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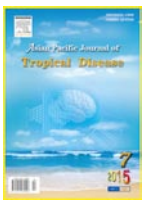

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TLC profiles and antibacterial activity of *Glinus oppositifolius* L. Aug. DC. (Molluginaceae) leaf and stem extracts against bacterial pathogens

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ABSTRACT

Objective: To determine the antibacterial activities and the thin-layer chromatography (TLC) fingerprint profiles of leaf and stem extracts of *Glinus oppositifolius* L. Aug. DC (*G. oppositifolius*).

Methods: The leaves and stems were extracted using chloroform, ethanol and methanol as solvents. The antibacterial activity of the extracts were evaluated through disc diffusion, minimum inhibitory concentration and bactericidal concentration assays against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, extended spectrum β-lactamase-producing, carbapenem-resistant Enterobacteriaceae, and metallo-β-lactamase-producing *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Acinetobacter baumanii* (*A. baumanii*). The TLC separation was carried out on leaf and stem ethanol extracts in ethyl acetate: *n*-hexane solvent system. Distinct spots were examined under visible light, UV 254 nm, UV 366 nm and after spraying with vanillin-sulfuric acid.

Results: The leaf extracts revealed antibacterial activities, inhibiting the growth of the non-resistant and multidrug-resistant strains of the Gram-negative bacteria *Escherichia coli*, *P. aeruginosa* and *A. baumanii*. The TLC fingerprint profiles demonstrated the presence of various phytochemicals in leaf and stem extracts. Leaf extracts exhibited more diverse constituents compared to stem extracts, but some constituents were similar in both plant parts.

Conclusions: *G. oppositifolius* leaf extracts can be used as new, alternative sources of antimicrobials against non-resistant and multidrug-resistant strains of the Gram-negative bacteria *Escherichia coli*, *P. aeruginosa* and *A. baumanii*. The TLC profiles represent the chemical integrity of *G. oppositifolius* leaf and stem extracts which form an important and powerful tool for standardization, authentication, quality control and determination of bioactive components of *G. oppositifolius* in any formulation and in powder form.

1. Introduction

The alarming increase in the incidence of new and re-emerging infectious diseases and the widespread, rapid development of multiple drug resistance in human pathogenic microorganisms against commonly used antibiotics have become one of the most serious public health concerns across the world. Factors that are responsible for the development of multiple drug resistance in human pathogenic microorganisms include the unscientific and impractical uses of commercial antimicrobial agents, the specific nature of the relationship of bacteria to antibiotics, host characteristics and environmental factors[1]. The emergence of multidrug-resistant (MDR) microorganisms brings about the necessity for a constant search for new antimicrobial substances from plant sources[2]. According to the Infectious Disease Society of America, MDR bacteria which pose great challenge in terms of management include the following: methicillin-resistant *Staphylococcus aureus* (*S. aureus*) (MRSA), vancomycin-resistant *Enterococcus* (VRE), extended spectrum β-lactamase-producing (ESβL+) and carbapenem-resistant Enterobacteriaceae (CRE),

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metallo-β-lactamase-producing (MβL+) *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Acinetobacter baumanii* (*A. baumanii*)[3].

Plants have been known for centuries as rich natural reservoir of medicinal agents[4]. The healing properties of medicinal plants lie on their bioactive compounds, mainly the secondary metabolites which can be divided into several general categories, namely, phenolics and polyphenols (composed of simple phenols and phenolic acids, quinones, flavones, flavonoids, and flavonols, tannins); and alkaloids; glycosides; terpenoids and essential oils. A wide range of substances from many plant species used in traditional medicine for the treatment of chronic and acute infectious diseases can be developed as new antimicrobial drugs[5,6]. Over the last few years, enormous studies have been conducted to identify plant-derived antibacterial agents for the treatment of various bacterial diseases. With the increasing use of plant extracts from medicinal and aromatic plants in the pharmaceutical, food and cosmetic industries, it is important that these plants be systematically investigated. Standardization and authentication of medicinal plant extracts and constituents are important prior to their formulation into medicines in order to ensure quality, safety and efficacy of herbal medicines and other formulations. To meet the universal standards of quality control of herbal formulations, the World Health Organization recommends that chemical fingerprinting methods be used for the identification of herbs and their constituents[7].

Glinus oppositifolius L. Aug. DC. (*G. oppositifolius*) syn. *Mollugo oppositifolia* and *Mollugo spergula* L. of the family Molluginaceae, commonly known in the Philippines as "sarsalida" or "malagoso" (Tagalog), "papait" (Iloko) and slender carpet weed (English) is known for its use in traditional medicine. The weed grows at low and medium altitudes throughout the Philippines and it also occurs in India to tropical Africa and Australia. There have been reports on its therapeutic value which include its analgesic, antidiabetic, anti-hyperlipidemic, antihelminthic, antidiarrhoeal, diuretic, antimalarial, antiviral, antioxidant and antimicrobial properties[8,9]. The shoot of *G. oppositifolius* is eaten occasionally as a vegetable even though it is bitter on account of its stomachic, aperient, and antiseptic properties. It can also improve digestion and can cure burning sensation, itchiness and other skin ailments. Although there have been reports on the antimicrobial activities of extracts of *G. oppositifolius*, this paper reports for the first time the antimicrobial potential of leaf and stem extracts of *G. oppositifolius* using clinical bacterial isolates of MDR bacteria and the thin-layer chromatography (TLC) fingerprint profiles of its extracts.

2. Materials and methods

2.1. Selection and collection of plant materials

Whole, vigorous plants of *G. oppositifolius* were harvested from plantations in Barrio Colibangbang, Paniqui, Tarlac, Philippines. The taxonomic identification of the plant was authenticated by a curator in the Botany Division of the National Museum, Manila where a voucher specimen of the herb was prepared and deposited.

2.2. Drying and extraction

In the laboratory, collected plant materials were washed thoroughly in running tap water to remove soil particles and other debris and shade-dried in an air-conditioned laboratory for 2-3 weeks. When dried, leaves and stems were segregated from each plant, after which were separately powdered by a dry mechanical grinder and then stored in airtight closed containers prior to analysis. One hundred grams of the different parts of *G. oppositifolius* was added separately to 500 mL ethanol, methanol and chloroform, and then soaked for 3 days. Removal of the plant residue from each of the solvents was done by filtration and the resulting filtrate was concentrated under reduced pressure at 40 ℃ on a rotary evaporator (Laborota 4001, Heidolph). The concentrated filtrates were transferred into Petri dishes and allowed to air-dry completely.

2.3. Selected microorganisms

The test organisms for *in vitro* antibacterial screening are presented in Table 1. Ten MDR bacterial strains 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) GP colorimetric identification card. The susceptibility patterns were obtained using Vitek® MS AST (BioMérieux, Marcy l'Etoile, France) following minimal inhibitory concentration (MIC) interpretative standards from the USA Clinical Laboratory Standard Institute M100-S25[10]. Four ATCC bacterial strains were used as controls.

2.4. Antibacterial assays of plant extracts

The plant extracts were tested for antibacterial activity using the disc diffusion method. The test organisms were sub-cultured 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. One hundred microliters (100 μL) of inoculum of test organism were 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 (dimethylsulfoxide) 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. Gentamicin was used as positive control for the Gram-negative bacteria [*Escherichia coli* (*E. coli*), *A. baumanii*, *Klebsiella pneumoniae* (*K. pneumoniae*), *P. aeruginosa*]; and, oxacillin for Gram-positive bacteria (*S. aureus* and VRE). Antibacterial activity was evaluated by

Table 1
Panel of test organisms for *in vitro* antibacterial screening.

Characteristics	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, CRO
Non-Enterobacteriaceae, MDR	MβL+ *P. aeruginosa*	64, Male, Blood	FEP, CTZ, IPM, MEM
Non-Enterobacteriaceae, MDR	MβL+ *A. baumanii*	53/Female, Blood	FEP, CTZ, IPM, MEM
Enterobacteriaceae, encapsulated, MDR	CRE, *K. pneumoniae*	58/Female, Sputum	AM, FEP, CTX, 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.

measuring the diameters of the zone of inhibition in mm against the test organisms.

The MICs of the plant extracts were determined in sterile 96-well microplates using the broth microdilution method of the Clinical Laboratory Standard Institute, M07-A8[10]. Each test was done in triplicate. The plant extracts were serially diluted to produce final concentrations of 19 μg/mL to 10 000 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 to 24 h in 5% sheep BAP culture. Suspension was adjusted to achieve a turbidity equivalent to 0.5 McFarland turbidity standard, which approximated 1.5 × 10^8 cells/mL. Within 15 min after standardization, 10 μL of the adjusted 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 to 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 endpoints, 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 minimum bactericidal concentrations (MBCs) were determined following the methods described previously with slight modifications[11]. Wells with no visible growth in MIC assays were sub-cultured using a 10 μL-inoculating loop onto a 5% sheep BAP at (35 ± 2) ℃ for 16 to 20 h of incubation. The MBC is defined as the lowest concentration of the extract that did not permit any growth.

2.5. TLC analysis

A TLC system equipped with a sample applicator was used for application of samples. Five microliters of leaf and stem ethanol extracts were separately applied on 5 × 17 cm chromatographic pre-coated silica gel plates (Merck, TLC grade) as the stationary phase. The chromatograms were developed in a twin trough glass chamber containing ethyl acetate and *n*-hexane (7:3, v/v) as the mobile phase. The plates were removed after the solvent front has moved about 15 cm from the original extract position, and subsequently allowed to dry. After drying, the spots on the developed plates were visualized under visible (white), short UV (254 nm) and long UV (366 nm) light. As post-derivatization, the plates were sprayed with vanillin-sulfuric acid reagent for color reaction and allowed to dry. A scanner was used for photo-documentation at UV 254 nm and UV 366 nm, and under visible light before and after application of vanillin-sulfuric acid reagent. The movement of each separating spot of the extract was expressed by its retention factor (R_f). Values were calculated for each spot for both leaf and stem ethanol extracts, using the formula:

R_f = distance travelled by the solute or spot distance travelled by the solvent

3. Results

3.1. Antibacterial activity of G. oppositifolius leaf and stem extracts

The antibacterial potential of the chloroform, ethanol and methanol extracts of *G. oppositifolius* leaves and stems were tested through disc diffusion assay against MDR bacteria, namely, four strains of

Table 2

Diameters of zone of inhibition, MIC and MBC of *G. oppositifolius* leaf extracts against MDR and ATCC (control) bacteria.

Bacterial strains	Chloroform			Ethanol			Methanol		
	Inhibition zone diameter (mm)	MIC (mg/mL)	MBC (mg/mL)	Inhibition zone diameter (mm)	MIC (mg/mL)	MBC (mg/mL)	Inhibition zone diameter (mm)	MIC (mg/mL)	MBC (mg/mL)
MRSA 1	-	-	-	-	-	-	-	-	-
MRSA 2	-	-	-	-	-	-	-	-	-
MRSA 3	-	-	-	-	-	-	-	-	-
MRSA 4	-	-	-	-	-	-	-	-	-
VRE	-	-	-	-	-	-	-	-	-
MβL+ *A. baumanii*	12	2.50	10.0	12	5.00	10.0	12	5.00	10.0
MβL+ *P. aeruginosa*	9	2.50	5.0	9	5.00	10.0	9	5.00	10.0
ESβL+ *E. coli*	-	5.00	10.0	-	5.00	10.0	-	5.00	10.0
ESβL+ *K. pneumoniae*	-	5.00	10.0	-	5.00	10.0	-	5.00	10.0
CRE *K. pneumoniae*	-	-	-	-	-	-	-	-	-
S. aureus ATCC 25923	-	-	-	-	-	-	-	-	-
E. coli ATCC 25922	10	1.25	5.0	10	5.00	10.0	10	5.00	10.0
K. pneumoniae ATCC BAA1705	-	1.25	10.0	-	5.00	10.0	-	5.00	10.0
P. aeruginosa ATCC 27853	8	2.50	5.0	8	5.00	5.0	8	5.00	10.0

-: No antibacterial activity; Susceptibility was difined as 8 mm diameter of zone of inhibition. No antibacterial activity was observed from the stem extracts.

MRSA, VRE, MβL+ *P. aeruginosa*, MβL+ *A. baumanii*, ESβL+ *E. coli*, ESβL+ *K. pneumoniae*, and CRE and *K. pneumoniae*. ATCC strains of *S. aureus*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* were also tested as controls. As shown in Table 2, among the three leaf extracts obtained using different solvents, the chloroform, ethanol and methanol leaf extracts exhibited zones of growth inhibition on MβL+ *P. aeruginosa*, MβL+ *A. baumanii*, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. Specifically, MβL+ *A. baumanii* had 12 mm, *E. coli* ATCC 25922 had 10 mm, MβL+ *P. aeruginosa* had 9 mm, and *P. aeruginosa* ATCC 27853 had 8 mm diameter of growth inhibition zone. All the stem extracts, however, did not inhibit growth of any bacteria tested.

3.2. MIC and MBC of G. oppositifolius leaf extracts

The MIC assay of *G. oppositifolius* chloroform, ethanol and methanol leaf extracts showed antagonistic activities against the Gram-negative bacteria, namely, MβL+ *A. baumanii*, MβL+ *P. aeruginosa*, ESβL+ *E. coli*, ESβL+ *K. pneumoniae*, including ATCC strains of *E. coli*, *K. pneumoniae* and *P. aeruginosa*, with MIC values ranging from 1.25 to 5.00 mg/mL of leaf extracts (Table 2). The strongest activity was exhibited by chloroform leaf extract against *E. coli* ATCC 25922 and *K. pneumoniae* ATCC BAA1705 with MIC value of 1.25 mg/mL, followed by *P. aeruginosa* ATCC 27853, MβL+ *P. aeroginosa* and MβL+ *A. baumanii* with MIC value of 2.50 mg/mL.

The MBC values were determined by sub-culturing the samples with no visible growth in the MIC assay. Overall, the MBC values were either 5 mg/mL or 10 mg/mL for the leaf extracts depending upon the extraction solvent, as well as the test bacterial strains, namely, MβL+ *A. baumanii*, MβL+ *P. aeruginosa*, ESβL+ *E. coli*, ESβL+ *K. pneumoniae*, including ATCC strains of *E. coli*, *K. pneumoniae* and *P. aeruginosa*.

3.3. TLC fingerprint profiles of leaf and stem extracts

The TLC profiles of leaf and stem ethanol extracts of *G. oppositifolius* are presented in Table 3. The photo-documentations of the TLC chromatograms are presented in Figures 1 and 2. Overall, more TLC spots were visualized in leaf ethanol extracts than in stem ethanol extracts under visible light, UV 254 nm, UV 366 nm, and after post-derivatization with vanillin-sulfuric acid reagent. Specifically, there were two spots in the leaf extract under visible light while none were seen in the stem extract. Under UV 366 nm, five spots were seen in the leaf extract while only one in the stem extract. After spraying with vanillin-sulfuric acid, seven spots were seen in the leaf extract while there were three in the stem extract. However, there were no spots visualized under UV 254 nm in both leaf and stem extracts.

Table 3

TLC profiles of leaf and stem ethanol extracts from *G. oppositifolius*.

R_f value	Plant part	Visible light	UV 254 nm	UV 366 nm	Vanillin-sulfuric acid spray
0.013	Leaf	-	-	Pink	Black
0.046	Leaf	-	-	Pink	-
0.060	Leaf	-	-	Blue	-
0.360	Stem	-	-	-	Gray
0.380	Leaf	-	-	-	Gray
0.560	Stem	-	-	-	Black
0.600	Leaf	-	-	-	Black
0.660	Leaf	-	-	-	Gray
0.660	Stem	-	-	-	Gray
0.710	Leaf	-	-	-	Green
0.730	Leaf	Light green	-	-	-
0.820	Leaf	-	-	Light blue	-
0.820	Stem	-	-	Light blue	-
0.860	Leaf	Light yellow	-	-	Gray
0.920	Leaf	-	-	Red	-
0.930	Leaf	-	-	-	Green

Mobile phase solvent system: ethyl acetate: *n*-hexane (7:3, v/v).

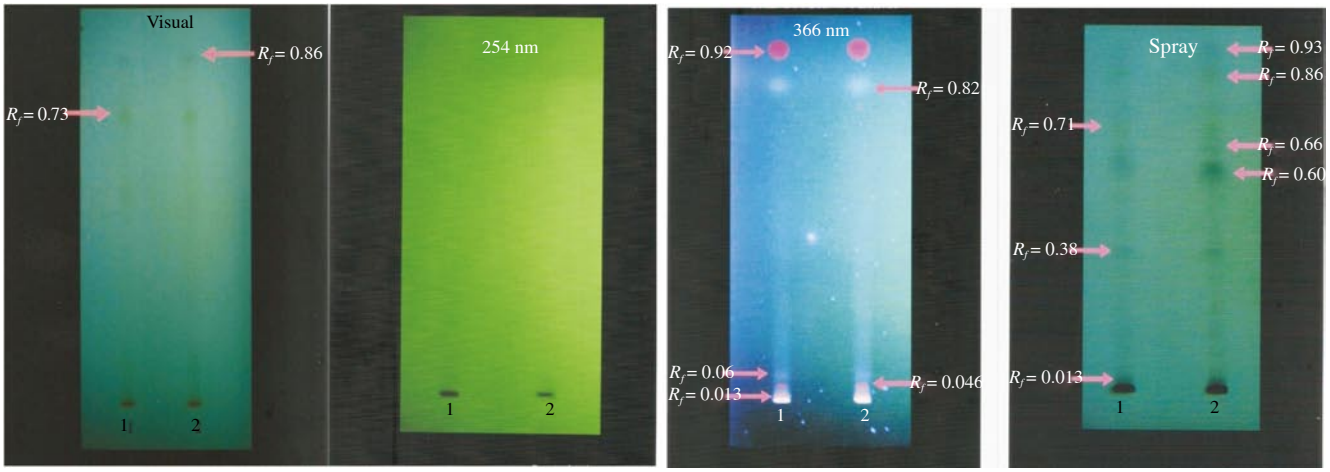

Figure 1. TLC chromatograms of *G. oppositifolius* leaf ethanolic extracts visualized under visible light, UV at 254 nm, UV at 366 nm and after post-derivatization with vanillin-sulfuric acid. Mobile phase: ethyl acetate: *n*-hexane (7: 3 v/v).

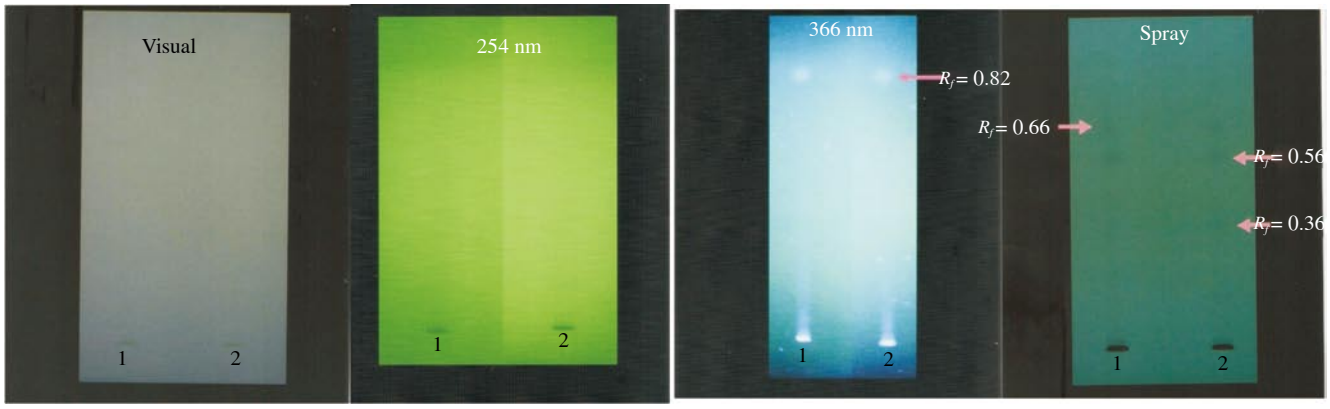

Figure 2. TLC chromatograms of *G. oppositifolius* stem ethanolic extracts visualized under visible light, UV at 254 nm, UV at 366 nm and after post-derivatization with vanillin-sulfuric acid. Mobile phase: ethyl acetate: *n*-hexane (7: 3 v/v).

4. Discussion

Due to enormously increasing cases of bacterial resistance to commercially available antibiotics, it is inevitable that medicinal plants be investigated as alternative sources of new metabolites which can be developed into new antibiotics and other antibacterial agents. Very few studies have been done on the antibacterial properties of the medicinal plant, *G. oppositifolius*[8,9]. The results of antimicrobial susceptibility, MIC and MBC assays on *G. oppositifolius* reported in this paper indicate that the leaf extracts can be an alternative source of antibacterial metabolites which can be developed into new antibacterial agents against the Gram-negative bacteria, namely, *E. coli* (ATCC and ESβL+), *K. pneumoniae* (ATCC and ESβL+), *P. aeruginosa* (ATCC and MβL+), and *A. baumanii* (MβL+). To our knowledge, this is the first report on the antibacterial activity of *G. oppositifolius* against MDR bacteria.

The varying degree of solubility of the antibacterial constituents in the leaf extracts with the three solvents used, *i.e.*, chloroform, ethanol and methanol, is a major factor behind the differences observed in the antibacterial activities of the leaf extracts against the bacterial strains tested. As shown by the MIC and MBC values, the leaf chloroform extract proved to have the greatest antibacterial potency against the Gram-negative bacteria, compared to the leaf ethanol and methanol extracts, thus indicating the presence of less polar antibacterial constituents. Nonetheless, the leaves also contain more polar antibacterial substances as demonstrated by the MIC and MBC values of leaf ethanol and methanol extracts.

The quality of herbal medicines relies on their bioactive constituents. Our previous findings on phytochemical screening of *G. oppositifolius* confirmed the presence of various classes of secondary metabolites in leaf and stem extracts, namely, flavonoids, terpenes, alkaloids, glycosides, tannins, phytosterols and saponins. The TLC profiles and images of chromatograms represent the chemical integrity of a specific plant extract[12]. Therefore, TLC fingerprinting serves as an important and powerful tool for standardization, authentication, determination of bioactive components, quality control and checking for adulterants of herbal medicines, drugs, other plant products, and pharmaceutical preparations obtained from medicinal plants[13], like *G. oppositifolius*. Distinct TLC spots on the silica gel plate representing isolated compounds with specific R_f values were observed under visible light, UV 254 nm, UV 366 nm and after spraying with vanillin-sulfuric acid. Most of the spots were visualized under UV and after spraying with vanillin-sulfuric acid. It was noteworthy that there were spots of similar color observed in both leaf and stem extracts which exhibited similar or closely similar R_f values. These included the following spots which were visualized

upon post-derivatization with vanillin-sulfuric acid spray: gray spots in R_f 0.36 and 0.38; black spots in R_f 0.56 and 0.60; and, gray spots in R_f 0.66. There were also light blue spots visualized in R_f 0.82 under UV 366 nm that were common to leaf and stem extracts. These spots with the same R_f value and with the same color are possibly the same or closely related compounds.

Phenolic compounds, being aromatic in structure, are known to show intense absorption in the UV region of the spectrum showing green, yellow, white to pale yellow, purple, pink, red, blue, grey, brown or black spots[14]. Most of these colors were observed in the TLC spots of *G. oppositifolius* leaf ethanol extracts. Certain chemical substances found in the leaf extracts that were isolated through TLC may be responsible for the antibacterial properties of *G. oppositifolius*. Each type of phenolics and polyphenols, the largest group of bioactive substances probably exhibit different mechanism(s) of action against microorganisms[5]. Depending upon the subclass of phenolic compounds, the antimicrobial action can be through enzyme inactivation and inhibition, complexation with cell wall, binding to proteins and adhesins, substrate deprivation, membrane disruption, and metal ion complexation. Moreover, the mechanism of action of alkaloids is probably due to intercalation into cell wall and/or DNA, while that of terpenoids and essential oils is probably attributed to membrane disruption.

All the leaf extracts obtained from chloroform, ethanol and methanol as solvents can inhibit the growth of ATCC and MDR strains of the Gram-negative bacteria *E. coli*, *P. aeruginosa* and *A. baumanii*. Thus, the leaf extracts can be used as new, alternative source of antimicrobials against non-resistant and MDR Gram-negative bacteria. The biochemical difference in the cell walls between Gram-negative and Gram-positive bacteria probably influences the growth responses of these bacteria on the antibacterial chemical constituents of *G. oppositifolius* leaf extracts. The outer lipid bilayer membrane of Gram-negative bacteria which is absent in Gram-positive bacteria is known to be composed of lipopolysaccharides, replacing the phospholipids in the outer side with phospholipids and lipoproteins on the inner side adjacent to the peptidoglycan. Knowing the specific mechanism of action of the antibacterial activity of *G. oppositifolius* leaf extracts and the chemical identity and characteristics of its specific antibacterial compounds will strengthen the potential of the medicinal plant as a novel agent against Gram-negative bacteria.

Conflict of interest statement

We declare that we have no conflict of interest.

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