Antimicrobial Activity of the Volatile Oil from the Leaves of Piper betle Linn.

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The study was carried out with the objective of evaluating the antibacterial and antifungal potentials and non-mutagenic activity of the pure volatile oil from the leaves of Piper betle vine, a traditional antimicrobial medicine. It was also the purpose of the study to determine the physicochemical properties and composition of the pure oil. The determined physical constants were congealing temperature (-4° C), melting point (8°C), specific gravity (0.9313), optical activity (+4.2307), and refractive index (1.4525). Twenty oil components were identified by gas chromatography-mass spectroscopy (GC-MS) analysis – among them are: 5-(2-propenyl)-1,3-benzodioxole, eugenol isomers, 3-carene and caryophyllene. In this study, the microbial activity of the pure oil from the leaves of P. betle was evaluated for potential antimicrobial activity using agar-well diffusion method. The antibacterial activity of the oil at 100µg/mL concentration were tested against Staphylococcus aureus, Escherichia coli and Bacillus subtilis while 20µg/mL, 30µg/mL and 40µg/ml concentrations of the oil were used against Streptococcus pyogenes. The antifungal activity of the oil at 100µg/mL was tested against Candida albicans and 2µg/mL, 10µg/mL and 15µg/mL concentrations against Trichophyton mentagrophytes. Zone of inhibition of the pure oil was compared with standards mupirocin for antibacterial activity and clotrimazole for antifungal activity. The results show active to very active inhibition of the bacterial and fungal growth against the test organisms. The antimicrobial action of the P. betle oil is due to bioactive constituents such as isosaffrol, eugenol and caryophyllene among others which are well known for their antibacterial and antifungal properties. P. betle oil does not possess carcinogenic and/or mutagenic activity as evident of lower number of micronucleus formed in rat bone marrow cells after treatment of the pure oil when compared to cyclophosphamide. The

mechanism of its antimicrobial activity is not yet well understood. Further studies on the pathway of microbial growth inhibition are recommended and preformulation data obtained in this study should be validated to establish specifications of the oil.

Keywords: Piper betle L. volatile oil, antibacterial activity, antifungal activity, GC-MS, non-mutagenic activity

INTRODUCTION

A large number of medicinal plants have been used for scientific researches in different areas of medicine. Researches are still underway for the discovery of their active constituents responsible for their medicinal and curative values. The latter part of the 1990s up to the present showed an unprecedented increase in the use of herbal remedies and herbal food supplement in the treatment as well as prevention of diseases. Global interest in traditional medicine has increased significantly in recent years, driven by global trends in health care (Yuan & Lin, 2002). Antimicrobials are one of our most important weapons in fighting bacterial and fungal infections and have greatly benefited the health-related quality of human life (Hsouna & Hamdi, 2012). However, over the past few decades, these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses not only because many of them produce toxic reactions, but also due to emergence of drug-resistant bacteria (Bhalodia & Shukla, 2011). Drugs derived from natural resources play a significant role in the prevention and treatment of human diseases. In the Philippines, traditional medicine is one of the primary healthcare systems. Natural products of plants may give a new source of antibacterial and antifungal agents with possible novel mechanisms of action (Bhalodia & Shukla, 2011). Plant extracts are widely claimed to have a broad-spectrum antibacterial and are considered as a main source for the search of lead compounds (Hsouna & Hamdi, 2012).

Piper betle L. according to Concha (1982) is locally known as buyo (Bik.), gaoed (Pang.), gaued (Ilk.), and ikmo (Tag.); in English it is known as betel leaf. It is a twining vine, climbing plant slightly woody at the base. It is probably a native of Malaysia but cultivated in India and most parts of the Philippines. The inflorescence is a spike; the male spikes are subpendulous and slender. The female spikes when mature are red, fleshy and oblong to elongate. The leaf is simple, glabrous, broadly ovate, base

cordate, apices rounded. Size and color of leaves are affected by light during cultivation. Odor is tar-like; taste pungent (Philippine pharmacopoeia 1, 2004).

P. betle L. commonly known as betel leaf has been used since ancient times. A mixture of betel leaf, betel nut and lime is still being used by old folks as a masticatory; it is believed to strengthen the teeth and protect the teeth from decay. It is useful in arresting secretions and is used in several common household remedies. The leaves have been used as an antiseptic for wounds and cuts and poultice for boils, used for relief of coughs, as an expectorant, for stomach ailments, diphtheria and inflammation (Quisumbing, 1978). The development of *P. betle* as a drug can contribute to the treatment of diseases caused by bacteria and fungi.

Previous studies on betel leaves, roots and whole extract showed a very strong antimicrobial activity (Jenie et al., 2001). The betel leaves contain a beneficial volatile oil and is known as the betel leaf essential oil. It is valued in Ayurveda as a stimulant, carminative, aromatic, and antiseptic. Volatile oils are usually obtained from plant parts containing the oil by steam distillation. The volatile oils are composed of several chemicals that form the odoriferous essences of a number of plants. They are also known as ethereal oils or essential oils (Tyler, Brady, & Robbers, 1988). This research utilized the extracted pure volatile oil from the leaves of *P. betle* to develop an antimicrobial agent. The betel plant grows abundantly in the Philippines thus it can be locally procured and processed. The volatile oils are composed of several chemicals that form the odoriferous essences of a number of plants.

Many studies proved that volatile or essential oils have potential use as antimicrobial agents whose importance grows as microbial resistance to antibiotics increases. Because of their potency and documented pharmaceutical efficacy, essential oils represent an important allopathic and herbal system of medicine. An antimicrobial agent is a substance that kills or inhibits the growth of microorganisms. This may be categorized on the basis of their antibacterial activity as either bacteriostatic or bactericidal and likewise antifungal activity as either fungistatic or fungicidal.

This research is specifically aimed to determine the percentage of oil that can be obtained from the leaves, and the quality characteristics of the volatile oil in the areas of physicochemical analysis, microbiological activity and antimutagenic activity. In this current investigation, a screening of the

volatile oil against pathogenic bacteria and fungi is done in order to detect new sources of antimicrobial agents.

MATERIALS AND METHODS

Collection of Plant Material

The mature Piper betle L. leaves were collected from La Union, Abra, Iloilo City, Palawan and Malaybalay. A representative of the whole plant was brought to the Philippine National Museum for authentication. The collected leaves were washed, dried and cut into small pieces.

Isolation of the Volatile Oil

One hundred sixty grams of cut leaves were subjected to hydro distillation using a Clavenger-type apparatus to extract the volatile oil. The volume of the oil was measured directly using a graduated cylinder. A small quantity of anhydrous sodium sulphate was added to remove any traces of water. The percentage yield (w/w) of the essential oil was calculated on dry basis, using the formula:

$$\%\ yield = \frac{weight\ of\ the\ volatile\ oil}{weight\ of\ the\ air\text{-}dried\ leaves} \times 100$$

Characterization of the Volatile Oil

Organoleptic Properties

The color and clarity of the volatile oil placed in transparent bottles were observed over a white background. The characteristic odor was determined by sniffing.

Solubility

The solubility of the oil was determined by mixing incremental volumes of the volatile oil on a 1:1 ratio of the following solvents: water, chloroform, ethyl alcohol, anhydrous ether and petroleum ether.

Specific Gravity

The determination of specific gravity was based on the procedures by Knevel and DiGangi (1977). The measurement of weight was performed using a Sartorius CP 135 balance. Three trials were conducted and the mean of the reading is considered as the specific gravity of the volatile oil.

Congealing Temperature

Congealing temperature was determined based on the procedures in United States Pharmacopeial Convention (2002). Ten millilitres of the volatile oil was placed in a test tube. The temperature was raised 4-5 degrees above the expected saturation point, allowed to cool, and the temperature at which the first crystals appear was noted as the congealing temperature.

Specific Rotation

The extent of optical activity of the oil was determined following the procedures of Knevel and DiGangi (1977) using an E. Harnack 220 polarimeter. The specific rotation was calculated using the formula provided by the United States Pharmacopeial Convention (2002). Determinations were done at 25° C. Readings from three conducted trials were obtained and the computed average was recorded as the specific rotation of the volatile oil.

Refractive Index

The refractive index of the volatile oil was measured using an Atago TM1 refractometer following the procedure provided by Knevel and DiGangi (1977). Three trials were performed and the mean of the readings was regarded as the refractive index of the volatile oil. The expected values are between 1.46 and 1.61 at 25°C (Tyler et al., 1988).

Chemical Analysis of the Piper betle Volatile Oil

The volatile oil was submitted to the National Chemistry Instrumentation Center (NCIC) at Ateneo de Manila, Quezon City for its chemical characterization by gas chromatography – mass spectrometry (GC-MS). The

analysis used a Hewlett Packard 5890 Series II gas chromatograph, equipped with a DB-1 fused silica capillary column (60m length; 0.25mm internal diameter; 0.25μm phase thickness), coupled with a Finnigan-MAT 95ST high-resolution mass spectrometer (70eV ionization voltage; 35-350 amu mass range; 1sec/decade scan rate). The carrier gas was used at 4mL/min purge flowrate and 10mL/min splitless flowrate. The temperature program is as follows: 50°C initial temperature, held for 1min, 50°C to 250°C at 4 °C/min. The NIST mass spectra library was used to match the mass spectra and only identities with Rfit values greater than 800 were considered as an acceptable match of identity. The reported amounts were based on the area of the identified peak relative to the area of all identified peaks. The mass spectrum and a list of possible identities of peaks were provided by NCIC after the analysis.

Antimicrobial Susceptibility Assay of the Piper betle Volatile Oil

Preparation of Volatile Oil Suspension and Controls

A 500mL volatile oil suspension with a concentration of 1000 μg/mL was made by suspending an equivalent volume of 500 mg of the volatile oil in isotonic saline containing 0.2% Tween 80. One hundred micrograms of this suspension was withdrawn to test the activity of the oil against *Staphylococcus aureus*, *Escherichia coli, Bacillus subtilis* and *Candida albicans.* Previous tests of the activity of 100 μg/mL volatile oil against *Streptococcus pyogenes* and *Trichophyton mentagrophytes* resulted to the formation of a zone of inhibition exceeding the size of a 90-mm petri dish, making it impossible to be measured pragmatically; thus, equivalent amounts of 20μg/mL, 30μg/mL, and 40μg/mL dilutions were prepared for *S. pyogenes* while corresponding dilutions of 2μg/mL, 10 μg/ml, and 15μg/mL were prepared for *T. mentagrophytes*.

The positive controls for the antibacterial and antifungal susceptibility tests were 2% (w/w) mupirocin cream and 10% (w/w) clotrimazole cream, respectively. Five hundred milligrams of each of the positive controls was suspended in 0.2% Tween 80 in saline solution to produce 10 mL of stock suspension with a concentration of 1000 μg/mL. Dilutions of the suspension were prepared matching the concentration of the volatile oil. An isotonic 0.2% Tween 80 solution served as the negative control.

Preparation of Bacterial and Fungal Cultures

Pure cultures of bacteria and fungi were obtained from the University of the Philippines Research Institute, Diliman, Quezon City. The test organisms included the bacteria *Staphylococcus aureus* UPCC 1143, *Bacillus subtilis* UPCC 1295, and *Escherichia coli* UPCC 1195; and the fungi *Candida albicans* UPCC 2168 and *Trichophyton mentagrophytes* UPCC 4193. The pure culture of *Streptococcus pyogenes* was obtained from the Benguet State University Microbiology Laboratory.

Mueller-Hinton broth cultures of *S. aureus*, *E. coli*, *B. subtilis*, and *S. pyogenes* were prepared and incubated for 18-24 hours at 35°C to allow sufficient growth. A vortex-mixed spore suspension of *T. mentagrophytes* in sterile isotonic Tween 80 solution was prepared from Sabouraud glucose slant culture incubated at room temperature for 3-5 days. A Sabouraud glucose broth culture of *C. albicans* was prepared and incubated for 18 hours at room temperature. The turbidity of the broth cultures and spores suspension was adjusted in comparison with the 0.5 McFarland standard prior to testing.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility assay (agar-well diffusion method) was performed based on the procedures by Quinto and Santos (2006). The bacterial isolates (*S. aureus, S. pyogenes, E. coli,* and *B. subtilis*) and fungal isolates (*C. albicans* and *T. mentagrophytes*) were inoculated on Mueller-Hinton agar and Sabouraud glucose agar plates, respectively. Three trials were prepared for each microorganism. Three 6-mm wells were created, each delivered with 100µL of either the oil or one of the controls using a micropipette. The plates were incubated for 18-24 hours at 35°C and at room temperature for plates containing bacteria and *C. albicans,* respectively, and at room temperature for 2-3 days for the plates containing *T. mentagrophytes*. After incubation, the size of zones of inhibition around the agar well was measured using a ruler. The zone of inhibition produced by the volatile oil suspension was compared with the positive and negative controls using statistical methods. The assessment of zone of inhibition diameters were based on the criteria by Quinto and Santos (2006).

Minimum Inhibitory Concentration Assay

Preparation of Piper betle Volatile Oil

Approximately 40 mg of the volatile oil was added to 10 mL Mueller-Hinton broth with a small amount of 0.1% Tween 80 to enhance the solubility of the volatile oil. The mixture was swirled, transferred to sterile test tubes, and agitated using a vortex mixer for 15sec. One millilitre of the suspension contains 4 mg of the volatile oil.

Preparation of Test Organisms

Five millilitres of Mueller-Hinton broth was inoculated with a loopful of the bacterial isolates (*B. subtilis, E. coli, S. aureus*, and *S. pyogenes*). A spore suspension of *T. mentagrophytes* using 0.05% Tween 80 saline solution was prepared. A loopful of *C. albicans* was inoculated into 5mL of Sabouraud glucose broth. The turbidity of each preparation was adjusted in comparison with 0.5 McFarland standard. The standardized inoculums were diluted to 20mL with Mueller-Hinton broth and 200mL with Sabouraud glucose broth for the bacterial and fungal isolates, respectively.

Determination of the Minimum Inhibitory Concentration, Minimum Bactericidal Concentration, and Minimum Fungicidal Concentration

The minimum inhibitory concentration (MIC) was determined by measuring the ability of a bacterial or fungal strain to grow in broth cultures containing different concentrations of the volatile oil. The turbidimetric method or tube dilution method, based on the procedures of Quinto and Santos (2006), was used to determine this parameter. Serial dilutions of decreasing concentrations of the volatile oil were first prepared in tubes containing Mueller-Hinton and Sabouraud growth medium. Then a known concentration of the prepared microorganism was added to each tube. The tubes were incubated and were examined for visible growth or turbidity. The lowest concentration of the volatile oil that prevents the growth of the microorganisms, seen as the absence of turbidity, is the MIC of the volatile oil (Nester et. al., 2004)

The minimum bactericidal concentration (MBC) of the volatile oil on the bacterial isolates was carried out by sub-culturing a small amount of the content of the tubes showing absence of visible growth or turbidity into Mueller-Hinton agar plates, incubation for 10-24 hours at 35°C, and observation for colonies after incubation period. The volatile oil concentration of the tube producing one colony or no colony on the Mueller-Hinton plates is the MBC of the volatile oil.

The minimum fungicidal concentration (MFC) was determined using similar procedures with MBC determination but using Sabouraud glucose agar and incubating *T. mentagrophytes* plates at 30°C for 3-5 days and *C. albicans* plates at 35°C for 18-24 hours.

Micronucleus Test for the Piper betle Volatile Oil

The assay was performed according to procedures by Bernas et al. (2005). Six healthy 8-week old laboratory-acclimatized mice were randomly grouped into 3, each receiving one of the preparations. The preparations used in the test were 2 mL of *P. betle* oil in 1 mL of 0.2% Tween 80 as test solution, 500 mg of cyclophosphamide powder in 20 mL of saline solution as positive control, and 5% Tween 80 solution as negative control. The preparations were administered intramuscularly to the mice at a volume not exceeding 2 mL/kg body weight for the test solution and 6 mg/kg body weight for the positive control. After two administrations of the preparations, the bone marrows of the test animals were collected, made into a smear and stained, followed by examination and scoring of the slides and computation of the mitotic index.

Statistical Analysis

The experimental results in the study were expressed as mean and standard deviation of three parallel measurements. The significance of differences was calculated using either Student's t-test or one-way analysis of variance at 0.05 level of significance. Regression equations were calculated by method of least squares and the differences between slope and elevations of the regression equations were tested using analysis of covariance. All graphical representations and statistical analysis were carried out using Excel® (Microsoft Corporation, USA).

RESULTS AND DISCUSSION

Extraction Yield and Physical Properties of the Volatile Oil

The air-dried leaves of *P. betle* were utilized to obtain the volatile oil by means of hydro distillation using Clavenger apparatus. The yield of the volatile oil depended on the situation of the vine, time of plucking, and the nature of material distilled. It was made sure that the leaves were taken from the upper part of the plant, were green, and have a soft texture. The essential oil yield was 1.4431%. The oil is colorless to pale yellow when freshly extracted, has a strong aromatic tar-like odor and pungent taste. It is miscible in organic solvents in the ratio of 1:1, but sufficiently soluble in water at 1:5 oil-water ratio to form a saturated solution and imparts its odor to water (Tyler et al., 1988).

Physical constants serve as a means of assessing the purity and quality of the volatile oil as well as for identification. Among the physical constants determined were: congealing temperature at -4°C (with a melting point of 8°C); specific gravity at 0.9313, optical activity at +4.2307. Specific gravity and optical activity both showed precision of tests based on the average which did not exceed the maximum of 2% relative standard deviation. The refractive index of 1.4525 slightly exceeded the limit due to changing temperature of the sample during the analysis.

Table 1. Specific gravity, refractive index, and optical activity of Piper betle volatile oil at various sample sites

Sample Site	Specific Gravity		Refractive Index		Optical Activity	
	Average	SD	Average	SD	Average	SD
Abra	0.9313	0.0053	1.4404	0.0141	+4.1877	0.1074
Iloilo	0.9313	0.0055	1.4535	0.0435	+4.2235	0.0620
La Union	0.9313	0.0054	1.4526	0.0327	+4.2235	0.0620
Malaybalay	0.9313	0.0056	1.4600	0.0479	+4.2593	0.0620
Palawan	0.9313	0.0053	1.4562	0.0377	+4.2593	0.0620
Mean	0.9313		1.4525		+4.2307	
%RSD	0.49%		2.21%		1.60%	

All physical data were measured at 25°C

SD – Standard deviation; %RSD – Relative standard deviation

The analysis (see Table 1) showed that the specific gravity, refractive index, and optical activity of the volatile oil from different collection sites were comparable.

Chemical Analysis of the Piper betle Oil

The GC-MS analysis of the volatile oil led to the identification of 20 different compounds (see Table 2) with the highest relative amount being 5-(2-propanol)-1,3-benzodioxole, also known as isosaffrol (48.37%), eugenol isomers (14.03% & 8.07%) and 3-carene (7.83%). Caryophyllene (1.76%) and α-caryophyllene (0.59%) were also found to be present.

Table 2. Chemical composition of Piper betle volatile oil

Retention Time (min)	Possible Identity	Relative Amounts (%)
11:05	2-methyl-5(1-methylethyl)- bicyclo[3.1.0] hex-2-ene	1.99
11:20	Alpha-pinene	1.42
11:51	Camphene	2.83
12:42	Beta-phellandrene	3.44
13:20	Beta-myrcene	0.41
13:55	Alpha-phellandrene	0.17
14:12	3-carene	7.83
14:32	1-methyl-4-(1-methyl ethyl)benzene	1.56
14:51	1-methyl-5-(1-metheyletenyl)cyclohexene	1.03
15:59	1-methyl-4-(1-methylethyl)-1,4-cyclohexadiene	0.51
17:09	2-carene	0.08
17:29	3,7-dimethyl-1,6-octadien-3-ol	0.18
20:32	4-methyl-1-(1-methylethyl)-3-cyclohexen-1-ol	2.12
23:33	4-(2-propenyl)phenol	2.92
24:26	5-(2-propenyl)-1,3-benzodioxole	48.47
26:50	Eugenol isomer	14.03
27:15	Eugenol isomer	8.07
28:17	Copaene	0.59
29:46	Caryophyllene	1.76
30:42	Alpha-caryophyllene	0.59

Antimicrobial Activity of the Piper betle Oil

Antibacterial Activity

Volatile oils are plant extracts that are widely claimed to have broad spectrum antibacterial activity. The activities of the *P. betle* oil against the test

microorganisms were qualitatively and quantitatively assessed by the size of the inhibition zones, minimum inhibitory concentration, and minimum bacterial/fungal concentration values. The mean of the zones of inhibition of S. *aureus, B. subtilis*, and *E. coli* including are shown in Figure 2.

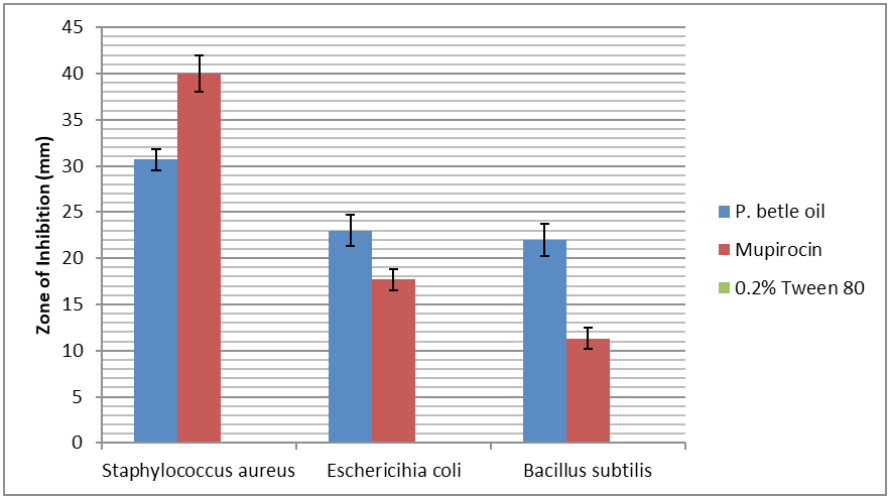

Figure 2. Inhibition sizes produced by 100µg/mL P. betle volatile oil, mupirocin, and 0.2 % Tween 80 on S. aureus, E. coli and B. subtilis.

The inhibition zone is the result of the interaction between the treatment and bacteria; its mean determines the effectiveness of the treatment against the target bacteria. All zones of inhibitions in the test bacteria due to either *P. betle* volatile oil and mupirocin were higher than or equal to 10mm which indicates its effectiveness against the test bacteria. No zone of inhibition was observed for the negative control.

The volatile oil exhibited a potent antimicrobial activity against both Gram-positive bacteria and Gram-negative bacteria – having inhibition zone diameters in the range of 20-32mm for former and 22-25mm for the latter. To determine if there is a significant difference in the efficacy of mupirocin and the *Piper betle* volatile oil against the test bacteria, t-test was performed.

The initial analysis showed that the *P. betle* volatile oil treatment had the largest inhibition diameter against *S. aureus* among the test bacteria but this activity is significantly lesser compared to mupirocin. On the other hand, the volatile oil exerted significantly greater activity against *E. coli* and *B. subtilis* compared to mupirocin treatment. The results partially agree with the findings of Hussain, et. al. (2010) as cited by Hsouna and Hamdi (2012) who

reported that Gram-positive bacteria are more sensitive to plant essential oils than Gram-negative bacteria. An exception is the Gram-positive *B. subtilis*, whose infections are among the most difficult to treat with conventional antibiotics, especially if complicated by multi-drug resistance; however, its potent inhibition by the volatile oil shows the latter's use to minimize drug resistance problems and protect foods from multiple pathogenic bacteria.

Table 2. Student's t-test analysis comparing inhibition sizes of P. betle oil and mupirocin (positive control) treatments

Bacteria	Piper betle Oil		Mupirocin		t	df	p-value*
	Mean	SD	Mean	SD			
Staphylococcus aureus	30.67	1.15	40.00	2.00	-7.00	4	0.002
Escherichia coli	23.00	1.73	17.67	1.15	4.44	4	0.011
Bacilus subtilis	22.00	1.73	11.33	1.15	8.88	4	0.001

Mean and standard deviation (SD) in mm

* Significant at $p<0.05$

Previous tests of the activity of 100 µg/mL volatile oil against *S. pyogenes* resulted to the formation of a zone of inhibition exceeding the size of a 90-mm petri dish, making it impossible to be measured pragmatically. It revealed that the volatile oil is very active against the Gram-positive streptococci. Therefore, further dilutions to 20 µg/mL, 30 µg/mL, and 40 µg/mL of the volatile oil were necessary to compare with the same concentrations of the positive control (mupirocin) to assess its activity.

Table 3. Assessment of activity of P. betle oil and mupirocin against S. pyogenes

Concentration	Piper betle Oil		Mupirocin	
	Mean ± SD	Assessment†	Mean ± SD	Assessment†
20µg/ml	18.13 ± 0.42	Active	21.33 ± 1.53	Very active
30µg/ml	26.5 ± 0.62	Very active	27.07 ± 1.01	Very active
40µg/ml	35.47 ± 0.68	Very active	35.83 ± 0.76	Very active

Mean and standard deviation (SD) in mm

†Inferential assessment based on following criteria by Quinto & Santos. (2006): <10mm – inactive; 10-13mm – partially active; 14-19mm – active ; >20mm – very active

The assessment of *Piper betle* oil reveals it to be active at 20µg/mL and very active at 30µg/mL and 40µg/mL as shown in Table 3. It also shows that

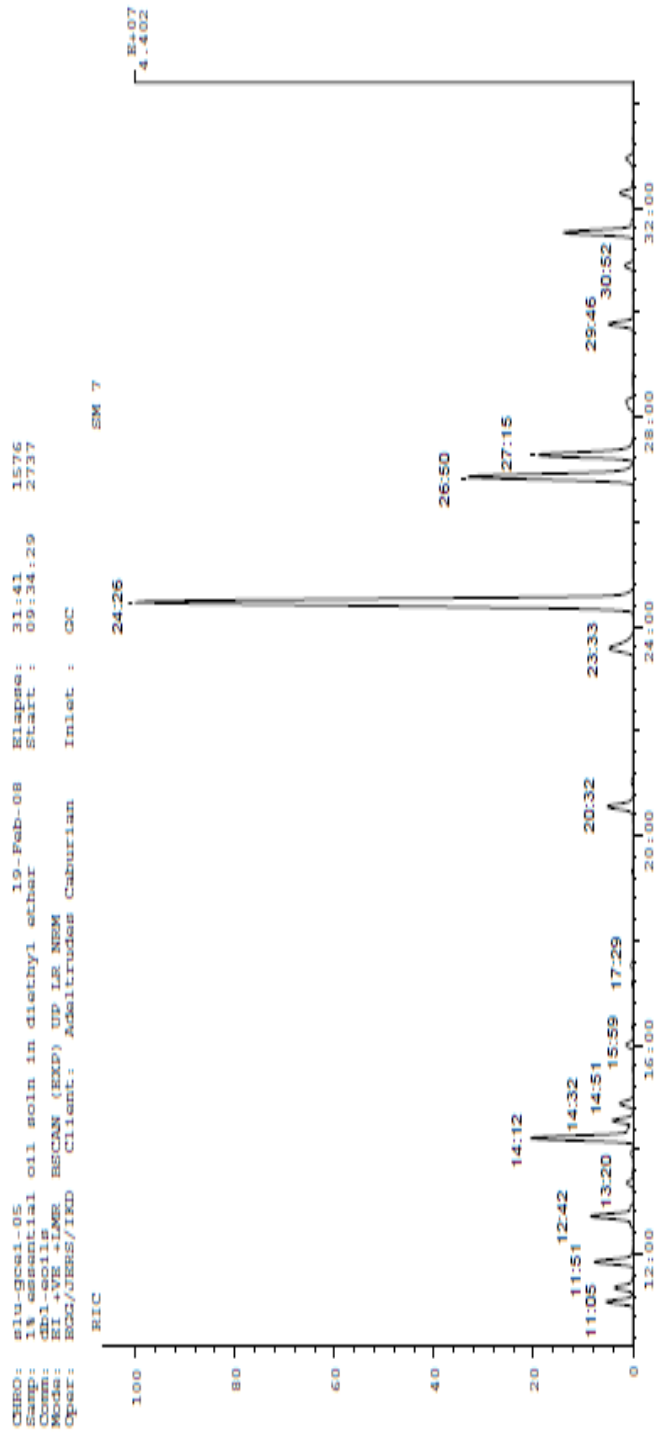

Figure 1. GC-MS spectrum of Piper betle volatile oil.

the antibacterial activity in terms of inhibition diameter is proportional to the concentration of the volatile oil.

A linear regression analysis was done to show similarities in slope and elevations of the lines representing the activity of P. betle oil and mupirocin against S. pyogenes. The regression lines are shown in Fig. 3.

For both *P. betle* volatile oil and mupirocin, there is a significant relationship between concentration and inhibition size, ($F(1,7)=1412.537$, $p<.001$; $F(1,7)=184.4217$, $p<.001$), and all regressions have the same slope ($F(1,14)=4.446$, $p=.053$). The mupirocin treatment has a statistically higher elevation, ($F(1,15)=7.694$, $p=0.014$) with a magnitude of difference of 1.4889. This indicates that the two treatments have the same increase in inhibition size per unit change in concentration with a 1.5mm difference in inhibition diameter at the same concentration. Since the difference in elevation is small, it still shows that the two treatments are still comparatively similar in their activity against *S. pyogenes*.

The antimicrobial action of the *P. betle* oil is due to bioactive constituents such as isosaffrol, eugenol, and caryophyllene among others which are well known for their antibacterial property (Evans, 2009). The intense antimicrobial activity of the volatile oil is in agreement with many studies in other plant species such as *Pelargonium graveolens, Eucalyptus alba, E. robusta* and *E. globulus* (Hsouna & Hamdi, 2012). Another theory in the study of Chakraborty and Shah (2011) cited in Pradhan, et.al. (2013) tried to explain that the bioactive molecule responsible for antibacterial activity is sterol which was obtained in large quantities in Piper betel extracts. The mode of action may be due to surface interaction of sterol molecules present in the extracts with the bacterial cell wall and membrane leading to alteration in the primary structure of cell wall and membrane permeability, ultimately leading to pore formation and degradation of the bacterial components. This mechanism is highly effective against the single-layered Gram-positive bacteria compared to Gram-negative bacteria with a multi-layered complex cell wall structure.

Antifungal Activity

The *P. betle* oil was also tested against fungal organisms *C. albicans* and *T. mentagrophytes.* The activity of 100µg/mL *P. betle* oil against *C. albicans* was compared with that of 1% clotrimazole (positive control). No

zone of inhibition was detected in 0.2 Tween 80 (negative control). The analysis found that P. betle volatile oil has significantly higher inhibition diameters (35.3333 ± 0.3333 mm) compared to clotrimazole (18 ± 7 mm), $t(4)=11.0864$, $p<.001$. This significant difference in treatment revealed that the fungal organism has higher susceptibility to the *P. betle* oil than to clotrimazole.

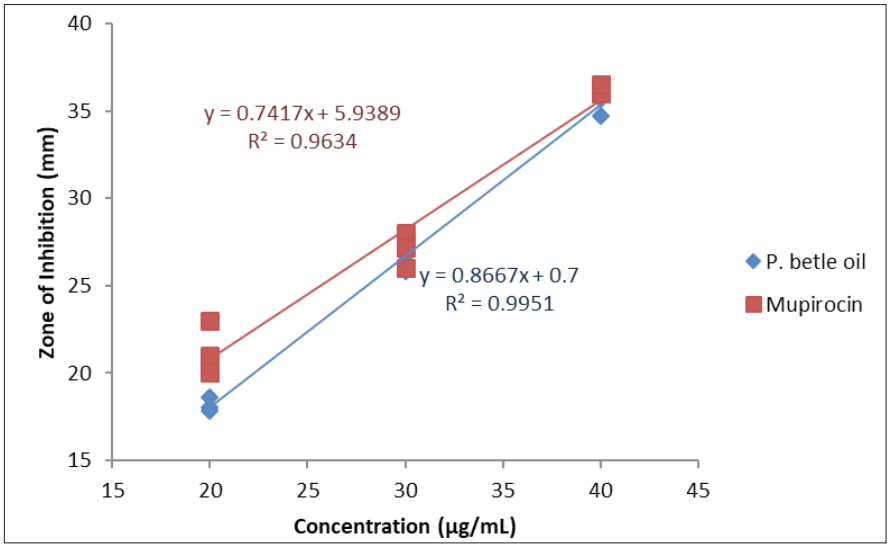

Figure 3. Scatter plot of inhibition zone produced by P. betle oil and control (mupirocin) on S. pyogenes.

Like its activity against the *S. pyogenes* bacteria, the pure volatile oil demonstrated a very high activity against *T. mentagrophytes*. Likewise, the size of the zone of inhibition produced by a concentration of 100 µg/mL of oil theoretically exceeded the size of the 90-mm Petri dish. For practical measurements, 2 µg/mL, 10 µg/mL, and 15 µg/mL were prepared to assess the activity of the oil when compared to the same concentration of the clotrimazole (positive control).

Table 4 shows the zone of inhibition of T. *mentagrophytes* as affected by different concentrations of the *P. betle oil* and the assessment of its activity.

The table above shows that *T. mentagrophytes* was found to be very susceptible to *P. betle* oil and clotrimazole in all concentrations. It also shows that the concentration of the treatments is proportional to the zone of inhibition diameter.

Table 4. Assessment of the activity of P. betle oil and clotrimazole against T. mentagrophytes

Concentration	Piper betle Oil		Clotrimazole	
	Mean ± SD	Assessment	Mean ± SD	Assessment
2 µg/ml	23.13 ± 1.03	Very active	24.5 ± 0.5	Very active
10 µg/ml	25.57 ± 0.55	Very active	25.4 ± 0.53	Very active
15 µg/ml	34.73 ± 1.67	Very active	32.36 ± 1.15	Very active

*Mean and standard deviation (SD) in mm
†Inferential assessment based on following criteria by Quinto & Santos (2006): <10mm – inactive; 10-13mm – partially active; 14-19mm – active ; >20mm – very active

A regression analysis was performed to check if the two treatments are similar in terms of the slope and elevation of their regression lines. The resultant lines are shown in Fig. 4.

A significant relationship between concentration and inhibition size has been found for both P. betle oil ($F(1,7)=24.4770$, $p=.002$) and clotrimazole ($F(1,7)=16.3559$, $p=.005$), and all regressions have the same slope ($F(1,14)=3.424$, $p=.085$), and elevation ($F(1,15)=0.130$, $p=.724$). The analysis shows that the two treatments have same rate increase in inhibition zone per change in concentration and exhibit no significant difference in inhibition diameter at the same concentration. It means that *P. betle* oil and clotrimazole are comparatively similar in their antifungal activity against T. mentagrophytes.

The mean values of the diameters of the zones of inhibition were greater than 10 mm for a 6 mm diameter agar well (Quinto & Santos, 2006) for both bacteria and fungi; they are effective against the test microorganisms. The activity is due to the constituents isosaffrole, eugenol isomers and caryophyllene. In one study, eugenol was identified as the antifungal principle in the oil (Pradhan, et,al., 2013). The *P. betle* oil may have the ability to permeate into the cell walls of the organisms or it has the ability to inhibit protein synthesis in the cell of the test organisms thereby inhibiting their growth and proliferation.

Minimum Inhibitory Concentration (MIC) of P. betle oil

Minimum inhibitory concentration is the lowest concentration that kills 99.9% of a bacterial or fungal population. Using the microdilution method,

the lowest concentration of the *P. betle* oil that inhibited the growth of the test organisms are: 62.50 µg/mL for *S. aureus*, 31.25 µg/mL for *S. pyogenes*, 125 µg/mL for both *E. coli* and *B. subtilis*, 250 µg/mL for *C. albicans,* and 1.95 µg/mL for *T. mentagrophytes*.

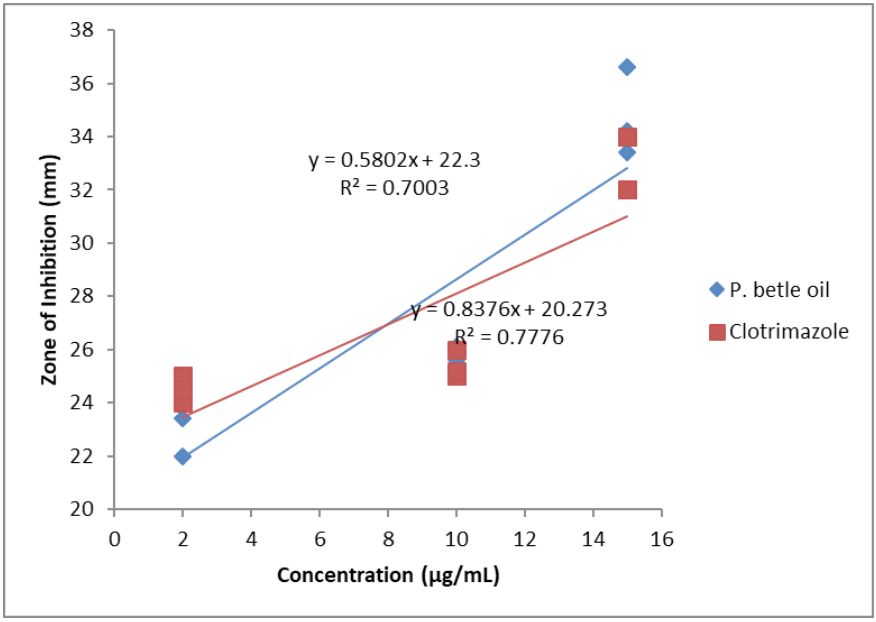

Figure 4. Scatter plot of inhibition zones produced by P. betle oil and control (clotrimazole) on T. mentagrophytes

The higher the value of the MIC for the test organism, the less resistant is the organism to the volatile oil, the lower the value the more sensitive is the organism to *P.betle oil* (Quinto & Santos, 2006). The findings specifically those for *S. pyogenes* (31.25 µg/mL) and *T. mentagrophytes* (1.95 µg/mL) are very useful in demonstrating the comparative activity of the volatile oil and the controls.

Minimum Bactericidal and Fungicidal Concentrations of P. betle Oil

The MBC and MFC were determined by inoculation of MIC tubes with no visible growth. It was found out that 62.50µg/mL was considered the MBC of the *P.betle* for *S. aureus;* 31.25 µg/mL for *S. pyogenes* and 125 µg/mL for *E. coli* and *B. subtilis*. The MFC for *C. albicans* and *T. mentagrophytes* are

250 μg/mL and 1.95 μg/mL, respectively. The MBC and MFC coincided with the MIC obtained.

Anticarcinogenic/Antimutagenic Effect of the P. betle Oil

The micronucleus test determines the capability of *P. betle* oil in inducing structural and/or numerical chromosomal damage. The average number of micronucleus formed in the bone marrow after treatment were obtained and compared with the cytotoxic drug cyclophosphamide and 5% Tween 80.

The analysis of variance showed that the effect of treatment on number of micronuclei was significant, $F(2,15)=707.64$, $p<.05$. Post hoc analyses were conducted given the statistically significant F-test. Specifically, Tukey HSD tests were conducted on all possible pairwise contrasts. The following pairs of groups were found to be significantly different ($p<.05$): *P. betle* volatile oil ($M=.517$, $SD=.0753$) and cyclophosphamide ($M=2.150$, $SD=.1225$), and cyclophosphamide and 0.5% Tween 80 ($M=.467$, $SD=.0516$). This showed that the induction of micronuclei by the *P. betle* oil and the negative control were comparable implying that the chemical constituents of the volatile oil have no carcinogenic nor mutagenic activity. This may be due to its antioxidant property or its ability to deactivate carcinogens, or by enhancing the tissue levels of protective enzymes in the body (Betel leaf cure, 2010). In a report by Indian Institute of Chemical Biology, as cited by Widowati, et. al. (2011), chlorogenic acid isolated from leaves of *P. betle* was shown to kill myeloid and lymphoid cancer cells but normal cells are unaffected. It induces apoptosis in human cancer cells transplanted in experimental nude mice but shows no effect on the growth of non-cancerous cells.

CONCLUSION

Piper betle is a twining plant native to Malaysia but found in most parts of the Philippines. The study on this medicinal herb reports the physical properties of the extracted volatile oil obtained from 5 specific places in the Philippines. Its chemical composition revealed 20 components. The antimicrobial activities of the volatile oil was also investigated and revealed a very important antimicrobial activity confirmed by its low

minimum inhibitory concentration (MIC). The volatile oil therefore can be used as a natural antimicrobial agent for the treatment of diseases caused by infectious bacteria and fungi. The volatile oil was also found to be non-mutagenic/carcinogenic as found in the micronucleus test. The mechanism of its antimicrobial activity is not yet well understood. Further studies on the pathway of microbial growth inhibition are recommended and preformulation data obtained in this study should be validated to establish specifications of the oil.

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