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Anti-infective Properties and Time-Kill Kinetics of *Phyllanthus muellerianus* and its Major Constituent, Geraniin

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Abstract

Microbial resistance to existing antimicrobial agents remains a global challenge. In recent years, there has been a significant upsurge in the search for newer antimicrobial agents from nature with plants becoming the major focus in most parts of the world due to the vast availability of plants, which have not been screened for their antimicrobial activity. Hence, the study investigates the antimicrobial properties of aqueous aerial part extract of *Phyllanthus muellerianus* (PLE) and its major constituent, geraniin. The agar well diffusion and micro-dilution methods as well as time-kill kinetic studies were used to determine the antimicrobial activity of PLE and geraniin against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* NCTC 10073, *Streptococcus pyogenes* (clinical isolate) and *Candida albicans* (clinical isolate). The mean zones of growth inhibition for PLE and geraniin were in the range of 12.0 ± 0.0 to 22.7 ± 0.3 and 12.0 ± 0.0 to 21.6 ± 0.3 mm, respectively. MIC of both PLE and geraniin ranged from 0.31 to 5 and 0.08 to 1.25 mg/mL (90 to 1310 µM), respectively whiles the minimum cidal concentrations were 5.0 to 50.0 and 2.5 to 10 mg/mL (2.62 to 10.5 mM), respectively. The time-kill kinetics study showed that PLE and geraniin may act as microbiostatic agents. Preliminary phytochemical screening of PLE showed the presence of alkaloids, glycosides, saponins, tannins, flavonoids and terpenoids. The observed antimicrobial activity of the extract, PLE, may be due in large proportion to its major constituent, geraniin.

Keywords: *Phyllanthus muellerianus*; Geraniin; Antibiotic resistance; Antibacterial; Antifungal

Introduction

Antibiotics are one of the most vital tools used in fighting bacterial infections and they have greatly improved the quality of health since their introduction in the fight against infectious disease. However, over the past few decades, these health benefits are under threat as many commonly used antibiotics have become less effective against known susceptible microbes due to emergence of antibiotic resistant bacteria [1-4].

Infectious diseases are ranked among wars and famines as one of the most serious factors that negatively influence the survival of man worldwide with developing countries facing the greatest impact of this menace [4-6]. In the developed nations, despite the tremendous advances made in the understanding of microorganisms and their control, incidence of epidemics due to drug resistant microorganisms and the emergence of new disease–causing microorganisms, pose huge public health concerns [3]. In addition, treatment option for some infections has become limited due to the emergence of multi-drug resistant strains [7,8]. Along with bacterial infections, the fungal infections are also a significant cause of morbidity and mortality despite advances in medicine and the discovery of new antifungal agents is increasingly becoming scarce [9,10].

In recent years, the search for newer antimicrobial agents from nature to combat the microbial menace has increased significantly [2,11,12]. Plants have been shown in several studies to be one of the most promising sources for obtaining natural compounds that can act as anti-infective agents. Several plants including *Pupalia lappaceae* (L) Juss. [13], *Strophanthus hispidus* DC [14], *Ocimum sanctum* L., *Eugenia caryophyllata* (L.) Baill., *Achyranthes bidentata* Blume and *Azadirachta indica* A. Juss. [15], *Centrosema pubescens* Benth. [16] *Myrianthus arboreus* P. Beauv [17] have been shown to possess antimicrobial activity in *in vitro* models. Again natural products obtained from plants such as hyperforin from *Hypericum perforatum* L. [18], hyperenone A and hypercalin B from *Hypericum acmosepalum N. Robson* [19] as well as essential oils from plants such as *Origanum vulgare* L. and *Thymus vulgaris* L., as well as their components, carvacrol and thymol have been shown to possess antimicrobial activity [20]. Other compounds such as sterols from *Laurencia papillosa* C. Agardh [21], *Ganoderma applanatum* (Pers.) Pat. [22] and *Curcubita maximus* Duch [23] have been identified to be responsible for the anti-infective property of these plants. However, antimicrobial activity of many medicinal plants has not been studied. This has necessitated the continuous search for medicinal plants with anti-infective property.

Phyllanthus muellerianus Kuntze Exell which belongs to the family Euphorbiaceae is locally used in Ghana and other parts of West Africa in treating microbial infections. Biologically, the methanol and ethyl acetate stem bark extracts [24] as well as oil extracted via hydro-distillation [25] from *P. muellerianus* have been reported for antibacterial activity. The aqueous extract of *P. muellerianus* has been identified to possess anti-diarrhoeal activity [26], antiplasmodial activity [27] and wound healing properties [28]. Geraniin is also reported to possess antiviral activity [29], inhibits TNF-α activity [30], antinociceptive activity [31] and antihypertensive effeet [32]. To confirm its ethnobotanical uses in the treatment of infections, we investigated the antimicrobial properties of aqueous aerial parts extract of *P. muellerianus* and its major constituent geraniin.

Materials and Methods

Plant collection

Aerial parts including the leaves of *P. muellerianus* were collected from Kuntunase (longitude 1.0° 28'18"W and latitude 6.0° 32'23"N), Ashanti Region, Ghana in February 2010. The plant was authenticated by Dr. Alex Asase of the Department of Botany, University of Ghana,

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Legon, Accra, Ghana. A voucher specimen (AA 102) was deposited in the Ghana Herbarium located in the same University.

Source of geraniin

Geraniin (96% w/w HPLC grade) was kindly provided by Prof. Dr. Andreas Hensel, Institute of Pharmaceutical Biology and Phytochemistry, University of Muenster, Muenster, Germany and had been isolated from the aqueous extract of the aerial parts of *P. muellerianus* and it was found to be the major compound (4.3% w/w, related to the dried plant material) [33].

Preparation of aqueous extract

Fresh matured aerial parts including the leaves of *P. muellerianus* were washed under running tap water to completely remove foreign materials and air dried at room temperature (25 to 28°C) for seven (7) days. The dried plant sample was pulverized into coarse powder with a lab mill machine (Christy and Norris, Chelmsford, England, UK). Four hundred (400) grams of the powdered plant material was suspended in 4 L of sterile distilled water and heated at 90°C for 15 min. The mixture was centrifuged at 6000 x g for 10 min and filtered through a Whatmann filter paper No. 10 (Sigma-Aldrich, Michigan, USA) with the aid of a vacuum pump (ABM, Greiffenberger Antriebstechnik GmbH, Germany). The filtrate was concentrated by the aid of a rotary evaporator (Rotavapor BÜCHI R-200 with heating bath B-490, Büchi, Konstanz, Germany) at 40°C under reduced pressure and lyophilized. The yield of the extract, related to the dried powdered plant material, was 14.1% w/w. The extract which will be referred to in this study as PLE was then stored at 4 to 8°C in the refrigerator in airtight containers.

Culture media and reference antibiotics

Nutrient agar and broth as well as Sabouraud dextrose agar and broth were purchased from Oxoid Limited, Basingstoke, United Kingdom. Ciprofloxacin and ketoconazole were obtained from Sigma-Aldrich, Michigan, USA.

Preliminary phytochemical screening of aqueous extract of *P. muellerianus* (PLE)

Qualitative phytochemical screening were performed on PLE using standard methods of analysis to determine the presence of secondary metabolites such as tannins, glycosides, saponins, anthraquinones, alkaloids, flavonoids, steroids and terpenoids [34,35].

HPLC finger-printing of PLE

HPLC profile of PLE was determined according to the method described by Agyare et al. [33]. The test was performed using an HPLC with a UV detector set at a wavelength of 280 nm. The chromatographic conditions included a flow rate of 3 mL/min and a pressure of 15 MPa. Chromolith® performance RP-18e Merck (100 x 4.6 mm) was used as stationary phase.

Test organisms

Gram-positive, Gram-negative bacteria and a fungus were used and these include our (4) typed strains of bacteria including *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* NCTC 10073 and clinical isolate of *Streptococcus pyogenes* as well as a clinical fungus (*Candida albicans*) were obtained from the Department of Biological Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana. They were maintained on nutrient agar slants containing 30% glycerol and stored at -4°C in a frost-free freezer in the Microbiology Laboratory of Department of Pharmaceutics, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana.

Susceptibility testing (Agar diffusion method)

Susceptibility of test organisms to PLE and geraniin was determined using the agar diffusion method [36,37]. Nutrient agar and potato dextrose agar were used for the determination of the antibacterial and antifungal activities, respectively. Twenty millilitres of freshly prepared sterile nutrient agar and potato dextrose agar were seeded with 100 µL (1×10^6 CFU/mL) of test bacteria and fungus, respectively and transferred aseptically into sterile petri dishes. In each of these petri dishes, six equidistant wells (10 mm) were cut out using sterile cork borer (number 5) and wells filled with 100 µL of 100, 50, 25 and 12.5 mg/mL of aqueous extract dissolved in sterile distilled water and allowed to diffuse for 1 h at room temperature (25°C). The zones of growth inhibition (including diameter of well) were measured after 24 h of incubation at 37°C for bacteria and 72 h post incubation at 28°C for the fungus. The procedure was performed in independent triplicates and the mean zones of growth inhibition determined. Ciprofloxacin and ketoconazole (Sigma-Aldrich, Michigan, USA) were used as reference antimicrobial agents against test bacteria and fungus, respectively. The same procedure was repeated for geraniin at concentrations of 10, 5, 2.5, 1.25 mg/mL.

Minimum inhibitory concentration (MIC) determination

Minimum inhibitory concentrations of the PLE and geraniin were determined by the microdilution method using the method described by Salie et al. [38] and Wiegand et al. [39]. Micro-titre plates (96-well) were filled with 100 µL of double strength nutrient broth. PLE-containing test solutions at differfent concentrations within the range of 0.1 to 10 mg/mL were prepared and microbial inoculum size of 20 µL (1.0×10^5 CFU/mL) was added to each well. Activity of the PLE was determined against test microbes after incubating at 37°C. After 24 h post incubation, the MIC was determined as the lowest concentration of extract that inhibited growth which was indicated by the absence of purple colouration upon the addition of 20 µL of 1.25 mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the medium in the wells and incubated at 37°C for 30 min (14). Ciprofloxacin at concentration range of 0.01 to 1.0 µg/mL and ketoconazole at concentration ranging from 1.0 to 10.0 µg/mL were used as standards. All tests were performed in triplicates (three independent experiments) to validate the results. The procedure was repeated for geraniin at concentration range of 0.02 to 5.0 mg/mL.

Determination of minimum bactericidal concentration and fungicidal concentration

Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of PLE and geraniin were determined according to the method described by Pfaller et al. [40]. Micro-titre plates (96-wells) were each filled with 100 µL of sterile double strength nutrient broth. A specified volume of the stock was added to each well to obtain a serial two-fold dilution of PLE in each well with concentrations within the range 1.0 to 100.0 mg/mL. An inoculum size of 20 µL (1.0×10^6 CFU/mL) of test organisms were added to the appropriately labeled wells and incubated at 37°C. After 24 h post incubation, aliquots (100 µL) were taken from the each well and inoculated into freshly prepared 10 mL nutrient broth. The inoculated nutrient broths were incubated at 37°C for 24 h. The MBC or MFC was determined as the least concentration of PLE at which no purple colouration was observed upon the addition of 20 µL of MTT (1.25 mg/mL) followed by incubation at 37°C for 30 min. The procedure was performed in triplicate. The same procedure was employed in determining the MBC

and MFC of geraniin using concentrations in the range of 0.3 to 20.0 mg/mL.

Time-kill kinetics studies of PLE and geraniin

Time-kill kinetics of PLE and geraniin were determined using the method described by Petersen et al. [41]. Concentrations equivalent to the MIC, twice the MIC and four times the MIC of PLE were prepared. Inoculum size of 1.0 x 10^5 CFU/mL was used. Concentrations of the extracts were prepared in test tubes containing sterile nutrient broth and inoculated with test microorganisms. Aliquots of 1.0 mL of the medium taken at time intervals of 0, 1, 2, 3, 4, 5, 6, 12 and 24 h were inoculated into nutrient agar and incubated at 37°C for 24 h. A control test (negative) was the test organisms alone without the extracts or reference antibiotic. The colony forming units (CFU) after incubating for 24 h was determined. The procedure was performed in triplicates (three independent experiments) and a graph of the log CFU/mL was then plotted against time. The same procedure was repeated for geraniin as described above.

Statistical analysis

Data obtained from study were analysed with Graph Pad Prism Version 5.0 for Windows (Graph Pad Software Inc, San Diego, CA, USA) statistical package programme by using using One-way ANOVA followed by Dunnett's *post hoc* test.

Results

Phytochemical screening of PLE

PLE was found to contain alkaloids (Dragendorff's test), glycosides (Fehling's test), saponins (frothing test), tannins (Ferric chloride test), flavonoids (2-aminoethyl diphenyl borate *'Naturstoff reagent A'* and ammonia tests) and terpenoids (Borntrager's test). However, steroids (Liebermann-Burchard's test) and anthraquinones (Salkowski's test) were found to be absent.

HPLC finger-printing of PLE

For characterisation of PLE, HPLC profile on reversed phase RP18 stationary phase was performed. The main peak in the chromatogram was identified as Geraniin (2 isomers) by spiking and co-injection with the respective reference compound, geraniin (HPLC purity, 96 %). The retention time (R_t) of geraniin was 6.4 min (Figure 1).

Antimicrobial activity of PLE and geraniin

Susceptibility testing (agar well diffusion): The mean zones of growth inhibition of PLE ranged from 12.0 ± 0.0 to 22.67 ± 0.3 mm against the test bacteria. In addition, the mean zones of growth inhibition for PLE against Gram-positive and Gram-negative bacteria were in the range of 12.0 ± 0.0 to 22.7 ± 0.3 mm and 13.0 ± 0.0 to 22.0 ± 0.33 mm, respectively. The mean zone of inhibition of PLE against C. albicans was between 12.0 ± 0.0 to 18.3 ± 0.3 mm (Table 1).

Furthermore, the mean zones of growth inhibition of geraniin against the test bacteria were from 12.0 ± 0.0 to 21.7 ± 0.33 mm. The mean zones of growth inhibition of geraniin against Gram-positive and Gram-negative bacteria were in the range of 12.0 ± 0.0 to 21.7 ± 0.3 mm and 12.3 ± 0.3 to 21.3 ± 0.3 mm, respectively. Against *C. albicans*, the mean zones of growth inhibition were 13.0 ± 0.6 to 18.3 ± 0.33 mm (Table 2).

MIC, MBC and MFC of PLE and geraniin: MIC of PLE against the test Gram-negative and Gram-positive bacteria was within the range of 0.31 to 5 mg/mL. The MIC of PLE against *C. albicans* was 0.5 mg/mL. The highest activity was observed against *S. aureus* and the lowest activity against *E. coli*. The MBC of PLE against test Gram-negative bacteria was found to be in the range of 10.0 to 50.0 mg/mL whiles the MBC of PLE against Gram-positive bacteria was in the range of 5.0 to 20.0 mg/mL. The MBC of PLE against *C. albicans* was 5.0 mg/mL (Table 3).

MICs of geraniin against test Gram-negative and Gram-positive bacteria were in the range of 0.08 to 0.31 mg/mL (90 to 330 µM) and 0.08 to 1.25 mg/mL (90 to 1310 µM), respectively. The MIC of geraniin against *C. albicans* was 0.16 mg/mL (170 µM). The highest activity was observed against *P. aeruginosa and S. pyogenes* whiles the lowest activity was observed against *E. coli*. The MBC of geraniin against the test Gram-negative and Gram-positive bacteria were between the ranges of 2.5 to 10.0 mg/mL (2.62 to 10.5 mM) and 1.25 to 5.0 mg/mL (1.31 to 5.25 mM), respectively whereas the MFC was 5.0 mg/mL (%.25 mM). The MIC of ciprofloxacin against test bacteria ranged from 0.1 to 0.25 µg/mL (0.3 t0 0.76 µM) whereas the MIC of ketoconazole against *C. albicans* was 5.0 µg/mL (9.41 µM) (Table 3).

MBC/MIC and MFC/MIC ratios of PLE and geraniin: The MBC/MIC ratio of PLE for Gram-negative bacteria was in the range of 10 to 32 whereas that of Gram-positive bacteria was 16. The MFC/MIC ratio of PLE against C. albicans was 10. The MBC/MIC ratio of geraniin for Gram-negtive bacteria was determined to be in the range of 8 to 31 whereas the MBC/MIC ratio of geraniin for Gram-positive bacteria was in the range of 8 to 16 (Table 4).

Time-kill kinetics of PLE and geraniin: Time-kill kinetics profiles of PLE against the test organisms were observed to be bacteriostatic. The time-kill kinetics profile of PLE against the test Gram-negative organisms (*E. coli* and *P. aeruginosa*) at test concentrations showed reduction in number of viable cells over the first three hours, followed by a gradual rise up to the 24th h when compared to the control (organisms without antimicrobial agent). The area under the curve, which is a measure of the activity of PLE over the 24 h period, showed that PLE at concentrations of 5, 10 and 20 mg/mL significantly (p<0.001) reduced the number of *E. coli* cells when compared to the control. Also the area under the curve (AUC) representing the activity of PLE against *P. aeruginosa* at concentrations of 0.31, 0.63 and 1.25 mg/mL revealed a significant (p<0.001) reduction in number of viable cells compared to the control (Figure 2).

Time-kill kinetics of PLE against the test Gram-positive bacteria (*S. aureus, B. subtilis and S. pyogenes*) showed a concentration dependent reduction in number of viable cells within the first 3 h followed by a gradual increase up to the 24th h. The AUC representing the activity of PLE against the test Gram-positive bacteria showed that PLE at concentrations of 0.31, 0.63 and 1.25 mg/mL reduced significantly (p<0.001) the number of *S. aureus* cells compared to the control whereas PLE at concentrations of 0.63, 1.25 and 2.5 mg/mL significantly (p<0.001) reduced the number *B. subtilis* cells when compared to the control. Furthermore, PLE at concentrations of 0.63, 1.25 and 2.5 mg/mL reduced significantly (p<0.001) the number of viable *S. pyogenes* cells compared to the control. Also, the time-kill kinetics of PLE at concentrations of 1.25, 2.5 and 5 mg/mL against *C. albicans* showed significant (p<0.001) reduction in number of viable cells (Figure 2).

Time-kill kinetics profiles of geraniin against test organisms were observed to be bacteriostatic. The time-kill kinetics profile of geraniin against test Gram-negative, Gram-positive bacteria and *C. albicans* showed a reduction in number of cells within the first 4 h and a gradual increase in number of cells after the 4th h to 24th h. The AUC for geraniin against *E. coli* at concentrations of 1.25, 2.5 and 5 mg/mL revealed that the number of *E. coli* cells significantly (p<0.001) reduced when compared to the control (Figure 3). For geraniin against

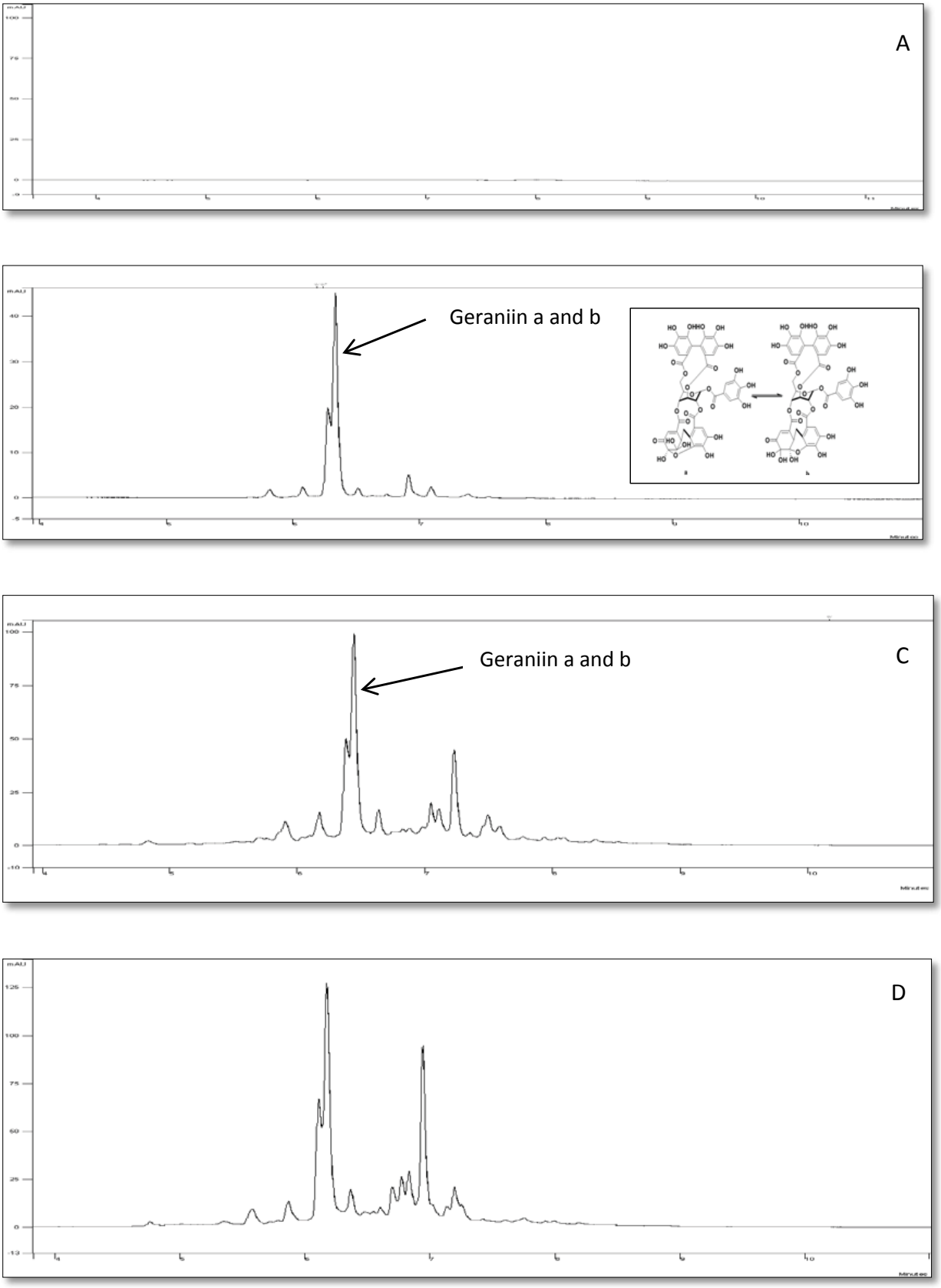

Figure 1: HPLC chromatogram of PLE. (A) solvent alone; (B): reference geraniin (100 µg/mL); (C): PLE (100 µg/mL) (D). PLE spiked with reference geraniin (100 + 100 µg/mL).

Conc (mg/mL)	Mean zone of growth inhibition (mm) ±SEM					
	Typed strains				Clinical strains	
	E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*	*S. pyogenes*	*C. albicans*
100	22.0 ± 0.0	20.33 ± 0.33	21.00 ± 0.0	22.67 ± 0.33	20.00 ± 0.58	18.33 ± 0.33
50	19.0 ± 0.0	19.00 ± 0.0	18.67 ± 0.33	19.67 ± 0.33	18.00 ± 0.00	14.00 ± 0.0
25	16.33 ± 0.33	16.00 ± 0.0	16.33 ± 0.33	16.00 ± 0.0	14.00 ± 0.58	12.00 ± 0.0
12.5	13.00 ± 0.57	13.00 ± 0.0	12.00 ± 0.0	14.00 ± 0.58	12.00 ± 0.0	na
Cipro (30 µM)	28.67 ± 0.67	21.33 ± 0.67	29.33 ± 0.67	20.67 ± 0.67	29.67 ± 0.33	nd
Keto (19 µM)	nd	nd	nd	nd	nd	26.00 ± 0.0

Recorded zone of growth inhibition= diameter of well plus zone of growth inhibition, diameter of well=10mm. Cipro: Ciprofloxacin, Keto: ketoconazole, nd: not determined, na: no activity. Value expressed as mean ± Standard Error Mean (SEM), n=3.

Table 1: Mean zones of growth inhibition of PLE against typed and clinical strains of test organisms.

Conc (mg/mL)	Mean zone of growth inhibition (mm) ±SEM					
	Typed strains				Clinical strains	
	E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*	*S. pyogenes*	*C. albicans*
10.0 (10.5 mM)	21.33 ± 0.67	20.00 ± 0.0	20.67 ± 0.33	21.67 ± 0.33	21.67 ± 0.88	18.33 ± 0.33
5.0 (5.25 mM)	18.00 ± 0.0	17.67 ± 0.33	17.67 ± 0.33	18.33 ± 0.33	18.67 ± 0.67	15.00 ± 0.0
2.5 (2.63 mM)	14.67 ± 0.33	15.67 ± 0.33	15.00 ± 0.00	15.67 ± 0.33	15.67 ± 0.33	13.00 ± 0.57
1.25 (1.32 mM)	na	12.33 ± 0.33	12.00 ± 0.0	13.67 ± 0.33	12.67 ± 0.33	na
Cipro (0.03 mM)	28.67 ± 0.67	21.33 ± 0.67	29.33 ± 0.67	20.67 ± 0.67	29.67 ± 0.33	nd
Keto (0.02 mM)	nd	nd	nd	nd	nd	24.33±0.33

Recorded zone of growth inhibition= diameter of well plus zone of growth inhibition, diameter of well=10mm. Cipro: Ciprofloxacin, Keto: ketoconazole, nd: not determined, na: no activity. Value expressed as mean ± standard error mean (SEM), n=3.

Table 2: Mean zones of growth inhibition of geraniin against typed and clinical strains of test organisms.

Test Organisms	Strain	PLE		Geraniin		Cipro MIC (µg/mL)	Keto MIC (µg/mL)
		MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)		
B subtilis	Typed	1.25	20.0	0.31 330 µM	5.0 5.25 mM	0.1 0.30 µM	nd
E. coli	Typed	5.0	50.0	1.25 1310 µM	10.0 10.5 mM	0.13 0.39 µM	nd
P. aeruginosa	Typed	0.31	10.0	0.08 90 µM	2.5 2.62 mM	0.25 0.76 µM	nd
S. aureus	Typed	0.31	5.0	0.16 170 µM	1.25 1.31 mM	0.25 0.76 µM	nd
S. pyogenes	Clinical	0.63	10.0	0.08 90 µM	2.5 2.62 mM	0.1 0.30 µM	nd
C. albicans	Clinical	0.5	5.0	0.16 170 µM	5.0 5.25 mM	nd	5.0 9.41 µM

nd: not determined, Cipro: Ciprofloxacin, Keto: ketoconazole, MIC: minimum inhibitory concentration, MBC: minimum bactericidal concentration

Table 3: MIC, MBC and MFC of PLE, geraniin and reference antibiotics.

Test organisms	MBC/MIC and MFC/MIC ratio	
	PLE	Geraniin
B. subtilis	16	16
E. coli	10	8
P. aeruginosa	32	31
S. aureus	16	8
S. pyogenes	16	31
C. albicans	10	31

PLE: Aqueous extract of the aerial parts of *P. muellerianus*, MIC: minimum inhibitory concentration, MBC: minimum bactericidal concentration

Table 4: MBC/MIC and MFC/MIC ratio of PLE and geraniin.

P. aeruginosa, significant reduction ($p<0.001$) in the number of viable cells at concentrations of 0.08, 0.16 and 0.32 mg/mL compared to the control was observed (Figure 3).

Also, the AUC revealed that, the number of viable *S. aureus* cells was significantly ($p<0.001$) reduced in geraniin concentrations of 0.08, 0.16 and 0.32 mg/mL compared to the control while geraniin reduced significantly ($p<0.001$) the number of *B. subtilis* cells at concentrations of 0.16, 0.32 and 0.64 mg/mL when compared to the control. Additionally, geraniin reduced significantly ($p<0.001$) the number of viable *S. pyogenes* cells compared to the control group. Geraniin at concentrations of 0.31, 0.63 and 1.25 mg/mL significantly ($p<0.001$) reduced the number of viable cells of *C. albicans* when compared to the control (Figure 3).

Discussion

Plants contain myriad of secondary metabolites such as alkaloids, flavonoids, tannins, glycosides, terpenoids and steroids which act either individually or in synergism to elicit a biological activity [42-

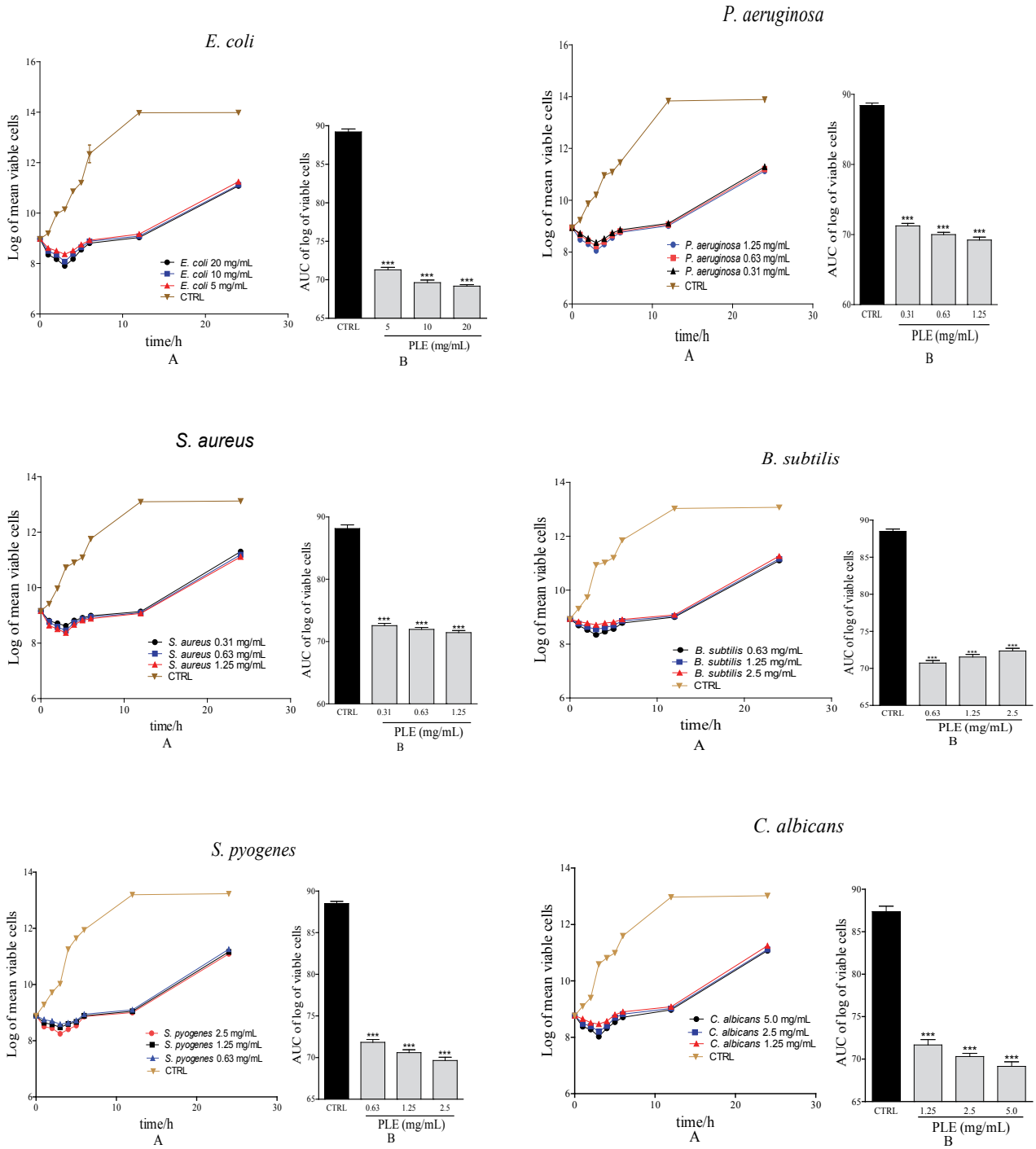

Figure 2: Time-kill kinetics of PLE against test microbes. **A**: time-kill kinetics curve, **B**: AUC of time-kill kinetics. PLE: aqueous extract of the aerial parts of *P. muellerianus*, CTRL: control.. Values are mean ±SEM, n=3. ***$p<0.001$ compared to the control (One-way ANOVA followed by Dunnett's *post hoc* test).

44]. Phytochemical screening of aqueous leaf extract of *P. muellerianus* (PLE) for secondary metabolites revealed the presence of alkaloids, glycosides, saponins, tannins, flavonoids and terpenoids. Steroids and anthraquinones were found to be absent in PLE. Bamisaye et al. [26] reported the presence of tannins, flavonoids, terpenoids, saponins and glycosides in the aqueous leaves and aerial parts extract of *P. muellerianus*. Doughari and Sunday [45] however, reported the presence of anthraquinones in the aqueous leaf extract in addition to alkaloids, tannins and flavonoids. The absence of anthraquinones in PLE may be due geographical location of the plant, the season and time of collection which are factors known to contribute to variations in the phytochemical constituents of same species of plants [46,47].

Although phytochemical screening for secondary metabolites can aid in the identification of plants, HPLC is preferred because it is

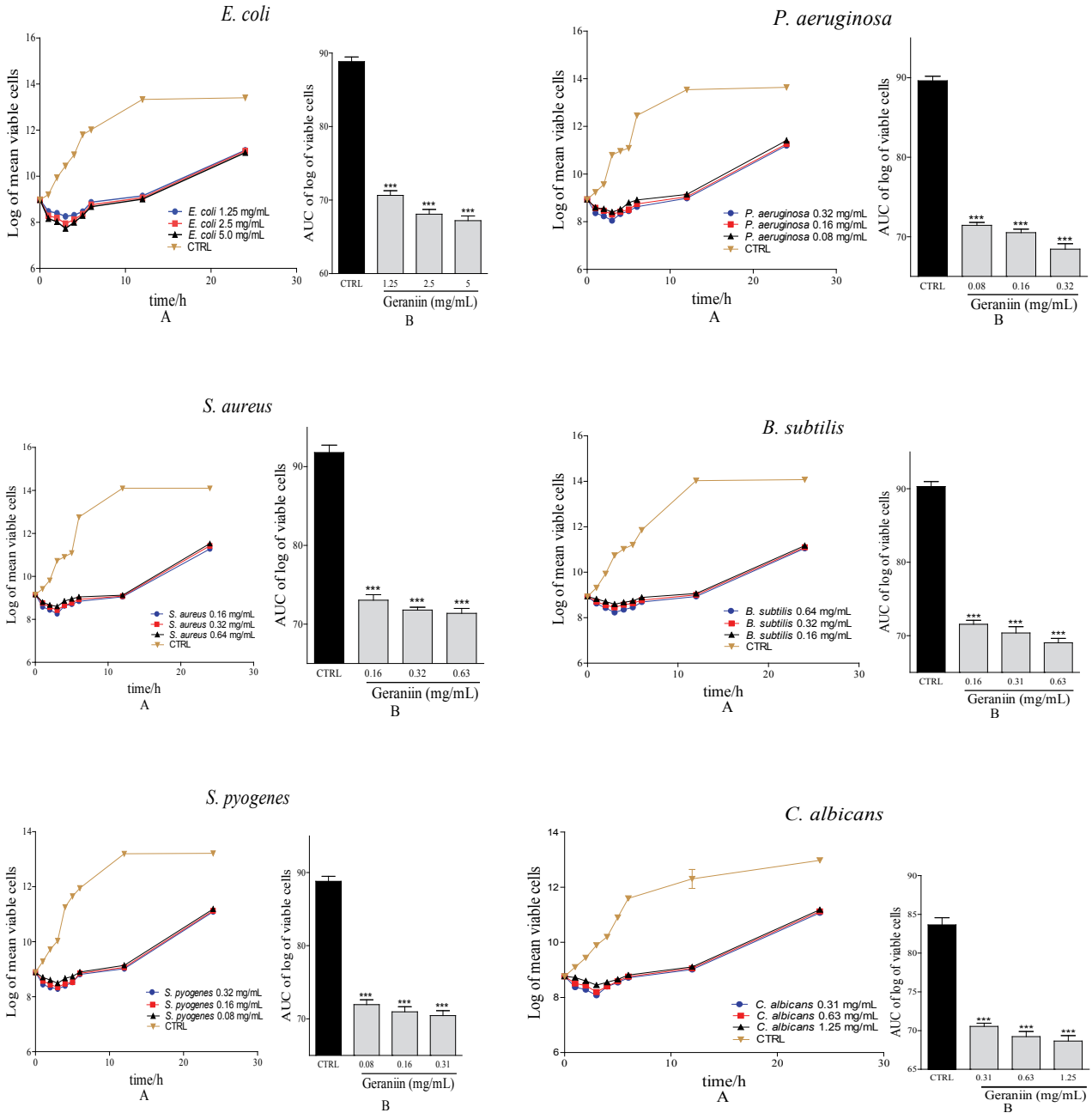

Figure 3: Time-kill kinetics of geraniin against test microbes. **A**: time-kill kinetics curve, **B**: AUC of time-kill kinetics. Values are mean ±SEM, n=3. ***p<0.001 compared to the control (One-way ANOVA followed by Dunnett's *post hoc* test). CTRL: control.

more specific. HPLC profiling of extract aids in easy identification and confirmation of the plant based on qualitative and quantitative analysis of specific phytochemicals. This provides adequate identification parameters to prevent alterations of formulated herbal products containing the extract. The profile also represents the complex chemical composition of the extract [48]. The chromatogram revealed two peaks which were identified to represent the two isomers of geraniiin (a and b) which is the major composition of PLE at a retention time R_t of 6.44 min (Figure 3).

Agar diffusion method has been employed frequently and is recommended as a good method for determining the relative potency of complex extracts and for establishing their antimicrobial spectrum [49,50]. PLE and geraniin were active against both Gram–positive and Gram–negative bacteria as well as antifungal activity (Tables 1 and 2). The demonstration of antibacterial activity against both Gram–positive and Gram–negative bacteria may be indication of the presence of broad-spectrum antimicrobial agent(s) or compound(s) [51]. The antimicrobial activity exhibited by PLE may likely be due to one or more of phytochemical constituents present in the plant [52,53] because phytochemical constituents such as tannins, flavonoids, alkaloids and glycosides, serve as defense mechanisms against predation by many microorganisms, insects and herbivores [52]. Again, recent studies have

indicated that tannins [54], alkaloids [55], saponins [56], glycosides [57], flavonoids [58] and terpenoids [59] possess antimicrobial activity and exert their effects by affecting the cell membrane integrity of the bacteria [60]. Furthermore, Assob et al. [24] and Doughari and Sunday [45] have shown that the methanol and ethyl acetate stem bark and aqueous leaf extract of *P. muellerianus*, respectively possess antibacterial activity and these support the findings of this study. Polyphenolic compounds have also been reported to possess antimicrobial activity which might be due to their ability to form complexes with bacterial cell wall which leads to inhibition of microbial growth [61]. Hence, this may account for the observed antimicrobial activity of the ellagitannin, geraniin which is a polyphenol. Gohar et al. [62] also reported that geraniin isolated from *Erodium glaucophyllum* was active against *E. coli*, *S. aureus* and *C. albicans* which is in agreement with our findings.

Studies have shown that plant extracts with MIC values between 2.5 and 8 mg/mL have led to the isolation of potent antimicrobial compounds [63-66]. This therefore suggests that PLE can be a source of potentially active antimicrobial compound. In addition, constituents or agents isolated from plants are routinely classified as potential antimicrobials on the basis of susceptibility tests that produce MICs of 100 to 1000 µg/mL [67]. This may suggest that geraniin can be classified as a potential antimicrobial agent.

Antimicrobials are usually regarded as bactericidal if the MBC/MIC or MFC/MIC ratio is ≤ 4 and bacteriostatic if >4 [68]. The ratios obtained for all the test organisms were above 4 which indicated that both PLE and geraniin were bacteriostatic and fungistatic in action against test organisms (Table 4). The bacteriostatic action of PLE and geraniin was also confirmed by the time-kill kinetic studies (Figures 2,3). Bacteriostatic or fungistatic antimicrobial agents only inhibit the growth or multiplication of microbes giving the immune system of the host time to clear the microbes from the system [69].

The observed antimicrobial activity of the PLE and geraniin against *S. aureus*, *S. pyogenes, P. aeruginosa*, *E. coli* and *C. albicans* suggest that that PLE and geraniin may play a significant role in the management and treatment of infected wounds. In most infected wounds, Gram-positive organisms especially *S. aureus* and *S. pyogenes* are implicated due to their ability to produce enzymes that destroy the extracellular matrix in wound bed [70]. Also, the most prevalent Gram-negative bacteria found in wound infections is *P. aeruginosa*. Wounds contaminated with *P. aeruginosa* are in most cases difficult to manage due to high intrinsic resistant factors and the ability of these organisms to form biofilms [71,72]. Although, *E. coli* is not a common pathogen of wounds, some reports have implicated *E. coli* as the third most prevalent microbe in wounds after *S. aureus* and *S. pyogenes* [73]. It is also interesting to note that fungi such as *C. albicans* are also believed to contaminate and colonize wounds [74]. This may suggest that PLE and geraniin may be useful in the management of infected wounds.

Conclusion

The aqueous leaf extract of *P. muellerianus* (PLE) was found to possess antimicrobial activity that may confirms its traditional use as anti-infective agent. The antimicrobial activity of *P. muellerianus* may largely be due to its major isolate, geraniin and they act by static means.

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