ANTIMICROBIAL ACTIVITIES OF *PIPER BETLE* L. LEAVES

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Abstract

Piper betle L. (Kun) is a well-known medicinal plant and widely distributed in Myanmar. In this study the leaves of *Piper betle* L. were collected from Yamethin Township, Mandalay Region, in the month of June to July, 2016. The extraction were done with 95%, 70%, 50% ethanol, aqueous and fresh juice. Endophytic microorganisms were also isolated from the leaves of *Piper betle*. Six endophytic bacterial strains and three endophytic fungal strains were obtained. Antimicrobial activities of leaf extract and endophytic microorganisms were tested on the seven pathogenic organisms by paper disc diffusion method. The ethanolic leaves extracts of 95% showed significant effect against *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus.* Endophytic bacterial strains TZ-2 and TZ-5 showed activities against *Candida albicans* but endophytic fungal strains did not show the effect against on seven pathogenic organisms.

Introduction

Piper betle L. (Kun) belongs to the Piperaceae family. The betel plant is an evergreen and perennial creeper (Houghton, 2001). The betel plant is indigenous to South and South East Asia (Mukherjee, 2000). Betel leaf is used as paan by Asian emigrants, with or without tobacco. The betel leaf is a heart shaped with different size (Dixit *et al*., 1995).

Betel leaf is traditionally known to be useful for the treatment of various diseases like bad breath, boils and abscesses, conjunctivitis, constipation, headache, itches, mastitis, mastoiditis, leucorrhoea, otorrhoea, swelling of gum, rheumatism, cuts and ayurvedic preparations (Sharma, 1991). Leaves were considered useful in treating bronchitis and dyspnea. The leaves were chewed by singers to improve their voice. The fruit of *Piper betle* employed with honey as a remedy for cough (Usmanghani *et al*., 1997).

Myanmar Traditional Medicine is the national heritage and have been existing since time immemorial. Eighty percent of Myanmar population live

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in countryside and there have many difficulties in gaining access to modern medicine. Thus, herbal medicines have become valuable and readily available resources for primary health care. Meanwhile, it has been realized that medicinal plants are invaluable resources for new pharmaceutical products and potential sources of new drugs as well as for economic development. A growing interest in the usage of 2 medicinal plants has created the need for scientific investigation. To enhance the quality and promote the systematic development of traditional herbal medicine assurance of the safety, quality and efficacy of medicinal plant has now become a key issue. *Piper betle* L. is an evergreen perennial creeper. Betel leaves can be used in many ways; for example as masticatory in betel quid, as spice, as poultice and as an applicant to the chest and abdomen. Myanmar people have the custom of offering the betel quid in traditional ceremonies (Kay Thwe Aung, 2008).

The fresh betel leaves possess antimicrobial, antifungal, antiseptic and antihelminthic effects (Chandra *et al*., 1987).The leaf has a significant antimicrobial activity against broad spectrum of microorganisms (Sarkar *et al*., 2013).The betel shows the antimicrobial activity against *Streptococcus pyrogen, Staphylococcus aureus, Proteus vulgaris, Escherichia coli, Pseudomonas aeruginosa* etc., and beside this, the leaf extract also possess the bactericidal activity against the urinary tract pathogenic bacteria (Agarwal and Singh, 2012).

In this work, the comparative study was done on antimicrobial activities of leaf extract and endophytic microorganisms were tested on seven pathogenic organisms.

Materials and Methods

Sample collection of *Piper betle* L.

Plants samples of *Piper betle* L. were collected from Yamethin Township, Mandalay Region, in the month of June to July, 2016.

Extraction of *Piper betle* L. leaves

Hundred grams of powdered leaves were soaked in 500 ml of aqueous and (95%, 70% and 50% ethanol), shaking 200rpm for seven days respectively. Hundred grams of fresh leaves were ground for fresh juice. Then the infusion was filtered by using filter paper and the residue was discarded. The solvent extracts were concentrated by using water bath at 70°C to

evaporate the solvent. After complete solvent evaporation, the crude extracts were weighed and recorded.

Preparation of *Piper betle* L. leaves extract for antimicrobial activity

Leaves extracts 0.1g, 0.5g and 1g were dissolved in 1 ml of aqueous, 95%, 70% and 50% ethanol and these were used for antimicrobial test.

The morphological characters of endophytic microorganisms

The morphological characters of endophytic microorganisms were studied under light microscope. According to the morphological characters, the isolated microorganisms were named TZ-1, TZ-2, TZ-3, TZ-4, TZ-5, TZ-6, TZ-7, TZ-8, and TZ-9.

Test organism for antimicrobial activities

Seven strains of clinical pathogen and one strain of phytopathogenic bacteria were obtained from Department of Medical Research (PyinOoLwin branch). These test organisms were sub cultured on nutrient agar slant for 24 hours. Then, these were inoculated into 5 ml of nutrient broth and incubated for 4-5 hour on shaker of 200rpm at room temperature.

Antimicrobial activity of leaves extract

The paper disc diffusion method (Atlas, 1993) was used to determine antibacterial activities of *Piper betle* L. leaves extract. Twenty-five milliliter of sterilized nutrient agar cool to 45°C and mixed with 20µl of test organisms. The mixture was poured onto each petri dishes, spread anticlockwise and allowed to stand for 15 minutes to solidify. The petri plates were placed in inverted position. Each disc with 20µl extract were gently pressed down on nutrient agar medium by using sterilized forceps. The plates were incubated at 37°C, overnight in incubator. Twenty microliter of 95% ethanol were applied on paper disc as control. Antibacterial activity showed as the zone of inhibition produced by plant extract. Zone of inhibition were assessed to evaluate by ruler and recorded in milliliter. This experiment was done in the Department of Medical Research (PyinOoLwin Branch) and it was carried out duplicate.

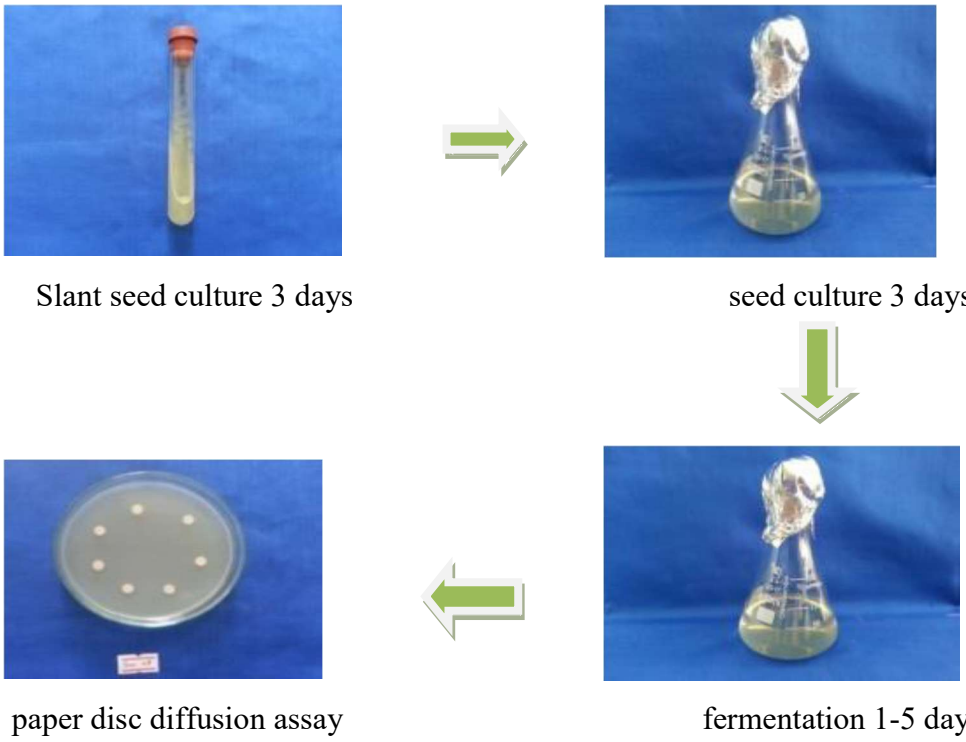


Figure 1. The antimicrobial activities by paper disc diffusion assay (Atlas, 1993)

Antimicrobial activity of endophytic microorganisms by paper disc diffusion assay

The isolated microorganism strains were grown on nutrient agar medium then one loop of isolated microorganisms was transferred into 50ml of seed medium and incubated at 30ºC for 3days. Five milliliter seed culture was transferred into 50ml fermentation medium and incubated at 30ºC for 5days on reciprocal shaker at 200 strokes/min. The fermentation was carried out for 5days. During the fermentation, the fermented broth between 1-5 days was applied on the paper disc (6mm in diameter) and allowed to dry in the air. For the antimicrobial activity, test organisms were separately inoculated in the nutrient broth for 4-5 hours, and 20µl of each test organisms were added to each medium, and then poured into plates. After solidification, paper disc impregnated with fermented broth were applied on agar plates and the plates were incubated for 24-36 hours and the inhibitory zones were recorded.

Medium used Antimicrobial activities test (Atlas, 1993, Phay N.1997)

Seed medium		**Fermentation medium**	
Composition per liter		Composition per liter	
Glucose	20.0g	Glucose	20.0g
Peptone	3.0g	Peptone	3.0g
KNO_3	1.0g	K_2HPO_4	0.1g
K_2HPO_4	0.1g	$MgSo_4$	0.1g
pH	6.5	$CaCo_3$	1.0 g
		pH	6.5

Results

The ethanolic leave extracts

The results of crude extract of *Piper betle* L. leaves by various ethanolic concentrations, aqueous and fresh juice. The highest amount of extracts 15.23g were obtained from aqueous, followed by 13.11g from 50% ethanol, 10.32g from 70% ethanol and lowest amount of extracts 10.21g from 95% ethanol.

Table(1) The crude extract of *Pipper betle* (L) leaves by various ethanol concentration

	Ethanol concentration	Crude extract (g)
1	95%ethanol	10.21
2	70%ethanol	10.32
3	50%ethanol	13.11
4	Aqueous	15.23

The morphological characters of isolated endophytic bacteria from leaf of *Piper betle* L.

The morphological characters of isolated endophytic bacteria from leaf of *Piper betle* L. were shown in table 2.

Table 2. Colony characters of isolated bacteria Morphology

	TZ – 1	TZ - 2	TZ - 3	TZ - 4	TZ - 5	TZ - 6
Color of colony	Cream	Pale yellow	Cream	Pale yellow	Pale yellow	Cream
Nature of colony	Irregular, Mucous present	Circular, Mucous present	Irregular, Mucous present	Circular, Mucous present	Irregular, Mucous present	Circular, Mucous present
Edges	Undulate	Entire	Undulate	Entire	Undulate	Entire
Size	2-3mm	3mm	1-2mm	0.5mm	1.5mm	2mm
Cell Shape	Chain	Short rod	Chain	Short rod	Short rod	Chain
Gram-staining	Positive	Negative	Positive	Negative	Negative	Negative
Oxygen requirements	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic
Translucency	Opaque	Translucent	Opaque	Translucent	Translucent	Opaque

Antimicrobial activities of ethanolic extracts *Piper betle* L.

Antimicrobial activities of various ethanol concentrations were carried out by paper disc diffusion method. Only 95% ethanol leaves extracts showed significant effect against *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus.* The tested leave extracts 0.1g dissolved in 1 ml of 95% of ethanolic extracts showed antimicrobial activity against on *Bacillus subtilis* (8mm), *Escherichia coli* (7mm), *Staphylococcus aureus* (8mm). The tested leaves extracts 0.5g dissolved in 1 ml of 95% of ethanolic extracts was showed effect on *Bacillus subtilis* (8mm), *Escherichia coli* (7mm), *Staphylococcus aureus* (9 mm), *Pseudomonas aeruginosa* (20mm). The tested leaves extracts 1gdissolved in 1 ml of 95% of ethanolic extracts

was showed significant effect against *Bacillus subtilis* (9mm), *Escherichia coli* (8mm), *Staphylococcus aureus* (9mm), *Pseudomonas aeruginosa* (22mm). The highest inhibitory zone size was recorded from 1g of 95% ethanolic extracts that against *Pseudomonas aeruginosa* (22mm). The fresh juice and aqueous extracts were not effect on test organisms. The diameters of inhibition zones that appeared were shown in Table 3, Figure 2.

Table3. Antimicrobial activity of different concentrations of leaves by 95% ethanol extract against clinical and pathogenic bacteria

No	Test organisms	Size of clear zone given by different concentration in 95% ethanol extract		
		0.1 g	0.5 g	1.0 g
1	*Aspergillus brasiliensis*	-	-	-
2	*Bacillus subtilis*	8 mm	8 mm	9 mm
3	*Candida albicans*	-	-	-
4	*Escherichia coli*	7 mm	7 mm	8 mm
5	*Pseudomonas aeruginosa*	-	20 mm	22 mm
6	*Staphylococcus aureus*	8 mm	9 mm	9 mm
7	*Salmonella enterica*	-	-	-

Size of paper disc = 6mm

Antimicrobial activities of isolated endophytic bacteria from leaf of *Piper betle*L.

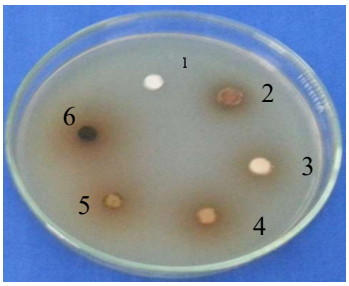
Antimicrobial activities of endophytic bacterial strains were tested on the seven pathogenic organisms by paper discs diffusion method. Endophytic bacterial strains TZ-2 and TZ-5 showed against *Candida albicans*. Days 2 fermentation of endophytic bacterial strain TZ-2 was showed antimicrobial activities against *Candida albicans* (15mm). Days 3 fermentation of endophytic bacterial strain TZ-2 was showed antimicrobial activities against *Candida albicans* (15mm). Days 4 fermentation of endophytic bacterial strain

TZ-2 was showed antimicrobial activities against *Candida albicans* (10mm). Days 5 fermentation of endophytic bacterial strain TZ-2 was showed antimicrobial activities against *Candida albicans* (10mm). Only days 2 fermentation of endophytic bacterial strain TZ-5 was showed antimicrobial activities against *Candida albicans* (10mm). The diameters of inhibition zones that appeared were shown in Table 4 and Figure 3.

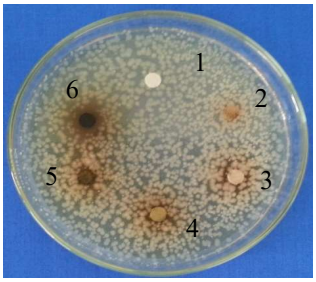
Table 4. Antimicrobial activities of TZ- 2

Test organisms	Antimicrobial activity in millimeter (mm)				
	Day1	Day2	Day3	Day4	Day5
Aspergillus brasiliensis	-	-	-	-	-
Bacillus subtilis	-	-	-	-	-
Candida albicans	-	15	15	10	10
Escherichia coli	-		-	-	-
Pseudomonas aeruginosa	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-
Salmonella enterica	-	-	-	-	-

Size of paper disc = 6mm



Zone of inhibition on *A. brasiliensis*



Zone of inhibition on *B. subt*

1=control

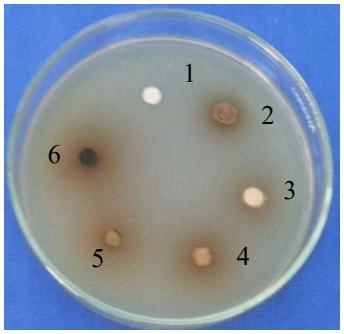
2=fresh juice

3=aqueous

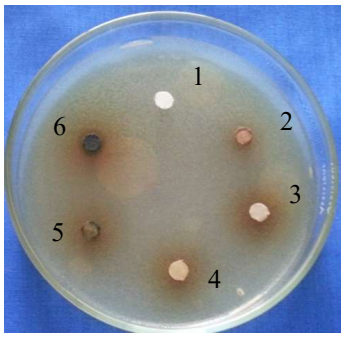
4=50%EtOH

5=70%EtOH

6=95%EtOH



Zone of inhibition on *C. albicans*



Zone of inhibition on *E. coli*

1=control

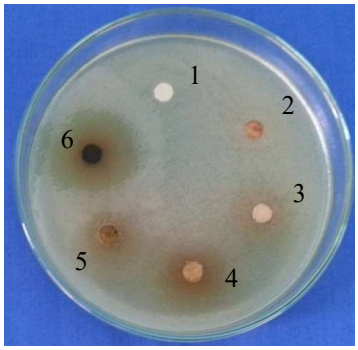
2=fresh juice

3=aqueous

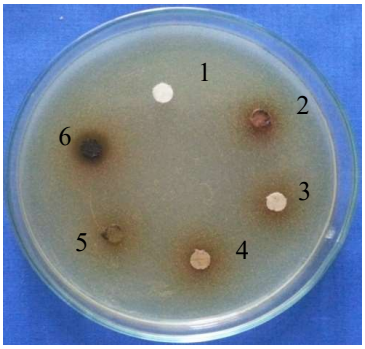
4=50%EtOH

5=70%EtOH

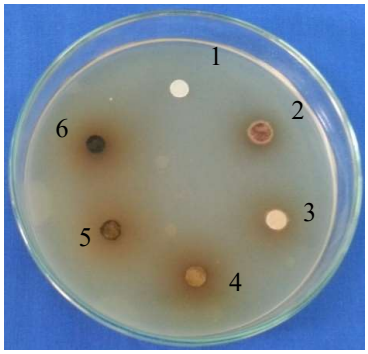
6=95%EtOH



Zone of inhibition on *P. aeruginosa*

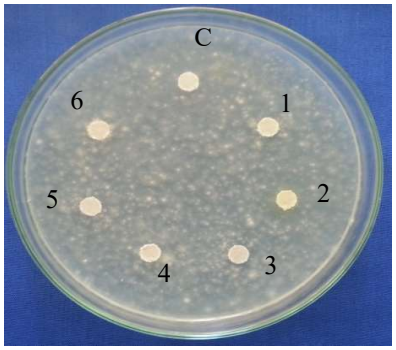


Zone of inhibition on *S. aureus*

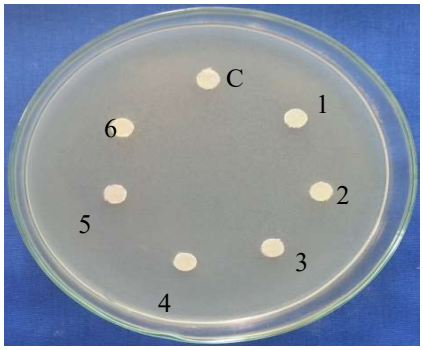


Zone of inhibition on *S. enterica*

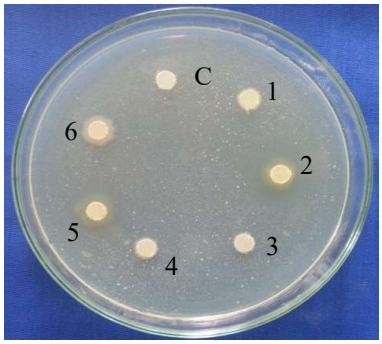
Figure 2. Antimicrobial activity provided by leaf extract 1.0 g dissolve in 95% ethanol



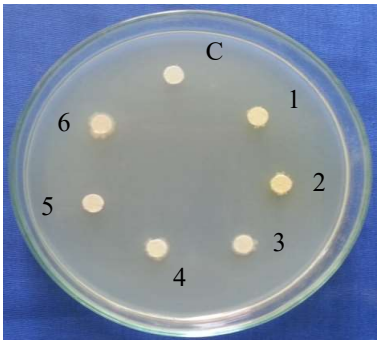
Zone of inhibition on *A. brasiliensis*



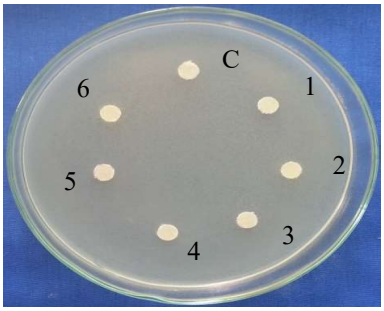
Zone of inhibition on *B. subtilis*



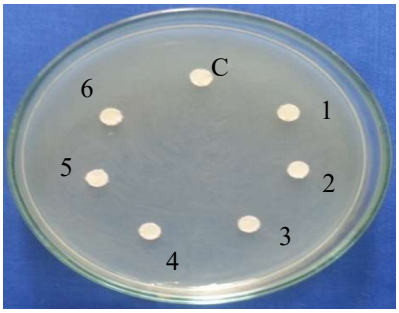
Zone of inhibition on *C.albicans*



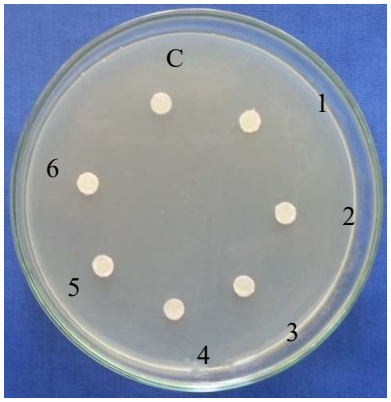
Zone of inhibition on *E.coli*



Zone of inhibition on *P.aeruginosa*



Zone of inhibition on *S.aureus*



Zone of inhibition on *S.enterica*

Figure 3. Antibacterial activity of isolated bacteria

Antimicrobial activities of isolated endophytic fungi from leaf of *Piper betle* L.

Antimicrobial activities of isolated endophytic fungal strains were tested on the seven pathogenic organisms by paper discs diffusion method. Endophytic fungal strains showed no effect on seven pathogenic organisms.

Discussion and Conclusion

The fresh leaves of betel was collected from Yamethin Township, Mandalay Region. The total of 3520g of fresh leaves were dried at 25°C for 20days for dry weight. The leaves dry weight of 438g were constant. The constant leaves dry weight 438g were powdered to use for extraction. The dry weight of leaves was significantly reduced 3520g to 438g. Each 100g of leaves powdered were mixed with 95%, 70%, 50% of ethyl alcohol and also aqueous. The results of crude extract of *Piper betle* L. leaves by various ethanolic concentration, aqueous and fresh juice were shown in Table (2). The highest amount of extracts 15.23g were obtained from aqueous, followed by 13.11g from 50% ethanol, 10.32g from 70% ethanol and lowest amount 10.21g from 95% ethanol respectively. The highest amount of extracts was recorded from aqueous. The aqueous extract of *Piper betle* L. leaf is higher than other ethanolic extracts. It may be due to the leaves constituents are more soluble in distilled water than ethanol.

Antimicrobial activities of various ethanol concentration were carried out by paper disc diffusion method. Only 95% ethanol leaves extracts showed significant effect against *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*. The tested leaves extracts (0.1g) 95% of ethanolic extracts was showed against on *Bacillus subtilis* (8mm), *Escherichia coli* (7mm), *Staphylococcus aureus* (8mm). The tested leaves extracts (0.5g) 95% of ethanolic extracts was showed effect on *Bacillus subtilis* (8mm), *Escherichia coli* (7mm), *Staphylococcus aureus* (9 mm), *Pseudomonas aeruginosa* (20mm). The tested leaves extracts (1g) 95% of ethanolic extracts was showed significant effect against *Bacillus subtilis* (9mm), *Escherichia coli* (8mm), *Staphylococcus aureus* (9mm), *Pseudomonas aeruginosa* (22mm). The highest inhibitory zone size was recorded from 1g of 95% ethanolic extracts that against *Pseudomonas aeruginosa* (22mm).The fresh juice and aqueous extracts were not effect on test organisms. Fresh juice did not show the inhibition zone on Escherichia coli, *Pseudomonas aeruginosa*, *Staphylococcus aureus* (Aye Thida Htun, 2015), but not agree with Ratnasooriya and Premakumara, 1997, they reported that the fresh betel leaves possess antimicrobial, antifungal, antiseptic and antihelminthic effects. The diameters of inhibition zones that appeared were shown in table 5, 6 and 7. According to the table control disc prepared solely with 95%, 70% and 50%ethanol showed no antimicrobial activity. Thus, it can be assumed that, the zone of inhibition could not be due to the ethanol solvent. Among the different extracts, only 95% ethanol leaves extracts showed significant effect against B*acillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*. Agarwal and Singh (2012) reported that the betel shows the antimicrobial activity against *Streptococcus pyrogen*, *Staphylococcus aureus, Proteus vulgaris, Escherichia coli, Pseudomonas aeruginosa*. The betel leaf has a significant antimicrobial activity against broad spectrum of microorganisms (Jesonbabu *et al*., 2012).

Six endophytic bacterial strains and three endophyti7[c fungal strains were also isolated from the leaves of *Piper betle*. Antimicrobial activities of endophytic bacterial strains were tested on the seven pathogenic organisms by paper discs diffusion method. Endophytic bacterial strains TZ-2 and TZ-5 showed against *Candida albicans*. Days 2 fermentation of endophytic bacterial strain TZ-2 showed antimicrobial activities against *Candida albicans* (15mm). Days 3 fermentation of endophytic bacterial strain TZ-2 was showed

antimicrobial activities against *Candida albicans* (15mm). Days 4 fermentation of endophytic bacterial strain TZ-2 was showed antimicrobial activities against *Candida albicans* (10mm). Days 5 fermentation of endophytic bacterial strain TZ-2 was showed antimicrobial activities against *Candida albicans* (10mm). Antimicrobial activities showed 15 mm in diameter against *Candida albicans* on days 2 and days 3 fermentation, but the activities reduced 10mm in diameter on days 4 and days 5 fermentation were observed. Only days 2 fermentation of endophytic bacterial strain TZ-5 was showed antimicrobial activities against *Candida albicans* (10mm). TZ-5 strain inhibited the growth of *Candida albicans* only on days 2 fermentation, but the activities did not show on days 3, days 4 and days 5 fermentation. In this study, seven test organisms were used and one out of seven was inhibited by endophytic bacterial strains TZ-2 and TZ-5 were observed. Endophytic fungal strains did not effect on seven pathogenic organisms. It is not agreed with Ali *et al*., 2010, stated that the leaf of *Piper betle* possess the antifungal activity against many fungal infections.

It is conclude that among the different ethanolic extracts, aqueous and fresh juice of *Piper betle* L. leaf, the antimicrobial activity of only 95% extract was effective against the test organisms such as Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus. Comparing with the antimicrobial activities of leaf extracts and endophytic microorganisms, it is observed that, the activity against the test organisms were not the same. The ethanolic leaf extract is more active than that of the endophytes. It is agreed with Jesonbabu *et al*., 2012 who stated that the leaf has a significant antimicrobial activity against broad spectrum of microorganisms. It could be suggested that the leaves of *Piper betle* L. can be useful as a medicine for some local diseases.

References

Agarwal, T and R. Singh. (2012). Evaluation of Antimicrobial Activity of *Piper betel* cultivars, Novus International Journal of Pharmaceutical Technology, 1(1), pp. 50-58.

Agarwal T., R. Singh, A.D. Shukla, I. Wari. and A. Gujrati. (2012). Comparative analysis of antibacterial activity of four *Piper betel* varieties, Advances in Applied Science Research, 3 (2), pp. 698-705.

Ali I., F. G. Khan, K.A. Suri, B.D. Gupta, N. K. Satti, P. Dutt, F. Afrin, G. N. Qaziand I.A. Khan. (2010). In vitro antifungal activity of hydroxyl chavicol isolated from *Piper betle* L., Annals of Clinical Microbiology and Antimicrobials, 9(7), pp1-9.

Atlas, M. Ronald. (1993). Handbook of Microbiological Media CRC Press, London

Aye Thida Htun and Saw Myat Thwe. (2015). Myanmar Health Sciences Research Journal, vol. 27, No.3.

Backer, C.A. (1968). Flora of Java, Neordhoff N. V. Groningen Company, Netherland.

Chandra T., J. Sadique and S. Somasundaram. (1987). Effect of Elipta alba on inflammation and liver injury, Fitoterapia, 58, pp.23-31.

Chopra RN, SL. Nayar and IC. Chopra. (1956). Glossary of Indian Medicinal Plants CSIR, New Delhi.

Dixit BS, R Banerji, Aminuddin and JK. Johri. (1995). Diosgenina Constituent of *Piper betle* L. root. Indian J Pharm Sci., 57(6): pp.263-264.

Harborne JB. (1984). Phytochemical Methods, 2nd Edition, Chapman and Hall, London.

Houghton PJ. (2001). Old yet new-pharmaceuticals from plants. J ChemEduc; 78:pp.175-184.

Hooker, J.D. (1875). Flora of British India, Vol.I.L, Reeve and Company, England.

Jesonbabu J., N. Spandana and K. A. Lakshmi. (2012). In vitro antimicrobial potentialities of chloroform extracts of Ethano medicinal plant against clinically isolated human pathogens, Int. J. Pharm. Pharm. Sci., 4(3), pp. 624-626.

Kay Thwe Aung. (2008). In vitro antibacterial activity of *Piper betle* L. (Kun)

Mukherjee PK. (2000). Quality control of herbal drugs. 1st ed. Business Horizons New Delh:Pharm

Sarkar M., P. Gangopadhyay and B. Basak. (2013). There versible antifertility activity of *Piper betel* Linn. on Swiss albino male mice, Contraception, 200, 62, pp. 271–274.

Sharma R. M. (1991). Contribution of Ayurveda towards management of bronchial asthma, Sachitra Ayurveda. 44, pp. 349-352.

Usmanghani K. A. Saeed and M. T. Alam (1997). Piper betle In, Indusvunic medicine, (University of Karachi Press), Karachi. pp. 340-341.