Evaluation of Antibacterial Activity of Crude Aqueous, Ethanolic and Methanolic Leaf Extracts of *Piper retrofractum* Vahl. and *Piper betle* L.

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**ABSTRACT**

*Piper retrofractum* and *P. betle* are commonly used as medicinal plants in the Philippines to treat several ailments including headache, fever, stomachache, cough, cuts and wounds. To determine how effective these plants are in treating microbial-caused ailments, this study was conducted. This primarily aimed to investigate the antibacterial activities of the crude aqueous, ethanolic and methanolic leaf extracts of these two plants using disc-diffusion assay and resazurin-based microtiter broth dilution method. Antibacterial tests against *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* showed that ethanolic and methanolic leaf extracts of *P. betle* had the highest zone of inhibition (i.e., 17.67 mm) against *E. coli* and *S. aureus*, respectively. The same was exhibited by methanolic extract of *P. retrofractum* against *S. aureus*. Maximum antibacterial activity was then recorded for methanolic and ethanolic leaf extracts of *P. betle* against *E. coli* and *S. aureus*, respectively at a Minimum Inhibitory Concentration (MIC) of 0.59 mg/mL. These results support the efficacy of these plants in treating skin and gastrointestinal diseases caused by *S. aureus* and *E. coli*, correspondingly.

**Key words:** Antibacterial, Disk diffusion assay, Microtiter broth dilution, Minimum inhibitory concentration, *Piper betle*, *Piper retrofractum*.

**INTRODUCTION**

The use of natural products with therapeutic properties is as ancient as human civilization and for a long time, plants are the main source of raw materials for most of these traditional medicines.[1,2] It was reported that about 80% of the world's population still rely on traditional plant-based medicines[3] which are being used for the treatment of many infectious diseases. Medicinal plants remain widely used as it provides safe and cost-effective remedies[4] with fewer to no known side effects.[5] The use of plants in traditional medicine is well known in rural areas of many countries. The Philippines is one of the Asian countries with diverse flora encompassing numerous species that are believed to possess medicinal properties.[6] Its utilization in local communities and by medicinal healers (locally known as *albularyo*) is a significant part of Philippine tradition and remains widely practiced to date; however, most of these claims lack validation of safety and efficacy.[7]

Examples of local medicinal plants are *Piper retrofractum* Vahl. and *P. betle* L. which are tropical vines belonging to the family *Piperaceae*. *P. retrofractum* (also known as Javaneese long pepper, or *litlit*) is relatively a less known spice. However, in traditional medicinal scope, this plant is often used as an anti-flatulent and expectorant.[8] More known member of family *Piperaceae* is *P. betle* (also known as betel nut or *ikmo*)[9,10] that is extensively cultivated throughout Southeast Asia.[11] Due to the strong pungent aromatic flavor of betel leaves, it is used as masticatory for oral hygiene and teeth preservation,[12] as a treatment to wounds and dyspnea and as an expectorant for inflammation and...
infection of the respiratory tract.\textsuperscript{[13,14]} Locally, these plants are known to treat ailments including headache, fever, stomachache, cough, cuts and wounds. To validate the efficacy of \textit{P. retrofractum} and \textit{P. betle} for human use, this study was conducted. This specifically encompasses determining the antibacterial potential of these two local medicinal plants.

**MATERIALS AND METHODS**

**Collection and Preparation of Leaf Samples**

Leaves of \textit{P. retrofractum} and \textit{P. betle} were collected from Tagaytay City area and were placed in clean plastic bags and stored in an ice cooler before transporting to the drying site.\textsuperscript{[15]} Leaves were cleaned with tap water to remove dirt and unwanted particles before air-drying under shade. Dried leaves were ground and pulverized using an electric blender.

**Crude Leaf Extraction**

Water, 95\% ethanol and 95\% methanol were used as solvents in preparing the medicinal plants for experimental use. Following Gakunga \textit{et al.}\textsuperscript{[16]} for every 100 g of powdered leaves, it was extracted in 500-mL solvent for 48 hr and was filtered using a muslin cloth afterward. Filtrates were transferred into clear, wide-mouthed glass vials and were oven-dried at 50°C until dried crude extract was left.\textsuperscript{[17]} Calculated volumes of 5\% DMSO (dimethyl sulfoxide) were then added into each vial\textsuperscript{[18,19]} to give a final crude extract concentration of 600 mg/mL. Crude extracts were finally sterilized by membrane sterilization\textsuperscript{[20,21]} and refrigerated prior to use.\textsuperscript{[22]} Sterility of extracts was ensured by plating them on Nutrient Agar (NA) every before any experiment was performed.

**Determination of Antimicrobial Activity of Crude Leaf Extracts**

**Test organisms**

The antimicrobial activity of the leaf extracts was tested against four pathogenic species, namely: Gram-positive: \textit{Enterococcus faecalis} ATCC 33186 and \textit{Staphylococcus aureus} ATCC 25923; Gram-negative: \textit{Escherichia coli} ATCC 25923 and \textit{Pseudomonas aeruginosa} ATCC 27853. Medical ailments associated with these pathogens are presented in Table 1. Bacterial cultures of these test organisms were maintained on Tryptone Glucose Yeast Extract Agar (TGYA). Following the procedures of Balinado and Chan,\textsuperscript{[23]} microbial cultures were allowed to grow in Nutrient Broth (NB) for 24 hr at 37°C. Resulting bacterial broth cultures were diluted in 0.9\% saline solution and their absorbance was compared spectrophotometrically to 0.5 McFarland\textsuperscript{[24]} turbidity standards to give an approximate cell density of 1-5 \times 10\(^6\) cells per mL at 530 nm.

**Disc diffusion assay**

Twenty microliters of previously prepared bacterial suspension were spread-plated on Mueller-Hinton Agar (MHA) plates and were incubated at 37°C for an hour. Sterile 6-mm filter paper discs impregnated each with 12 \(\mu\)L of the prepared crude extract (\textit{i.e.,} 7.2 mg of plant extract per disc) were placed onto resulting agar plates together with two other discs. These discs were impregnated with 5\% DMSO and an antibiotic (\textit{i.e.,} 2 \(\mu\)g/mL clindamycin for \textit{S. aureus} and \textit{E. faecalis} and 5 \(\mu\)g/mL ciprofloxacin for \textit{E. coli} and \textit{P. aeruginosa}) to serve as negative and positive controls, respectively. All tests were performed in triplicates. MHA plates were incubated at 37°C for 24 hr. Antimicrobial activity was assessed by measuring the resulting zones of inhibition in millimeter.\textsuperscript{[25]}

**Microtiter Broth Dilution Method**

Minimum Inhibitory Concentration (MIC) of plant extracts that showed inhibitory activity in the disc diffusion assay was determined using a microtiter broth dilution broth method. This followed the protocol presented by Balinado and Chan.\textsuperscript{[23]} Each of the 96-well microplates was first filled with 75 \(\mu\)L NB. Seventy-five microliters of the previously prepared crude extract were then dispensed into the first well of the microplate from where a two-fold serial dilution began and which terminated at the 10\(^{th}\) well.

**Table 1: Test organisms with medical ailments associated with these pathogens.**

<table>
<thead>
<tr>
<th>Test Organism</th>
<th>Associated Ailment</th>
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<tbody>
<tr>
<td>\textit{Staphylococcus aureus}</td>
<td>abrasion, abscesses (boils), bloodstream infections, furuncles, lacerations, pneumonia</td>
</tr>
<tr>
<td>\textit{Enterococcus faecalis}</td>
<td>endocarditis, bacteremia, intra-abdominal, periodontitis, pelvic abscesses, soft tissue infections, urinary tract infections</td>
</tr>
<tr>
<td>\textit{Escherichia coli}</td>
<td>bacteremia, myalgia, intestinal infection, soft tissue infections, urinary tract infection</td>
</tr>
<tr>
<td>\textit{Pseudomonas aeruginosa}</td>
<td>endocarditis, cystic fibrosis, pneumonia, superficial skin infection, \ tussis, urinary tract infection</td>
</tr>
</tbody>
</table>

Note: Underlined ailments were locally known to be addressed by \textit{P. retrofractum} and \textit{P. betle}.
Seventy-five microliters of each test organism were transferred in each well. This gave the following plant extract concentrations (in mg/mL) per well: 300.00, 150.00, 75.00, 37.50, 18.75, 9.38, 4.69, 2.34 and 1.17. The remaining two wells in each row of the microplate were allotted for negative (i.e., plant extract replaced with 5% DMSO) and positive (i.e., plant extract replaced with 50 µL of antibiotics: 2 µg/mL clindamycin for S. aureus and E. faecalis and 5 µg/mL ciprofloxacin for E. coli and P. aeruginosa) controls. Lastly, 10 µL of filter membrane-sterilized resazurin solution (Preparation: 300 mg of resazurin dissolved in 40 mL sterile water) was added into each well. All tests were performed in triplicates and incubated for 24 hr at 37°C. MIC values were recorded as the least amount of plant extract capable of preventing visible microbial growth organism[26] or a no change in resazurin dye color from purple to pink or to uncolored.[27]

RESULTS

Disc diffusion assay revealed that only ethanolic and methanolic leaf extracts of both P. retrofractum and P. betle had inhibitory activity against all the test organisms used. For P. betle (Figure 1), the highest inhibition (17.67 mm) was exhibited by ethanolic and methanolic leaf extracts against E. coli and S. aureus, respectively. The smallest inhibition was, on the other hand, exhibited by ethanolic extract against E. faecalis. The recorded highest inhibition for E. coli was found to be not significantly different (p < 0.05) with the positive control (16.30 mm), while for S. aureus, the activity was found to be significantly higher than the positive control (11.67 mm). For P. retrofractum (Figure 2), the highest inhibition was exhibited against S. aureus by methanolic (17.67 mm) and ethanolic (13.50 mm) leaf extracts. On the other hand, the smallest inhibition was exhibited by ethanolic leaf extract against P. aeruginosa (5.00 mm). The recorded highest inhibition for S. aureus was found to be significantly different from the positive control (11.67 mm).

All the negative control discs impregnated with 0.5 percent DMSO did not produce any zones of inhibition on any of the ATCC bacterial isolates. The crude extracts that showed inhibitory activity in the disc diffusion assay were further screened for their antibacterial potential by determining their Minimum Inhibitory Concentrations (MICs) using microtiter broth dilution method. As shown in Table 2, ethanolic and methanolic leaf extracts of P. betle showed promising activity as they inhibited the growth of the four test organisms at a very low concentration. The highest activity of ethanolic extracts (MIC=0.59 mg/mL) was observed against E. coli and S. aureus, while for methanolic extracts (0.59 mg/mL), it was recorded against S. aureus only. These were also identified as the maximum activities against these test organisms. High antibacterial activity of 9.38 mg/mL was observed both in ethanolic and methanolic extracts against E. faecalis, 18.75 mg/mL in methanolic extract against S. aureus and 150.00 mg/mL of both extracts against P. aeruginosa.

Similarly, ethanolic and methanolic extracts of P. retrofractum showed promising activities as they inhibited the growth of the test organisms at a very low concentration. The highest activity of ethanolic and methanolic extracts was found against E. coli (MIC=0.59 mg/mL). These were also identified as the maximum activities against these test organisms. High antibacterial activity of 9.38 mg/mL was observed for ethanolic extract against P. aeruginosa and methanolic extract against S. aureus; 18.75 mg/mL of methanolic and ethanolic extracts against E. faecalis and S. aureus, respectively; and 75.00 mg/mL for methanolic extract against P. aeruginosa.

DISCUSSION

Results of this study were similarly obtained by Rahman et al.[28] wherein P. betle methanolic and ethanolic extracts were observed to have antibacterial activity against food and waterborne pathogenic bacteria...
such as S. aureus, Bacillus cereus, E. coli, Salmonella typhi, P. aeruginosa and Bacillus subtilis.

It was observed in this study that methanolic extracts of P. betle were the best extract in inhibiting the growth of Gram-positive bacteria, while aqueous and ethanol extracts were less effective as antibacterial agents except against S. aureus. Likewise, the study by Mohtar et al.[30] revealed that methanolic extract of P. betle had a significant antibacterial activity on S. aureus. The chavibetol component contained in the methanolic extract of P. betle was higher and this compound may help in the killing mechanism of P. betle towards certain organisms. In addition, a study conducted by Khan and Kumar[31] showed that ethanolic and methanolic extracts of P. betle leaves were effective against E. coli, P. aeruginosa and S. aureus although methanolic extract was found to be more effective. On the other hand, Mohtar et al.[32] further revealed that ethanolic extract of P. betle leaves had higher antibacterial activity inhibiting Vibrio parahaemolyticus as compared to methanol extract. The antimicrobial activity of the ethanolic extract of P. betle against V. cholera ATCC 6395, E. coli ATCC 25922, E. coli O175: H7 12049, Shigella dysenteriae-1-MJ-84 and S. aureus ATCC 25923 was also recorded by Mahfuzul et al.[33]

Meanwhile, the antibacterial effect of P. retrofractum against S. aureus, P. aeruginosa and E. coli was similarly presented by Jamal et al.[34] and Salleh et al.[35] This activity was found to be associated with the essential oil component of its leaves. In addition, Biswas et al.[36] noted the role of chabbarin in the medicinal property of P. chaba (i.e., a synonym of P. retrofractum). It was found to be responsible for its activity against E. coli and P. aeruginosa. Its inhibition of E. coli and S. aureus growth was also supported by the study of Naz et al.[37]

CONCLUSION

Crude ethanolic and methanolic leaf extracts of P. betle and P. retrofractum had high antibacterial activity against E. coli, E. faecalis, P. aeruginosa and S. aureus. Among all the extracts used, the maximum activity was recorded against E. coli and S. aureus using methanolic and ethanolic P. betle leaf extracts, respectively. The inhibitory activities exhibited by P. betle extracts against the test organisms supported its role in treating bacterial infections in general, while its specific activity against S. aureus explains its efficacy in dealing with skin infections.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS


SUMMARY

This study reports on the antibacterial activity of crude aqueous, ethanolic, and methanolic leaf extracts of P. retrofractum and P. betle collected from Tagaytay City, Cavite, Philippines. The results of the disc diffusion assay and the resazurin-based microtiter broth dilution method showed that ethanolic and methanolic extracts of both plants were inhibitory against all the test isolates used: E. coli, P. aeruginosa, E. faecalis, and S. aureus. These greatly inhibited the growth of E. coli at a concentration as low as 0.59 mg/mL. The same was observed against S. aureus using the ethanolic P. betle extract.
REFERENCES


