Original Research Article

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.*

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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

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infection of the respiratory tract.[13,14] Locally, these plants are known to treat ailments including headache, fever, stomachache, cough, cuts and wounds. To validate the efficacy of *P. retrofractum* and *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 *P. retrofractum* and *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.[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 *et al.*[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.[17] Calculated volumes of 5% DMSO (dimethyl sulfoxide) were then added into each vial[18,19] to give a final crude extract concentration of 600 mg/mL. Crude extracts were finally sterilized by membrane sterilization[20,21] and refrigerated prior to use.[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: *Enterococcus faecalis* ATCC 33186 and *Staphylococcus aureus* ATCC 25923; Gram-negative: *Escherichia coli* ATCC 25923 and *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,[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[24] turbidity standards to give an approximate cell density of 1-5 x 10^6 cells per mL at 530 nm.

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

Test Organism	Associated Ailment
Staphylococcus aureus	abrasion, abscesses (boils), bloodstreams infections cellulitis, furuncles, lacerations, pneumonia
Enterococcus faecalis	endocarditis, bacteremia, intra-abdominal, periodontitis, pelvic abscesses, soft tissue infections, urinary tract infections
Escherichia coli	bacteremia, myalgia, intestinal infection, soft tissue infections, urinary tract infection
Pseudomonas aeruginosa	endocarditis, cystic fibrosis, pneumonia, superficial skin infection, tussis, urinary tract infection

Note: Underlined ailments were locally known to be addressed by *P. retrofractum* and *P. betle.*

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 µL of the prepared crude extract (*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 (*i.e.,* 2 µg/mL clindamycin for *S. aureus* and *E. faecalis* and 5 µg/mL ciprofloxacin for *E.coli* and *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.[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.[23]

Each of the 96-well microplates was first filled with 75 µL 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 10th well.

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 and 0.59. 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).

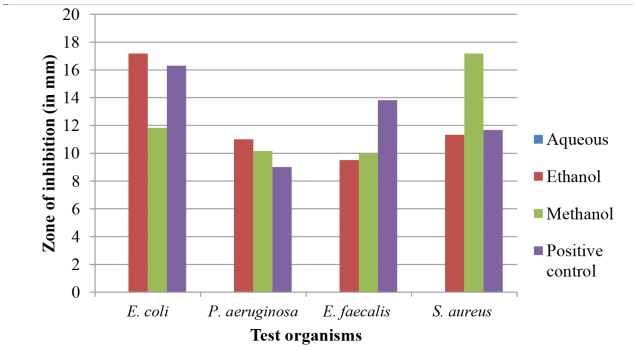

Figure 1: Mean zone of inhibition of crude leaf extracts of *P. betle* against *E. coli, E. faecalis, S. aureus* and *P. aeruginosa.*

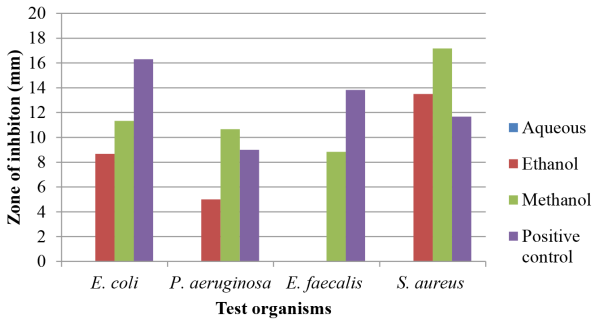

Figure 2: Mean zone of inhibition of crude leaf extracts of *P. retrofractum* against *E. coli, E. faecalis, S. aureus* and *P. aeruginosa.*

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

Table 2: Minimum inhibitory concentrations (MIC, in mg/mL) of crude leaf extracts of *P. betle* and *P. retrofractum* against *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus*.

Plant	Extraction Solvent	Minimum Inhibitory Concentration (MIC, in mg/mL)			
		E. coli	*P. aeruginosa*	*E. faecalis*	*S. aureus*
P. betle	Aqueous	-	-	-	-
	Ethanol	0.59	150.00	9.38	0.59
	Methanol	0.59	150.00	9.38	18.75
P. retrofractum	Aqueous	-	-	-	-
	Ethanol	0.59	9.38	-	18.75
	Methanol	0.59	75.00	18.75	9.38

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.*[29] 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[30] 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.*[29] 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.*[31]

Meanwhile, the antibacterial effect of *P. retrofractum* against *S. aureus, P. aeruginosa* and *E. coli* was similarly presented by Jamal *et al.*[32] and Salleh *et al.*[33] This activity was found to be associated with the essential oil component of its leaves. In addition, Biswas *et al.*[34] 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.*[35]

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

ATCC: American Type Culture Collection; **DMSO:** Dimethyl Sulfoxide; **MIC:** Minimum Inhibitory Concentration; **MHA:** Mueller-Hinton Agar; **NA:** Nutrient Agar; **NB:** Nutrient Broth; **TGYA:** Tryptone Glucose Yeast Extract.

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 inihibited 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.

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