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Antimicrobial monomeric and dimeric diterpenes from the leaves of *Helichrysum tenax* var *tenax*

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Abstract

The hexane extract of fresh air-dried leaves of *Helichrysum tenax* (Asteraceae) afforded *ent*-beyer-15-en-19-ol (**1**), its 4-epimer *ent*-beyer-15-en-18-ol (**2**), 15β,16β-epoxide-*ent*-beyeran-19-ol (**3**), as well as (**4**) consisting of two units of (**1**) linked as a diester of malonic acid, and (**5**), a compound. Its constituents are (**1**) and (**3**) also linked as a diester of malonic acid. The leaves of the plant are densely covered in fine glandular trichomes. These are extremely sticky and exude a mixture of the above diterpenes. Antimicrobial tests showed that (**1**), in particular, was highly active (3.1 and 3.6 μg/ml) against *Bacillus cereus* and *Staphylococcus epidermidis*, respectively.

Keywords: *Ent*-beyer-15-en-18-and 19-ols; 15β,16β-epoxide-ene-beyeran-19-ol; Malonic acid diesters of diterpenes; *Helichrysum tenax*; Antimicrobial

1. Introduction

Helichrysum tenax var. *tenax* M.D. Hend. (Asteraceae) occurs as a conspicuous subshrub up to 1.5 m tall with each branch topped by a rosette of leaves. The bright green leaves are extremely sticky. The plant flowers in spring producing glossy yellow bracts (Fig. 1). It occurs on high ground (up to 2150 m) from the Eastern Cape Province to KwaZulu-Natal (Pooley, 2003). The sticky glandular trichomes are readily visible to the naked eye, particularly on the underside of the leaves. The nature of the trichomes as seen by the electron microscope are shown in Fig. 2. *Helichrysum* leaves are frequently covered in fine hairs, but few are as sticky as those found in *H. tenax*, and hence the designation 'tenax', i.e., holding fast, is very appropriate. It was this property of the plant which triggered the present investigation, particularly since nothing was known about the nature and function of the exudates from the trichomes. Killick (1990), records in his book that "insects get stuck on the leaves, but the plant is not insectivorous as was once speculated".

A great deal has been written about the formation and function of plant volatiles (Werker, 1993) and a recent review (Pichersky and Gershenson, 2002) serves as a good summary on current views in the area.

Plants synthesize and emit large quantities of volatile organic compounds. Terpenoids and fatty acid derivatives figure prominently among these. These volatiles, such as are found in the trichomes of *H. tenax*, appear to protect plants by deterring herbivores and by attracting the enemies of herbivores. Indeed, Pichersky and Gershenson (2002) go as far as saying "now that we have recognized that herbivore-induced plant volatiles appear to mediate both direct and indirect defenses, and even signal to

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Fig. 1. *Helichrysum tenax* in the wild.

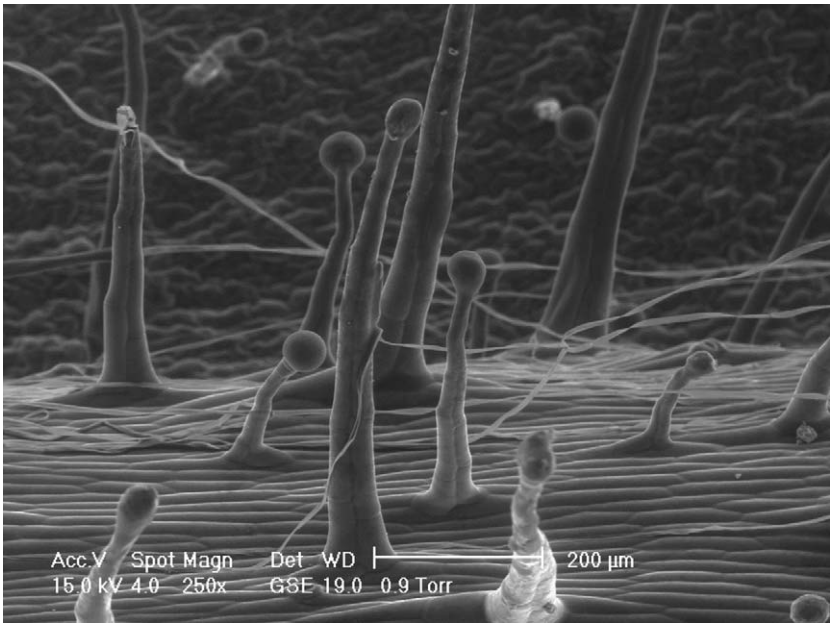


Fig. 2. Low vacuum electron microscopy on trichomes found on leaves of *Helichrysum tenax*.

nearby plants, interest in their evolutionary origin and value to the plant seems certain to increase''.

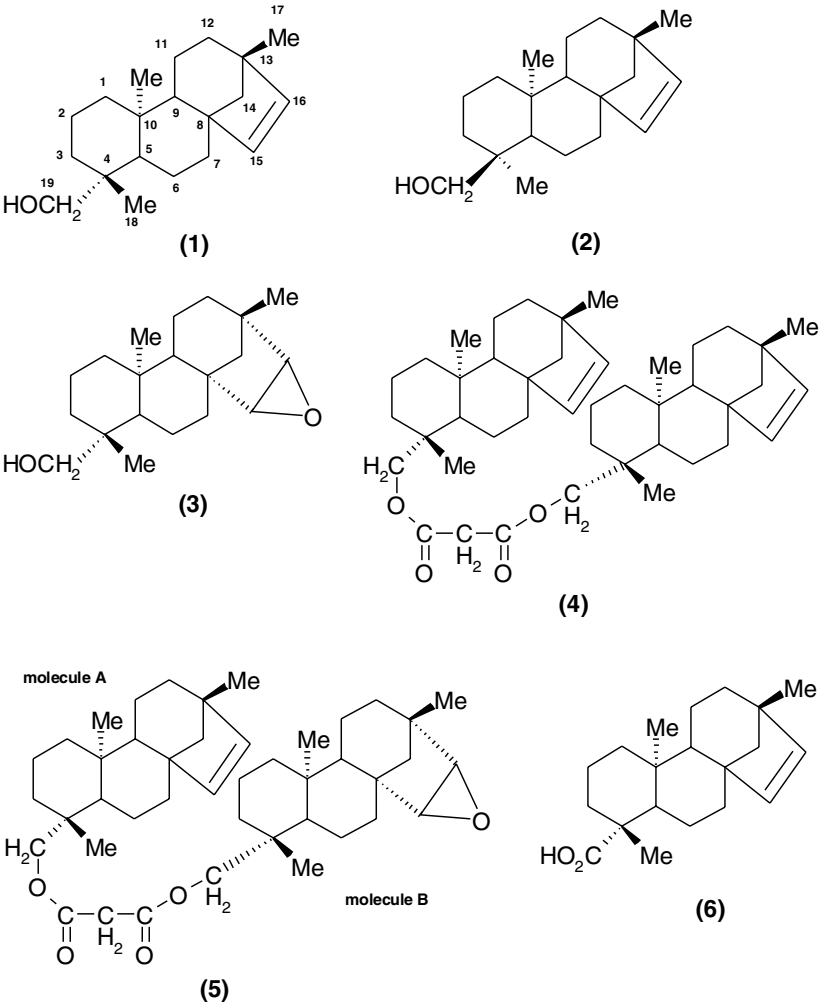
It should be noted that *Helichrysum* species are widely distributed in South Africa and are known for their antimicrobial properties (e.g., Afolayan and Meyer, 1995; Lourens et al., 2004). Their medicinal uses vary, e.g., the plants are burned as incense, or used to cure coughs and colds, but their medicinal use often depends on local availability rather than preference for a particular species (Van Wyk et al., 1997).

2. Results and discussion

2.1. Characterization of compounds

The compounds (**1**), (**2**) and (**3**) all consist of variations of the *ent*-beyer-15-en-19-ol structure, first isolated by McCrindle et al. (1968) from the trunkwood of *Erythroxylon monogynum*. In this connection the spectroscopic data recorded by San Martin et al. (1980, 1983) for the diterpenoids from *Baccharis tola* proved to be very helpful

especially since one of the compounds isolated by the authors was 15β, 16β-epoxide-*ent*-beyeran-19-ol, identical to our (**3**).



The pioneering work of Ferdinand Bohlmann et al. (1977, 1982) on the constituents of the South African Asteraceae, particularly the genus *Helichrysum*, provided valuable clues for the identification of (**4**) and (**5**). Compound (**4**) is identical to the compound named nidoanomalin by Bohlmann and Wagner (1982), from the Cape plant *Nidorella anomala* (Asteraceae). Subsequent to this finding further bis-diterpene compounds have been isolated, for example, from *Corymbium villosum* (Asteraceae) (Zdero and Bohlmann, 1988), and from three *Calceolaria* species (Scrophulariaceae) (Chamy et al., 1989, 1991; Piovano et al., 1989; Silva et al., 1993). The linking acid is usually malonic acid.

The proton spectrum of compound (**5**), which consists of two non-identical halves (Molecule A and Molecule B), the ^{13}C spectrum (Table 1) proved to be particularly helpful. The carbon shifts of twenty of the carbon atoms were directly superimposable on (**1**), *ent*-beyeren-19-ol. The remaining carbons showed small differences in chemical shift to (**1**) but quite marked discrepancies for carbon atoms 8, 9, 13, 14, 15 and 16. When one considers that compound (**5**) as assigned by us, has an epoxy ring at C-15, C-16 (molecule B), then it is not surprising that the carbon atoms adjacent to the epoxy ring system should have different chemical shifts. The strong shielding effect of the epoxy group on C-14 from 61.0 ppm in (**4**) to 46.6 ppm in (**5**), was particularly noticeable. As anticipated, the presence of the epoxy group in (**5**) also shifts C-15 and C-16 very far upfield compared to (**4**).

The proton spectrum of (**5**) proved equally informative. Inspection showed that, despite the presence of two diterpene units, only *one* of them (molecule A) possesses the C-15, C-16 double bond. Also, while two C-19 methylene groups are present (in molecule A as well as molecule B), these are not exactly superimposable as in (**4**). They appear as two doublets of doublets in which one set of doublets are superimposed on one another the other set is slightly displaced. This is in keeping with the fact that the two halves of the parent molecule are not identical.

The proton spectrum of (**5**) (Table 2), is in complete accord with the proposed structure. The HMQC experiments proved conclusively the connectivities of C-14 to C-8, C-13, C-15 and C-16. Further strong connectivities

Table 1
^{13}C data for compounds (**4**) and (**5**) 125 MHz, $CDCl_3$

Atom (nature)	Compound (**4**)	Compound (**5**)	
	(A and B identical)	Molecule A	Molecule B
1 (CH_2)	38.9	38.9	39.1
2 (CH_2)	18.1	18.1	18.0
3 (CH_2)	36.1	36.0	35.2
4 (Quat.)	37.0	37.0	36.9
5 (CH)	57.0	56.7	56.4
6 (CH_2)	20.3	20.3	19.8
7 (CH_2)	37.5	37.4	37.4
8 (Quat.)	49.0	48.9	44.1
9 (CH)	52.8	52.8	56.7
10 (Quat.)	37.1	37.1	36.9
11 (CH_2)	20.1	20.0	19.3
12 (CH_2)	33.1	33.1	33.4
13 (Quat.)	43.6	43.5	39.0
14 (CH_2)	61.0	61.0	46.6
15 CH=	134.9	134.8	60.0 (CH–O)
16 CH=	136.5	136.5	55.7 (CH–O)
17 (CH_3)	24.9	24.8	21.4
18 (CH_3)	27.4	27.4	27.4
19 (CH_2-O)	68.2	68.2	68.1
20 (CH_3)	15.7	15.7	16.2
21 (CO.O)	166.7	166.7	166.7
22 (CH_2)	41.8	41.7	–

were discernable for C-10 to the methyl group at C-20 and C-4 (in both molecule A and molecule B) and to C-18 and C-19.

The full ^{13}C and proton spectra for (**5**) are shown in Tables 1 and 2. The proton spectrum of (**4**) is also included since the data recorded in Bohlmann's paper (1982) is incomplete and contains some omissions and errors. In Table 2 the chemical shifts are given for all protons of (**4**) but H-1 to H-11 form part of a largely unresolved area where *J* values were difficult to determine.

Bohlmann (1982) named the compound (**4**) from *N. anomala*, nidoanomalin. In his case spectroscopy did not afford a molecular ion peak (M^+ 644) but gave a peak at *m/z* 330 rationalized as a McLafferty fragmentation product. By contrast, high resolution mass spectrometry on our compound (**4**) produced a reasonable (10% rel. abundance) mol. ion peak at 644.48633, in keeping with a molecular formula of $C_{43}H_{64}O_4$. For compound (**5**) a molecular ion peak at *m/z* 660.47749 (rel. abundance 28%) was found by high resolution mass spectrometry, consistent with a molecular formula of $C_{43}H_{64}O_5$.

2.2. Antimicrobial tests

The antimicrobial test results on all five compounds described here are listed in Table 3. Nine test pathogens were selected and some noteworthy conclusions can be drawn. Compounds (**1**) in particular (19-ol), but also (**2**) (18-ol), show very good inhibition of *Bacillus cereus* and *Staphylococcus epidermidis* (3.1 and 3.6 μg/ml, respectively). The inhibition of the latter pathogen is of considerable interest since a common source of nosocomial infections of hospitalized patients in Intensive Care Units are *S. epidermidis* and *Staphylococcus aureus* (Donowitz et al., 1982).

The recent review by Gibbons (2004) on anti-staphylococcal plant natural products, including a range of diterpenes, draws attention to the good activity of beyerenoic acid (**6**) (Zamilpa et al., 2002) against *S. aureus* and *Enterococcus faecalis*. This compound is identical to (**1**), but the C-19 CH_2OH group has been replaced by a carboxyl functionality. For this reason we considered whether compound (**4**) with an ester functionality attached to C-19 would show enhanced activity. This proved *not* to be the case for any of

Table 2
^1H NMR spectra for compounds (**4**) and (**5**). (500 MHz, $CDCl_3$)

	(**4**)	(**5**)	
	Molecule (A) and (B) identical	Molecule (A)	Molecule (B)
H-1 (CH_2)	0.86 (2H, *m*)	0.84 (2H, *m*)	1.84 (2H, *m*)
H-2 (CH_2)	1.37 (2H, *m*)	1.37 (2H, *m*)	1.37 (2H, *m*)
H-3 (CH_3)	1.34(2H, *m*)	1.34 (2H, *m*)	1.34 (2H, *m*)
H-5 (CH)	1.08(1H, *m*)	1.08 (1H, *m*)	1.13 (1H, *m*)
H-6 (CH_2)	1.46(2H, *m*)	1.48 (2H, *m*)	1.49 (2H, *m*)
H-7 (CH_2)	1.66 (2H, *m*)	1.62 (2H, *m*)	1.62 (2H, *m*)
H-9 (CH)	0.99 (1H, *m*)	0.96 (1H, *m*)	0.99 (1H, *m*)
H-11 (CH_2)	1.46 (2H, *m*)	1.48 (2H, *m*)	1.50 (2H, *m*)
H-12 (CH_2)	1.26 (2H, *m*)	1.23 (2H, *m*)	1.22 (2H, *m*)
H-14 (CH_2)	14a 1.44 (1H, *d*, $J = 11.1$)	14a 1.44 (1H, *d*, $J = 11.1$)	14a 0.50 (1H, *d*, $J = 10.8$)
	14b 1.02 (1H, *d*, $J = 11.1$)	14b 1.02 (1H, *d*, $J = 11.1$)	14b 1.13 (1H, *d*, $J = 10.8$)
H-15 (CH=)	5.64 (1H, *d*, $J = 5.60$)	5.64 (1H, *d*, $J = 5.60$)	3.00 (1H, *d*, $J = 2.95$)
H-16 (CH=)	5.46 (1H, *d*, $J = 5.60$)	5.45 (1H, *d*, $J = 5.60$)	3.36 (1H, *bs*)
H-17 (CH_3)	0.99 (3H, *s*)	0.98 (3H, *s*)	1.00 (3H, *s*)
H-18 (CH_3)	0.95 (3H, *s*)	0.94 (3H, *s*)	0.95 (3H, *s*)
H-19 (CH_2O)	19a 4.34 (1H, *d*, $J = 10.9$)	4.35 (1H, *d*, $J = 10.9$)	4.33 (1H, *d*, $J = 10.9$)
	19b 3.93 (1H, *d*, $J = 10.9$)	3.93 (1H, *d*, $J = 10.9$)	3.93 (1H, *d*, $J = 10.9$)
H-20 (CH_3)	0.74 (3H, *s*)	0.72 (3H, *s*)	0.92 (3H, *s*)
H-22 (CH_2)	3.35 (2H, *s*)	3.36 (2H, *bs*)	Common to A and B

Table 3
MIC values (μg/ml) for compounds (**1**)–(**5**) from *Helichrysum tenax*

Pathogen	Control	(**1**)	(**2**)	(**3**)	(**4**)	(**5**)	Hexane extract
Enterococcus faecalis ATCC 29212	0.625	31.2	>250	468	250	625	31
Staphylococcus aureus ATCC 6538	0.30	57	31	156[a]	>250	>1250	3.6
Bacillus cereus ATCC 11778	0.80	3.1	3.6	19.5	62.5	>1250	57
Staphylococcus epidermidis ATCC 2223	0.156	3.6	31.3	235	62.5	313	27.1
Cryptococcus neoformans ATCC 90112	0.313	62.5	93.8	–	125	78.1	1000
Candida albicans ATCC 10231	2.5	62.5	>250	–	>250	82.1	2000
Escherichia coli ATCC 11775	0.313	104	93.8	625	>250	313	4000
Klebsiella pneumoniae NCTC 9633	0.80	62.5	46.8	313[a]	250	313	600
Pseudomonas aeruginosa ATCC 9027	0.156	41.5	41.5	156	62.5	156	300

Tests were done at least in duplicate and ciproflaxin (for bacteria) and amphotericin B (for yeasts) were used as controls.
[a] Single determinations.

the pathogens examined. The crude hexane extract was very effective (3.6 μg/ml) against *S. aureus*.

2.3. Nature and function of glandular trichomes

Reference has been made in the introduction to the prominent glandular trichomes found on the leaves of *H. tenax*, and to their extreme stickiness. Our interest in the extremely sticky exudates from the trichomes was heightened by an article on the resinous exudates from the leaves of the Chilean shrub *Fabiana densa* var. *ramulosa* (Solanaceae) (Erazo et al., 2002). Furthermore, Erazo et al. (2002) found that alkaline hydrolysis of the two major constituents in the extract afforded ent-beyer-15-en-18-ol (i.e our compound (**2**)). These authors state that their *ent*-beyer-15-en-18-ol showed no antimicrobial effects against any of the microorganisms tested and they include some used in our study (see above). The parent compounds in the plant were identified as the succinoyl and oxaloyl esters of (**2**). The two esters, as well as the resinous leaf extract, inhibited growth of *S. aureus* with the succinoyl ester achieving a value of <10 μg/ml.

We were curious to determine whether our two dimeric compounds (**4**) and (**5**) (both non-crystalline) originated from the glandular trichomes themselves or whether the leaves as such contained the compounds. To this end, fresh green leaves of *H. tenax* were submerged in hexane for 30 s, after which the hexane solution was concentrated under vacuum and the residue examined by TLC. The five major compounds with their characteristic Rf's and colour reactions were all present. This is a good indication of the diterpenes residing in the trichomes, in agreement with the earlier findings of Wollenweber (1984).

Afolayan and Meyer (1995) have studied in detail the morphology and ultrastructure of the glandular trichomes found in *Helichrysum aureonitens*. They identified the 3,5,7-trihydroxyflavone galangin, a well-known broad spectrum antimicrobial agent from the leaf trichomes (Afolayan and Meyer, 1997) from this source.

Our own findings and those listed above indicate that the glandular trichomes in the plants examined secrete a variety of antimicrobial substances. Current opinion is that the exudates act as a deterrent to predators. In the case of *H. tenax* and *F. densa* (Erazo et al., 2002) the exudates are very sticky. Our own observations indicate that it is the 'dimeric' terpenes (**4**) and (**5**) which have this particular property. Why this should be a desirable property in a plant which does not appear to be carnivorous, remains unsolved.

3. Experimental

3.1. General experimental procedures

^{1}H and ^{13}C NMR spectra were recorded on a Varian 500 MHz instrument. Mass spectra (EI) were obtained from a Kratos MS 80RF double-focussing magnetic sector instrument at 70 eV. A Perkin–Elmer model 241 polarimeter was used to measure optical rotations. Thin layer chromatography was done on Merck silica gel 60 F_{254}. TLC plates were dipped in an 8% solution of *para*-anisaldehyde in EtOH/H_2SO_4 and warmed gently with a heat gun for colour formation.

3.2. Plant material

Mature plants of *H. tenax* var. *tenax* were collected in October 2004 in the Monk's Cowl region of the Drakensberg. A flowering plant was identified by Prof. Trevor Edwards, curator of the Bews Herbarium at the University of KwaZulu-Natal, Pietermaritzburg. The specimen was deposited in the herbarium under the no. SED9.

3.3. Extraction and isolation

Leaves from mature plants were allowed to dry out in the shade (196 g). Extraction with hexane afforded 25.6 g of solid residue. This residue was pale yellow while subsequent extractions with more polar solvents (CH_2Cl_2, EtOAc and EtOH) gave considerably darker residues. Hexane extract (5 g) was subjected to an initial separation on silica gel (Merck No. 9385) using a short glass column (7 × 4 cm) and eluted with ether.

Five 50 ml fractions were collected, with the bulk of the material eluting in fraction 3 (2.2 g) and 4 (2.05 g). Subsequently fraction 3, which contained all the compounds of interest, was used for further fractionation. Using a preparative silica gel column (6 × 15 cm), fraction (3), (1.0 g) was separated using hexane–EtOAc (400:70) as eluent. Initially 10 ml fractions (1–10) were collected and this was subsequently decreased to 3 ml (11–75). Compound (**4**) was present in fractions 10–35, compound (**5**) in 43–73, compounds (**1**) and (**2**) in 75–90 and (**3**) in 90–110. Compounds (**4**), (**1**) and (**2**) were major constituents (200, 220, 106 mg resp.) and could be obtained pure by one further chromatographic separation. By contrast, (**5**) and (**3**) were present in only low levels and afforded 71 and 10 mg, respectively. The latter two were finally obtained in pure form by further separations on the chromatotron (hexane–EtOAc, 400:70). The 1 g starting material afforded 53 mg of (**5**) and 6 mg of (**3**). In the hexane–EtOAc solvent the five compounds had the following Rf's: 0.81 (**4**), 0.53 (**5**), 0.38 (**1**), 0.34 (**2**) 0.13 (**3**). With the anisaldehyde reagent (**3**) and (**5**) gave an intense dark blue colour, whereas (**4**), (**1**) and (**2**) were brown first and changed to a purple colour. None of the compounds fluoresce under short-wave UV light so the anisaldehyde reagent was crucial for their detection.

Compounds (**1**), (**2**), (**3**) and (**4**) are all known, being *ent*-beyer-15-en-19-ol, *ent*-beyer-15-en-18-ol, 15β,16β-epoxide-*ent*-beyeran-19-ol and nidoanomalin, respectively.

Nidoanomalin (**4**). Bohlmann (1982) describes this compound as a colourless gum with $[\alpha]^{\lambda}_{24^\circ} = \frac{589}{+19.7}, \frac{578}{+19.7}, \frac{546}{+22.1}, \frac{436\ \mathrm{nm}}{+37.6}$ ($CHCl_3$; *c*. 0.29). After repeated purification we obtained (**4**) as a white semi-crystalline solid mp 115–125 °C.

We recorded the following optical rotations: $[\alpha]^{\lambda}_{25^\circ} = \frac{589}{+27}, \frac{578}{+28.7}, \frac{546}{+32.6}, \frac{436\ \mathrm{nm}}{+58.4}$ ($CHCl_3$; c.0.23) *ent*-Beyer-15-en-19-ol, 15β,16β-epoxide-*ent*-beyeran-19-ol-diester of malonic acid (**5**).

Colourless, viscous oil, which solidifies; IR $\nu^{CCl_4}_{max}$, cm^{-1}: 2950, 1744.1728 (ester), 1422; EIMS *m*/*z* (rel, int): 660 [M^+] (28), 642 (18), 388 (27), 270 (38), 135 (100), 105 (97); HREIMS *m*/*z*: found 660.47749 $C_{43}H_{46}O_5$ requires 660.47538. $[\alpha]^{\lambda}_{24^\circ} = \frac{589}{+21.9}, \frac{578}{+22.9}, \frac{546}{+26.2}, \frac{436\ \mathrm{nm}}{+46.2}$ ($CHCl_3$; c. 0.29). ^{13}C and proton spectra are recorded in Tables 2 and 3.

3.4. Antimicrobial analysis

Culture, media preparation and minimum inhibitory concentration (MIC) assays were undertaken according to methodology adopted from NCCLS (2003) guidelines (Carson et al., 1995; Eloff, 1998). Nine test pathogens (Table 3) were selected for MIC investigation against the isolated compounds and crude hexane extract. Ciproflaxin for bacteria and amphotericin B (for yeasts) were included as positive controls at a starting concentration of 0.01 and 0.1 mg/mL, respectively. Where possible, assays were undertaken in triplicate.

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