Bioactive constituents from *Malvastrum coromandelianum* (L.) Garcke leaf extracts

Mutalib A. Aderogba[a,b], Balungile Madikizela[b], and Lyndy J. McGaw[b,*]

[a]Department of Chemistry, Obafemi Awolowo University, Ile-Ife, Nigeria.

[b]Phytomedicine Programme, Department of Paraclinical Sciences, University of Pretoria, Private Bag X01, Onderstepoort 0110, South Africa.

*Corresponding Author
E-mail:lyndy.mcgaw@up.ac.za

Highlights

• *Malvastrum coromandelianum* is used to treat infectious diseases and inflammatory conditions.

• Two compounds were isolated for the first time from the 80% methanol leaf extract.

• Apigenin-7-*O*-*β*-6″(*p*-coumaroyl)-glucopyranoside and apigenin-8-C-glucopyranoside (vitexin) were identified.

• Compounds had strong antimycobacterial and anti-inflammatory activity.

• Bioactive compounds were not cytotoxic to Vero cells.

Abstract

Malvastrum coromandelianum leaf extracts are used to treat infectious diseases and various inflammatory conditions in folk medicine. This study was designed to isolate its bioactive secondary metabolites and determine their potential bioactivity. The crude leaf extract obtained from 80% methanol was successively partitioned with n-hexane, dichloromethane, ethyl acetate, n-butanol and water.

The 80% methanol extract, four solvent fractions and the two compounds isolated from selected fractions were assayed for antimycobacterial activity (against *Mycobacterium aurum*, *M. fortuitum* and *M. smegmatis*), and anti-inflammatory activity (against the 15-lipooxygenase enzyme). The isolated compounds were further tested for cytotoxicity against Vero monkey cells. Structures were elucidated using Nuclear Magnetic Resonance spectroscopic methods.

The ethyl acetate fraction, followed by the butanol fraction, showed the highest antimycobacterial and anti-inflammatory activities, and their fractionation afforded apigenin-7-*O*-*β*-6"(*p*-coumaroyl)-glucopyranoside (**1**) and apigenin-8-C-glucopyranoside, vitexin (**2**). These compounds are reported from *M. coromandelianum* for the first time. Both compounds showed interesting antimycobacterial activity against at least one *Mycobacterium* strain with MIC values ranging from 0.03-0.25 mg/ml. Anti-inflammatory activity shown by apigenin-7-*O*-*β*-6"(*p*-coumaroyl)-glucopyranoside and vitexin was noteworthy with IC_{50} values of 6.67 and 8.02 µg/ml respectively. Bioactive compounds were not cytotoxic towards Vero cells with the lowest LC_{50} value=105.40 µg/ml.

This study provides support for the use of this plant to treat infectious diseases, and as an anti-inflammatory agent. The isolated compounds may serve as possible leads for antimycobacterial or anti-inflammatory products.

Keywords: *Malvastrum coromandelianum*; apigenin glycosides; antimycobacterial, anti-inflammatory, toxicity

1. Introduction

Malvastrum coromandelianum (L.) Garcke is an invasive weed, commonly known as false mallow, belonging to the Malvaceae family (Saxena and Rao, 2018). The plant extracts are used as anti-inflammatory and analgesic remedies in folk medicine while the flowers are used to treat cough, chest and lung diseases (Devi and Kumar, 2018). In previous pharmacological studies, water extracts of *M. coromandelianum* exhibited antidiabetic, anti-inflammatory and analgesic activities. The extract did not demonstrate significant toxicity (Khonsung et al., 2006). The plant extracts also had antinoceceptive and antibacterial activities against methicillin resistant *Staphylococcus aureus* (Sanghai et al., 2013, Devi and Kumar, 2018). The chloroform extract of the leaf also had antifungal activity (Mushtaq et al., 2012).

Phytoconstituents of members of the Malvaceae family are characterized by flavonoid glycosides, especially flavonols and flavones. Flavones with additional oxygenation at C-8 and C-5′ are common secondary metabolites from this family indicating their chemotaxonomic importance. The presence of diverse flavonoids among the familys could explain the broad biological activities and the various uses of their extracts in traditional medicine for various disease conditions (Vadivel et al., 2016).

Previous phytochemical studies revealed the presence of quercetrin and a mixture of cis- and trans-tiliroside as the antioxidant constituents of the plant (Devi and Kumar, 2018). High performance thin layer chromatography analysis of the petroleum ether extract revealed the presence of β-sitosterol (Sanghai et al., 2013). Gas chromatography-mass spectrometry analysis of the aqueous methanol leaf extract indicated the presence of guanosine and 9,12,15-octadecatrienoic acid as the major phytoconstituents (Saxena and Rao, 2018).

In our quest for novel bioactive compounds that are safe and effective in the management of infectious diseases and inflammatory conditions, we investigated the leaf extracts of *M. coromandelianum* that are used in related conditions in folk medicine for its bioactive constituents.

2. Materials and methods

2.1 General

All solvents used in this study were laboratory grade reagents. Column chromatography was carried out using either silica gel (60-230 mesh, Merck, Darmstadt, Germany) or Sephadex LH-20. All thin layer chromatography (TLC) analyses of samples were performed at room temperature using pre-coated plates (Merck, silica gel 60 F_{254}). Detection of spots on TLC plates was performed by viewing under ultraviolet light using a Camag Universal UV lamp TL-900 at 254 nm. Nuclear Magnetic Resonance (NMR) ^{1}H (400 MHz) and ^{13}C (100 MHz) spectra were recorded at the Department of Chemistry, University of Pretoria, South Africa on a Bruker Avance III 400 MHz NMR Spectrometer (Bruker, Germany).

2.2 Plant material

The leaves of *M. coromandelianum* were collected in August 2017 at Oduduwa Hall, Obafemi Awolowo University (O.A.U), Ile-Ife, Nigeria. The plant was authenticated at the Department of Botany, O. A. U., Ile-Ife, Nigeria, by Prof Augustine O. Isichei. Voucher specimen (IFE-17699) was deposited at the Ife Herbarium, in the Department of Botany, O.A.U, Ile-Ife. The leaves were air dried at room temperature for two weeks in the laboratory and ground to powder. The powdered material was stored in a dark container until use.

2.3 Extraction

Powdered material (1.1 kg) was extracted with 80% methanol (10 L) with occasional shaking for 24 h. The extract was filtered and the filtrate collected was concentrated *in vacuo* using a rotary evaporator at 45 ℃. This was repeated three times for the powdered material. This afforded the crude extract of the plant (52 g).

2.3.1 Solvent partitioning of the crude extracts

The crude extract obtained (50 g) was suspended in distilled water in a separatory funnel (5 L) and successively partitioned with *n*-hexane, dichloromethane (DCM), ethyl acetate, butanol, and water respectively. This gave five solvent fractions of *M. coromandelianum* leaf extracts.

2.4 Bioassays

2.4.1 Antimycobacterial assay

Non-pathogenic *Mycobacterium* strains, two from the American Type Culture Collection (ATCC), and one from the National Collection of Type Cultures (NCTC); *M. fortuitum* (ATCC 6841) and *M. smegmatis* (ATCC 1441), and *M. aurum* (NCTC 10437) were used to determine the antimycobacterial activity of the crude extract, fractions, and compounds. The *Mycobacterium* cultures were grown in Middlebrook 7H9 broth supplemented with 10% (v/v) oleic acid, albumin, dextrose and catalase (OADC) and glycerol. All the cultures were maintained on Middlebrook agar supplemented with glycerol and OADC supplement. *M. fortuitum* and *M. smegmatis* were incubated for 24-48 h, while *M. aurum* was incubated for 3 days at 37ºC. All the test inocula were adjusted to McFarland standard 1, equivalent to 3.0×10^8, and diluted to a final density of 5×10^5 CFU/ml using Middlebrook 7H9 broth supplemented with glycerol and OADC . A microdilution method in a 96 well microtitre plate was used to determine the MIC values of the methanol extract,

fractions, and compounds from the plant following a method by Jadaun et al. (2007) with slight modifications. Briefly, test samples were prepared in 10% dimethylsulfoxide (DMSO) and water (for water extract), serially diluted twofold (100 µl) down the wells of a 96 well microtitre plate with OADC supplemented Middlebrook 7H9 broth. Positive controls, isoniazid, streptomycin and rifampicin, and negative controls which included 10% DMSO, water, inoculum and OADC supplemented Middlebrook 7H9 broth were used in this assay. *Mycobacterium* cultures (100 µl) were added to all the wells, except for the broth only wells. The microtitre plates were sealed with parafilm and incubated at 37°C for 24-48 h (*M. smegmatis* and *M. fortuitum*), and 3 days for *M. aurum*. Concentrations were tested in triplicate and the experiments were repeated thrice. MIC values were determined by adding 40 µl of 0.2 mg/ml p-iodonitro-tetrazolium chloride (INT) solution. After the addition of INT, when the colour became visible in the untreated control wells, MIC values were read as the concentrations where a marked reduction in colour formation corresponding to inhibition of mycobacterial growth was noted.

2.4.2 Inhibition of 15-lipoxygenase (15-LOX) *enzyme*

A 96 well microtitre plate was used to determine the inhibitory activity of samples from *M. coromandelianum* against the 15-LOX enzyme using a method described by Pinto et al. (2007) with some modifications. An aliquot of 15-LOX enzyme (40 µl) was preincubated with 20 µl test sample (starting from 1 mg/ml) at room temperature for 5 min. Then the reaction was initiated by adding 40 µl of linoleic acid (final concentration, 140 mM) in borate buffer (50 mM, pH 7.4) in all the wells except the blanks (background), and the mixture was incubated at 25ºC in the dark for 20 min. Quercetin (1 mg/ml) was used as a positive control, while 15-LOX solution and buffer wells were used as a negative control. After the incubation period, linoleic acid (40 µl) was added to the blanks and the reaction was terminated by adding freshly prepared FOX reagent (40 µl) comprising of xylenol orange (100 mM), sulphuric acid (30 mM), iron (II) sulfate (100 mM) in methanol/water (9:1) in all the wells. A microplate reader at 560 nm was used to measure the complex Fe^{3+}/xylenol orange formation. The 15-LOX inhibitory activity was determined by calculating the percentage inhibition of hydroperoxide production using the following formula:

$$\%\text{inhibition} = \left\{\frac{(Acontrol - Ablank) - (Asample - Ablank)}{(Acontrol - Ablank)}\right\} X\,100$$

Where Acontrol= absorbance of control, Ablank= absorbance of blank, Asample= absorbance of sample. The results were expressed as IC_{50}, which is the concentration of a test sample that resulted in 50% inhibition of 15-LOX enzyme plotted on a graph.

2.4.3 The MTT cytotoxicity assay

The [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay was used to determine the cytotoxicity of compounds following a method by Mosmann (1983). The cytotoxicity of compounds was evaluated against African green monkey kidney (Vero) cells obtained from the Department of Veterinary Tropical Diseases (University of Pretoria), South Africa. Briefly, cells cultured in sterile minimal essential medium (MEM) supplemented with 0.1% gentamicin and 5% foetal calf serum (FCS) in a 75 cm^2 flask, incubated at 37 °C in 5% CO_2 for a week were harvested using 3 ml of trypsin-EDTA, centrifuged for 5 min at 200×g, counted using a Neubauer haemocytometer, and resuspended in MEM to a concentration of 1×10^5 cells per well. A 96 well flat bottomed sterile microtitre plate was used for the study. Cells (100 µl) were added to all the wells of columns 2-11 at a final concentration of 10 000 cells per well, and 200 µl of MEM was added to wells of columns 1 and 12. The microtitre plates were incubated in 5% CO_2 at 37°C overnight. Test samples were added to wells (100 µl) in quadruplicate. A positive control, doxorubicin hydrochloride, and negative controls (untreated cells, MEM, and DMSO) were also included. The microtitre plates were incubated at 37°C in 5% CO_2 for 48 h. After the incubation period, the test samples were aspirated from the wells, and the cells were washed with phosphate buffered saline (PBS). Fresh MEM (200 µl) and 30 µl of 5 mg/ml MTT dissolved in PBS were added to all the wells and the microtitre plates were incubated for 4 h. Following 4 h incubation at 37°C in 5% CO_2, the medium was removed and DMSO (50 µl) was added to each well, before the plates were shaken gently to dissolve the crystals. The amount of MTT reduction was measured at a wavelength of 570 nm (reference wavelength of 630 nm) using a microplate reader, and wells in columns 1 and 12 containing MEM only were used as blanks. The results were recorded and expressed as percentage of the control wells and LC_{50} values. The LC_{50} values were calculated as the concentration of the compound that resulted in 50% reduction of absorbance compared to untreated cells. From the MIC of the compounds against tested *Mycobacterium* strains and LC_{50} values, the selectivity index values for each sample were calculated using the following formula (Shai et al., 2008):

$$SI = \frac{LC50}{MIC}$$

2.5 Isolation of bioactive constituents

2.5.1. Compounds 1 and 2

Fractionation of the ethyl acetate fraction (3.3 g) was carried out on column chromatography using silica gel (60-200 mesh) as a stationary phase. Different solvent gradients starting with n-hexane: ethyl acetate (3:2) followed with an increasing gradient of ethyl acetate in n-hexane up to 100% were used. This was followed with an increasing gradient of methanol in ethyl acetate up to 50%. The eluant was collected using test tubes (15 ml each). A total of 101 test tubes were collected and analysed on TLC (Thin Layer Chromatography) plates developed with DCM/methanol (9:1 and 4:1 respectively). Four column fractions were obtained: A_1-A_4. Fraction A_3 gave a single spot on the TLC plate and this yielded compound **1** (50 mg). Fractionation of A_4 (1.2 g) on a silica gel column using different solvent gradients of DCM and methanol starting with 9:1 solvent mixture afforded one major subfraction that was purified on Sephadex LH-20 column using DCM:methanol (4:1). This yielded compound **2** (42 mg).

2.5.2 Compound 2

Open column fractionation of the butanol fraction (8.7 g) using silica gel (60-200 mesh) as a stationary phase and eluted with n-hexane: ethyl acetate (1:1) ratio followed with an increasing gradient of ethyl acetate up to 100%. The elution was continued with ethyl acetate: methanol mixture, starting with 10% methanol and followed with an increasing gradient of methanol up to 60%. A total of one hundred test tubes were collected and analysed on TLC plates using dichloromethane/methanol (8.5:1.5 and 7:3 respectively). Three column fractions were obtained B_1-B_3. Purification of fraction B_2 (0.7 g) was carried out on a Sephadex LH-20 column using DCM/methanol (7.5:2.5) followed with an increasing gradient of methanol up to 40%. Analysis of the test tube fractions collected on TLC plates using dichloromethane/methanol (7.5:2.5) gave more of compound **2** (49 mg).

3. Results and discussion

Phytochemical investigation of the ethyl acetate and butanol fractions of an aqueous methanol crude extract from *Malvastrum coromandelianum* afforded two compounds.

3.1 Structure elucidation of isolated compounds

*3.1.1 Compound **1***

In the ^{1}H NMR spectrum of compound **1**, the splitting patterns of the proton signals confirmed the presence of two AA'BB' units (1,4-disubstituted benzene ring). This was indicated by two doublet of doublets. One was assigned as the ring B of a flavonoid unit (J = 8.8 Hz). The second (J = 8.8 Hz) was assigned as part of a *p*-coumaroyl group due to the presence of the trans-disubstituted double bond protons signals (J = 16.0 Hz). The spectrum also showed the presence of two meta related protons of the ring A (J = 2.4 Hz) of the flavonoid and a singlet assigned as H-3 proton signal. The flavonoid nucleus was identified as a flavone- apigenin. The anomeric proton (H-1") of the glucosyl appeared as a doublet (J = 7.6 Hz) which indicated beta configuration of the sugar. The ^{13}C NMR spectrum of this compound revealed the presence of 26 carbon signals. These were assigned as follows: six carbon signals to a glucopyranosyl group, thirteen carbon signals to the apigenin nucleus and the remaining seven signals were classified as the *p*-coumaroyl group signals. Compound **1** was determined to be apigenin-7-*O*-β-6"(*p*-coumaroyl)-glucopyranoside. This compound has recently been reported as an antioxidant agent with significant neuroprotective activity from *Clematis tangutica* (Maxim.) Korsh (Ranunculaceae). The spectral data are in agreement with the literature (Cail et al., 2016). A closely related analogue of compound (**1**), apigenin-3-*O*-β-6" (*p*-coumaroyl) glucopyranoside (tiliroside) has recently been reported from *Malvastrum coromandelianum* (Devi and Kumar, 2018). Compound **1** ^{1}H NMR spectrum (DMSO-d_6, 400 MHz), δ 12.96 (1H, s, 5-OH chelated), 7.94 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.50 (1H, d, J =16.0 Hz, H-3‴), 7.37 (2H, d, J = 8.8 Hz, H-5‴, 9‴), 6.93 (2H, d, J = 8.8 Hz, H-3′, 5′), 6.82 (1H, s, H-3), 6.81 (1H, d, J = 2.4 Hz, H-8), 6.67 (2H, d, J = 8.8 Hz, H-6‴, 8‴), 6.47 (1H d, J = 2.0 Hz, H-6), 6.34 (1H, d, J = 16.0 Hz, H-3‴), 5.17 (1H, d, J = 7.6 Hz, H-1″), 4.48-3.34 (m, H-2″ – H-6″). The ^{13}C NMR (DMSO-d_6, 100 MHz), δ: 162.8 (C-2), 103.1 (C-3), 182.1 (C-4), 157.0 (C-5), 99.5 (C-6), 164.4 (C-7), 94.8 (C-8), 161.5 (C-9), 105.5 (C-10), 121.1 (C-1′), 128.7 (C-2′), 116.1 (C-3′), 161.2 (C-4′), 116.1 (C-5′), 128.7 (C-6′), 99.5 (C-1″), 73.0 (C-2″), 76.3 (C-3″), 70.1 (C-4″), 73.9 (C-5″), 63.5 (C-6″), 166.6 (C-1‴), 113.8 (C-2‴), 145.1 (C-3‴), 125.0 (C-4‴), 130.2 (C-5‴), 115.8 (C-6‴), 159.8 (C-7‴), 115.8 (C-8‴), 130.2 (C-9‴), (Figure 1).

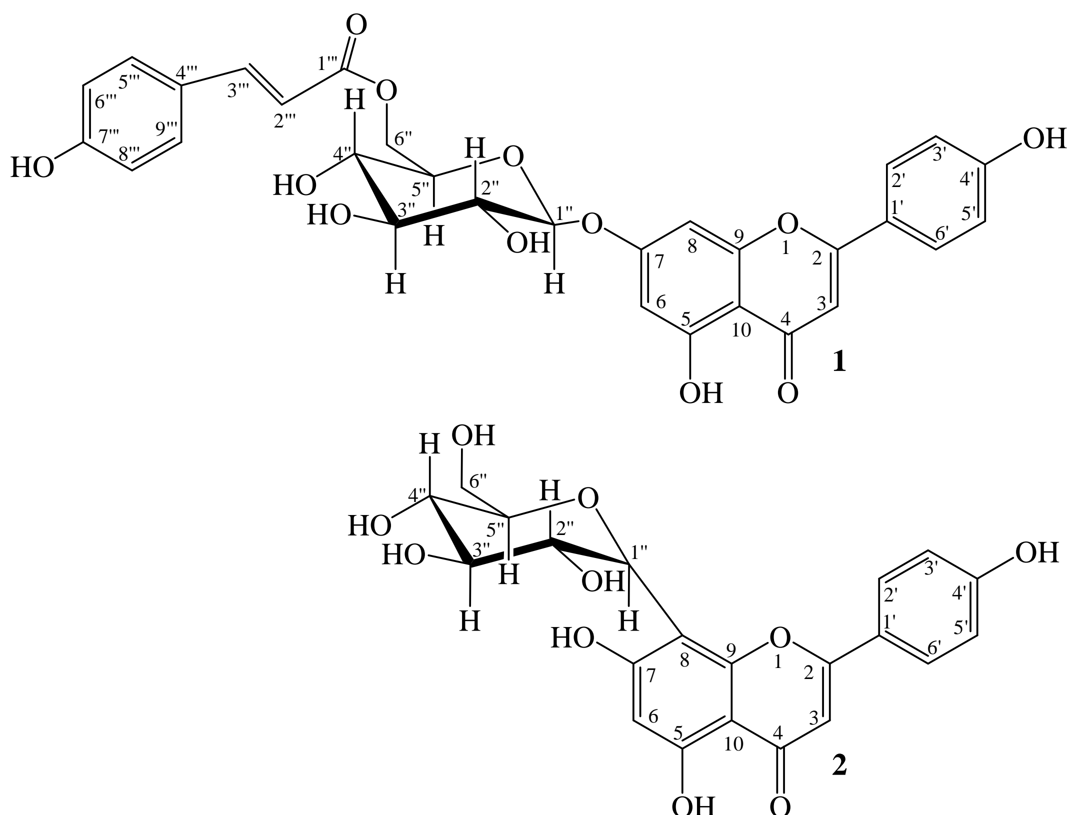

Figure 1: Bioactive compounds from *Malvastrum coromandelianum* leaf extract

*3.1.2. Compound **2***

The ^{1}H NMR (DMSO-d_6, 400 MHz) spectrum of compound **2** showed characteristic splitting pattern of a flavone-apigenin closely related to that of compound **1**. In addition, there was the presence of anomeric proton signal (1H d, J = 5.6 Hz, H-1''). Absence of meta related proton signals in the spectrum suggested apigenin-C-glucoside. This was confirmed and the position of the substitution was determined from the ^{13}C NMR spectrum data and comparison of the NMR data with the literature (Harbone and Mabry, 1982). Compound **2** was identified as apigenin-8-C-glucoside (vitexin) and the spectra data are in agreement with the literature. This compound had previously been reported from the Malvaceae family (Vadivel, 2016). The ^{1}H NMR spectrum (DMSO-d_6, 400 MHz), δ 13.17 (1H, s, 5-OH chelated), 8.02 (2H, d, J = 9.2 Hz, H-2′, 6′), 6.90 (2H, d, J = 8.4 Hz, H-3′, 5′), 6.78 (1H, s, H-3), 6.27 (1H, s, H-6), 5.07 (1H, d, J = 5.6 Hz, H-1″), 5.03-3.74 (glucosyl-H, m, H-2″ – H-6″). The ^{13}C NMR (DMSO-d_6, 100 MHz), δ: 164.1 (C-2), 102.5 (C-3), 182.2 (C-4), 160.5 (C-5), 98.2 (C-6), 162.7 (C-7), 104.1 (C-8), 156.1 (C-9), 104.7 (C-10),

121.7 (C-1′), 129.1 (C-2′), 116.0 (C-3′), 161.5 (C-4′), 116.0 (C-5′), 129.1 (C-6′), 73.5 (C-1″), 70.9 (C-2″), 78.7 (C-3″), 70.6 (C-4″), 81.9 (C-5″), 61.4 (C-6″), (Figure 1).

3.2 Biological assay results

3.2.1 Antimycobacterial results of M. coromandelianum

Nontuberculous mycobacteria are opportunistic pathogens that have potential to cause various diseases in multiple organs of both humans and animals. It has recently been highlighted that there is a rise in the prevalence of nontuberculous mycobacterial diseases, hence they are recognised as an emerging public health concern (Shen et al., 2018). Several strains of nontuberculous mycobacteria have developed drug resistance, for example a culture of *M. fortuitum* was reported by Shen et al. (2018) to be highly resistant to isoniazid, rifampin, ethambutol, clofazimine, ethionamide, and rifabutin in a drug susceptibility study. Therefore, research on antimycobacterial activity of plants against nontuberculous mycobacteria is important. The antimycobacterial results of crude extracts, fractions and compounds of the leaf methanol extract of *M. coromandelianum* are presented in Table 1. The criteria described in literature to determine good antimycobacterial activity for test samples (crude extract or compound) were followed to describe our findings. In this study a wide range of MIC values from 0.03 to >2.50 mg/ml against the strains tested was observed. The crude extract of *M. coromandelianum* showed moderate antimycobacterial activity with MIC values ranging from 0.31-0.63 mg/ml. There is no known previous report of antimycobacterial activity of *M. coromandelianum*. In a study by Sittiwet et al. (2008) it was reported that the water extract of this plant exhibited slight antibacterial activity against methicillin sensitive and methicillin resistant *Staphylococcus aureus* with MIC values ranging from 2.50-5.00 mg/ml. The ethyl acetate *M. coromandelianum* crude extract was reported to have antibacterial activity showing 15.66 mm inhibition zone against *S. aureus* (Silva et al., 2017). It is expected that isolated compounds should possess better antibacterial activity than the crude extract as the compound would be more concentrated, thus further fractionation of *M. coromandelianum* methanol extract was done in our study. Amongst the five fractions tested for antimycobacterial activity, the ethyl acetate fraction showed the best activity against all strains tested with MIC values ranging from 0.08-0.31 mg/ml, followed by butanol with MIC values ranging from 0.16-0.63 mg/ml. This led to selection of both ethyl acetate and butanol fractions of *M. coromandelianum* for isolation of compounds responsible for antimycobacterial activity observed

in our study. Overall, the best MIC value was observed with vitexin, with the lowest MIC value of 0.03 mg/ml against *M. fortuitum*, which was 21 times better than that of the crude extract. Activity of vitexin against *M. fortuitum* is very interesting as this *Mycobacterium* strain is reported to be the main rapidly growing mycobacterial species responsible for extrapulmonary diseases (Shen et al., 2018). Vitexin also showed some antimycobacterial activity against *M. smegmatis* and *M. aurum* with MIC values of 0.06 and 0.13 mg/ml respectively. Vitexin is an important flavonoid compound derived from several plant species. It has been reported to be of pharmacological importance possessing biological activities such as anticancer, antinociceptive, anti-inflammatory, antispasmodic, antihypertensive, and antioxidant, thus great attention has been given to the compound by the science community (Venturini et al., 2018). Antimycobacterial activity shown by apigenin-7-*O*-β-6''(*p*-coumaroyl)-glucopyranoside with MIC value=0.13 (against *M. fortuitum*) and 0.25 mg/ml (against both *M. smegmatis* and *M. aurum*) was not interesting. Based on our literature search, there were no known reports of the antibacterial potential of both the compounds isolated in this study. Further studies are needed to determine the antimycobacterial effect of vitexin against other strains of *Mycobacterium* including pathogenic species.

Table 1: Antimycobacterial and 15-LOX inhibitory activity of *Malvastrum coromandelianum* leaf extracts, fractions and compounds

Test substance	Extract/fraction	*Mycobacterium* strains			Inflammatory enzyme
		Mf	*Ms*	*Ma*	15-LOX
		MIC values mg/ml			IC_{50} (µg/ml)
M. coromandelianum	Crude extract (80% methanol)	0.63	0.31	0.63	77.52±1.31
	Hexane fraction	0.63	0.63	0.31	60.07±1.02
	DCM fraction	0.31	0.63	0.63	62.40±1.73
	EtOAc fraction	0.08	0.16	0.31	40.63±0.78
	Butanol fraction	0.16	0.63	0.31	56.74±0.41
	Water fraction	>2.50	>2.50	>2.50	308.51±1.18
Apigenin-7-*O*-β-6''(*p*-coumaroyl)-glucopyranoside	-	0.13	0.25	0.25	6.67±0.24
Apigenin-8-C-glucopyranoside	-	0.03	0.06	0.13	8.02±1.08
Isoniazid	-	0.16	0.31	0.31	-
Streptomycin	-	4.88×10^{-3}	1.00×10^{-2}	1.00×10^{-2}	-
Rifampicin	-	7.623×10^{-5}	3.05×10^{-4}	6.10×10^{-4}	-
Quercetin	-	-	-	-	16.08±1.44

DCM=dichloromethane, EtOAc= Ethyl acetate, *Mf=Mycobacterium fortuitum*, *Ms=Mycobacterium smegmatis*, *Ma=Mycobacterium aurum*, 15-LOX=15 lipoxygenase, -=not determined, MIC=minimum inhibitory concentration, IC_{50}= the concentration of the test samples that resulted in 50 % inhibition of 15-LOX enzyme.

3.2.2 Anti-inflammatory results

One of the most prominent uses of *M. coromandelianum* is as an anti-inflammatory. Hence, one of the objectives of our study was to determine the anti-inflammatory potential of this plant, which was achieved by using the 15-LOX inhibition model. The results of the anti-inflammatory activity of *M. coromandelianum* extracts are presented in Table 1 as IC_{50} values. The tested samples of *M. coromandelianum* had IC_{50} values ranging from 6.67-308.51 µg/ml. A study conducted by Khonsung et al. (2006) showed that *M. coromandelianum* crude extract (leaf water) had anti-inflammatory activity on carrageenin-induced hind-paw edema in rats, however in our study the same solvent extract showed anti-inflammatory activity that was not interesting against the 15-LOX enzyme. The 15-LOX inhibitory activity of *M. coromandelianum* leaf methanol extract was not interesting in this study with IC_{50} value=77.52 µg/ml, however it was fractionated using solvents of different polarities to determine if anti-inflammatory activity would improve. Although having weak 15-LOX inhibition, out of the five fractions tested, the ethyl acetate fraction followed by the butanol fraction showed better inhibition of the 15-LOX enzyme with IC_{50} values=40.63 and 56.74 µg/ml respectively. This was better than the activity of the crude extract, hexane, DCM, butanol, and water fractions. This led to isolation of compounds from the ethyl acetate and butanol fractions responsible for the anti-inflammatory activity observed. Apigenin-7-O-β-6''(p-coumaroyl)-glucopyranoside showed interesting anti-inflammatory activity with IC_{50} =6.67 µg/ml, and it was the best activity in this study. Nazemiyeh et al. (2007) reported that a fraction from *Stachys schtschegleevii* Sosn. containing acylated glycosyl flavonoids including apigenin-7-O-β-6''(p-coumaroyl)-glucopyranoside exhibited significant anti-inflammatory activity by reducing mean maximal oedema response from 85.00% in the control to 74.10% and 48% with 15 and 30 mg/kg, respectively. Abdallah et al. (2015) reported that apigenin-7-O-β-6''(p-coumaroyl)-glucopyranoside showed a significant decrease of IL-1β, IL-6, TNF-ά and PGE_2. Vitexin, a compound found in both the ethyl acetate and butanol fractions of *M. coromandelianum* showed interesting LOX inhibitory activity (IC_{50}=8.02 µg/ml) which was comparable to that of the positive control, quercetin. A study by Rosa et al. (2016) demonstrated the anti-inflammatory activity of vitexin and mechanism of action in *in vitro* and *in vivo* experimental models. Vitexin was reported to reduce TNF-α, IL-1β and NO in the peritoneal cavity of LPS challenged mice. In the same study, the compound was reported to reduce the release of IL-1β, TNF- ά, PGE2, NO and to increase the IL-10 release by LPS activated RAW 264.7 cells (Borghi et al., 2013; Rosa et al., 2016). The

mechanism of action of vitexin was associated with inactivation of important signalling pathways eg P38, ERK1/2, and JNK, which act on transcription factors for eliciting induction of inflammatory response (Rosa et al., 2016). More research on the anti-inflammatory potential of apigenin-7-*O*-*β*-6''(*p*-coumaroyl)-glucopyranoside is required, and the mechanism of action should be determined.

3.2.3 Cytotoxicity results

Apigenin-7-*O*-*β*-6''(*p*-coumaroyl)-glucopyranoside and vitexin were investigated for cytotoxicity against Vero monkey kidney cells, and the results are presented in Table 2 as LC_{50} and selectivity index values. Both compounds were not cytotoxic towards the Vero Monkey cells with LC_{50} values=105.40 (apigenin-7-*O*-*β*-6''(*p*-coumaroyl)-glucopyranoside) and 413.79 µg/ml (vitexin). Selectivity index values as high as 13.79 for vitexin demonstrated that the compound was more antimycobacterially active and less toxic towards the tested cells. Vitexin has been shown to be cytotoxic toward cancer cells, namely human breast, liver, cervix, and colon cancer cells (Ganesan and Xu, 2017). However, the compound was reported as non-cytotoxic against RAW 264.7 cells with LC_{50} value=200 µg/ml, supporting the current safety findings of this compound (Rosa et al., 2016). More toxicity studies of the compounds, which should include *in vivo* studies and genotoxicity, are required.

Table 2: Cytotoxicity activity and selectivity index values of the compounds from *Malvastrum coromandelianum* leaf extracts

Compound	LC_{50} values (µg/ml)	Selectivity Index values		
	Vero cells	*Mf*	*Ms*	*Ma*
Apigenin -7-O-β-6''(p-coumaroyl)-glucopyranoside)	105.40±1.02	0.81	0.42	0.42
Apigenin-8-C-glucopyranoside	413.79±0.67	13.79	6.90	3.18
Doxorubicin	7.00×10^{-3}±0.50	-	-	-

Mf=Mycobacterium fortuitum, *Ms=Mycobacterium smegmatis*, *Ma=Mycobacterium aurum*, LC_{50}= the concentration of the test samples that resulted in 50 % reduction of absorbance compared to untreated cells.

4. Conclusion

Two compounds were isolated from the leaf extracts of *M. coromandelianum* and characterized as apigenin-7-*O*-*β*-6''(*p*-coumaroyl)-glucopyranoside and apigenin-8-C-glucoside (vitexin). According to our knowledge, the compounds are reported from this plant species for the first time. Although the methanol extract of *M. coromandelianum* did not show interesting activities in the parameters tested, the compounds isolated from this plant showed interesting antimycobacterial

and anti-inflammatory activities, and were not cytotoxic towards Vero mammalian cells. Further studies on antimycobacterial activity against pathogenic strains and genotoxicity of both compounds, as well as anti-inflammatory activity of apigenin-7-*O*-*β*-6"(*p*-coumaroyl)-glucopyranoside are required.

Acknowledgements

Prof M.A. Aderogba is grateful to the University of Pretoria, South Africa for the award of Visiting Professorship Fellowship 2018 at the Department of Paraclinical Sciences, Phytomedicine Programme, Faculty of Veterinary Sciences. Dr Mamoalosi Selepe, Department of Chemistry, University of Pretoria conducted the Nuclear Magnetic Resonance (NMR) experiments of the isolated compounds. Dr Kirk Marat, Department of Chemistry, University of Manitoba provided the SpinWork NMR Software version 4.2.7 (2017).

Dr B. Madikizela is grateful to National Research Foundation (NRF) South Africa (SFP14062571657) for financial support.

The National Research Foundation (Grant number UID 105993) provided research funding to Prof L.J. McGaw.

References

Abdallah, H.M., Almowallad, F.M., Esmat, A., Shehata, I.A., Abdel-Sattar, E.A., 2015. Anti-inflammatory activity of flavonoids from *Chrozophora tinctorial*. Phytochemistry Letters 13 74-80.

Borghi, S.M., Carvalho, T.T., Staurengo-Ferrari, L., Pinge-Filho, P., Casagrande, R., Verri, W.A., 2013. Vitexin inhibits inflammatory pain in mice by targeting trpv1, oxidative stress, and cytokines. Journal of Natural Products 76, 1141-1149.

Cail, M., Ma, Y., Zhang, W., Wang, S., Wang, Y., Tian, L., Peng, Z., Wang, H., Qingrong, T., 2016. Apigenin-7-*O*-β-D-(-6"-*p*-coumaroyl)-glucopyranoside treatment elicits neuroprotective effect against experimental ischemic stroke. International Journal of biological Sciences 12(1): 42-52.

Devi, S., Kumar, V., 2018. Comprehensive analysis of cis and trans-tiliroside and quercetrin from *Malvastrum coromandelianum* and their antioxidant activities. Arabian Journal of Chemistry, https://doi.org/10.1016/j.arabjc.2018.01.009 (Article in Press).

Ganesan, K., Xu, B., 2017. Molecular targets of vitexin and isovitexin in cancer therapy: a critical review. Annals of New York Academy of Sciences 1401, 102-113.

Harbone, J,B., and Mabry, T.J., 1982. The flavonoids: advances in research. Chapman and Hall, USA.

Jadaun, G.P.S., Agarwal, C., Sharma, H., Ahmed, Z., Upadhyay, P., Faujdar, J., Gupta, A.K., Das, R., Gupta, P., Chauhan, D.S., Sharma, V.D., Katoch, V.M., 2007. Determination of ethambutol MICs for *Mycobacterium tuberculosis* and *Mycobacterium avium* isolates by resazurin microtitre assay. Journal of Antimicrobial Chemotherapy 60, 152-155.

Khonsung, P., Nantsupawat, S., Jesadanont, S.N., Chantharateptawan, V., Panthong, A., 2006. Anti-inflammatory and analgesic activities of water extract of *Malvastrum coromandelianum* (L.) Garcke. Thailand Journal of Pharmacology 28(3), 8-15.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. Journal of Immunological Methods 65, 263-271.

Mushatq, S., Haider, M. S., Ali, A., Javed, A., Khokhar, I., Mukhtar, I., 2012. *In vitro* comparative screening of antibacterial and antifungal activities of some common weeds extracts. Pakistan Journal of Weed Science. Research 18(1), 15-25.

Nazemiyeh, H., Maleki, N., Mehmani, F., Kumarasamy, Y., Shoeb, M., Garjani, A., Sarker, S.D., 2007. Assessment of anti-inflammatory properties of ethyl acetate extract of *Stachys schtschegleevii* Sosn. DARU 15(4), 174-182.

Pinto, M.C., Tejeda, A., Duque, A.L., Macias, P., 2007. Determination of lipoxygenase activity in plant extracts using a modified ferrous oxidation. Xylenol orange assay. Journal of Agriculture and Food Chemistry 55, 5956-5959.

Rosa, S.I.G., Rios-Santos, F. Balogun, S.O., de Oliveira Martins, D.T., 2016. Vitexin reduces neutrophil migration to inflammatory focus by down-regulating pro-inflammatory mediators via inhibition of p38, ERK1/2 and JNK pathway. Phytomedicine 23, 9-17.

Sanghai, D.B., Kumar, S.V., Srinivasan, K.K., Aswatharam, H.N., Shreedhara, C.S., 2013. Pharmacognostic and phytochemical investigation of the leaves of *Malvastrum coromandelianum* (L.) Garcke. Ancient Science of Life 33(1), 39-44.

Shai, L.J., McGaw, L.J., Aderogba, M.A., Mdee, L.K., Eloff, J.N., 2008. Four pentacyclic triterpenoids with antifungal and antibacterial activity from *Curtisia dentata* (Burm.f) C.A. Sm. leaves. Journal of Ethnopharmacology 119, 238-244.

Shen, Y., Wang, X., Jin, J., Wu, J., Zhang, X., Chen, J., Zhang, Wenhong, 2018. *In vitro* susceptibility of *Mycobacterium abscessus* and *Mycobacterium fortuitum* isolates to 30 antibiotics. BioMed Research International, https://doi.org/10.1155/2018/4902941.

Silva, ACP; Hellmann, J.; Velasquez, P.A.G., 2017. Antibacterial activity evaluation of raw extracts and fractions of plants *Malvastrum coromandelian* and *Alternanthera tenella*. In: 29th Brazilian Congress of Microbiology, Foz do Iguaçu.

Sittiwet, C., Jesadanont, S., Pongpech, P., Naenna, P., Pongsamart, S., 2008. Antibacterial activity of *Malvastrum coromandelianum* Garcke against methicillin-sensitive and methicillin resistant *Staphylococcus aureus* Research in Bacteriology 1(1), 42-45.

Saxena, S., Rao, P.B., 2018. GC-MS screening of bioactive constituents and antioxidant profiling in an invasive weed, *Malvastrum coromandelianum* (L.) Garcke. The Pharma Innovation Journal 7(4), 738-746.

Vadivel, V., Sriram, S., Brindha, P., 2016. Distribution of flavonoids among Malvaceae family members – A review. International Journal of Green Pharmacy 10(1), 33-45.

Venturini, C.L., Macho, A., Arunachalam, K., de Almeida, D.A.T., Rosa, S.I.G., Pavan, E., Balogun, S.O., Damazo, A.S., de Oliveira Martins, D.T., 2018. Vitexin inhibits inflammation in murine ovalbumin-induced allergic asthma. Biomedicine and Pharmacotherapy 97, 143-151.