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RESEARCH ARTICLE

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Antifungal Compounds from the Leaves of *Rhynchosia minima*

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Rhynchosia minima, commonly known as jumby bean, is used as a remedy for respiratory ailments in various parts of the world. It is also used by South African traditional healers to treat heart or chest pain. This study aimed to investigate the bioactive constituents of the leaf extracts of *R. minima* against selected fungal isolates that have been identified as risk factors in respiratory illness. *Rhynchosia minima* leaves were extracted sequentially using hexane, dichloromethane, ethyl acetate and methanol in increasing order of polarity. The extracts were subjected to repeated chromatographic techniques, for phytochemical isolation. The extracts and isolated compounds were screened against *Candida albicans* and *Cryptococcus neoformans* by determining the minimum concentration that inhibited fungal growth. Six flavonoids, one norisoprenoid and one cyclitol were isolated and characterized by 1D and 2D NMR and HR-ESI-MS. The extracts obtained in the study had moderate to weak antifungal activities, with MICs ranging from 312.5 to 1250.0 μg/mL against both fungi. Four isolated compounds were also screened, with two of them exhibiting activity against *C. albicans* (MIC = 6.25 μg/mL) that was comparable to amphotericin B, the positive control. These two compounds also had better antifungal potential against *C. neoformans* with an MIC = 6.25 μg/mL, compared to the MIC of 12.5 μg/mL of amphotericin B. Seven of the eight isolated compounds were obtained from the extracts of *Rhynchosia minima* for the first time. Two of the isolated compounds demonstrated activity comparable or superior to amphotericin B activity. The notable potency displayed by these compounds warrants further investigation on their development as antifungal agents.

Keywords: antifungal activity, cyclitol, flavonoid, norisoprenoid, *Rhynchosia minima*.

Introduction

Invasive fungal infections (IFIs) are a scourge to human health as they result in high rates of morbidity and mortality.[1,2] *Candida* and *Cryptococcus* species are the leading causes of these infections. People with compromised immune systems, e.g., organ transplant, HIV/AIDS and cancer patients, are susceptible to contracting these IFIs, which often lead to life-threatening conditions.[3,4] Proper management of these opportunistic infections requires a swift diagnosis and commencement of antifungal therapy.

Cryptococcus neoformans is a major cause of infectious morbidity and mortality, resulting in an estimated 181,000 deaths every year.[5] Cryptococcosis, caused by the fungus *Cryptococcus neoformans*, can be expressed in different forms depending on how it was contracted. Infections may begin in the lungs and spread to other parts of the body such as the central nervous system, urinary system and skin. The pulmonary form often presents in patients with symptoms such as coughing, chest pains, fatigue, skin rash and even bruises.[6]

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Candida species are commonly found in the human gut and can cause a wide range of diseases. Candidiasis is a leading fungal infection caused by *Candida* species.[7] It occurs when the sterile sites of organs such as the lung, eye, liver, spleen, bone, kidney etc., are invaded. *Candida albicans* is the most common cause of invasive candidiasis, and diagnosis can be challenging as symptoms are not specific.[8]

Excellent progress have been made in diagnosing and treating IFIs, however mortality rates are still high in patients of high risk.[9] Drugs presently used in IFI treatment have limitations in terms of their bioavailability, narrow spectrum and absence of oral or intravenous preparations.[10] There is also a significant spike in antifungal resistance, especially by *Candida* species, which is of great concern to scientists and clinicians.[11,12] Due to these reasons, novel antifungal agents with fewer side effects are needed.

Nature has always been a good source of drugs and lead compounds.[13] Polyenes and echinocandins are two classes of successful antifungal agents that are natural products or their derivatives.[14–16]

The genus *Rhynchosia* (Fabaceae) consists of about 230 species.[17] They are widely distributed in the tropical and subtropical regions of the world. Nearly half of the species are found in Africa, and approximately 73 species are native to Southern Africa.[18] The genus is utilized as pasture plants as well as for ornamental purposes. Diverse ethnic groups have used different parts of the plants to treat ailments such as the common cold, rheumatic pains, peptic ulcer, arthritis, migraine, bronchitis, conjunctivitis and asthma.[19–22] It is administered as an abortifacient, used to induce labour and treat wounds, boils and skin diseases.[23–25] Phytochemical studies have revealed that *Rhynchosia* is a rich source of flavonoids and flavonoid glycosides; polyphenols and sterols have also been isolated from them.[26]

Rhynchosia minima (L.) DC. is a flowering plant commonly called least snout bean and jumby bean. It is used to treat skin diseases, particularly boils, in Zimbabwe.[27] Yi Tiao Gen, an herbal medicine prepared from the roots of *R. minima* is used to treat upper respiratory ailments, swelling and joint pains.[28] According to ethnomedicinal reports provided by a South African traditional healer, the leaves are used to treat and prevent heart or chest pain and diseases. The seeds extract was found to have agglutinating effects against human red blood cells.[29]

R. minima is used to treat some of the symptoms observed in IFI patients; hence this research sought to isolate and screen the identified compounds from the leaves against *C. albicans* and *C. neoformans*.

Results and Discussion

Chemistry

The leaf extracts of *R. minima* were subjected to chromatographic purifications, which led to the isolation and characterization of 6 flavonoids, a norisoprenoid and a cyclitol.

Compound **1** was isolated as a yellow solid. The molecular formula was established as $C_{18}H_{16}O_7$ based on ^1H and ^{13}C spectra and the HR-ESI-MS (*m/z* 345.0983 $[M+H]^+$) data. The HR-ESI-MS showed a molecular ion peak at *m/z* 345.0983 $[M+H]^+$ (calculated for $C_{18}H_{17}O_7$, 345.0974 $[M+H]^+$). The proton NMR showed the presence of an AB spin system at δ_H 6.34 (1H, d, $J=2.2$, H-6) and δ_H 6.44 (1H, d, $J=2.2$, H-8), consistent with the *meta* doublets on ring A. The chemical shifts δ_H 7.68 (1H, d, $J=2.1$, H-2′), δ_H 6.96 (1H, d, $J=8.6$, H-5′) and δ_H 7.72 (1H, dd, $J=8.6$, 2.1, H-6′) of the B-ring protons were consistent with the ABX spin system. Furthermore, compound **1** possessed three methoxy groups at δ_H 3.86 (3H, s), 3.87 (3H, s) and 3.98 (3H, s), which were assigned to positions C-3, C-7 and C-4′, respectively, based on their HMBCs. Compound **1** was identified as ayanin (*Figure 1*), with the data consistent with literature values.[30,31]

Compound **2**, a colorless solid, was assigned a molecular formula $C_{16}H_{12}O_6$, determined by the HR-ESI-MS (*m/z* 301.0713 $[M+H]^+$, calculated for $C_{16}H_{13}O_6$, 301.0712 $[M+H]^+$). The ^1H-NMR spectrum displayed signals characteristic of a flavonoid moiety. A para-substituted ring B, evident by δ_H 7.40 (2H, d, $J=8.5$, H-2′, 6′) and δ_H 6.91 (2H, d, $J=8.5$, H-3′, 5′) showed the presence of an AA′BB′ spin system. The singlet at δ_H 7.86 was characteristic of H-2 of an isoflavone, and δ_H 6.52 was attributed to H-8 based on the HMBC. In addition, a methoxy group and a phenolic hydroxy group signals were observed at δ_H 4.03 (OCH_3-6) and δ_H 13.10 (s, 5-OH), respectively. Compound **2** was established as tectorigenin.[32,33]

Compound **3** was obtained as a white amorphous powder with the molecular formula of $C_{11}H_{16}O_3$ as determined by its HR-ESI-MS (*m/z* 197.1182 $[M+H]^+$, calculated for $C_{11}H_{17}O_3$, 197.1178 $[M+H]^+$) data. The signals of an olefinic proton [δ_H 5.68 (1H, s, H-3)], two methylene protons [δ_H 1.97 (1H, dt, $J=14.4$, 2.6, H-5α), δ_H 1.52 (1H, dd, $J=14.4$, 3.9, H-5β), δ_H 2.45 (1H, dt, $J=14.0$, 2.6, H-7α) and δ_H 1.775 (1H, dd, $J=14.0$, 3.9, H-7β)], a methine proton δ_H 4.32 (1H, quint, $J=3.3$, H-6),

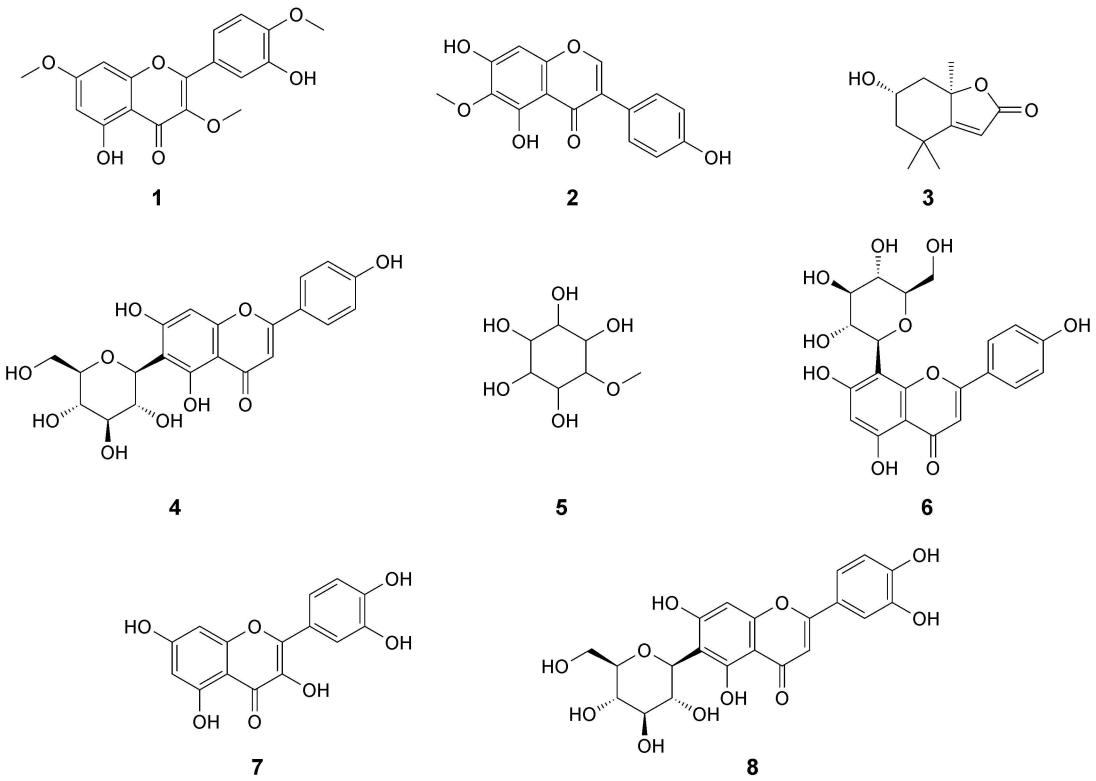

Figure 1. Structures of ayanin (**1**), tectorigenin (**2**), loliolide (**3**), isovitexin (**4**), pinitol (**5**), vitexin (**6**), quercetin (**7**) and isoorientin (**8**) isolated from *R. minima*.

a geminal dimethyl group [δ_H 1.46 (3H, s, H-10), δ_H 1.26 (3H, s, H-11)] and a methyl group [δ_H 1.77 (3H, s, H-12)] were observed on the proton NMR. The ^{13}C-NMR spectrum showed distinctive signals of a lactone carbonyl, olefinic carbon and hydroxylated carbon at δ_C 172.1 (C-2), δ_C 112.9 (C-3) and δ_C 66.8 (C-6), respectively. Compound **3** was characterized as loliolide, and its analytical data is consistent with those previously reported.[34,35]

Compound **4** was obtained as a yellow solid. The molecular formula was determined as $C_{21}H_{20}O_{10}$ by HR-ESI-MS measurement with the molecular ion peak at *m/z* 433.1134 $[M+H]^+$ (calculated for $C_{21}H_{21}O_{10}$, 433.1135 $[M+H]^+$). A pair of doublets at δ_H 6.91 (2H, d, $J=8.4$, H-3′, 5′) and δ_H 7.82 (2H, d, $J=8.4$, H-2′, 6′) and a singlet at δ_H 6.49 (1H, H-8) were seen on the proton NMR spectrum. A signal characteristic of H-3 flavones was observed at δ_H 6.58 (1H, s, H-3). The anomeric proton of the hexose sugar was observed at δ_H 4.91, and other proton signals of the sugar appeared between δ_H 3.43 to δ_H 4.17. The carbon signals attributed to the hexose sugar appeared between δ_C 62.8 to δ_C 80.1. Compound **4** was identified as isovitexin, and the observed data was consistent with literature values.[36,37]

Compound **5** was isolated as a white powder. The molecular formula was indicated to be $C_7H_{14}O_6$ by its positive HR-ESI-MS with *m/z* 217.0678 $[M+Na]^+$ (calculated for $C_7H_{14}O_6Na$, 217.0688 $[M+Na]^+$). The methoxy proton was observed at δ_H 3.43, the other protons were detected ranging from δ_H 2.99 to δ_H 3.62 with some of the signals obscured by the solvent peaks, and the hydroxy protons were visible as doublets from δ_H 4.34 to δ_H 4.73. Compound **5** was elucidated as pinitol as it had consistent NMR data with literature.[38,39]

Compound **6** was purified as a yellow amorphous solid with the molecular formula $C_{21}H_{20}O_{10}$, the same as compound **4**, which was deduced by HR-ESI-MS (*m/z* 433.1137 $[M+H]^+$, calculated for $C_{21}H_{21}O_{10}$, 433.1135 $[M+H]^+$) data. Two doublet signals at δ_H 6.89 (2H, d, $J=8.4$, H-3′, 5′) and δ_H 8.02 (2H, d, $J=8.4$, H-2′, 6′), characteristic of AA′BB′ splitting pattern on the B-ring of flavonoids were observed on the proton NMR. Two singlet signals were observed at δ_H 6.77 and δ_H 6.26 and were assigned to positions H-3 and H-6, respectively, based on HMBC and HSQC correlations.

The beta-anomeric proton of the sugar unit was detected at δ_H 4.68 ($J=9.8$) while other sugar protons appeared between δ_H 3.23 to δ_H 3.83. Compound **6**, a C-glycoside had six carbon signals belonging to the sugar moiety that appeared at δ_C 61.2 (C-6″), δ_C 70.5 (C-4″), δ_C 70.8 (C-2″), δ_C 73.3 (C-1″), δ_C 78.6 (C-3″) and δ_C 81.8 (C-5″). Compound **6** was established as vitexin, and the spectral data agreed with previously reported ones.[40,41]

Compound **7** was obtained as a yellow amorphous powder. The molecular formula was assigned as $C_{15}H_{10}O_7$ based on the HR-ESI-MS data (*m/z* 303.0510 $[M+H]^+$, calculated for $C_{15}H_{11}O_7$, 303.0505 $[M+H]^+$). The proton NMR spectrum showed signals resulting from a meta-coupled aromatic proton at δ_H 6.18 (1H, d, $J=1.9$, H-6) and δ_H 6.38 (1H, d, $J=1.9$, H-8), attributed to the A-ring. The proton NMR also revealed the presence of an ABX spin pattern at δ_H 7.73 (1H, d, $J=2.0$, H-2′), 7.63 (1H, dd, $J=8.5$, 2.0, H-6′) and δ_H 6.88 (1H, d, $J=8.5$, H-5′). Compound **7** was elucidated as quercetin, and it is consistent with known data.[42]

Compound **8** was isolated as a light-yellow substance. The compound's molecular formula was indicated as $C_{21}H_{20}O_{11}$ by its positive HR-ESI-MS with *m/z* 449.1089 $[M+H]^+$ (calculated for $C_{21}H_{21}O_{11}$, 449.1084 $[M+H]^+$). The H-3 of flavones has a peculiar signal observed at δ_H 6.55 (1H, s, H-3), and the signal at δ_H 6.49 (1H, s, H-8) was attributed to the proton on ring A. A signal at δ_H 7.37 (1H, s, H-2′) slightly overlapping with a doublet of doublet at δ_H 7.39 (1H, dd, $J=8.1$, 2.0, H-6′) was also observed on the proton NMR spectrum. In addition, an ortho-doublet was observed at δ_H 6.90 (1H, d, $J=8.1$, H-5′). The anomeric proton of the glucopyranose moiety was observed at δ_H 4.89 while other protons appeared between δ_H 3.44 to δ_H 4.17. The NMR and HR-ESI-MS data were used in combination with literature[37,43] and the compound was identified as isoorientin.

The leaves, flowers, roots, bark and seeds of the *Rhynchosia* genus have been investigated for their phytochemistry. This has resulted in the isolation of flavones, isoflavones, flavanones, isoflavanones, flavanonols and flavonoid glycosides.[44–46] Polycyclic alcohols and some biphenyls were also isolated in the studies.[47,48] Phytochemical investigation of *R. minima* has resulted in the isolation of *C*-glycosylated flavonoids and sterols.[29,49,50] Despite these studies, this is the first study reporting the isolation of the flavonoids ayanin and tectorigenin in the genus. The norisoprenoid loliolide, has never been isolated from the *Rhynchosia* genus. Vitexin and isoorientin are *C*-glycosylated flavonoids that have been isolated in the genus but not in *R. minima*. Of the eight compounds isolated in this study, only isovitexin has been previously isolated from this species.

Biological Activity

There are no previous pharmacological studies on the antifungal activity of *R. minima* extracts. However, a few species in the *Rhynchosia* genus have been reported to possess varying degrees of antifungal activity against selected fungal strains.[19,51,52] Minimum Inhibitory Concentration (MIC) experiments of the extracts were carried out against *C. albicans* and *C. neoformans*. According to Kuete,[53] plant extracts with MIC values lesser than 100 μg/mL are deemed significantly active, MIC values from 100 to 625 μg/mL are moderately active, while MIC values greater than 625 μg/mL exhibit a weak activity. The hexane, DCM and AcOEt extracts were moderately active against *C. albicans* (*Table 1*), with the hexane extract (MIC = 312.50 μg/mL) having the best activity while the MeOH extract exhibited weak activity. Against *C. neoformans*, the hexane, DCM and MeOH extracts, with MIC values of 625.00 μg/mL were moderately active, and the AcOEt extract (MIC = 1250.00 μg/mL) was weak.

Compounds **1**, **3**, **5** and **7** were also screened against both fungal isolates (*Table 1*). Pure compounds with MIC values lesser than 10 μg/mL are significantly active, MIC values ranging from 10 to 100 μg/mL are moderately active, while MIC values greater than 100 μg/mL are weak.[53] Compounds **3** and **7** exhibited antifungal activity comparable to the standard drug, amphotericin B, against *C. albicans* with MIC = 6.25 μg/mL. Compounds **3** and **7** also had better activity

Table 1. Antifungal activity (MIC in μg/mL) of the extracts and selected compounds.

Compounds	*Candida albicans* ATCC 10231*	*Cryptococcus neoformans* ATCC 32045*
1	62.50	125.00
3	6.25	6.25
5	125.00	125.00
7	6.25	6.25
Hexane	312.50	625.00
DCM	625.00	625.00
AcOEt	625.00	1250.00
MeOH	1250.00	625.00
Amphotericin B	6.25	12.50

*Standard deviation (SD) was 0.00 in all cases. Two independent experiments in triplicates (n = 6) were performed.

(MIC = 6.25 μg/mL) against *C. neoformans* than the positive control, amphotericin B (MIC = 12.50 μg/mL). Compound **5** was the least potent, with MIC of 125 μg/mL against both fungi, while compound **1** had moderate activity (MIC = 62.5 μg/mL) against *C. albicans*.

Flavonoids, a class of secondary metabolites that are widely found in plants, have been identified for their diverse biological activities.[54–56] The antifungal activities of flavonoids have also been documented.[57–59] Grayer and Harborne posited that flavonoids play a significant role in defending plants against microbial attack.[60] A study carried out by Sudheeran *et al.*[61] revealed that glycosylated flavonoids possess strong antifungal properties and serve to protect fruits containing them against fungal attacks. Some glycosylated flavonoids isolated from *Colubrina breggii* were reported to show good antifungal activities (MIC values ranging from 16 to 63 μg/mL) against clinical isolates of *Candida* species.[62] Glycosylated flavonoids isolated from other medicinal plants also had good antifungal properties against *C. albicans*.[63] Even though the *C*-glycosylated flavonoids in this study were not screened, Latte and Kolodziej[64] reported that vitexin, isovitexin and isoorientin were inactive at concentrations ranging from 1.7 to 2.3 μM against *C. albicans* and *C. neoformans*.

Quercetin isolated in our study exhibited a strong antifungal activity with an MIC of 6.25 μg/mL against *C. albicans* and *C. neoformans*; a better activity was previously reported in which quercetin isolated from *Leucosidea sericea* had an MIC of 3.9 μg/mL against *C. albicans*.[42] A study conducted by Karioti and coworkers[65] revealed that loliolide had moderate activities against selected fungal isolates with MIC ranging from 3.75 to 10 nmol/mL. Loliolide isolated from the Chinese liverwort *Tritomania quinquedentata* were not active (MIC_{80} > 128 μg/mL) against five strains of *C. albicans*.[66] Loliolide obtained from *Ocotea minarum* had weak anitifungal activites (MIC 100 μg/mL) against three strains of *Candida* (*C. albicans* inclusive) and *C. neoformans*.[67] There could be a variety of reasons for the observed differences in the antifungal activity of loliolide isolated from other sources as against the one reported in this study. Various strains of *C. albicans* and *C. neoformans* were used in the reported studies; hence we could emphasize the differences in the sensitivities of the strains against loliolide. Pinitol isolated from *Artemisia* species moderately inhibited the growth of *C. albicans*.[68]

Conclusion

This is the first time that ayanin, tectorigenin, loliolide, pinitol, vitexin, quercetin and isoorientin are reported from *Rhynchosia minima*. The antifungal activities of the solvent extracts of *R. minima* leaves were investigated in this study, owing to its use in traditional medicine. The solvent extracts showed moderate antifungal activity, and this could be the rationale behind the use of the plant by traditional healers. Loliolide and quercetin isolated from the plant exhibited better activity than amphotericin B against *C. neoformans*, while their activities were comparable to amphotericin B against *C. albicans*. Further studies will be carried out to determine if the isolated compounds are non-toxic and selectively act against the fungi.

Experimental Section

General Experimental Procedures

1D and 2D NMR were acquired at room temperature on either a Bruker Avance III 400 MHz or a Bruker Avance III HD 500 MHz spectrometer. The chemical shifts (δ) were reported in parts per million, relative to the solvent residual peaks ($CDCl_3$, δ_H 7.26 and δ_C 77.16; MeOD, δ_H 3.31 and δ_C 49.00 or (D_6) DMSO, δ_H 2.50 and δ_C 39.52). Solvents used to prepare the extracts and for chromatographic fractionation/purification were purchased from Radchem (Pty) Ltd. (Johannesburg, South Africa). Formic acid (LC/MS grade) used as an additive during LC/MS-SPE purification and deionized water were purchased from Microsep (Pty) Ltd (Johannesburg, South Africa).

HPLC/MS-SPE-NMR analyses of the fractions were performed on an Agilent-1260 Infinity HPLC coupled to a Bruker AmaZon SL ion trap, MS Bruker Prospect II SPE Interface and Bruker Sample Pro Tube. HR-ESI-MS were recorded on a Waters Acquity UPLC system (Waters Corp., MA, USA) coupled to a QTOF Synapt G2 HDMS using electrospray ionization in the positive and negative modes.

Column chromatographic separations were performed using silica gel 60 (0.063–0.200 mm, Merck) and Sephadex LH-20 (Pharmacia). Fractions collected from the columns were monitored using thin-layer chromatography plates (silica 60 F_{254}, Merck) and observed under UV light (254 and 366 nm).

Plant Collection and Preparation

The plant material, collected at Mabopane, Pretoria North, in January 2018 was supplied by Mr. Monare, a traditional healer. The plant was authenticated by Ms. Magda Nel as Rhynchosia minima var prostrata at the H.G.W.J. Schweickerdt Herbarium, University of Pretoria. The voucher specimen was deposited as PRU 127912.

Finely ground *R. minima* leaves (1 kg) were extracted sequentially using hexane, dichloromethane (DCM), ethyl acetate (AcOEt) and methanol (MeOH) (5×5 L each) by subjecting to percolation for 24 h at room temperature, with occasional shaking. The solvents were filtered through Whatman filter paper (No 1). The crude extracts were obtained by concentrating to dryness under reduced pressure at 40 °C, using a rotary evaporator. The yields recovered were 21 g (2.1 %), 11 g (1.1 %), 5 g (0.5 %) and 90 g (9 %) for hexane (3A), dichloromethane (3B), ethyl acetate (3C), and methanol (3D) extracts, respectively. The extracts were stored in glass vials at 4 °C until further use.

Isolation of Compounds

The DCM extract (9.79 g) was fractionated using a silica gel column chromatography (CC) and eluted with hexane – AcOEt (8:2) in an increasing gradient to 100 % AcOEt. The column was then washed with 5 % methanol to obtain sixteen fractions (9A to 9P). Fraction 10 (9J, 205 mg) was subjected to CC on silica gel and eluted with DCM – acetone (9:1) with an increasing gradient up to 100 % acetone, which yielded ten subfractions 20A to 20J. Subfractions 20D (17.4 mg) and 20E (4.7 mg) gave compound **1**. Subfraction 20H (8.9 mg) was subjected to LC-SPE to obtain compound **2** (0.2 mg). Fraction 12 (9L, 290 mg) was loaded on a silica gel CC and eluted with DCM – AcOEt (5:5) with an increasing gradient up to 100 % AcOEt. This gave rise to eight subfractions (21A to 21H). Subfraction 21G (26.2 mg) was loaded on a Sephadex LH-20 column and eluted with acetone – DCM (1:1) to obtain six subfractions 23A to 23F. Subfractions 23D (5.2 mg) and 23E (5.7 mg) gave compound **3**.

The AcOEt extract (3.4 g) was fractionated with a silica gel CC and eluted with 100 % DCM with an increasing gradient of MeOH up to 100 %. This yielded fifteen fractions (33A to 33O). Fractions 33J to 33L showed similar TLC profiles and 33J and 33K were combined. These combined fractions gave a solid precipitate that was insoluble in a MeOH/chloroform ($CHCl_3$) mixture. The soluble portion was loaded on a Sephadex LH-20 column and eluted with MeOH – $CHCl_3$ (7:3) to provide seven subfractions (35A to 35G). Subfraction 35E (23.9 mg) was further purified on a Sephadex LH-20 column and eluted with 100 % MeOH to obtain 36C as compound **4** (3.2 mg). Subfraction 35D (125.7 mg) was washed sequentially with DCM, acetone and MeOH, thereby providing compound **5** (19.9 mg).

The MeOH extract (90 g) was suspended in distilled water in a 2 L separating funnel which was then partitioned with DCM, AcOEt and butanol (BuOH). This afforded: DCM (3.6 g, 4 %), AcOEt (1.6 g, 1.8 %) and BuOH (12.9 g, 14.3 %) fractions. The AcOEt fraction (802 mg) was packed on a silica gel CC, eluted with DCM – MeOH (40:1) with an increasing gradient up to 1:1; six subfractions (45A to 45F) were obtained. A solid precipitated out of 45C, which gave rise to compound **6** (4.2 mg) after a thorough washing in MeOH. Subfraction 45B (82.1 mg) was loaded on a Sephadex LH-20 column and eluted with 100 % MeOH to obtain compound **7** (1.1 mg). The BuOH fraction was subjected to LC-SPE and yielded compound **8** (0.1 mg).

LC-SPE Isolation of Compounds **2** *and* **8**

A Phenomenex Luna 5 µm polar column was used for LC-SPE purification. Thirty microlitres of 10 mg/mL solution of subfraction 20H was injected. The mobile phase consisted of 0.1 % formic acid in methanol (solvent C) and 0.1 % formic acid in water (solvent D) at a flow rate that differed for each sample. To aid trapping, water was continually injected at a 1.5 mL/min flow rate using the Knauer pump. The peaks eluting from the HPLC were trapped continuously on the Spark Holland GP SPE cartridges using the multi-trapping function. The adsorbed compound was eluted with acetonitrile from the cartridges and evaporated. The sample was dried under a high vacuum and redissolved in the appropriate deuterated solvent before NMR analyses.

Compound **2** was isolated with an HPLC flow rate of 0.4 mL/min, using the following method: linear gradient from 0 to 12 min (10–95 % C), 12–20 min (95 % C), 20–22 min (95–100 % C), 22–26 min (100 % C), 26–28 (100–10 % C) and 28–30 (10 % C).

Compound **8** was isolated with an HPLC flow rate of 0.7 mL/min: linear gradient from 0 to 6 min (10–40% C), 6–15 min (40% C), 15–23 min (40–50% C), 23–25 min (50–100% C), 25–31 min (100% C), 31–33 min (100–5% C) and 33–35 (5% C).

Fungal Test Organisms

The fungal isolates *Candida albicans* (ATCC 10231) and *Cryptococcus neoformans* (ATCC 32045) were retrieved from the culture collection of the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria. The fungi were maintained in Sabouraud Dextrose (SD) agar (Oxoid, Basingstoke, UK).

Antifungal Activity by Minimum Inhibitory Assay

The antifungal activity of the extracts and isolated compounds were determined using a previously described method.[69] The crude extracts and compounds were solubilized in acetone to a concentration of 10 mg/mL and 1 mg/mL, respectively and a two-fold serial dilution using sterile distilled water was performed. Overnight cultures of *Candida albicans* and *Cryptococcus neoformans*, appropriately adjusted to 0.5 McFarland standard in Sabouraud broth, were added to the wells. Amphotericin B was used as the positive control, while acetone was the negative control. Forty µL of 0.2 mg/mL *p*-iodonitrotetrazolium violet (INT) dissolved in sterile water was added to each microplate well as an indicator of growth. The plates were incubated for 48 h at 35 °C. The minimum inhibitory concentration (MIC) was recorded as the lowest concentration of the extracts or compounds that inhibited antifungal growth.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Author Contribution Statement

AHA, MAS and NO designed the project. AHA conducted the phytochemical investigation and wrote the draft manuscript. IMF performed the biological assay. MAS and NO supervised the chemistry aspect, while LJM supervised the biology aspect of the work. All authors reviewed, edited, and approved the manuscript for submission.

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