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Characterization and Evaluation of Antimicrobial Activity of the Essential Oil from the Leaves of *Piper betle* L.

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Abstract

The Betel oil from the leaves of *Piper betle* L obtained through steam distillation was characterized for its organoleptic properties and physicochemical constants. Freshly extracted Betel oil is pale yellow, soluble in common organic solvents and very slightly soluble in water. Its specific gravity is 0.9313; with 1.4526 as refractive index; +4.249 optical activity and ester value of 101. The chemical components of the oil identified via Gas Chromatography-Mass Spectroscopy consist of 5-(2-propenyl)-1, 3-benzodioxole, eugenol isomer and caryophyllene among others. The minimum effective concentration (MIC) of the oil was determined using Dilution method. The oil was found to have significant antibacterial and antifungal activity against *Staphylococcus aureus, Streptococcus pyogenes, Candida albicans* and *Trichophyton mentagrophytes* with MIC values of: 125 µg/ml; 15.60 µg/ml; 250µg/ml and 195µg/ml respectively. Antimicrobial activity was tested by agar diffusion method utilizing the same test organisms. The zones of growth inhibition measured to the nearest mm. were: 67.50 mm for *S. aureus*; 90 mm for S. *pyogenes, C. albicans* and *T. mentagrophytes.*

Key words: essential oil, antimicrobial activity, dilution method, agar diffusion, MIC

Introduction

It is well known that intensive use of an antibiotic is often followed by the appearance of resistant strains. In view of this, the search for new antimicrobial agents continues unabated. Medicinal plants are promising resources. The use of medicinal plants as screening pool for novel antibiotics has several advantages related to safety, availability, and minimizing the risk of side effects and addiction (Lee et al., 2003). The World Health Organization adopted a major policy change in accepting that most developing nations would have to make use of more traditional medical practices for primary health care. (Yuan and Lin, 2005).

Piper betle is a glabrous climbing vine belonging to the family Piperaceae. It is abundantly distributed in many Asian countries. The leaves have been used in traditional medicine as carminative, stimulant, antiseptic, antifungal, and antibacterial agent. The volatile oil known as Betel oil is the chief constituent of the leaves. *Piper betle* L. can be of great benefit in treating diseases caused by bacteria and fungi. Previous studies on the betel leaves, roots and whole extract (mixture of volatile and non-volatile) of the green variety showed a very strong antimicrobial activity (Jenie , 2001).

The oil was characterized by determining the physical and chemical properties. Antimicrobial activity was evaluated by the lowest concentration that will inhibit the growth of the test organisms by Dilution method. The zone of growth inhibition caused by the Betel oil in the Agar diffusion test was also measured as an indication of its activity.

An antimicrobial agent is a substance that kills or inhibits the growth of microorganisms. It may be categorized on the basis of their antibacterial activity as either bacteriostatic or

bactericidal. Some volatile oils have antifungal activity. These are agents capable of destroying or inhibiting the growth of fungi. These agents may either be fungicidal or fungistatic. The study was conducted to prove that the volatile oil of the vine is the chief constituent that causes its antimicrobial activity.

Figure 1 *Piper betle L.*Plant

Materials and Methods

Collection and Preparation of Plant Materials

The mature *Piper betle L*. leaves was collected from La Union, Abra, Iloilo City, Palawan and Malaybalay, Bukidnon. A representative of the whole plant was brought to the Philippine National Museum for authentication. The collected leaves were washed, dried between filter papers and air-dried. The leaves were cut into small pieces for extraction using steam distillation and isolated by rotary evaporation. The percentage yield of the oil was computed on the basis of the air-dried material.

Physical and Chemical Characterization of the Betel Oil

Physical characterization of the *Piper betle* L. volatile oil consists of the description and determination of attributes.

Organoleptic Properties

The volatile oil was placed in a transparent bottle over a white background and the color and clarity were observed; the characteristic odor was determined by sniffing; and to determine its characteristic feel to the touch, it was rubbed between fingers.

Solubility

The solubility of the *Piper betle* volatile oil was determined by mixing increment volumes of the volatile oil in specified volumes of the following solvents: water, chloroform, alcohol, anhydrous ether and petroleum ether**.**

Specific Gravity

Specific gravity is an important criterion of the quality and purity of volatile oils. The actual weight or the tare of a vial or was determined accurately using a Sartorius CP135 Balance. The vial was filled with water and weighed. The procedure was repeated using the *Piper betle* volatile oil in place of water. The specific gravity of the oil is expressed as the ratio of the weight of the volume of oil to that of an equal volume of pure water when both are determined at 25^{o}C (Knevel and DiGangi, 1977).

Specific Rotation

Both the degree of rotation and its direction are important criteria of purity. The extent of optical activity of oil was determined by a polarimeter (E. Harnack 220) which measured the degree of rotation. The zero point of the polarimeter was adjusted and determined. The previously cleaned and dried polarimeter tube was filled with 10% alcoholic solution of the volatile oil. The analyzer was rotated until equal illumination of light of the two halves of the visual field is achieved (Knevel and DiGangi, 1977).
The angles of rotation was determined and the specific rotation was calculated (USP 25, 2002).

$$[\alpha]= t/\lambda = 100\alpha/lcd$$

Where:

$[\alpha]$ = Specific rotation at λ , t = temperature, α = observed rotation in degrees, c = concentration
l = path length in dm, λ = wavelength of light used in nm, d = specific gravity of the oil

Refractive Index

The index of refraction is a physical constant frequently made use of in determination of the identity and purity of volatile oils.The test plate was attached to the refracting prism of the Atago DTM 1 refractometer provided with the test plate, by moistening the test plate with the liquid and pressing it against the refractive prism. The light was focused on the test plate. The instrument was adjusted until the borderline of the critical angle coincides with the cross hairs in the telescope, and the reading of the refractive index was taken. The test plate was removed, cleaned, and 2-3 drops of the *Piper betle* volatile oil was placed on the prism and the prism was clamped together firmly. The light source was fixed so that the light is reflected through the prisms and the instrument adjusted until the borderline between the light and dark halves of the field of view exactly coincides with the cross hairs of the telescope. The refractive index was then read. The values should be between 1.46 and 1.61 at 25^{O}C (Knevel and DiGangi, 1977).

Chemical Analysis of the Piper betle Volatile Oil

The *Piper betle* volatile oil was brought to the National Chemistry Instrumentation Center (NCIC) at Ateneo de Manila, Quezon City for its chemical characterization using Gas Chromatography- Mass Spectroscopy**.**

*Ester content (Saponification Value***)**

Determination of the total esters serves to detect adulteration and to establish the quality and purity of valued oils.
About 1.5 to 2 grams of the oil, accurately weighed was placed in a 250-mL Erlenmeyer flask to which 10 mL of neutralized alcohol and 2 drops of phenolphthalein TS was added drop

wise. Then 0.1N sodium hydroxide solution was added until a faint pink color appeared. After adding 25.0 mL of 0.5N alcoholic potassium hydroxide, a reflux condenser was connected and was then heated on a boiling water bath for 1 hour. The mixture was allowed to cool, about 20 mL of water and 3 drops of phenolphthalein TS was then added and the excess alkali was titrated with 0.5N hydrochloric acid. A blank test was performed and the total ester was calculated using the following formula (British Pharmacopoeia, 1980):

$$\text{Ester Value} = \frac{(\text{mL of 0.5NHCl} - \text{mL blank}) \times 0.5\text{N HCl} \times 56.11\text{mg/mEq}}{\text{Weight of sample}}$$

Preliminary Screening of the Antimicrobial Activity of Piper betle volatile oil

Assay for the Antibacterial and Antifungal Activity Using Agar-well Diffusion Method

Agar plates were prepared using Mueller Hinton agar for *Staphylococcus aureus* and *Streptococcus pyogenes* and Sabouraud glucose agar for *Candida albicans* and *Trichophyton mentagrophytes* in triplicate. The agar plates were swabbed with the test organisms. Six wells were made with a sterile cork borer. With a micropipette, 100 µL of the oil was measured and dripped directly into the well until a concave rim is achieved. The plates were incubated at 35^{o}C for bacteria and 37^{o}C for molds and yeasts containing plates. The results were observed after 18-24 hours for bacteria and yeast and 2-3 days for molds. The zone of growth inhibition was measured in millimeters using a caliper or ruler. The measurements were recorded (Quinto, *et al*., 2005)

Assay for the Determination of the Minimum Inhibitory Concentration

The lowest concentration or dilution of the volatile oil in a serial dilution sequence that results in the absence of observable growth is reported as the minimal inhibitory concentration, MIC. The lowest concentration or dilution that kills 99.9% of the test organism is reported as the minimal bactericidal concentration, MBC; minimal fungicidal concentration, MFC, if the test organism used is a yeast or mold.

Determination of the MIC (Bacteria) by Dilution Method

Thirteen screw-capped test tubes (13mm x 100 mm) were sterilized and numbered individually. One mL of Mueller-Hinton/Sabouraud glucose broth was introduced into tubes #2 to #11. To tube #12, 2.0 mL of Mueller-Hinton/Sabouraud broth was introduced; 1 mL of the *Piper betle* volatile oil was pipetted into tube #1 and #2 and capped, it was vortexed for 5 seconds; 1.0 mL was withdrawn from the contents of tube #2 and transferred to tube #3, after capping the tube and mixing by shaking the contents, 1.0 mL from the contents of tube #3 was withdrawn and transferred to tube #4, the tube was capped, shaken and mixed well. This process was continued until 1.0 mL was withdrawn from tube #9 and subsequently added to tube #10, capped and shaken. One mL of the diluted inoculum was introduced into tubes #1 to #11 and to tube #13. To tube #13, 1.0 mL of the antibiotic standard was added. The tubes were incubated at 35^{o}C for 18 to 24 hours. After incubation the tubes were examined for bacterial or fungal growth. This can be visible as turbidity in the tube or as whitish pellet at the bottom of

the tube. The tube with the lowest concentration of the volatile oil at which no growth or turbidity is observed was reported as the MIC against the organism *(*Quinto and Santos, 2006)

Determination of Minimal Bactericidal Concentration (MBC)

The tube with the lowest concentration of the *Piper betle* volatile oil that gave no visible growth/ turbidity and the succeeding tube with visible growth were selected. The tubes were gently shaken to homogenize the contents. A 0.01 mL of the contents of each tube was sub cultured by streaking on Mueller-Hinton agar plates.. The plates were inverted and incubated for 18-24 hours at 35^{o}C. The plates were observed for growth of colonies after the incubation period. The volatile oil concentration of the tube producing one colony or no colony at all on Mueller-Hinton plates was reported as the minimum bactericidal concentration, MBC. The MBC is used to measure the ability of the volatile oil to kill the test organisms (Quinto and Santos, 2006).

Determination of Minimal Fungicidal Concentration (MFC) (Fungi and Yeast)

The same procedure for MBC determination for bacteria was done using Sabouraud glucose agar plates instead of Mueller–Hinton agar. The plates were inverted and incubated at room temperature or at 30^{o}C or lower for 3 to 5 days. The plant concentration of the tube producing one colony or no colony at on Sabouraud glucose agar was reported as the MFC. The MFC is the last dilution or the minimum concentration of plant extract from the MIC tubes resulting in growth of 1 CFU or no growth in the Sabouraud glucose agar plates (Quinto and Santos, 2006).

Results and Discussions

Collection and Gathering of Material

The matured leaves were collected from La Union, Abra, Iloilo, Palawan and Malaybalay. A total of 33.759 kilograms was gathered. Approximate weights are shown as follows:

Place	Approximate Weights (Kg)
Abra	1.200
Iloilo	3.100
La Union	25.250
Malaybalay	3.250
Palawan	0.959
Total	33.759

A representative of the plant sample was authenticated by the Philippine National Museum in Manila.

Percentage Yield of the Volatile Oil

The air dried leaves were subjected to steam distillation in order to extract the volatile oil. Two hundred fifty six (256) mL was obtained from about 18 kilograms of air dried leaves. The steam distillation process had a standard deviation of 0.0951675, which is equivalent to 6.5946% relative standard deviation (RSD). The % of RSD indicates that experimental method

of steam distillation is imprecise due to wastage (specification: precision = maximum of 2%). Table 10 shows an average percentage yield of 1.4431% of the oil from 5 batches.

Table 1.

Percentage yield (v/wt) Volatile Oil in *Piper betle* Leaves.

Batch Number	Wt. of Leaves (Kg)	Volume of Oil	Percentage Yield (%v/w)
1	4.9833	73.00	1.4649
2	1.0248	14.50	1.4149
3	0.6067	9.70	1.5988
4	1.0960	15.00	1.3686
5	10.5258	144.00	1.3681
		Σ = 256.20	Ave =1.4431

Physical Properties and Constants of the Piper betle L. Volatile Oil

Organoleptic evaluation of the volatile oil was done and the following properties were noted: color, odor, taste and feel to the touch. The solubility in different solvents such as water, ethyl alcohol, chloroform, anhydrous ether and petroleum ether were determined. Physical constants such as specific gravity, optical rotation and refractive index of the volatile oil were also determined.

Table 2 shows the physical properties of the volatile oil. It is colorless to pale yellow when freshly extracted but acquires a darker yellow to orange on exposure to light and heat. It has a strong aromatic odor, pungent taste and is greasy to the touch. Pure volatile oils are colorless or with a yellowish tinge when freshly prepared. Their taste vary; sweet, mild, pungent, hot acrid, caustic or burning. They have a characteristic aroma or odor (Tyler, et al., 1988). The *Piper betle* volatile oil possesses the characteristics of most volatile oil.

Table 2.
Organoleptic Evaluation of *Piper betle* Volatile Oil

Organoleptic Property	Description
Color	Colorless to pale yellow
Odor	Strong aromatic odor
Taste	Pungent
Characteristic feel	Greasy

Table 3 shows that the *Piper betle* volatile oil is miscible in all proportions in organic solvents like ethyl alcohol, chloroform, anhydrous ether and petroleum ether. It is immiscible in water in the ratio of 0.1:0.1 but was soluble in 50.0 mL of water or no separation of phase was observed. Most volatile oils are miscible in organic solvents but sufficiently soluble in water but sufficiently soluble to form a saturated solution and impart its odor to the water. The *Piper betle* volatile oil also possesses this property of volatile oils (Tyler, et al., 1988).

Table 3.
Solubility of *Piper betle* Volatile Oil

Solvent	Volume Ratio (Oil:solvent) mL	Description
Water	1:1	immiscible
	1:5	No separation of phases but cloudy
Ethyl alcohol	1:1	miscible
Chloroform	1:1	miscible
Anhydrous ether	1:1	miscible
Petroleum ether	1:1	miscible

Physical constants serve as a means of assessing the purity and quality of the volatile oil as well as for identification. The specific gravity, optical activity and refractive index were determined. The table below shows the average values obtained from these determinations**.**

Table 4.
Physical constants of the *Piper betle* Volatile Oil at 25^{o}C

Trial	Specific gravity	Optical Activity	Refractive Index
1	0.9271	+4.295	1.4207
2	0.9164	+4.295	1.4860
3	0.9205	+4.188	1.4512
Average	0.9313	+4.259	1.4526

The average specific gravity of the volatile oil by the pycnometer method is 0.9313, the optical activity determined by a polarimeter (Model: E. Harnack 220) is +4.259 and the refractive index using a refractometer (Model: Atago DTM-1) is 1.4526. The determinations were done at 25^{o}C. The precision of the physical analysis was evaluated by determining the standard deviation and percentage relative deviation (%RSD). Statistical evaluation of the result shows that the precision of test for specific gravity was satisfactory because it did not exceed the maximum of 2%. Optical activity was also satisfactory with a %RSD = 1.68%. The test for refractive index slightly exceeded the maximum limit, the wavelength (289 nm) being held constant, the variability of the test may be due to the changing temperature of the sample.

Table 5
Precision of the physical analysis of *Piper betle* volatile oil

Physical Analysis	Standard deviation	%RSD
Specific gravity	0.0054	0.58
Optical activity	+4.259	1.68
Refractive index	0.0327	2.25

The specific gravity of official volatile oils varies approximately 0.842 to 1.172. Majority of the volatile oils are lighter than water and this indicates the *Piper betle* volatile oil with a specific gravity of 0.9246 is lighter than water and is within the range of specific gravity of volatile oils.

The rotary power of volatile oil varies within relatively wide limits. It can either be dextrorotatory or levorotatory. This may serve as an index of purity and identity of volatile oils. The obtained specific rotation indicates that the volatile oil is dextrorotatory.

The index of refraction is made use of in the determination of the identity and purity of volatile oils. It is commonly measured at 25^{o}C. The index of refraction does not vary greatly with different official volatile oils. The values are between 1.4600 and 1.6100. In some case refractive index may serve in the detection of extraneous matter. (Knevel and Digangi, 1977). The *Piper betle* volatile oil has a refractive index of 1.4626 which is within the range of refractive indices of volatile oils.

Chemical Analysis of Piper betle Volatile Oil

The *Piper betle* volatile oil was brought to the National Chemistry Instrumentation Center (NCIC) for instrumental analysis using Gas Chromatography-Mass Spectrometry to identify the different constituents of the volatile oil. The following table shows the peaks of the sample with their corresponding retention times, possible identities, and relative amounts of each volatile compound.

Table 6.
Chemical Composition of *Piper betle* Volatile Oil (NCIC)

Retention time (min)	Possible Identity	Rfit	Relative Amounts
11: 05	2-methyl-5(1-methylethyl)-bicyclo[3.1.0]hex-2-ene	989	1.99
11::20	Alpha-pinene	989	1.42
11: 51	Camphene	988	2.83
12: 42	Beta-phellandrene	980	3.44
13: 20	Beta-myrcene	971	0.41
13: 55	Alpha-phellandrene	968	0.17
14: 12	3-carene	980	7.83
14: 32	1-methyl-4-(1-methyl ethyl)benzene	985	1.56
14: 51	1-methyl-5-(1-metheyletenyl)cyclohexene	939	1.03
15: 59	1-methyl-4-(1-methylethyl)-1,4-cyclohexadiene	980	0.51
17: 09	2-carene	965	0.08
17: 29	3,7-dimethyl-1,6-octadien-3-ol	959	0.18
20: 32	4-methyl-1-(1-methylethyl)-3-cyclohexen-1-ol	889	2.12
23: 33	4-(2-propenyl)phenol	940	2.92
24: 26	5-(2-propenyl)-1,3-benzodioxole	978	48.47
26: 50	Eugenol isomer	967	14.03
27: 15	Eugenol isomer	957	8.07
28: 17	Copaene	976	0.59
29: 46	Caryophyllene	969	1.76
30: 52	Alpha -caryophyllene	977	0.59

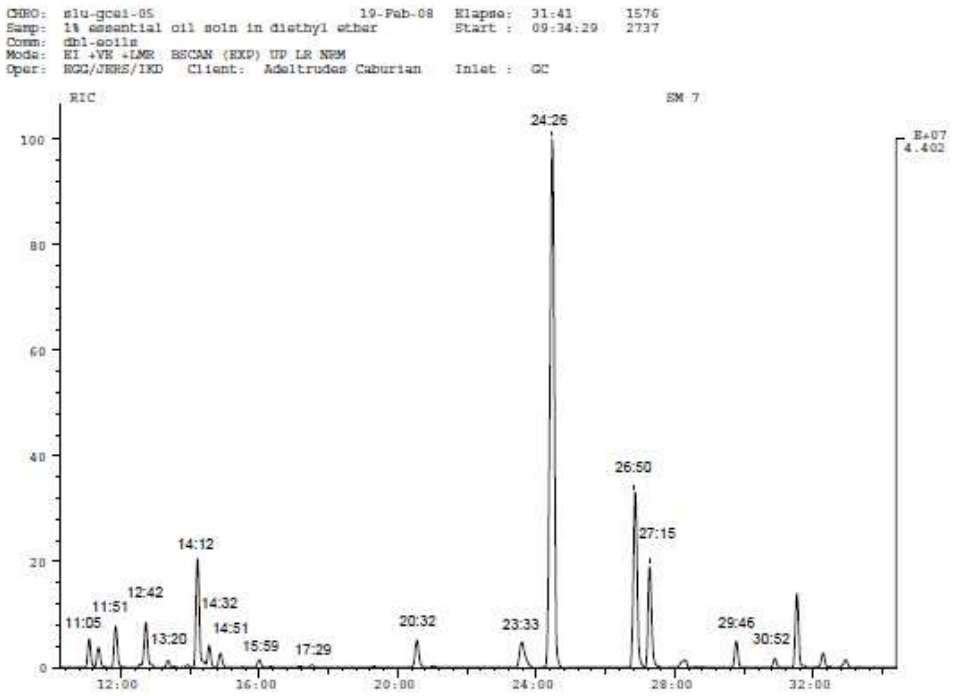

Figure 2 GC-MS Spectrum of the Betel Oil (NCIC)

Table 6 shows that the compounds with the highest retention time are the following: 5-(2-propenyl)-1, 3-benzodioxole (48.47), eugenol isomers (14,03 and 8.07 respectively) and 3-careen (7.83). Figure 2 is the spectrum of the constituents of the Betel oil showing the different peaks.

Screening of the Antimicrobial Activity of Piper betle Volatile Oil

Agar-well diffusion method was used to evaluate the antibacterial and antifungal activity of the volatile oil. This method is based on the ability of the volatile oil to diffuse through the agar medium that is heavily seeded with test organisms. The diameter of the clear zone surrounding the well containing the volatile oil was measured. This indicated the extent of the inhibitory power of the *Piper betle* oil against the test organisms namely: *Staphylococcus aureus* (UPCC4193), pure culture of *Streptococcus pyogenes*, *Candida albicans* (UPCC 2168) and *Trichophyton mentagrophytes* (UPCC4193). The test organisms were obtained from UP Research Institute, Diliman Quezon City and the pure culture of *Streptococcus pyogenes* was obtained from Saint Louis University Hospital of the Sacred Heart. The measured zones of growth inhibition of *Staphylococcus aureus* and *Streptococcus pyogenes* are shown in the following table.

Table 7.
Zones of growth inhibition of *Staphylococcus aureus, Streptococcus pyogenes* as affected by *Piper betle* volatile oil in mm.

Replication	Microorganisms	
	Staphylococcus aureus	*Streptococcus pyogenes*
1	76.00	90.00
2	66.00	90.00
3	67.50	90.00
Mean	67.50	90.00

Based on the Table 7 the volatile oil produced the highest mean zone of growth inhibition against *S. pyogenes* with a mean of 90.00 mm. To further analyze and interpret the data gathered, the mean zones were subjected to a T-test procedure to determine if there is a significant difference in the zones of inhibition produced by the volatile oil against the bacterial test organisms.

Table 8.
Test of the significance on the difference of zone of growth inhibitions of *S. aureus* and *S. pyogenes* using T-test procedure

Type of bacterial organisms	Mean	Mean difference	T-value	Tabular value	Interpretation
Staphylococcus aureus	67.50	22.50	4.959	2.776	Significant
Streptococcus pyogenes	90.00				

The T-value of 4.959 obtained is greater than the tabular value 2.776. This indicates that there is a significant difference in the mean zones of growth organisms as affected by the volatile oil. This therefore implies that the antibacterial activity of the volatile oil against *S. pyogenes* is greater than in *S. aureus.*

Table 9.
Zone of growth inhibition of *C. albicans* and *T. mentagrophytes* as affected by Piper betle oil in mm

Replication	Microorganisms	
	Candida albicans	*Trichophyton mentagrophytes*
1	90.00	90.00
2	90.00	90.00
3	90.00	90.00
Mean	90.00	90.00
Computed value: N/A	Tabular value: N/A	Interpretation: N/A

The zone of growth inhibition for both is 90.00 mm. This implies that the antifungal activity of the volatile oil towards the test organisms are comparable or the same.

The pure volatile oil was found to be very effective against the test organisms. It either was able to permeate into the cell walls of the organisms or was able to inhibit protein synthesis in the cell of the test organisms thereby inhibiting their growth and proliferation.

Determination of the Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration is the lowest concentration that kills 99.9% of bacteria and fungi. The MIC of the volatile oil was determined by micro dilution method using two-fold dilution for the dilution series. Table 16 shows the results of the MIC of the test organisms.

Table 10.
MIC results for *S. aureus, S. pyogenes, C. albicans* and *T. mentagrophytes* (DOST- Benguet)

Conc. (µg/mL) Dilution level	Growth of Test Organisms			
	Staphylococcus aureus	*Streptococcus pyogenes*	*Candida albicans*	*Trichophyton mentagrophytes*
1000.00	-	-	-	-
500.00	-	-	-	-
250.00	-	-	-	-
125.00	-	-	+	-
62.50	+	-	+	-
31.30	+	-	+	-
15.60	+	-	+	-
7.80	+	+	+	-
3.90	+	+	+	-
1.95	+	+	+	-

Inferences from the above assay table: the minimum inhibitory concentration of *Staphylococcus aureus* is 125 µg/mL, *Streptococcus pyogenes* 15.60 µ/mL, Candida *albicans* 250µg/mL and Trichophyton *mentagrophytes* is 1.95 µg/mL.

Conclusion

Medicinal plants have played a vital role in treating diseases and in promoting health of mankind for a long time. They continue to become an important source of medicinal agents. The utilization of local sources as alternative drug can be more or equally effective as the synthetic counterpart.

The ability to assure the physical and chemical properties of an active pharmaceutical ingredient in a drug product is critical for regulatory approval and therapeutic success. Physical constants such as specific gravity, refractive index, optical rotation obtained confirms that the Betel oil is pure and possesses the characteristics of a volatile oil

The dilution method and the disc diffusion method/agar well diffusion are used to determine the antimicrobial activity of the oil. The study showed that the Betel oil has a minimum inhibitory concentration for *Staphylococcus aureus* at 125 µg/mL, *Streptococcus pyogenes* 15.60 µg/mL, Candida *albicans* 250 µg/mL and Trichophyton *mentagrophytes* at1.95 µg/mL. The zone of growth inhibition of 67.50 mm for *S. aureus*, 90 mm for *S.pyogenes*, *C. albicans* and *T. mentagrophytes* demonstrated that Betel oil is a very effective antimicrobial agent.

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