

An antifungal active extract from the aerial parts of *Galenia africana*

F.A. Vries¹, H. El Bitar², I.R. Green³, J.A. Klaasen⁴, W.T. Mabusela^{3,4*}, B. Bodo³ & Q. Johnson^{4,5}

¹Disease Management Division, Agriculture Research Council Infruitec – Nietvoorbij,
Private Bag x 5013, Stellenbosch,; 7599

²Laboratoire de Chimie des Substances Naturelles, ESA 8041 CNRS, 63 rue Buffon, 75005, Paris France

³Department of Chemistry, University of the Western Cape, Private Bag x 17, Bellville, 7535, South Africa

⁴South African Herbal Science and Medicine Institute, University of the Western Cape, Private Bag X 17,
Bellville, 7535, South Africa

⁵Department of Medical Biosciences, University of the Western Cape, Private
Bag X 17, Bellville, 7535, South Africa

*corresponding author: W T Mabusela, Department of Chemistry, University of the Western Cape, Private Bag X17, Bellville, 7535, South Africa; email: wmabusela@uwc.ac.za; tel:+27 21 9593052; fax :+27 21 9593055

Abstract

Dried samples of *Galenia africana* were sequentially extracted with hexane, chloroform, ethanol and distilled water. The ethanol extracts showed antifungal activity using the agar well diffusion method. Ethanol extracts were purified using silica gel column chromatography and thin – layer chromatography. Four fractions showed antifungal activity as evidenced by a clear zone of inhibition. One of these fractions inhibited the growth of fourteen fungal isolates with *Alternaria* sp., *Fusarium equiseti*, *Fusarium graminearum*, *Fusarium verticillioides* and *Phaeomonilla chlamydospora* being the most sensitive ones. The minimum inhibitory concentration for fungi showed values ranging from 10.0 to 2.5%. Spectroscopic analysis of the bioactive fraction of *G. africana* resulted in the identification of flavonoid structures as major components of this fraction.

Key words Index – *Galenia africana*; Aizoaceae; antifungal activity; flavonoids.

Introduction

Galenia africana L., locally known as “kraalbos” or “geelbos”, and belonging to the family Aizoaceae, is a dominant plant throughout Namaqualand, South Africa. “Kraalbos” is an aromatic, woody perennial sub-shrub, growing 0.5 - 1 m high, having oppositely arranged green leaves (5 cm long and hairless) which turn yellow with age (1). Due to severe drought and overgrazing, “kraalbos” is almost the only pasture plant on resource-poor farms in Namaqualand. The plant has been associated with liver damage and severe ascites, a condition commonly referred to as “waterpens” in sheep and goats. Indigenous tribes chewed the plant to relieve toothache and it was said to blister the mucous membrane of the mouth if used excessively. The plant was also used in the treatment of venereal diseases and a decoction as a lotion for skin diseases, including ringworms, and for the relief of inflammation of the eyes (2).

The objectives of this study were to verify the implicated antifungal activities of the *G. africana* extract and to isolate, purify and characterise the natural products contained in the plant material.

Results and Discussion

The ethanol extract, which was obtained as a yellow solid, was fractionated on a silica gel column to yield fractions as depicted in Table 1. Although it would have been most ideal to work with human fungal pathogens, the ready availability of plant fungal pathogens in our lab persuaded us to use the latter specimens. Thus the combined fractions 1 – 29, 30-34 and 35 – 72 exhibited antifungal activity against *F. verticillioides*. Combined fractions (1 – 29)

were rechromatographed and gave rise to 3 subfractions (1-3; 7-9 and 11-13) in which fractions 11 – 13 (0.0110 g) retained the antifungal activity (Table 2). Similar treatment of combined fractions (30 – 34) resulted in one subfraction 5 – 11 (0.0078 g), which did not show any antifungal activity. Combined fractions 35 – 72 were also rechromatographed, using CH₂Cl₂ – EtOAc - Hexane (50: 20: 30) as eluent to afford major subfractions 9 – 29 (0.9616 g), which showed significant antifungal activity as evidenced by a clear zone of inhibition. The inhibition zones ranged between 3.1 – 6.0 mm for subfractions 9 – 29 and 1.0 – 3.0 mm for subfractions 11 – 13.

Table 1. Antifungal activity of combined fractions against the growth of *Fusarium verticillioides*

Combined fraction ^a	Incubation time (day)		
	3	5	Control
1-29	++	++	- ^b
30-34	++	+ ^c	-
35-72	++ ^d	++	-
79-105	-	-	-
124-159	-	-	-

^a Fractions combined according their similarity showed on TLC plates (solvent system CH₂Cl₂-EtOAc- hexane 75:10:15).

^b - = no inhibition zones present.

^c + = clear zones of inhibition between 1.0 – 3.0 mm.

^d ++ = clear zones of inhibition between 3.1 – 7.0 mm.

Table 2. Antifungal activity of rechromatographed fractions against the growth of *Fusarium verticillioides*

Combined ^a fractions	Rechrom. ^b fractions	Weight (g)	Incubation time (days)		
			3	5	Control
1-29	1-3	0.0449	- ^c	-	-
	7-9	0.0117	-	-	-
	11-13	0.0110	+ ^d	+	-
30-34	5-11	0.0078	-	-	-
35-72	9-29	0.9616	++ ^e	++	-

^a Fractions were combined according their similarity on TLC (solvent system CH₂Cl₂-EtOAc- hexane 75:10:15).

^b These fractions were the result of rechromatography on silica gel, using CH₂Cl₂-EtOAc-hexane 50:20:30 as eluent).

^c - = no inhibition zones present.

^d + = clear zones of inhibition between 1.0 – 3.0 mm.

^e ++ = clear zones of inhibition between 3.1 – 5 mm.

The rechromatographed fractions (9-29) was effective against most of the fungal isolates, where *Alternaria* sp., *F. equiseti*, *F. verticillioides*, *Botrytis cinerea* and *Cylindrocarpon* sp. showed clear zones of inhibition up to 5 days, while *P. chlamydospora* was the most sensitive in showing inhibition zones up to 9 days (Table 3). All control wells were overgrown by the

fungal isolate being tested.

Table 3. Effect of the rechromatographed fractions (9-29) on the growth of different fungal species cultured on PDA (20 ml agar) with initial inoculum levels of 1×10^6 spores/ml at 27 °C for 2-10 days

Rechromatographed fractions ^a	Fungal isolates	Incubation time (days)							control
		2	3	5	7	9	10		
9-29	<i>Alternaria</i> sp.	+	+	+ ^b					- ^c
	<i>Botrytis cinerea</i>			++	-	-	-	-	-
	<i>Cylindrocarpon</i> sp.			++	-	-	-	-	-
	<i>Eutypa lata</i>	+	-	-					-
	<i>Fusarium equiseti</i>	++ ^d	+	+					-
	<i>Fusarium graminearum</i>	+	-	-					-
	<i>Fusarium oxysporum</i>	++	-	-					-
	<i>Fusarium solani</i>	++	-	-					-
	<i>Fusarium verticillioides</i>	++	+	+					-
	<i>Penicillium expansum</i>				-	-	-	-	-
	<i>Phaeoconiella chlamyospora</i>			+	+	+	-	-	-

^a Combined fractions 35 – 72 were rechromatographed using CH₂Cl₂-EtOAc- Hexane 50: 20: 30 as the eluent and afforded a major subfractions 9 – 29 (0.9616 g).

^b + = clear zones of inhibition between 1- 3 mm.

^c - = no inhibition zones present.

^d ++ = clear zones of inhibition ranging from 3.1 – 5 mm.

For *Alternaria* sp, *F. oxysporum* and *F. verticillioides* a MIC of 5% was required to prevent the organisms from growing, whereas a figure of 2.5% was obtained for *Cylindrocarpon* sp., *F. equiseti*, *F. graminearum* and *P. chlamyospora* (Table 4). *F. solani* required a higher concentration (10%) of the active fraction to prevent fungal growth. A concentration of 0.625% and 1.25%, respectively was required to inhibit the growth of *E. lata* and *B. cinerea*.

Table 4. The minimum inhibitory concentration (MIC) of the rechromatographed fractions (9-29) tested against different fungal isolates

Fungal isolates	MIC (%)	Inhibition zones (mm)
<i>Alternaria</i> sp.	5	2.2
<i>Botrytis cinerea</i>	1.25	1.0
<i>Cylindrocarpon</i> sp.	2.5	2.4
<i>Eutypa lata</i>	0.625	0.7
<i>Fusarium equiseti</i>	2.5	3.2
<i>Fusarium graminearum</i>	2.5	1.9
<i>Fusarium oxysporum</i>	5	0.3
<i>Fusarium solani</i>	10	0.6
<i>Fusarium verticillioides</i>	5	0.4
<i>Phaeoconiella chlamyospora</i>	2.5	1.1

The active fraction was inoculated onto individual filter paper disks, whereupon it displayed potent antifungal activity on agar plates seeded with conidia of the various fungal isolates that were tested (Table 5). The fraction showed potent activity as was measured by a zone of inhibition surrounding each disk at low concentrations against *B. cinerea* (0.312%), as well as *Alternaria* sp., *E. lata*, *F. equiseti*, *F. graminearum* and *F. pseudograminearum* (0.625%). The fraction was also inhibitory to the growth of *Cylindrocladium* and *F. tricinctum* at a MIC of 5%. The MIC for *Cylindrocarpon*, *F. oxysporum* and *P. expansum* was 2.5%, whereas a concentration of 1.25% was required to inhibit the growth of *F. verticillioides* and *P. chlamydosporum*.

Table 5. Effect of the rechromatographed fractions (9-29) on the growth of different fungal species by the filter paper method on PDA plates (20 ml agar) with initial inoculum levels of 1×10^6 spores/ml at 27 °C for 5 days

Rechrom fractions ^a	Fungal isolates	MIC ^b	Inhibition zones (mm)
9-29	<i>Alternaria</i> sp.	0.625	0.5
	<i>Botrytis cinerea</i>	0.312	0.6
	<i>Cylindrocarpon</i> sp.	2.5	4.2
	<i>Cylindrocladium</i> sp.	5	0.4
	<i>Eutypa lata</i>	0.625	1.8
	<i>Fusarium equiseti</i>	0.625	3.2
	<i>Fusarium graminearum</i>	0.625	3.4
	<i>Fusarium pseudograminearum</i>	0.625	0.8
	<i>Fusarium oxysporum</i>	2.5	0.4
	<i>Fusarium solani</i>	10	0.3
	<i>Fusarium tricinctum</i>	5	1.6
	<i>Fusarium verticillioides</i>	1.25	0.5
	<i>Penicillium expansum</i>	2.5	0.9
	<i>Phaeomoniella chlamydospora</i>	1.25	2.2

^a Combined fractions 35 – 72 were rechromatographed using CH₂Cl₂-EtOAc- hexane 50: 20: 30 as the eluent and afforded a major subfractions 9 – 29 (0.9616 g).

^b MIC = Minimal inhibitory concentration

The filter paper disk method was found to be more sensitive than the agar well diffusion method in the detection of antifungal activity. Several different methods exist to test the susceptibility of microorganisms against antimicrobial compounds. These include broth dilution (3), agar – diffusion (4), disk diffusion (5), and microtiter bioassays (6). These bioassays were found to be useful in detecting proper MIC zones.

Structural analyses

The ESI mass spectrum of **1** displayed the protonated molecular [M+H]⁺ ion at *m/z* 271 and together with the ¹H- and ¹³C-NMR data, indicated the molecular formula C₁₆H₁₄O₄. The ¹³C-NMR spectrum showed signals for 16 carbons among which was a methoxyl group at 56 ppm. In the ¹H-NMR spectrum (Table 6) the basic structure for a flavanone was readily recognized from an ABX spin system at 2.81 (dd, 17.2, 2.9 Hz) ; 3.07, (dd, 17.2, 13.0 Hz) and 5.41 (dd, 13.0, 2.9 Hz) characteristic of protons at –3 and –2 respectively. A chelated hydroxyl group was observed at 12.00 ppm, and assigned from the HMBC spectrum at -5 on A-ring which had in addition two *meta* coupled protons at 6.06 and 6.07 (d, 2.3 Hz each). The

methoxyl group (δ_{H} 3,79 ppm) was assigned to position -7 from the HMBC correlations and the NOEs interactions with both H-6 and H-8. The B-ring was a non substituted phenyl ring. The structure of **1** was thus 5-hydroxy-7-methoxy-flavanone or pinostrobin (7).

Table 6: ^1H NMR (400 MHz) data of Compounds 1 and 2

H No.	COMPOUNDS					
	1			2		
	δ	m	J (Hz)	δ	m	J (Hz)
2	5.41	dd	13.3, 3.0	5.77	dd	13.3, 3.0
3e	2.81	dd	17.2, 3.0	2.94	dd	17.3, 3.0
3a	3.07	dd	17.2, 3.0	3.09	dd	17.3, 13.3
6	6.06	d	2.3	6.09	d	2.3
8	6.05	d	2.3	6.07	d	2.3
2'	7.42	m	-	-	-	-
3'	7.42	m	-	6.86	dd	8.1, 1.5
4'	7.42	m	-	7.24	ddd	8.1, 8.1, 1.5
5'	7.42	m	-	6.76	ddd	14.5, 1.5
6'	7.42	m	-	7.34	dd	14.5, 1.5
OMe-7	3.79	s	-	3.80	s	-
OH-5	12.00	s	-	12.01	s	-

Compound **2** gave a $[\text{M}+\text{H}]^+$ ion at m/z 287 in the ESI MS and was assigned the formula $\text{C}_{16}\text{H}_{14}\text{O}_5$ as 16 carbons were depicted in the ^{13}C -NMR spectrum and 14 protons in the ^1H -NMR spectrum. This spectrum was also typical of a flavanone structure, with H-2 at 5.70 (dd 13.3, 3.0 Hz) and H-3 at 2.94 (dd 17.3, 3.0 Hz) and 3.09 (dd 17.3, 13.3 Hz). The chelated hydroxyl group (δ_{H} 12,01) was assigned to -5 and the methoxyl group (δ_{H} 3.80) to -7 from the HMBC data. The remaining hydroxyl group was linked at -2' on B-ring from long rang correlations and thus compound **2** was identified as dihydroechinoidinin (**8**) recently isolated from the Acanthaceae *Andrographis echoides*.

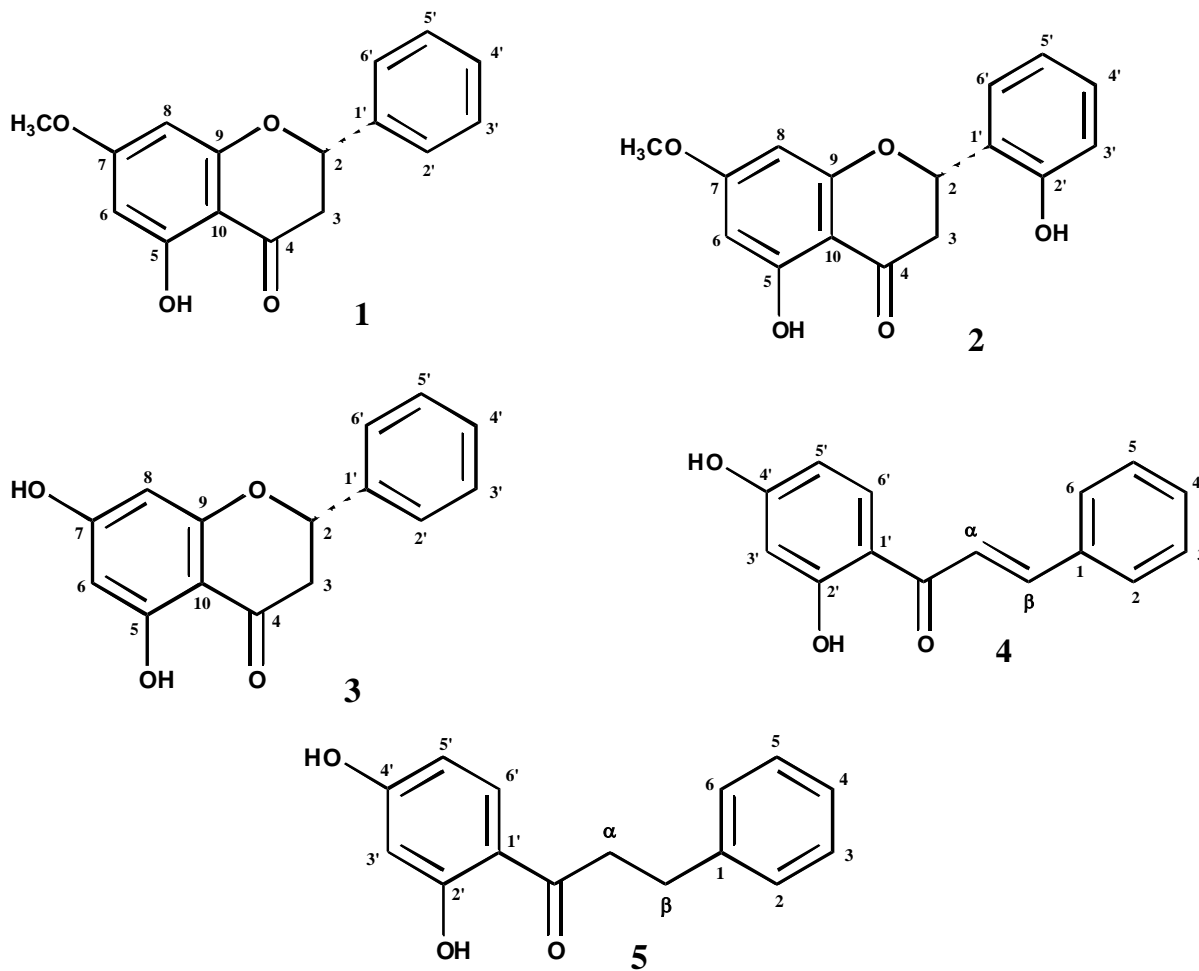
Compound **3** had the $[\text{M}+\text{H}]^+$ ion at m/z 257 ($\text{C}_{15}\text{H}_{12}\text{O}_4$) in the ESI MS and was identified as 5,7-dihydroxy-flavanone or pinocembrin (**9**) from NMR data. Compound **4** gave yellow crystals and analyzed as ($\text{C}_{15}\text{H}_{12}\text{O}_3$) from ESI MS ($[\text{M}+\text{H}]^+$ ion at m/z 241) and NMR data which allowed it to be identified as 2',4'-dihydroxychalcone, with *trans* stereochemistry of the $\alpha\beta$ double bond ($J = 15.5$ Hz) (**9**). The strongly chelated 2'-hydroxy group was depicted at 13,36 ppm.

Compound **5** was characterized as the $\alpha\beta$ -dihydro-derivative of **4**, showing the $[\text{M}+\text{H}]^+$ ion at m/z 243 ($\text{C}_{15}\text{H}_{12}\text{O}_4$) in the ESI MS, and the $\alpha\beta$ -ethylenic protons of **4** being replaced in the ^1H -NMR spectrum (Table 7) by two methylenes at δ_{H} 3.22 and 3.03, each as a triplet ($J = 7$ Hz) and were observed in the ^{13}C -NMR spectrum at $\delta_{\text{X}}\text{H}$ 39.7 and 30.4 ppm, respectively. It was identified as 2',4'-dihydroxydihydrochalcone (**10**).

Table 7: ¹H NMR (400 MHz) data of Compounds 4 and 5

H No.	COMPOUNDS					
	4			5		
	δ	m	J (Hz)	δ	m	J (Hz)
2,6	7.63	m	-	7.23	m	-
3,5	7.41	m	-	7.29	m	-
4	7.40	m	-	7.20	m	-
a	7.55	d	15.5	3.22	t	7.0
b	7.87	d	15.5	3.03	t	7.0
3'	6.41	d	2.4	6.35	d	2.4
5'	6.42	dd	8.8, 2.4	6.33	dd	8.4, 2.4
6'	7.82	d	8.8	7.61	d	8.4
OH-2'	13.36	s	-	12.71	s	-

Finally, compound **6** was identified as lupeone from spectral data.



Conclusion

The results obtained serve to validate the antifungal properties of the plant. Such properties may be associated with the flavonoid structures characterised. Furthermore, it is apparent that the antifungal activity probably requires a specific combination of these flavonoids rather than any single one of these compounds, given that none of them have been previously implicated in this way. Although the antifungal activity was tested mainly against plant pathogens, it may be anticipated that similar activity might be displayed against human pathogens as well.

Materials and Methods

General procedures

¹H and ¹³C NMR spectra were recorded either (1D ¹³C) on a Bruker AC 300 spectrometer, equipped with an Aspect 3000 computer using DISNMR software or (2D spectra) on a Bruker Avance 400 spectrometer operating at 400.13 MHz (2D spectra). The coupling constant used to establish the necessary delay for the selection of the proton coupled to the carbon in the HSQC spectrum was 135 Hz, corresponding to a delay of 3.7 ms ; the delay for the HMBC spectra was 70 ms corresponding to a long-range coupling constant of 7 Hz. The phase sensitive ROESY experiments were obtained with mixing time of 150 ms. Mass spectra were recorded on an API Q-STAR PULSAR of Applied Biosystem.

Fungal isolates

Compounds were tested for antifungal activity against 14 different fungal species: *Alternaria* sp., *Botrytis cinerea*, *Cylindrocladium* sp., *Cylindrocarpon* sp., *Eutypa lata*, *Fusarium equiseti*, *F. oxysporum*, *F. graminearum*, *F. pseudograminearum*, *F. solani*, *F. tricinctum*, *F. verticillioides*, *Penicillium expansum* and *Phaeoemoniella chlamydospora*. The cultures were obtained from the culture collection of the Disease Management Division, Agricultural Research Council (ARC) Infruitec - Nietvoorbij, South Africa and from the Programme on Mycotoxins and Experimental Carcinogenesis (PROMECA), Medical Research Council (MRC), South Africa. *Fusarium* spp. was maintained on carnation leaf agar (CLA) and the other fungi on potato dextrose agar (PDA). The PDA plates were incubated at 25 °C in the dark whereas the CLA plates were incubated at 22 °C under a white fluorescent light with a 12: 12 light: - dark photoperiod for 7-14 days.

Preparation of fungal spore suspensions

Standardised conidial suspensions were prepared. The spores were harvested by adding 5 ml of sterile distilled water to each CLA plate and the conidia were aseptically dislodged with a sterile inoculating loop into the water. Spore suspensions were aseptically filtered through two layers of sterile muslin cloth to remove mycelial debris. The spore suspensions were microscopically adjusted with the aid of a Neubauer haemocytometer to give a final concentration of approximately 1×10^6 spores/ml.

Plant material

Aerial parts of *G. africana* were collected in Namaqualand, in April and December 1999, and the identity of the plant was authenticated by Mr. F. Weitz (Herbarium, Department of Botany, University of the Western Cape, Bellville). The dried plant material was separately ground in a mill into a fine powder and stored at 4 °C until required. Voucher specimens are kept in the UWC herbarium (Vries 1; herb no 6595).

Extraction and isolation

Powdered, dried plant material (200 g) was sequentially extracted with various solvents

(hexane, chloroform, ethanol and sterile distilled water) and the extracts were filtered through Whatman No. 4 filter paper. The EtOH extract was concentrated on a rotary evaporator to obtain 11 g of an olive-yellow solid, which was subjected to column chromatography (particle size of silica gel 0.063 - 0.2 mm) using CH₂Cl₂-EtOAc-Hexane [75:10:15] as eluent. Fractions were analysed by thin-layer chromatography (TLC), pooled into subfractions according to their behavioural similarity on TLC plates (same solvent system as for column chromatography, spots were visualized under UV light) and screened for antifungal activity. The active fraction/s were pooled and repeatedly rechromatographed on silica gel (solvent system CH₂Cl₂-EtOAc-hexane 50:20:30), followed by a redetermination of the antifungal activity.

The active major fraction, which appeared as a yellow-green solid, was soluble in EtOH, MeOH CH₂Cl₂ and THF, but insoluble in hexane and partially soluble in water. A portion of this material (2.52 g) was further chromatographed on silica gel column eluting initially with CH₂Cl₂, and later on with increasing proportions of MeOH. Fractions GA1 to GA9, eluted with CH₂Cl₂, all gave strong colour responses with the vanillin- acid spray reagent. A 10 gave a strong colour response, while the rest showed only faint responses and were thus not further investigated. Components which could be positively identified, were isolated as follows. Fractions GA 2 (11 mg) and GA 3 (26 mg) which had similar TLC profiles were individually chromatographed on a silica gel column, eluting with EtOAc/cHex : 5/95. From both fractions was obtained the known triterpene lupeone **6**, 7 mg, in addition to the flavanone **1**, 9 mg. The major fractions GA 7 (0.48 g), GA8 (1.46 g) and GA 9 (0.34 g) were shown by TLC and NMR to be mixtures containing varying proportions of chalcone, dihydrochalcone and flavanone. Further fractionation into pure compounds **2** to **5** was achieved through column chromatography on Sephadex LH 20 using methanol as eluent. Due to almost identical chromatographic mobilities of the compounds **2** to **5**, they were recovered after repeated chromatography, and so in pure enough form for complete characterisation in the respective yields of 15.0, 3.0, 5.0 and 10.3 mg.

Chemicals

All the organic solvents were of analytical grade and distilled prior to use during chromatography.

Agar well diffusion method

Antimicrobial activity was measured by the agar well diffusion method. Petri dishes (90 mm) containing 20 ml agar (PDA) were surface-inoculated with 200 µl of the prepared spore suspension (1×10^6 spores/ml) and evenly spread with a L-shaped glass rod across the agar surface and allowed to dry in a laminar flow hood. Four 5 mm diameter wells were aseptically made in each of the inoculated agar plates using a pre-sterilised cork-borer. The fraction (1 g) was dissolved in 9 ml CH₂Cl₂. Each well was loaded with 50 µl of the fraction selected. The fraction was allowed to evaporate to dryness in a laminar flow hood. Plates were inverted and incubated at 27 °C for 5 days. The inhibition zones were measured in millimeters from the edge of the well and the means were determined. Triplicate determinations were made.

Determination of minimum inhibitory concentration (MIC)

Once the sensitivity range for each organism was known, the MIC's was determined by the agar well diffusion method. Two-fold serial dilutions of the active fraction (0.156, 0, 315, 0.625, 1.25, 2.5, 5 and 10%) were prepared in 9 ml of CH₂Cl₂ and used against the test

organism. The MIC is defined as the lowest concentration of the active fraction capable of preventing growth of an organism. Triplicate determinations were made.

Filter paper disks method

The active residue (1 g) was dissolved in 9 ml CH₂Cl₂ before the assay. Aliquots of the active residue (50 µl) were pipetted onto individual sterile analytical grade filter paper disks (Whatman No. 4, 10 mm diameter) in individual Petri dish lids and dried for 30 min in a laminar flow hood.

Filter paper disks without the active residue represent the control disks. Four disks were placed equidistant from one another on the surface of inoculated PDA plates with standardised fungal spore suspensions (200 µl).

The bioassay plates were incubated for 5 days at 27 °C and examined for the presence of a zone of inhibition surrounding the disk, which is evidence of the inhibition of germination and a measure of antifungal activity. Triplicate determinations were made.

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