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ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES IN TERPENOIDS FROM CAMEROONIAN *OCIMUM CANUM* SIMS. AND *XYLOPIA AETHIOPICA* (DUN.) A. RICH#

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ABSTRACT
Bioassay-guided fractionations were carried out on the extracts of aerial parts of *Ocimum canum* and fruits of *Xylopia aethiopica*. From the methanol extract of *Ocimum canum*, a new nerolidol derivative, 6,10-dimethyl-2-methylene-5,7,10-trihydroxydodeca-2,11-diene, was isolated along with eight known compounds, viz. salvigenin, nevadensin, valaran-4,11-diol, lupeol, 24-ethylcholest-4-ene-3-one, 4'-methoxyapigenin, β-sitosterol and stigmasterol. The extract of *Xylopia aethiopica* afforded mainly known diterpenes kauranol and xylopic acid. The structures of the new and known compounds were elucidated with the help of various spectroscopic techniques and chemical modifications. Some fractions of both the plants possessed a moderate antibacterial activity against all the bacteria tested viz. Gram positive (*M. luteus*, *S. aureus*) and Gram negative (*K. pneumoniae*, *P. aeruginosa* and *S. typhimurium*). When tested for their cytotoxic potential, only one fraction displayed a significant cytotoxicity against breast cancer MCF-7 and liver cancer WRL-68 cell lines with IC_{50} of 9.8 and 8.6 µg/mL, respectively. Therefore, the results of the antimicrobial and cytotoxicity activities performed on the extracts, fractions and isolated compounds from both plants showed a possible synergism among the constituents.

KEYWORDS: *Ocimum canum*, *Xylopia aethiopica*, bioassay-guided fractionation, 6,10-dimethyl-2-methylene-5,7,10-trihydroxydodeca-2,11-diene, cytotoxicity, antimicrobial activity.

1. INTRODUCTION
It is well established from ancient time that the plants have been rich source of biomedical molecules (Majeed et al., 2021, Okou et al. 2019, Padmavathi et al. 2023). In continuation of our interest on bioactive compounds derived from medicinal and aromatic plants (Misra et al. 2013; Wouatsa et al. 2013a, 2013b; Misra et al., 2019, Balkrishna et al., 2018, Balkrishna and Misra, 2018a, Balkrishna and Misra, 2018b, Balkrishna and Misra, 2017, Misra et al., 2023, Verma et al., 2023), we now report the results of the bioassay-guided investigations on Cameroonian *Ocimum canum* Sims. (Lamiaceae) and *Xylopia aethiopica* **(**Dun.) A. Rich. (Annonaceae). Plants belonging to the genus *Ocimum*, have been shown to harbour triterpenoids, flavonoids, phenylpropanoids, etc. (Kelm et al. 2000). *Ocimum canum* known as 'kotimandjo' in Cameroon is an aromatic shrub employed mostly for its medicinal properties and as spice in various ethno-dietery soups. Previous studies on its extracts showed a wide range of activities including antioxidant, anti-inflammatory, larvicidal, insecticidal, and antimicrobial properties (Nyarko et al. 2002; Devi et al. 2009). Earlier investigations on its phytoconstituents showed the presence of oleanolic acid, ursolic acid, salvigenin and nevadensin (Xaasan et al. 1980). Other investigations on the components of this aromatic shrub have focused only on its essential oil constituents (Ngassoum et al. 2004; Oussou et al. 2007).

With regard to *Xylopia aethiopica* commonly known as 'luteh' in Cameroon and is used in traditional medicine for managing various ailments including skin infections, fever, stomachache, bronchitis, biliousness and dysentery (Burkhill 1985; Iwu 1993). Previous studies on its chemical constituents have reported oxoaporphine alkaloids altogether with kaurane, trachylobane diterpenes, sitosterol glucoside and essential oil (Ngouela et al. 1998, Harrigan et al. 1994; Koba et al. 2008). Some of the studies have showed its wide spectrum of biological activities (Choumessi et al. 2012a, 2012b).

In this paper, we report the results of the bioassay-guided fractionation of the extracts of these two medicinally important plants which led to the isolation of a

sesquiterpene derivative of nerolidol as new compound, 6,10-dimethyl-2-methylene-5,7,10-trihydroxydodeca-2,11-diene from the methanol extract of *O. canum*. The details of the isolation, structure elucidation and biological activities testing are described, herein.

2. RESULTS AND DISCUSSION

The methanol extract of *O. canum* and its fractions (Experimental) were tested for their antibacterial and cytotoxic activities (Tables- 1 and 2). The isolation of the bioactive compounds contained in the methanol extract of *O. canum* using several chromatographic techniques yielded a new nerolidol derivative namely 6,10-dimethyl-2-methylene-5,7,10-trihydroxydodeca-2,11-diene (**1**) along with eight known compounds viz. salvigenin (**2**), nevadensin (**3**), valaran-4,11-diol (**4**), lupeol, 24-ethylcholest-4-ene-3-one, 4'-methoxyapigenin, β-sitosterol and stigmasterol. The identification of the known compounds was done by comparison of their spectral data with those reported in the literature (Kulkarni et al. 1964; Hikino et al. 1966; Krishnan and Nair, 1999; Jassbi et al. 2002) and the data available with us whereas the structure of compound 1 was established by spectroscopic methods which is discussed in this paper.

Compound **1,** a viscous liquid, showed IR bands at 3369 for OH, 2925 for C-C bond along with 1456, 1372, 1254 cm^{-1}. Its ESI-MS showed $[M]^+$ at m/z 256 and $[M-H]^+$ at m/z 255; HR-ESI-MS confirmed $[M]^+$ at m/z 256.2033 for $C_{15}H_{28}O_3$ which matched well for calculated at m/z 256.2032). Its 1HNMR showed a doublet (J=9 Hz) at δ 5.88 for H-11 along with a singlet at δ 5.06 and a doublet (J= 9.0 Hz) at δ 5.20 for H-12 supported by a methyl singlet at δ 1.32 for H-15 indicating a terminal CH_3-COH-CH=CH_2 group. Two broad singlets at δ 4.08 and 4.06 accounted for CH-OH at C-5 and C-7 with their α-orientation which was supported by NOESY experiments. Another set of signals at δ 1.78 singlet, δ 4.85 singlet broad and δ 4.97 singlet broad indicated a terminal group of CH_3-C=CH_2 for C-1, C-2 and C-13a, C-13b, respectively. Rest of the 1HNMR and 13CNMR data as given in Table-3 followed by 1H1H COSY experiments, supported its structure as **1**. On acetylation, it gave **1a** showing spectral data which were compatible for its structure as **1** (Experimental).

The extracts and fractions of *O. canum* were screened for their antibacterial and cytotoxic activities. The results displayed in Tables- 1 and 2 showed that fractions 9 to 15 possessed a moderate antibacterial activity (Table- 1) against all the bacteria tested viz. Gram positive (*M. luteus*, *S. aureus*) and Gram negative (*K. pneumoniae*, *P. aeruginosa* and *S. typhimurium*). When tested for their cytotoxic potential, only fraction 13 displayed a significant cytotoxicity against breast cancer MCF-7 and liver cancer WRL-68 cell lines with IC_{50} of 9.8 and 8.6 µg/mL, respectively (Table- 2). With regard to the activity of the isolated compounds, compound **1** showed a weak antibacterial activity with MIC >500µg/mL (Table- 4) whereas the inseparable mixture of flavonoids i.e. salvigenin (**2**) and nevadensin (**3**) isolated from fraction 13 was found active (Table- 4) against Gram negative bacteria *S. typhymurium* (MIC 187.5 µg/mL) and *P. aeruginosa* (MIC 312.5 µg/mL), therefore suggesting that the antimicrobial activity of the methanol extract of *O. canum* may result from the synergism among these flavonoids. However, when tested for their cytotoxic potential against breast cancer MCF-7, they failed to show any activity.

The bioassay-guided investigation carried out on the extracts of *X. aethiopica* revealed its hexane extract as the most active (Table 5) against Gram positive *S. aureus* and *S. mutans* with MIC of 416.67 and 333.33 µg/mL, respectively. The solvent partition of the methanol extract of *X. aethiopica* with $CHCl_3$, EtOAc and butanol decreased the antibacterial activity as well as the cytoxicity of the fractions obtained (Tables- 5 and 6). The fractionation of the hexane extract through column chromatography afforded known diterpenes (Figure 1) kauranol (**5**) and xylopic acid (**6**) whose structure was deduced by comparison of their spectral data with those reported in the literature (Ekong and Ogun., 1968; Ekong et al. 1969; Hasan et al. 1982). With regard to the biological activities (Table- 4), compound **6** and its epoxy derivative **6a** (Figure 1) prepared through oxidation with m-CPBA were tested for antibacterial activity. The derivatization of **6** into **6a** failed to enhance the antibacterial potential of **6**. None of the compounds (**6** and **6a**) displayed a significant antibacterial activity (MIC ≥ 500 µg/mL).

3. Experimental

3.1. General experimental procedures

1H and 13C NMR spectra were obtained with a FT-NMR 300 MHz equipped with a 5 mm 1H and 13C (ATP) probe operating at 300 and 75 MHz, respectively, with TMS as internal standard. Chemical shifts were reported in δ (ppm) and coupling constants (J) were measured in Hz. QTOF-HRMS spectra were recorded on Agilent 6520-QTOF LC/MS having an ESI source in positive mode. Dry Nitrogen was used with 11 LPM flow rate for ionization at 350 Dc. The ESIMS was recorded on API-3000 LC-MS/MS ABSCIEX in 1.0 ppm solution through MS grade acetonitrile-water with injection mode: infusion through motor driven hardware syringe. UV analysis was carried out on a Spectronic Genesys 2 spectrophotometer and IR was recorded with FT-IR Perkin Elmer instrument. Optical rotations were taken with a Horiba SEPA-300 polarimeter. Melting points were determined on a Fisher-Johns melting point apparatus and were uncorrected.

Precoated aluminium sheets silica gel 60 F254 TLC plates were used to check the purity of compounds and preparative TLC was performed on glass plates of silica gel 60 (20x20 cm, Merck). Flash chromatography was performed with a Buchi Pump manager C-615 flash model operating with two pump modules C-605. Spots

were viewed under UV lamp (254 nm and 365 nm) and sprayed with Anisaldehyde- sulfuric acid reagents. All reagents used, were of analytical grades.

3.2. Plant material

The aerial parts of *Ocimum canum* were collected at Douala, Littoral Region of Cameroon in August 2009 while the dried fruits of *Xylopia aethiopica* were purchased from the general spice market of Douala in December 2009. The plants were identified at the National Herbarium of Cameroon against samples deposited under the voucher specimen number 26804SFR/Cam and 16419SFR/Cam for *O. canum* and *X. aethiopica*, respectively.

3.3. Extraction and fractionation

3.3.1. Extraction and fractionation of O. canum

The dried aerial parts of *O. canum* (180 g) were successively extracted following the methodology described in Misra et al. (2013) and yielded 2 and 14 g of hexane and methanol extracts, respectively. Silica gel column chromatography (221 g of silica 100-200 mesh) of the methanol extract (9.42 g) with a mixture of *n*-hexane, EtOAc and MeOH afforded 250 fractions of 100 mL each which were further regrouped into 19 major fractions following their TLC pattern. Prep. TLC of fraction 4 (*n*-hexane-EtOAc, 9:1) in *n*-hexane-EtOAc, 4:1 gave lupeol (2.4 mg) and 24-ethylcholest-4-ene-3-one (10 mg). Fraction 6 was made up of mixture of stigmasterol and β-sitosterol. Subsequent flash chromatography of fraction 9 (*n*-hexane-EtOAc, 4:1) with *n*-hexane and EtOAc at 2.5mL/mn as flow rate yielded 4'-methoxyapigenin (5.4 mg) and β-sitosterol (40 mg). Fraction 12 (284 mg, *n*-hexane-EtOAc, 7:3) was separated through column chromatography (10 g, silica gel 100-200 mesh) using *n*-hexane and EtOAc as eluting solvents. Seventy (70) fractions (ca. 50 mL) were collected and pooled into 5 sub-fractions. Sub-fraction 3 eluted in *n*-hexane-EtOAc (4:1) afforded **1** (14 mg, Rf= 0.57 in *n*-Hexane-EtOAc 3:2). From fraction 13 (60 mg, *n*-hexane-EtOAc, 7:3), **2** and **3** were obtained as an inseparable mixture (10 mg) after repetitive flash chromatography followed by prep.TLC in *n*-hexane-EtOAc (1:1) yielded pure compounds. Fraction 15 (123 mg, *n*-hexane-EtOAc, 3:2) was subjected to flash chromatography (20 g, 230-400 mesh, 15 x 100 mm glass column C-690 Sepacore Buchi fitted with a precolumn of ID 15 mm) with *n*-hexane as solvent A and EtOAc as solvent B. The flow rate was set up to 3 mL/ min., five sub-fractions were obtained and prep.TLC of the fourth sub-fraction using MeOH-DCM-EtOAc (3:8:10) gave compound **4 (**12 mg).

3.3.2. Extraction and fractionation of X. aethiopica

The successive extraction of the dried and ground fruits of *X. aethiopica* (124 g) with *n*-Hexane and MeOH yielded 5 and 48 g of hexane and methanol extracts, respectively. The methanol extract (25 g) was further partitioned with $CHCl_3$ (400 ml), EtOAc (400 ml) and BuOH (200 ml) yielding three principal fractions. Before the fractionation through column chromatography, an aliquot of the hexane extract of *X. aethiopica* was analyzed by GC/MS. GC/MS analysis revealed the presence of two major diterpenes of $[M]^+$ 272 and 303 found at 46.83 and 10.45 %, respectively. Therefore, recrystallization with EtOAc of the hexane extract (5 g) of *X. aethiopica* was carried out which yielded compound **5 (**207.5 mg). Further, 2.51 g of the un-crystallised hexane extract were subjected to column chromatography (147 g of silica 60-120 mesh) eluting with *n*-hexane and EtOAc. Ninety (90) fractions of 100 mL each were collected and pooled into seven fractions based on their similarity on TLC. Recrystallization of fractions 5 (*n*-hexane-EtOAc 19:1 and 37:3) and 6 (*n*-hexane-EtOAc 37:3 and 9:1) in EtOAc afforded crystals of **5** (65.8 mg, Rf= 0.57 in *n*-Hexane-EtOAc 1:1) and **6** (239.7 mg).

3.4. 6,10-dimethyl-2-methylene-5,7,10-trihydroxydodeca-2,11-diene (1)

Green viscous liquid *(*Rf= 0.57 in *n*-Hexane-EtOAc 3:2); $[\alpha]_D^{24.6}$: +10.90° (*c* 0.0011, MeOH), IR (KBr) ν_{max}: 3369 (OH), 2925 (C-C), 1456, 1372, 1254 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, δ in ppm, J in Hz) and ^{13}C NMR (75 MHz, $CDCl_3$): Table 3; ESI-MS $[M]^+$ m/z 256 , $[M–H]^+$ m/z 255; HR-ESI-MS $[M]^+$ m/z 256.2033 (calcd. for $C_{15}H_{28}O_3$ 256.2032).

3.5. Acetylation of compound 1

The acetylation of compound **1** (2.2 mg) was done following the procedure already described (**Wouatsa et al. 2013a**) and yielded **1a**.

3.6. 6,10-dimethyl-2-methylene-5,7,10-triacetoxydodeca-2,11-diene (1a)

Oily substance. ^1H NMR (300 MHz, $CDCl_3$, δ in ppm, J in Hz): 1.25 (3H, s, H-15), 1.29 (3H, d, J = 6 Hz, H-14), 1.60 (1H, m, H-9β), 1.62 (1H, m, H-8α), 1.65 (1H, s, H-4α), 1.72 (1H, s, H-4β), 1.75 (3H, s, H-1), 1.78 (1H, m, H-8β), 2.09 (3H, s, OAc), 2.35 (1H, m, H-6), 2.45 (1H, m, H-9α), 2.46 (1H, s, H-3α), 2.50 (1H, s, H-3β), 4.94 (1H, d, J = 9Hz, H-12β), 5.10 (1H, s, H-12α), 5.16 (1H, s, H-5), 5.18 (1H, s, H-7), 5.91 (1H, d, J = 9Hz, H-11).

3.7. Exocyclic methylene double bond epoxidation of compound 6

Compound **6 (**9.8 mg, 0.027 mmole) was taken in 8mL of dichloromethane and stirred in ice bath. Then, 6.8 mg (1.5 equivalent) of m-CPBA (meta- chloroperoxybenzoic acid) and 7.3 mg (3 equivalent) of sodium bicarbonate were added. The reaction mixture was stirred on ice bath for 30h. The reaction was worked up by removing the organic phase from the aqueous phase and dried over anhydrous sodium sulphate. The removal of the solvent *in vacuo* yielded oily compound **6a** (9.0 mg, 91.38%), the epoxy derivative of compound **6**.

3.8. Epoxy-xylopic acid (6a)

Oily substance. ^1H NMR (300 MHz, $CDCl_3$, δ in ppm, J in Hz): 0.94 (3H, s, H-20), 1.22 (3H, s, H-18), 2.16 (3H,

s, OAc), 2.18 (1H, sbr, H-13), 2.82 (2H, m, H-17), 5.15 (1H, s, H-15).

3.9. Biological screening

3.9.1. Antibacterial screening

The *in vitro* antimicrobial activity of the extracts, fractions and isolated compounds of *O. canum* and *X. aethiopica* was performed by micro dilution technique as already reported (**Misra et al. 2013**).

3.9.2. Cytotoxicity

The cytotoxicity of the extracts, fractions and isolated compounds of *O. canum* and *X. aethiopica* was evaluated by MTT assay. The IC_{50} values were determined against a panel of four cell lines following the methodology previously described (**Misra et al. 2013**).

Table 1: MIC of the methanol extract and column chromatography fractions of *O. canum*.

	MIC in µg/mL				
	KP	**PA**	**STM**	**ML**	**SA96**
O. canum extract	1000	833.33±288.68	1000	500	>1000
Fractions					
3	>1000	>1000	>1000	>1000	nt
4	>1000	>1000	>1000	500	nt
6	>1000	>1000	>1000	>1000	nt
9	416.67 ±144.34	>1000	1000	500	1000
10	416.67±144.34	>1000	500	291.67 ±190.94	>1000
11	500	>1000	500	208.33±72.17	nt
12	500	>1000	500	104.67±36.08	250
13	500	>1000	666.67 ±288.67	125	nt
14	833.33±288.68	1000	500	125	>1000
15	1000	>1000	833.33±288.68	>1000	>1000
16	1000	>1000	1000	>1000	>1000
Control					
Kanamycin	4.85	62.5	0.52	4.16	6.94

nt= not tested, KP: *Klebsiella pneumoniae*, ML: *Micrococcus luteus*, PA: *Pseudomonas aeruginosa*, SA96: *Staphylococcus aureus* MTCC96, STM: *Salmonella typhimurium.*

Table 2: IC_{50} of the methanol extract and column chromatographic fractions of *O. canum*.

	IC_{50} in µg/mL			
	MCF-7	**CaCO2**	**WRL-68**	**PC-3**
Extract				
O. canum	192	nt	16.8	87
Fractions				
3	nt	nt	nt	nt
4	nt	nt	82	88
6	nt	nt	nt	nt
9	nt	nt	83	nt
10	78	78	80	88
11	77	77	64	96
12	73	84	9.2	82
13	9.8	64	8.6	66
14	67	91	9.8	63
15	100	nt	47	87
16	nt	nt	nt	nt
Doxorubicin	0.85	3.5	2.1	5.0

nt: not tested. Cell lines: WRL-68= *Liver cancer*, CaCO2= *colon cancer*, MCF-7= *breast cancer*, PC-3= *prostate cancer.*

Table 3: 1HNMR and 13CNMR data of compound 1.

Position	^{1}H data			^{13}C data	
	δ	Multiplicity	Stereochemistry	δ	Multiplicity
1	1.78	s	-	18.3	CH_3
2	-	-	-	147.8	C
3	2.46	s	α	38.6	CH_2
	2.50		β		
4	1.65	s	α	29.7	CH_2
	1.72		β		
5	4.08	s	β	76.6	CH
6	2.29	m	β	28.67	CH
7	4.06	s	β	76.1	CH
8	1.62	m	α	30.0	CH_2
	1.78		β		
9	2.45	m	α	38.1	CH_2
	1.60		β		
10	-	-	-	73.3	C
11	5.88	d (J = 9 Hz)	-	145.3	CH
12	5.06	s	α	112.3	CH_2
	5.20	d (J = 9 Hz)	β		
13	4.85	s	α	111.4	CH_2
	4.97		β		
14	1.29	d (J = 6 Hz)	α	18.16	CH_3
15	1.32	s	β	28.45	CH_3

Table 4: MIC of isolated compounds 1, 2+3, 6, 6a.

Compounds	MIC in µg/mL				
	PA	STM	SA96	KP	SM
1	>500	>500	>500	nt	>500
2+3*	312.5±265.17	187.5±88.39	500	>500	>500
6	>500	>500	nt	nt	nt
6a	>500	>500	>500	>500	500
Kanamycin	62.5	0.52	6.94±2.40	4.85±3.19	0.86±0.30

***** Compounds 2 and 3 were obtained as an inseparable mixture. nt= not tested, KP: *Klebsiella pneumoniae* MTCC109, PA: *Pseudomonas aeruginosa* MTCC741, STM: *Salmonella typhimurium.* MTCC1251, SA96: *Staphylococcus aureus* MTCC96, SM: *Streptococcus mutans* MTCC890.

Table 5: MIC of the extracts and fractions of *X. aethiopica.*

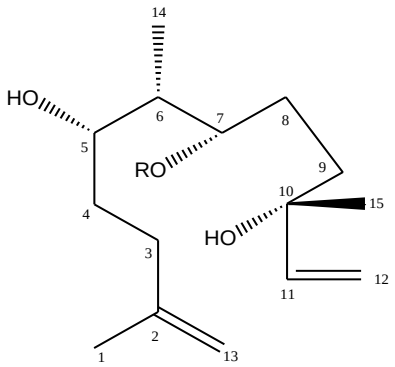
	MIC in µg/mL						
	KP	PA	STM	ML	SA96	BS	SM
Extracts							
XAM	>1000	>1000	>1000	1000	>1000	>1000	1000
XAH	nt	>1000	nt	>1000	416.67±144.34	>1000	333.33±144.34
Fractions							
XAM-C	nt	>1000	>1000	nt	>1000	>1000	>1000
XAM-E	>1000	>1000	>1000	nt	>1000	>1000	>1000
XAM-B	>1000	>1000	>1000	833.33±288.68	>1000	>1000	1000
Control							
Kanamycin	4.85	62.5	0.52	4.16	6.94	0.53	0.86

XAM= *X. aethiopica* methanol extract, XAH= *X. aethiopica* hexane extract, XAM-C= *X. aethiopica* chloroform fraction, XAM-E= *X. aethiopica* ethyl acetate fraction, XAM-B= *X. aethiopica* butanol fraction.
nt= not tested, BS: *Bacillus subtilis*, ML: *Micrococcus luteus*, SA96: *Staphylococcus aureus* MTCC96, SM: *Streptococcus mutans*, KP: *Klebsiella pneumoniae*, PA: *Pseudomonas aeruginosa*, STM: *Salmonella typhimurium.*

Table 6: IC_{50} of the methanol extract and fractions of *X. aethiopica.*

	IC_{50} in µg/mL			
	MCF-7	**CaCO2**	**WRL-68**	**PC-3**
Extracts				
XAM	130	152	16.6	104
Fractions				
XAM-C	96	NA	62	78
XAM-E	97	NA	79	78
XAM-B	NA	NA	NA	NA
andard				
Doxorubicin	0.85	3.5	2.1	5.0

XAM= *X. aethiopica* methanol extract. Cell lines: WRL-68= *Liver cancer cell line*, CaCO2= *colon cancer*, MCF-7= *breast cancer*, PC-3= *prostate cancer*.



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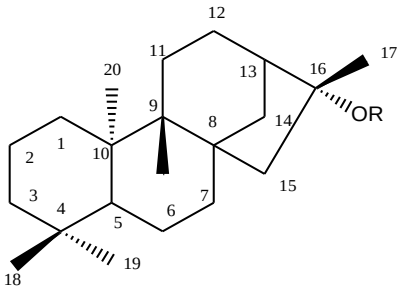
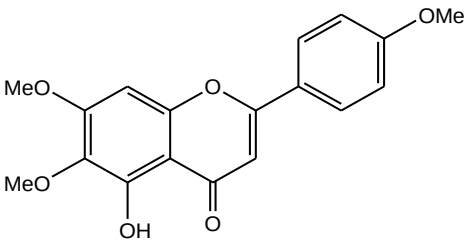
1a, R= OAc



5, R= H
5a, R= OAc



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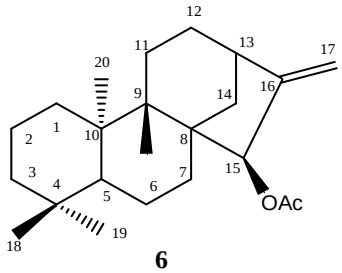

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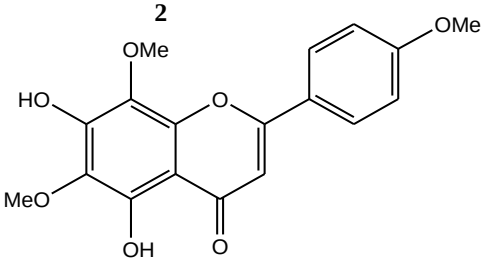

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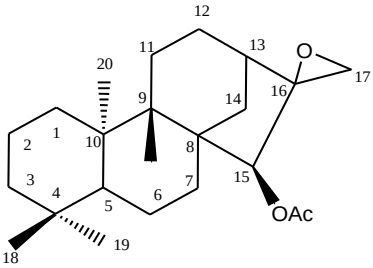

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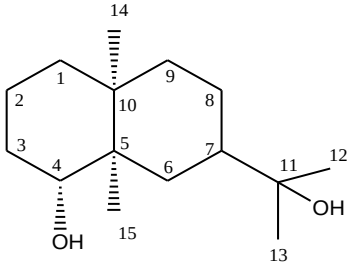

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Figure 1: Structures of compounds 1-6.

4. CONCLUSION

The bioassay-directed investigation of the extracts of two Cameroonian medicinal and aromatic plants namely *O. canum* and *X. aethiopica* led to the isolation of one new nerolidol derivative 2,11-dodecadiene-5,7,10-trihydroxy-6,10-dimethyl-2-methylene (**1**) from the methanol extract of *O. canum* altogether with eight known compounds of various types viz. sesquiterpene, triterpene, flavonoids, steroid and sterols.

In the case of *X. aethiopica*, known diterpenes kauranol and xylopic acid were isolated and identified. Some fractions showed moderate antibacterial activity against all the bacteria tested whereas a fraction exhibited a significant cytotoxicity against breast and liver cancer cell lines.

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