SUPPLEMENTARY MATERIALS

Isolation and antimicrobial activities of a novel discolornolide and other compounds from Monanthotaxis discolor

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Abstract

Characterisation of a novel discolornolide (1) isolated from a first time investigation of Monanthotaxis discolor is described. Other 6 known compounds, karatavin (2), N-acetylanonaine (3), quercetin-3-O--arabinose (4), stigmasterol (5), a mixture of stigmasterol and β-sitosterol (6) and octahydro-5-isopropyl-3-methyl-2-methyleneazulene-8,8-diol (7) isolated are also reported. The structures were established by spectroscopic methods. Citotoxicities and antimicrobial activities of the compounds and crude extracts are also reported whereby compound 1 showed in vitro antifungal activity against Candida albicans and Aspergillus niger at concentrations of 0.13 and 0.17 mg/ml with zones of inhibitions of 7.0 and 5.5 mm respectively. The compound also showed cytotoxic activity in the brine shrimp test with LC₅₀ of 5.88 µg/ml. Also compound 4 exhibited antibacterial activity against E. coli and S. aureus. The compound also exhibited cytotoxic activities in the brine shrimp test with LC₅₀ of 24.73 µg/mL. The crude extracts exhibited varying citotoxic and in vitro antimicrobial activities.

Keywords: antimicrobial; cytotoxicity; discolornolide; Monanthotaxis; Tanzania
3. Experimental

3.1 Chromatography and Spectroscopy experiments

Column chromatography was carried out using silica gel of 230-400 mesh (Merck) and Sephadex® LH-20 (Pharmacia). Fractions were monitored by thin layer chromatography (TLC) (60 F254, Merck) developing with different solvent systems and spots visualized under UV light at 254 nm and 366 nm. Detection was done under UV and thereafter spraying with p-anisaldehyde reagent and heating at 105 °C. Vacuum liquid chromatography (VLC) used for fractions and was carried out using normal phase silica gel of particles size 400 mesh ASTM (Merck) applying gradient elution. The vacuum was generated from a membrane pump. The Melting Points were determined using electrothermal melting point apparatus and are uncorrected. The IR spectra were recorded on Perkin Elmer FT-IR spectrum 100 spectrophotometer fitted with UATR with absorptions given in wave number (cm⁻¹). UV/Vis spectra were run on a scanning spectrophotometer Shimadzu UV-2101PC using chloroform or methanol of which maximum absorption (λmax) are given in nm. Mass spectra were recorded under electron impact (EI) at 70 eV.

1H and 13C NMR spectra were recorded on a Bruker 300 spectrophotometer operating at 300 MHz for 1H NMR and 75 MHz for 13C NMR. CDCl₃ or MeOH or DMSO were used as solvents. Chemical shift are given in δ value relative to an internal standard TMS (δ = 0 ppm) for 1H NMR or δ value relative to the internal standard CDCl₃ for 13C NMR. All 13C spectra were obtained as 13C decoupled spectra. The 1H and 13C NMR spectra data are summarized in table S1.

3.2 Plant Material

The leaves, root and stem barks of *Monathotaxis discolor* were collected from Chimila Scarp Forest Reserve in Mbeya region, Tanzania. The plant material materials were identified in the field by a botanist and the identification was confirmed at the herbarium of the Department of Botany of the University of a Dar es Salaam where a voucher specimen was deposited under number FMM 3403 T3.

3.3 Extraction and isolation

The air-dried and pulverized plant material was soaked twice consecutively in petroleum ether, dichloromethane and methanol for 48 hrs twice at room temperature (25-30 °C). The extracts
were concentrated using a rotary evaporator and fractionated by VLC over silica gel. Repeated column chromatography on silica gel and Sephadex® LH-20 (Pharmacia) yielded compounds 1 – 7.

3.4 Brine shrimp lethal testing
The brine shrimp test (BST) was used to evaluate the citotoxicity of crude extracts and pure compounds. It was carried out using brine shrimps (Artemisia salina) larvae as test organisms using the standard method. (Meyer et al. 1982). Table 2 & 3 show the BST results of crude and the test pure compounds.

3.5 Anti-microbial Assays
Antimicrobial activity of the plant crude extracts, some VLC fractions and pure compounds were evaluated by the agar well diffusion method as describe by Meyer et al. (1982) both in bacteria and fungi tests and determination of minimum inhibition concentration (MIC) for the active samples. Antimicrobial analyses were carried out using the agar well diffusion method (Doughari 2006; Mbwambo et al. 2007). The agar well diffusion method was used to determine in vitro antifungal activity of crude extracts, semi-purified fractions and pure compounds (Ndukwe et al. 2007).

The crudes of dichloromethane, methanol, petroleum ether of root and stem bark of M. discolor extracts exhibited higher antimicrobial activity (Table S4). Virtually no activity was observed against P. aeruginosa and Candida albicans. The exhibited antimicrobial activity of the root bark extract of M. discolor can be attributed to the presence of T-cadinol, γ-cadinene, elemene, and α-muurolene since these constituents have been reported to exhibit antibacterial activity against Acetobacter calcoaceticus, Bacillus subtilis, Clostridium sporogenes, Clostridium perfringens, Escherichia coli, Salmonella typhii, Staphylococcus aureus, and Yersinia enterocolitica (Kirichenko, Orlova, & Kurilov, 2008). The methanol extract of the root bark of M. discolor showed high level of cytotoxicity against brine shrimp larvae. Among the tested bacteria for this extract mild activity exhibited on S. aureus whereas no activity was observed from any of the tested fungi. The ethanol extract of the M. discolor stem showed growth inhibition only to S. aureus at mean IZ 14 mm, with the most active fraction exhibiting a mean inhibition zone ranging from 2.5 to 5.0 mm. The crude extract of M. discolor showed antimicrobial activity against S. aureus only. However, only compound 1 and 3 showed activity against both bacteria and fungi (Table 5).
3.6 The minimum inhibitory concentrations (MIC)

The minimum inhibitory concentrations (MIC) were determined using the micro plate serial dilution method. A duplicated experiment was done for each extract/ pure compound against each organism. Nutrient broth was prepared and 1 mL of it was transferred into each of the test tubes (Perez et al.1990). For antifungal MIC, Malt Extract Broth (MEB) was prepared according to the manufacturer’s instructions and dispensed in capped test tubes to about 10 mL in each tube. For the determination of antmycotic activity, all the fungal isolates, were first adjusted to the concentration of $10^6$ CFU/mL of subcultured fungal colonies were then asceptically transferred into the broth and adjusted to a turbidity equivalent to 0.5 McFarland standard. The results for tested compound 1, 2 and 3 showed mild activity against tested fungi and bacteria (Table 6).

Legend for table
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Figure S2: The $^{13}$C NMR spectrum for discolornolide
Figure S3. The DEPT-135° spectrum for discolornolide
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Table S1. $^1$H and $^{13}$C NMR spectral data, and HMBC interactions for compound 1
| H/C | δ_H | J (Hz) | δ_C | HMBC (1H→13C) |
|-----|-----|--------|-----|----------------|
| 1   | --- | 119.8  | --- | ---            |
| 2   | 7.12| s      | 137.9| 4, 15          |
| 3   | --- | 117.7  | --- | ---            |
| 4   | 6.07| s      | 130.7| 3.5            |
| 5   | --- | 169.9  | --- | ---            |
| 6   | --- | 137.5  | --- | ---            |
| 7   | 6.43| s      | 114.2| 5, 9           |
| 8   | --- | 205.8  | --- | ---            |
| 9   | 3.04| overlap| 54.1 | 8, 12          |
| 11  | --- | 154.2  | --- | ---            |
| 12’ | 3.21| dd, 18.0, 5.4 | 35.9 | 9, 11          |
|     | 2.94| dd, 18.0, 2.4 |      |                |
| 13  | 2.78| m      | 25.9 | 6, 8, 12, 14,  |
| 14  | 0.72| d, 6.6 | 14.1 | 9, 12          |
| 15  | 2.28| s      | 13.8 | 1, 2, 5        |
| 16  | 2.06| s      | 8.1  | 5, 8, 9        |
Table S2. Brine shrimp lethality tests for crude extract and some VLC fractions

| S/N | Sample     | LC50 (μg/mL) | 95% (CI)       |
|-----|------------|--------------|----------------|
| 1.  | MDLED-3    | 418.051      | 320.015 - 738.498 |
| 2.  | MDLW       | 90.108       | 53.492 - 195.361  |
| 3.  | MDRD       | 41.794       | 36.135 - 48.352   |
| 4.  | MDRM       | 13.560       | 12.176 - 15.009   |
| 5.  | MDSE       | 250.478      | 215.265 - 310.784 |

MD = Monathotaxis discolor, the subsequent letters stand for plant part followed by extracting solvent and the numerical stands for fraction/potion number i.e. L= leaves, R= root, S= stem bark, E=ethanol, W= water, D= dichloromethane, M= ethanol

Table S3. Brine shrimp lethality test for pure compounds

| Compound | LC50 (μg/mL) | 95% (CI)       |
|---------|--------------|----------------|
| 1       | 5.888        | 3.571 - 8.578  |
| 4       | 24.737       | 10.402 - 58.984 |
| 5       | 57.754       | 33.094 - 154.940 |

CI= Confidence interval

Table S4. Antimicrobial activities of crude extracts

| Sample | E. coli | S. aureus | P. aeruginosa |
|--------|---------|-----------|--------------|
|        | IZ\text{mean} (mm) | AI | IZ\text{mean} (mm) | AI | IZ\text{mean} (mm) | AI |
| MDRD   | 8.0     | 0.33      | 5.0          | 0.21 | - | - |
| MDRM   | -       | -         | 5.0          | 0.21 | - | - |
| MDSE   | -       | -         | 14.0         | 0.58 | - | - |
| Amp.   | 22.0    | 1.0       | 24.0         | 1.0  | 21.5 | 1.0 |

IZ\text{mean}= Mean of inhibition zone, AI = Activity index, N/A= not applicable, '-' = No activity; Amp.= Ampicillin,
Table S5. Antimicrobial activities of pure compounds: Antimicrobial tests done in duplicate

| Sample | Bacteria | | | | Fungi | |
|---|---|---|---|---|---|---|
| | | E. coli | S. aureus | P. aeruginosa | C. albicans | A. niger |
| | | IZ\_mean (mm) | AI | IZ\_mean (mm) | AI | IZ\_mean (mm) | AI |
| 4 | 5.0 | 0.23 | 4.0 | 0.20 | - | - | - | - |
| 1 | - | - | 5.5 | 0.27 | - | - | 7.0 | 0.44 | 5.5 | 0.31 |
| 3 | - | - | 5.5 | 0.27 | 7.0 | 0.33 | - | - | 4.0 | 0.22 |
| 5 | - | - | - | 3.0 | 0.14 | - | - | - | - | - |
| Amp. | 22.0 | 1.0 | 20.5 | 1.0 | 21.5 | 1.0 | N/A | N/A |
| Fluc. | N/A | N/A | N/A | N/A | 16.0 | 1.0 | 18.0 | 1.0 |

IZ: inhibition zone, AI: activity Index (i.e IZ\_mean of sample divided by IZ\_mean of the standard), Amp : Ampicillin, Fluc. : Fluconazole

Table S6. The MIC test for both Fungi and Bacteria

| Compound | Fungi | Bacteria | |
|---|---|---|---|
| | C. albicans | A. niger | S. aureus | P. aeruginosa |
| | MIC( mg /mL) | MIC( mg /mL) | MIC (mg /mL) | MIC (mg /mL) |
| 1 | 0.13 | 0.17 | 0.17 | Not done |
| 3 | 0.17 | 0.25 | 0.25 | 0.25 |
| 5 | 0.17 | Not done | Not done | 0.25 |
| Fluc. | 0.5 | 0.5 | N/A | N/A |
| Amp. | N/A | N/A | 0.5 | 0.5 |

IC: inhibition concentration initial conc.0.5mg/mL; Not done: because they did not exhibit activity in preliminary assays; MIC: initial concentration of the sample divide by number of serial dilution before deep-purple coloration; Fluc. : fluconazole, Amp.: ampicillin N/A: Not applicable
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Properties of the other compounds isolated from *M. discolor*

**Karatavin (2):** Pale yellow oil; yield 147 mg; TOFMS-EI for \(C_{29}H_{48}O\) m/z 220 (M^+161); UV (CHCl3) \(\lambda_{\text{max}}\) (log ε) 230.60 (1.8); [α]_D^25 - 0.07, (c. 0.5, CHCl3) IR (film), \(v_{\text{max}}\) cm\(^{-1}\) 3411, 2955, 2868, 1668, 1464, 1432, 1367, 1297, 1235, 1191, 1142, 1096, 1072, 1010, 904, 878 and 769; TOF MS-EI m/z 222.19, C\(_{13}H_{26}O_2\); \(^1\)H NMR (CDCl\(_3\), 300 MHz): \(\delta\) 1.98 (2H, m, H-1), 5.55 (1H, s, H-2), 1.32 (1H, m, H-4α), 1.47 (1H, m, H-4β), 1.03 (1H, m, H-5), 1.37 (1H, m, H-6α), 1.92 (1H, m, H-6β), 1.41 (1H, m, H-7α), 1.74 (1H, m, H-7β), 1.11 (1H, m, H-9), 1.95 (1H, m, H-10), 2.18 (1H, m, H-11), 0.79 (3H, d, 6.9 Hz, H-12), 0.94 (3H, d, 6.9 Hz, H-13), 1.22 (3H, br, s, H-14), 1.67 (3H, m, H-15). \(^{13}\)C NMR (CDCl\(_3\), 300 MHz): \(\delta\) 30.9 (C-1), 122.7 (C-2), 134.3 (C-3), 19.8 (C-4), 46.7 (C-5), 22.6 (C-6), 40.3 (C-7), 70.7 (C-8), 47.9 (C-9), 26.2 (C-11), 15.2 (C-12), 21.4 (C-13), 28.5 (C-14), 23.7 (C-15).

**N-Acetylanonaione (3):** Brown crystals; yield, 2.2 mg; mol. wt C\(_{19}H\_17NO_3\); \(^1\)H NMR (CDCl\(_3\), 300 MHz): \(\delta\) 6.59 (1H, s, H-3), 2.65, 2.86 (2H, dd, 15.6, 18.4 Hz, H-4), 3.30, 3.99 (2H, dd, 15.6, 18.4 Hz, H-5), 5.21 (1H, d, 12.4 Hz, H-6a), 2.73, 3.13 (2H, d, 14.4, 13.6 Hz, H-7), 7.26–7.33 (1x3H, m, H-8,9,10), 8.11 (1H, d, 8 Hz, H-11), 5.98 (2H, s, OCH\(_2\)O), 2.23 (3H, s, C(O)CH\(_3\)). \(^{13}\)C NMR (CDCl\(_3\), 300 MHz): \(\delta\) 143.1 (C-1), 126.0 (C-1a), 130.5 (C-1b) 146.8 (C-2), 107.3 (C-3), 135.8 (C-3a), 30.8 (C-4), 42.2 (C-5), 50.5 (C-6a), 33.5 (C-7), 117.6 (C-7a), 126.9–128.9, (C-8,9,10), 126.9 (C-11), 117.6 (C-11a), 100.91 (OCH\(_2\)O), 22.6 (C(O)CH\(_3\)), 169.2 (C(O)).

**Quercetin-3-O-D-arabinose (4):** Yellow powder; yield, 34 mg; react with anisaldehyde- green then orange; TOFMS-EI for \(C_{20}H_{19}O_{11}\) m/z 434 (M^+302); m.p 215.2–216. \(^0\)C, UV (MeOH) \(\lambda_{\text{max}}\) (log ε) 342.20 (5.0); [α]_D^25 - 41.6, (c. 0.5, MeOH) IR (film), \(v_{\text{max}}\) cm\(^{-1}\) 3443 (br,m), 3297 (br,m), 2962 (br,m), 1650, 1599, 1552, 1498, 1448, 1360 (s), 1305, 1268, 1194, 111, 1042, 1005, 943, 924, 827, 803 and 772 ; \(^1\)H NMR (CDCl\(_3\), 300 MHz): \(\delta\) 6.39 (1H, d, 1.8 Hz, H-6), 6.21 (1H, d, 1.8 Hz, H-8), 6.90 (1H, d, 8.4 Hz, H-2'), 7.52 (1H, d, 1.8 Hz, H-5'), 7.49 (1H, dd, 1.8, 2.4 Hz, H-6'), 5.49 (1H, s, H-1'), 4.30 (2H, d, 4.8 Hz, H-2''), 3.90 (1H, t, 3.0, 4.8 Hz, H-3'), 3.85 (1H, dd, 4.2, 6.6 Hz, H-4'). 3.50 (2H, d, 4.2 Hz, H-5'). \(^{13}\)C NMR (CDCl\(_3\), 300 MHz): \(\delta\) 157.2 (C-2), 138.5 (C-3), 178.6 (C-4), 104.2 (C-4a), 157.9 (C-5), 98.5 (C-6), 164.8 (C-7), 93.4 (C-8), 161.7 (C-8a), 121.7 (C-1'), 115.0 (C-2'), 144.9 (C-3'), 148.5 (C-4'), 115.4 (C-5'), 121.5 (C-6'), 108.1 (C-1"), 81.9 (C-2'"), 77.3 (C-3'"), 86.6 (C-4'"), 61.1 (C-5'').

**Stigmasterol (5 & 6):** White powder; yield 96 mg; m.p 162.1–162.6 \(^0\)C, TOFMS-EI for \(C_{29}H_{48}O\) m/z 412 (M^+, 55); IR (film), \(v_{\text{max}}\) cm\(^{-1}\) 3362, 3194, 3063, 2970, 2916, 2900, 2865, 1686, 1604, 1571, 1550, 1436, 1379, 1340, 1215, 1142, 1098, 1041, 986, 929, 881, 858, 789, 760 and 739; \(^1\)H NMR (CDCl\(_3\), 300 MHz): \(\delta\) 0.68, 0.79, 0.82, 0.86, 0.92, 1.02 (each 3H, Me x 6), 3.53 (1H, m, H-3), 5.36 (1H, t, H-22), 5.15 (1H, s, H-22), 5.01 (1H, s, H-23); \(^{13}\)C NMR (CDCl\(_3\), 300 MHz): \(\delta\) 140.8 (C-5), 138.3 (C-22), 129.4 (C-3), 121.7 (C-6), 71.9 (C-3), 56.8 (C-14), 55.9 (C-17), 51.2 (C-24), 50.1 (C-9), 5.8 (C-25), 40.6 (C-13), 39.8 (C-20), 37.3 (C-12), 36.5 (C-4), 36.2 (C-1), 36.1 (C-10), 33.9 (C-8), 31.9 (C-7), 29.2 (C-16), 28.4 (C-2), 26.1 (C-28), 24.3 (C-15), 21.2 (C-21), 20.3 (C-11), 19.80 (C-27), 19.1 (C-26), 18.7 (C-19), 12.0 (C-29), 11.9 (C-18).

**Octahydro-5-isopropyl-3-methyl-2-methyleneazulene-8,8-diol (7):** Colourless oil 57 mg; molecular formula C\(_{15}H_{26}O_2\); TOFMS-EI for m/z 220 (M^+81), IR (film), \(v_{\text{max}}\) cm\(^{-1}\) 3425,
2929, 2868, 1711, 1666, 1635, 1452, 1374, 1148, 1095, 907, 886, 758; $^1$H NMR (CDCl$_3$, 300 MHz): δ 1.05- 1.68 (1H, m, H-1α), 1.92-1.96 (1H, m, H-1β), 0.68-0.73 (1H, m, H-3), 2.01 – 2.09 (1H, m, H-4α), 2.42 – 2.46 (1H, dd, J = 6, H-4β), 0.44 – 0.51 (1H, m, H-5), 0.98 -1.02(1H, m, H-6α), 1.96 – 1.99(1H, m, H-6β), 2.03 – 1.09 (1H, m, H-7α), 2.40 – 2.44 (1H, m, H-7β), 1.32- 1.35(1H, m, H-9), 2.17 – 2.42 (1H, m, H-10), 1.50(1H, m, H-11), 1.29 (3H, s, H-12), 1.06 (3H, s, H-13), 1.05 (3H, s, H-14), 4.67(1H, d, H-15α), 4.70(1H, d, H-15β); $^{13}$C NMR (CDCl$_3$, 300 MHz): δ 26.7(C-1), 153.4(C-2), 27.3 (C-3), 38.8 (C-4), 29.9 (C-5), 24.8(C-6), 41.7 (C-7), 80.9 (C-8), 54.3 (C-9), 53.4 (C-10), 26.2 (C-11), 26.05 (C-12), 28.6 (C-13), 16.3 (C-14), 106.2 (C-15).