower and leaf extracts of *Gliricidia sepium* from South India. *International Journal of Current Pharmaceutical Research* 2010; 2(3): 18-20.
5. Ahsan MR, Islam KM, Haque ME & Mossaddik MA. *In vitro* antibacterial screening and toxicity study of some different medicinal plants. *World Journal of Agricultural Sciences* 2009; 5(5): 617-621.
6. Farnsworth NR & Bunyapraphatsara N. Thai Medicinal Plants. Medicinal Plant Information Center, Faculty of Pharmacy, Mahidol University, Thailand, 1992; 402.
7. Kay Thwe Aung. *In vitro* antibacterial activity of *Piper betle* Linn.(Kun) *Thesis*, M.Med.Sc (Pharmacology), University of Medicine (MDY), 2009.

8. Dassanayake MD. A Revised Handbook to the Flora of Ceylon. Vol. VI, Amerind Publishing Co. Pvt. Ltd., New Delhi, 1987; 274-289.
9. World Health Organization. Quality control methods for medicinal plant materials, Geneva, 1998; 28-46.
10. British Pharmacopoeia. The pharmaceutical Press, HMSO, London: The general medical Council, 2007.
11. World Health Organization. Basic Laboratory Procedures in Clinical Bacteriology. 2nd ed, Geneva, 2003; 103-119.
12. Cruickshank R, Duguid JP & Marmion BP. Medical Microbiology. 12th ed. Churchill Living Son Ltd,, 1974; 2.
13. Suppakul P, Ead NS & Pheopuritham P. Anti-microbial and antioxidant activities of betel oil. *Journal of Natural Science* 2006; 40: 91-100.