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Antibacterial activities of ethanol extracts of Philippine medicinal plants against multidrug-resistant bacteria

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ABSTRACT

Objective: To investigate the antibacterial activities of crude ethanol extracts of 12 Philippine medicinal plants.

Methods: Crude ethanol extracts from 12 Philippine medicinal plants were evaluated for their antibacterial activity against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, extended spectrum β-lactamase-producing, carbapenem-resistant Enterobacteriaceae and metallo-β-lactamase-producing *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

Results: The leaf extracts of *Psidium guajava*, *Phyllanthus niruri*, *Ehretia microphylla* and *Piper betle* (*P. betle*) showed antibacterial activity against the Gram-positive methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus*. *P. betle* showed the highest antibacterial activity for these bacteria in the disk diffusion (16-33 mm inhibition diameter), minimum inhibitory concentration (19-156 μg/mL) and minimum bactericidal concentration (312 μg/mL) assays. *P. betle* leaf extracts only showed remarkable antibacterial activity for all the Gram-negative multidrug-resistant bacteria (extended spectrum β-lactamase-producing, carbapenem-resistant Enterobacteriaceae and metallo-β-lactamase-producing) in the disk diffusion (17-21 mm inhibition diameter), minimum inhibitory concentration (312-625 μg/mL) and minimum bactericidal concentration (312-625 μg/mL) assays.

Conclusions: *P. betle* had the greatest potential value against both Gram-negative and Gram-positive multidrug-resistant bacteria. Favorable antagonistic activities were also exhibited by the ethanol extracts of *Psidium guajava*, *Phyllanthus niruri* and *Ehretia microphylla*.

1. Introduction

Antibiotic resistance is a problem that continues to challenge the healthcare sector in a large part of the world in both developing and developed countries. The spread of multidrug-resistant (MDR) bacteria in hospital and community settings remains a widely unresolved problem and a heavy burden to health services[1]. Despite advances in antibiotic therapy, infectious complications remain an important cause of mortality and morbidity among hospitalized patients. Although medical practitioners can resort to second- or third-choice drugs for treating these patients, the use of these synthetic drugs may subject the patient to a higher risk, due to the possibility of the drugs producing more harmful side effects. To address this challenge, actions must be taken to reduce this problem, such as controlling the use of antibiotics, understanding the genetic mechanisms of resistance and developing new antibiotics and new therapeutic strategies. Advances in identifying new sources of natural products with antimicrobial activities and expanding antibiotic chemical diversity are providing chemical leads for new drugs[2].

The vast majority of modern medications were derived originally

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from ancient herbal traditions. The practices of plant-based traditional medicine are founded on hundreds of years of belief and observations, which predate the development of modern medicine. Medicinal plants have been used for centuries as remedies for human diseases as they contain components of therapeutic value. There are numerous plant natural products which have antifungal, antibacterial and antiprotozoal activities that could be used either systemically or locally[3]. Several plants containing volatile oils, polyphenols and alkaloids as active constituents are utilized as popular folk medicines, while others gained popularity in the form of finished products collectively named phytomedicines[4]. During the second half of the 20th century, the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the classical antibiotics led researchers to investigate the antimicrobial activities of medicinal plants. Antimicrobials of plant origin have enormous therapeutic potential[3]. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials.

Traditionally used medicinal plants produce a variety of compounds of known therapeutic properties. One of the vital activities possessed by these medicinal plants is antimicrobial. The scarcity of infective diseases in plants is in itself an indication of the successful defense mechanisms developed by them[5]. The substances that can either inhibit the growth of bacteria or kill them, with no toxicity or minimum toxicity to host cells are considered candidates for developing new antimicrobial drugs[6]. Some of the bioactive compounds could hinder the life processes of disease-causing bacteria, either by itself or in combination with other therapeutic agents[7]. In recent years, antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world[8-10]. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug resistant microbial pathogens. However, very little information is available on such activity of medicinal plants[6]. There have been numerous documentations found in the medical literature concerning the significance of traditional medicinal plants as alternatives to synthetic antibacterial and antifungal medications[11-17]. Most of these published works come from many countries that are still practicing the use of herbal medicine for the treatment of various diseases for practical and economic reasons. These studies are valuable resources for local medical scientists who seek to explore and substantiate the antibacterial and antifungal activities of Philippine medicinal herbs, particularly against MDR bacteria. Knowledge on the different antimicrobial assays and the plants' bioactive compounds are vital for the design of future studies[2].

The Philippines is one of the Asian countries with a diverse flora, and numerous species are believed to possess curative properties. However, most of these claims lack scientific validation. The use of herbal medicines in the pre-Spanish era had been widespread, although its documentation was only initiated during the Spanish occupation, with the main practitioners being called the *mediquillos* (herbal scientists) or *herbolarios*[18]. In 1997, the Philippine Government passed Republic Act 8423, also known as the "Traditional and Alternative Medicine Act of 1997," an act creating the Philippine Institute of Traditional and Alternative Health Care to accelerate the development of traditional and alternative health care in the Philippines[19]. Most noteworthy objectives include "the encouragement of scientific research on traditional and alternative health care systems that have direct impact on public health care, and the promotion of the use of traditional, alternative, preventive and curative health care modalities that have been proven safe, effective, cost-effective, and they are consistent with government standards on medical practice."

The Infectious Disease Society of America has considered the following bacteria as especially challenging in terms of management: methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), extended spectrum β-lactamase (ESβL)-producing and carbapenem-resistant Enterobacteriaceae (CRE), metallo-β-lactamase (MβL)-producing *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Acinetobacter baumannii* (*A. baumannii*)[20]. In Asia, the same emerging MDR bacteria have turned into an immense threat. There have also been distressing reports regarding the occurrence of ESβL-producing Enterobacteriaceae in fresh imported culinary herbs from Thailand, Vietnam and Malaysia, which are usually consumed without appropriate heating[21]. Even *A. baumannii*, over the last 10 years, has emerged as one of the most problematic bacteria since it exhibits a wide spectrum of antimicrobial resistance mechanisms, such that treatment has been limited to a few antibiotics[22]. VRE likewise has emerged as one of the difficult-to-treat bacteria that cause several life-threatening infections, and for which a definitive and successful therapy remains elusive from the reach of an ever-growing number of patients[23]. The advent of these MDR bacteria has solicited some inquiries with regard to the future of therapeutic drugs in chemotherapy. In line with the aforementioned findings, it is crucial that therapeutic antimicrobial studies should be more focused on the inhibition of these medically significant bacteria[24].

This study aimed to investigate the antibacterial activities of crude ethanol extracts of 12 Philippine medicinal plants, namely, *Cassia alata* L. (locally known as "akapulko") (*C. alata*), *Psidium guajava* L. (locally known as "bayabas") (*P. guajava*), *Piper betle* L. (locally known as "ikmo") (*P. betle*), *Vitex negundo* L. (locally known as "lagundi") (*V. negundo*), *Mitrephora lanotan* (locally known as "lanotan") (*M. lanotan*), *Zingiber officinale* Rosc. (locally known as "luya") (*Z. officinale*), *Curcuma longa* (locally known as "luyang dilaw") (*C. longa*), *Tinospora rumphii* Boerl (locally known as "makabuhay") (*T. rumphii*), *Moringga oleifera* (locally known as "malunggay") (*M. oleifera*), *Phyllanthus niruri* (locally known as "sampa-sampalukan") (*P. niruri*), *Centella asiatica* (locally known as "takip kuhol") (*C. asiatica*), and *Ehretia microphylla* Lam. (locally known as "tsaang gubat") (*E. microphylla*) against different MDR bacteria isolated from a tertiary medical center in the Philippines. Selection of the plants was based on their documented medicinal values. All 12 plants have traditionally known antibacterial and antifungal activities, with no extensive bioassay studies performed locally. In order to identify the plants with potential bioactive molecules of pharmaceutical importance in the management of MDR bacteria, *in vitro* methods of evaluation on test organisms were performed using the plant extracts.

2. Materials and methods

2.1. Plant materials

The 12 medicinal plant samples tested in this study are shown in Table 1. These were collected from different regions of the Philippines. All the plant materials were identified and compared

to voucher specimens at the Herbarium of the Institute of Biology, University of the Philippines Diliman for authentication. The different plant parts were washed with water to remove all unwanted materials, air-dried, pulverized in a mill and stored in a sterile air-tight container until further use.

Table 1

Philippine medicinal plants assayed against different MDR bacteria.

Scientific name	Family	Local name	Plant part used	Place of collection
P. guajava	Myrtaceae	Bayabas	Leaves	Tacloban, Leyte
M. oleifera	Moringaceae	Malunggay	Leaves	Cainta, Rizal
T. rumphii	Menispermaceae	Makabuhay	Stem	Tacloban, Leyte
P. niruri	Euphorbiaceae	Sampa-sampalukan	Aerial parts	Tacloban, Leyte
C. asiatica	Apiaceae	Takip kuhol	Leaves	Luisiana, Laguna
P. betle	Piperaceae	Ikmo	Leaves	General Nakar, Quezon
C. longa	Zingiberaceae	Luyang dilaw	Rhizome	Luisiana, Laguna
Z. officinale	Zingiberaceae	Luya	Rhizome	Luisiana, Laguna
V. negundo	Verbenaceae	Lagundi	Leaves	Roxas, Palawan
M. lanotan	Annonaceae	Lanotan	Leaves	Mt. Isarog, Camarines Sur
E. microphylla	Boraginaceae	Tsaang gubat	Leaves	Tanay, Rizal
C. alata	Fabaceae	Akapulko	Leaves	Angono, Rizal

2.2. Plant extract preparation

Plant extracts were prepared in accordance to the methods of Basri and Fan with minor modifications[25]. Briefly, 150 g of each powdered plant material was soaked in 500 mL of ethanol for 24 h with shaking. The resultant extracts were centrifuged at 3000 r/min for 5 min at 4 ℃. The supernatant was filtered through Whatman filter paper No. 1 (Whatman, UK), while the residues were used for a second extraction with 300 mL of ethanol. After the second extraction, the filtrates were concentrated under reduced pressure using a rotary evaporator at 50 ℃. The crude extracts were collected and allowed to dry at room temperature.

2.3. Test microorganisms

The panel of test organisms for primary *in vitro* antibacterial screening in this study is summarized in Table 2. About 10 MDR bacteria were isolated from patients of the Makati Medical Center, Makati City, Philippines. All isolates were identified by automated biochemical tests using Vitek®MS (bioMérieux, Marcy l'Etoile, France) Gram-positive colorimetric identification card. The susceptibility patterns were obtained using Vitek®MS aspartate aminotransferase (bioMérieux, Marcy l'Etoile, France) following minimum inhibitory concentration (MIC) interpretive standards from the Clinical Laboratory Standard Institute M100-S25[26].

2.4. Antibacterial assay of plant extracts

The plant extracts were tested for antibacterial activity using the disk diffusion method. The test organisms were subcultured in 5% sheep blood agar plate (BAP) for 24 h at (35 ± 2) ℃. The colonies were inoculated in normal saline solution. The turbidity was then adjusted to equal the turbidity of 0.5 McFarland standard giving a final inoculum of 1.5×10^8 CFU/mL. About 100 μL of inoculum of test organism was spread on Mueller-Hinton agar plate (Remel™, Thermo Fisher Scientific, USA). Sterile 6 mm paper disks (Becton Dickinson and Company, USA) with the plant extracts (200 μg) or solvent blank (dimethyl sulfoxide) were then placed on the inoculated plates. The plates were incubated at (35 ± 2) ℃ for 16 to 24 h. Representative antibiotics of the test isolates based on susceptibility patterns were used as positive controls (Table 2). Antibacterial activities were evaluated by measuring the diameters of zones of inhibition in mm against the test organism.

2.5. Determination of MIC and minimum bactericidal concentration (MBC) of the plant extracts

The MICs of the plant extracts were determined in sterile 96-well microplates using the broth microdilution method of the Clinical and Laboratory Standards Institute, M07-A8[26]. Each test was done in triplicate. The plant extracts were serially diluted to produce final concentrations of 19 μg/mL to 10000 μg/mL. Cation-adjusted Mueller-Hinton broth (Becton Dickinson and Company, USA) was used as diluent. The set-up included bacterial growth controls in wells containing 10 μL of the test inoculum and negative controls without bacterial inoculum. Reference drug controls were likewise included in the set-up.

The inoculum was prepared by direct saline suspension of isolated bacterial colonies selected from an 18-24 h 5% sheep BAP culture. Suspension was adjusted to achieve a turbidity equivalent to 0.5 McFarland turbidity standard, which approximated 1.5×10^8 CFU/mL. Within 15 min after standardization, 10 μL of the adjusted

Table 2

Panel of test organisms for primary antibacterial *in vitro* screening.

Characteristic	Species	Source	Antibiotic resistance pattern
Gram-positive coccus	*S. aureus*	ATCC 29223	Susceptible
Enterobacteriaceae	*E. coli*	ATCC 25922	Susceptible
Enterobacteriaceae, encapsulated	*K. pneumoniae*	ATCC BAA-1705	Susceptible
Non-Enterobacteriaceae	*P. aeruginosa*	ATCC 27853	Susceptible
Gram-positive coccus, MDR, #1	MRSA	12/Male, wound	AM, FOX, OX, P
Gram-positive coccus, MDR, #2	MRSA	69/Male, wound	AM, FOX, OX, P
Gram-positive coccus, MDR, #3	MRSA	42/Male, blood	AM, FOX, OX, P
Gram-positive coccus, MDR, #4	MRSA	35/Female, sputum	AM, FOX, OX, P
Enterobacteriaceae, MDR	ESβL, *E. coli*	55/Female, blood	AM, FEP, CTX, CTZ, CRO
Enterobacteriaceae, encapsulated, MDR	ESβL, *K. pneumoniae*	25/Female, urine	AM, FEP, CTX, CTZ, CRP
Non-Enterobacteriaceae, MDR	MβL, *P. aeruginosa*	64/Male, blood	FEP, CRZ, IPM, MEM
Non-Enterobacteriaceae, MDR	MβL, *A. baumannii*	53/Female, blood	FEP, CRZ, IPM, MEM
Enterobacteriaceae, MDR	CRE, *E. coli*	48/Male, urine	AM, FEP, CTX, CTZ, CRO, IPM, MEM
Enterobacteriaceae, encapsulated, MDR	CRE, *K. pneumoniae*	58/Female, sputum	AM, FEP, CTZ, CTZ, CRO, IPM, MEM
VRE	VRE	45/Male, urine	P, VA

AM: ampicillin; FEP: cefepime; CTX: cefotaxime; FOX: cefoxitin; CTZ: ceftazidime; CRO: ceftriaxone; IPM: imipenem; MEM: meropenem; OX: oxacillin; P: penicillin; VA: vancomycin; *S. aureus*: *Staphylococcus aureus*; *E. coli*: *Escherichia coli*; *K. pneumoniae*: *Klebsiella pneumoniae*.

Table 3

*Diameters of zones of inhibition of ethanol extracts from 12 medicinal plants against clinical isolates of MDR bacteria and ATCC reference strains (mm).

Plant extracts	MRSA 1	MRSA 2	MRSA 3	MRSA 4	VRE	MβL *P. aeruginosa*	MβL *A. baumannii*	ESβL *E. coli*	ESβL *K. pneumoniae*	CRE *K. pneumoniae*	*S. aureus* (ATCC 25923)	*E. coli* (ATCC 25922)	*K. pneumoniae* (ATCC BAA1705)	*P. aeruginosa* (ATCC 27853)
C. alata	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. asiatica	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. longa	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. microphylla	19	14	12	9	12	-	12	-	-	-	12	-	-	10
M. lanotan	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M. oleifera	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. niruri	9	11	9	9	13	-	-	-	-	-	14	-	-	-
P. betle	32	34	28	34	28	17	23	20	20	21	30	16	17	17
P. guajava	18	16	18	13	12	-	-	-	-	-	14	-	-	-
T. rumphii	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V. negundo	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Z. officinale	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*: Zone size includes 6-mm disk; -: No zone of inhibition.

Table 4

MIC of ethanol extracts from 12 medicinal plants against clinical isolates of MDR bacteria and ATCC reference isolates (μg/mL).

Plant extracts	MRSA 1	MRSA 2	MRSA 3	MRSA 4	VRE	MβL *P. aeruginosa*	MβL *A. baumannii*	ESβL *E. coli*	ESβL *K. pneumoniae*	CRE *K. pneumoniae*	*S. aureus* (ATCC 25923)	*E. coli* (ATCC 25922)	*K. pneumoniae* (ATCC BAA1705)	*P. aeruginosa* (ATCC 27853)
C. alata	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. asiatica	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. longa	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. microphylla	1250	1250	2500	1250	1250	-	5000	-	-	-	1250	-	-	2500
M. lanotan	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M. oleifera	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. niruri	5000	2500	2500	5000	1250	-	-	-	-	-	2500	-	-	-
P. betle	156	156	156	78	19	312	625	312	625	312	312	625	1250	625
P. guajava	1250	625	1250	625	5000	-	-	-	-	-	1250	-	-	-
T. rumphii	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V. negundo	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Z. officinale	-	-	-	-	-	-	-	-	-	-	-	-	-	-

-: No antibacterial activity.

Table 5

MBC of ethanol extracts from 12 medicinal plants against clinical isolates of MDR bacteria and ATCC reference isolates (μg/mL).

Plant extracts	MRSA 1	MRSA 2	MRSA 3	MRSA 4	VRE	MβL *P. aeruginosa*	MβL *A. baumannii*	ESβL *E. coli*	ESβL *K. pneumoniae*	CRE *K. pneumoniae*	*S. aureus* (ATCC 25923)	*E. coli* (ATCC 25922)	*K. pneumoniae* (ATCC BAA1705)	*P. aeruginosa* (ATCC 27853)
C. alata	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. asiatica	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. longa	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. microphylla	1250	2500	2500	1250	1250	-	5000	-	-	-	2500	-	-	2500
M. lanotan	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M. oleifera	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. niruri	5000	2500	2500	5000	1250	-	-	-	-	-	2500	-	-	-
P. betle	312	156	156	78	19	312	625	625	625	625	312	625	1250	625
P. guajava	2500	1250	1250	625	5000	-	-	-	-	-	1250	-	-	-
T. rumphii	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V. negundo	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Z. officinale	-	-	-	-	-	-	-	-	-	-	-	-	-	-

-: No antibacterial activity.

inoculum was added into each well containing 100 μL plant extract in the dilution series, and mixed. The sealed microdilution trays were incubated at (35 ± 2) ℃ for 16-20 h in an ambient air incubator.

The MIC was determined by selecting the lowest concentration of plant extract that completely inhibited the growth of the organism in the well as detected by the unaided eye. To determine the growth end points, the amount of growth in the wells containing the plant extracts was compared with the amount of growth in the growth-control well (no plant extracts) used in each set of tests. For a test to be considered valid, acceptable growth (2 mm button or definite turbidity) must occur in the growth-control well.

The MBC was determined following the methods described by Irobi and Daramola with slight modifications[27]. Wells with no visible growth in MIC assays were subcultured using a 10 μL inoculating loop onto a 5% sheep BAP at (35 ± 2) ℃ for 16-20 h incubation. The MBC was defined as the lowest concentration of the extract that did not permit any growth.

3. Results

3.1. Disk diffusion method

The disk diffusion method for antimicrobial susceptibility testing was initially performed to determine the antibacterial activities of the 12 medicinal plants against MDR bacteria,

namely, MRSA, VRE, MβL *P. aeruginosa* and *A. baumannii*, ESβL *E. coli*, ESβL *K. pneumoniae* and CRE *K. pneumoniae*. ATCC control strains of *S. aureus*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* were also included. Of the 12 medicinal plants, the ethanolic extracts of *E. microphylla*, *P. niruri*, *P. guajava* and *P. betle* showed inhibition of bacterial growth against some or all of the test organisms (Table 3). The plant extracts exhibited inhibition zones ranging from 9 mm to 34 mm diameter, with the most noteworthy results shown by *P. betle* (Figure 1). Cefoxitin 30 μg disk (14 mm) showed a resistant result. The diameters included the 6 mm filter paper disk.

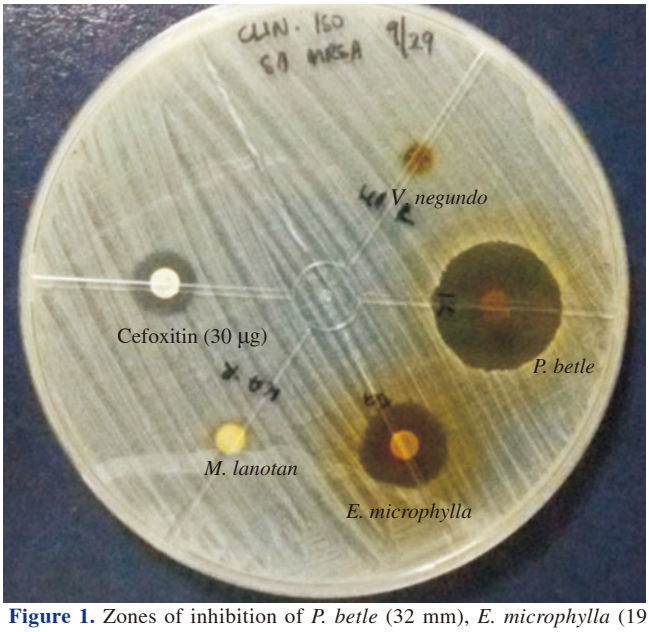

Figure 1. Zones of inhibition of *P. betle* (32 mm), *E. microphylla* (19 mm), *V. negundo* (0 mm) and *M. lanotan* (0 mm) against clinical isolate MRSA 1.

The ethanolic extract prepared from the *P. betle* leaf demonstrated inhibition zones greater than 15 mm in diameter, as shown in Table 3, with the greatest zones (28-34 mm) produced against the clinical strains of VRE and MRSA and for the *S. aureus* ATCC control strain. The leaf extract likewise showed growth inhibition against all Gram-negative clinical MDR bacterial strains and their respective ATCC controls tested, with zones ranging from 16 mm to 23 mm. On the other hand, *P. guajava* and *P. niruri* produced zones of growth inhibition against both clinical MRSA and VRE and ATCC strain of *S. aureus*, but failed to inhibit the growth of Gram-negative organisms tested. *E. microphylla* likewise exhibited inhibition zones for clinical strains of MRSA, VRE and *S. aureus* ATCC 25923. In addition, it inhibited the Gram-negative MβL *A. baumannii* and *P. aeruginosa* ATCC 27853.

Results of the disk diffusion assay showed that the most commonly inhibited bacteria by the ethanolic plant extracts were the clinical MRSA and VRE strains, and the ATCC control *S. aureus*, followed by MβL *A. baumannii* and *P. aeruginosa* ATCC 27853.

3.2. MIC

The MIC assay of the *P. betle* leaf extract consistently showed strong antagonistic activities against all microorganisms tested, with MIC values ranging from 19-1 250 μg/mL (Table 4). The strongest activity was exhibited against the clinical strains of Gram-positive bacteria: VRE (19 μg/mL), MRSA 4 (78 μg/mL), MRSA 1-3 (each with MIC of 156 μg/mL) and *S. aureus* ATCC 25923 (312 μg/mL). For the Gram-negative bacteria, the most common MIC value was 625 μg/mL for MβL *A. baumanni*, ESβL *K. pneumoniae*, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, followed by 312 μg/mL for MβL *P. aeruginosa*, ESβL *E. coli* and CRE *K. pneumoniae*. The *K. pneumonia* ATCC BAA-1705 only showed MIC greater than 1 000 μg/mL (1 250 μg/mL).

P. guajava and *P. niruri* similarly showed good patterns of inhibition against all Gram-positive bacteria tested, although the MIC values were much higher than those for *P. betle*. The MIC values ranged from 625-5 000 μg/mL and 1 250-5 000 μg/mL, respectively (Table 4). Similar to *P. niruri*, the ethanol extract from *E. microphylla* showed MIC values of 1 250-5 000 μg/mL, with the lowest MICs for MRSA, VRE and *S. aureus* ATCC 25923 and the highest MIC for MβL *A. baumannii*.

3.3. MBC

The MBCs were established by subculturing the samples with no visible growth in the MIC assays. Most of the MBC and MIC values for the ethanol plant extracts were closely similar, with only *P. niruri* showing exactly identical values for both assays (Table 5). Slightly higher MBC values for MRSA 1 (312 μg/mL; MIC 156 μg/mL), ESβL *E. coli* (625 μg/mL; MIC 312 μg/mL) and CRE *K. pneumoniae* (625 μg/mL; MIC 312 μg/mL) were obtained with *P. betle*. For *P. guajava*, the MBC values slightly differed for MRSA 1 (2 500 μg/mL; MIC 250 μg/mL) and MRSA 2 (1 250 μg/mL; MIC 625 μg/mL) strains, whereas *E. microphylla* had slightly higher MBC values for MRSA 2 (2 500 μg/mL; MIC 1 250 μg/mL) and ATCC *S. aureus* control strain (2 500 μg/mL; MIC 1 250 μg/mL).

4. Discussion

The current problem associated with emerging MDR bacteria presents a serious global medical crisis, requiring constant surveillance, which continuously challenges the scientific community[24]. The diminishing efficacy and increasing toxicity of synthetic drugs further aggravate this problem, thus, scientists are directed to seek more natural or organic materials for solutions. Traditional medicine has been practiced worldwide for centuries, particularly the application of herbal plants for therapeutic purposes. Philippines possess a rich source of medicinal plants, although not as extensive as India or China, enough to provide us with alternative remedies[18]. The 12 plants have been used locally for their traditional medicinal properties, all of which have documented antimicrobial activities. However, their efficacies against MDR bacteria have not been studied. In developing countries, it is imperative that effective but less expensive antibacterials should be developed to accommodate all

patients, regardless of financial status, in order to eliminate some of the human factors that can cause MDR.

In recent years, there are increasing published reports showing successful antimicrobial activities of various traditional medicinal plants against MDR bacteria, such as MRSA, ESβL, VRE, and MDR *P. aeruginosa*[9,28-38]. In the present study, favorable antagonistic activities against various MDR bacteria were exhibited by the ethanol extracts of four Philippine medicinal plants, namely, *P. betle*, *P. guajava*, *P. niruri* and *E. microphylla*.

As previously mentioned, the medicinal plants have known antimicrobial properties, but their efficacies against MDR bacteria have not been well-documented in the local medical literature or in other countries. *P. guajava* has been separately documented to be inhibitory against MRSA[39], MDR *V. cholera*[40] and MDR *S. aureus*[41]. In relation to the extraction method, it was reported that only ethanol extracts showed antagonistic activities against Gram-negative bacteria[10,42], while Mishra and Babele documented that the highest antimicrobial activity was obtained with the methanol extract of *P. guajava* leaf against drug resistant and susceptible *E. coli* and *P. aeruginosa*[43]. In the present study, the ethanol extract from the *P. guajava* leaf demonstrated significant inhibitory activities against MRSA and VRE and no activity against the Gram-negative bacteria tested. MICs and MBCs obtained in the current study are almost similar to the values obtained from another study by Esimone against MRSA, which ranged from 1 250-5 000 μg/mL for the water extracts, and 625-2500 μg/mL for the methanol extracts[39].

P. niruri is traditionally used for the treatment of kidney stones, gallstones, wound healing, genito-urinary tract infections and sexually transmitted diseases[18,44,45]. Bokarey *et al*. determined that different extracts from the *P. niruri* plant were effective against common human bacteria such as *S. aureus*, *E. coli*, *Proteus vulgaris* and *P. aeruginosa*[46]. Our present study is the first to show the antibacterial activity of *P. niruri* against MRSA and VRE.

P. betle is a multifunctional medicinal plant, which is cultivated throughout the Philippines and can also be found in other countries, such as Malaysia and India. The leaves have been traditionally used to control caries, periodontal disease and prevention of halitosis and is proven to have significant gastrointestinal and hepatoprotective effects[47,48]. The 80% methanol and 70% ethanol leaf extracts have been reported to be effective against strains of *S. aureus*, *E. coli* and *P. aeruginosa*, with the methanolic extract exhibiting wider zones of inhibition in the modified agar well diffusion method by Khan and Kumar[49]. The zones of inhibition for the methanolic extract ranged from 15 to 29.5 mm, whereas the ethanol extracts showed inhibition zones ranging from 14 to 17 mm. MIC for both extracts ranged from 2 to 8 196 μg/mL. A similar study against the three bacteria has been performed but with the addition of two extraction methods: cold aqueous and ethyl acetate extracts of several Indian varieties of *P. betle* leaves[50]. Another study showed the efficacy of the ethanolic leaf extract against *K. pneumoniae* and *P. vulgaris*, wherein the MIC value obtained for the two bacteria was 25 μg/mL[51]. In a more recent study related to the activities of 90% methanol and 90% ethanol leaf extracts of *P. betle* against clinical oral isolates (*S. aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Bacillus subtilis*, *Listeria monocytogenes*, *E. coli*, *Salmonella typhimurium*, *Salmonella enteritidis*, *K. pneumoniae* and *P. aeruginosa*), the extracts demonstrated good antimicrobial activities against the Gram-positive and Gram-negative bacteria, except for *P. aeruginosa*[52]. At present, studies on the antimicrobial activities of *P. betle* against MRSA, ESβL, CRE, VRE, or MβL-producing *P. aeruginosa* and *A. baumannii* have been noted in scientific literature. The results of the present study showed that *P. betle* had the greatest potential value against both Gram-negative and Gram-positive MDR bacteria, exhibiting wide zones of growth inhibition in the disk diffusion assay and with the lowest concentrations of the extract required to inhibit the growth and affect death of the pathogens, as supported by the MIC and MBC assays, respectively.

E. microphylla, *P. guajava* and *P. niruri* also showed significant antagonistic activities against the Gram-positive bacteria, but the limited or lack of activity against Gram-negative bacteria has reduced their value as an ideal or broad spectrum antibacterial for MDR microorganisms. Nevertheless, they can still be developed as second or third choices for the treatment of susceptible MDR bacteria. In addition, *E. microphylla* could provide us with an alternative treatment for MβL-producing *A. baumannii* and *P. aeruginosa*. *In vitro* antimicrobial studies regarding *E. microphylla* are rarely encountered. It has been surmised that the antibacterial activities of herbal plant extracts and essential oils are focused on the structures and cellular membranes and due to the presence of various bioactive compounds and extensive chemical profiles, it is likely that the antimicrobial potency is not just caused by one solitary mechanism but rather by several events at a cellular level[53].

The outer membrane found in the Gram-negative cell wall is composed of structural lipopolysaccharides which render the cell wall impermeable to lipophilic solutes, unlike Gram-positive bacteria which do not have this outer membrane. This morphologic difference influences their reaction to antibacterial agents. In addition, Gram-negative bacteria also have inherent overexpressed or multiple efflux pumps that prevent the intracellular accumulation of antibacterial agents. Thus, there is a need to discover and develop antibacterial agents that are capable of bypassing or suppressing the efflux pumps and that could also re-establish the antibacterial potency of older antibiotics[54]. Although previous studies on *P. guajava*[10,42] and *P. niruri*[46] reported significant antagonistic activities against Gram-negative bacteria, the results of the present study did not show the efficacies of these two plants on the Gram-negative bacteria tested. The variation in sensitivities could be attributed to the reinforced defense mechanisms acquired by the MDR bacteria.

However, this study resolutely established the efficacy of the ethanolic leaf extract of *P. betle* against known Gram-positive and

Gram-negative MDR bacteria, indicating that the plant produces compounds that are able to combat the bacterial defenses. Henceforth, the results justify that further investigation should be performed on *P. betle*. Additional or complementary studies are necessary concerning the application of different extraction methods, phytochemical screening, phytophysical analysis, bioassay-guided isolation, purification and quantification of the bioactive components, including the detection of toxicity, side effects and pharmacokinetic properties in preparation for its *in vivo* assessment. Completion and favorable outcomes obtained from succeeding studies would definitely strengthen its potential as a novel and cost-effective agent against MDR bacteria.

Conflict of interest statement

We declare that we have no conflict of interest.

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