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RESEARCH PAPER

PHYTOCHEMICAL COMPOSITION AND *IN VITRO* ANTIBACTERIAL ACTIVITIES OF *MILLETTIA CHRYSOPHYLLA* AND *MILLETTIA ZECHIANA*

Beverly Egyir[1], Kofi Baffour-Awuah Owusu[2], Mitsuko Ohashi[2], Mary Anti Chama[3]*

[1]Bacteriology Department, Noguchi Memorial Institute for Medical Research, University of Ghana
[2]Parasitology Department, Noguchi Memorial Institute for Medical Research, University of Ghana
[3]Department of Chemistry, School of Physical and Mathematical Sciences, University of Ghana

***Corresponding author: machama@ug.edu.gh**

ABSTRACT

Millettia chrysophylla (MC) and Millettia zechiana (MZ) are two medicinal plants distributed in Africa. MZ is used traditionally to treat infectious diseases. Current literature survey suggests no scientific studies on MC and phytochemical studies on MZ were scanty with no biological activities. In vitro antibacterial activities of the ethanol extracts of the leaves (L), stems (S), and roots (R) for both plants, and flowers (F) and twigs (T) for MZ were tested against eight laboratory bacteria strains and 28 clinical isolates of Methicillin-Resistant Staphylococcus aureus (MRSA). The chemical profile of the extracts was obtained by Gas Chromatography Mass Spectrometry (GCMS) whiles characterisation of the alkaloid, flavonoid, and tannin contents were also determined. MCS extract showed a strong and broad activity against S. epidermidis (8.79 µg/mL), E. coli (3.9 µg/mL), S. enteritidis (5.11 µg/mL) and B. cereus (5.33 µg/mL). Broad bactericidal activity against the MRSA strains: MR21, MR4 and MR19 with respective IC_{50} values of 72.30, 86.45 and 97.76 µg/mL were showed by MCL extract. The major components identified from the GCMS analysis included 17-octadecenoic acid (39.46%), 17-octadecynoic acid (27.90%) n-decanoic acid (27.88%), (Z, Z)-9,12-octadecadienoic acid (27.02%), (Z)-18-octadec-9-enolide (24.46%), and n-hexadecanoic acid (20.87%). MCL indicated 6.97±0.62 mg CA/g, 3.75 ± 0.12 mg TA/g, and 3.58 ± 0.18 mg RU/g for the respective contents of alkaloids, tannins, and flavonoids. These findings have given insights into the phytochemicals of M. chrysophylla and the antibacterial activities of leaves, stems and roots of M. chrysophylla and the twigs of M. zechiana for further drug discovery research.

Keywords: *Millettia chrysophylla*; *Millettia zechiana;* bacteria; Methicillin-Resistant *Staphylococcus aureus* (MRSA); phytochemicals

INTRODUCTION

Millettia chrysophylla Dunn and *M. zechiana* Hams, are two plant species of the Fabaceae family which grow in some parts of Africa (Banzouzi *et al*., 2008; Irvine, 1961; Hutchinson *et al*., 1954). The Millettia genus appears as liana, shrub or tree occurring in tropical and subtropical Africa, Asia, and Australia with about 260 species (Banzouzi *et al*., 2008; Lemmens, 2008). In Sub-Saharan Africa, 64% of the species are used medicinally as antimicrobials, antitumour, antidiabetic, insecticidal agents, as well as remedies for reproductive, and respiratory disorders (Banzouzi *et al*., 2008; Zirihi *et al*., 2005). *M. zechiana*, is used locally to manage pulmonary, naso-pharyngeal, and gonorrheal infections (Bouquet and Debray, 1974; Burkill, 1995; Okafor and Ham, 1999). For *M. chrysophylla*, despite its documentation in the African Traditional Pharmacopoeia, there are no medicinal uses apart from its use as a fish poison (Allen and Allen, 1981) with no phytochemical and biological studies.

Microbial infection and antimicrobial resistance have become worldwide issues threatening the fight against infectious agents particularly the resistant ones (WHO, 2020). Currently, infection associated with Coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has claimed the lives of over two million people globally (WHO, 2021) in a year. The need for antibiotics to curb infectious diseases cannot be over emphasized especially for the success of major surgeries and cancer chemotherapies. Methicillin-Resistant *Staphylococcus aureus* (MRSA), one of the leading agents that cause nosocomial and community infections in humans, creates significant health care challenges in many parts of the world. MRSA expresses methicillin-resistance by producing penicillin-binding protein (PBP2′) that has a decreased binding affinity to *beta*-lactam antibiotics (Reynolds and Brown, 1985; Utsui and Yokota, 1985). While attempts made to cure resistant bacteria strains have proven difficult, the use of plant-base alternatives with antibacterial properties have been suggested as potential remedy to antimicrobial resistance bacteria strains (Atta-ur-Rahman *et al*., 2005; Magi *et al*., 2015; Seebaluck-Sandoram *et al*., 2018; Shalayel *et al*., 2017). *M. chrysophylla* and *M. zechiana* species can play important roles as sources of antibacterial agents. This study investigated the *in vitro* antibacterial activities of the ethanol extracts of *M. chrysophylla* and *M. zechiana* against laboratory and clinical bacteria strains of MRSA as potential sources of alternate antibiotics as well as the chemical compositions of the two plants.

MATERIALS AND METHODS

Plant collection

Different parts of *Millettia chrysophylla* (MC) and *Millettia zechiana* (MZ) plants including the leaves (MCL, MZL), stem barks (MCS, MZS), roots (MCR, MZR), flowers (MZF) and twigs (MZT) were collected in the Ashanti region of Ghana on September 14 and November 19 of 2015. The plants were identified by Mr. John Ntim-Gyakari formerly of Kumasi Forestry Commission. The respective Voucher specimens, MC-MCF001 and MC-MZF001, have been deposited at the National Herbarium (GC) of the University of Ghana at the Department of Plant Science and Environmental Biology. Each plant part was air dried for four weeks and pulverized for further extraction.

Extraction of plant material

Two hundred grams (200 g) of each of the pulverized plant materials of *M. chrysophylla* (MCL, MCS, MCR), and *M. zechiana* (MZL, MZS, MZR, MZT and MZF) was exhaustively

extracted by cold percolation with a total of 4 L of 96% ethanol. Each extract was filtered and concentrated after 48 h to crude extracts coded as MCL, MCS, and MCR for the ethanol extracts of the leaf, stem bark, and root of *M. chrysophylla*, respectively and MZL, MZS, MZR, MZT, and MZF for the ethanol extracts of the leaf, stem bark, root, twig, and flower of *M. zechiana* respectively.

Phytochemical Analyses

Preliminary phytochemical screening

The various plant extracts of both *M. chrysophylla* and *M. zechiana* were screened for the presence of different classes of secondary metabolites including alkaloids, flavonoids, phenols, saponins, tannins, anthocyanins, anthraquinones, sterols, and terpenoids using similar methods described by Harborne (1998), Trease and Evans (2002), and Stahl (1969).

Quantitative phytochemical analysis

Due to the presence of alkaloids, tannins and flavonoids phytochemicals obtained from the qualitative screening in all the extracts and the fact that some of these phytochemicals are noted for their antimicrobial properties necessitated their selection for quantification to ascertain the significance of any antimicrobial activity or otherwise.

Total alkaloid content

Total alkaloid content was quantified by spectrophotometric method based on the reaction between alkaloid and bromocresol green (BCG) as described by Ganapaty *et al*. (2013). Stock solution of each ethanol extract was prepared by dissolving 25 mg of the extract in 25 mL of ethanol. Volume of 1.0 mL of each stock solution of the extract was dissolved in 1.0 mL of 2 M HCl and filtered. The resulting solution was transferred into a 100 mL separating funnel, and 5.0 mL of BCG solution along with 5.0 mL of the phosphate buffer (adjusted to neutral pH with 0.1 M NaOH) was added. The mixture was shaken, and the complex formed was extracted with 10.0 mL of chloroform. The chloroform extract of concentration 0.1 mg/mL was collected, and the absorbance measured at 470 nm. Stock solution (1.0 mg/mL) of caffeine was used as a reference standard for constructing a calibration curve. Volumes of 0.5, 1.5, 2.5 and 5.0 mL of the stock caffeine solution were taken through the same procedure as for the extracts to give respective concentrations of 0.05, 0.15, 0.25 and 0.50 mg/mL. The absorbance of each concentration was measured at 470 nm. Total alkaloid content in each extract was expressed in terms of Caffeine equivalents (mg of CA/g of plant extract).

Total tannin content

The method proposed by Graham (1992) was used to quantify tannin content in each extract. To 0.1 mL of each extract (from the stock solution of 1.0 mg/mL ethanol), 6.9 mL of distilled water, 1.0 mL of 0.008 M potassium ferric cyanide and 1.0 mL of 0.2 M ferric chloride in 0.1 M HCl solutions were added and mixed. The absorbance of each of the resulting blue colored solution (0.01 mg/mL) was measured at 700 nm wavelength. Tannic acid was used as standard for constructing a calibration curve. Volumes of 0.1, 0.3, 0.5 and 1.0 mL from the tannic acid stock solution of 5 ppm were subjected to the same procedure as were the extracts to give corresponding concentrations of 0.06, 0.16, 0.27, and 0.51 ppm respectively. The absorbances were measured at 700 nm wavelength. Concentration of tannin in each extract was extrapolated from the calibration curve of the standard tannic acid. Total tannins in extracts were expressed in terms of tannic acid equivalents (mg of TA/g of plant extract).

Total flavonoid content

Total flavonoid content was quantified by spectrophotometric method reported by Jaradat *et al*. (2015) with slight modifications.

Serial dilutions with concentrations 50, 100, 200, 400, and 1000 ppm were prepared from a stock solution (1.0 mg/mL) of the reference standard, Rutin in methanol. From each solution, 0.5 mL was taken and mixed with 3.0 mL of methanol, 0.2 mL of 10% $AlCl_3$, 0.2 mL of 1.0 M sodium acetate and 5.0 mL of distilled water in a beaker to give respective final concentrations of 2.8, 5.6, 11.2, 22.5 and 56.2 ppm. The solutions were incubated at room temperature for 30 min. This procedure was repeated for all extracts using 0.5 mL of the stock solution (1.0 mg/mL) in methanol to give a resulting concentration of 0.562 mg/mL for each extract solution. The blank solution was prepared from a mixture of distilled water, methanol, 10% $AlCl_3$ and sodium acetate. Absorbance for each solution was measured at a wavelength of 415 nm. Total flavonoid content in each extract was expressed as Rutin equivalents (mg of RU/g of plant extract).

Gas Chromatography Mass Spectrometry (GC-MS) analyses

Plant extracts were each reconstituted in ethyl acetate for the analyses. The GC-MS analysis was performed on an Agilent Technology brand of model 7890B GC coupled to an Agilent Technology model 7000C GC-MS triple Quad detector (CA, USA). Separation was achieved on a VF-5ms (Agilent, Santa Clara, CA, USA) capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). The GC operating conditions were as follows: Oven temperature was held at 70°C for 2 min, increased from 70-150°C at a rate of 25°C min^{-1}, 150-200°C at a rate of 3°C min^{-1} and 200-300°C at a rate of 8°C min^{-1}, with a final isothermal held for 15.6 min. Helium was used as carrier gas. The samples were injected splitless (typically 2 µL) with the injector temperature at 250°C. The MS was operated in the electron impact mode at 70 eV and scanned from 40 to 600 Da. Data were acquired and processed with the Masshunter software (Agilent Technology CA, USA). Individual constituents of each extract were identified by their retention times and compared with mass spectra data from the National Institute of Standards and Technology (NIST) library available with the GC-MS system. The GC retention times were expressed in minutes and cited on each respective mass spectrum.

Bacteria Isolates

Twenty-eight MRSA isolates recovered from clinical and non-clinical sources from previous studies (Egyir *et al*., 2015) in Ghana and laboratory bacteria species including three Gram-positive strains; *Staphylococcus aureus* (ATCC 29213), *Bacillus cereus* (ATCC 14579), and *Staphylococcus epidermidis* (ATCC 12228) and five Gram-negative strains; *Eschericha coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella paratyphi* B (ATCC 9150), *Shigella flexneri* (ATCC 12022) and *Salmonella enteritidis* (ATCC 13076) were used in the current study.

Preparation of bacteria inoculum and extracts

Bacteria species were plated on Mueller-Hinton agar (Sigma-Aldrich, USA) and incubated overnight at 37°C. Three individual colonies from each bacteria plate were selected, transferred into microbiological culture broth and incubated overnight at 37°C for bacteria to reach the log phase of growth. Stock solution of each extract was prepared at concentration of 10 mg/mL in dimethyl sulfoxide (DMSO). The extracts were vortexed, and filter sterilized into vials through 0.45 µm millipore filters under sterile conditions and stored at – 20°C until use.

Antibacterial assay by the Alamar Blue method

The antibacterial activity of all extracts was carried out by slight modification of the Alamar Blue assay (Life Technologies, USA) to determine the Inhibitory Concentrations, the standard Minimal Inhibitory Concentrations

(MIC) and Minimal Bactericidal Concentrations (MBC).

Determination of the Inhibitory concentration

The log phase bacteria were diluted with sterile saline to achieve a turbidity of 0.5 McFarland standard of an approximate concentration of 2 x 10^8 CFU/mL. The bacteria were then diluted to the working concentration of each strain. Log phase of bacteria at a concentration range of 1 x10^2 to 1x10^6 CFU/mL were incubated with different concentrations of extracts (0 – 400 µg/mL), and 10% Alamar Blue® reagent at 37 ºC for 6-8 h. Absorbance was read at 540 nm with a reference wavelength of 595 nm, and half maximal inhibitory concentration (IC_{50}) values of extracts were calculated by the plot of a growth curve. Ampicillin and ciprofloxacin were used as positive controls for the standard bacteria and MRSA isolates, respectively.

Determination of Standard minimal inhibitory and bactericidal concentrations

In the determination of the bactericidal and bacteriostatic properties of the extracts, bacteria cells were seeded with different concentrations of extracts and 10% Alamar Blue® as described above. The reducing power of cells which converts the Alamar Blue component resazurin to the pink resorufin was used to determine the Minimum Inhibitory Concentration (MIC) of the extracts. The least concentration of extracts with no observable color change was noted as the MIC. In the determination of the Minimum Bactericidal Concentration (MBC), all concentrations of extracts where there was no observable color change were streaked on a Mueller-Hinton agar plate and incubated at 37 ºC overnight and the least concentration of extracts with no bacteria growth was noted as the MBC.

Statistical analysis

Half of the maximal inhibitory concentration (IC_{50}) values were calculated by the plot of a growth curve using the GraphPad Prism (v.7). Data from dose-response experiments were represented as the percent of inhibition compared to control. Absorbance obtained were normalized and IC_{50} value for each compound against a strain was calculated by fitting the data to a non-linear regression curve, fixing the top and bottom of the curve at 100 and 0 percent, respectively. The IC_{50} values correspond to the concentration that would yield an inhibition of 50% which means the nonlinear regression algorithm also estimates a 95% confidence interval for the IC_{50}.

RESULTS AND DISCUSSION

Phytochemical Analysis

The preliminary phytochemical screening of the different extracts revealed the presence of tannins, cardiac glycosides, alkaloids, steroids, flavonoids and leucoanthocyanins, in all the extracts except saponins which were present in only MCR extract (Table 1). Terpenoids were present in all extracts except MZR, MZS and MCS.

Table 1: Phytochemical constituents present in *M. zechiana* and *M. chrysophylla* extracts

Phytochemicals	MZR	MZT	MZS	MZL	MZF	MCR	MCS	MCL
Tannins	+	+	+	+	+	+	+	+
Cardiac Glycosides	+	+	+	+	+	+	+	+
Terpenoids	_	+	_	+	+	+	_	+
Alkaloids	+	+	+	+	+	+	+	+
Saponins	_	_	_	_	_	+	_	_
Flavonoids & leucoanthocyanins	+	+	+	+	+	+	+	+
Steroids	+	+	+	+	+	+	+	_
Anthraquinones & anthracene derivatives	–	–	–	–	–	–	–	–

+ Present – Absent

MCS: Ethanol extract of Millettia chrysophylla stem bark, MCL: Ethanol extract of M. chrysophylla leaf, MCR: Ethanol extract of M. chrysophylla root, MZS: Ethanol extract of Millettia zechiana stem bark, MZL: Ethanol extract of M. zechiana leaf, MZR: Ethanol extract of M. zechiana root, MZT: Ethanol extract of M. zechiana twig, MZF: Ethanol extract of M. zechiana flower

There were no anthraquinones and anthracene derivatives in all extracts. Due to the presence of alkaloids, tannins and flavonoids phytochemicals obtained from the qualitative screening in all the extracts and the fact that some of these phytochemicals are noted for their antimicrobial properties necessitated their selection for quantification to ascertain the significance of any antimicrobial activity or otherwise. The leaf extract of MC had the highest total alkaloid content (6.97 mg CA/g) followed by leaves of MZ (4.73 mg CA/g) with the least content obtained for the flowers of MZ (1.79 mg CA/g), Table 2. The highest flavonoid (3.58 mg RU/g) content was obtained for leaf extract of MC and the highest tannin content was obtained for the stem extract of MZ (4.49 mg TA/g), Table 2.

Table 2. Total flavonoid, tannin, and alkaloid contents of *M. zechiana* and *M. chrysophylla* extracts.

Extract	Total Flavonoid Content (mg RU/g) ±SD	Total Tannin content (mg TA/g) ±SD	Total Alkaloid content (mg CA/g) ±SD
MCL	3.58±0.18	3.75±0.12	6.97±0.62
MCS	1.44±0.24	2.66±0.20	3.79±0.20
MCR	0.44±0.12	1.67±0.14	2.97±0.26
MZT	2.95±0.30	3.79±0.16	3.91±0.34
MZS	1.10±0.28	4.49±0.10	3.61±0.28
MZR	0.91±0.28	2.64±0.16	3.97±0.12
MZL	2.15±0.20	2.06±0.10	4.73±0.40
MZF	2.54±0.34	4.08±0.24	1.79± 0.24

MCS: Ethanol extract of *Millettia chrysophylla* stem bark, MCL: Ethanol extract of *M. chrysophylla* leaf, MCR: Ethanol extract of *M. chrysophylla* root, MZS: Ethanol extract of *Millettia zechiana* stem bark, MZL: Ethanol extract of *M. zechiana* leaf, MZR: Ethanol extract of *M. zechiana* root, MZT: Ethanol extract of *M. zechiana* twig, MZF: Ethanol extract of *M. zechiana* flower.

Alkaloids are reported to show various biological activities including antiviral, antibacterial, anti-inflammatory, and antitumor properties (Henriques et al., 2004; Shirwaikar et al., 2006; de Sousa et al., 2008), while flavonoids have antioxidant, anti-inflammatory and anticancer activities (Ogunleye and Ibitoye, 2003). Tannins also act as anti-inflammatory and anticancer agents (Mota *et al.,* 1985; Ruch *et al.,* 1989) and saponins maintain blood cholesterol level with antimicrobial properties (Cheeke, 2005). Several flavonoid compounds have previously been isolated from Millettia species including *M. zechania.* Astragalin, quercitrin, isoquercitrin, 3-hydroxy-4'-methoxyflavone and 3-0-α-L-rhamnosekampferol (Parvez and Ogbeide, 1990) have been isolated from the aerial parts of *M. zechania*. The flowers have also seen the isolation of 7-O-β-D-glucoside-8-hydroxyquercetin and 3-methyletherquercetin (Ogbeide and Parvez, 1992). Anthocyanins from the aerial parts of *M. zechiana* including cyanin, 3, 5-di-O-β-D-glucosidemalvidin and 3-0-α-L-rhamnosepelargonidin (Parvez and Ogbeide, 1990) have also been isolated. These indeed confirmed the characterization of flavonoid which gave higher amounts in the aerial parts of the two plants, with the roots (MCR = 0.44 mg RU/g, MZR = 0.91 mg RU/g) having the least amounts, Table 2.

In vitro antibacterial activities of extracts

To determine the antibacterial activity of the extracts, the bacteria species were challenged with the crude extracts from *M. chrysophylla* and *M. zechiana* within the concentration range of 0 to 400 µg/mL. Three (3) out of the eight (8) extracts tested; MCS, MCL and MZT showed significant inhibition with IC_{50} values below 10 µg/mL against five (5) of the bacteria; *S. aureus, B. cereus, S. epidermidis, E. coli* and *S. enteritidis* compared to the standard drug Ampicillin (0.17 – 2.82 µg/mL), Table 3.

Table 3: IC_{50} (µg/mL) of *M. zechiana* and *M. chrysophylla* ethanol extracts against ATCC and MRSA bacteria strains

Extracts	*E. coli*	*P. aeruginosa*	*S. paratyphi B*	*S. enteritidis*	*S. flexneri*	*S. aureus*	*B. cereus*	*S. epidermidis*
MCS	3.90	>100	>100	5.11	>100	>100	5.33	8.79
MCR	24.47	>100	>100	>100	>100	84.86	18.47	>100
MCL	>100	>100	>100	>100	>100	9.50	>100	86.72
MZS	>100	>100	>100	>100	>100	>100	>100	>100
MZR	>100	25.08	>100	>100	>100	>100	>100	>100
MZL	>100	54.54	>100	>100	>100	24.04	30.73	49.00
MZF	43.07	59.46	>100	>100	>100	>100	62.69	>100
MZT	>100	>100	>100	>100	>100	>100	>100	8.61
Ampicillin	2.30	2.82	2.68	2.73	1.59	0.17	0.26	0.36

MCS: Ethanol extract of *Millettia chrysophylla* stem bark, MCL: Ethanol extract of *M. chrysophylla* leaf, MCR: Ethanol extract of *M. chrysophylla* root, MZS: Ethanol extract of *Millettia zechiana* stem bark, MZL: Ethanol extract of *M. zechiana* leaf, MZR: Ethanol extract of *M. zechiana* root, MZT: Ethanol extract of *M. zechiana* twig, MZF: Ethanol extract of *M. zechiana* flower.

Interestingly, the stem bark of *M. chrysophylla* (MCS) showed strong and broad activity against three Gram-negative and one Gram-positive bacteria: *E. coli* (3.90 µg/mL), *S. enteritidis* (5.11 µg/mL), *S. epidermidis* (8.79 µg/mL) and *B. cereus* (5.33 µg/mL) out of the eight strains. The leaves of the plant (MCL) showed narrow activity against *S. aureus* (9.50 µg/mL) only (Table 3). Among the *M. zechiana* (MZ) extracts, the twigs (MZT) indicated significantly high activity against *S. epidermidis* (8.61 µg/mL) only, while the leaves (MZL) showed broad but moderate activity against one Gram-negative; *P. aeruginosa* (54.54 µg/mL) and three Gram-positive; *S. aureus* (24.04 µg/mL), *B. cereus* (30.73 µg/mL) and *S. epidermidis* (49.00 µg/mL) bacteria. Moderate activity (IC_{50}, 10 – 25 µg/mL) was also recorded for the MCR extracts against *E. coli* and *B. cereus* and MZR against *P. aeruginosa* (25.08 µg/mL). MZF also showed moderate activity against *E. coli* (43.07 µg/mL), *P. aeruginosa* (59.46 µg/mL) and *B. cereus* (62.69 µg/mL). MZS extract did not show activity against any of the tested bacteria (Table 3). The results suggested that the ethanol extract of the leaves, stems and roots of *M. chrysophylla* act as better antibacterial agents compared to extracts of *M. zechiana*. None of the extracts was active against *S. paratyphi B* and *S. flexneri*.

To determine the inhibitory concentration (bacteriostatic) and bactericidal activity of the extracts against MRSA strains, 28 MRSA isolates from previous studies (Egyir *et al.,* 2015) were tested with varying concentrations of *M. chrysophylla* and *M. zechiana* extracts. One MRSA isolate, MR4, originating from wound infection was susceptible to the leaves of both plant extracts (MCL and MZL) at IC_{50} levels of 86.45 and 93.88 µg/mL respectively (Table 4). The bacteria isolate MR21 also originating from wound infection was also susceptible to the leaves of *M. chrysophylla* (MCL) with a better IC_{50} value of 72.13 µg/mL compared with isolate MR4. Extracts from roots of *M. zechiana* (MZR) and leaves of *M. chrysophylla* (MCL) showed activity against MR19 isolated from nasal swab with IC_{50}

values of 87.90 and 97.76 µg/mL respectively (Table 4).

Table 4: IC_{50} (µg/mL) of *M. zechiana* and *M. chrysophylla* ethanol extracts against Ghanaian MRSA isolates

MRSA Isolates	Origin	MCS	MCR	MCL	MZS	MZR	MZL	MZF	MZT	CIPRO
MR1	Unknown clinical infection	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR2	Eye infection	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR3	Eye infection	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR4	Wound infection	>100	>100	86.45	>100	>100	93.88	>100	>100	0.02
MR5	Nasal swab	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR6	Nasal swab	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR7	Wound	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR8	Blood	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR9	Wound	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR10	Blood	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR12	Wound	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR12	Wound	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR13	Unknown clinical infection	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR14	Blood	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR15	Wound infection	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR16	Blood	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR17	Nasal swab	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR18	Nasal swab	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR19	Nasal swab	>100	>100	97.76	>100	87.90	>100	>100	>100	0.02
MR20	Nasal swab	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR21	Wound infection	>100	>100	72.13	>100	>100	>100	>100	>100	0.02
MR22	Wound	>100	>100	>100	>100	>100	>100	>100	>100	0.02

MR23	Soft tissue infection	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR24	Nasal swab	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR25	Wound infection	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR26	Urinary tract infection	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR27	Nasal swab	>100	>100	>100	>100	>100	>100	>100	>100	0.02
MR28	Unknown clinical infection	>100	>100	>100	>100	>100	>100	>100	>100	0.02

MRSA: Methicillin-resistant Staphylococcus aureus, CIPRO: Ciprofloxacin,

MCS: Ethanol extract of *Millettia chrysophylla* stem bark, MCL: Ethanol extract of *M. chrysophylla* leaf, MCR: Ethanol extract of *M. chrysophylla* root, MZS: Ethanol extract of *Millettia zechiana* stem bark, MZL: Ethanol extract of *M. zechiana* leaf, MZR: Ethanol extract of *M. zechiana* root, MZT: Ethanol extract of *M. zechiana* twig, MZF: Ethanol extract of *M. zechiana* flower.

The frequency of activity shown by *M. chrysophylla* leaves (MCL) against the MRSA isolates was higher than the other extracts confirming the activity against standard laboratory strains. In all the MRSA tests, ciprofloxacin was used as the positive control with IC_{50} value of 0.02 µg/mL. MIC and MBC tests were not carried out for the MRSA strains because from the inhibitory activity assay (Table 4), most of the extracts showed higher IC_{50} values than the concentration range within which they were tested, except for MCL (MR4 = 86.45 µg/mL; MR19 = 97.79 µg/mL and MR21 = 72.13 µg/mL), MZL (MR4 = 93.88 µg/mL) and MZR (MR19 = 87.90 µg/mL) extracts which even had IC_{50} values higher than 70 µg/mL.

In vitro* determination of MIC and MBC standard bacteria strains of ethanol extracts of *M. chrysophylla* and *M. zechiana

The ability of the extracts to inhibit bacterial growth based on their bacteriostatic (MIC) and bactericidal (MBC) properties were investigated *in vitro* within the concentration range of 0 to 400 µg/mL against the standard laboratory strains, Table 5. The stem bark extract of *M. chrysophylla* (MCS) showed significant MIC and MBC activities with respective values of 25 µg/mL and 100 µg/mL against *E. coli, S. enteritidis, B. cereus* and *S. epidermidis*, which corresponded to the inhibitory ability of the extract (Tables 3 and 4).

Table 5: MIC and MBC (µg/mL) of *M. chrysophylla* and *M. zechiana* ethanol extracts against ATCC bacteria strains

Extract Test	*E. coli*		*P. aeruginosa*		*S. paratyphi B*		*S. enteritidis*		*S. flexneri*		*S. aureus*		*B. cereus*		*S. epidermidis*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MCS	25	100	>100	-	>100	-	25	100	>100	-	>100	-	25	>100	25	100
MCR	>100	-	>100	-	>100	-	>100	–	>100	-	>100	-	50	>100	>100	-
MCL	>100	-	>100	-	>100	-	>100	-	>100	-	50	>100	>100	-	>100	-
MZS	>100	-	>100	-	>100	-	>100	-	>100	-	>100	-	>100	-	>100	-
MZR	>100	-	>100	-	>100	-	>100	-	>100	-	>100	-	>100	-	>100	-
MZL	>100	-	>100	-	>100	-	>100	-	>100	-	50	>100	100	>100	100	>100
MZF	>100	-	>100	-	>100	-	>100	-	>100	-	>100	-	100	>100	>100	-
MZT	50	>100	>100	-	>100	-	>100	-	>100	-	>100	-	>100	-	25	100
Ampicillin	>100	-	>100	-	>100	-	>100	-	>100	-	25	100	>100	-	>100	-

MIC: Minimal inhibitory concentration, MBC: Minimal bactericidal concentration,

MCS: Ethanol extract of *Millettia chrysophylla* stem bark, MCL: Ethanol extract of *M. chrysophylla* leaf, MCR: Ethanol extract of *M. chrysophylla* root, MZS: Ethanol extract of *Millettia zechiana* stem bark, MZL: Ethanol extract of *M. zechiana* leaf, MZR: Ethanol extract of *M. zechiana* root, MZT: Ethanol extract of *M. zechiana* twig, MZF: Ethanol extract of *M. zechiana* flower. – implies not tested for extracts with MIC > 100µg/mL

Similarly, the leaf (MCL) and root (MCR) extracts of *M. chrysophylla* each showed moderate MIC (50 µg/mL) and MBC (>100 µg/mL) activities against *S. aureus* and *B. cereus* respectively (Table 5). Twigs of *M. zechiana,* which had IC_{50} value of 8.61 µg/mL (Table 3) showed significant MIC and MBC activities against *S. epidermidis* at 25 µg/mL and 100 µg/mL, respectively. The extracts also showed moderate MIC activity against *E. coli* with IC_{50} of 50 µg/mL (Table 5). For the standard drug Ampicillin, all MIC and MBC values except for *S. aureus* (MIC: 25 µg/mL; MBC: 100 µg/mL), were above 100 µg/mL. With reference to Table 3, the half maximal inhibitory activity (IC_{50}) values (a quantitative determination) of the positive control drug Ampicillin, was higher against all the bacteria strains used. However, in Table 5, which presents the MIC and MBC of the extracts and Ampicillin, the MIC of Ampicillin against all the bacteria strains except *S. aureus* was >100 µg/mL. MIC, which is the Minimum Inhibitory Concentration is a qualitative determination that gives the least concentration of test extracts or compounds with no observable colour change in the colour of the Alamar blue dye, from blue resazurin to pink resorufin. Thus, the poor MIC activity of Ampicillin could be due to factors such as the bacteria strains may have developed some resistance to Ampicillin over time hence Ampicillin is not lethargic against them but still has bacteriostatic potency, or reduction of potency may have occurred due to factors such as storage, usage, and handling. Therefore, it can only be speculated that Ampicillin is not as potent as what it is expected of as a first line antibiotic and consequently the need to discover new antibiotics with higher potencies.

This is the first report of the antibacterial activity of both *M. chrysophylla* and *M. zechiana* even though some species of the genus have been studied for their antimicrobial properties.

Among the species of the genus Millettia, the ethanol extract of the leaf, the methanol extract of the root and the aqueous extracts of *Millettia abonensis* have been investigated for their antibacterial activities against some clinical isolates with MIC of 12.5 mg/mL on *Klebsiella pneumoniae*, 50.0 mg/mL on *Staphylococcus aureus* and 12.5 mg/mL on *Pseudomonas aeruginosa* (Onyegeme-Okerenta and Okafor, 2014). The crude extracts of *Millettia oblata* showed activity against three gentamycin sensitive *S. aureus*, *B. pumilus*, and *E. coli* with MIC of 613 µg/mL for each of the tested bacteria (Kamau, 2012). The leaf extracts of *Millettia peguensis* inhibited the growth of *B. cereus, S. aureus, Micrococcus mucilaginosus, Klebsiella pneumoniae, K. terrigena*, *Pseudomonas aeruginosa,* and *E. coli* with values comparable to the standard Ampicillin used (Packiyalakshmi, *et al.,* 2017). Bactericidal activity has been reported for the isoflavones *3R*(-)-isomillinol-B and *3R*(-)-vestitol from *M. racemosa* (Rao and Krupadanama, 1994).

In this current study, the stem bark, roots and leaves of *M. chrysophylla* indicated activities below 10 µg/mL against five bacteria strains; *E. coli* (3.90 µg/mL), *S. enteritidis* (5.11 µg/mL), *S. epidermidis* (8.79 µg/mL), *B. cereus* (5.33 µg/mL) and *S. aureus (9.50* µg/mL*)* with significant MIC and MBC activities below 100 µg/mL. Likewise, the twigs of *M. zechiana* (MZT) indicated significantly high activity against *S. epidermidis* (8.61 µg/mL) with MIC and MBC activities of 25 and 100 µg/mL respectively. Thus, the stem bark, roots and leaves of *M. chrysophylla* and the twigs of *M. zechiana* merit further isolation and identification of the active compounds. The high potencies obtained for the extracts involved in this study has given insights into the antibacterial potential of the plants which could lead to the discovery of compounds that can be developed into antibiotics.

Gas Chromatography-Mass Spectrometry profile of extracts

Gas Chromatography-Mass Spectrometry profiles of the ethanol extracts of both plants were obtained. Majority of the phytochemical classes identified included fatty acids and fatty acid esters. Results of the retention times, molecular formula, molecular mass, and percentage content of individual compounds obtained based on the GC retention times and comparison of their mass spectra with NIST library have been tabulated in Tables 6 and 7. The GC-MS analysis of all five extracts of *M. zechiana* identified 26 different compounds. The leaf ethanol extract of *M. zechiana* (MZ), Table 6, identified (*Z*)-18-octadec-9-enolide (24.46%), *n*-decanoic acid (14.37%), decanoic acid, ethyl ester (12.13%) and eugenol (10.53%) as the major components. *n*-Decanoic acid (25.24%), (*Z*, *Z*) 9,12-octadecadien-1-ol (11.42%) and 15-hydroxypentadecanoic acid (10.76%) constituted the major components from the stems while those from the roots were (*Z*, *Z)*-9,12-octadecadienoic acid (22.20%), *n*-hexadecanoic acid (13.83%) and dimethylsulfoxonium formylmethylide (12.18%). The flowers indicated major components as *n*-decanoic acid (27.88%), (*Z*, *Z)*-9,12-octadecadienoic acid (27.02%) and 15-hydroxypentadecanoic acid (9.35%). In addition, there were 30 major identified compounds from the three extracts (MCL, MCS, MCR) of *M. chrysophylla* (Table 7). The most abundant compounds were 17-octadecenoic acid (39.46%) from the leaves, 17-octadecynoic acid (27.90%) from the stem and *n*-hexadecanoic acid (20.87%) from the roots. This is the first report of the phytoconstituents of *M. chrysophylla*.

Table 6: The major chemical constituents of the ethanol extracts of leaf, stem, root, flower, and twig of *Millettia zechiana* (MZ) from GC-MS data

Extract	RT	Compound name	Formula	M.W	% Content
Leaf	7.903	Eugenol	$C_{10}H_{12}O_2$	164.20	10.53
	21.956	n-Decanoic acid	$C_{10}H_{20}O_2$	172.26	14.37
	22.283	Decanoic acid, ethyl ester	$C_{12}H_{24}O_2$	200.32	12.13
	25.081	Phytol	$C_{20}H_{40}O$	128.17	4.03
	25.928	(Z)-18-Octadec-9-enolide	$C_{18}H_{32}O_2$	280.40	24.46
	25.982	cis-Vaccenic acid	$C_{18}H_{34}O_2$	282.46	6.30
	26.076	Ethyl Oleate	$C_{20}H_{38}O_2$	310.50	9.35
	26.304	15-Hydroxypentadecanoic acid	$C_{15}H_{30}O_3$	258.40	7.00
	26.507	Nonanoic acid, 5-methyl-, ethyl ester	$C_{12}H_{24}O_2$	200.32	7.15
	33.994	Squalene	$C_{30}H_{50}$	410.73	1.24
Stems	21.699	n-Decanoic acid	$C_{10}H_{20}O_2$	172.26	25.24
(MZS)	22.125	Decanoic acid, ethyl ester	$C_{12}H_{24}O_2$	200.32	4.02
	25.557	9,12-Octadecadien-1-ol, (Z, Z)	$C_{18}H_{34}O$	266.50	11.42
	25.661	cis-Vaccenic acid	$C_{18}H_{34}O_2$	282.46	7.65

	25.829	1,3,12-Nonadecatriene	$C_{19}H_{34}$	262.50	3.87
	25.938	Oleic Acid	$C_{18}H_{34}O_2$	282.47	2.69
	26.116	15-Hydroxypentadecanoic acid	$C_{15}H_{30}O_3$	258.40	10.76
	26.414	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	312.50	2.48
Roots	4.125	Dimethylsulfoxonium formylmethylide	$C_4H_8O_2S$	120.17	12.18
(MZR)	4.432	Dimethyl sulfone	$C_2H_6O_2S$	94.13	6.69
	21.655	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	13.83
	25.715	9,12-Octadecadienoic acid (Z, Z)	$C_{18}H_{32}O_2$	280.40	22.2
	25.804	cis-Vaccenic acid	$C_{18}H_{34}O_2$	282.46	8.42
	26.176	Octadecanoic acid	$C_{18}H_{36}O_2$	284.48	9.37
	31.578	Docosanoic acid	$C_{22}H_{44}O_2$	340.58	4.67
	32.930	3(2H)-Benzofuranone, 6-methoxy-2-[(4 – methoxyphenyl)methylene]-, E	$C_{17}H_{14}O_4$	282.29	2.22
	38.293	Dihydrofuro[2,3 – e] phenalene, 6-methoxy-8 – oxo-2,3,3,10-tetramethyl-4,5,7-trihydroxy	$C_{20}H_{20}O_6$	356.38	4.14
	39.610	Cyclotrisiloxane, hexamethyl	$C_6H_{18}O_3Si_3$	222.00	5.33
Flowers	4.169	Dimethyl sulfone	$C_2H_6O_2S$	94.13	3.08
(MZF)	22.036	n-Decanoic acid	$C_{10}H_{20}O_2$	172.26	27.88
	22.199	Nonanoic acid, 5-methyl-, ethyl ester	$C_{12}H_{24}O_2$	200.32	6.04
	25.873	9,12-Octadecadienoic acid (Z,Z)	$C_{18}H_{32}O_2$	280.40	27.02
	25.967	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1 – (hydroxymethyl)ethyl ester	$C_{21}H_{38}O_4$	354.52	7.22
	26.007	Ethyl Oleate	$C_{20}H_{38}O_2$	310.50	3.20
	26.304	15-Hydroxypentadecanoic acid	$C_{15}H_{30}O_3$	258.40	9.35
	26.457	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	312.50	2.90
	29.131	7-Methyl-Z-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	268.40	1.27

M.M = Molecular mass

Table 7: The major identified chemical constituents of the ethanol extracts of leaf, stem, and roots of *Millettia chrysophylla* (MC) from GC-MS data

Extract	RT	Compound name	Formula	M.M	% Content
Leaves (MCL)	18.144 22.45 22.63 25.440	Oxirane hexadecyl- n-Decanoic acid nonanoic acid, 2,4-dimethl-, methyl ester Phytol	$C_{18}H_{36}O$ $C_{10}H_{20}O_2$ $C_{12}H_{24}O_2$ $C_{20}H_{40}O$	268.48 172.26 200.32 128.17	1.32 17.06 4.47 3.36
	26.375	17-Octadecenoic acid	$C_{18}H_{32}O_2$	282.46	39.46
	26.845	Formamide, N-(4-[2-(1,1-dimethylethyl)-5-oxo-1,3-dioxolan-4-yl]butyl)	$C_{12}H_{21}NO_4$	278.50	16.63
	29.378 34.292 35.033 42.814	Cyclopentadecanone, 2-hydroxy Squalene Carbonic acid, decyl undecyl ester (-)-Isolongifolol, methyl ether	$C_{15}H_{28}O_2$ $C_{30}H_{50}$ $C_{22}H_{44}O_3$ $C_{16}H_{28}O$	240.38 410.73 356.60 236.39	1.81 6.10 1.33 1.06
Roots	4.099	Dimethyl Sulfoxide	C_2H_6OS	78.13	17.46
(MCR)	4.417	Dimethyl sulfone	$C_2H_6O_2S$	94.16	13.29
	21.566	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	20.87
	25.497	9,12-Octadecadienoic acid (Z, Z)-	$C_{18}H_{32}O_2$	280.40	9.87
	25.616	Oleic Acid	$C_{18}H_{34}O_2$	282.47	10.23
	26.027	Octadecanoic acid	$C_{18}H_{36}O_2$	284.48	7.56
	32.089	4,6-Diamino-3-[p-methoxyphenyl]-1-methylpyrazolo[3,4-d] pyrimidine	$C_{13}H_{14}N_6O$	270.29	13.40
	32.92	(*E*)-3(2H)-Benzofuranone, 6-methoxy-2-[(4-methoxyphenyl)methylene]	$C_{17}H_{14}O_4$	282.29	7.32
Stems MCS	10.217	Bicyclo[5.2.0]nonane, 4 – methylene-2,8,8-trimethyl-2 – vinyl	$C_{15}H_{24}$	204.35	1.52
	18.231	Cyclopentadecanone, 2 – hydroxy	$C_{15}H_{28}O_2$	240.38	1.33
	18.701	Undecanoic acid, 11 – mercapto	$C_{11}H_{22}O_2S$	218.36	1.55
	21.996	n-Decanoic acid	$C_{10}H_{20}O_2$	172.26	22.27

	22.514	10-Bromodecanoic acid, ethyl ester	$C_{12}H_{23}BrO_2$	279.21	1.51
	24.205	n-Decanoic acid	$C_{10}H_{20}O_2$	172.26	1.71
	25.968	17-Octadecynoic acid	$C_{18}H_{32}O_2$	280.45	27.9
	26.332	10-Hydroxydecanoic acid	$C_{10}H_{20}O_3$	188.26	7.76
	32.286	Normorphine	$C_{16}H_{17}NO_3$	271.31	1.75
	35.721	cis-1-Chloro-9-octadecene	$C_{18}H_{35}Cl$	286.92	7.49
	41.797	(-)-Isolongifolol, methyl ether	$C_{16}H_{28}O$	236.39	1.94
	42.858	Longifolenaldehyde	$C_{15}H_{24}O$	220.35	10.73
	44. 171	Cyclobuta[1,2:3,4] dicycloocte ne-1,7(2H,6bH)-dione, dodecahydro-, (6a. alpha.,6b.alpha.,12a.alph a.,12b.beta.)-	$C_{16}H_{24}O_2$	248.35	1.26

M.M = Molecular mass

The MCS extract, which showed very high and broad potency contained *n*-decanoic acid (22.26%) and 17-octadecynoic acid (27.9%) as the highest components. *n*-Decanoic acid which also occurred in the leaves inhibited *Streptococcus gordoni, S. sanguinis and S. mutans* (Huang *et al*., 2011) in previous studies. Significant amount of 9,12-octadecadienoic acid (Z, Z) (10.87%), oleic acid (10.23%) and octadecanoic acid (7.56 %) which were obtained from the roots of *M. chrysophylla* have been showed to have antibacterial activities (Agoramoorthy *et al*., 2007; Gehan *et al.,* 2009; Awa *et al.,* 2012; Ukil, *et al.,* 2016). The most active MZ extract was the MZL, notable with the major constituents as (*Z*)-18-octadec-9-enolide (24.46%), *n*-decanoic acid (14.37%), decanoic acid ethyl ester (12.13%) and eugenol (10.53%). (*Z*)-18-Octadec-9-enolide which was significantly present in *Imperata cylindrica* plant was active against the bacteria *P. aeruginosa, K. pneumoniae* and *B. subtilis* (Lalthanpuii, *et al.,* 2019). Several Gram-negative and Gram-positive bacteria such as *E. coli*, *P. vulgaris*, *S. typhi*, *P. fluorescens, S. aureus, L. plantarum, B. subtilis, S. epidermidis, S. pneumoniae, B. bronchiseptica, B. cereus* and *S. mutans* have been studied to be susceptible to eugenol (Mak *et al.,* 2019). Most antibacterial activities of compounds including fatty acids are due to the presence of their free hydroxyl groups (Laekeman *et al*., 1990) via different mechanisms of action against the bacteria. While some antibacterial compounds may act as inhibitors of cell wall biosynthesis (Olasupo *et al.,* 2004), protein biosynthesis (Yoon *et al.,* 2018) or membrane functions (Devi *et al.,* 2013; Biasi-Garbin *et al.,* 2015), others inhibit nucleic acid synthesis (Lee and Jin, 2008), metabolic pathways (Devi et al., 2013; Galbraith and Miller, 1973) and ATP Synthase (Ali *et al.,* 2005).

CONCLUSION

This finding provided the first information on the phytochemical profile of *M. chrysophylla* and the antibacterial potential of *M. chrysophylla and M. zechiana*. Since *M. chrysophylla* extracts showed very good antibacterial activity (IC_{50} <10.0 µg/mL) against five bacteria species, and the leaf extract inhibited three of the MRSA isolates gave credence that *M. chrysophylla* was a

better potential antibacterial agent than *M. zechiana*. The study provided the scientific basis for the folkloric use of *M. zechiana* and the evidence that *M. chrysophylla* can also be used folklorically to treat infectious diseases since there were no documented medicinal use of the plant in the African pharmacopoeia. Additionally, this study provided baseline information that could support the development of chemotherapy suitable for the African sub-region.

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DECLARATION OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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