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Research article

The attenuation of antibiotic resistant non-albicans *Candida* species, cytotoxicity, anti-inflammatory effects and phytochemical profiles of five *Vachellia* species by FTIR and UHPLC–Q/Orbitrap/MS

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ABSTRACT

This work investigated the antifungal, cytotoxic and LPS-induced anti-inflammatory effects of five *Vachellia* species (*V. karroo*, *V. kosiensis*, *V. sieberiana*, *V. tortalis* and *V. xanthophloea*). The antifungal activity of the aqueous-methanolic extracts were performed using the broth dilution method against four non-albicans *Candida* species (*C. glabrata*, *C. auris*, *C. tropicalis* and *C. parapsilosis*). The cytotoxic and anti-inflammatory effects of the extracts were evaluated on African green monkey Vero kidney cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay and the 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA) method. The fourier-transform infrared spectroscopy (FTIR) and Q Exactive plus orbitrap™ Ultra-high-performance liquid chromatography-mass spectrometer (UHPLC-MS) analysis was conducted to evaluate phytochemical constituents of the extracts. The plant extracts selected in this study displayed potency against the *Candida* species tested, with MIC values ≤0.62 mg/mL for *V. karroo*, *V. kosiensis* and *V. xanthophloea*. A dose-dependent cell viability was observed on Vero cells with all extracts showing LC_{50} values >20 μg/mL. Extracts tested at 10 μg/mL elicited a significant decrease in lipopolysaccharide (LPS)-induced reactive oxygen species (ROS) in Vero cells with *V. sieberiana*, *V. tortilis*, *V. karroo*, *V. kosiensis* and *V. xanthophloea* displaying inhibitory percentages of 35%, 32%, 55%, 52% and 49%, respectively. Characterisation of functional groups representing compounds in the extracts demonstrated the presence of different classes of compounds of the aliphatic, sugar and aromatic types. The Q Exactive plus orbitrap™ mass spectrometer enabled tentative identification of three major compounds in the extracts, including epigallocatechin, methyl gallate and quercetin amongst others. Based on the mass spectrometer results, it is postulated that quercetin found mostly in active extracts of *V. karroo*, *V. xanthophloea,* and *V. kosiensis* may be responsible for the observed antifungal and anti-inflammatory activity. This data demonstrates that the *Vachellia* species that were investigated could potentially be promising candidates for the management of fungal infections and related inflammation.

1. Introduction

Candidiasis is the world's third most common infection caused by opportunistic *Candida* species such as *Candida albicans*, *C. glabrata*, *C. tropicalis, C. parapsilosis, C. dubliniensis, C. guilliermondii, C. krusei*, *C. kefyr* and the currently globally emerging *C. auris*. These *Candida* species are amongst the top ten pathogens causing severe mucosal infections as well as fatal invasive bloodstream infections (Ciurea et al., 2020). Although *Candida albicans* is the most prevalent of these fungal species, there has been a shift towards highly virulent non-albicans species which has resulted in increased death rates, patient hospitalization and higher healthcare costs (Friedman and Schwartz, 2019). In recent years, there have been increasing concerns regarding the alarming rise of non-albicans *Candida* species in different areas worldwide, in addition to antifungal resistance which poses serious healthcare challenges. Paradoxically, the use of broad-spectrum antibiotics plays a major role in the success of these opportunistic fungal strains in immunocompromised patients with underlying diseases including HIV/AIDS, tuberculosis and other respiratory infections (Lamoth et al., 2018; Köhler et al., 2015). Multi-drug resistance of *Candida* species to azoles

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(miconazole, econazole, clotrimazole, flucozole, posaconazole, voriconazole), echinocandins (caspofungin, micafungin, and anidulafungin), allylamines and thiocarbamates have been reported (Berkow and Lockhart, 2017; Pristov and Ghannoum, 2019; Fan et al., 2019) and for this reason, the discovery and development of new antifungal therapeutic agents is significant.

During *Candida* infection, defence mechanisms via phagocytosis are initiated by innate immune cells, including macrophages and neutrophils. The production of reactive oxygen species (ROS) increases at this stage, which play a crucial role as antimicrobial agents (Dantas et al., 2015). It should be noted however that lowered production of ROS can lead to adaptation and resistance to the fungicidal properties of ROS. As a strategy to evade the toxic exposure to ROS, fungal species have the reported ability to convert harmful hydrogen peroxide (H_2O_2) to H_2O, or to modify its morphology for survival. Survival strategies which are also attributed to highly resistant phenotypes include the formation of biofilms and transition from planktonic to sessile forms (Silva et al., 2017). Studies have shown that ageing yeast cells accumulate ROS, which disrupt a diverse array of biological processes in host cells. The production of ROS by innate immune cells is necessary as it creates a toxic environment resulting in oxidative stress and contributes to combating the progression of *Candida* infections, although the production of ROS should however be in balance with antioxidants to avoid the cytotoxic effects of ROS on normal immune cells (Phillips et al., 2003). Uncontrolled increases in ROS may result in inflammatory responses in host cells, which impair host defences by damaging cellular components including DNA, mitochondria, protein and lipids (Vafa et al., 2002). In addition, overproduction of ROS may result in stimulation of antioxidant defence mechanisms and cell death can occur either by apoptosis or necrosis (Perrone et al., 2008).

Medicinal plants have played a crucial role in providing mankind with therapeutic agents to treat various diseases. Natural products are a source of compounds such as phenols, terpenoids, flavonoids, alkaloids and tannins, with distinct mechanisms of action, good bioavailability and very low toxicity *in-vitro* (Rein et al., 2013). Several studies have shown that medicinal plants have the potential of inhibiting fungal strains including *Candida* species (Rein et al., 2013; Pedroso et al., 2019; Steenkamp et al., 2007). Moreover, terpenoids from plants have been reported to inhibit the 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase in fungal strains resulting in antifungal activity (Zore et al., 2011). Carvalho et al. (2018) demonstrated that gallic acid and tannic acid isolated from the ethyl acetate fraction of roots from *Cochlospermum regium* bind to the ergosterol of the fungal membrane, thereby elucidating the antifungal mechanism. Hence, plants with diverse active compounds may potentially provide solutions to the growing problem of combating resistant organisms. In this study, the antifungal activity of five *Vachellia* species was investigated against *Candida* species, and in addition, the anti-inflammatory and cytotoxicity properties were also evaluated. The genus *Vachellia* previously known as *Acacia* belongs to the family Fabaceae, subfamily Leguminosae, which are flowering plants, commonly known as thorn trees. They are characterized by their capitate inflorescence and spinescent stipules which distinguishes them from the genus *Senegalia* (Dyer, 2017). The African species of *Vachellia* includes *V. karroo*, *V. nilotica*, *V. xanthophloea* and *V. sieberiana* have a long history of use in traditional medicine (Kyalangalilwa et al., 2013). Medicinal uses of *Vachellia* species include the treatment of diarrhoea, fever, sore throat and diabetes amongst others (Table 1). Several compounds produced by *Vachellia* species including saponins, tannins, phenols, flavonoids (apigenin, luteolin and quercetin), sugars, anthocyanins, terpenoids, lactones, polyphenols and amino acids have been reported to contribute positively to *in-vitro* biological activities (Pietta, 2000). Primary and secondary compounds may be classified as pro-oxidants (metal chelators) or oxidants which are important in the regulation of ROS that play a role in the pathogenesis of various diseases (Kyalangalilwa et al., 2013; Pietta, 2000). In this study, we evaluated anticandidal activity against four non-albicans candida species namely, *C. glabrata*, *C. tropicalis, C. parapsilosis* and *C. auris*. The anti-inflammatory properties of plant extracts were evaluated using the LPS-induced reactive oxygen species assay, and furthermore, extracts were subjected to FTIR and UHPLC-MS for phytochemical characterization.

2. Materials and methods

2.1. Cells, Candida species, and reagents

African green monkey kidney (Vero) cells were purchased from Cellonex Separation Scientific SA (Pty) Ltd. (Roodepoort, South Africa). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were from Celtic Molecular Diagnostics SA (Pty) Ltd. (Cape Town, South Africa). Methanol, Dimethyl sulfoxide (DMSO), Sabouraud dextrose agar/broth (SDA/SDB), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and 2′,7′-Dichlorofluorescin Diacetate (H_2DCF-DA) reagent were purchased from Sigma-Aldrich® (Darmstadt, Germany). The *Candida* strains were obtained from the American Type Culture Collection (ATCC) and included *C. glabrata* ATCC 15126, *C. parapsilosis* ATCC 22019, *C. auris* CDC B11903, and *C. tropicalis* ATCC 1369.

2.2. Plant collection and extraction

Leaves of five *Vachellia* species (Table 1) were collected from the University of Pretoria (Hatfield Campus: 25° 45′ 10.92″ S, 28° 13′ 47.40″ E) in Gauteng Province of South Africa. The leaves were air-dried at room temperature and pulverized to a fine powder using a grinding mill (IKA™ MF10 Mill, Munich, Germany). Fifty grams (50 g) of powdered plant material was extracted with 500 mL of 50% aqueous methanol, filtered using a Buchi® filtration system and concentrated under speed-vacuum (EZ-2plus GeneVac™ evaporator, St. Louis, MO, USA). The concentrated crude extracts were dissolved in 5% dimethylsulfoxide (DMSO) in ultrapure water and filtered through a 0.2 μm sterile syringe filter to obtain a final stock solution of 10 mg/mL.

2.3. Minimum inhibitory concentration determination

Antifungal activity was assessed using the broth microdilution method described by the Clinical and Laboratory Standards Institute with slight modifications (CLSI, 2008). Pure colonies of an overnight fungal strain cultured on Sabouraud dextrose (SDA) were inoculated in Sabouraud dextrose broth (SDB) and incubated for 24 h at 37 °C. The culture was then diluted with sterile 0.85% sodium chloride (NaCl) solution at a 1:1000 ratio. The absorbance of the culture (0.25–0.28), equivalent to 0.5 McFarland solution, was measured at 530 nm using a spectrophotometer (Masoko et al., 2007; Asong et al., 2019). Sterile 96 well plates were filled with 100 μL SDB, followed by a serial dilution of extracts and amphotericin-B (AMB). The fungal inoculum (100 μL) was then added, resulting in a final extract and AMB concentration range of 0.019–2.5 mg/mL. Microtiter plates were covered with para-film and incubated for 24 hours at 37 °C. After incubation, 50 μL of the fluorometric indicator resazurin (0.2 mg/mL) was added to each well and the minimum inhibitory concentration (MIC) was determined by observing the colour change of the indicator dye after 1h. Resazurin is reduced to resorufin (pink-colour) by mitochondrial oxidoreductases which is indicative of viability while a dark blue colour signifies inhibition. The MIC was defined as the lowest antifungal concentration that maintained a blue colour, which is indicative of fungal inhibition.

2.4. MTT cytotoxicity assay

The cytotoxic activity of the compounds were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay following a method by (Mosmann, 1983), with slight

Table 1. *Vachellia* species selected in this study and their traditional uses to treat various ailments in different countries.

Plant name	Medicinal uses	Plant parts used	Country of use/References
V. sieberiana	Convulsions, sedative (mental illness), dizziness	Bark	Zimbabwe (Stafford et al., 2005)
	Fever	Leaves, Bark, Roots	South Africa (McGaw et al., 2008)
	Sore throat	Root	Nigeria (Kubmarawa et al., 2007)
	Diarrhoea	Bark	South Africa (Cock and Vuuren, 2020)
V. xanthophloea	Diabetes	Bark	Zimbabwe (Stafford et al., 2005)
	Emetic/cathartic, sickle cell anaemia	Roots	Tanzania (Johns et al., 1994)
	Fatigue, indigestion, skin disorders	Bark, Roots	Kenya (Muthee et al., 2011)
	Febrile, fevers, gingivitis, high cholesterol,	Leaves, Bark, Roots	South Africa (Corrigan et al., 2011)
	Malaria, emetic, mouth sores, pharyngitis, tuberculosis symptoms	Bark	South Africa (McGaw et al., 2008)
V. karroo	Diarrhoea, dysentery, gastrointestinal, venereal diseases	Leaves, Roots	South Africa (Corrigan et al., 2011; Mulaudzi et al., 2011)
	Fractures, diarrhoea	Bark	South Africa (Merwe et al., 2001)
	Aphrodisiac, sexually transmitted infections, urinary schistosomiasis	Bark, Roots	Zimbabwe (Merwe et al., 2001; Chigora et al., 2007)
V. kosiensis	No literature on the medicinal uses found		
V. tortilis	Cough	Bark	Nigeria (Kubmarawa et al., 2007)
	Stomach-ache, digestive	Fruits	Yemen (Al-fatimi et al., 2007)
	Indigestion, malaria, strengthen bones, kidney cleanser	Roots	Kenya (Koch et al., 2005)
	Diarrhoea	Branch tips	South Africa (Merwe et al., 2001)

modifications. African green monkey kidney (Vero) cells were maintained in DMEM supplemented with 10 % FBS and 1 % penicillin/streptomycin in culture flasks incubated at 37 °C with 5% CO2. When the cells reached 85 % confluency, cells were detached using 2 % trypsin and a cell count was performed using a handheld automated cell counter (Scepter 3.0™, Merck, Burlington, MA, USA). Vero cells were seeded at 1×10^4 cells/well overnight at 37 °C in 5 % CO2-incubator to allow cell attachment and after 24 hours, the extracts or doxorubicin (positive control) were administered with different concentrations of extracts ranging from 0.78 to 1000 mg/mL. After a further 24-hour incubation, 20 μL of MTT solution (5 mg/mL in PBS) was added into all the wells, followed by 4-hour incubation. DMSO (100 μL) was then added to dissolve the formazan crystals and the absorbance was measured at 570 nm using a microplate reader.

2.5. Measurement of reactive oxygen species in Vero cells

Mitochondrial ROS levels in a Vero cell line was measured using the 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA) fluorescent probe (James et al., 2015). Vero cells were seeded in a 96 well plate and incubated for 24 hours at 37 °C in 5% CO2. The extracts (10 μg/mL) and LPS (1 μg/mL) were added into the 96 well plate and further incubated for 24 hours. The media was then aspirated, followed by the addition of H_2DCF-DA (10 μM) for 30 min in the dark and the absorbance of the fluorescence was measured using a microplate reader at 485 and 535 nm excitation and emission, respectively. Data was processed and analysed using ANOVA, which was followed by Duncan's multiple comparison test.

2.6. FTIR spectrometry

Fourier transform infrared (FTIR) spectroscopy (Vertex 7, Bruker) was used to determine reflectance spectra of the extracts. Dried powdered extracts were placed into the sample chamber of the FTIR and the spectra were recorded in the range of 4500–500 cm^{-1}. Measurements were averaged to 32 scans with a resolution of 4 cm^{-1}. Data were processed using OriginPro 8.1 software (Northampton, Massachusetts, USA) and results obtained from the graphs were compared with data from previously reported publications.

2.7. UHPLC-Q Exactive-Orbitrap-MS analysis

The extracts were separated and analysed using a Q Exactive plus orbitrap™ mass spectrometer coupled with a Thermo Scientific Dionex Ultimate™ 3000 UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA). The Exactive™ plus LC-MS was equipped with a heated electrospray ionization probe and optimum source (with capillary temperature of 290 °C, sheath gas flow, 50 arbitrary units, spray voltage of 3 kV, auxillary temperature of 400 °C). Full MS sim and Full MS/data-dependent (dd-ms^2) with positive or negative polarity switching over a scan range from 100 to 1500 *m/z* with mass accuracy of less than 5 ppm was used to perform analysis. The mass spectrometer was operated at a resolution of 70 000 FWHM in full scan-mode, with automatic gain control target set at $1.0 \times 10^6$and maximum injection time of 100 ms A C18 analytical column (4.6 × 150 mm, 3.5 μm) was used to separate the extracts. The mobile phase consisted of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B). The linear gradient elution mode was as follows: 5% (B) at 0min to 100% (B) at 20min. The mobile phase flow rate of 0.9 mL min^{-1}, sample injection volume of 10 μL and column temperature of 25 °C were used. Data processing was performed using the XCaliber software version 3.0 (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.8. Statistical analyses

Experimental absorbances were quantified with a microplate reader (VarioSkan Flash, Thermo Fisher Scientific, Vantaa, Finland). Data analysis was performed using GraphPad Prism version 8.2 software (GraphPad Software, CA, USA). One-Way Analysis of Variance was used to compare means and the results were expressed as the mean ± SD. Differences between means were determined using the Duncan's multiple range test.

Table 2. Antifungal activity of five *Vachellia* plants.

Plants	MIC (mg/mL)			
	C. glabrata	*C. auris*	*C. tropicalis*	*C. parapsilosis*
V. karroo	**0.62**	**0.31**	**0.31**	**0.20**
V. kosiensis	1.25	1.25	**0.62**	**0.62**
V. sieberiana	2.50	2.50	1.25	1.25
V. tortilis	>2.50	>2.50	2.50	2.50
V. xanthophloea	**0.62**	**0.31**	**0.20**	**0.20**
Amphotericin-B*	3.12	0.78	<0.02	<0.02

The values in bold are considered as significant antimicrobial activity with (MIC ≤0.62 mg/mL).
* Data represented in μg/mL.

3. Results and discussion

3.1. Antifungal activity

The aqueous-methanolic extracts of five *Vachellia* species exhibited antifungal activities against four non-albicans *Candida* species. As shown in Table 2 and Figure 1, *V. Karroo* and *V. xanthophloea* displayed strong inhibitory activity with MIC values ≤0.62 mg/mL for all tested *Candida* species, followed by *V. kosiensis* with MIC values of 1.25 mg/mL against *C. glabrata* and *C.auris*, with the best activity observed against *C. tropicalis* and *C. parapsilosis* (MIC = 0.62 mg/mL). *V. tortilis* failed to inhibit *C. glabrata* and *C. auris* but inhibited the growth of *C. tropicalis* and *C. parapsilosis* at the highest tested concentration (MIC = 0.25 mg/mL). The antifungal activity of *V. karroo* has been extensively reported (Mulaudzi et al., 2011; Maroyi, 2017), and the outcomes of our study are consistent with those of (Mulaudzi et al., 2011) where four *V. karroo* extracts showed MIC values ≤6.25 mg/mL against *Candida albicans*. In their study, the activity observed could be attributed to a high content of compound classes such as phenolics (12.11 ± 0.08 mgGAE/g), flavonoids (7.74 ± 0.16 μgCAE/g) and gallotannin (30.54 ± 9.81 μgGAE/g). The positive control (Amphotericin-B) showed growth inhibitory activity with a MIC value range of 0.02–3.12 μg/mL.

3.2. MTT cytotoxicity assay

The cytotoxic effect of extracts was performed by MTT colorimetric assay. Tested at a varying concentration range (0.78–100 μg/mL), the extracts demonstrated non-toxic results in a dose-dependent manner where a decrease in cell viability was observed with increasing concentrations of the extract. Lethal concentrations (LC_{50}) that reduce 50% of cell viability were recorded (Table 3). LC_{50} values less-than 20 μg/mL were considered toxic according to the United States National Cancer Institute (NCI) criteria for cytotoxicity (Abdel-Hameed et al., 2012). Compared to the positive control (Doxorubicin = 7.3 μg/mL), *V. sieberiana* and *V. tortilis* extracts were the least toxic against the Vero cells with LC_{50} values of 123.00 ± 5.85 and 150.60 ± 4.63, respectively. This was followed by *V. xanthophloea* (42.6 ± 4.00 μg/mL), *V. karroo* (26.70 ± 1.30) and *V. kosiensis* (30.9 ± 0.55) which exhibited low cytotoxic LC_{50} values compared to *V. sieberiana* and *V. tortilis.*

3.3. Measurement of reactive oxygen species

Determining the ability of the extracts to reduce the production of ROS was investigated using the H_2DCF-DA method utilising a non-toxic concentration against Vero cells, where the generation of ROS was induced by stimulating Vero cells with LPS. This experiment allowed us to demonstrate for the first time that the *Vachellia* species tested were able to reduce the oxidative stress induced by an inflammatory stimulus. As shown in Figure 2, after treatment with LPS, elevated ROS was observed. However, the addition of extracts significantly suppressed the elevation of ROS although. *V. sieberiana* and *V. tortilis* exhibited lower ROS inhibitory potentials of 35% and 32%, respectively. The other extracts of *V. karroo*, *V. kosiensis* and *V. xanthophloea* substantially decreased ROS by 55%, 52%, 49%, respectively. These results highlight the anti-inflammatory potential of *Vachellia* species to reduce the inflammatory mediators induced by oxidative stress. Since *V. karroo, V. kosiensis* and *V. xanthophloea* demonstrated significant antifungal activity and reduced ROS levels, while *V. sieberiana* and *V. tortilis* displayed lower activities, it can be hypothesized from the results obtained that reduction of ROS is important in promoting cell survival and contributes to antifungal potency.

Previous studies on *Acacia confusa* extract resulted in the isolation of melanoxetin, which demonstrated significant (98%) suppression of the production of the inflammatory mediators prostaglandin E_2 (PGE_2) and nitric oxide (NO) *in-vitro* in LPS-activated mouse macrophage cells. Melanoxetin exhibited an IC_{50} value of 6.9 μM and was comparable to quercetin with an IC_{50} value of 6.4 μM (Jæger et al., 2019). Another study revealed the anti-inflammatory potential of fractions extracted from *Acacia mearnsii*, which decreased ROS in LPS-stimulated RAW 264.7 macrophage cells and further inhibited the production of NO. ROS, including singlet oxygen, hydrogen peroxide and hydroxyl radicals, are metabolic by products from endogenous or exogenous sources in *Candida* infections. A balance between ROS and antioxidants should however be maintained, since the disruption of this balance due to an overproduction of ROS is related to cellular damage due to oxidative stress (Perrone et al., 2008). Several studies have demonstrated that commercial antifungal agents, such as miconazole (azoles), echinocandins and liposomal induce ROS in fungal biofilms (Donato et al., 2015). Therefore, in our study, lowering of ROS in Vero cells by the *Vachellia* extracts suggest that these extracts have cytoprotective properties against the toxic effects of ROS on normal cells and maintain an antioxidative homeostasis which may contribute to the antifungal effects.

Table 3. Cytotoxicity effects five *Vachellia* species. The values with $LC_{50} > 20$ μg/mL are considered as non-toxic. Data is expressed as Lethal concentration (LC_{50}) values (μg/mL) ± standard deviation (SD) obtained by non-linear regression analysis of three separate experiments.

Plants	LC_{50} ± SD (μg/mL)
	Vero cells
V. karroo	26.70 ± 1.30
V. kosiensis	30.90 ± 0.55
V. sieberiana	123.00 ± 5.85
V. tortilis	150.60 ± 4,63
V. xanthophloea	42.60 ± 4.00
Doxorubicin	7.30 ± 3.50

3.4. FTIR spectrometry

The phytochemical analysis of the functional groups in five *Vachellia* species was evaluated using FTIR spectroscopic analysis. FTIR is an analytical technique that can be used for routine quantitative and qualitative analyses, which measures the vibration of a molecule excited by infrared radiation at a specific range of wavelengths (Santos et al., 2019). The FTIR spectra of the *Vachellia* extracts are shown in Figure 3. Characteristic peaks appeared at ~3286–3306 cm^{-1} and were assigned to the O–H group in a spectrum of the extracts which may possibly represent phenols, carboxylic acids and alcohols. The spectra of *V. karroo, V. kosiensis* and *V. xanthophloea* showed absorption at ~2923 and 2853 cm^{-1} indicating the symmetric stretching of C–H vibration of an aromatic stretch, while *V. sieberiana* and *V. tortilis* displayed a single bend at 2922 cm^{-1} representing C–H stretching (Mohan Reddy et al., 2021). Absorption at ~1797–1695 was present in the *V. karroo, V. kosiensis* and *V. xanthophloea* spectra, while *V. sieberiana* and *V. tortilis* showed absorbance at 1604–1608 cm^{-1}, with these peaks attributed to C=O stretching vibration of carboxylic acids. Peaks at ~1444 $cm^{-1}$and 1348 cm−1, unique in *V. karroo, V. kosiensis* and *V. xanthophloea*, are related to the scissoring vibration of C–C bonds for aromatics and the N–O symmetric stretch of nitro-compounds (Trzebiatowska-Gusowska et al., 2010). Bands at 1068 cm^{-1} and 1034 cm^{-1} were ascribed to C–O–C stretching vibration and C–OH bending vibration as well as the aliphatic alcohol, C–O stretch observed near 1015 cm^{-1}. These peaks may be attributed to saccharides, glycosides and amino acids. Peaks detected at ~ 835 cm^{-1} – 690 cm^{-1} were assigned to the aromatic compounds (Oladunmoye et al., 2018). The spectral data obtained from the FTIR shows the presence of different compound classes such as flavonoids, terpenoids, phenolics, alkaloids and polysaccharides indicated by the presence of alkanes, alkenes, alcohols and ethers, aldehydes and carboxylic acids. The data presented here is in accordance with previous findings where *V. tortilis* was characterized and functional compounds such as aldehydes and

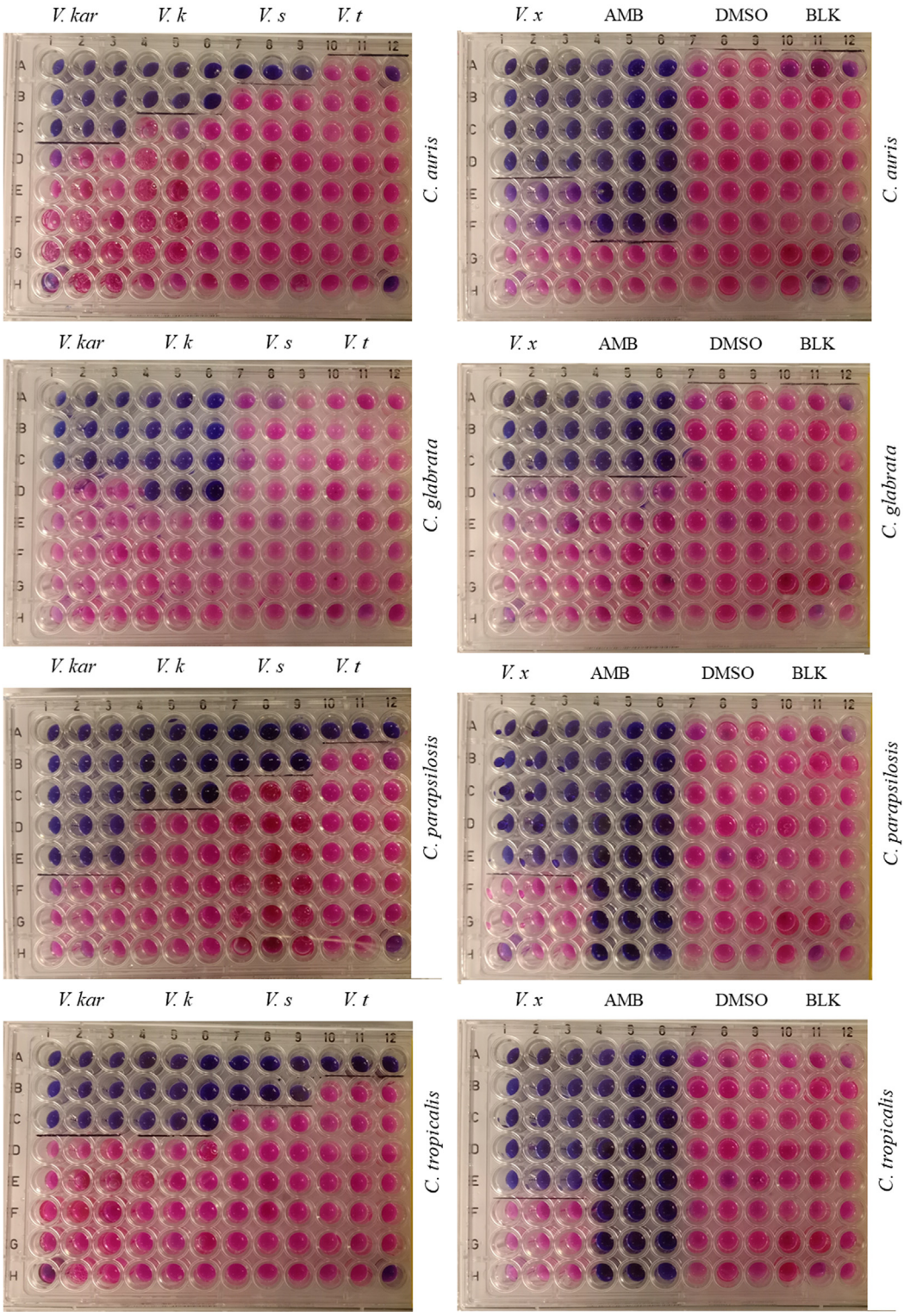

Figure 1. Photographs showing the minimum inhibitory concentrations (MIC) results of five *Vachellia* species evaluated using a two-fold broth microdilution method in 96 well microtiter plates (CLSI, 2008). The non-fluorescent blue dye resazurin is converted into bright pink-fluorescent resorufin in the presence of metabolically active living *Candida* cells. Wells A – H contain the serial diluted concentrations of extracts ranging from 0.019 - 2.5 mg/mL. Extracts and controls were tested in triplicates. *V.kar* = *V. karroo*, *V.k* = *V. kosiensis*, *V.s* = *V. sieberiana*, *V.t* = *V. tortalis* and *V.x* = *V. xanthophloea*, AMB = Amphotericin B, DMSO = 5 % Dimethylsulfoxide, BLK = Blank (*Candida* only).

alkanes were detected (Charis et al., 2020). Other studies on *Vachellia* species have reported the presence of numerous compounds such as phenolics, flavonoids, gallotannin (Mulaudzi et al., 2011), epicatechin, epigallocatechin and β-sitosterol from *V. Karoo* (Nyila et al., 2012) in addition to apigenin-6,8-bis-C-β-d-glucopyranoside (vicenin), Rutin (quercetin 3-O-rutinoside), oleic, linolenic and 1,3-di-O-galloyl-4, 6-(-)-hexahydroxydiphenoyl-β glucopyranose from *V. tortilis* (Yadav et al., 2013). Zeuko (2017) detected the presence of saponin, tannins, cardiac glycosides, steroidal ring, resins and carbohydrates from a *V. sieberiana* acetone extract.

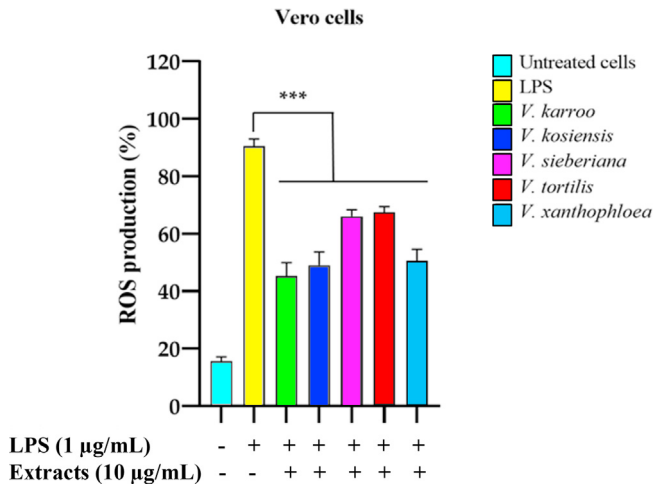

Figure 2. Upregulation of ROS following lipopolysaccharide (LPS) stimulation and inhibitory effects of ROS production by five *Vachellia* extracts on Vero cells. A control group (untreated cells) was included in the experiment to show a normal cell growth which was comparable to the extracts and LPS treated group. The experiments were conducted in triplicates and expressed as the mean ± SD. ***$P < 0.001$ compared with the LPS-treated group.

3.5. UHPLC-Q exactive-orbitrap-MS analysis

The phytochemical content in five *Vachellia* extracts was assessed using UHPLC-MS in addition to the FTIR. Our primary analysis aimed to putatively identify major compounds that could potentially contribute to the antifungal activity from five *Vachellia* extracts. The high resolution UHPLC-MS revealed among others, three prominent polyphenolic compounds in the extracts, tentatively identified on the basis of their mass-to-charge ratio (*m/z*) and fragmentation patterns (Table 4). Mass-to-charge ratio (*m/z*) and fragmentation patterns of these compounds were compared and confirmed using published literature. Figure 4 shows their retention time (min) and mass-to-charge ratio (*m/z*) mass spectra, while Figure 5 shows the extracted chromatograms of these compounds with fragmentation patterns. Retention times for epigallocatechin, methyl gallate, and quercetin using the conditions detailed in section 2.7 were 12.06, 1.67, and 13.19 min, respectively. Interestingly, all three compounds were detected in *V. karroo* and *V. xanthophloea* at high concentrations, while *V. kosiensis* contained epigallocatechin and quercetin. On the other hand, the less active sample, *V. sieberiana* displayed low concentrations of quercetin, while epigallocatechin, methyl gallate, and quercetin were not detected in *V. tortilis* extract. These results suggest that the observed antifungal and anti-inflammatory activities may be mainly due to quercetin acting in synergy with the other compounds in the extracts. to combat fungal growth and ROS.

4. Discussion

Today there are increasing numbers of fungal infections in humans with many of these organisms becoming multidrug resistant, which has stimulated scientific research to investigate solutions to this problem. There has been interest in alternative antimicrobials from herbal extracts for the treatment and management of fungal infections caused by *Candida* species, particularly plant-based medicine, which can potentially provide a rich source of diverse secondary molecules which serve as lead compounds for drug discovery. Previous studies have highlighted the presence of phenolic acids, terpenes, and flavonoids to mention a few in *Vachellia* spp. and these type of compounds have been reported to exhibit various biological activities including antimicrobial, anticancer, anti-inflammatory and antiviral properties (Maroyi, 2017).

In this study, the assessment of antifungal activity indicated that the *Vachellia* species tested could potentially be a significant source of natural anticandidal therapeutic agents. Three *Vachellia* species, namely *V. karroo*, *V. kosiensis* and *V. xanthophloea* displayed strong potency against four *Candida* species, where the results were compared to a commercial antifungal agent, Amphotericin-B, which demonstrated strong inhibitory properties. Evolution in natural product research has shown that incorporation of medicinal plants into nanoparticles is beneficial in increasing the efficacy of these plant extracts. Coumarin and its complexes have been shown to inhibit fungal pathogens such as *C. albicans, C. tropicalis*, and *Aspergillus fumigatus*, where elevation of ROS was responsible for the reduction of the fungal viability. Furthermore, the extracts tested in this study exhibited LC_{50} values greater than 20 µg/mL, which according to the recognized cytotoxicity safety standard are

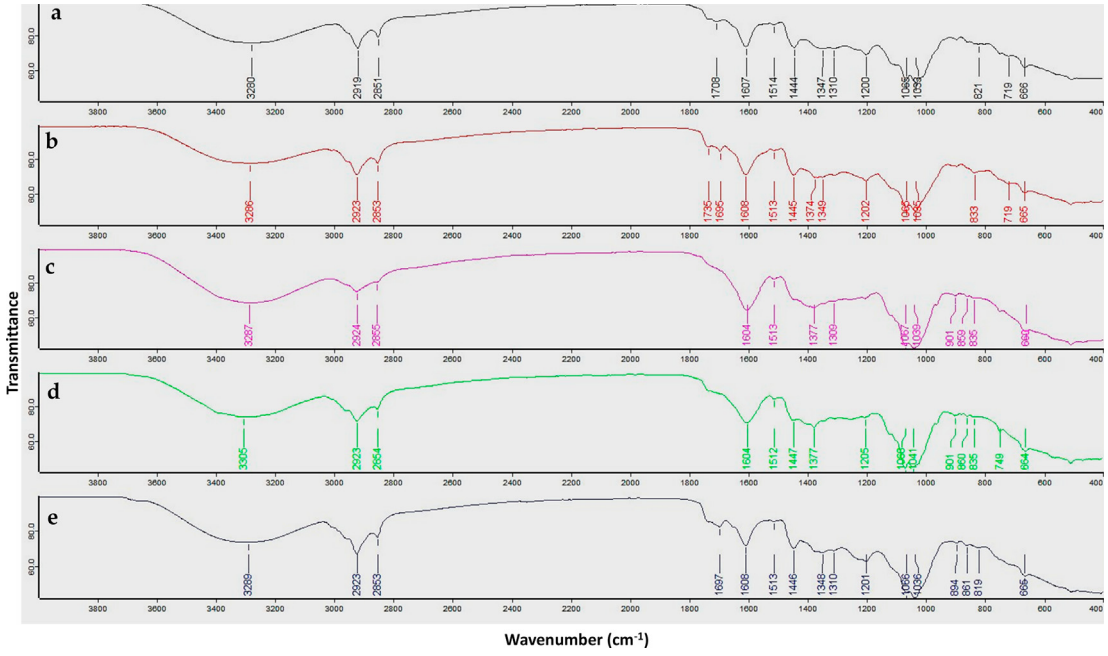

Figure 3. Fourier-transform infrared spectroscopy (FTIR) spectra of five *Vachellia* samples. *V. karroo* (a), *V. kosiensis* (b), *V. tortilis* (c), *V. sieberiana* (d), *V. xanthophloea* (e).

Table 4. Major compounds tentatively identified in negative mode characterised by Q Exactive plus orbitrap™ mass spectrometer coupled with a Thermo Scientific Dionex Ultimate™ 3000 UHPLC system.

Retention Time (min)	Tentative compounds	Empirical formula	Mass (*m/z*)	Fragmentation ion	References
12.06	Epigallocatechin	$C_{15}H_{14}O_7$	306.19235	109.0253, 151.0933, 168.2001, 203.0457, 221.1808, 269.2362, 287.1442, 269.0319	(Yuzuak et al., 2018)
1.67	Methyl gallate	$C_8H_8O_5$	184.06458	101.2185, 124.1692, 128.6672, 153.1128	(Jiamboonsri et al., 2015)
13.19	Quercetin	$C_{15}H_{10}O_7$	302.16901	121.0302, 151.2012, 167.0155, 178.5689, 225.0342, 273.0678	(Chen et al., 2015)

regarded as non-toxic. In addition, *V. karroo*, *V. kosiensis* and *V. xanthophloea* extracts lowered the LPS-induced ROS production in Vero cells, although a lower reduction was observed for *V. tortalis* and *V. seberiana* extracts. This may suggest a cytoprotective property in Vero cells by these extracts against oxidative stress. To support the findings of our investigation, several studies have highlighted the cytoprotective properties of flavonoids especially quercetin and epigallocatechin, which have been found to exhibit antioxidant, hepatoprotective, and genoprotective properties (Saccol et al., 2020; Kim et al., 2019). Literature has shown that the elevation of ROS by phagocytes and neutrophils is important for fungicidal properties, however, it is necessary that the detrimental effects of ROS-toxicity is minimized to maintain viability in normal cells such as hepatocytes and kidney cells, since increased ROS can disrupt cellular functions.

In an attempt to elucidate the compounds from five *Vachellia* extracts with antifungal and anti-inflammatory properties, extracts were subjected to UHPLC-MS analyses. Three prominent compounds, epigallocatechin, methyl gallate, and quercetin were tentatively identified. Previous studies have shown the presence of these compound in *Vachella* species, and their biological activities have been demonstrated. Nyila et al. (2012) screened the ethyl acetate and chloroform extracts of *A. karroo* against *Listeria monocytogenes*. This study led to the isolation of epicatechin, β-sitosterol and epigallocatechin, which reduced the viability of *L. monocytogenes* with a MIC value range of 0.03–0.500 mg/mL. In their review article, Aboody & Mickymaray (2020) outlined the antifungal potency and possible mechanisms of flavonoids. Among the listed flavonoids, quercetin exhibited antifungal activity against *C. albicans, C. glabrata, C. krusei, C. parapsilosis, C. tropicalis, Trichophyton rubrum, Trichosporon beigelii* with MIC values ranging from 31.2 – 125 μg/mL, while epigallocatechin gallate also demonstrated activity against *C. albicans* (MIC = 15–30 μg/mL). Possible mechanisms of action include the induction of mitochondrial dysfunction, plasma membrane disruption, inhibition of cell wall formation, cell division, RNA and protein synthesis, and the efflux

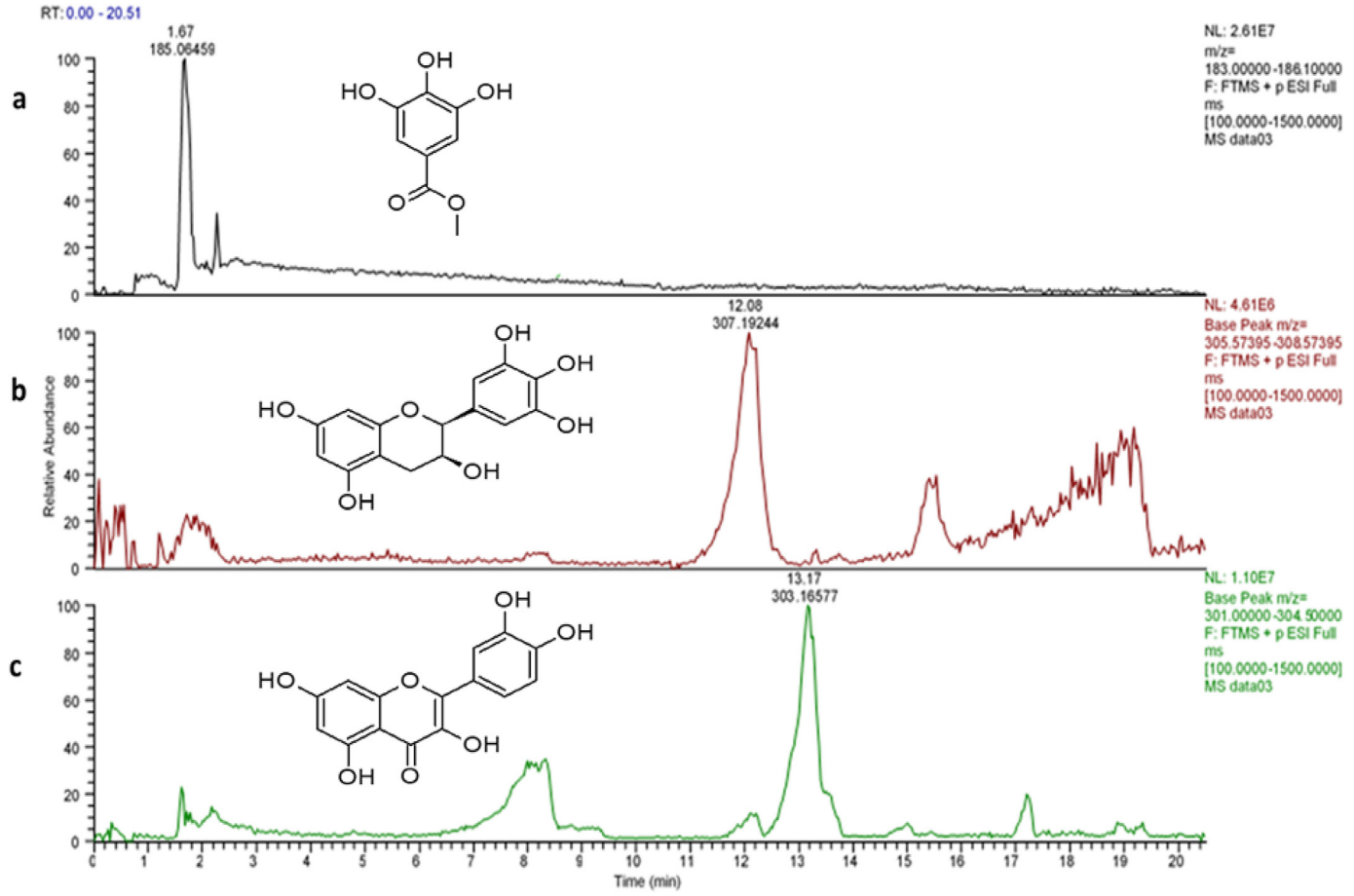

Figure 4. HPLC-MS spectra showing molecular masses with respective chemical structures of prominent compounds: (a) methyl gallate, (b) epigallocatechin and (c) quercetin tentatively identified from five *Vachellia* aqueous-methanol extracts.

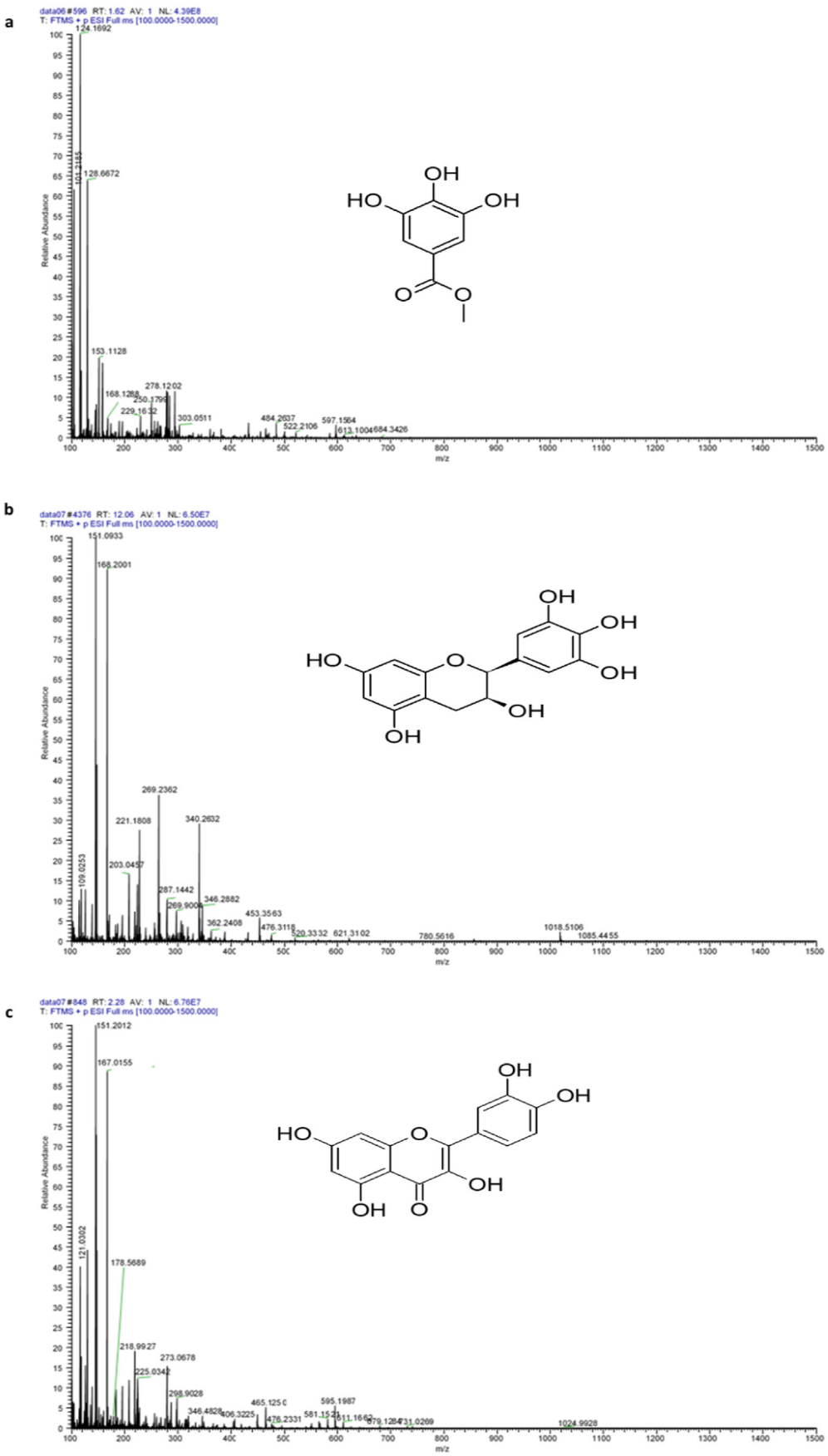

Figure 5. HPLC-MS spectra showing chromatograms with respective chemical structures of prominent compounds: (a) methyl gallate, (b) epigallocatechin and (c) quercetin tentatively identified from five *Vachellia* aqueous-methanol extracts.

mediated pumping system (Kwun and Lee, 2020; Aboody and Mickymaray, 2020).

5. Conclusion

Species of the genus *Candida* are among the leading causes of Candidiasis, and they often show resistance to one or more existing antibiotics. However, plant-based medicine used in traditional practices could be a rich source of therapeutic drug leads. Among five tested aqueous-methanolic extracts, *V. karroo*, *V. kosiensis* and *V. xanthophloea* displayed potency against *Candida* species and were capable of reducing LPS-induced ROS. Data acquired from UHPLC-MS analyses suggest that compounds such as epigallocatechin, methyl gallate, and quercetin are responsible for this antifungal and anti-inflammatory activity. The findings in this study support the use of *Vachellia* species in traditional healing as a source of bioactive compounds to treat and manage several human diseases. Further investigation is warranted, especially for non-albicans species, as their incidence is increasing, and they have shown resistance to several therapeutic agents. Metabolomic research to identify compounds responsible for the reduction of free radicals to better understand their mechanism of action in immune responses are therefore required in future studies.

Declarations

Author contribution statement

Garland Kgosi More: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Christinah Ramakwala Chokwe: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Stephen Meddows-Taylor: Conceived and designed the experiments; Analyzed and interpreted the data.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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