Cytotoxicity and antimicrobial activity of isolated compounds from *Monsonia angustifolia* and *Dodonaea angustifolia*

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Highlights

- ·Four compounds (**1**, **2**, **3** and **4**) were isolated from *Monsonia angustifolia.*
- ·Compound **5** was isolated from *Dodonaea angustifolia*.
- ·Compounds (**1-5**) were screened for antimicrobial activity and tested for cytotoxicity.
- ·All compounds were non-toxic to MDCK and VERO cells.

Abstract

Ethnopharmacological relevance: *Monsonia angustifolia* is traditionally used to treat anthrax, heartburn, diarrhea, eye infections and hemorrhoids. *Dodonaea angustifolia* is frequently used as a treatment for dental pain, microbial infections and jungle fever. The two plant species were selected due to the presence of secondary metabolites such as coumarins, flavonoids, terpenoids, saponins and polyphenolics from the crude extracts, which exhibit pharmacological significance. The pure isolated compounds from the crude extracts are known for their diverse structures and interesting pharmacophores.

Aim : To isolate and identify antibacterial and antifungal chemical constituents from *Monsonia angustifolia* and *Dodonaea angustifolia* plant extracts and evaluate the cytotoxicity of pure compounds from the crude extracts.

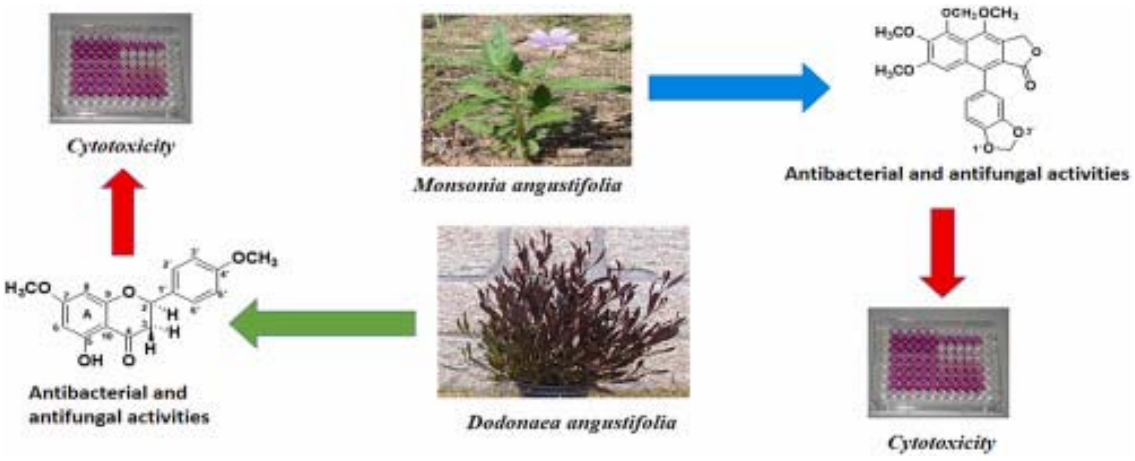
Materials and methods: Extractives from *M. angustifolia* and *D. angustifolia* plants were isolated using chromatographic techniques and structures were elucidated based on NMR, IR and MS spectroscopic techniques. A microplate serial dilution method was used to evaluate the antibacterial activity of extracts and pure compounds against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and antifungal activity against *Candida*

albicans and *Cryptococcus neoformans*. The cytotoxicity was determined using the 3-(4, 5-dimethylthiazol)-2, 5-diphenyl tetrazolium bromide (MTT) assay.

Results: The dichloromethane, ethyl acetate and methanol crude extracts from the plants exhibited significant inhibition of microbial growth. The phytochemical investigation of these active crude extracts led to the isolation of five pure active compounds, 5-methoxyjusticidin A (**1**), *cis*-phytyl diterpenoidal fatty acid ester (**2**), stigmasterol (**3**), *β*-sitosterol (**4**) and 5-hydroxy-7,4'-dimethoxyflavone (**5**). Stigmasterol (**3**) showed good antifungal activity against *Cryptococcus neoformans* with a minimum inhibition concentration (MIC) of 25 µg/mL and *Candida albicans* (MIC = 50 µg/mL).

Conclusion: Compounds (**1**–**5**) isolated from *Monsonia angustifolia* and *Dodonaea angustifolia* showed antibacterial and antifungal activities and were non-toxic against Madin-Darby canine kidney (MDCK) cells and VERO monkey kidney (VERO) cells.

Graphical abstract



Keywords: Monsonia angustifolia; Dodonaea angustifolia; Antibacterial activity; Antifungal activity; Chemical constituents

1. Introduction

Since ancient days medicinal plants were used to treat many infirmities, particularly infectious diseases such as the common cold, influenza and diarrhea (Mitscher et al., 1987; Hutchings and Van Staden, 1994). South Africa is rated third in the world for its rich earthly biodiversity (Griffiths et al., 2010; Tolley et al., 2019). The use of traditional remedies is essential in the lives of African people; their use is attributable to affordability and accessibility with approximately 3000 medicinal plants used for medicinal purposes (Drewes, 2010). The genera *Monsonia* and *Dodonaea* are South African medicinal plants with a wide range of medicinal applications. Venter (1979) reported *Monsonia* as one of five genera recognized in the Geraniaceae family and consists of 25 species distributed in Africa and Asia.

For many years, *Monsonia* species were used as herbal teas and vegetation (Venter, 1979). The infusions and extraction of the leaves, roots, stem barks and flowers of *Monsonia* species including *M. angustifolia* are traditionally used to treat heartburn, diarrhea, eye infections, hemorrhoids, sores, ulcers, flatulence, influenza, dysentery and minor stomach ailments (Roberts, 1990; Khorombi, 2006; Fouche et al., 2015).

The acetone/hexane leaf extract of *M. angustifolia* was reported to inhibit bacterial growth of *Pseudomonas aeruginosa* (MIC = 2.50 mg/mL) and *Saphylococcus aureus* at MIC = 2.50 mg/mL (Matotoka and Masoko, 2017). Fouche et al. (2016) investigated the acetone crude extract of *M. angustifolia* (whole plant) which showed anthelmintic activity against *Haemonchus contortus* at (56% ±6%) and the cytotoxicity on Vero African Green Monkey Kidney cells, which showed low toxicity (LC_{50} =120.37 ± 4.06 µg/mL). Khorombi (2006) and Fouche et al. (2015) isolated five lignan compounds from the aerial parts of *M. angustifolia* namely, 5-methoxyjusticidin A, justicidin A, suchilactone, chinensinaphthol and retrochmensmaphthol methyl ether. In addition, Fouche et al. (2015) evaluated the isolated lignans *in vivo* and *in vitro* bioassays for erectile dysfunction and libido. From the findings, it was concluded that the lignans could be considered for the treatment of erectile dysfunction as well as for increasing male libido. Lyimo et al. (2003) reported that the whole plant of *M. angustifolia* is a potential source of crude fiber and is rich in vitamin C, calcium and proteins. Chun et al. (2017) discovered that the ethanol extracts of *M. angustifolia* exhibited a similar function as *M. brevirostrate* at suppressing the production of a *β*–amyloid protein, thereby presenting the use of the plant extracts for prevention, improvement and treatment of dementia.

The genus *Dodonaea* consists of more than 70 species including *D. angustifolia,* which is endemic to Australia (Nissim-levi et al., 2013). The decoctions of *D. angustifolia* plants are frequently used as a treatment for different diseases, infections, injuries, dental pain and jungle fever (Mengiste et al., 2012; Rao et al., 2015; Melaku et al., 2017; Akhalwaya et al., 2018). The leaves, stems, roots and flowers are used in the preparation of traditional medicine to treat several ailments by topical application of the extracts on the skin or the plant decoctions may be taken orally (Beshah et al., 2020; Patel and Coogan, 2008). The extracts of this plant species were reported to have several biological activities against various infections and diseases. Beshah et al. (2020) reported that different methods such as vacuum liquid chromatography (Mostafa et al., 2014), serial extraction (Teffo et al., 2010) and bioassay-guided extraction (Melaku et al., 2017; Teffo et al., 2010) were used for isolating new active compounds and for identifying the classes of compounds present in *Dodonaea* species.

Ngabaza (2016) reported that the phytochemical analysis of *D. angustifolia* produced an anticariogenic constituent, 5,6,8-trihydroxy-7,4'-dimethoxyflavone, which showed high antimicrobial activity (MIC = 0.05 mg/mL) against *Streptococcus mutans* (ATCC 10923). The methanol crude extract of *D. angustifolia* and 5,6,8-trihydroxy-7,4'-dimethoxyflavone were evaluated against human embryonic kidney (HEK) 293 cells and showed low cytotoxicity with LC_{50} values of 0.09 mg/mL and 0.03 mg/mL respectively.

The species *M. angustifolia* and *D. angustifolia* (Fig. 1) are recognized as valuable resources of natural antimicrobial compounds for this study due to their medicinal applications in

inhibiting microbial growth. The effect of plant decoctions on bacteria and fungi was studied by a great number of researchers all over the world (Nayan et al., 2011).

Fig. 1. Whole plants of *D.angustifola* and *M.angustifolia*.

The useful information about the health benefits of different plant-based natural products and their use as sources of antifungal, antibacterial and antiviral drugs was made readily available by researchers (Clark, 1996).

People are not immune to diseases and with age, the human immune system deteriorates and weakens hence susceptible to viral, fungal, bacterial and other infections. Scientific studies showed that over time the human body gradually develops resistance to some antibiotics currently in use and this resistance crisis was attributed to the overuse and misuse of antimicrobial medications and evolving of bacterial and fungal infections (Berkowitz, 1995; Boucher et al., 2009; Ventola, 2015). Antimicrobial activity of the crude extracts of *Monsonia* and *Dodonaea* species were carried out in the past years to identify classes of compounds such as lignans, flavonoids, steroids, saponins present in the crude extracts (Khorombi, 2006; Fouche et al., 2015; Matotoka and Masoko, 2017). Much work has been done previously on the aerial plant parts of *M. angustifolia* (Chun et al., 2017; Fouche et al., 2015; Khorombi, 2006) and there is a minimal investigation done on the stem barks of this plant species.

The leaf content of *D. angustifolia* is known for the presence of flavonoids and their derivatives, which are the major bioactive components (Beshah et al., 2020), hence the study was carried out to exploit phytochemicals with antifungal and antibacterial activity from the stem barks of *M. angustifolia* and leaves of *D*. *angustifolia*.

2. Materials and methods

2.1. Plant collection and identification

The stem barks of *M. angustifolia* were collected from Limpopo, South Africa (22^{o} 56S, 30^{o} 28E) and provided by the Council for Scientific and Industrial Research South Africa (CSIRSA) in Pretoria. The leaves of *D. angustifolia* were collected from Watercombe Farm Indigenous Tree Nursery in Pretoria, South Africa (51.816^{o} N, 2.0804^{o} W). The stem barks of *M. angustifolia* and the leaves of *D. angustifolia* were identified at the South African National Biodiversity Institute (SANBI) in Pretoria as *Monsonia angustifolia* E. Mey. Ex A. Rich (Genspec number: 3925000) and *Dodonaea viscosa* Var*. angustifolia* (L.f.). (Genspec number: 4831000) respectively.

2.2. Experimental procedure

2.2.1. Extraction

Two different approaches of extraction were used to achieve maximum efficient results. The stem barks of *M. angustifolia* were sequentially extracted using hexane, dichloromethane, ethyl acetate and methanol and the leaves of *D. angustifolia* were extracted with methanol.

2.2.1.1. Extraction of Monsonia angustifolia

The stem bark of *M. angustifolia* (1.5 kg) was cut into smaller pieces, then air-dried for seven days and grounded using a hammer mill equipped with a mesh pore size of 2 mm. The dried ground plant material in the extraction vessel was extracted with organic solvents of increasing polarity (100% hexane, 100% dichloromethane, 100% ethyl acetate and 100% methanol) through a sequential extraction method.

2L of each solvent and the ground plant material were added in a 5L round bottom flask and placed on an IKA HS 501 horizontal shaker and stirred occasionally at 110 rpm (revolutions per minute) for 24 h. The filtrate was filtered through a Whatman No.1 filter paper and concentrated using a rotary evaporator to obtain the crude extract (Zhang et al., 2018). The crude extract yields obtained were (hexane, 28.99 g), (dichloromethane, 17.43 g), (ethyl acetate, 18.17 g) and (methanol, 22.58 g).

2.2.1.2. Extraction of Dodonaea angustifolia

The ground dried leaves of *D. angustifolia* (500 g) were extracted with 100% methanol (1.5 L) using a shaker for 72 h, then filtered and concentrated under reduced pressure at 35 °C and afforded (70 g), representing 14% of the green solid. The methanol extract (65 g) was used for fractionation and separation (Dhanarasu, 2012).

2.2.2. Fractionation and separation

The crude extracts (dichloromethane, ethyl acetate and methanol) were fractionated using column chromatography over silica gel (Kiesel 60, Macherey-Nagel, Germany) and eluted

with increasing polarity of 10% hexane/ethyl acetate solvent system to 100% ethyl acetate for *M. angustifolia* and with increasing polarity of 10% petroleum ether/ethyl acetate to 100% ethyl acetate for *D. angustifolia* methanol extract. About 400 fractions of 100 mL volume were collected, evaporated and concentrated under the fume hood to about 15 mL volume and spotted on Alugram Aluminum base sheets (TLC plate) covered with silica gel 60 F_{254} (Macherey-Nagel). The plates were eluted by the use of an adequate solvent system. The fractions were observed under UV light and further sprayed with the *p*-anisaldehyde sulphuric acid reagent to identify non-UV active compounds. Based on their TLC profiles, the fractions were subjected to further purification using a small column (Dhanarasu, 2012). Repeated column chromatography of *M. angustifolia* (dichloromethane, ethyl acetate and methanol) extracts resulted in the isolation of four compounds, a lignan, 5-methoxyjusticidin A (**1**), an ester which is *cis*-phytyl diterpenoidal fatty acid ester (**2**) and two well-known steroids, stigmasterol (**3**) and *β*-sitosterol (**4**). Repeated column chromatography of the methanolic extract of *D. angustifolia* yielded a flavonoid, 5-hydroxy-7,4′- dimethoxyflavone (**5**).

2.2.3. Fourier-Transform Infrared Spectroscopy (FTIR)

Qualitative analysis was performed at Tshwane University of Technology (TUT) using Fourier Transform Infrared (FTIR) instrument (PerkinElmer UATR two FT-IR spectrometer) at a wavelength ranging from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}. Small portions (1.0 mg dissolved in 0.5 mL of Chloroform) of the isolated compounds (**1**–**5**) were placed separately in a single reflection diamond sample hole. The activity was carried out for the identification of functional groups present in pure isolated compounds. The resulting spectrum of the analyzed compounds consisted of many absorption bands of varying intensities, this technique works by absorption of IR radiation by organic compounds through excitation, vibration and rotation transitions.

2.2.4. Nuclear Magnetic Resonance (NMR) spectroscopy

All the NMR techniques were performed and recorded with gradient enhancement using a premium shield NMR spectrometer instrument (400/54/ASP Varian 400 MHz) at the Tshwane University of Technology, Pretoria, Department of Chemistry. Approximately 5.0–7.0 mg of each pure compound (**1**–**5**) was dissolved in deuterated chloroform solvent ($CDCl_3$).

The chemical shifts of the solvent ($CDCl_3$) were recorded at δ_H 7.2 ppm for proton NMR spectra (^{1}HNMR) and δ_C 77.0 ppm for carbon NMR spectra (^{13}CNMR).

2.2.5. High-resolution LC-MS analysis

This study was carried out at Rhodes University, Department of Chemistry, using a standard protocol. The compounds (**1**–**5**) were analyzed on a Bruker Daltonics Compact QTOF Mass Spectrometer using an electrospray ionization probe in positive mode. The Mass Spectrometer was coupled to a Thermo Scientific Ultimate 3000 Dionex UHPLC system consisting of an RS Auto Sampler WPS-3000, Pump HPG-3400 RS, and detector DAD-3000 RS, using an Acclaim RSLC 120 C18, 2.2 µm, 2.1 × 100 mm (P/N 068982) column at 40 °C. A

binary solvent mixture was used consisting of Water-Acetonitrile (10:90, v/v) each solvent containing 0.1% of formic acid. The initial conditions were 100% A at a flow rate of 0.2 mL/min and were maintained for 1 min. The run time was 5 min and the injection volumes ranged between 1 and 3 µL.

2.3. Bacterial and fungal test organisms

The antibacterial and antifungal assays of compounds (**1**–**5**) were carried out in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria.

Three bacterial microorganisms, *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC25922) and *Pseudomonas aeruginosa* (ATCC 27853) and two fungal cultures, *Cryptococcus neoformans* isolated form the lungs of a Cheetah and *Candida albicans* isolated from the pharynx of a Gouldian finch were used for antimicrobial testing. The fungal isolates used were obtained from the culture collection of the Phytomedicine Programme. Isolates in −80°C glycerol stocks were resuscitated in Sabouraud dextrose agar and broth before use for assays.

2.3.1. Quantitative antibacterial assay by minimum inhibitory assay

The antibacterial assay was carried out only for isolated compounds (**1**–**5)** and for *M. angustifolia* crude extracts (dichloromethane, ethyl acetate and methanol). The crude extract of *D. angustifolia* was erroneously omitted, hence the antibacterial activity of the crude extract for *D. angustifolia* is not reported for this assay in Table 2.

Table 1. ^{13}C (δ_c) (100.6 MHz) NMR data for compounds **1**–**5** in $CDCl_3$.

N0	Compound 1	Compound 2	Compound 3	Compound 4	Compound 5
1.	169.6 C	61.2 CH_2	37.2 CH_2	37.2 CH_2	–
2.	–	118.2 CH	31.5 CH_2	31.6 CH_2	78.9 CH
3.	66.5 CH_2	142.6 C	71.7 CH	71.7 CH	43.2 CH_2
3a.	120.6 C	16.4	–	–	–
4.	149.2 C	39.8 CH_3	42.2 CH_2	42.2 CH_2	195.9 C
4a.	122.1 C	–	–	–	–
5.	148.0 C	25.0 CH_2	140.7 C	140.7 C	164.1 C
6.	144.8 C	36.6 CH_2	121.7 CH	121.7 CH	95.1 CH
7.	153.0 C	32.6 CH_2	31.8 CH_2	31.8 CH_2	167.9 C
7a.	–	19.7	–	–	–
8.	103.6 CH	37.4 CH	31.6 CH	29.7 CH	94.2 CH
8a.	133.4 C	–	–	–	–
9.	135.5 C	24.7 CH_3	50.0 CH	50.0 CH	162.8 C
9a.	129.9 C	–	–	–	–
10.	62.1 CH_3	37.4 CH_2	36.4 C	36.4 C	103.1 C
11.	62.4 CH_3	32.7 CH_2	21.1 CH_2	21.0 CH_2	–
11a.	–	17.7	–	–	–
12.	61.4 CH_3	37.3 CH_2	39.7 CH_2	39.7 CH_2	–
13.	55.7 CH_3	24.4 CH	42.2 C	42.2 C	–
14.	–	39.3 CH_3	56.8 CH	56.7 CH	–
15.	–	28.6 CH_2	24.3 CH_2	25.9 CH_2	–
15a	–	22.6 CH_3	–	–	–

16.	–	22.6 CH_3	28.2 CH_2	28.2 CH_2	–
17.	–	–	56.7 CH	56.0 CH	–
18.	–	–	11.8 CH_3	36.4 CH_3	–
19.	–	–	19.0 CH_3	19.3 CH_3	–
20.	–	–	36.1 CH	36.1 CH	–
21.	–	–	19.3 CH_3	27.1 CH_3	–
22.	–	–	138.2 CH	45.7 CH_2	–
23.	–	–	129.2 CH	23.0 CH_2	–
24.	–	–	45.8 CH	12.2 CH	–
25.	–	–	29.2 CH	29.2 CH	–
26.	–	–	19.8 CH_3	20.4 CH_3	–
27.	–	–	18.9 CH_3	19.8 CH_3	–
28.	–	–	23.0 CH_2	19.3 CH_2	–
29.	–	–	11.9 CH_3	11.8 CH_3	–
30.	–	–	–	–	–
1′	–	173.9 C	–	–	130.3 C
2′	101.2 CH_2	34.4 CH_3	–	–	127.7 CH
3′	–	–	–	–	114.2 CH
3a′	149.0 C	–	–	–	–
4′	110.5 CH_3		–	–	160.0 C
4′-OCH_3	–	–	–	–	55.6 CH_3
5′	128.3 C	–	–	–	114.2 CH
6′	123.5 CH	–	–	–	127.7 CH
7′	108.2 CH	–	–	–	–
7a′	147.5 C	–	–	–	–
7′-OCH_3	–	–	–	–	55.6 CH_3

Table 2. Antibacterial and antifungal activity of extracts and compounds.

Sample	***E. coli* (µg/mL)**	***S. aureus* (µg/mL)**	***P. aeruginosa* (µg/mL)**	***C. albicans* (µg/mL)**	***C. neoformans* (µg/mL)**
			Crude extracts		
Dichloromethane (*M. angustifolia*)	2500	2500	312.5	312.5	625.0
Ethyl acetate (*M. angustifolia*)	2500	2500	625.0	312.5	312.5
Methanol (*M. angustifolia*)	1250	1250	312.5	156.25	312.5
Methanol (*D. angustifolia*)	–	–	–	78.0	78.0
			Isolated compounds		
Compound (**1**)	>100	>100	>100	>100	>100
Compound (**2**)	>100	>100	>100	>100	>100
Compound (**3**)	>100	>100	>100	50	25
Compound (**4**)	>100	>100	>100	>100	>100
Compound (**5**)	>100	>100	>100	62.5	250
Gentamicin	0.39	0.39	0.39	–	–
Amphotericin-B	–	–	–	6.25	12.5

(−): Not determined; Gentamicin: Positive control, Amphotericin-B: Positive control, MIC: Minimum inhibitory concentration values given as (µg/mL).

A 96-well micro plate serial dilution method was used to evaluate the antibacterial activity (Eloff, 1998) of the crude extracts and pure compounds 5-methoxyjusticidin A (**1**), *cis*-phytyl diterpenoidal fatty acid ester (**2**), stigmasterol (**3**), *β*-sitosterol (**4**), and 5-hydroxy-7,4'-dimethoxyflavone (**5**) against *Escherichia coli* (ATCC 25922)*, Staphylococcus aureus* (ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 27853). The crude extracts and isolated compounds (**1**–**5**) were dissolved in acetone to a concentration of 10 mg/mL and 100 µL of each test substance was serially diluted two-fold with water in wells of a microtitre plate (Fig. 2) (Masoko et al., 2005). Subsequently, 100 µL of a 3 h old culture of one Gram-positive bacterium, *Staphylococcus aureus* (ATCC 29213) and two Gram-negative bacteria, *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) grown at 37 °C in Múller-Hinton broth (Merck chemicals) were added to each well. Microbes were adjusted to a McFarland 1 standard equivalent to approximately 10^8 cfu/mL. The covered microplates were incubated overnight at 37 °C. Gentamicin and acetone served as positive and negative controls respectively.

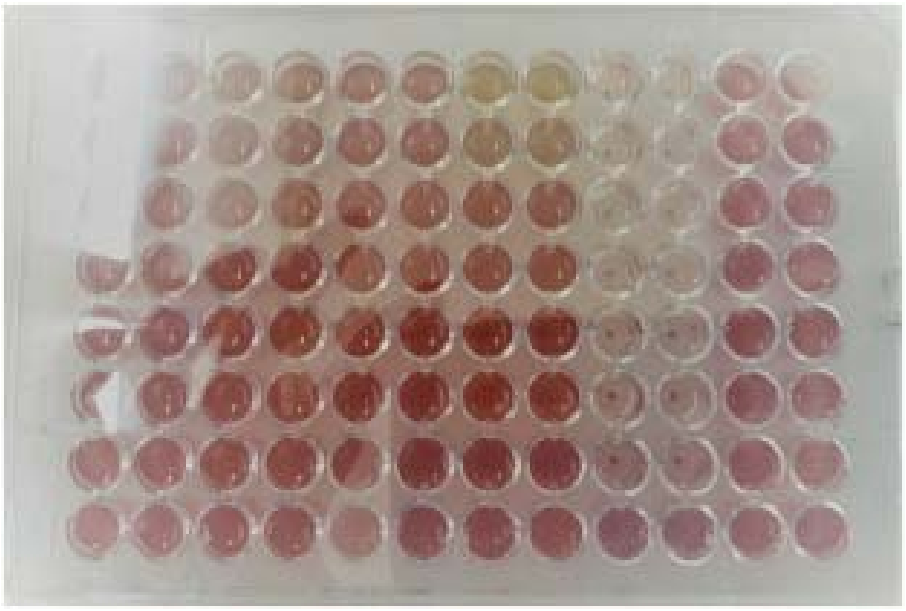


Fig. 2. Compounds (**1**–**5**) added with bacterial cultures, *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* into the wells of the 96-well microtitre plate.

The presence of bacterial growth was detected upon addition of tetrazolium salts: 40 µL of *p*-iodonitrotetrazolium violet (INT) (Sigma Aldirich, S.A.) dissolved to a concentration of 0.2 mg/mL in water and added to the microplate wells and incubated at 37 °C for 10–30 min (Eloff, 1998). According to Eloff (1998) and Masoko et al. (2005) tetrazolium salts are frequently used to indicate biological activity because the colorless compound acts as an electron acceptor and is reduced to a colored product by biologically active organisms.

2.3.2. Quantitative antifungal assay by minimum inhibitory assay

The method previously used for antibacterial activity by Eloff (1998) was used for determining antifungal activities (Masoko et al., 2005). The crude extracts and isolated compounds 5-methoxyjusticidin A (**1**), *cis*-phytyl diterpenoidal fatty acid ester (**2**),

stigmasterol (**3**), *β*-sitosterol (**4**), and 5-hydroxy-7,4'-dimethoxyflavone (**5**) were dissolved in acetone to a concentration of 10 mg/mL and serially diluted twofold with water in microtitre plates as before (total volume in the well was 100 µL). A single colony of fungal cultures, *Cryptococcus neoformans* and *Candida albicans* were transferred from Sabouraud Dextrose (SD) agar plates into fresh SD using a sterile swab and incubated at 37 °C for 24 h. Amphotericin-B served as the positive control. As an indicator of growth, 40 µL of 0.2 mg/mL INT dissolved in water was added to each of the microplate wells (Eloff, 1998; Masoko et al., 2005). The covered microplates were incubated for a day at 37 °C at 100% relative humidity. Minimum inhibitory concentration (MIC) was recorded as the lowest concentration of the extracts and compounds that inhibited antifungal growth after 24 h (Tripathi, 2013).

2.3.3. Cytotoxicity of isolated compounds

Two different cell lines, Madin-Darby canine kidney (MDCK) cells and Vero monkey kidney (VERO) cells were used to test for the cytotoxicity of isolated compounds (**1**-**5**). The specific cells were chosen based on the availability of the respective cells at the time of screening of the samples in this study. The cell toxicity assay was carried out only for pure compounds (**1**–**5**). The cytotoxicity of 5-methoxyjusticidin A (**1**), *cis*-phytyl diterpenoidal fatty acid ester (**2**), stigmasterol (**3**) and *β*-sitosterol (**4**) to Madin-Darby canine kidney (MDCK) cells and 5-hydroxy-7,4'-dimethoxyflavone (**5**) to Vero monkey kidney (VERO) cells was determined using the 3-(4, 5-dimethylthiazol)-2, 5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983; Mcgaw et al., 2007). The cells were maintained in minimal essential medium (MEM, Highveld Biological, South Africa) supplemented with 5% foetal calf serum (Adcock-Ingram) and 0.1% gentamicin (Virbac) in a 5% CO_2 incubator at 37 °C. Cell suspensions were prepared from 70 to 80% confluent monolayer cultures and plated at a density of 5×10^4 cells into each well of sterile 96-well microtitre cell culture plates, which were incubated for 24 h at 37 °C before exposure to the compounds (**1**–**5**) (Famuyide et al., 2019). The isolated compounds (**1**–**5**) were dissolved in acetone (10 mg/mL) and appropriate dilutions were prepared in MEM and added to the wells. Cells were exposed to different concentrations of the compounds (**1**–**5**) (200, 150, 100, 50 and 12.5 µg/mL) for 48 h. Doxorubicin (Pfizer) and acetone served as positive and negative controls respectively. After incubation for 48 h the wells were rinsed twice with 200 µL of phosphate-buffered saline (PBS, Sigma) and 200 µL of fresh medium was dispensed into the wells. Then 30 µL of MTT (Sigma) dissolved in PBS (5 mg/mL) was added to each well and the plates were further incubated for 4 h at 37 °C. Each concentration of compounds (**1**–**5**) was tested in quadruplicate and the assay was repeated three times. The concentration causing 50% inhibition of cell viability (LC_{50}) was calculated using linear regression of the absorbance plotted against the logarithm of the compound concentration.

3. Results

3.1. Structure elucidation

5-Methoxyjusticidin A (**1**), *cis*-phytyl diterpenoidal fatty acid ester (**2**), stigmasterol (**3**) and *β*-sitosterol (**4**) were isolated from the stem barks of *M. angustifolia* and 5-hydroxy-7,4'-dimethoxyflavone (**5**) was isolated from the leaves of *D. angustifolia* (Fig. 3).

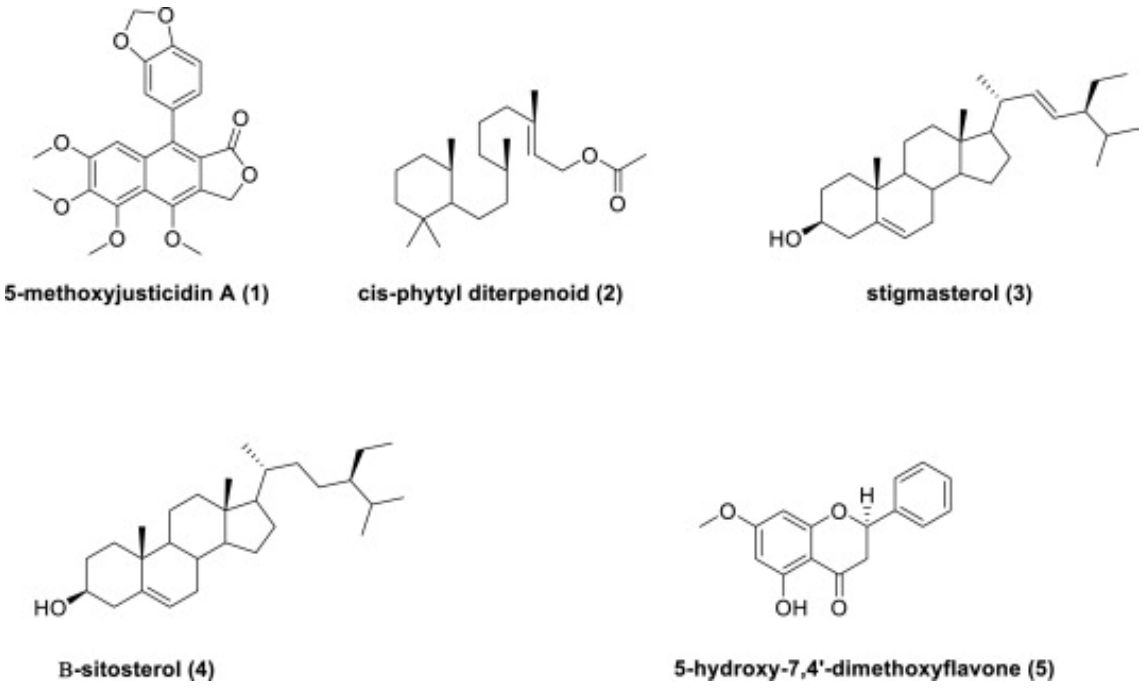


Fig. 3. Structures of isolated compounds from *M. angustifolia* and *D. Angustifolia*.

5-Methoxyjusticidin A (**1**) was obtained as a cream-white amorphous solid with a melting point ranging from (181–189 °C). The HR-ESI-MS showed a *pseudo*-molecular ion peak of $[M+H]^+$ at *m/z* = 425.5003 which corresponded with a molecular formula of $C_{23}H_{20}O_8$. The IR spectrum displayed absorption bands at 1767 (C=O), 2927, 2854 (C–H) and 1614 (C=C) cm^{-1}. The ^{13}C NMR spectrum exhibited 23 carbon resonances and the presence of a cyclic 5-membered ring carbonyl carbon (C=O) which was observed at δ_C 169.6 ppm assigned to C-3a. Furthermore, the ^1H NMR spectrum showed the presence of four methoxy resonances at δ_H 3.98; 3.94; 4.01 and 3.74 ppm assigned to H-10; H-11; H-12 and H-13 respectively. The ^1H and ^{13}C NMR data reported by (Khorombi, 2006; Fouche et al., 2015) were consistent with those of compound **1** and identified as 5-methoxyjusticidin A (**1**).

Cis-phytyl diterpenoidal fatty acid ester (**2**) was isolated as a white powder. The HR-ESI-MS spectrum exhibited a *pseudo*-molecular ion peak of $[M+H]^+$ at *m/z* 339.2155 consistent with a molecular formula of $C_{22}H_{42}O_2$. The IR spectroscopic analysis displayed absorption bands at 2921 and 2854 cm^{-1} due to (CH) stretches. The absorption band at 1742 cm^{-1} confirmed the presence of a carbonyl carbon (C=O) stretch and frequencies at 1465 and 1379 cm^{-1} provided evidence for the presence of a (C=C) stretch. The ^1H NMR spectrum showed six methyl proton resonances assigned to protons (3H-2'; 3H-3a; 3H-7a; 3H-11a; 3H-15a and 3H-16) at δ_H 2.28; 1.67; 0.89; 0.86; 0.84 and 0.88 ppm respectively. A broad singlet peak at δ_H 1.23 ppm was assigned to superimposed methylene proton resonances, H-1; H-4; H-5; H-6; H-8; H-9; H-10; H-12; H-13 and H-14. The ^1H and ^{13}C NMR spectral data were compared with those found in the literature for *cis*-phytyl diterpenoidal fatty acid ester (**2**) (Rasool et al., 1991).

Stigmasterol (**3**) was obtained as a white crystalline solid, with melting point recorded between 129 and 132 °C. The HR-ESI-MS spectrum displayed a *pseudo*-molecular ion peak of

$[M+H]^+$ at *m/z* 413.3845 which is consistent with a molecular formula of $C_{29}H_{48}O$. The IR spectrum exhibited absorption bands at 3423; 2927 and 2851 cm^{-1} due to (OH) and aliphatic (CH) stretching respectively. The absorption band at 1716 cm^{-1} is consistent with a (C=C) stretch. The ^1H NMR spectra showed six methyl proton resonances; two tertiary methyl proton resonances appeared at δ_H 0.83 and δ_H 0.81 ppm assigned to 3H-26 and 3H-27 respectively, one secondary methyl group assigned to 3H-29 appeared at δ_H 0.85 ppm and three primary methyl groups ascribed to 3H-21; 3H-18 and 3H-19 appeared at δ_H 0.90; δ_H 0.66 and δ_H 0.91 ppm respectively. The data obtained from the ^{13}C NMR spectrum displayed twenty-nine carbon resonances which showed downfield alkene carbon resonances at δ_C 138.2 ppm and δ_C 129.2 ppm ascribed to C-22 and C-23 respectively. The ^{13}C NMR further displayed six methyl groups assigned to C-26; C-27; C-29; C-21; C-18 and C-19 at δ_C 19.8; δ_C 18.9; δ_C 11.9; δ_C 19.3; δ_C 11.8 and δ_C 19.0 ppm respectively, which is characteristic of the steroid. The results further confirmed the structure with the reported IR, ^1H and ^{13}C NMR spectral data as stigmasterol (**3**) (Luhata and Munkombwe, 2015).

β-sitosterol (**4**) was obtained as a cream white solid, the melting point of this compound was recorded at (134–136 °C). The HR-ESI-MS spectrum displayed a *pseudo*-molecular ion peak $[M+H]^+$ at *m/z* 415.2770 which is consistent with a molecular formula of $C_{29}H_{50}O$. *β*-sitosterol (**4**) is a derivative of stigmasterol (**3**), however, the downfield ethylene carbons ascribed to carbon resonances C-22 (δ_C 138.4 ppm) and C-23 (δ_C 129.2 ppm) observed in stigmasterol (**3**) are absent. The ^{13}C NMR spectrum displayed upfield methylene carbon resonances at C-22 (δ_C 45.7 ppm) and C-23 (δ_C 23.0 ppm). The ^1H NMR spectrum revealed the absence of ethylene protons at H-22 (δ_H 5.33 ppm) and H-23 (δ_H 5.28 ppm) which are observed in stigmasterol (**3**) and showed the presence of methylene protons at H-22 (δ_H 1.28 ppm) and H-23 (δ_H 1.21 ppm), confirming the compound as *β*-sitosterol (**4**) (Nyingo et al., 2016).

5-hydroxy-7,4′-dimethoxyflavone (**5**) was obtained as a cream-white amorphous solid with a melting point (116–117 °C). The HR-ESI-MS spectrum displayed a *pseudo*-molecular ion of $[M+H]^+$ at *m/z* 301.9516 corresponding to the molecular formula $C_{17}H_{16}O_5$. The IR spectrum exhibited absorption bands at 3395; 2928; 2857 and 1578 cm^{-1} due to (OH), (CH_2), (CH_3) and (C=C) stretching respectively. The spectrum showed the presence of a carbonyl group (C=O) at 1642 cm^{-1}. The ^{13}C NMR spectral data showed the presence of seventeen carbon resonances. The ^{13}C NMR spectrum further revealed the presence of a hydroxyl carbon resonance attached to a benzene ring at δ_C 164.1 ppm assigned to a carbon resonance C-5, which is typical of a flavonoid. The ^{13}C NMR spectrum further revealed one methylene carbon resonance assigned to C-3 (δ_C 43.2) ppm and four methine aromatic carbon resonances on a *para*-substituted B-ring at δ_C 127.7 (C-2′; C-6′) and δ_C 114.2 (C-3′; C-5′) ppm. The ^1H and ^{13}C NMR spectral data reported by Rossi et al. (1997); Mangoyi et al. (2015) were comparable with the data obtained for the isolated compound 5-hydroxy-7,4′-dimethoxyflavone (**5**).

3.2. Spectroscopic data

3.2.1. 5-Methoxyjusticidin A (1)

Compound (**1**); *5-methoxyjusticidin A*, cream white solid (5.0 mg), HR-ESI-MS $[M+H]^+$ *m/z* 425.5003, $C_{23}H_{20}O_8$, (calcd 424.1158). IR (cm^{-1}): 2927, 2854 (C–H), 1767 (C=O), 1614 (C=C); $[\alpha]_D$ = + 42.16 (c = 0.06, $CHCl_3$); mp 181–189 °C, NMR data in Table 1 alongside Supplementary S1.1-S1.5.

3.2.2. Cis-phytyl diterpenoidal fatty acid ester (2)

Compound (**2**); *Cis-phytyl diterpenoidal fatty acid ester*, white crystal solid (7.0 mg), HR-ESI-MS $[M+H]^+$ *m/z* 339.2155, $C_{22}H_{42}O_2$, (calcd 338.5677), IR (cm^{-1}) 2921, 2854 (C–H), 1742 (C=O), 1465, 1379 (C=C), $[\alpha]_D$ = + 48.0 (c = 0.20, $CHCl_3$). NMR data in Table 1 alongside Supplementary S2.6-S2.10.

3.2.3. Stigmasterol (3)

Compound (**3**); *Stigmasterol* [(3*β*,22E)-Stigmasta-5,22-dien-3-ol], white solid (5.0 mg), HR-ESI-MS $[M+H]^+$ *m/z* 413.3845, $C_{29}H_{48}O$ (calcd 412.3705). IR (cm^{-1}) 2930, 2851 (C–H), 3420 (O–H), 1719 (C=C), $[\alpha]_D$ = −3.52 (c = 1.21, $CHCl_3$); mp 129–132 °C, NMR data in Table 1 alongside Supplementary S3.11-S3.15.

3.2.4. β-Sitosterol (4)

Compound (**4**); *β-Sitosterol* [(3*β*)-Stigmast-5-en-3-ol], colourless substance (8.0 mg), HR-ESI-MS $[M+H]^+$ *m/z* 415.2770, $C_{29}H_{50}O$ (calcd 414.3836). IR (cm^{-1}): 2927, 2851 (C–H), 1745 (C=C), 3423 (O–H), $[\alpha]_D$ = +53.5 (c = 0.49, $CHCl_3$); mp 134–136°C, NMR data in Table 1 alongside Supplementary S4.16-S4.20.

3.2.5. 5-hydroxy-7,4'-dimethoxyflavone (5)

Compound (**5**); *5-hydroxy-7,4'-dimethoxyflavone*, white solid (8.0 mg), HR-ESI-MS $[M+H]^+$ *m/z* 300, $C_{17}H_{16}O_5$. IR (cm^{-1}): 2928, 2857 cm^{-1} (C–H), 1578 cm^{-1} (O–H), 1642 cm^{-1} (C=O), $[\alpha]_D$ = + 67.6 (c = 0.22, $CHCl_3$); mp 116–117 °C, NMR data in Table 1 alongside Supplementary S5.21-S5.25. The ^{13}C NMR data for compounds (**1**–**5)** are provided in Table 1 and shown in Fig. 3

3.3. Antibacterial activity

The antibacterial activity of *M. angustifolia* crude extracts and isolated compounds (**1**–**5**) is given in Table 2. The minimum inhibitory concentration (MIC) for the crude extracts against Gram-negative bacteria ranged between 312.5 and 2500 µg/mL and 1250–2500 µg/mL for Gram-positive bacterium. *P. aeruginosa* with MIC value of 312.5 µg/mL showed lower resistance to the methanolic crude extract of *M. angustifolia* than the Gram-positive bacterium, *S. aureus* and Gram-negative bacterium, *E. coli* both with MIC values of 1250 µg/mL. The results demonstrated that the crude extracts (dichloromethane, ethyl acetate and methanol) had moderate activity against selected bacteria and synergistic

effect, while the pure isolated compounds (**1**–**5**) from the same crude extracts showed no significant activity against tested microbes with MIC values > 100 µg/mL.

3.4. Antifungal activity

The isolated compounds (**1**–**5**) and crude extracts of *M. angustifolia* and *D. angustifolia* were tested against *C. albicans* and *C. neoformans* (Table 2). The results showed that the crude extracts displayed good antifungal activity with *C. albicans* overall being most susceptible. The compound 5-hydroxy-7,4'-dimethoxyflavone (**5**) isolated from the leaves of *D. angustifolia* showed moderate activity against *C. albicans* and *C. neoformans* with MIC values of 62.5 and 250 µg/mL respectively. Stigmasterol (**3**) displayed good antifungal activity with MIC values of 25 µg/mL for *C. neoformans* and 50 µg/mL for *C. albicans.* The compounds, 5-methoxyjusticidin A (**1**), *cis*-phytyl diterpenoidal fatty acid ester (**2**) and *β*-sitosterol (**4**) showed no significant activity (MIC >100 µg/mL) against selected fungi.

3.5. Cytotoxicity activity

The cytotoxicity of compounds, 5-methoxyjusticidin A (**1**), *cis*-phytyl diterpenoidal fatty acid ester (**2**), stigmasterol (**3**) and *β*-sitosterol (**4**) against Madin-Darby canine kidney cells and 5-hydroxy-7,4'-dimethoxyflavone (**5**) against VERO monkey kidney cells is given in Table 3. It was necessary to determine the cell toxicity (LC_{50}) of plant extracts and compounds to check how lethal the samples possible are to mammalian cells. According to Kuete et al. (2011) an LC_{50} value above 20 µg/mL indicated that the compounds were relatively non-toxic to cells and an $LC_{50} \leq 20$ µg/mL was considered cytotoxic. The compounds 5-methoxyjusticidin A (**1**) and *β*-sitosterol (**4**) displayed the highest LC_{50} of 81.25 and 196.86 µg/mL respectively, while *cis*-phytyl diterpenoidal fatty acid ester (**2**), stigmasterol (**3**) and 5-hydroxy-7,4'-dimethoxyflavone (**5**) showed LC_{50} values greater than 200 µg/mL.

Table 3. Cytotoxicity LC_{50} (µg/mL) of isolated compounds against MDCK and VERO cells.

Compounds	**Cells**	**LC_{50} (µg/mL)**
Compound (**1**)	MDCK	81.25
Compound (**2**)	MDCK	>200
Compound (**3**)	MDCK	>200
Compound (**4**)	MDCK	196.86
Compound (**5**)	VERO	>200
Doxorubicin (Positive control)	MDCK	3.04

Key: MDCK: Madin-Darby canine kidney, VERO: Vero monkey kidney.

LC_{50}: Concentration inhibiting 50% of growth, values given as (µg/mL).

4. Discussion

The five compounds (**1**–**5**) were isolated from the stem barks of *M. angustifolia* and leaves of *D. angustifolia*, the structures of the isolated metabolites were elucidated using various spectroscopic techniques. The identified compounds (**1**–**5**) were compared to data from previously identified structural databases.

The minimum inhibitory concentration (MIC) of compounds (**1**–**5**) and dichloromethane, ethyl acetate and methanol crude extracts was determined. The crude extracts of *M. angustifolia* showed moderate antimicrobial activity against selected microorganisms and the crude extract of *D. angustifolia* exhibited good antifungal activity against selected fungi (Table 2). For antibacterial assay, the well-known active antibiotic, gentamicin widely used to treat a wide range of bacterial infections (Chen et al., 2014) was used as a positive control. Acetone was used as a negative control, this solvent easily dissolved compounds (**1**–**5**) and was nontoxic to bioassay systems (Masoko et al., 2005). For antifungal assay, the antibiotic amphotericn B was used as a positive control and has been widely used in medicinal applications for fungal infections (World Health Organization, 2010). Gram-positive bacteria are generally more susceptible to antimicrobial substances than are Gram-negative pathogens due to the different cell wall structures of the two groups (Vlietinck et al., 1995). These differences may be attributed to the unique feature Gram-negative bacteria cell wall has which, in contrast to those of Gram-positive bacteria, consists of a hydrophilic lipopolysaccharide outer layer that shows high resistance to antibacterial agents, and also the presence of some enzymes in the periplasmic space which neutralizes antibacterial compounds (Djihane et al., 2017)

However, in this study, the opposite was observed for Gram-negative bacterium, *P. aeruginosa* (MIC = 312.5 µg/mL) which showed lower resistance to the *M. angustifolia* methanol extract than the Gram-positive bacterium, *S. aureus* (MIC = 1250 µg/mL) (Table 2). The results prove that the *M. angustifolia* methanol extract possesses great inhibitory activity against a well-known multi-drug resistant Gram-negative bacterium, *P. aeruginosa*.

The antibacterial and antifungal MIC values of 5-methoxyjusticidin A (**1**) was >100 µg/mL, establishing the antimicrobial activity of this compound as insignificant against the tested microbes. *Cis*-phytyl diterpenoidal fatty acid ester (**2**) also showed no significant antimicrobial activity against tested microbes (MIC>100 µg/mL). This compound is reported for the first time from the stem barks of *M. angustifolia*. Other bioassay studies showed that *cis*-phytyl diterpenoidal fatty acid ester had anti-inflammatory properties and inhibited the inflammatory response through reducing cytokine production and oxidative stress (Silva et al., 2014). Stigmasterol (**3**) showed no significant antibacterial activity but good antifungal activity against *Cryptococcus neoformans* (MIC = 25 µg/mL) and *Candida albicans* (MIC = 50 µg/mL). Stigmasterol (**3)** is a well-known phytosterol reported to have a major function to maintain the structure and physiology of cell membranes and is used to increase the phytosterol content (Cabral and Klein, 2017). Zeb et al. (2017) isolated stigmasterol from the roots of *Indigofera heterantha* and showed significant anti-inflammatory activity. The MIC values of *β*-sitosterol (**4**) for both antibacterial and antifungal activities were >100 µg/mL, establishing the antimicrobial activity of the compound against the selected microbes as insignificant. This compound is a derivative of stigmasterol (**3**) and is among the phytosterols found in many different plants known to have many health benefits including the control of heart diseases, lowering cholesterol content (Ganesan andKumaresan, 2017) and inhibition of colon cancer (Baskar et al., 2010).

The flavonoid, 5-hydroxy-7,4'-dimethoxyflavone (**5**) was isolated for the first time from the leaves of *D. angustifolia* and showed good antifungal activity and insignificant antibacterial activity against tested microbes (Table 2). Bohm (1968) first identified this compound as a

major constituent of the alkali-soluble fraction of the extract of *Pityrogramma calomelanos* and Rossi et al. (1997) isolated this compound from the trunk and stem barks of *Aniba riparia*. In this study, the antifungal activity (MIC = 62.5 µg/mL) of 5-hydroxy-7,4′-dimethoxyflavone (**5**) against *C. albicans* isolated from the pharynx of a gouldian finch was compared to the antifungal activity (MIC = 22.5 µg/mL) of 5-hydroxy-7,4′-dimethoxyflavone previously isolated from the leaves of *Combretum zeyheri* by Mangoyi et al. (2015) against *C. albicans*. The authors did not specify the strain of the *Candida* used. According to Mangoyi et al. (2015), the mechanism of action of 5-hydroxy-7, 4′-dimethoxyflavone as an antifungal includes inhibition of *C. albicans*' ergosterol synthesis, drug efflux pumps as well as the antioxidant enzymes. Contrast between the current study and literature data (Mangoyi et al*.,* 2015) of 5-hydroxy-7,4′-dimethoxyflavone (**5**) isolated from two different plant species, we can conclude that compound (**5**) is an effective antifungal agent against *C. albicans* regardless of the *Candida* strain that was used.

Renal cells are choice models in drug pharmacokinetic and toxicity studies. To determine cell toxicity (LC_{50}), the Madin-Darby canine kidney cells and Vero monkey kidney cells were exposed to different concentration range of compounds from high to low (200, 150, 100, 50 and 12.5 µg/mL). The concentration of 12.5 µg/mL was the lowest concentration employed in the study.

The choice to test compounds (**1**-**4**) isolated from *M. angustifolia* on Vero cells was based on the low level of toxicity the acetone crude extract of *M. angustifolia* had on Vero cells (LC_{50} = 120.37 µg/mL) as previously reported by (Fouche et al., 2016). In this study, the isolated compounds (**1**–**4**) from the dichloromethane, ethyl acetate and methanol crude extracts of *M. angustifolia* were also nontoxic to Vero cells. The selection to test compounds (**5**) on Madin-Darby canine kidney cells was based on the fact that, literature studies revealed no information on the cytotoxicity of crude extracts and isolated compounds from *D. angustifolia* to these cells. According to our knowledge, this appears to be the first study to determine the cytotoxicity of the investigated compounds (**5**) to these cells. All compounds (**1**–**5**) investigated were relatively non-toxic to selected cells with $LC_{50} \leq 20$ µg/mL (Table 3).

5. Conclusion

The dichloromethane, ethyl acetate and methanol extracts and isolated compounds, 5-methoxyjusticidin A (**1**), *cis*-phytyl diterpenoidal fatty acid ester (**2**), stigmasterol (**3**), *β*-sitosterol (**4**), and 5-hydroxy-7,4′-dimethoxyflavone (**5**) were investigated for their antimicrobial activity against test fungi (*C. albicans* and *C. neoformans*) and select bacteria (*E. coli*, *S. aureus* and *P. aeruginosa*). Despite the medicinal importance and good biological activity of compounds (**1**–**5**) previously reported in other studies, the compounds (**1**–**5**) showed insignificant antimicrobial activity against selected microbes in this study, except for stigmasterol (**3**) and 5-hydroxy-7,4′-dimethoxyflavone (**5**) which exhibited strong antifungal activity.

The compounds, *cis*-phytyl diterpenoidal fatty acid ester (**2**) and 5-hydroxy-7,4′-dimethoxyflavone (**5**) were reported for the first time in this study. Further antimicrobial screening of compounds (**1**–**5**) against different pathogenic species of fungi and bacteria will

be conducted, since the extracts from which these compounds were isolated displayed promising antimicrobial activity.

CRediT authorship contribution statement

Zenande K.S. Mcotshana: Data curation, Writing – original draft, Investigation. **Lyndy J. McGaw:** screening of samples and data interpretation. **Douglas Kemboi:** structure characterization & editing. **Gerda Fouche:** structure elucidation. **Ibukun M. Famuyide:** screening of samples and data interpretation. **Rui W.M. Krause:** NMR acquisition, structure characterization. **Xavier Siwe-Noundou:** MS interpretation. **Vuyelwa Jacqueline Tembu:** Conceptualization, of study, Supervision, Writing – review & editing.

Declaration of competing interest

Authors declare no competing interests.

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