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Research article

Volatiles from African species of *Croton* (Euphorbiaceae), including new diterpenes in essential oil from *Croton gratissimus*

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ABSTRACT

The chemistry of essential oils from the leaves of three African species of *Croton* (*C. gratissimus*, *C. pseudopulchellus* and *C. sylvaticus*) is comprehensively characterised. Two new volatile diterpenes were isolated from *C. gratissimus* and the structures assigned using 1D and 2D NMR. One is a furanyl-halimane methyl ester (**1**) assigned as 12-β-furanyl-halima-5,9-dien-4-methylcarboxylate (gratissihalimanoic ester); the other is an abietane ketone (**2**) assigned as *ent*-abiet-8(14), 13(15)-dien-3-one, which we have named gratissimone. High relative abundance of diterpenes in a hydrodistilled essential oil is rare and may be considered an interesting discovery. Known non-volatile diterpenes were also isolated, which were assigned as crotohalimaneic acid (**3**) and hardwickiic acid (**4**). All diterpenes occur in fresh leaves prior to distillation and extract into apolar or moderately polar solvents, which demonstrates that the two volatiles are not generated during the hydrodistillation. At this stage it is not clear how widespread this diterpene essential oil chemotype is within the species distribution or if any therapeutic effects can be attributed to them. No antimicrobial activity was observed at 1 mg/ml against a range of bacterial strains.

1. Introduction

Species of *Croton* feature prominently in the traditional pharmacy of several of the world's cultures, making up a large part of the Amazonian therapies of South America and to a lesser extent traditional Chinese medicine (TCM) and Ayurvedic practices of Asia. Several records can also be found for Central and North America (Salatino et al., 2007), Australia and Africa, the last-mentioned being the focus of the current study. More than 20 species of *Croton* are used in Africa and Madagascar for medicinal purposes (Neuwinger, 2000).

Two species of *Croton* are cultivated to supply an international trade of fixed oils (non-volatile oils) that can be expressed from the seeds. The species endemic to the African continent is *C. megalocarpus* Hutch., which is widely distributed from the DR Congo, east to Kenya and as far south as Mozambique and South Africa. The oil from *C. megalocarpus* is used as a biofuel but acts as a laxative if ingested. The other *Croton* oil is of Indian origin, obtained from the seeds of *C. tiglium* L. This species is a source of a tumour promoting phorbol ester (12-O-tetradecanoylphorbol-13-acetate), which is currently used in research for the induction of tumour growth. The oil of *C. tiglium* is a skin irritant; the irritation is caused mainly by the high concentrations of the phorbol esters. Its traditional use as a laxative continues today in Chinese medicine (Salatino et al., 2007; Wink and Van Wyk, 2008).

The phorbol esters of *C. tiglium* are only a minor representation of the other classes of terpene described in the genus. A number of shikimate-derived compounds have been described, which include phenolics such as flavonoids, lignoids and proanthocyanidins. Triterpenoids, such as the lesser known pentacyclic and steroidal compounds acetyl aleuritolic acid and lophenol respectively, have been described and less often alkaloids. Thus, the chemistry of *Croton* is diverse, but the greater research focus has been on the classes of diterpene that predominate in the metabolomics profile of the majority of the species. The clerodane or halimane type diterpene is overwhelmingly the most common class reported in *Croton* (Salatino et al., 2007).

The three species investigated in the current study are endemic to southern and eastern Africa. They are *C. gratissimus* Burch., *C. pseudopulchellus* Pax and *C. sylvaticus* Hochst. All three of these species have been used in the African traditional pharmacy (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996; Neuwinger, 2000).

Croton gratissimus is currently known under two varieties, i.e., var.

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gratissimus and var. *subgratissimus* (Prain) Burtt Davy. Due to the complete overlap in the distribution areas of the two varieties, their identification is somewhat ambiguous. There is currently no published data describing chemical distinctions between the two and no chemotypes have yet been described. An unpublished account of the chemical character of the essential oil of a single specimen from eastern Pretoria in South Africa gave a predominantly monoterpenoid essential oil comprised of α-pinene, α-phellandrene, Z- and E-β-ocimene and germacrene-D (Van Vuuren, 2007; Van Vuuren and Viljoen, 2008).

Previous phytochemical studies on *C. pseudopulchellus* include the isolation of several *ent*-kaurenoic acid derivatives from the stem bark (Langat et al., 2012) but the volatile compounds have apparently not yet been studied from specimens collected in southern Africa. A Kenyan specimen yielded an oil rich in limonene, linalool and caryophyllene (Odalo et al., 2005).

The uses of *C. gratissimus* and *C. pseudopulchellus* in traditional aromatherapy, skin care and perfumery, as well as their vernacular names (respectively 'lavender croton' or 'small lavender fever berry') indicate that it would be interesting to know the chemical character of volatiles and from where this apparent lavender smell originates. Furthermore, knowledge of the volatile components of these species may be relevant in the context of the use of steam baths, chest rubs and smoke inhalation to treat respiratory complaints.

2. Materials and methods

2.1. General

Plant material from natural populations was collected with permissions and permits; *C. gratissimus* from Venda, near Kundun Village (Limpopo Province, South Africa) and also from the Pretoria Botanic Gardens (Gauteng Province, South Africa). The material of *C. pseudopulchellus* also came from the vicinity of Kundun village, a few km from the *C. gratissimus* population. Leaves from *C. sylvaticus* were harvested from private land in Kwa-Zulu Natal. Voucher specimens have been deposited in the herbarium of the University of Johannesburg (JRAU) with accession numbers NJS001-006.

Essential oils were produced by continuous hydrodistillation in a Clevenger apparatus fitted with a water-cooled mantle (design unpublished) over 3 h and 48 h periods using carefully air-dried leaves from the three species. Crude essential oils were characterised by GC-MS and NMR. GC-MS operating conditions were as follows: A Shimadzu 2010 with detector interface at 250 °C; ion source 200; injector temperature 200 °C; carrier gas helium; 1 μl injections with a split ratio (1:20); fitted with an OV-1 (WCOT) (non-polar) column. Column flow was at 1 ml/min; column ramp: 60 °C (no hold), 5 °C per min then held at 280 °C for 5 min. Identification of compounds were made by comparing the mass spectra and retention indices (calculated relative to n-alkanes) with the National Institute of Standards and Technology (NIST) library and Adams (2007). High resolution MS for the two diterpenes were produced at the Mass Spectrometry Unit, Central Analytical Facility, University of Stellenbosch using a Waters Micromass GCT Premier Mass Spectrometer (GC-TOF MS). See supplementary files (MS data – gratissimone and gratissihalimanoic ester).

Unknown compounds in the essential oils were isolated and elucidated using NMR. A dichloromethane solvent extract of fresh leaves was also made for direct injection into the GC-MS to confirm the presence of **1** and **2** in fresh leaves and another prominent non-volatile diterpene **3** was isolated when the solvent extracted residue was subjected to chromatographic separation. All isolations were made using polar stationary phase (silica gel) column chromatography with mobile phase 10% (v/v) ethyl acetate in cyclohexane for **1** and **2** (essential oils), and 15% (v/v) ethyl acetate in cyclohexane for **3** and **4** (solvent extracted residue). Diterpenes **2** and 'iso-**2**' coeluted in chromatography so the mixture was subjected to crystallisation steps to achieve separation of iso-**2** (non-crystaline) and **2** (crystalised), using a H_2O in methanol combination (2:8, H_2O:MeOH) and slow evaporation. Only **2** was successfully isolated, but iso-**2** remained in a mixture with **2** so only ^{13}C spectra can be provided, with no structure successfully assigned.

The NMR instrument was a 500 MHz Bruker Avance (Bruker, Germany) and diterpenes were dissolved in *d*-chloroform. 1D (^1H, ^{13}C and DEPT135) and 2D-NMR experiments (^1H–^1H COSY, ^1H–^{13}C HSQC, ^1H–^{13}C HMBC and ^1H–^1H NOESY) were used to elucidate structures **1** and **2** using standard Bruker pulse sequences. See supplementary files for ^1H and ^{13}C spectra of compounds **1** and **2** (**1**: ^1H gratissihalimanoic ester, ^{13}C gratissihalimanoic ester; **2**: ^1H gratissimone, ^{13}C gratissimone). Optical rotations were calculated on Polartronic H532.

Compound **1**; gratissihalimaneic ester, (17-α, 18-β)-12-β-furanylhalima-5,9-dien-4-methylcarboxylate. Isolated as a clear viscous oil. $[\alpha]_D^{23}$-24° (c 0.2, MeOH). GC-HREI-MS$^+$ found 314.1889, $C_{20}H_{26}O_3$ requires 314.1882; MS (EI) *m/z* 314 (M^+ 23), 299 (8), 255 (44), 173 (100), 159 (11), 145 (10), 131 (10), 81 (8) (see supplementary files); NMR see Table 2.

Compound **2**; gratissimone, assigned as (5S,9R,10S)-*ent*-abiet-8(14), 13(15)-dien-3-one. Isolated as white crystalline solid. $[\alpha]_D^{23}$ + 108° (c 0.2, MeOH). GC-HREI-MS$^+$ found 286.2295, $C_{20}H_{30}O$ requires 286.2297; MS (EI) *m/z* 286 (M^+ 83), 271 (22), 243 (17), 201 (10), 148 (47), 135 (100), 119 (36), 105 (40), 91 (44), 79 (26) (see supplementary files); NMR see Table 2.

Compound *iso*-**2**; iso-gratissimone. Partly isolated as clear oil. GC-HREI-MS$^+$ found 286.2295, $C_{20}H_{30}O$ requires 286.2297; MS (EI) *m/z* 286 (M^+ 100), 271 (16), 243 (49), 201 (10), 187 (10), 173 (10), 157 (15), 145 (15), 136 (31), 105 (29), 91 (19), 81 (18) (see supplementary files); CNMR, 216.9, 145.8, 135.4, 122.2, 120.8, 51.6, 50.2, 47.7, 38.2, 34.97, 34.9, 34.87, 27.5, 25.1, 24.3, 22.8, 22.3, 21.5, 20.95, 13.5.

Compound **3** and **4**; crotohalimaneic acid. NMR spectra were matched exactly with spectra published in Roengsumran et al. (2004) and (Salatino et al., 2007) respectively.

X-ray Crystal Structure of **2** (Fig. 1). Single-crystal X-ray diffraction data on compound **2** were collected at 100 K using Mo Kα radiation and a Bruker APEX-II CCD diffractometer. A crystal with approximate dimensions 0.143 × 0.143 × 0.112 mm^3 was coated with high-viscosity microscope oil (MiTeGenDualThickness Micro-Mount, coated in a layer of Paratone oil), mounted on a glass rod and positioned in the cold stream (-170 °C) on the diffractometer. Using Olex2, the structure was solved with the ShelXT structure solution program using Intrinsic Phasing and refined with the ShelXL refinement package using Least Squares minimisation. Crystal data for **2** (M =286.44 g/mol): monoclinic, space group $P2_1$ (no. 4), a = 8.2928(9) Å, b = 11.8775(13) Å, c = 8.7522(9) Å, β = 105.104(3)°, V = 832.29(15) $Å^3$, Z = 2, T = 100(2) K, μ(MoKα) = 0.068 mm^{-1}, *Dcalc* = 1.143 g/cm^3, 11691 reflections measured (4.82° ≤ 2Θ ≤ 56.648°), 2983 unique (R_{int} = 0.0241, R_{sigma} = 0.0233) which were used in all calculations. The final R_1 was 0.0348 (I > 2σ(I)) and wR_2 was 0.0959 (all data). Flack parameter = 0.3(7). The crystal structure was deposited at the Cambridge Crystallographic Data Centre, with CCDC 1538534 as the assigned deposition number.

2.2. Antimicrobial assay (minimum inhibitory concentration determination)

Diterpenes **1** and **2** were subjected to antimicrobial testing against Gram-negative bacterial strains; *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, and Gram-positive strains; *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228 and *Bacillus cereus* ATCC 11175. Starting concentrations were 1 mg/ml and the method used followed that described by Eloff (1998).

3. Results and discussion

3.1. Antimicrobial activity of 1 and 2

No antimicrobial activity was observed at 1 mg/ml against the organisms tested.

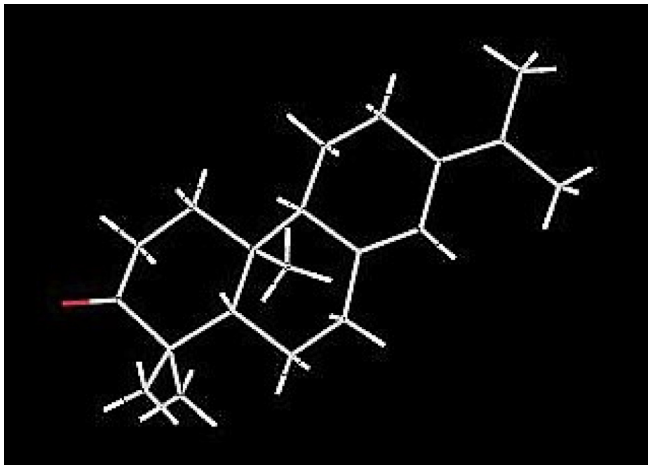

Fig. 1. Crystal structure of gratissimone (2).

3.2. Structural assignment of *1* and *2*

Using NMR, chemical assignments were made for the two heavier major unknown volatile compounds detected by GC-MS. These diterpenes occur in the hydrodistilled essential oils at varying relative abundances (Table 1) in all three specimens sampled from the Pretoria population of *C. gratissimus* but were detected in only trace quantities in the Venda population of the same species.

The two isolated diterpenes were assigned as 12-β-furanyl-halima-5,9-dien-4-methylcarboxylate (**1**) and (5S,9R,10S)-*ent*-abiet-8(14), 13(15)-dien-3-one (**2**) by 2D-NMR and the structure and stereochemistry of **2** was confirmed by x-ray crystallography (Fig. 1). ^{13}C and ^{1}H spectroscopic assignments are provided in Table 2. Shorter names for **1** and **2** are gratissihalimanoic ester and gratissimone respectively. Structure **3** (crotohalimaneic acid) was isolated from a dichloromethane extract of the leaves to provide stereochemical insight into **1** and NOESY couplings were able to confirm this.

Assignment of **1** (Fig. 2) as a C20 diterpenoid methyl ester is slightly unusual because the addition of a methyl ester to a terpene usually generates adducts with a carbon number indivisible by five; the carbon number of the isoprene unit. In the case of a diterpene it would therefore occur as a C21 molecule (4 X 5 + 1). At first it was hard to know if the current structure **1** was either a labdane or halimane derivative. Both of these normally have a quaternary methyl group attached at position 10 (labdane) or 9 (halimane); however, **1** has an olefinic bridgehead at position 9(10) with no methyl attachment. This might point toward a reason for the unusual carbon number of this molecule. This also made it difficult to know if the biosynthetic precursor is a labdane or halimane.

Table 1
Composition of essential oils from *Croton gratissimus*. Specimens sampled from Pretoria Botanical Gardens are labelled 'P-A, B, C' with the numerical value 3 or 48 indicating hours of distillation time. Specimens from Venda are denoted with 'V-A, B, C' again with numerical values reflecting distillation time.

Percentage of total yield	AI	Pub. AI	P-A48	P–B48	P–C48	P–C3	V-A3	V–B3	V–C3	V–C48
Yield % g/g	-	-	1.02	1.48	1.22	0.38	1.34	1.41	0.12	1.53
α-Thujene	920	924	-	-	-	-	2.5	2.8	2.8	-
α-Pinene	927	932	-	-	-	-	6.2	5.5	6.1	-
Camphene	944	946	-	-	-	-	0.4	0.5	0.5	-
Sabinene	965	969	-	-	-	-	0.7	1.4	0.8	-
β-Pinene	971	974	-	-	-	-	0.9	0.6	1.7	-
1-Octen-3-ol	976	974	-	1.6	-	-	-	-	-	-
β-Myrcene	981	988	-	-	-	-	1.0	1.0	1.3	-
α-Phellandrene	1004	1002	-	7.0	-		41.6	46.2	49.1	35.5
α-Terpinene	1011	1013	-	-	-	-	0.6	0.7	0.6	-
p-Cymene	1021	1020	-	9.1	-	-	8.2	6.6	5.0	7.6
Limonene	1022	1024	-	-	-	-	1.6	1.6	1.5	-
β-Phellandrene	1024	1025	-	-	-	-	-	3.4	3.0	-
Z-β-Ocimene	1027	1032	-	-	-	-	0.9	1.5	-	3.4
E-β-Ocimene	1040	1044	-	0.8	-	-	1.8	2.1	1.2	-
γ-Terpinene	1051	1054	-	-	-	-	1.8	2.0	1.8	-
Terpinolene	1080	1086	-	-	-	-	0.8	1.0	0.9	-
Linalool	1095	1095	-	1.0	-	-	-	-	-	-
Camphor	1145	1141	-	1.7	-	-	0.9	1.4	1.4	-
α-Terpineol	1193	1186	-	1.2	-	2.0	-	-	-	-
α-Copaene	1375	1374	4.6	3.0	2.7	2.4	2.2	1.6	1.9	3.1
β-Bourbonene	1383	1387	4.6	4.0	2.1	2.3	2.4	0.6	1.3	2.6
E-Caryophyllene	1419	1417	11.2	8.3	4.8	9.6	0.8	0.5	4.4	6.7
β-Cupaene	1429	1430	0.4	0.8	-	0.3	-	-	-	-
α-Caryophyllene	1454	1455	2.3	3.6	-	2.8	0.4	-	1.6	2.6
9-epi-*E*-Caryophyllene	1462	1464	1.0	-	-	0.9	-	-	-	-
Germacrene D	1480	1484	16.9	5.9	5.7	24.1	10.5	4.7	5.7	9.0
Bicyclogermacrene	1497	1500	1.3	0.7	-	0.8	1.1	0.6	0.7	0.9
α-Muurolene	1498	1500	-	-	-	-	0.6		0.3	0.5
γ-Cadinene	1511	1513	0.3	0.5	-	0.3	-	0.4	0.3	0.4
δ-Cadinene	1517	1522	3.0	1.9	1.8	1.3	1.7	0.9	1.1	1.6
Elemol	1546	1548	2.6	1.1	-	6.7	-	-	0.3	2.7
Spathulenol	1575	1577	-	0.5	0.8	4.1	0.1	-	-	0.2
Caryophyllene oxide	1581	1582	1.8	1.1	1.1	6.6	0.1	-	-	0.3
γ-Eudesmol	1630	1630	3.9	1.0	1.3	1.4	0.2	-	-	2.1
β-Eudesmol	1653	1649	7.5	2.5	3.0	7.2	t	2.8	-	0.1
Nerolidylisobutyrate	1789	1783	2.9	2.4	-	-	0.8	-	1.5	4.5
Unknown	1860	-	-	2.2	3.7	0.6	-	2.3	-	-
Gratissihalimanoic ester (**1**)	2179	NMR	12.9	1.6	4.7	1.9	-	-	-	-
Unknown	2222	-	1.7	0.4	1.8	-	-	-	-	-
Unknown	2295	-	-	0.8	3.7	-	-	-	-	-
iso-Gratissimone	2352	Epi	6.7	0.3	2.2	-	-	-	-	-
Gratissimone (**2**)	2369	NMR	1.0	20.8	58.8	15.9	t	t	t	t
Unknown	2420	-	-	1.3	1.3	1.6	-	-	-	-

Due to the occurrence of furanyl-halimanes in other African *Croton* species we have described **1** as a halimane derivative. Due to the isolation of **3** and **4** in the current study, and to biosynthetic constraints, our assignment of **1** as a halimane is tentatively confirmed. This was further iterated by NOESY coupling of both the methyl-methoxy protons (at carbon 20) and methyl protons (at position 17) with the α-H on carbon 11 (2.06 ppm). Another NOESY coupling was observed for methyl protons at 18 with the β-H at carbon 8.

The HRMS of compound **1** gave a molecular formula of $C_{20}H_{26}O_3$. The index of hydrogen deficiency (IHD) = 8. ^{13}C spectra indicates a carbonyl group in the acid region (δ 178.0) and eight olefinic carbons (δ 138.5, 117.5, 138.0, 125.8, 125.0, 111.2, 142.8 and 139.0), which gives four C=C double bonds and one C=O double bond, giving a total of five hydrogen substituted bonds, with the remainder of three (from IHD = 8) indicating a tricyclic molecule. The mass spectrum of the molecule shows a molecular ion at m/z 255.1748, corresponding to the removal of an acid ester group by alpha cleavage (expected m/z 59.0133, observed m/z 59.0141).

Table 2
NMR spectral values of **1** and **2**.

Gratissihalimanoic ester (1)				Gratissimone (2)		
Position	δ_C	δ_H (*J* in Hz)	HMBC	δ_C	δ_H (*J* in Hz)	HMBC
1	25.9	2H - m, 1.96 & m, 2.51	2	38.0	2H - m, 1.50 & ddd, 2.0 (13.3, 5.6, 3.8)	2, 5, 9
2	20.3	2H - m, 1.53 & m, 1.67	3	34.9	2H - td, 2.3 (3.8, 14.7) & dt, 2.65 (5.6, 14.7)	1, 20
3	34.8	2H - m, 1.50 & m, 2.11	18	216.9	-	1, 2, 18, 19
4	48.0	-	2, 3, 6, 18	48.1	-	2, 6, 18, 19,
5	138.5	-	7, 18	55.4	1H brd, 1.9 (9.8)	1, 6, 7, 18, 19, 20
6	117.5	1H dd, 5.30 (2.2, 6.1)	7, 8	23.4	2H - m, 1.51 & m,1.57	5, 7, 9, 16, 17
7	30.2	2H - m, 1.89 & m, 2.25	6, 8, 17	35.8	2H - dd, 2.17 (9.8, 15.5) & brd, 2.4 (15.5)	6, 14
8	31.3	1H m, 2.02	7, 11, 17	137.6	-	6, 7, 9, 11, 14, 16,
9	138.0	-	11, 17	50.5	1H t, 1.5 (11.7)	1, 7, 11, 12, 14, 20
10	125.8	-	11	38.1	-	1, 2, 5, 9, 20
11	32.6	2H - m, 2.06 & m, 2.52	12	22.8	2H - m, 1.41 & m, 1.8	9, 12
12	24.7	2H t, 2.49 (7.42)	11,	25.9	2H - dt, 1,87 (14.1, 3.6) & dt, 2.5 (14.1, 5.6)	9, 11, 14
13	125.0	-	12, 14, 15, 16	128.1	-	11, 12, 14, 16, 17
14	111.2	1H brs, 6.30	12, 15, 16	122.7	1H s, 6.26	7, 9, 12,
15	142.8	1H brs, 7.35	14, 16	124.3	-	12, 14, 16, 17
16	139.0	1H brs, 7.23	12, 14, 15	20.5	3H s, 1.71 *a	17
17	16.6	3H d, 0.86 (6.93)	7, 8	19.8	3H s, 1.75 *a	16
18	26.4	3H s, 1.32	3	25.9	3H s, 1.1 *b	5, 19
19	178.0	-	18, 20	22.5	3H s, 1.07 *b	5, 18
20	52.1	3H s, 3.70	-	15.0	3H s, 0.96	1, 5, 9

* Assignments may be interchangeable.

The aromatic proton shifts in the ^1H spectrum (6.30, 7.23 and 7.35 ppm) demonstrate the presence of an aromatic moiety. Coupling constants were too low to be resolved on our 500Mhz instrument but ^1H–^1H COSY couplings were observed between protons shifted to 6.30 and 7.35 ppm. The HSQC spectrum identified carbon positions 14 (δ 111.2), 16 (δ 139.0) and 15 (δ 142.8) as the location of the aromatic proton attachments respectively. On the HMBC spectrum all of these aromatic protons coupled to olefinic carbons at positions 13 (δ 125.0), 14, 15 and 16. Two of the protons at shifts of 6.30 and 7.23 ppm coupled long-range to the methylene carbon at position 12 (δ 24.7). Since only four olefinic carbons are present in the aromatic moiety it indicates the possibility of a furan. The broad apparent singlets on the ^1H spectra were poorly resolved but are evidently second order. HSQC places the protons at position 14 (6.30 ppm) and 15 (7.35 ppm), which is consistent with the appearance of a β-substituted furan moiety.

Furthermore, the next major ion in the HRMS spectrum at m/z 173.1325 corresponds to further removal of a non-radical methyl furan (radical + H) (expected m/z 173.1330) by beta cleavage along the carbon chain of the assigned structure. But the furanyl methyl radical itself can also be seen at m/z 81.0337 (expected m/z 81.0340) (see supplementary files). The carbon shifts at positions 15 (δ 142.8) and 16 (δ 139.0) are slightly downfield for sp^2 hydridised olefins, consistent with the presence of an electronegative neighbour. Since there are no ^{13}C shifts in the alcohol or ether region it makes sense that the remaining oxygen atom in the molecular formula (after the ester group) belongs to a heterocyclic group and in this case it is clearly a furan. Thus, we argue for the presence of a furanyl ethyl moiety.

HMBC and COSY helped to elucidate the remainder of the furanyl ethyl moeity: methylene 12 (δ 24.7) coupled by HMBC to two of the protons in the furan moiety (δ 6.30 and 7.23). COSY correlations demonstrated protons attached at positions 11 and 12 to be part of the same spin system and HMBC showed long range couplings of these protons to fully substituted olefinic carbons at positions 9 (δ 138.0) and 13 (δ 125.0). Since olefinic carbon at position 13 is part of the furan, position 9 corresponds to where the furanyl ethyl moiety attaches to the bicyclic section of the molecule.

HMBC showed that the olefinic carbon at position 10 (δ 128.5) and the tertiary carbon at position 8 (δ 31.3) coupled to protons at position 11. Thus, it is clear that the furanyl ethyl moiety attaches at position 9 adjacent to the 6, 7, 8 proton spin system on one side and the bridgehead of the bicyclic molecule (position 10) on the other.

1D and 2D-NMR experiments identified a quaternary methyl (δC 26.4, 3H s δH 1.32), a tertiary methyl (δC 16.6, 3H d δH 0.86), a carbonyl in the ester region (δ 178.0), a methyl ester carbon (δC 52.1, 3H s δH 3.70), six methylenes (δ 25.9, 20.3, 34.8, 30.2, 32.6 and 24.7), a quaternary (δ 48.0) and tertiary carbon (δ 31.3), four fully substituted olefinic carbons (δ 138.5, 138.0, 125.8 and 125.0) and another four sp^2 hydridised (δ 117.5, 111.2, 142.8 and 139.0). The two fully substituted olefinic carbons at positions 5 and 10 were established as the bridgehead of the bicyclic moiety on the basis that, after ignoring carbons assigned for the furanyl methyl and its position of attachment, the remaining substituted carbons were assigned as secondary methyl (position 8) and tertiary methyl carbonyl (position 4) respectively, with only methylenes and an sp^2 hybridised olefin remaining.

Correlations in the COSY spectrum demonstrated two spin systems on the bicyclic moiety, on either side of the bridgehead carbons. The previously mentioned methylenes 6, 7 and 8 made up one of the spin systems. ^1H spectra displayed that protons on the tertiary methyl at position 17 coupled to the methine proton at 8. HMBC couplings from C9 to H17 confirmed that this spin system was adjacent to the furanyl ethyl moiety attached at olefinic 9, as previously mentioned.

The other spin system on carbons 1, 2 and 3 are on the other ring and are

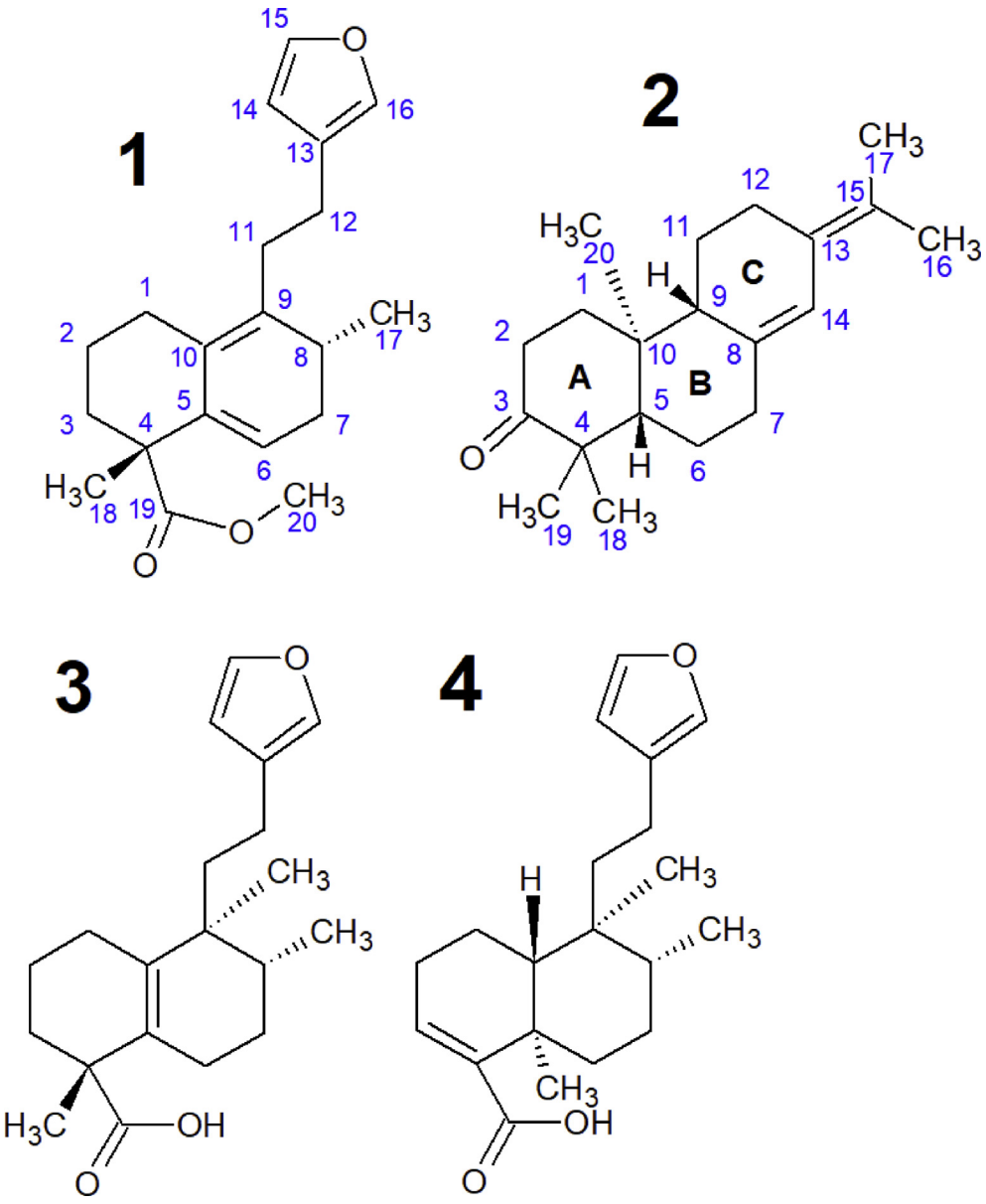

Fig. 2. Structures 1–4; 1) gratissihalimanoic ester, 2) gratissimone, 3) crotohalimaneic acid and 4) hardwickiic acid.

attached in that order. Further information from the HMBC spectra established the position of the carbonyl group relative to this spin system: the methyl ester protons couple to the carbonyl carbon and the carbonyl group is coupled to the quaternary methyl protons at position 18. The remaining fully substituted carbon at position 4 was established to be the position of attachment of this methyl group and the carbonyl previously discussed. Lastly, HMBC coupling of the methyl carbon at position 18 to the protons at position 3 established proximity to the 1, 2, 3 proton spin system, with position 3 being the adjacent carbon. This gave the final structure **1** (Fig. 2).

The occurrence of structures similar to our furan halimane **1** in *Croton* has been reported in several species. The halimane crotohalimaneic acid (**3**) was reported in *C. persimilis* Müll. Arg. (=*C. oblongifolius* Roxb.) and the clerodane hardwickiic acid (**4**) was isolated from *C. californicus* Müll. Arg., *C. draco* Schltdl. and *C. aromaticus* L. (Salatino et al., 2007). Both **3** and **4** were also reported in the root bark of a Kenyan specimen of *C. sylvaticus* by a research team in Nairobi (Ndunda et al., 2015). As previously mentioned, the stereochemical assignment of **3** provided insight into that of **1**, where the methyl groups positioned at 17 and 18 were confirmed to be in a relative *trans* configuration by a NOESY experiment.

Compound **2** was isolated as a crystalline solid, producing clear needle-like crystals. The HRMS of compound **2** gave a molecular formula of $C_{20}H_{30}O$. The assignments were first made by NMR using 1D and 2D experiments, such as DEPT, COSY, HSQC and HMBC. ^{13}C spectra displayed a carbonyl at δ 216.9 which is evidently a ketone, since no proton attachment could be seen on the ^{1}H or HSQC spectra. Four olefinic carbons could be seen; three fully substituted (δ 137.6, δ 124.3 and δ 128.1) and one sp^2 hybridised (δ 122.7). The attached proton gave a singlet at 6.26 ppm.

The index of hydrogen deficiency = 6. With four olefinic carbons (two double bonds = 2) and one C=O double bond (the ketone = 1) it is evidently another tricyclic molecule (6–1 – 2 = 3).

Five methyl singlets were observed; three quaternary and two further downfield at 1.71 ppm and 1.75 ppm, shifted downfield through attachment to an olefinic carbon. In HMBC the olefinic carbons demonstrating the strongest coupling to these downfield methyl protons were at 13 (δ 128.1) and 15 (δ 124.3). The two methyl groups were recognised to be part of a dimethyl methylidene group by examination of the HMBC couplings from methyl 3H proton singlets at 1.71 ppm and 1.75 ppm to the methyl carbons at δ 19.8 and δ 20.5 respectively (methyl carbons 16 and 17), showing that the two methyl groups are attached to the same

carbon, enabling the ^{3}J couplings. The position of attachment was established as olefinic carbon at position 15 (δ 124.3) since carbon 13 (δ 128.1) showed HMBC coupling to protons at position 11 (1.41 ppm and 1.8 ppm) and 12 (1.87 ppm and 2.5 ppm) whereas carbon 15 only coupled to protons at 12 but not 11.

Just to clarify, COSY spectra indicated that the proton at methine carbon 9 (1.5 ppm, δC 50.5) is part of a spin system that includes protons on carbons 11, (1.41 ppm and 1.8 ppm) and 12 (1.87 ppm and 2.5 ppm) in the order 9, 11, 12. This gives the total sequence of carbons as 9, 11, 12, 13, 15, 16/17. Since HMBC couplings from methyl carbons at 16/17 could also be seen to the sp^2 proton (6.26 ppm) it is certain that the two double bonds are adjacent and therefore conjugated. This clarifies the assignment of ring c.

From the remaining three quaternary methyl groups it was determined that one was angular and the remaining two were part of a dimethyl moiety. This was confirmed by HMBC which gave proton to carbon correlations between methyl carbons at 18 (δ 25.9) and 19 (δ 22.5) to methyl protons at 1.1 ppm and 1.07 ppm respectively. As previously mentioned, ^{3}J couplings are through a common carbon. In this case, the quaternary carbon attachment was assigned at position 4 (δ 48.1) by HMBC couplings from carbon 4 to the methyl protons (at 1.1 ppm and 1.07 ppm). These methyl protons also correlated in HMBC to the carbonyl at position 3 (δ 216.9) and the methine at position 5 (δ 55.4). This places the quaternary dimethyl carbon (position 4) between the ketone moiety and a bridgehead carbon. Furthermore, a proton-proton spin system attached to the two methylene carbons at positions 1 and 2 (δ 38.0 and δ 34.9 respectively) has long range correlations to the carbonyl carbon, the bridgehead carbon at position 5 and the bridgehead at position 10 (δ 38.1). This gave complete assignment of ring A (Fig. 2). HMBC gave a coupling of the quaternary carbon at position 10 with the methyl protons at position 20 (0.96 ppm), which established the position of the angular methyl (carbon 20).

Lastly, the COSY experiment demonstrated another proton-proton spin system attached to carbons 5 (δ 55.4), 6 (δ 23.4) and 7 (δ 35.8). HMBC correlation of olefinic carbon 8 (δ 137.6) to the protons in this spin system, mainly at positions 6 and 7, clarifies the connection of ring c (at position 8) through ring b to ring a (position 5). Finally, HMBC couplings from carbon at position 10 (δ 38.1) to the proton at position 9 (1.5 ppm) gave the structure **2** (Fig. 2).

The stereochemistry of compound **2** was confirmed by crystallography (Fig. 1). From XRD the stereochemistry of the molecule is enantiomeric to abietane and so compound **2** was assigned as an *ent*-abietane derivative with 5S, 9R and 10S stereocentres, numerated according to abietane. The assignment of **2** as an enantiomer of abietane is supported by the optical rotation at +108°, which is consistent with structures having the same three stereocentres (Anthonsen and Bergland., 1973) and opposite of its negative enantiomeric form (De Pascual Teresa et al., 1978). An isomer of **2** was also partially isolated but was not clean enough for assignment, however ^{13}C spectra are provided in materials and methods as *iso*-**2**. Relative ^{13}C shifts indicate different positions for the double bonds.

3.3. Characterisation of essential oils

Surprisingly the volatile oils of many of the species in *Croton* have not been comprehensively characterised but this may be a consequence of a lack of commercial interest. A glance over the literature reveals that the greater focus has been on the linalool-rich essential oil of *C. cajucara* Benth., followed by *C. zehntneri* Pax & K. Hoffm., *C. nepetifolius* Baill. and *C. flavens* L. All of these species are endemic to the South American region. Others from the same region (South America) and other parts of the world have been described and the data are summarised by Salatino et al. (2007).

In the current study, both **1** and **2** were detected in essential oils as major components of essential oils from *C. gratissimus* growing in Pretoria, and in one case **2** made up approximately 60% of the composition (Table 1). Two populations of *C. gratissimus* were sampled by us, one from Pretoria as previously mentioned, containing the new diterpenes, and one from the Venda region, which did not contain the new diterpenes (except for **2** in trace amounts). Essential oils from both populations were distilled for 3 h and 48 h using a Clevenger apparatus to determine if distillation time impacted upon relative abundance of diterpene. This was confirmed but the effect was not as pronounced as anticipated, since diterpene accumulation probably plateaued after 10hr. The chemotype from Pretoria which yielded the diterpenoid oil (specimen C) had 30–40% of **2** after only 3 h of distillation when compared to 48 h. The chemotype from Venda had higher sesquiterpene content after 48 h but no diterpenes were observed, aside from trace quantities of **2**.

Two varieties are known in *C. gratissimus*, i.e., var. *gratissimus* and var. *subgratissimus* (Prain) Burtt Davy. An attempt was made in the current study to correlate our chemical data with the varieties that have been described previously, however no conclusion could be drawn. The phenotypic characters of the Pretoria population were an intermediate of the two varieties, having dull olive-green upper leaf surfaces but no hairs. The Venda population clearly displayed characters of *C. gratissimus* var. *gratissimus* and so we determined it to belong to that variety. Most of the unpublished chemical studies of *C. gratissimus* essential oil describe a similar profile to the Venda variety in the current study, lacking the new diterpenes (Van Vuuren, 2007; Kamatou et al., 2016). Generally essential oils are dominated by the monoterpenes α-pinene, α-phellandrene and ocimene. The diterpenoid essential oil from the Pretoria population is therefore an unusual chemical variant within the species. The authors conclude that further research is necessary to clarify if the intermediate morphology in the Pretoria population correlates with this unusual chemistry.

Essential oil from the other two species, *C. pseudopulchellus* and *C. sylvaticus* (Table 3), were not unusual. A predominantly monoterpenoid

Table 3
Composition of essential oils from *Croton pseudopulchellus* (CP1, CP2 and CP3) and *Croton sylvaticus* (CS1 and CS2).

	AI	Pub.AI	CP1	CP2	CP3	CS1	CS2
Yield % g/g	-	-	1.96	1.46	1.57	0.06	0.08
α-Thujene	923	924	2.1	2.9	3.4	-	-
α-Pinene	930	932	11.1	20.7	19.4	-	-
Camphene	946	946	4.1	9.0	7.9	-	-
Sabinene	966	969	0.4	0.4	0.3	-	-
β-Pinene	972	974	5.6	8.0	5.9	-	-
β-Myrcene	981	988	16.2	5.3	14.5	-	-
α-Phellandrene	1001	1002	0.4	0.1	0.3	-	-
α-Terpinene	1011	1013	0.4	0.4	0.4	-	-
p-Cymene	1018	1020	1.4	1.7	1.5	-	-
Limonene	1023	1024	3.6	1.7	-	-	-
β-Phellandrene	1024	1025	22.8	6.8	20.0	-	-
Z-β-Ocimene	1027	1032	-	0.8	-	-	-
E-β-Ocimene	1038	1044	0.3	0.7	0.2	-	-
γ-Terpinene	1051	1054	1.9	1.6	1.9	-	-
Terpinolene	1080	1086	0.1	0.1	-	-	-
Linalool	1093	1095	0.8	0.6	-	-	-
Borneol	1169	1170	12.3	25.9	16.0	-	-
α-Terpineol	1178	1186	0.3	0.4	-	-	-
Bornyl acetate	1282	1285	1.0	3.0	2.3	-	-
α-Copaene	1375	1374	0.5	0.6	0.6	6.2	2.2
β-Bourbonene	1383	1387	0.5	0.5	0.6	6.9	-
E-Caryophyllene	1420	1417	3.8	2.2	2.1	6.1	5.1
β-Cupaene	1429	1430	0.2	0.2	-	-	-
α-Caryophyllene	1455	1455	1.7	1.0	0.9	-	-
9-epi-E-Caryophyllene	1462	1464	0.1	-	-	-	-
γ-Muurolene	1474	1478	0.5	0.4	0.3	-	-
Germacrene D	1481	1484	3.3	1.9	1.7	-	-
γ-Amorphene	1492	1495	0.2	0.2	-	-	-
Bicyclogermacrene	1495	1500	0.3	0.1	-	68.6	81.8
α-Muurolene	1498	1500	0.3	0.2	-	-	-
Bornyl isovalerate	1507	1520	0.2	0.3	-	-	-
γ-Cadinene	1513	1513	0.4	0.3	0.2	-	-
δ-Cadinene	1518	1522	1.1	1.0	-	7.4	3.4
Spathulenol	1578	1577	0.05	0.1	-	-	-
Caryophyllene oxide	1582	1582	0.1	0.2	1.0	-	-
β-Eudesmol	1656	1649	0.3	-	-	-	-

AI is retention index, Pub. AI is published retention index.

composition was observed for essential oils from the leaves of *C. pseudopulchellus* with α-pinene, β-myrcene, β-phellandrene and borneol as major components. Borneol may be considered a contributor in the therapeutic efficacy described for the species (Sadgrove et al., 2011). A low yield of a sesquiterpenoid oil was collected from *C. sylvaticus* leaves which was almost entirely comprised of bicyclogermacrene.

Although linalool is common in *Croton* essential oils (Salatino et al., 2007) its occurrence in the three species studied was negligible (Tables 1 and 3). *Crotons* generally acquire the colloquial name of 'lavender bush' or something to that effect. Despite this, the aroma is not comparable to that of lavender and so this vernacular may have been adapted from the association with the mere tradition of using lavender for such aromatic preparations where *Croton* has been traditionally used in African aromatherapy.

Therapeutic uses of the bark of *C. gratissimus* and *C. sylvaticus* are similar (summarised in Neuwinger, 2000) but their leaves are used in different ways. A steam bath with the aromatic leaves of *C. gratissimus* is prepared to treat coughs and colds. The leaves are also used to prepare a perfume which involves extraction into oil or animal fat (Smith, 1966). The lesser aromatic leaves of *C. sylvaticus* are used as a poultice on swellings or taken as a purgative tea (Van Wyk and Van Wyk, 1997; Neuwinger, 2000; Van Wyk et al., 2009).

Traditional uses of leaves of *C. pseudopulchellus* in Zimbabwe and Tanzania have some degree of similarity to *C. gratissimus*; aromatic leaves are utilised as a perfume by rubbing an oil or fat extraction onto the body, while leaf infusions are taken orally to treat coughs and colds. Leaves are also used fresh, by rubbing them on the chest for the same respiratory afflictions (Burkill, 1994). There are also some differences in the use of leaves between the two species; *C. pseudopulchellus* leaves are uniquely used as vapour steams to treat syphilitic ulcers (Bally, 1937; Watt and Breyer-Brandwijk, 1962). Furthermore, to combat fever, dried leaves are dipped in coconut oil and heated on hot embers and the fumes inhaled (Chhabra et al., 1990). Other plant parts are utilised; powdered root is either sniffed to treat colds or drunk with leaf decoctions to alleviate headache.

4. Conclusion

The current study aimed to chemically characterise the volatiles and other lipophilic compounds that could be involved in therapeutic outcomes using the three species, *C. gratissimus*, *C. pseudopulchellus* and *C. sylvaticus*. The realisation of chemotypes within *C. gratissimus* complicates the interpretation of ethnobotanical information, since traditional records don't acknowledge chemotypes or geographical provenances and specificity of therapeutic use. Furthermore, the new volatile diterpenes were not active against bacteria at the concentrations tested, so further research is necessary to know if any importance can be attributed in therapeutic outcomes to these diterpenes. The discovery of predominantly bicyclogermacrene in *C. sylvaticus* leaves motivates for research on fixed components, since the main volatile is common. Some therapeutic efficacy from use of *C. pseudopulchellus* can be explained by the presence of borneol, but research on non-volatile lipophilic compounds in this species will certainly be interesting.

Declarations

Author contribution statement

Nicholas Sadgrove: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ben-Erik Van Wyk, Lee Madeley: Performed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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