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ISOLATION AND CHEMICAL STRUCTURE OF HIGHLY OXYGENATED MONOTERPENOIDS AND ANTIBACTERIAL EVALUATION OF NEW COMPOUNDS, EXTRACT AND ESSENTIAL OIL OF CAMEROONIAN *CHENOPODIUM AMBROSIOIDES* L.

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ABSTRACT
The methanol extract of the aerial part of Cameroonian *Chenopodium ambrosioides* yielded two new highly oxygenated monoterpenoids viz, 1,2,3,4,8-pentahydroxy-*trans*-menthane (**1**) and 2,3,4,8-tetrahydroxy-*trans*-menthane (**2**). In addition, 2-palmitoylglycerol has been isolated for the first time from this plant along with several sterols and fatty acids. Structures of these compounds were deduced on the basis of spectroscopic evidences and chemical modifications. Its essential oil after GC/MS analysis showed that α-terpinene, *trans*-ascaridole and p-cymene were the major constituents. The antibacterial activity of its essential oil and methanol extract was screened by disc diffusion and agar hole assays, respectively. Likewise, MIC of the methanol extract and compounds **1** and **2** was also determined following the standard procedure. The isolated compounds and the essential oil showed negligible antibacterial activity against a panel of ten pathogenic Gram negative and Gram positive bacteria.

KEYWORDS: *Chenopodium ambrosioides*, oxygenated monoterpenoids, 1,2,3,4,8-pentahydroxy-*trans*-menthane (1S*,2R*,3R*,4S*)-p-menthane-1,2,3,4,8-pentol), 2,3,4,8-tetrahydroxy-*trans*-menthane (2R*,3R*,4S*)-p-menthane-2,3,4,8-tetraol), extract, essential oil, antibacterial test.

1. INTRODUCTION
Medicinal and aromatic plants have been source of large number of bioactive natural products.[1–3] *Chenopodium ambrosioides* L., belonging to the Family Chenopodiaceae, is an annual, sometimes perennial plant known as Mexican tea or wormwood. Native to tropical America, this erect herb of up to 1 m tall is widely distributed in the tropics and subtropics, especially in America and Africa.[4] Its leaves are alternate, lanceolate to lance-elliptical and the margins are wavy, toothed to lobed. One of the main features of the plant is the presence of strongly scented, tiny, golden-colored and glandular resinous dots on the leaves.[5] In Cameroon, *C. ambrosioides* is called 'nvengmemmya' and is widely used in traditional medicine as vermifuge. Other ethnopharmacological uses include anthelmintic, carminative, anti-malarial, emmenagogue, female infertility. Earlier phytochemical investigations on *C. ambrosioides* revealed that its leaves contained essential oils, terpenes, sterols, phenols and flavonoids[6–11] while its fruits harbor flavonol glycosides.[12,13] Several studies demonstrated that the extracts of this plant have a wide variety of biological activities such as sedative, anthelmintic, anti-inflammatory, insectidicidal, antifungal and anti-nociceptive properties.[14–20] The oxygenated monoterpenoid, ascaridole, has been shown to be most bioactive component of this plant.[15,21] Despite its widespread biological activity, no study has been carried out on its biologically active phyto-constituents. Hence, in continuation of our interest on the investigation of medicinal and aromatic plants.[3,22–29] We hereby report the results of the bioassay-guided investigations on the methanol extract and essential oil of aerial parts of *C. ambrosioides*, a medicinally important plant from the western region of Cameroon.

2. METHODOLOGY
2.1. General experimental procedures
Optical rotations were taken with a Horiba SEPA-300 polarimeter. Melting points were determined on a Fisher-

Johns melting point apparatus and were uncorrected. UV analysis was carried out on a Spectronic Genesys 2 spectrophotometer and IR was recorded with FT-IR Perkin Elmer instrument. ^{1}H and ^{13}C NMR spectra were obtained with a FT-NMR 300 MHz equipped with a 5 mm ^{1}H and ^{13}C (ATP) probe operating at 300 and 75 MHz, respectively, with TMS as internal standard. Chemical shifts are reported in δ and coupling constants in Hz. The ESIMS was recorded on API-3000 LC-MS/MS ABSCIEX in 1.0 ppm solution through MS grade methanol with injection mode: infusion through motor driven hardware syringe. QTOF-HRMS spectra were recorded on Agilent 6520-QTOF LC/MS having an ESI source in positive mode. Dry Nitrogen was used with 11 L/min flow rate for ionization at 350 Dc. GC and GC/MS of the essential oil were recorded under the same conditions on the instruments described earlier.[22] Flash chromatography was performed with a Buchi Pump manager C-615 flash model operating with two pump modules C-605. Precoated aluminium sheets silica gel 60 F254 TLC plates (Merck) were used to check the purity of compounds. Spots were viewed under UV lamp (254 nm and 365 nm) and sprayed with Anisaldehyde-sulfuric acid reagent. All reagents used, were of analytical grades.

2.2. Plant material

The aerial parts of *Chenopodium ambrosioides* were collected with the help of Dr Tacham Walter in September 2009 in Bamoubou, a village located in the mountains of West region of Cameroon. The identification of the plant was done at the National Herbarium of Cameroon against voucher specimen number 23696SFR/Cam.

2.3. Extraction and isolation

The dried and ground aerial parts (612 g) of *C. ambrosioides* were extracted with *n*-hexane (2 x 24 h) yielding 5 g of extract. Subsequent extraction performed with methanol (3 x 24 h) gave 21 g of extract.

2.3.1. Hexane extract

For the analysis of the non polar constituents, an aliquot of the hexane extract (17.5 mg) was derivatized with H_2SO_4/Toluene/MeOH (1:10:20) as described previously[23] for the analysis of fatty acids by GC and GC/MS. The identified constituents are presented in Table 1.

2.3.2. Methanol extract

19 g of the methanol extract was fractionated by column chromatography using silica gel (458 g, 100-200 mesh) packed in *n*-hexane. Elution was performed with *n*-hexane and the polarity was increased with EtOAc in increasing order of its ratio from 9:1, 4:1, 7:3, 3:2, 1:1 to 0:1. Seventy-eight (78) fractions each of 250 mL were collected and further pooled into 11 fractions based on their TLC profile. Fraction 7 (*n*-hexane-EtOAc, 2:3) was further subjected to flash chromatography (120 g, 230-400 mesh, 36 × 230 mm glass column C-690 Sepacore Buchi) with *n*-hexane as solvent A and EtOAc as solvent B. The flow rate was set up to 6 mL/min. A total of 87 sub-fractions (each of 50 mL) were collected and pooled into nine fractions (7A to 7I). Prep. TLC of fraction 7H (40 mg, *n*-hexane-EtOAc, 1:4) in EtOAc gave 2-palmitoylglycerol (18 mg**)**. Fraction 8 (253 mg, *n*-hexane-EtOAc, 3:7) after column chromatography on silica gel (26 g, 100-200 mesh) using *n*-hexane and EtOAc as eluting solvents gave 8 fractions (8fA-8fH). Fractions 8fC and 8fD afforded crystals of **1 (**41.9 mg, Rf= 0.55, *n*-hexane-EtOAc 1:4). Further column chromatography of fraction 8fG (78.8 mg) using silica gel (8 g, 230-400 mesh) with *n*-hexane and EtOAc gave 8 fractions (8G1-8G8). Fraction 8G7 after subsequent prep. TLC in EtOAc afforded **2** (13.5 mg, Rf= 0.50, *n*-hexane-EtOAc 1:4). Additionally, fraction 9 (*n*-hexane-EtOAc, 1:4) yielded **1** (13.0 mg).

2.4. 1,2,3,4,8-Pentahydroxy-trans-menthane (1): White crystals; mp 148°C; $[\alpha]_D^{23.7}$ +34.58º (*c* 0.0004, $CHCl_3$); UV ($CHCl_3$) λmax (abs.) 241.0 (0.721), 340.3 (0.062) nm; IR (KBr) ν_{max}: 3600-3300, 2922, 1718, 1376, 1171 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$, δ in ppm, J in Hz): 1.28 (3H, s, H-10), 1.29 (1H, m, H-6α), 1.30 (3H, s, H-7), 1.40 (3H, s, H-9), 1.69 (1H, m, H-5α), 2.05 (1H, m, H-5β), 2.10 (1H, m, H-6β), 3.57 (1H, d, J= 9.0 Hz, H-2), 4.17 (1H, d, J= 9.0 Hz, H-3); ^{13}C NMR (75 MHz, $CDCl_3$): 93.3, s (C-1), 86.0, s (C-4), 73.6, d (C-2), 72.0, s (C-8), 67.7, d (C-3), 30.1, t (C-6), 25.4, q (C-9), 25.5, q (C-10), 24.4, t (C-5), 20.4, q (C-7); $^{1}H^{1}H$ COSY: H-2 at δ 3.57 --- H-3 at δ 4.17; HMBC: H-3→C-2, C-4, C-8; H-2→C-7, C-1, C-3; HR-(+)ESI-MS *m/z:* 220.1305 $[M]^+$ (20) (calcd. for $C_{10}H_{20}O_5$ 220.1306); ESI-MS *m/z*: 220 $[M]^+$ (100), 184 $[M–2H_2O]^+$ (20), 166 $[184–H_2O]^+$ (8), 148 $[166–H_2O]^+$ (35), 125 (30).

2.5. Acetylation of compound 1

Compound **1 (**10 mg) was taken in 2 mL of acetic anhydride and one drop of pyridine. The mixture was heated at 60 °C with a calcium chloride guard tube for 5 h. Thereafter, acetic anhydride and pyridine were removed in vacuo yielding compound **1a** (8.0 mg).

2.6. 2,3-Diacetoxy-1,4,8-trihydroxy-trans-menthane (1a): Viscous liquid (R_f = 0.88 (*n*-hexane-EtOAc 1:9); ^{1}H NMR (300 MHz, $CDCl_3$, δ in ppm, J in Hz): 1.24 (3H, s, H-10), 1.28 (1H, m, H-6α), 1.36 (3H, s, H-7), 1.41 (3H, s, H-9), 1.66 (1H, m, H-5α), 2.09 (1H, m, H-5β), 2.10 (6H, s, 2 OAc), 2.14 (1H, m, H-6β), 4.79 (1H, d, J= 9.0 Hz, H-2), 5.28 (1H, d, J= 9.0 Hz, H-3).

2.7. 2,3,4,8-Tetrahydroxy-trans-menthane (2): oily; ^{1}H NMR (300 MHz, $CDCl_3$, δ in ppm, J in Hz): 1.15 (3H, d, J= 7.0 Hz, H-7), 1.21 (3H, s, H-10), 1.42 (3H, s, H-9), 1.62 (1H, m, H-5α), 1.78 (1H, m, H-6α), 1.80 (2H, m, H-1 and H-5β), 1.82 (1H, m, H-6β), 3.75 (1H, d, J= 9.0 Hz, H-2), 4.25 (1H, d, J= 9.0 Hz, H-3); ^{13}C NMR (75 MHz, $CDCl_3$): 82.4, s (C-4), 77.6, d (C-2), 75.3, s (C-8), 71.4, d (C-3), 34.3, t (C-5), 32.3, q (C-6), 28.5, q (C-1), 28.4, q (C-9), 26.3, q (C-10), 23.6, q (C-7); $^{1}H^{1}H$ COSY: H-1 at

δ 1.80 --- H-7 at δ 1.15, H-2 at δ 3.75 and H-6α at δ 1.78; H-2 at δ 3.75 --- H-1 at δ 1.80, H-3 at δ 4.25; HMBC: H-3→C-2, C-4, C-8; H-2→C-7, C-1, C-3; H-1→C-7, C-2, C-6; HR-(+)ESI-MS *m/z:* 204.1356 $[M]^+$ (20) (calcd. for $C_{10}H_{20}O_4$ 204.1357); ESI-MS *m/z*: 204 $[M]^+$ (100), 168 $[M–2H_2O]^+$ (20), 150 $[168–H_2O]^+$ (8).

2.8. Essential oil extraction

Fresh aerial parts *C. ambrosioides* (7 kg) collected in November 2009 from Bafoussam in West region of Cameroon were used for the extraction of its essential oil. The extraction was done through hydrodistillation and the essential oil was further analyzed through GC and GC/MS as already described.[22]

2.9. Biological screening

The antibacterial activity of the essential oil and methanol extract of *C. ambrosioides* was screened by disc diffusion and agar hole assays, respectively as already described.[22] Likewise, MIC of the methanol extract and compounds **1** and **2** was also determined following the procedure previously reported.[22]

3. RESULTS

The methanol extract after rigorous purifications by chromatographic methods yielded mainly three compounds, viz., **1**, **2** and 2-palmitoylglycerol. The structure of 2-palmitoylglycerol was confirmed by comparing its ^1H and ^{13}C NMR data with the values earlier reported.[22] To the best of our knowledge, 2-palmitoylglycerol is being reported for the first time from this plant. However, the structure elucidation of new compounds, **1** and **2,** was done mainly by spectroscopic methods which are discussed in this paper.

3.1. Compounds identified from n-hexane extract

The *n*-hexane extract showed complex mixture of fatty acids. Therefore, in order to identify them, a portion of extract was methylated and analyzed by GC and GC/MS. The results, as given in Table 1, showed the presence of 1,7-dimethoxy-1,6-heptadiene, 6-octadecenoic acid methyl ester, 12-methyltetradecanoic acid methyl ester, stearic acid methyl ester, oleic acid methyl ester, palmitic acid methyl ester and docosanoic acid methyl ester.

3.2. Essential oil of C. ambrosioides

From the light brown colored essential oil of *C. ambrosioides* (yield: 1.75 mg/g; $d^{20°}$: 0.8998, $\alpha_D^{20°}$: 0.492, $n_D^{20°}$: 1.482), forty three constituents (Table 2) were identified by GC and GC/MS representing 92.84% of the oil with monoterpenoids (91.87%) and sesquiterpenes in minute amount (0.97%). The main constituents identified in the essential oil on the basis of their Kovats index in GC and mass spectra were: α-terpinene 32.86%, p-cymene 31.08% and *trans* ascaridole 13.88%.

3.3. Biological screening

3.3.1. Compounds 1 and 2

Compounds **1** and **2** isolated from the Cameroonian *C. ambrosioides* did not show significant antibacterial activity (MIC >500 µg/mL) against *Klebsiella pneumoniae* MTCC109 and *Pseudomonas aeruginosa* MTCC741.

3.3.2. Essential oil

The essential oil of *C. ambrosioides* was found inactive when tested for antibacterial activity against a panel of ten pathogenic Gram-negative and Gram-positive bacteria.[22] Which could be attributed to the predominance of lesser active monoterpene hydrocarbons in the oil.

4. DISCUSSION

4.1. Structure elucidation of compounds 1 and 2

Compound **1** (Fig. 1) showed IR bands at 3600-3300cm^{-1} for OH groups along with bands at 2922, 1376 and 1171 cm^{-1}. Its HRMS showed $[M]^+$ at *m/z* 220.1305 which indicated its molecular formula as $C_{10}H_{20}O_5$. Its ^1H NMR spectrum showed two doublets (J= 9.0 Hz) for CH at δ 3.57 and 4.17 coupling with each other along with another set of four CH overlapping multiplets at δ 1.29, 1.69, 2.09 and 2.10. After acetylation of **1** into **1a**, these CH doublets (J= 9.0 Hz) shifted from δ 3.57 in **1** to δ 4.79 in **1a** and from δ 4.17 in **1** to δ 5.28 in **1a**. Three methyl singlets at δ 1.30, 1.40 and 1.28 appeared slightly downfield in the ^1H NMR spectrum of **1**, clearly suggesting that C-8 and C-1 positions are oxygenated. Its ^{13}C NMR spectrum showed ten signals with three methyl at δ 20.4, 25.4 and 25.5, two methylene at δ 24.4 and 30.1, two CHOH at δ 67.7 and 73.6 and three C-OH at δ 72.0, 86.0 and 93.3 suggesting for a cyclic monoterpenoid with menthane skeleton. These data clearly indicated that **1** belongs to the menthane skeleton of monoterpenoids with the oxygenation of C-2 and C-3 to CHOH. Three singlets at δ 93.3, 86.0 and 72.0 supported that it has three tertiary hydroxyl groups at C-1, C-4 and C-8. The presence of the three methyl groups at C-7, C-9 and C-10 were supported by the quartets at δ 20.4, 25.5 and 25.4 in its ^{13}C NMR spectrum. A perusal of the literature on this plant showed that a compound with 1,2,3,4-tetrahydroxy-*p*-menthane (**3**) has earlier been reported by Ahmed (2000) from the aerial parts of the Egyptian *C. ambrosioides*. The main difference between **1** reported herein from the Cameroonian specie and **3** was that the former had an additional tertiary hydroxyl group. The only possible position for the third tertiary hydroxyl group happens to be C-8. Rest of the signals matched well with the known tetrahydroxy menthane (**3**) reported by Ahmed (2000). The stereochemistry at C-1 and C-4 for methyl and isopropyl groups was possibly *trans*, as in case of **3,** because the chemical shifts of C-1 and C-4 were relatively deshielded reported by Bohlmann. In the $^1H^1H$ COSY spectrum of **1**, H-2 at δ 3.57 showed correlations with H-3 at δ 4.17. In its HMBC spectrum, **1** showed main correlations of H-3 at δ 4.17 with C-4 at δ 86.0, C-8 at δ

72.0, C-2 at δ 73.6 and H-2 at δ 3.57 with C-7 at δ 20.4, C-3 at δ 67.7 and C-1 at δ 93.3. In the NOESY spectrum of **1**, H-9 at δ 1.40 did not show any correlation with H-3 at δ 4.17 indicating that the possible configuration at C-8 is S.[23] These data supported the structure of **1** as 1,2,3,4,8-pentahydroxy-*trans*-menthane or (1S*,2R*,3R*,4S*)-p-menthane-1,2,3,4,8-pentol.

Compound **2** (Fig. 1) showed IR bands at 3600-3300 cm^{-1} for OH groups along with bands at 2922, 1376 and 1171 cm^{-1}. Its HRMS showed $[M]^+$ at *m/z* 204.1356 which indicated its molecular formula as $C_{10}H_{20}O_4$. Compound **2** had almost similar ^1H NMR spectral data as of **1** with the only difference that **2** had a multiplet at δ 1.80 which showed correlations with H-7, H-6α and H-2 in its $^1H^1H$ COSY spectrum. This difference was supported by its ^{13}C NMR spectrum which showed only two C-OH signals at δ 82.4 and 75.3 along with a CH signal at δ 28.5 for C-1. These data clearly supported that it is a tetrahydroxylated menthane having its structure as 2,3,4,8-tetrahydroxy-*trans*-menthane or (2R*,3R*,4S*)-p-menthane-2,3,4,8-tetraol.

4.2. Essential oil

The GCMS data showed that the essential oil of *C. ambrosioides* from West Cameroon is of mixed α-terpinene/ p-cymene chemotype because the concentration of these two principal components is almost equal. Previous studies have shown that two main chemotypes of *C. ambrosioides* existed, namely, ascaridole-rich chemotype.[7,31,32] and α-terpinene-rich chemotype.[11,33,34]

4.3. Biological screening

When tested against *Klebsiella pneumoniae* MTCC109 and *Pseudomonas aeruginosa* MTCC741, Compounds **1** and **2** did not show significant antibacterial activity (MIC >500 µg/mL). When tested for antibacterial activity against a panel of ten pathogenic Gram-negative and Gram-positive bacteria[22], the essential oil of *C. ambrosioides* was found inactive. Normally, the oxygenated terpenoids show antibacterial activity. Therefore, the poor activity in this oil could be attributed to the predominance of lesser active monoterpene hydrocarbons in the oil.

Table 1: Fatty acid composition in methylated hexane extract of *C. ambrosioides* identified by GC and GC/MS.

Compound	GC/MS Retention time	% in *n*-hexane extract*
12-Methyltetradecanoic acid, methyl ester	45.45	2.85
Palmitic acid methyl ester	50.18	32.00
6-Octadecenoic acid, methyl ester	56.00	20.01
Oleic acid methyl ester	56.20	12.84
Stearic acid methyl ester	56.83	5.63
Docosanoic acid, methyl ester	60.00	2.14

Unidentified 24.53 %

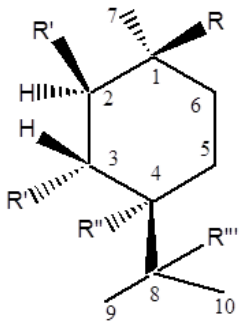
* The percentage is given by the GC/MS spectra

Table 2: Chemical constituents from the essential oil of *C. ambrosioides.*

Compounds	KI	Percentage (%)*
α-Phellandrene	1003	0.08
δ-3-Carene	1011	0.35
α-Terpinene	1018	32.86
p-Cymene	1026	31.08
Limonene	1030	0.85
γ-Terpinene	1069	0.50
p-Cresol	1076	0.09
Fenchone	1083	0.39
Terpinolene	1089	0.14
Linalool	1100	0.87
cis-Thujone	1103	0.10
Fenchol endo	1114	0.38
p-Mentha-1,5,8-triene	1130	2.08
Citronellal	1154	0.14
Isoborneol	1156	1.51
Borneol	1164	0.11
Terpinen-4-ol	1178	0.23
Carvomenthone	1181	0.14
α-Terpineol	1189	0.20
Myrtenol	1193	0.38
Methylchavicol	1200	0.13
Trans- Pulegol	1213	0.12

Isobornyl formate	1233	1.18
Ascaridole cis	1242	0.32
Sabinene hydrate acetate trans	1254	0.09
Geraniol	1257	0.49
Geranial	1272	0.78
Safrole	1284	0.09
Pregeijerene	1287	0.82
Thymol	1292	0.16
Carvacrol	1300	0.46
Ascaridole trans	1308	13.88
Terpinylacetate trans-dihydro-alpha	1317	0.26
1,3,8-p-Menthatriene	1321	0.50
α-Cubebene	1353	0.11
Eugenol	1357	0.26
Neryl acetate	1366	0.11
α-Copaene	1379	0.15
Longifolene	1400	0.08
α-Gurjunene	1415	0.08
α-Humulene	1456	0.08
Z-Nerolidol	1534	0.12
Caryophyllene acetate	1700	0.09
Class composition#		
Monoterpene hydrocarbons		69.21
Oxygenated monoterpenes		22.61
Sesquiterpene hydrocarbons		0.50
Oxygenated sesquiterpenes		0.47

KI: Kovats Index
* The percentage is given by the GC/MS spectra.
# for each class of compounds, the sum of the percentages of all the constituents present is calculated.



1, R= R'= R''= R'''= OH
1a, R= R''= R'''= OH, R'= OAc
2, R'= R''= R'''= OH, R= H
3, R= R'= R''= OH, R'''= H

Figure 1.
Structure of compounds **1-3**

5. CONCLUSION

The phytochemical investigation of the aerial parts of Cameroonian *C. ambrosioides* yielded two new highly oxygenated monoterpenoids namely, 1,2,3,4,8-pentahydroxy-*trans*-menthane (**1**) and 2,3,4,8-tetrahydroxy-*trans*-menthane (**2**). Known compounds identified from this plant included sterols, fatty acids and 2-palmitoylglycerol which have been isolated and reported for the first time from this plant. Besides, the chemical analysis of its essential oil revealed the presence of α-terpinene, *trans*-ascaridole and p-cymene as its main constituents. The essential oil, extract and compounds **1** and **2** did not show significant antibacterial activity.

Credit authorship contribution statement

N. A. Vyry Wouatsa: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. Laxminarain Misra: Conceptualization, Data curation and interpretation, Methodology. Francois Tchoumbougnang: Plant collection and identification. Venkatesh Kumar R: Writing, review and editing.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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