Pharmacological properties and radical scavenging potential of 5-hydroxy-4',5',6,7-tetramethoxyflavone (5-demethyl sinensetin) obtained from *Loxostylis alata* (Anacardiaceae)

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Abstract

Loxostylis alata A. Spreng. ex Rchb. is used in South African traditional medicine to alleviate labour pain and to boost the immune system. A polymethoxyflavone (PMF), identified as 5-hydroxy-4',5',6,7-tetramethoxyflavone (5-demethyl sinensetin) isolated from the ethyl acetate extract of *L. alata,* was evaluated for its *in vitro* pharmacological properties. Antibacterial activity of 5-demethyl sinensetin was determined using a microdilution assay against clinical bacterial isolates from hens' eggs including: *Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis*, *Salmonella* Typhimurium and *Stenotrophomonas maltophilia*. Antioxidant activity was determined using free-radical scavenging assays. The anti-inflammatory activities of the compound were determined by evaluating its inhibitory effect on the activity of the pro-inflammatory enzyme 15- lipoxygenase, as well as its ability to inhibit nitric oxide

(NO) release from macrophages. Furthermore, it was investigated for potential *in vitro* cytotoxic effects using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) test against African green monkey (Vero) kidney and Madin-Darby bovine kidney (MDBK) cell lines. 5-Demethyl sinensetin had antibacterial activity against the tested bacteria with minimum inhibitory concentration (MIC) values ranging from 0.312 to 0.500 mg/mL. Strains of *Salmonella* Typhimurium were more susceptible than the other pathogens with MIC = 0.312 mg/mL. The 5-demethyl sinensetin had good antioxidant activity with IC_{50} values of 1.23 ± 0.15 µg/mL using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging method and 0.14 ± 0.09 µg/mL with the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method compared to the positive controls ascorbic acid and Trolox. The 5-demethyl sinensetin inhibited nitric oxide production in a dose-dependent manner in LPS-stimulated RAW 264.7 macrophages. Nitric oxide (NO) production was inhibited by 87.9% at a concentration of 50 µg/mL with no toxic effect on the macrophages (viability of > 80%), while inhibiting the metabolism of 15-lipoxygenase (15-LOX) with an IC_{50} = 3.26 ± 0.35 µg/mL in comparison to the positive control quercetin. On the other hand however, cytoxicity to Vero (LC_{50} = 0.010 mg/mL) and MDBK (LC_{50} = 0.014 mg/mL) cells was observed with 5-demethyl sinensetin. The compound 5-demethyl sinensetin had potent antioxidant/anti-inflammatory properties, although it was toxic to the cell lines used in this study. There is therefore a need for further research to explore the potential anticancer properties of this important plant species.

Keywords: *Loxostylis alata*; Polymethoxyflavones; 5-Hydroxy-4',5',6,7-tetramethoxyflavone (5-demethyl sinensetin), Antimicrobial activity; Antioxidant; Anti-inflammatory activity; Cytotoxicity.

1. Introduction

Medicinal plants have been a valuable source of potential therapeutic agents for millennia (Cragg et al., 2014). Secondary metabolites from plants represent important leads for novel antimicrobial agents (Pan et al., 2013). However, 95% of the Earth's biodiverse flora and fauna is yet to be evaluated for biological activity (David et al., 2015). Owing to the high challenges of identification and characterization of bioactive constituents, the interest in natural product-based drug discovery has been reported to be gradually declining (Atanasov et al., 2015). With the approximately 310 000 plant species that have been described so far (Atanasov et al., 2015), it has been previously reported that only an estimated 6% of existing plant species have been systematically investigated pharmacologically and only around 15% phytochemically screened (Cragg and Newman, 2013). However, current ongoing research efforts suggest an increase in the percentage of better characterised species (Pan et al., 2013). Natural products are believed to possess properties that have evolved over time and are fully "optimized for serving different biological functions such as binding to specific target proteins or other biomolecules" (Appendino et al., 2010). Discovery of antimicrobials from medicinal plants, especially when supported by well documented ethnopharmacological information about the traditional use (Nasir et al., 2015), is still a worthwhile research area. It has been reported that approximately 80% of the over 100 antimicrobial compounds derived from plants, were used ethnobotanically for the conditions for which the pure compounds are effective (Fabricant and Farnsworth, 2001, Atanasov et al., 2015).

Over the last decade, microorganisms have been quick to develop varied resistance mechanisms resulting in fatality for millions globally, and such resistance has become a public health concern (Corrêa et al., 2020). This emergence of bacterial resistance to current antibiotics is rapidly accelerating, necessitating the development of novel

antibiotics, which has proven to be a rather slow process (Jubeh et al., 2020). The scientific community however, has been meticulously exploring natural resources, particularly plants (Sasidharan et al., 2011, Ivanisova et al., 2017) and fungi (Abdalla et al., 2020) in the search for new, safer and more efficient drugs. Previous reports support the potential of natural products, compounds or molecules extracted from these natural sources, to act as significantly safer antimicrobial agents (Corrêa et al., 2020).

Loxostylis alata A. Spreng. ex Rchb is a member of the family Anacardiaceae (Coates Palgrave, 2002). The bark and leaves of *L. alata* are used in South African traditional medicine to boost the immune system (Pell, 2004) and to relieve labour pain during childbirth (Pooley, 1993). Compounds including ginkgol and ginkgolic acid (Drewes et al., 1998), lupeol and β-sitosterol (Suleiman, 2010) were previously isolated from the plant. Previous studies on crude extracts of *L. alata* reported antimicrobial and antioxidant activities in fractions of the plant (Suleiman et al., 2012b), treatment of aspergillosis in a chicken model (Suleiman et al., 2012a) and subsequently, pure compounds isolated from *L. alata* (Suleiman, 2010).

In our previous study, ten South African plant species were found to be active against various species of the bacterial pathogen *Salmonella. Loxostylis alata* had promising activity and two pure compounds were isolated and characterized from the ethyl acetate fraction following bioassay-guided fractionation (Gado et al., 2021). In the current report, one of these compounds, namely 5-hydroxy-4',5',6,7-tetramethoxyflavone (5-demethyl sinensetin) was evaluated further for pharmacological properties and antioxidant activities.

2. Materials and methods

2.1 Compound source

Leaves of *Loxostylis alata* A. Spreng. ex Rchb (PRU 124357) were collected from the Manie van der Schijff Botanical Garden of the University of Pretoria, South Africa. The plant was identified by Ms. Magda Nel from the Department of Plant and Soil Science, University of Pretoria. The pure compound used in this study was isolated from leaves of *L. alata* as previously reported (Gado et al., 2021).

2.2 Test organisms

Pathogenic species commonly associated with opportunistic and foodborne infections were selected for this study. Five strains, namely *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Stenotrophomonas maltophilia,* isolated from commercial hens' eggs and two resistant strains of *Salmonella* Typhimurium isolated from large animals were included for antimicrobial screening. All the bacterial strains were subcultured from the original culture, stored at −80°C on ceramic beads in cryoprotective media (Pro-Lab Diagnostics Microbank) and maintained on Müller-Hinton (MH) agar (Merck, South Africa) plates at 4°C.

2.3 Microdilution assay for MIC determination

The bacterial cultures were grown by overnight incubation with shaking (MRC orbital shaker incubator; United Scientific, South Africa) at 37°C in MH broth (Merck, South Africa). After 18 h to 20 h incubation, each bacterial stain was adjusted using a spectrophotometer (Epoch microplate reader: BioTek, United States) at a wavelength of 560 nm, to McFarland standard 1, equivalent to 3×10^8 colony forming unit (cfu)/mL. The two-fold serial dilution microplate method (Eloff, 1998) was used to determine the MIC values. Briefly, aliquots (100 µL) of 5 mg/mL solution of compound dissolved in

50% acetone were serially diluted with sterile distilled water in 96-well microtitre plates (Lasec, South Africa). A 100 µL aliquot of bacterial suspension was added to each well. Controls included sterilised distilled water (negative control) and acetone (Minema, South Africa) as solvent control, while gentamicin (Virbac, South Africa) was used as positive control. After 24 h of incubation (IncoTherm, Labotec) of the compound with bacterial cultures at 37°C, 40 µL of 0.2 mg/mL p-iodonitrotetrazolium (INT, Sigma-Aldrich, United States) was added and incubated further for 30 min to 1 h (Eloff, 1998) until optimal colour development. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a formazan product by biologically active organisms (Eloff, 1998). Tests were carried out in triplicate and the MIC was recorded as the lowest concentration of the compound that inhibited bacterial growth (Eloff, 1998).

2.4 Measurement of antioxidant activity

2.4.1 The 2, 2-diphenyl-1-picrylhydrazyl radical scavenging assay

The DPPH scavenging capacity of the compound was evaluated using the method described by Brand-Williams *et al*. (1995) with some modifications. Briefly, the extracts (1 mg/mL) were serially diluted in 40 µl methanol (Minema, South Africa) in 96 well-microtitre plates. Then, 160 µl of a solution of 63.4 µM/mL DPPH (25 µg/mL) was added to the sample and the mixture was allowed to stand at room temperature (±20^{o}C in the dark for 30 min). The absorbance of the resulting solution was measured at 517 nm using a microplate reader (Epoch: BioTek, United States). Ascorbic acid (Adminide, South Africa) and Trolox (6–hydroxyl–2,5,7,8-tetramethlchromane-2-carboxylic acid) (Sigma-Aldrich, United States), both at a stock concentration of 1 mg/mL (serially diluted as for the plant extracts), were used as positive controls, 100% methanol (Minema, South Africa) as negative control and sample without DPPH as

blank (Dzoyem et al., 2014b). Samples were replicated and tested three times. The percentage of DPPH scavenging capacity was calculated at each concentration according to the formula (1) below:

$$\text{Scavenging capacity } (\%) = 100 - \left[\frac{\text{Abs (sample)} - \text{Abs (sample blank)}}{\text{Abs (control)} - \text{Abs (control blank)}} \times 100 \right]$$

The concentration of the extract leading to 50% reduction of DPPH colour (IC_{50}) was also determined by plotting the graph of percentage DPPH scavenging capacity against the different concentrations of the compound (Dzoyem et al., 2014b).

2.4.2 The 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ABTS radical scavenging assay

The ABTS radical scavenging capacity of the compound was measured as described by Re et al. (1999) with some modifications. The ABTS radical was produced by reacting a solution of ABTS (7 mM) (Sigma-Aldrich, United States) with a solution of potassium persulfate (2.45 mM) (Sigma-Aldrich, United States) at room temperature (±20^{o}C) for 12 h. The absorbance of the ABTS radical produced was adjusted to 0.70 ± 0.02 at 734 nm (Epoch microplate reader: BioTek, United States) before use. The extracts (1 mg/mL) were serially diluted in methanol (40 µl) in 96 well-microtitre plates and 160 µl of ABTS solution added to each well. The absorbance was measured after 5 min at 734 nm using a microplate reader (Epoch, BioTek, United States). Ascorbic acid (Adminide, South Africa) and Trolox (Sigma-Aldrich, United States) were used as positive controls, 100% methanol (Minema, South Africa) as negative control and sample without ABTS as blank. Samples were tested three times The percentage of ABTS scavenging capacity was calculated at each concentration according to the

formula (1) above and the IC_{50} values were calculated from the graph plotted as inhibition percentage against the concentration (Dzoyem et al., 2014b).

2.5 Maintenance of cell lines

African green monkey (Vero) kidney (ATCC® CCL-81™), Madin-Darby bovine kidney (MDBK) (ATCC® CCL-22™) and RAW 264.7 murine macrophage (ATCC® TIB-71™) cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, USA). Vero cells were maintained in Minimal Essential Medium (MEM, Whitehead Scientific, South Africa) containing 4.5 g/L glucose and 4 mM L-glutamine (Whitehead Scientific, South Africa) supplemented with 1% gentamicin and 5% foetal calf serum (FCS, Highveld Biological, South Africa). The MDBK cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Whitehead Scientific, South Africa) supplemented with 10% heat inactivated foetal bovine serum (FBS, Sigma-Aldrich, United States) and 1% penicillin/streptomycin/fungizone solution (PSF; Invitrogen, Cergy-Pontoise, France). The RAW 264.7 murine macrophage cells were maintained in DMEM high glucose (4.5 g/L) containing L-glutamine (Whitehead Scientific, South Africa) and supplemented with 10% FBS and 1% PSF. All cell lines were maintained at 37^{o}C in a 5% CO_2 incubator (Hera Cell 150, ThermoScientific Germany). Cells were passaged three times weekly by trypsinization with trypsin/ethylenediaminetetraacetic acid (EDTA) solution (Invitrogen, Cergy-Pontoise, France) into 75 cm^2 culture flasks (Whitehead Scientific, South Africa).

2.6 Cytotoxicity of 5-demethyl sinensetin

The 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction assay (Mosmann, 1983) was used to determine the cytotoxicity of the compound against viable cell growth of Vero and MBDK cells. Briefly, cells of a subconfluent

culture were harvested and centrifuged (Universal 320R, Labotec South Africa) at 200 x g for 5 min and re-suspended in a growth medium (MEM for Vero cells and DMEM for MBDK cells) to a density of 1×10^5 cells/mL. One hundred µL of the cell suspension were seeded into wells of 96-well microtitre plates (Lasec, South Africa) and incubated in a 5% CO_2 humidified environment (Hera Cell 150, ThermoScientific Germany) at 37°C for 24 h. Test compound was dissolved in dimethyl sulphoxide (DMSO) (BDH, South Africa) to a concentration of 2 mg/mL and diluted in growth medium to decreasing concentrations of the test sample (200, 150, 100, 50, 20 and 10 µg/mL). After seeding, the cells were treated with the prepared compound of increasing concentrations. Doxorubicin hydrochloride (Pfizer Laboratories, South Africa) was used as a positive control while untreated cells (no compound added to the cells) and 1% DMSO served as negative controls. After incubation for 48 h at 37°C with 5% CO_2 (Hera Cell 150, ThermoScientific Germany), the culture medium was aspirated, washed with 200 µL of phosphate buffered saline (PBS) pH 7.4 (Whitehead Scientific, South Africa) and replaced with 200 µL of fresh MEM and 30 µL MTT (Sigma-Aldrich, South Africa; stock solution of 5 mg/mL in PBS). Plates were further incubated for 4 h at 37ºC in a 5% CO_2 incubator (Hera Cell 150, ThermoScientific Germany). After 4 h of incubation, the growth medium in each well was carefully removed using a suction pump (Integra, USA) and the resulting formazan crystals were dissolved in 50 µL of 100% DMSO (BDH, South Africa). The absorbance was measured spectrophotometrically (Biotek Synergy, USA) at 570 nm. Each compound concentration was tested in quadruplicate and the assays were repeated three times. The viability of treated cells was calculated for each concentration using the formula:

Percentage cell viability = (Mean absorbance of sample / Mean absorbance of control) * 100

The 50% lethal concentrations (LC_{50}) for both Vero and MDBK cells were determined by plotting the graph of viability rate versus the concentrations. The selectivity index values were calculated by dividing cytotoxicity LC_{50} values by the MIC values in the same units (mg/mL).

2.7 Determination of anti-inflammatory activity

2.7.1 Nitric oxide inhibitory assay

Inhibition of nitric oxide (NO) production by mouse macrophages (RAW 264.7) was determined (Joo et al., 2014) by measuring the accumulation of nitrite, an indicator of NO, after 24 h of lipopolysaccharide (LPS) treatment with or without the compound or quercetin (Sigma-Aldrich, United States) (positive control) using Griess reagent (Sigma-Aldrich, United States) (Njoya et al., 2017). Briefly, RAW 264.7 macrophages were seeded at a density of 2×10^4 cells per well in 96-well microtitre plates (Lasec, South Africa) and the cells were allowed to attach overnight at 37ºC in a 5% CO_2 incubator (Hera Cell 150, ThermoScientific Germany). The cells were activated by incubation in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, United States) containing 5 µg/mL lipopolysaccharide (LPS) (Sigma-Aldrich, United States) alone (control) and treated simultaneously with different concentrations (1.6, 12.5, 50 and 100 µg/mL) of the samples dissolved in dimethyl sulfoxide (DMSO) (BDH, South Africa). Quercetin (Sigma-Aldrich, United States) was used as a positive control. After 24 h of incubation at 37ºC in a 5% CO_2 incubator (Hera Cell 150, ThermoScientific Germany), 100 µL of supernatant from each well of the 96-well microtitre plates (Lasec, South Africa) were transferred into new 96-well microtitre plates (Lasec, South Africa) and an equal volume of Griess reagent (Sigma-Aldrich, United States) was added. The absorbance of the mixture was determined at 550 nm on a microplate reader (Biotek Synergy, USA) after 10 min of incubation at room temperature (±20ºC).

The quantity of nitrite was determined from a sodium nitrite standard curve. The percentage of NO inhibition was calculated based on the ability of each sample to inhibit nitric oxide production by RAW 264.7 macrophages compared with the control (cells treated with LPS without samples) (Njoya et al., 2017). Subsequently, the cell viability was determined using the MTT assay as described in section 2.6 above.

2.7.2. Lipoxygenase inhibition assay

The lipoxygenase inhibitory (LOX) activity was determined spectrophotometrically (Lyckander and Malterud, 1992) with an assay based on the formation of the complex Fe3+/xylenol orange (del Carmen Pinto et al., 2007). Briefly, 20 µL of Tris-HCl (Sigma-Aldrich, United States) Buffer (pH 7.4) was added to all wells of a 96-well microtitre plate (Lasec, South Africa). A volume of 20 µL of the compound (1 mg/mL) was added to the first row of the microplate followed by two-fold serial dilution down the columns. The compound was added to the sample and the sample blank wells. Quercetin (Sigma-Aldrich, United States) served as the positive control and the buffer was used as a negative control. After the serial dilution, 40 µL of the lipoxygenase enzyme (Sigma-Aldrich, United States) was added to each well and the plates were incubated at room temperature (±20ºC) for 5 min. After incubation, 40 µL of linoleic acid (Sigma-Aldrich, United States; final concentration, 140 µM) prepared in Tris-HCl (Sigma-Aldrich, United States) buffer (50 mM, pH 7.4) was added to the wells (except for the blanks). The plates were incubated at ±20ºC for 20 min in the dark. Thereafter, 100 µL of freshly prepared ferrous oxidation−xylenol orange (FOX) reagent [sulfuric acid (30 mM) (Sigma-Aldrich, United States), xylenol orange (100 µM) (Sigma-Aldrich, United States), iron (II) sulfate (100 µM) (Sigma-Aldrich, United States) in methanol (Minema, South Africa)/water (9:1)] was added to all wells. Samples were tested in triplicate.The plates were incubated at ±20ºC for 30 min in the dark and 40 µL of

linoleic acid (Sigma-Aldrich, United States) was added to the blanks. The absorbance was measured at 560 nm using a BioTek Epoch microplate reader (Biotek Synergy, USA).

Statistical analysis

Data are presented as means of three replicate determinations ± standard error of mean (SEM).

3. Results

3.1 Minimum inhibitory concentration

In vitro antibacterial activity of 5-demethyl sinensetin against *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis, Salmonella* Typhimurium and *Stenotrophomonas maltophilia* was determined. The results of the MIC values are presented in Table 1. 5-Demethyl sinensetin inhibited the *S.* Typhimurium isolates at 0.312 mg/mL, compared to the positive control gentamicin (MIC = 0.004-0.031 mg/mL). On the other hand, *E. cloacae*, *E. coli*, *K. pneumoniae*, *P. mirabilis* and *S. maltophilia*, were all inhibited at the MIC value of 0.625 mg/mL, while the positive control gentamicin ranged from MIC = 0.004- 0.031 mg/mL.

Table 1: Minimal inhibitory concentration (MIC in mg/mL) of 5-demethyl sinensetin screened against seven bacterial strains

Samples	**Bacterial strains MIC (mg/mL)**						
	E. cl	***E.coli***	***K. p***	***P. m***	***S.*T 1**	***S.*T 2**	***S. m***
5-demethyl sinensetin	0.625	0.625	0.625	0.625	0.312	0.312	0.625
Gentamicin	0.008	0.004	0.004	0.004	0.031	0.004	0.004

E.cl = Enterobacter cloacae, *E. coli = Escherichia coli*, *K. p = Klebsiella pneumoniae*, *P. m = Proteus mirabilis, S*.T 1= *Salmonella* Typhimurium B1781, *S*.T 2= *Salmonella* Typhimurium B2692 and *S. m = Stenotrophomonas maltophilia*

3.2 Radical scavenging potential

The free radical scavenging potency of 5-demethyl sinensetin is presented in Table 2. The compound demonstrated good antioxidant capacity with the inhibitory concentration (IC_{50}) of 1.23 ± 0.15 µg/mL and 0.14 ± 0.09 µg/mL using the DPPH and ABTS assays respectively. However, the positive controls had higher IC_{50} values for both the DPPH (ascorbic acid: 1.59 ± 0.04 µg/mL and Trolox: 3.04 ± 1.42 µg/mL) and ABTS (ascorbic acid: 1.71 ± 0.32 µg/mL and Trolox: 2.40 ± 0.87 µg/mL) assays.

Table 2: Antioxidant and lipoxygenase inhibitory activity (IC_{50} in µg/mL) of 5-demethyl sinensetin and positive controls (trolox, ascorbic acid and quercetin)

Assay	IC_{50} (µg/mL) ± SEM		
	DPPH inhibition	**ABTS inhibition**	**LOX inhibition**
5-demethyl sinensetin	1.23 ± 0.15	0.14 ± 0.09	3.26 ± 0.35
Ascorbic acid	1.59 ± 0.04	1.71 ± 0.32	ND
Trolox	3.04 ± 1.42	2.40 ± 0.87	ND
Quercetin	ND	ND	43.06 ± 0.52

Data are presented as means of triplicate measurements ± standard error, ND = not determined

3.3 Anti-inflammatory activity of 5-demethyl sinensetin

The anti-inflammatory activity of 5-demethyl sinensetin was evaluated using the 15-LOX model of inhibition and the activities of inducible nitric oxide synthase. The result presented in Table 2 suggests that 5-demethyl sinensetin may possess some anti-inflammatory activity. This is seen with the 15-LOX inhibitory activity of IC_{50} = 3.26 ± 0.35 µg/mL compared with quercetin (IC_{50} = 43.06 ± 0.52 µg/mL) used as a positive control.

A concentration-dependent inhibition of NO production was observed. 5-Demethyl sinensetin had noteworthy NO inhibition, inhibiting up to 50% of NO production even

at the lowest concentration of 1.6 µg/mL (IC_{50} > 1.6 ug/mL), while inhibition above 70% was observed at 50 µg/mL.

3.4 Cytotoxic activity of 5-demethyl sinensetin

Table 3 shows the results for cytotoxicity of 5-demethyl sinensetin represented as LC_{50} values and the respective selectivity index (SI) values, which is the representation of the relationship between antibacterial activity and cytoxicity. LC_{50} values >0.1 mg/mL for plant extracts are considered non-cytotoxic (Kuete, 2010). 5-Demethyl sinensetin had LC_{50} values of 0.010 ± 0.001 mg/mL and 0.014 ±0.113 mg/mL against Vero and MDBK cells respectively, compared to doxorubicin (positive control) which was 0.007 ±0.003 mg/mL and 0.008 ±0.001 mg/mL against the same cells.

Table 3: LC_{50} and selectivity index values of 5-demethyl sinensetin against Vero monkey kidney cells and Madin-Darby bovine kidney (MDBK) cells

Samples	LC_{50} values (mg/ml)		Selectivity Index value													
			E. cl		*E. c*		*K. p*		*P. m*		*S*. T1		*S*. T2		*S. m*	
	Vero	MDBK	Vero	MDBK	Vero	MDBK	Vero	MDBK	Vero	MDBK	Vero	MDBK	Vero	MDBK	Vero	MDBK
5-demethyl sinensetin	0.010 ± 0.001	0.014 ± 0.113	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.05	0.03	0.05	0.02	0.03
Doxorubicin	0.007± 0.003	0.008 ± 0.001	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Vero= Vero monkey kidney cells, MDBK= Madin-Darby Bovine kidney cells, *E.cl = Enterobacter cloacae*, *E. coli = Escherichia coli*, *K. p = Klebsiella pneumoniae*, *P. m = Proteus mirabilis, S*.T 1= *Salmonella* Typhimurium B1781, *S*.T 2= *Salmonella* Typhimurium B2692 and *S. m = Stenotrophomonas maltophilia,* ND = not determined

4. Discussion

The quantitative MIC assay used in this study to measure antimicrobial activity is based on the principle of contact of a test organism to a series of dilutions of test substance. This MIC methodology is widely used and an accepted criterion for measuring the susceptibility of organisms to medicinal plants. The search for novel chemotherapeutic agents commences with the identification of a potential antimicrobial compound, which in this case, is the polymethoxylated flavone (PMF) 5-demethyl sinensetin isolated from *L. alata*. The potency, otherwise referred to as MIC expressed in mg/mL, is the measure of the strength of the activity of a substance against an organism. Promising bioactive compounds exert antimicrobial activity at lower MIC values of 0.01 mg/mL (Van Vuuren, 2008). The antimicrobial activity observed against the strains tested in this study suggests poor antibacterial potential of 5-demethyl sinensetin, although it should be borne in mind that the total antimicrobial activity obtained with the crude extract could be as a result of the complex interaction between the components in which synergistic or antagonistic actions intervene (Tacchini et al., 2020). There are scarce reports on antimicrobial activity of 5-demethyl sinensetin and to the best of our knowledge this is the first report of antibacterial activity of 5-demethyl sinensetin against the tested bacterial strains (*E. cloacae*, *E. coli*, *K. pneumoniae*, *P. mirabilis, S.* Typhimurium and *S. maltophilia*). A study on two PMF monomers (nobiletin and tangeretin; members of the same class as sinensetin) had *in vitro* growth inhibition of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens,* each with an MIC = 3.6 mg/mL (Yao et al., 2012) which is a high MIC reflecting little activity and further corroborates our findings. Cell pyknosis and eventually death in bacteria is the proposed mode of action of polymethoxylated flavones (PMFs); this is achieved by causing reduction in chitin production, resulting

in permeability changes of the cytomembrane and increasing fragility of cell walls (Yao et al., 2012). Although the antibacterial activity of 5-demethyl sinensetin was not noteworthy, other biological activities ascribed to flavones including antioxidant, anti-inflammatory, antitumor, anti-allergic considerations may be of interest.

As has already been stated, the bark and leaves of *L. alata* have been used therapeutically in South African traditional medicine to boost the immune system. The effects presented by the extracts from different parts of the *L. alata* are yet to be elucidated. Our data reveal evidence that 5-demethyl sinensetin (as a constituent of *L. alata*) can exhibit antioxidant and anti-inflammatory activities in a biological system, hence providing scientic evidence to support the traditional claims of the plants' efficacy.

In the present study, two colorimetric antioxidant assays based on the principle of colour change of the free radicals DPPH and ABTS were used to quantify the antioxidant property of 5-demethyl sinensetin. These assays are typically based on the scavenging capacity of radicals which are converted into a colourless product (Njoya et al., 2017). The degree of this discoloration corresponds to the amount of ABTS or DPPH that has been scavenged. Antioxidant activity is the ability of a bioactive compound to maintain cell structure and function by not only scavenging free radicals, but by effectively inhibiting lipid peroxidation reactions and preventing other oxidative damage (Zou et al., 2016). Many biological functions including anti-inflammation and, even more importantly, the prevention of many chronic diseases, has been associated with antioxidant activity (Rajendran et al., 2014). Therefore, identification of natural sources of antioxidants, especially from plants rather than synthetic sources, is of great relevance to human health (Zou et al., 2016). Values obtained in this study showed a dose dependent percentage inhibition of DPPH and

ABTS reduction with remarkable results for 5-demethyl sinensetin, with its free radical scavenging activity better than that of ascorbic acid and Trolox, the positive controls. This activity can be ascribed to the antioxidant ability for which flavonoids are generally known among plant secondary metabolites (Hu et al., 2016). Flavonoids are reported to play a direct role in scavenging reactive oxygen species which, while reducing peroxide formation *in vivo* and thereby improving the body's antioxidant enzyme activity, they can also counteract lipid oxidation *in vitro* (Zou et al., 2016). The presence of a hydroxyl group in 5-demethyl sinensetin is considered a structural enhancer responsible for its antioxidative activity and other effects (Lai et al., 2015). Findings obtained in this study further support previous reports of antioxidative properties of the widely studied class of PMFs (Li et al., 2009, Xu et al., 2017) and for 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (Akowuah et al., 2005).

Inflammation is one of the first but comprehensive and complex physiological and immune responses, playing a defensive function from inside the body against metabolic stress, injury or potentially harmful pathogens (Ondua et al., 2019). Preliminary assessment of anti-inflammatory activity of 5-demethyl sinensetin was carried out using nitric oxide (NO) inhibitory and lipoxygenase inhibition assays. Nitric oxide is considered an important inflammatory mediator molecule involved in a broad array of biological processes (Assemian et al., 2019). Nitric oxide is able to modify proteins, lipids and DNA, and it even alters their functions and immunogenicity as well as regulation of apoptotic death and cell viability (Boscá et al., 2005). The NO produced in large amounts by inducible nitric oxide synthase (iNOS) is known to be responsible for the vasodilation and hypotension observed during septic shock and inflammation (Yang et al., 2009). Thus, inhibitors of iNOS may be useful candidates for the treatment of inflammatory diseases resulting in the overproduction of NO.

Activation of RAW 264.7 macrophages with lipopolysaccharide (LPS) induces the production of NO. The extent of NO production is determined by measuring the concentration of nitrite, a stable oxidised product of NO (Dzoyem et al., 2017). First, we investigated the viability of cells treated with 5-demethyl sinensetin. The MTT assay was carried out to evaluate the viability/ cytotoxicity of the pure compound in RAW 264.7 macrophage cells. In this study, it was observed that 5-demethyl sinensetin had good NO inhibition, where 50 µg/mL inhibited NO production above 70%, which is considered as very potent (Yang et al., 2009), and unlikely to be due to toxicity as cell viability was above 80%. On the other hand, however, dose-dependent cytoxicity on the cell line was observed by 5-demethyl sinensetin and the quercetin control. The observed effect of the 5-demethyl sinensetin and quercetin on cell viability may be due to macrophage toxicity; the consequence of this damage is a decrease in cell viability (Souza et al., 2017). Previous studies have demonstrated that treatment with LPS in macrophages leads to impairment of the electron transfer system, thus increasing the rate of oxygen radical·production, thus damaging lipids, proteins, and DNA (Mills et al., 2016).

Lipoxygenase (LOX) enzymes are postulated to play an important role in the physiology of several inflammatory diseases (Kumar et al., 2013). They are present in the human body and play an important role in the stimulation of inflammatory reactions. Reactive oxygen species, when in excess amounts, can cause inflammation that stimulates a release of cytokines and subsequent activation of LOXs (Lončarić et al., 2021). The 15-LOX is an important enzyme involved in the synthesis of leukotriene from arachidonic acid. Leukotrienes are mediators of many pro-inflammatory compounds and targeting 15-LOX is considered as one of the therapeutic strategies in the management of inflammatory conditions (Dzoyem et al., 2014a, Ondua et al.,

2019). Plant phytochemicals have been shown to play an important defensive role, which could help in the prevention of diseases caused by oxidative stress. The anti-inflammatory activities of natural compounds have been reported in several studies and have been observed in numerous preclinical studies (Ravipati et al., 2012). A review of the inhibition of LOX by plant extracts prepared from various plant families (Lončarić et al., 2021) suggested that the selection of appropriate solvents for compound extractions, as well as the corresponding phytochemical profiles, play important roles in LOX inhibition. While some extracts show better inhibitory activities than others, greater LOX inhibitory activity is anticipated from isolated compounds rather than crude extracts (Lončarić et al., 2021).

An *in vivo* study of the anti-inflammatory and analgesic activities of the crude acetone extract of *L. alata* using experimental models reported a dose-dependent (50-200 mg/kg) inhibition of induced oedema and pain. The findings from the study support the anti-inflammatory and analgesic effects of *L. alata* and provides a rationale for its traditional use against pain and inflammation (Suleiman et al., 2015).

The compound 5-demethyl sinensetin belongs to the group of flavones referred to as ‘polymethoxyflavones’ (PMFs) which are almost exclusively found in the citrus genus (Li et al., 2007). Results obtained in this study support the numerous studies and reports of the good anti-inflammatory properties of flavonoids of the PMF class (Li et al., 2009, Gosslau et al., 2014, Lai et al., 2015, Gao et al., 2018, Wang et al., 2018). Flavonoids play a key role in decreasing the arachidonic acid cascade via cyclooxygenase or lipoxygenase pathways, which leads to a reduced inflammatory response (Mulaudzi et al., 2013). The mechanism of action of many anti-inflammatory compounds is thought to be via their free radical scavenging activities or inhibition of pro-inflammatory enzymes such as cyclooxygenase (COX), lipoxygenase (LOX), NF-

κB and mitogen-activated protein kinases (MAPKs) (Arulselvan et al., 2016). Flavonoids are known to interfere with the different stages of the conversion of arachidonic acid to hydroperoxyeicosatetraenoic acids (HPETEs), which are reduced to mono-HETEs or diHETEs and leukotrienes (known natural mediators of hypersensitivity and inflammation) via COX or LOX pathways to prevent inflammatory responses (Mulaudzi et al., 2013). Therefore the inhibition of the synthesis of the mediators of many pro-inflammatory and allergic reactions is considered to be one of the therapeutic strategies in the management of inflammatory conditions (Adebayo et al., 2015). The PMFs have been reported to exert anti-inflammatory potential through inhibiting gene expression of pro-inflammatory cytokines and enzymes involved in inflammation (Gao et al., 2018) and also inhibiting the release of the important inflammatory mediator, PGE2 (Li et al., 2009).

Rational investigation of pure compounds as potential new drug leads will include assessment of their efficacy and safety to verify traditional claims (Atanasov et al., 2015). *In vitro* cytotoxicity studies on potential plant compounds are therefore a prerequisite for further clinical trials. A high selectivity index value has been recommended as an indicator of safety of a test sample on cells (Elisha et al., 2017). The higher the selectivity index value of a compound the greater is its potential as a new antimicrobial lead (Madikizela and McGaw, 2018). Based on the standard stated above, 5-demethyl sinensetin was relatively toxic to both Vero and MDBK cell lines. The selective index values obtained in this study, with values all less than 1, suggest that 5-demethyl sinensetin may be cytotoxic to normal cells and antibacterial activity may therefore be due to general metabolic toxicity. A literature search provided evidence of a number of studies on the selective cytotoxicity of the PMFs and their

antiproliferative activity on a variety of cancer cell lines (Kim et al., 2010, Dong et al., 2014, Chien et al., 2015, Gao et al., 2018).

5. Conclusion

Though the antibacterial activity of 5-demethyl sinensetin may not be remarkable, this study provided evidence that 5-demethyl sinensetin can serve as a potent antioxidant agent, while preliminary anti-inflammatory properties observed will require further research. Cytotoxicity to cells should be further explored for antiproliferative and anti-cancer research. Further safety evaluation such as genotoxicity and toxicity testing using bioorganisms is recommended. Another focus for further studies should be on the determination of the precise molecular mechanism of action of 5-demethyl sinensetin.

Abbreviations

ABTS: 2,2‘-azinobis-(3-ethyl benzothiazoline 6-sulfonic acid) diammonium salt, ATCC: American Type Culture Collection; CFU: Colony forming units; DMEM: Dulbecco's Modified Eagle's Medium; DMSO: Dimethylsulphoxide; DPPH: 2, 2, diphenyl-1-picrylhydrazyl; EDTA: Ethylenediaminetetraacetic acid; FBS: foetal bovine serum; FCS: foetal calf serum; FOX: Ferrous oxidation-xylenol orange; HCL: Hydrochloric acid; HPETEs: hydroperoxyeicosatetraenoic acids; INT: *p*-iodonitro-tetrazolium violet; LOX:lipoxygenase; LPS: lipopolysaccharide; MH: Mueller-Hinton; MDBK: Madin-Derby Bovine Kidney; MEM: minimal essential medium; MIC: minimal inhibitory concentration; MTT: 3-(4,5-dimethylthiazolyl-2)-2.5-diphenyltetrazolium bromide; NO: Nitric oxide; PBS: phosphate buffered saline; PMF: Polymethoxyflavones; PSF: penicillin/streptomycin/ fungizone solution; SI: Selectivity index.

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