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Antimicrobial acylphloroglucinols and dibenzyloxy flavonoids from flowers of *Helichrysum gymnocomum*

Siegfried E. Drewes [a,*], Sandy F. van Vuuren [b]

[a] School of Chemistry, University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209 Pietermaritzburg, South Africa
[b] Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of Witwatersrand, 7 York Road, Parktown, 2193 Johannesburg, South Africa

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ABSTRACT

From the dichloromethane extract of the flowers of *Helichrysum gymnocomum* (Asteraceae) two known flavonoids, **4** and **5**, and a known acylphloroglucinol, **3B**, were isolated. In addition to **1** and **2**, the 4′,6′-dibenzyloxy-2′-hydroxy derivative of 2′,4′,6′-trihydroxychalcone and 5,7-dibenzyloxy derivative of pinocembrin, respectively, are reported in Nature for the first time. A compound **3A**, related to **3B** has the structure 2-methyl-1-[2,4,6-trihydroxy-3-(2-hydroxy-3-methyl-3-butenyl)phenyl]-1-propanone. Compounds **1, 2, 3A, 3B, 4** and **5** have MIC values below 64 μg/ml against a selection of pathogens, with **3B** having the highest sensitivity (6.3–45 μg/ml) for eight of the ten pathogens tested, including *Staphylococcus aureus* (6.3 μg/ml) and methicillin and gentamycin resistant strain of *S. aureus* (7.8 μg/ml). With the exception of **2**, the other compounds had notable activity (45–63 μg/ml) towards *Pseudomonas aeruginosa*.

1. Introduction

Some 600 species of *Helichrysum* (Asteraceae) occur in Africa, of which some 244 species are found in South Africa (Pooley, 2003). The plants occur as herbs and shrubs. Often the leaves and flowers are pleasantly scented and may be burnt by the indigenous people to fumigate a sick room or to invoke the goodwill of the ancestors (Pooley, 2003). Mixed with fat, the plant produces a soothing ointment. Other applications include the use of various parts of the plant to treat coughs and colds and particularly to combat the infection of wounds (Pooley, 2003; Bremner and Meyer, 2000). *Helichrysum gymnocomum*, the subject of this investigation, is a perennial herb which grows profusely in the KwaZulu-Natal Drakensburg. The flowers are pleasantly scented. The above properties, coupled with its long flowering season (February–July) (Hilliard, 1997) make the plants an obvious choice for medicinal and ritual purposes.

The chemistry of a large number of *Helichrysum* species from diverse areas of South Africa has been studied at length by Bohlmann and Mahanta (1979), Bohlmann and Abraham (1979), Bohlmann and Hoffmann (1979). More recent research on the genus has focused on the antimicrobial properties of the phloroglucinol derivatives in the genus (Meyer et al., 1997; Bremner and Meyer, 2000; Drewes et al., 2006).

On the whole researchers have utilized only the leaves, stems and roots of *Helichrysum* plants. Since flowers have long been known to be particularly rich sources of biologically active compounds (Swerdlow, 2000), this paper reports on the constituents in the flowers of *H. gymnocomum*. This decision was further reinforced by the findings of a recent paper (Appendino et al., 2007) in which the authors describe the isolation of arzanol, a phloroglucinol α-pyrone having anti-inflammatory and anti-HIV properties. Arzanol was extracted from the bright yellow flowerheads of *Helichrysum italicum* growing in Sardinia.

2. Results and discussion

2.1. Determination of structure

From the yellow flowers of *H. gymnocomum,* three known compounds were isolated. These were 3-methoxyquercetin **4** (Bouktaib et al., 2002), the 4′-*O*-glucose derivative of 2′-hydroxy-6′-methoxy chalcone **5** (Wright, 1976), and the acylphloroglucinol derivative **3B**, 3-[3′,3′-dimethylallyl-(1′)]-1-isobutyrylphloroglucinol (Bohlmann and Mahanta, 1979). Comparison of spectral data of our compounds with those published in the literature together with high resolution mass spectral analysis of **4, 5** and **3B** confirmed the identity of the isolated compounds (see Fig. 1).

The compounds **1** (2′-hydroxy-4′,6′-dibenzyloxychalcone) and **2** (5,7-dibenzyloxyflavanone) are new in Nature although their 'parent' compounds (2′,4′,6′-trihydroxychalcone and

* Corresponding author. Tel.: +27 33 260 5243; fax: +27 33 260 5009.
E-mail address: drewes@ukzn.ac.za (S.E. Drewes).

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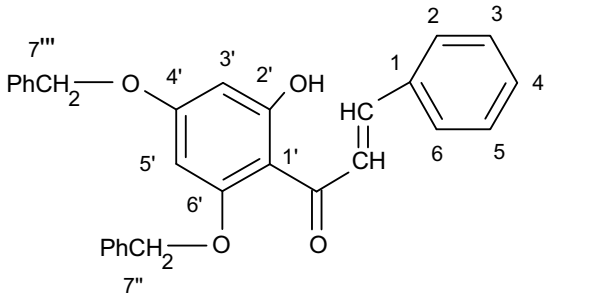
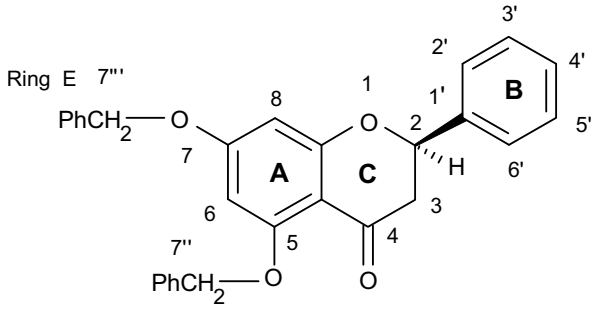


(1)



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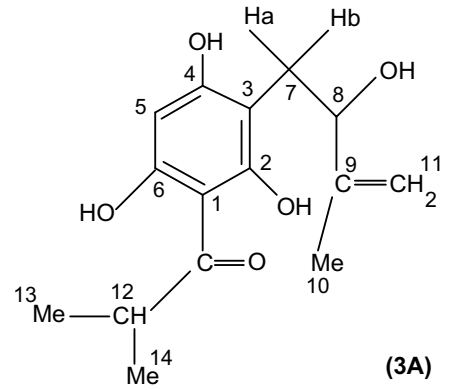
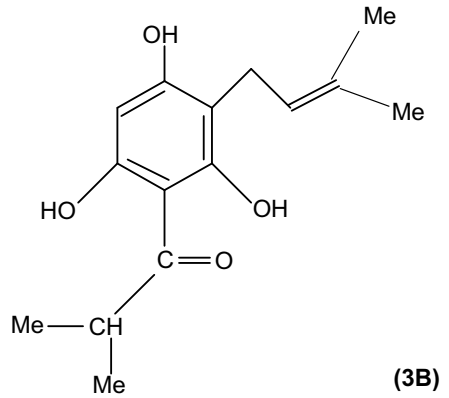


(3A)



(3B)

Fig. 1. Structures of compounds (**1**), (**2**), (**3A**) and (**3B**).

5,7-dihydroxyflavanone, respectively) are well known (Linstedt, 1951; Jurd and Horowitz, 1961). It is interesting to note that the recent findings on benzyloxy derivatives of a range of chalcones have shown them to be potent inhibitors of interleukin-5 (Yang et al., 2007). The NMR data for compounds **1** and **2** are shown in Table 1.

The new compound **3A** reported here using IU PAC nomenclature, is 1-[2,4,6-trihydroxy-3-(2-hydroxy-3-methyl-3-butenyl)phenyl]-1-propanone. It is close in structure to **3B**. The latter was first isolated from *H. gymnocomum* (Bohlmann and Mahanta, 1979) and later from *H. platypterum* (Bohlmann and Zdero, 1979). In both instances, leaves and stalks of the plants were extracted. A synthesis of **3B** was subsequently published (Kuhnke and Bohlmann, 1985). Accurate ^{1}H and ^{13}C NMR data have been reported by Bremner and Meyer (2000). Comparison of our detailed spectral data for **3B**, coupled with a high resolution mass analysis, leave no doubt that it is identical to the compound described in the literature as cited here.

Compound **3A** was only separated with great difficulty from **3B**. When pure, it crystallized in very fine needles, m.p. 133 °C. Its structure was established using ^{1}H and ^{13}C NMR techniques including COSY, DEPT, HSQC and HMBC programmes. Comparison of the proton spectra of **3A** and **3B** shows immediately that both compounds contain a phloroglucinol ring with the identical substituent (2-methylpropan-1-one) at C-1 and a singlet aromatic proton, C-5, at 5.89 ppm. The single difference is the replacement of the prenyl side chain at C-3 with a 2-hydroxy-3-methyl-3-butenyl moiety in **3A**. Since the methylene group at C-7 (using the "simpler" numbering of the side chain) is adjacent to the chiral centre at C-8, the two protons are diastereotopic, and give rise to a clear doublet of doublets (Table 2). For purposes of comparison the ^{13}C spectrum of **3B** is also included in the table. The isolation of **3A** neatly supplements the existence of a series of highly active antimicrobial phloroglucinol derivatives starting with **3B** (Bohlmann and Mahanta, 1979), followed by the acylphloroglucinols from *Hypericum foliosum* and *Hypericum beanii* (Gibbons et al., 2005; Shiu and Gibbons, 2006), and very recently the isolation of arzanol (Appendino et al., 2007) from *H. italicum*. Apart from all these compounds being based on phloroglucinol, they have other structural features of interest. Thus, the acyl phloroglucinol from *H. foliosum* (Gibbons et al., 2005) contains a five carbon epoxy side chain which chemically is not far removed from the CH_2–CH(OH)C-(Me)=CH_2, group present in **3A**. In fact, the one could be a precursor of the other.

2.2. The dibenzyloxy derivatives 1 and 2

The existence of the dibenzyloxy derivatives in the yellow flowers (and not in the leaves and stems) of *H. gymnocomum* was unexpected. While the benzyloxy group is a familiar protecting group for phenolic OH's, its existence in Nature is not recorded. The antimicrobial test results (Table 3) reveal high activity against several pathogens (Section 2.3). At this stage it is not known to what extent the existence of the benzyloxy group, as well as the presence of the unsubstituted B-ring in both **1** and **2**, play a role in influencing antimicrobial activity. It is noteworthy that Yang et al. (2007) reported that benzyloxy derivatives of some chalcones are potent inhibitors of interleukin-5.

2.3. Antimicrobial test

The antimicrobial activity of the crude *H. gymnocomum* extract exhibited a broad spectrum of activity with minimum inhibitory concentration (MIC) values ranging from 312 to 1000 μg/ml depending on the pathogen studied. According to Fabry et al. (1998), extracts having MIC values below 8000 μg/ml possess some antimicrobial activity. MIC values below 1000 μg/ml are considered noteworthy (Gibbons, 2004; Rios and Reico, 2005). Thus, the crude extract having activities of 1000 μg/ml or lower against all the pathogens studied demonstrated potential anti-infective properties.

Table 1
NMR data (500 MHz, $CDCl_3$) benzyloxy derivatives **1** and **2**

Compound **1**				Compound **2**			
Atom	δH	Carbon	δC	Atom	δH	Carbon	δC
3′	6.19(*d*, *J* 6.2)	1′	106.6	2	5.43(*dd*, *J* 13.4, 2.8)	2	79.3
5′	6.23(*d*, *J* 6.2)	2′	162	3a	2.82(*dd*, *J* 16.6, 2.80)	3	45.7
				3b	3.06(*dd*, *J* 16.6, 13.4)		
1	7.08–7.96*m*	3′	92.8	6	6.25(*d*, *J* 2.3)	4	189.5
2/6	7.08–7.96*m*						
3/5	7.08–7.96*m*	4′	165.5	8	6.26(*d*, *J* 2.3)	5	161.1
4	7.08–7.96*m*	5′	95.29	2′/6′	7.43(*m*, B ring)	6	95.2
1″	7.08–7.96*m*	6′	169.1	3′/5′	7.43(*m*, B ring)	7	164.9
2″/6″	7.08–7.96*m*						
3″/5″	7.08–7.96*m*	1	136.1	4′	7.43(*m*, B ring)	8	94.8
4″	7.08–7.96*m*	2/6	128.2–129.1	2″/6″	7.60(*m*, D ring)	9	164.9
7″	5.06(*s*)	3/5	128.2–129.1	3″/5″	7.60(*m*, D ring)	10	106.5
1‴	7.08–7.96(*m*)	4	128.2–129.1	4″	7.60(*m*, D ring)	1′	138.7
2‴/6‴	7.08–7.96(*m*)						
3‴/5‴	7.08–7.96*m*	1″	135.5	7″	5.17(*s*)	2′/6′	125.5–128.8
4‴	7.08–7.96(*m*)	2″/6″	128.2–129.1	2‴/6‴	7.40(*m*, E ring)	3′/5′	125.5–128.8
7‴	5.11(*s*)	3″/5″	128.2–129.1	3‴/5‴	7.40(*m*, E ring)	4′	125.5–128.8
∝	7.89(*d*, *J* 15.6)	4″	128.2–129.1	4‴	7.40(*m*, E ring)	1″	136.4
β	7.72(*d*,*J* 15.6)	7″	71.7	7‴	5.05(*s*)	2″/6″	125.5–128.8
OH	14.6(*s*)	1‴	135.6			3″/5″	125.5–128.8
		2‴/6‴	128.2–129.1			4″	125.5–128.8
		3‴/5‴	128.2–129.1			7″	70.4
		4‴	128.2–129.1			1‴	135.7
		7‴	70.6			2‴/6‴	125.5–128.8
		∝	127.5			3‴/5‴	125.5–128.8
		β	143.0			4‴	125.5–128.8
		C=O	192.9			7‴	70.3

Table 2
NMR data (500Mz CD_3 OD) of **3A** and **3B**

Compound **3A**				Compound **3B**	
Atom	δC	δH	HMQC(H–C)	δC	δH
1	105.8	–		104.5	
2	165.2	–		165.3	
3	110.6	–		108.1	
4	164.3	–		163.4	
5	95.6	5.88(*s*)	C-4, C-6	95.0	5.88(*s*)
6	162.3	–		161.1	
7a	29.9	2.93(*dd*, *J* 14.1, 4.6)	C-8, C-9, C-11	22.2	3.17(*d*, *J* 7.1)
7b		2.71(*dd*, *J* 14.1, 7.8)			
8	77.2	4.27(*dd*, *J* 7.8, 4.6)	C-11, C-7, C-10	124.6	5.18(*t*, *J* 7.1)
9	148.8	–	C-7	131.0	
10	18.1	1.79(*s*)	C-11, C-8	17.8	1.64(*s*)
11	110.6	4.73,4.84(*bs*) *J* <.1)	C-10	25.9	1.73(*s*)
12	39.9	4.00(*sep*, *J* 6.7)	C-13	38.9	4.08(*sep*, *J* 7.1)
13	19.7	1.12(*d*, *J* 6.7)	C-12, C=O	19.8	1.12(*d*,*J* 6.7),
14	18.1	1.12(*d*, *J* 6.7)	C=O	17.8	1.12(*d*, *J* 6.7)
C=O	211.9	–		211.7	

Compounds having antimicrobial activities less than 64 μg/ml are accepted as having notable antimicrobial activity (Gibbons, 2004) and those compounds exhibiting activity at concentrations below 10 μg/ml are considered "clinically significant" (Gibbons, 2004; Rios and Reico, 2005). The isolated compounds **1**, **2**, **3A**, **3B**, **4** and **5** from *H. gymnocomum* had MIC values below 64 μg, with **3B** having an MIC value of 7.8 μg/ml against a selection of pathogens (including *Staphylococcus aureus* and the *S. aureus* methicillin and gentamycin resistant strain). Notable activity (less than 64 μg/ml) is shown in bold in Table 3. Compound **3B** exhibited the highest sensitivity with activities 6.3–45 μg/ml against eight of the ten pathogens tested. Acylphloroglucinols are known to be microbiologically active (Gibbons, 2004) and previous antimicrobial studies against isolated acylphloroglucinols have been demonstrated by Gibbons et al. (2005), where anti-staphylococcal activity (16–32 μg/ml) was noted. Further anti-staphylococcal activity (16–256 μg/ml) on isolated acylphloroglucinols was demonstrated by Shiu and Gibbons (2006). By comparison with these previous studies, compound **3B** showed excellent anti-staphylococcal activity (6.3–9.8 μg/ml). The Gram-positive micro-organisms tested were all susceptible to **3B** at concentrations less than 10 μg/ml with *S. aureus* (ATCC 12600) having the highest sensitivity (6.3 μg/ml). By comparison, a test done by Bremner and Meyer (2000) on **3B** (isolated as a light orange powder from *Helichrysum kraussi*) exhibited higher MIC values (100 μg/ml) against seven bacteria. In our tests on **3B**, the five Gram-positive pathogens tested were all susceptible at concentrations of less than 10 μg/ml with *S. aureus* (ATCC 12600) having the highest sensitivity at 6.3 μg/ml. Compound **2**, the benzyloxy derivative of pinocembrin, and **3A**, both afforded MIC values of 7.8 μg/ml against the yeast *Cryptococcus neoformans* (ATCC 90112). It is also interesting to note that all the compounds with the exception of compound **2** exhibited notable activity (45–63 μg/ml) towards the pathogen *Pseudomonas aeruginosa*. The *Helichrysum* genus has been used extensively for the treatment of wounds and topical infections (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996). The high activities noted for both *P. aeruginosa* and *S. aureus* (illustrated for compounds **3A** and **3B**) thus correlates well with the ethno-botanical use of the plant. This also supports the recent findings by van Vuuren et al. (2006) for related phloroglucinols isolated from *Helichrysum cymosum* as well as other publications on antimicrobial phenolics from *Helichrysum* species and other plant sources (Bharate et al., 2007; Tomaś-Barberań et al., 1990).

3. Experimental

3.1. General

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Mass spectra were recorded on a Kratos MS 80 RF double-focusing magnetic sector instrument at 70 eV. Optical rotations were measured on a Perkin Elmer 127 machine.

Table 3
The mean minimum inhibitory concentration (μg/ml) of *Helichrysum gymnocomum* extract and isolated compounds

Pathogen	Crude extract	Compounds						Control[a]
		1	**2**	**3A**	**3B**	**4**	**5**	
Enterococcus faecalis ATCC 29212	1000	83.0	125.0	**63.0**	**7.8**	125.0	250.0	0.6
Staphylococcus epidermidis ATCC 2223	1000	166.6	208.0	125.0	**9.8**	125.0	250.0	1.0
Staphylococcus aureus ATCC 12600	1000	125.0	250.0	**63.0**	**6.3**	125.0	250.0	1.3
Staphylococcus aureus (methicillin and gentamycin resistant) ATCC 33592	312.5	**63.0**	185.7	**63.0**	**7.8**	**63.0**	187.5	0.5
Bacillus cereus ATCC 11778	666.6	104.3	125.0	458.3	**7.8**	125.0	250.0	0.8
Escherichia coli ATCC 8739	888.3	125.0	250.0	166.6	125.0	125.0	187.5	0.6
Klebsiella pneumoniae ATCC 700603	888.3	**63.0**	125.0	375.0	187.5	**63.0**	125.0	0.5
Pseudomonas aeruginosa ATCC 7858	562.5	**45.0**	81.6	**63.0**	**45.0**	**63.0**	**45.0**	3.1
Cryptococcus neoformans ATCC 90112	500.0	85,0	**7.8**	**7.8**	**26.0**	117.0	**46.9**	3.1
Candida albicans ATCC 10231	830.0	**63.0**	125.0	125.0	**23.8**	92.5	92.5	2.0

[a] Ciprofloxacin and amphotericin B served as controls for bacteria and yeasts, respectively.

Nuclear magnetic resonance spectra were recorded on a Varian 500 spectrometer operating at 499.98 for ^{1}H and 125.73 MHz for ^{13}C nuclei. Flash column chromatography was used for initial purification of fractions. Subsequent separations were accomplished by centrifugal separation (Chromatotron) using circular plates coated with preparative silica gel (2 mm thickness). Thin layer chromatography (TLC) plates (Kieselgel 60 F_{254}, 0.25 mm) were used to examine separated fractions. Visualization of spots was achieved by dipping plates in anisaldehyde/H_2SO_4 reagent and heating.

3.2. Plant material

The flowers of *H. gymnocomum* were collected in May 2006 in the Monk's Cowl area of the KwaZulu-Natal Drakensberg. A voucher specimen (flowers, leaves and stems) was deposited in the Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg and lodged under the label S.E. Drewes, No 11. Identification was done by research staff at National Biodiversity Institute in Pretoria, South Africa.

3.3. Extraction isolation and identification of components

The dried yellow flowers of the plant (100 g) were extracted at room temperature with CH_2Cl_2/MeOH (1:1) for five days. Concentration under vacuum afforded a reddish brown powder (8.5 g). Examination by TLC showed the presence of at least six compounds (orange or yellow after visualization). The R_f's in EtOAc/hexane (1:1) were spread over a range: 0.60 (red), 0.37 (major orange spot), 0.22 (light orange), 0.16 (intense yellow) and baseline (orange–yellow). Subsequent separation with different solvent systems would show that the two highest R_f components consisted of more than one compound.

The red brown powder (410 mg) was separated by column chromatography (EtOAc/hexane 1:1) initially, but with subsequent increase in polarity into four fractions: High R_f (*A*, 47 mg), middle R_f (*B*, 71 mg), lower R_f (*C*, 25 mg) and baseline material (*D*, 70 mg).

Fraction A was run two times on the chromatotron using EtOAc/hexane (1:4), to yield two compounds, clearly separated from one another, and subsequently identified as compound **1**, (9 mg, yellow crystals m.p., 119 °C, higher R_f) and compound **2**, (8.6 mg white crystals m.p., 115 °C; $[\alpha]_D^{25}$ = +9.2 °C; *c*, 0.027). Based on the work of Arakawa and Nakazaki, 1960 this suggests an *R*-configuration for **2**. Extended exposure of the extract led to the decomposition of **1** and **2**.

Both **1** and **2** were further characterized by high resolution mass spectrometry. For **1**, *m/z* 436.16642 M^+ (calculated for $C_{29}H_{24}O_4$ 436.16746) and **2**, *m/z* 436.16940 M^+ (calculated for $C_{29}H_{24}O_4$ 436.16746). ^{1}H and ^{13}C spectra are collated in Table 1.

Fraction B consists of a minor component **3A** and a major one, **3B**. From the impure fraction (B, above 71 mg) repeated chromatotron separation (ether/hexane, 3:1) afforded pure **3A** (8 mg, buff rosettes, m.p. 133 °C; $[\alpha]_D^{25}$ = −16.6 °C; *c*, 0.072) and pure **3B** (17 mg, pale orange prisms, m.p. 115 °C, softened at 105 °C).

For **3A,** HRMS showed *m/z*: 280.13051 M^+ (calculated for $C_{15}H_{20}O_5$ 280.13105). ^{1}H and ^{13}C spectral data are collated in Table 2.

Compound **3B** was first isolated from *H. gymnocomum* by Bohlmann and Mahanta (1979) as a mixture of two compounds and again later in 1979 (Bohlmann and Zdero). HRMS showed *m/z* 264.13559 M^+ (calculated for $C_{15}H_{20}O_4$ 264.13604) leaving no doubt as to its identity (Table 2).

Fraction C (25 mg) was obtained pure after one chromatotron separation (EtOAc/hexane, 3:2) and proved to be 3-methoxyquercetin (12 mg, m.p. 273 °C) designated as compound **4**. High resolution mass spectrometry gave an M^+ at 316.05831 and a molecular formula of $C_{16}H_{12}O_7$.

Fraction (D) (71 mg) was purified on a chromatotron plate (2 mm) using ether/MeOH (20:3) as an eluent. The yellow band at R_f 0.64 was collected (25 mg) to afford **5** as a glass, m.p. 279 °C (decomposition). It proved to be identical to the chalcone glucoside isolated by Wright (1976) from flowers of *Helichrysum cooperi*. HRMS showed conclusively that it had an M^+ of 448.13694 consistent with a molecular formula of $C_{22}H_{24}O_{10}$.

3.4. Materials and methods for antimicrobial tests

Culture inoculum determination, media preparation and assays were undertaken according to the NCCLS (2003). The MIC assay for determining antimicrobial activity was adopted from Eloff (1998). All stock cultures were obtained from the National Health Laboratory Services with the exception of *Candida albicans,* which was obtained from the South African Bureau of Standards, and the methicillin and gentamycin resistant strain of *S. aureus,* obtained from Davies Diagnostics. Micro-well MIC's were performed with ten test micro-organisms comprising of five Gram-positive strains (*Bacillus cereus* ATCC 11778, *Enterococcus faecalis* ATCC 29212, *Staphylococcus epidermidis* ATCC 2223, *S. aureus* ATCC 12600 and methicillin and gentamycin resistant strain of *S. aureus* ATCC 33592), three Gram negative strains (*Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 7858) and two yeast strains (*C. neoformans* ATCC 90112 and *C. albicans* ATCC 10231). The crude *H. gymnocomum* extract and isolated compounds **1**, **2**, **3A**, **3B**, **4** and **5** were diluted in acetone so that starting concentrations of 32 or 2 mg/ml, respectively, were introduced into the first well of a microtitre plate. The starting concentrations were diluted two fold in each successive serial dilution. All bacterial cultures were subcultured from stock agar plates and grown in

Tryptone Soya broth for 18 h. The yeasts were incubated for a further 24 h. The cultures were introduced into all wells of the microtitre plate, yielding an approximate inoculum size of 1×10^8 colony forming units (CFU)/ml. Optimal incubation conditions followed; 37 °C for 24 h for bacteria and 48 h for the yeasts. Commercial antimicrobials (ciprofloxacin for bacteria and amphotericin B for yeasts) at starting concentrations of 0.01 and 0.10 mg/ml, respectively, were included as positive controls in all MIC repetitions to validate microbial sensitivity. After incubation, a 0.2 mg/ml *p*-iodonitrotetrazolium violet solution was introduced into all inoculated wells and examined after 6 h (bacteria) or 24 h (yeasts) to determine a colour change in relation to the concentration of microbial growth. Assays were repeated to ensure standard error variation of not more than one dilution factor was obtained.

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