CHEMICAL INVESTIGATION AND ANTIOXIDANT ACTIVITY OF *Piper betel* L. (KUN) AND ITS POSSIBLE USE IN DRUGS FOR DENTAL AND SKIN INFECTIONS

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Abstract

Today, there are various types of infections. Among them, dental infections and skin infections are very common in developing countries. Myanmar medicinal plant; *P.betel* (KUN) used for the treatment of dental infections and skin infections, was screened for antimicrobial activity by agar disc diffusion technique. Polar and non-polar solvents were employed for the extraction of leaves of *P. betel.* Essential oil was obtained from leaves by steam distillation. The antimicrobial activity of crude extracts and essential oil of *P. betel* was studied on seven species of *Staphylococcus aureus* cultured from plaque of gingivitis and five strains of organisms which usually cause skin infections. It was observed that extracts and essential oil of *P. betel* were effective against all the tested microorganisms (11 mm - 40 mm). The minimum inhibitory concentration (MIC) of the active extract was evaluated by agar disc diffusion method. Acute toxicity of 70 % ethanol and watery extracts of the leaves evaluated by the methods of Organization for Economic Cooperation and Development (OECD) guidelines showed no toxicity. Eugenol (0.1 %) was isolated from essential oil of *P. betel* and identified by UV, FT IR and EI-MS. In addition, an ointment was formulated with essential oil of *P. betel* and *in-vivo* test was performed on induced open wound infected with *Staphylococcus aureus* on rats. The ulcer healing was observed within 9 days. Moreover, *in-vitro* antioxidant activity of ethanol and watery extract were determined by Dot-Blot and DPPH staining method. The results thus indicated the possible uses of *P. betel* leaf in the formulations of drug for dental and skin infections.

Keywords: antimicrobial, essential oil, dental infection, skin infection, *Piper betel*

Introduction

In Myanmar, some herbal medicinal plants are effective against bacteria in oral cavity and are used in the treatment of oral diseases. Skin infections are treated by many antibiotic ointments, creams and other topical

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applications. However, some bacteria are resistant to some antibiotics which may delay healing. Thus ointments and topical applications prepared by using extracts of medicinal plants that are very common and easily available is desirable (Mi Mi Htwe *et al*., 2001).

Piper betel L. belongs to the family Piperaceae and also known as KUN in Myanmar. It is a common plant cultivated in Asia countries. *P. betel* leaves are used in folk medicine for the treatment of various disorders and are commonly chewed among Asians (Arambewela, 2006). It is an indigenous medicinal plant and is relatively cheap, easily available and less expensive. Although antibacterial activities of petroleum ether (60 - 80 ℃), methanol and watery extracts of *P. betel* (KUN) were reported in Myanmar (Hnin Hnin Aye, 2002), no paper has been published regarding the evaluation of antimicrobial activity against infectious bacteria causing dental and skin infections. Thus the present study focused on the investigation of antimicrobial activity of the essential oil and solvent extracts of *P. betel* on microorganisms from dental infections and skin infections, with the aim of making economic production of antimicrobial and preparation of ointment for wounds and sores. Therefore, the present study is expected to provide valuable information in traditional and alternative medicine practice.

Materials and Methods

Sample

The plant sample, the leaves of *P.betel* (KUN) was collected from Tontay Township, Yangon Region, in January and February 2016. Botanical identification of plant sample was confirmed by Daw Khin Lay, Associate Professor and Head, Department of Pharmacognosy, University of Pharmacy, Yangon.

Preparation of crude extracts

Dried powders of leaves were extracted with petroleum ether (60 - 80 ℃), 95 % ethanol and water using Soxhlet apparatus. Extraction time with each solvent was 6 h. After removing each solvent by rotary evaporator, the crude extract was dried and kept in desiccator.

Extraction of essential oil from *P. betel* L.

Extraction of essential oil from *P. betel* was carried by steam distillation. The dried powder (100 g) and distilled water (500 mL) were placed in 1 L round bottomed - flask. The flask was fitted for steam distillation and heated. The steam was passed into the flask. The condensed oil and water were collected in the flask and the oil was extracted with petroleum ether (60 – 80 ℃) in a separating funnel. The PE extract was dried over anhydrous sodium sulphate. After filtration, the filtrate was evaporated to get the essential oil which was weighed and kept in air tight bottle for further analysis (Pauli, 2001).

Antimicrobial activity

A sterile cotton swab was dipped into the bacterial suspension and streaked evenly three times by rotating the plates through an angle of 60 ℃ onto the surface of the nutrient agar plate before the plates were seeded. After the inoculum has dried for a few minutes, the dried discs impregnated with plant extract were placed on the agar with a flamed forceps and gently pressed down to ensure contact. A control disc, impregnated with solvent only and clinical drug discs were also included. Plates were incubated at 37 ℃ in an incubator within 30 min after inoculation. After overnight incubation, the zone of inhibition diameters (including 6 mm disc) were measured (WHO, 2003).

Determination of minimum inhibitory concentration (MIC) of the active extracts by agar disc diffusion method

In order to determine the least concentration of extracts that inhibit the growth of microorganisms, the specific concentrations of extracts were prepared in serial dilution with respective solvents (e.g. ethanolic extract with ethanol). The crude extract (0.1 g) was dissolved in 1 mL of respective solvent as a stock solution. And then 500 mL was carried over from the stock solution and diluted with 500 mL of respective solvent to obtain the following concentrations: (10, 5, 2.5 and 1.25 mg/mL) and used to testing with micro-organisms. The bacterial broth suspension was streaked onto the surface of the medium. After the inoculum was dried for five minutes, the dried discs incorporated with plant extracts which were diluted into various concentrations. Then, these discs were placed on the agar with a flamed

forceps and gently pressed down to ensure contact. The plates were incubated at 37 ℃ in an incubator within 30 min after inoculation. After overnight incubation, the lowest dilution of the plant extracts where organisms could not grow was taken as the MIC (Stokes *et al*., 1993).

Acute toxicity study of both 70 % ethanol and watery extracts on albino mice

To determine the consequence of utilization of the plant, the acute toxicity test was done to determine the nature and degree of toxicity produced by the plant extracts as the medium lethal doses (LD50). In this study, acute toxicity of 70 % ethanol and watery extracts of P. betel leaves were determined on albino mice, at Laboratory Animal Services Division, Department of Medical Research (DMR), and Yangon. Acute toxicity of different doses of the extracts was evaluated by the methods of OECD (Organization for Economic Cooperation and Development) Guidelines. They were fasted for 18 h before giving the extracts. Group (A) mice were orally administrated with ethanol extract of 2000 mg/kg dose. Group (B) mice were given orally with ethanol extract of 5000 mg/kg dose. Group (C) mice were orally administrated with watery extract of 2000 mg/kg dose. Group (D) mice were also given orally with watery extract of 5000 mg/kg dose. Group (E) mice performed as a control group and they were treated with clean water and normal animal food. All groups of mice were kept in the three mouse cages in the separated room at the room temperature of 26 ± 1 ℃. After administration of extract on each group of animals were observed first 6 hours continuously for mortality and behavior changes. Then check the animals each 24 h for fourteen days. The mortality during this period was noted (OECD, 2000).

***In- vivo* evaluation the healing activity of *Staphylococcus aureus* induced open wounds**

Both sexes of adult rats (154 g to 225 g), comprising four animals per group were used in the experiments: open wounds to be infected were made, using a modified method (Skerman, 1967). Inoculation of the wounds with *Staphylococcus aureus* was done as described above method. The flanks were chosen for the sites of operation. The areas were initially shaved with a razor blade and then sterilized by applying serially with methylated sprit.

Longitudinal incisions of three centimeters length was made in each sterilized area, the depth incision cutting through some fibers of the muscular layer. The bacterial suspension of *Staphylococcus aureus* 0.2 mL was induced into the incision by the syringe. The wounds were daily observed and dressed with either the test drug or just sterile dressing. Estimation of wound healing was made by measuring the length of unhealed portion of the wound, in centimeter. At the same time, evidence of induration, erythematous area and the presence of pus were checked and noted.

Rapid screening of antioxidant activity by Dot-Blot and DPPH staining method

In this study, the antioxidant potential of ethanol and watery extracts of *P. betel* leaves was evaluated by Dot-Blot and DPPH staining method (Soler-Rivas *et al*., 2000). Drops of each sample were carefully loaded on TLC plate and allowed to dry (3 min). Drops of each sample were loaded in order of decreasing concentration along the row. The sheet bearing the spots was placed upside down for 10 s in a DPPH solution. The stained silica layer revealed a purple background with white spots at the location of the drops, which showed radical scavenger capacity. Tested amounts were 400, 200,100, 50, 25, 12.5, 6.25 µg for each extracts.

Isolation and characterization of eugenol from *P. betel* L.

Eugenol was isolated from the essential oil of *P. betel* by column chromatographic method using toluene and ethyl acetate (9:1). It is clear to pale yellow oily liquid. It is slightly soluble in water and organic solvents. It has a pleasant spicy, clove-like odour.

Results and Discussion

Dental infection

Antimicrobial activity of petroleum ether, 95 % ethanol, watery extract and essential oil of *P. betel* was tested on seven species of *Staphylococcus aureus* isolated from plaque of gingivitis (Figures 1, 2 and Table 1). It was observed that all the extracts showed antimicrobial activity on the tested

bacteria strains (11 mm - 40 mm). Among them, essential oil (40 mm) has higher antimicrobial activity than the other extracts.

Skin infection

The screening of antimicrobial activity of crude extracts (PE, 95 % ethanol, water) and essential oil of *P. betel* was also tested on five strains of bacteria (*Candida albicans*, *Escherichia coli*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*) which caused skin infection. From the experimental results, all the extracts showed antimicrobial activity against all tested organisms (11 mm - 40 mm) (Figures 3, 4 and Table 2). It is clearly seen that essential oil also shows significant antimicrobial activity against the organisms which can cause skin infections.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations of crude extracts (PE, 95 % ethanol, water) of *P. betel* were determined by agar disc diffusion method. It was found that MIC of PE extract on *S. aureus* from plaque of gingivitis was 5 mg/mL. It was also observed that the MICs of petroleum ether extract of *P. betel* were 5 mg/mL for *C. albicans*, 2.5 mg/mL for *K. aerogenes* and *S. aureus*. It did not give inhibition zone in concentration of 10 mg/mL on *P. aeruginosa*. Alcoholic extract and watery extract were 5 mg/mL for *P.aeruginosa* and *C. albicans* and 2.5 mg/mL for *K. aerogenes*. From the above data, the crude extracts of *P. betel* leave were more effective on *K. aerogenes* and *C. albicans*. These two organisms are the most common bacteria which can cause skin infections (Figures 5, 6 and Table 3).

Acute toxicity

Acute toxicity screening of 70 % ethanol and watery extract of P. betel leaves were done with the dosage of 2000 mg/kg and 5000 mg/kg body weight in each group of albino mice. The condition of mice groups was recorded after fourteen days' administration. The results showed no lethality of the mice was observed up to fourteen days' administration. Each group of animals were also observed still alive and did not show any visible clinical

symptoms of toxicity like restlessness, respiratory disorders, convulsion, aggressive activities, coma and death.

Wound healing activity

From the antimicrobial results, ointment was formulated by the fusion of essential oil of *P. betel* leaves and was tested on rats because of its strong activity against bacterial infection. The effect of ointment with the essential oil of *P. betel* leaves on open wound is shown in Figure 7. By *in - vivo* test, the oil of *P. betel* accelerated the rate of wound healing in infected rats when compared with the controls. In the control group of infected rats which did not receive any drug treatments, the healing activity was not observed after 9 days. Treatment with the ointment of oil of *P. betel* showed the complete healing in 9 days.

Antioxidant activity

To make a semi-quantitative visualization possible, ethanol and watery extracts were detected on the TLC plate by Dot - Blot and DPPH staining method. The appearance of white colored spots has a potential value of antioxidant activity. After staining with DPPH solution, white spots were appeared from 400 µg down to 6.25 µg of ethanol and watery extracts (Figure 8). From the experimental results, *P. betel* leaves extract were found to possess the antioxidant activity.

Identification of compound

The antimicrobial active oil of *P. betel* was separated on silica gel by using toluene: ethyl acetate (9:1). Eugenol (0.1 %) was obtained and the purity of compound was checked on TLC. It was identified by spectroscopic methods namely UV and FT IR and EI - MS. The EI - MS data clearly showed the molecular ion peak of eugenol at m/z 164 as well as the diagnostic fragments for eugenol at m/z 149, 137, 131 and 121.

Eugenol: UV λ_{max}^{PE} 280 nm (Figure 9), FT IR υ_{max}^{KBr} cm^{-1}: 3514 (ν_{O-H}), 1637 ($\nu_{C=C}$), 1268, 1234 (ν_{C-O-C}), 1634 (ν_{C-O-H}) (Figure 10), EI-MS m/z: 164, 149, 137, 131,121 (Figure 11)

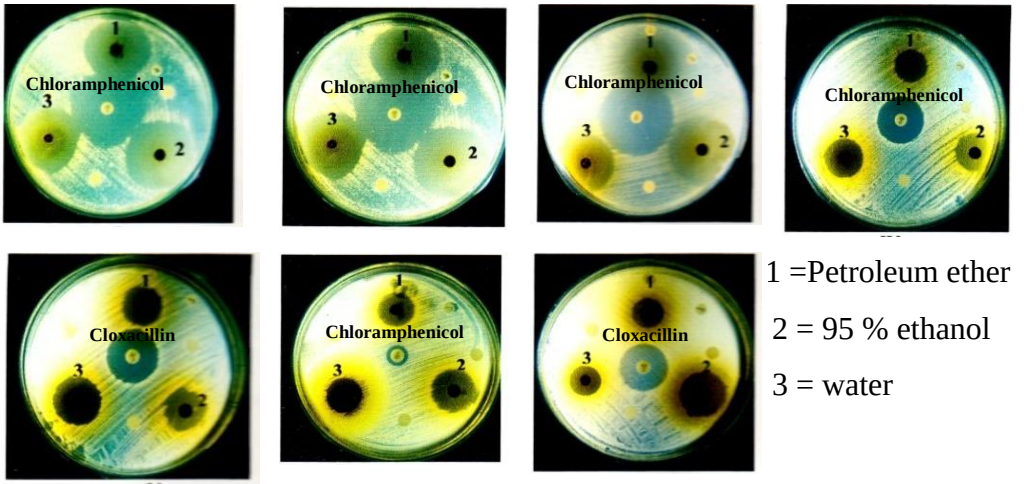


Figure 1: Sensitivity of crude extracts of *P. betel* on 7 species of *Staphylococcus aureus* from plaque of gingivitis

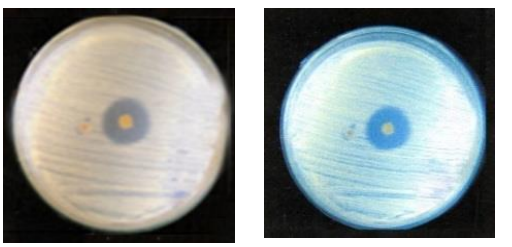
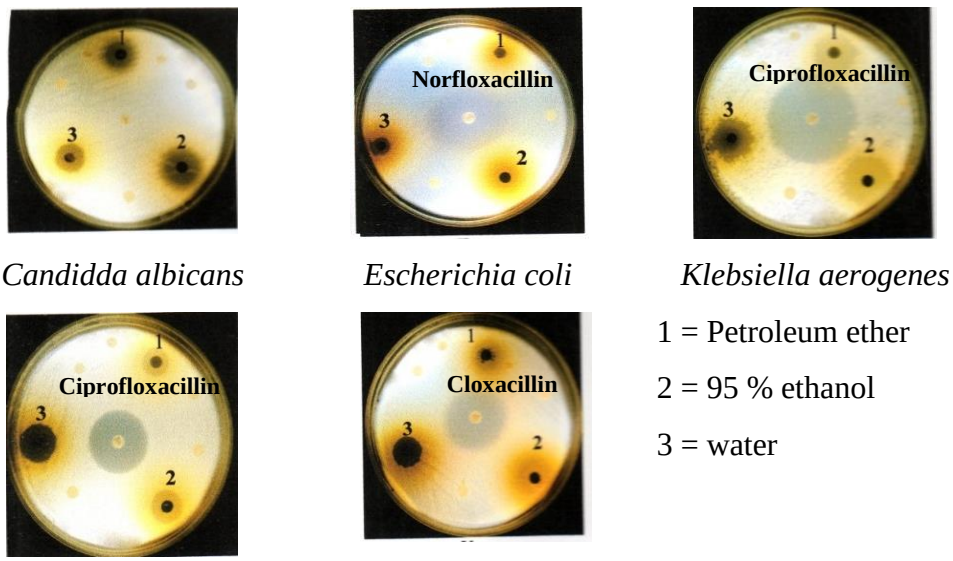


Figure 2: Sensitivity of essential oil of *P. betel* on *Staphylococcus aureus* from plaque of gingivitis

Table 1: Antimicrobial Activity of Different Extracts and Essential Oil of *P. betel* on *Staphylococcus aureus* from Plaque of Gingivitis

Tested Organisms	Diameter of zone of inhibition (mm)				
	PE	95 % Ethanol	Water	Essential oil	Antibiotic
S. aureus 1	35	33	33	11	42 (C)
S. aureus 2	20	20	23	20	45 (C)
S. aureus 3	30	33	30	40	40 (C)
S. aureus 4	32	33	30	20	28 (C)
S. aureus 5	15	15	20	16	35 (OB)
S. aureus 6	19	22	19	17	13 (C)
S. aureus 7	19	22	19	18	28 (OB)

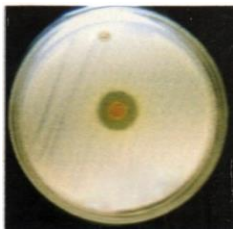
C = Chloramphenicol, OB = Cloxacillin, disc diameter = 6 mm



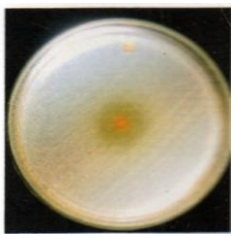
Candidda albicans *Escherichia coli* *Klebsiella aerogenes*

Pseudomonas aeruginosa *Staphylococcus aureus*

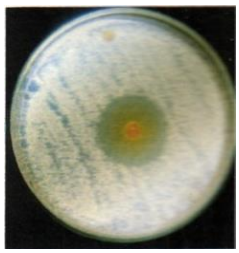
Figure 3: Sensitivity of crude extracts of *P. betel* on micro-organisms which cause skin infection



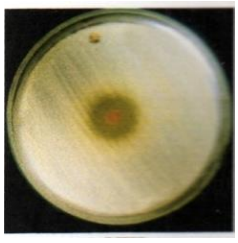
Candidda albicans



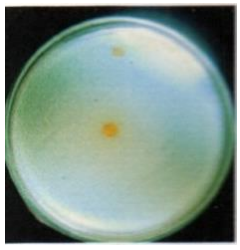
Escherichia coli



Klebsiella aerogenes

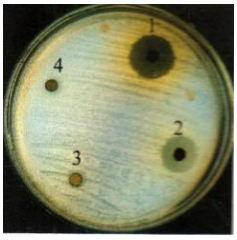


Pseudomonas aeruginosa

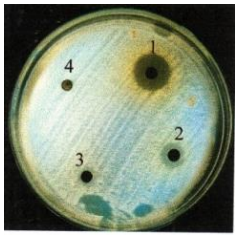


Staphylococcus aureus

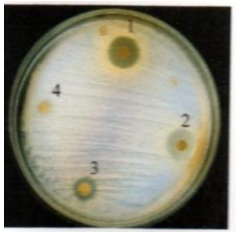
Figure 4: Sensitivity of essential oil of *P. betel* on micro-organisms which cause skin infection



Petroleum ether



95 % ethanol



Water

1=10 mg/mL

2=5 mg/mL

3=2.5 mg/mL

4=1.25 mg/mL

Figure 5: Minimum inhibitory concentrations of crude extracts of *P. betel* on *Staphylococcus aureus* from plaque of gingivitis

Table 2: Antimicrobial Activity of Different Extracts and Essential Oil of *P. betel* on Micro-organisms of Skin Infections

Tested Organisms	Inhibition zone diameter (mm) of different extracts and control				
	PE	**95 % Ethanol**	**Water**	**Essential oil**	**Antibiotic**
Candida albicans	35	33	33	11	42 (C)
Escherichia coli (ATCC)	20	20	23	20	45 (C)
Klebsiella aerogenes	30	33	30	40	40 (C)
Pseudomonas aeruginosa	32	33	30	20	28 (C)
Staphylococcus aureus	15	15	20	16	35 (OB)

disc diameter = 6 mm, C = Chloramphenicol, OB = Cloxacillin

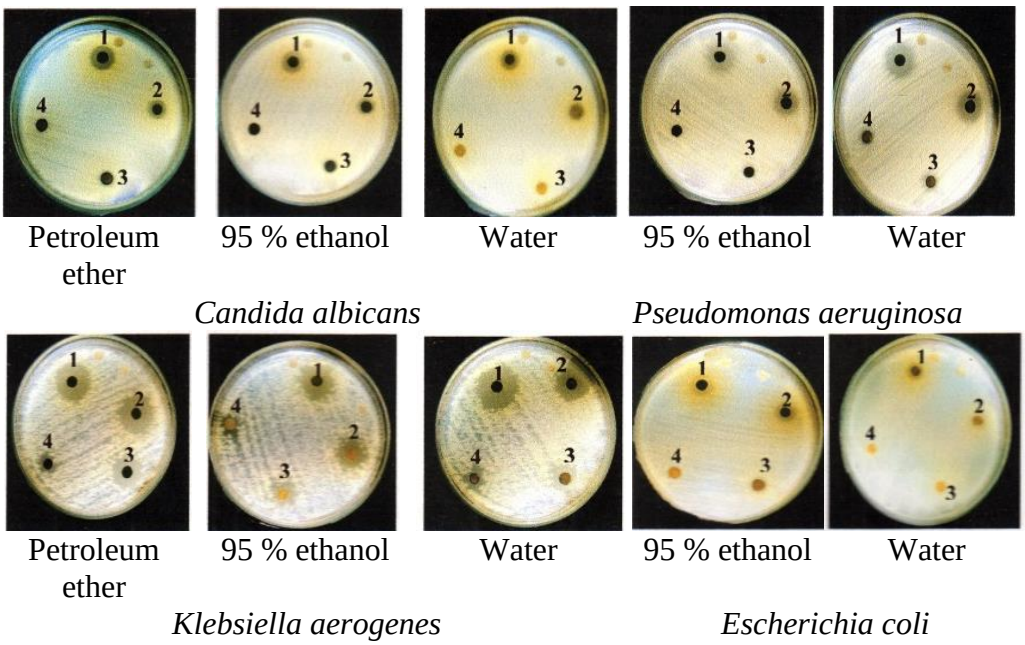


Figure 6: Minimum inhibitory concentrations of crude extracts of *P. betel* on microorganisms which cause skin infection

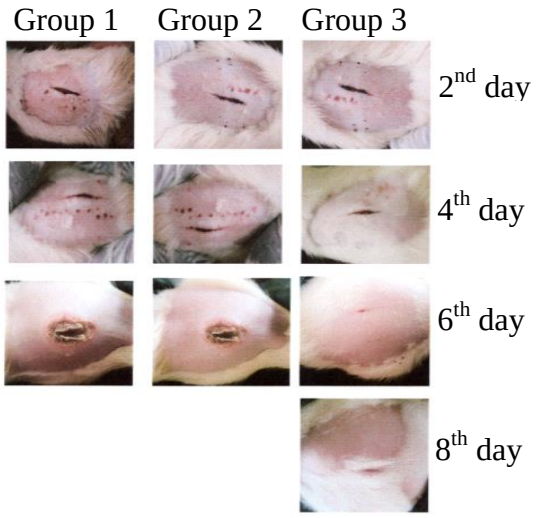


Figure 7: Process of the rate of wound healing of essential oil of *P. betel* on rats

Table 3: Minimum Inhibitory Concentration of Different Extracts of *P. betel*

Tested Organisms	Zone Inhibition Diameters (mm) of different extracts (mg/mL)											
	PE extract				95 % ethanol extract				Watery extract			
	10	5	2.5	1.25	10	5	2.5	1.25	10	5	2.5	1.25
**Staphylococcus aureus*	12	10	-	-	15	13	7	-	9	-	-	-
***Pseudomonas aeruginosa*	-	-	-	-	10	9	-	-	8	-	-	-
***Candida albicans*	12	8	-	-	13	9	-	-	11	7	-	-
***Klebsiella aerogenes*	20	21	17	-	24	17	14	-	17	15	13	-
***Escherichia coli*	-	-	-	-	9	10	-	-	9	-	-	-

disc diameter = 6 mm, * dental infections, ** skin infections

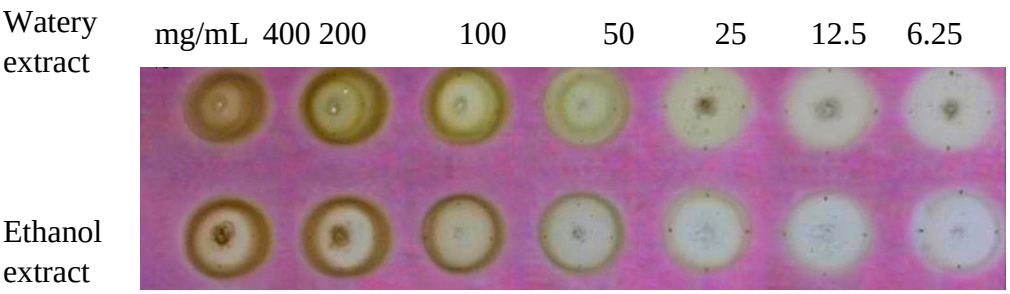


Figure 8 : Antioxidant activity of water and ethanol extract of *P. betel* by Dot-Blot and DPPH staining method

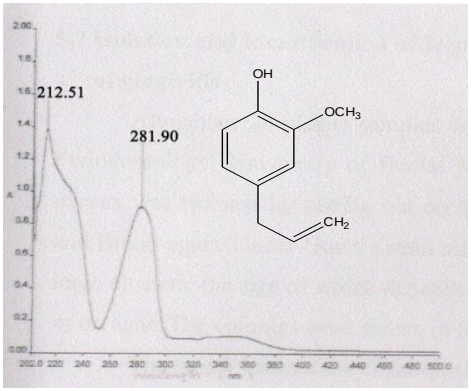


Figure 9: UV spectrum of isolated eugenol from *P. betel*

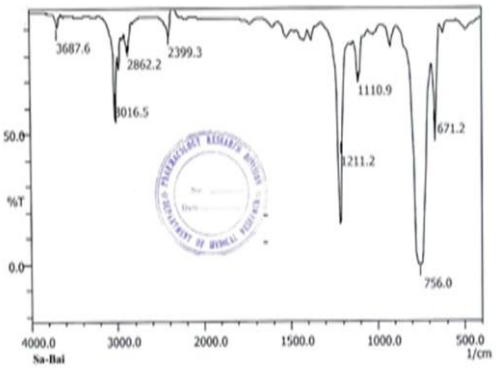


Figure 10: FT IR spectrum of the isolated eugenol from *P. betel*

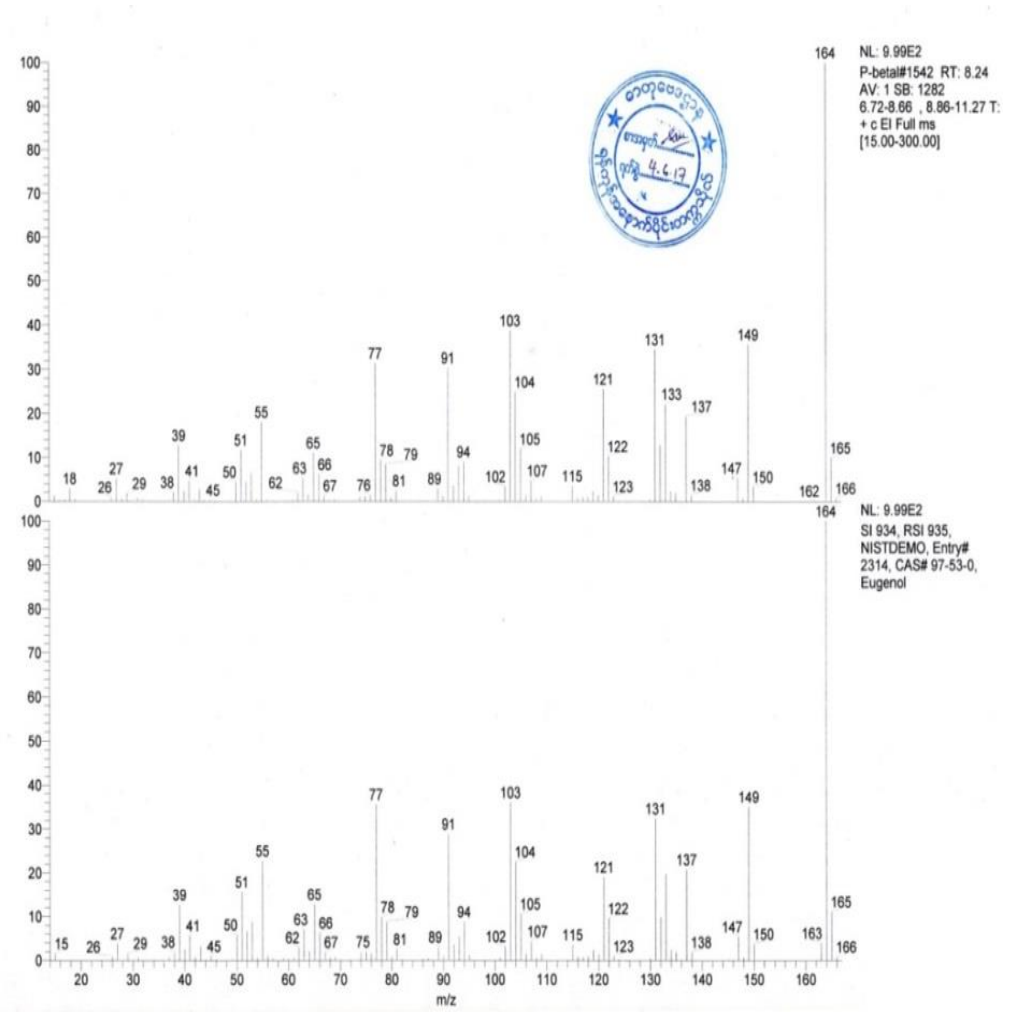


Figure 11: EI -MS spectrum of the isolated eugenol from *P. betel*

Conclusion

From the present research work, the following conclusions can be drawn.

Crude extracts have been prepared from *P. betel* by using polar and nonpolar solvents. Antimicrobial activity of crude extracts and essential oil were screened by agar disc diffusion technique on seven species of

Staphylococcus aureus isolated form plaque of gingivitis and five strains of organisms which usually cause skin infections. The minimum inhibitory concentration (MIC) of the 95 % ethanol extracts was 2.5 mg/mL for *Staphylococcus aureus* (dental infection) and 5 mg/mL for *Candida albicans* (skin infection).

Even with the dose up to 2000 mg/kg and 50000 mg/kg body weight administration, there was no lethality at the day fourteen. The ointment preparation with the essential oil of *P. betel* leaves promoted healing of wounds infected with *Staphylococcus aureus* in rats.

The evaluation of *in - vitro* antioxidant activities of extracts (ethanol, water) showed up to 6.25 µg/mL. Based on this information, it could be concluded that *P. betel* leaf is natural source of antioxidant of high importance. The essential oil of *P. betel* was fractionated by column chromatography to give eugenol (0.1 %). The isolated compound was confirmed by spectroscopic method (UV, FT IR and EI - MS).

From the experimental results, the extracts of *P. betel* leaves are suitable for economic production of antimicrobial agent for oral preparations. Moreover, the crude extracts are also more useful in skin infections caused by bacteria. The essential oil from *P. betel* will be useful medicament in wounds and sores as an alternative use in rural area especially where expensive antibiotics are not available.

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References

Arambewela, L. (2006). "A Potential Natural Antioxidant". *International Journal of Food Science & Technology*, vol.41, pp. 10-14

Hnin Hnin Aye. (2002). *Antituberculosis Activities and Chemical Investigation of Myanmar Traditional Medicine Used for the Treatment of Tuberculosis*. PhD Dissertation, Department of Chemistry, University of Yangon

Mi Mi Htwe, Mar Mar Nyein, Khin Chit, Mu Mu Sein Myint and Aye Than. (2001). "*In vitro* Antibacterial Activity of Some Indigenous Plants and Effect on *in vivo Staphylococcal* Induced Wounds". *Myanmar Health Science Research Journal*, vol.13 (1-3), pp. 32-37

OECD (2000). *Guidance Document on Acute and Toxicity*. Environmental Health and Safety Monograph Series on Testing and Assessment, No 24 (Organization for Economic Cooperation and Development).

Pauli (2001). "Antimicrobial Properties of Essential Oil Constituents". *J. Aromatherapy*, vol. 11(3),
pp. 126-133

Skerman. V. B. D. (1967). *A Guide to the Identification of Bacteria*. Baltimore: 2nd Ed, Williams and Wilkins Co

Soler – Rivas. C., Espin, J.C. and Wichers, H.J. (2000). "An Easy and Fast Test to Compare Total Free Radical Scavenger Capacity of Food Stuffs", *Phytochem. Annal*; **11,** p. 330

Stokes, E. J, Ridgway, G. L. and Wren, M. W. D. (1993). *Laboratory Control of Antimicrobial Chemotherapy, In: Clinical Microbiology*. London: 7th Ed, Edward Arnold, pp. 258-263

WHO. (2003). *Staining Techniques In: Guideline on SOP for Microbiology*. New Delhi: pp. 19-22