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A-type doubly linked proanthocyanidin trimer and other metabolites from *Canthium venosum* fruits, and their biological activities

Appolinaire Kene Dongmo[a], Raymond Ngansop Nono[a], Fatma Kaaniche[b,c], Isabelle Kamga Mawabo[d], Marcel Frese[c], Jean Rodolphe Chouna[a,*], Celine Nguefeu Nkenfou[d], Bruno Ndjakou Lenta[e], David Ngnokam[a], Pépin Nkeng-Efouet-Alango[a], Norbert Sewald[c]

[a] Department of Chemistry, Faculty of Science, University of Dschang, P.O. Box 67, Dschang, Cameroon
[b] Team Natural Substances (LR17ES08), Laboratory of Organic Chemistry, University of Sfax, Faculty of Sciences, BP. 1171, 3000, Sfax, Tunisia
[c] Organic and Bioorganic Chemistry, Department of Chemistry, Bielefeld University, P.O. Box 100131, 33501, Bielefeld, Germany
[d] Department of Biology Higher Teacher Training College, University of Yaoundé 1, PO Box 47, Yaoundé, Cameroon
[e] Department of Chemistry, Higher Teacher Training College, University of Yaoundé 1, PO Box 47, Yaoundé, Cameroon

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ABSTRACT

Phytochemical investigation of *Canthium venosum* fruits led to the isolation of a new doubly linked A-type proanthocyanidin trimer: epicatechin-(2*β*→*O*→7, 4*β* →8)-catechin-(5→*O*→2*β*, 6→4*β*)-epicatechin [venosumtannin A-1 (**1**)], along with twenty known compounds **2-21**. Allyl (**9a**) and acetyl (**9b**) derivatives of **9** were prepared. The structures of compounds were established using comprehensive spectroscopic analysis including 1D NMR, 2D NMR (COSY, HMQC, HMBC, and NOESY) and circular dichroism (CD), and by comparison with the corresponding literature data. The antioxidant, cytotoxic, acetylcholinesterase and antibacterial activities of some of the isolated compounds were investigated. In the acetylcholinesterase inhibitory activity test, compounds **2** and **9** (IC_{50}: 0.03 ± 1.22 × 10^{-3} and 0.04 ± 1.23 × 10^{-3} *μ*M) were more active than the references (eserine and tacrine; IC_{50}: 0.77 ± 1.84 × 10^{-3} and 0.15 ± 1.04 × 10^{-3} μM respectively).

1. Introduction

The genus *Canthium* (Rubiaceae) consists of approximately 110 species, which are mainly found in the tropical and subtropical areas of Asia and Africa. *Canthium venosum* (Oliv) Hiern is a scandent shrub of up to 7 m high, or liana with more or less dense crown, horizontally-spreading, distributed from Senegal to Sudan (Arbonnier, 2000; Patro et al., 2014). Its leaves are traditionally used in the treatment of rheumatism, the mixture of leaves and wood are used for the treatment of intercostal pains. The roots are used as tonic. The fruits are edible in some areas (Arbonnier, 2000). Previous investigation on some *Canthium* species revealed the presence of iridoides (Joubouhi et al., 2015), coumarins, benzoic acid derivatives, lignans, flavonoids, terpenoids, and alkaloids (Sangare, 2003). Some *Canthium* species are reported to exhibit: nematocidal (Wabo et al., 2010), hypocholesterolaemic (Bandara et al., 2009), antioxidant (Kumar et al., 2008), antimicrobial (Yang et al., 2010), antiplasmodial and antibacterial activities (Akomo et al., 2009). In our continuing search for bioactive compounds from plants, we investigated the fruits of *C. venosum* and report here on the isolation and structure elucidation of a new doubly linked A-type proanthocyanidin trimer (**1**) together with the antioxidant, cytotoxic, acetylcholinesterase inhibition effect and the antibacterial activities of some of the isolated compounds.

2. Results and discussion

The dry powder of *Canthium venosum* fruits was extracted with MeOH. The resulting crude extract was further extracted using ethyl acetate (EtOAc) and *n*-BuOH. Repeated and successive column chromatography on silica gel, Sephadex LH-20 and reverse phase HPLC of the EtOAc fraction led to the isolation and characterization of a new compound (**1**) (Fig. 1), together with 20 known compounds, namely, ixoratannin A-2 (**2**), cinnamtannin B-1 (**3**) (Idowu et al., 2010), proanthocyanidin A-1 (**4**), proanthocyanidin A- 2 (**5**), epicatechin-(2*β*→*O* →7, 4*β*→8)-*ent*-epicatechin (**6**) (Lou et al., 1999), epicatechin-(2*β*→*O*→7, 4*β*→6)-*ent*-epicatechin (**7**) (De Oliveira et al., 2009), betulin (**8**), betulinic acid (**9**), ursolic acid (**10**), ursolic acid-3-*O*-glucoside (**11**) (Mahato and Kundu, 1994), kaempferol-3-*O*-*α*-L-rhamnopyranoside (**12**) (Cardoso et al., 2013), kaempferol-3-*O*-*α*-L-arabinopyranoside (**13**) (Gudej, 2003), kaempferitrin (**14**) (Cuyckens et al., 2001), kaempferol-

* Corresponding author.
E-mail address: jean.chouna@univ-dschang.org (J.R. Chouna).

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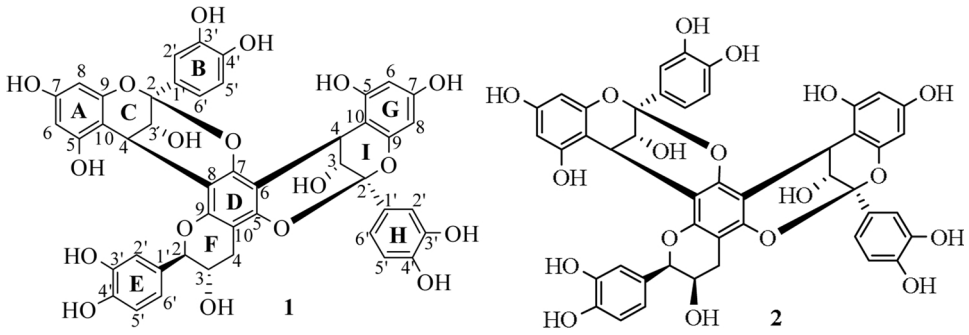

Fig. 1. Structure of compounds 1 and 2.

3-*O*-*α*-L-arabinopyranosyl-7-*O*-*α*-L-rhamnopyranoside (**15**) (Dong et al., 2017), epicatechin (**16**) (Xu et al., 2010), 3,5-dihydroxybenzoic acid (**17**) (Guria et al., 2013), *β*-sitosterol (**18**), *β*-sitosterol 3-*O*-*β*-*D*-glucopyranoside (**19**), stigmasterol (**20**), stigmasterol 3-*O*-*β*-*D*-glucopyranoside (**21**) (Khatun et al., 2012).

Compound **1** was obtained as a white powder with $[\alpha]_D^{20}$ + 33.5 (*c* = 1 mg/ml, MeOH). Its HRESIMS in the positive mode showed an $[M+H]^+$ ion at *m/z* 863.1801 (calcd 863.1823 for $C_{45}H_{35}O_{18}$, corresponding to 29 degrees of unsaturation). In the ^{1}H NMR data of compound **1** (Table 1) two AX systems at δ_H 4.39 (1H, d, *J* = 3.5 Hz) / 4.31 (1H, d, *J* = 3.4 Hz) and an AX at δ_H 4.38 (1H, d, *J* = 3.5 Hz) / 4.17 (1H, d, *J* = 3.5 Hz) corresponding to the C-ring and I-ring, respectively. Two *meta*-coupled pairs of protons resonating at δ_H 5.95 (1H, d, *J* = 2.3 Hz) / 6.14 (1H, d, *J* = 2.3 Hz) and δ_H 5.93 (1H, d, *J* = 2.3 Hz) / 6.05 (1H, d, *J* = 2.3 Hz) correspond to the A-ring and G-ring, respectively. Two AMX systems and one AA'X system were also observed in the aromatic shift range δ_H 6.80 – 7.32. They were assigned to the B-ring (7.31, d, *J* = 2.1 Hz, 1 H; 6.91, d, *J* = 8.3 Hz, 1 H; 7.21, dd, *J* = 8.4, 2.2 Hz, 1 H), H-ring (7.17, d, 2.1 Hz, 1 H; 6.84, d, 8.3 Hz, 1 H; 7.06, dd, 8.3, 2.2 Hz, 1 H), and E-ring (6.86, d, *J* = 8.2 Hz, 1 H; 6.97, d, *J* = 8.4 Hz, 1 H; 6.86, dd, *J* = 8.3, 2.2 Hz, 1 H) respectively. The ^{13}C NMR spectrum of compound **1** (Table 1) shows 42 signals. The intensities of some signals suggested that they represent more than one carbon. The signal at δ_C 115.7 was assigned to three carbons and the one at δ_C 98.5 represents two carbons. In addition, signals of two ketal carbons at δ_C 101.3 and 100.5 as well as of three oxymethine carbons at δ_C 67.2, 68.1 and 67.4 were observed. These data are characteristic of an A-type proanthocyanidin trimer in which one unit of flavan is doubly bound to the other two units. (Jacques et al., 1974; Lou et al., 1999; Kamiya et al., 2001; Liu et al., 2007; Idowu et al., 2010). Linear oligomeric proanthocyanidins are generally joined between C-4 of one of the flavan units and C-6 or C-8 of the other, and when doubly bound, the second junction usually results from condensation between the C-2 of the C-ring of the starting unit and the hydroxyl group at C-5 or C-7 of D-ring. It is for this reason that the ^{1}H NMR spectrum of a trimer formed in this manner will always show a single signal of unsubstituted C-6 or C-8 proton (Idowu et al., 2010). One of the peculiarities of compound **1**, is the absence of any singlet signal and the appearance of four sets of *meta*-coupled protons in the upper aromatic region (Idowu et al., 2010). The HMBC spectrum further confirmed the A-type trimeric proanthocyanidin skeleton through the correlation (Fig. 2) observed between the proton at C-4 (δ_H 4.39) of the upper unit and C-7 (δ_C 146.8), C-8 (δ_C 108.4) and C-9 (δ_C 149.4) of the middle unit; between proton at C-4 (δ_H 4.38) of the lower unit and the carbons C-6 (δ_C 108.3), C-7 (δ_C 146.8), of the middle unit; between proton at C-3 (δ_H 4.31) of the upper unit and carbon C-8 (δ_C 108.4) of the middle unit, and between proton at C-3 (δ_H 4.17) of the lower unit and C-6 (δ_C 108.3) of the middle unit. All these data are close to those of ixoratannin A-2 (**2**) (Idowu et al., 2010). The difference was observed in the middle unit, where in **1**, the chemical shifts of C-2 (δ_C 83.7) and C-3 (δ_C 68.1) were significantly different for what has been observed in ixoratannin A-2 (**2**), which appeared at δ_C 81.8 (C-2)

Table 1
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) data of compounds **1–2** (CD_3OD).

Cycle		1		2	
	N^0	^{1}H (*J* in Hz)	^{13}C (ppm)	^{1}H (*J* in Hz)	^{13}C (ppm)
Uper Unit					
C	2		101.3		101.1
	3	4.31 (*d*, 3.4)	67.2	4.22 (*d*, 3.4)	67.5
	4	4.39 (*d*, 3.5)	29.4	4.52 (*d*, 3.3)	29.4
A	5		156.9		157.1
	6	5.95 (*d*, 2.3)	98.5	5.99 (*d*, 1.6)	98.3
	7		158.3		158.2
	8	6.14 (*d*, 2.3)	96.8	6.14 (*d*, 2.3)	96.5
	9		154.0		154.0
	10		103.4		103.5
B	1′		131.4		131.6
	2′	7.31 (*d*, 2.1)	115.7	7.28 (*d*, 2.2)	115.6
	3′		147.2		147.2
	4'		145.9		146.8
	5′	6.91 (*d*, 8.3)	116.0	6.88 (*d*, 8.4)	115.9
	6'	7.21 (*dd*, 8.4, 2.2)	119.8	7.18 (*dd*, 8.4, 2.2)	119.7
Middle Unit					
F	2	4.80 (*d*, 7.6)	83.7	4.99 (*brs*)	81.8
	3	4.12 (td,7.9, 5.3)	68.1	4.24 (*m*)	66.7
	4*β*	3.04 (*dd*, 16.6, 5.3)	28.6	3.06 (*dd*, 17.2, 4.9)	29.7
	4*α*	2.63 (*dd*, 16.5, 8.3)		2.77 (*dd*, 17.2, 3.1)	
D	5		150.1		150.6
	6		108.3		108.1
	7		146.8		146.9
	8		108.4		109.1
	9		149.4		150.5
	10		103.6		103.2
E	1′		130.8		130.9
	2′	6.86 (*d*, 8.2)	116.4	7.10 (*d*, 2.0)	115.8
	3′		146.4		145.9
	4'		146.7		146.3
	5′	6.97 (*d*, 8.4)	115.3	6.80 (*d*, 8.2)	116.0
	6'	6.86 (*dd*, 8.3, 2.2)	120.1	6.97 (*dd*, 8.3, 2.2)	120.4
Lower Unit					
I	2		100.5		100.4
	3	4.17 (*d*, 3.5)	67.4	4.17 (*d*, 3.5)	67.6
	4	4.38 (*d*, 3.5)	29.6	4.36 (*d*, 3.5)	29.6
G	5		156.7		156.6
	6	5.93 (*d*, 2.3)	98.5	5.94 (*d*, 2.1)	98.3
	7		158.3		158.1
	8	6.05 (*d*, 2.3)	96.7	6.02 (*d*, 1.9)	96.4
	9		153.8		153.9
	10		103.5		103.6
H	1′		132.0		132.1
	2′	7.17 (*d*, 2.1)	115.7	7.17 (*d*, 2.2)	115.8
	3′		146.9		146.9
	4'		145.7		145.7
	5′	6.84 (*d*, 8.3)	115.7	6.83 (*d*, 8.4)	115.7
	6'	7.06 (*dd*, 8.3, 2.2)	119.9	6.97 (*dd*, 8.3, 2.2)	119.9

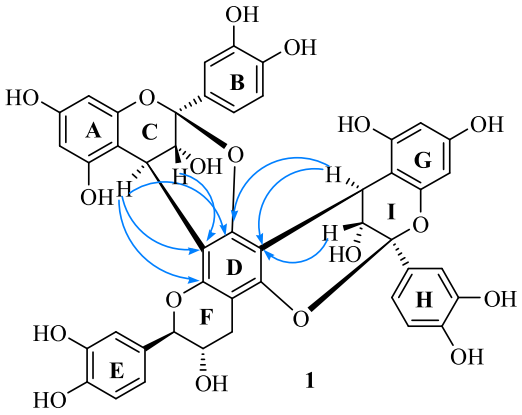

Fig. 2. Key HMBC correlations of compound **1.**

and 66.7 (C-3). In addition, the ^{1}H NMR spectrum also showed difference; in **1**, the proton at C-2 of the middle unit, δ_H 4.80 resonates as a doublet (J = 7.6 Hz), which revealed that H-2 and H-3 are *trans* to each other (catechin), compared to that of ixoratannin A-2 (**2**) which appear as broad singlet and therefore has epicatechin as middle unit (Lou et al., 1999). The β-orientations at C-4 of the interflavan linkages were deduced from the diagnosis of a positive Cotton effect observed in the region 220–240 nm of the CD spectrum (Idowu et al., 2010; Botha et al., 1981). Based on the above data, compound **1** was characterized as [epicatechin-(2β→*O*→7, 4β→8)-catechin-(5→*O*→2β, 6→4β)-epicatechin], and given the trivial name venosumtannin A-1.

Some of the isolated compounds and extracts were tested for their antioxidant, cytotoxic, and antibacterial activities as well as for acetylcholinesterase inhibition (Table 2 and 3).

The DPPH antioxidant activity results (Table 2) showed that, the MeOH extract, EtOAc and n-BuOH sub-fractions exhibited significant antioxidant activities with EC_{50} values of 7.38 ± 0.06, 6.33 ± 0.02 and 5.14 ± 0.02 μM. These results are due to their polyphenolic character and indicated that the fractionation of the extract may be one of the factors that allowed increasing activity. The increase in activity was also supported by the good antioxidant potential of some of the isolated polyphenols (**1**, **2, 6**, and **16**) that exhibited activities with EC_{50} values 5.50 ± 0.04, 3.59 ± 0.04, 12.18 ± 0.06 and 8.42 ± 0.06 μM respectively. The activities of the trimeric oligomer **1**, and **2** were higher than that of dimeric **6** or monomeric **16**. This observation was partially in accordance with Pedan et al. (2016) who indicated that oligomeric proanthocyanidins had a stronger antioxidant capacity than their monomers, and the EC_{50} values were highly correlated to total phenolic and flavonoid contents (Chen et al., 2016). In addition, proanthocyanidins were one of the most potent antioxidants in nature with chemoprotective properties against free radicals and oxidative stress (Liu and White, 2012).

Compound **2** showed negligible cytotoxicity and the other compounds were inactive. In recent studies, proanthocyanidin demonstrated strong cytotoxicity towards cancer cells HT29 (IC_{50} 7.0 ± 0.3 μg/mL), HepG2 (IC_{50} 16 μg/mL) and HCT116 (IC_{50} 20 μg/mL) and weak cytotoxic activities towards the non-malignant Chang cells (IC_{50} 48 μg/mL) (Lai et al., 2017). In addition, other studies showed a selectivity in the cytotoxic effect of the procyanidin trimers in gastric and colon cancer cell lines (Navarro et al., 2017). Some of the isolates were also evaluated for their inhibitory effect towards acetylcholinesterase *in vitro* (Table 2). These analyses showed that compounds **2** and **9** showed potent inhibition towards acetylcholinesterase (IC_{50} values of 0.03 ± 1.22 × 10^{-3} and 0.04 ± 1.23 × 10^{-3} μM, respectively) and were found to be more active than the reference compounds, eserine and tacrine (IC_{50} values 0.77 ± 1.84 × 10^{-3} and 0.15 ± 1.04 × 10^{-3} μM, respectively).

The MeOH extract, EtOAc and *n*-BuOH subfractions and some of the isolates **1-3**, **9**, **9a**, **9b**, **14** and **16** were tested against two Gram positive bacteria: *Salmonella typhi* (clinical isolate) and *Staphylococcus aureus* ATCC 25923 and three Gram negative bacteria: *Streptococcus pneumoniae* ATCC 491619, *Salmonella enterica* NR 13555 and *Pseudomonas aeruginosa* HM 801. All the tested samples showed weak to moderate activity (MIC values ranging from 250 to 500 μg/mL) compared to the reference compound ciprofloxacin as described by Kuete and Efferth (2010). The MeOH extract, EtOAc and *n*-BuOH subfractions were not active on *Streptococcus pneumoniae* and *Salmonella enterica*, however the best potency (250 μg/mL) was observed with the MeOH extract on *Salmonella typhi* and *Pseudomonas aeruginosa* and with n-BuOH extract on *Salmonella typhi*. None of the tested compounds were active against *Salmonella enterica*, while compounds **3**, **9a** and **14** were also not active against *Salmonella typhi*. Compounds **9** exhibited the best potency (250 μg/mL) on *Streptococcus pneumoniae*, while **1**, **9a**, **14** and **16** were inactive. Only compound **3** exhibited activity against *Staphylococcus aureus*, while **3** and **9b** were active on *Pseudomonas aeruginosa*.

These data provide evidence, for the first time, of the diversity of phenolic acids in *Canthium venosum*, and the potential health effects of *Canthium venosum* fruit extracts. This plant was evaluated as a source of potential antioxidants, acetylcholinesterase inhibitors, as well as antitumoral compounds.

Table 2
Antioxidant activity, cholinesterase inhibition and cytotoxicity of some isolated compounds.

Activity Compounds/ extracts	Antioxidant EC_{50} (μM)	Anticholinesterase IC_{50} (μM)	Cytotoxicity (KB-3-1) IC_{50} (μM)
1	5.50 ± 0.04	NA	–
2	3.59 ± 0.04	0.03 ± 1.22 × 10^{-3}	> 100
6	12.18 ± 0.06	NA	NA
9	–	0.04 ± 1.23 × 10^{-3}	NA
16	8.42 ± 0.06	NA	NA
MeOH extract*	7.38 ± 0.06	NA	NA
EtOAc extract*	6.33 ± 0.02	NA	–
n-BuOH extract*	5.14 ± 0.02	NA	–
BHA**	0.13 ± 0.01	–	–
BHT**	0.46 ± 0.01	–	–
Eserine**	–	0.77 ± 1.84 × 10^{-3}	–
Tacrine**	–	0.15 ± 1.04 × 10^{-3}	–
Griseofulvin**	–	–	19.00 ± 1.06

EC_{50} = Concentration of an antioxidant drug that gives half maximal response (μ**M**); **IC_{50}** = Concentration of acetylcholinesterase inhibitor drug that gives half maximal response (μ**M**); (*) = contentration in μg/mL; (**) = European Pharmacopoeia (**EP**) Reference Standard. **NA**: Not active; BHA = Butylhydroxyanisol; and BHT = Butylatedhydroxytoluene.

3. Experimental

3.1. General experimental procedures

The ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance DRX 500 spectrometer at 500 MHz and 125 MHz respectively. ESI-HR-MS spectra were recorded on a Synapt G2Si -Q-IMS-TOF-mass spectrometer. Column chromatography (open column) was performed over silica gel Merck 60 (0.063-0.200 mm). Precoated aluminum backed silica gel 60 F254 sheets were used for TLC. Size exclusion column chromatography was performed using Sephadex LH-20. The TLC spots were visualized under UV light (254 and 365 nm); H_2SO_4 and anisaldehyde-sulfuric acid were used as spraying reagents. Optical rotation was performed on an Autopol IV at 20 °C. CD spectra were recorded in methanol (11.6 × 10^{-6} μmol/L) on a Jasco 810 spectrophotometer. The UV data was recorded on PhotoLab 6600 UV-VIS 143320661 2.17-WTW-2.20 in MeOH (1 mg/mL).

3.2. Plant material

C. venosum fruits were collected in Bangoua (Western Region of Cameroon), in November 2016 and identified at the Cameroon National Herbarium, Yaoundé, with voucher specimen N°: 8645 (16589/SRF/CAM).

3.3. Extraction and isolation

The air-dried and ground fruits of *Canthium venosum* (6 kg) were extracted with methanol (18 L) at room temperature for 72 h. The filtrate obtained was concentrated under vacuum to dryness to yield a residue of 480 g. The MeOH extract (340 g) was suspended in distilled water (900 mL) and re-extracted with EtOAc (800 mL, 102 g) and *n*-butanol (1200 mL, 85 g) respectively. The EtOAc sub-extract (100 g) was chromatographed over silica gel (0.063-0.200 mm) using *n*-hexane/CH_2Cl_2 (from 5 to 100 %), CH_2Cl_2/MeOH (from 1 to 10 %) systems, with increasing polarity gradient. This resulted to eleven (11) major fractions (F1-F11) based on their TLC profile. Fraction F3 (2 g) was processed further through column chromatography over silica gel (0.063-0.200 mm) with *n*-hexane/EtOAc (from 20 to 100 %) in increased gradients to afford **18** (12 mg); and **20** (5 mg). F8 (1.3 g) was processed further through column chromatography over silica gel (0.063-0.200 mm) with *n*-hexane/EtOAc (from 80 to 100 %) and EtOAc/MeOH (from 1 to 5 %) in increased gradients and afforded **19** (6 mg) **21** (9 mg) and **11** (7 mg). Fraction F6 (1.5 g) was subjected to silica gel (0.063-0.200 mm) column chromatography using CH_2Cl_2/MeOH (99:1, 98:2 and 95:5) to obtain **8** (17 mg); **9** (450 mg) and **10** (25 mg). Fraction F7 (1 g) was separated on Sephadex LH-20 using CH_2Cl_2/MeOH (85/15) to provide **12** (12 mg); **13** (6 mg); **14** (18 mg), **15** (14 mg) and a subfraction F7a (300 mg) and F7b **(**120 mg). The F7a fraction was processed further through HPLC using the H_2O-ACN gradient method from 0 to 25 % ACN in 100 min to give **3** (4 mg, t_R 41.35 min), **1** (14 mg, t_R 52.34 min); **2** (7 mg, t_R 55.61 min). Fraction F9 (800 mg) was purified on silica gel (0.063-0.200 mm) column chromatography using CH_2Cl_2/MeOH (from 0.5–10%) with increasing polarity gradient to afford **4** (8 mg); **5** (5 mg); **16** (6 mg); **17** (3 mg) and mixture of **6** and **7** (12 mg).

3.4. Venosumtannin A-1 (*1*)

White powder; $[\alpha]_D^{20}$ + 33.5 (*c* 1 mg/mL, MeOH); UV (MeOH) λ_{max} (log ε) 228 (2.61) and 289 (1.17) nm; CD $[\theta]_{287}$ – 69261, $[\theta]_{271}$ –146554, $[\theta]_{237}$ +1.14249, $[\theta]_{223}$ +605682, $[\theta]_{207}$ – 5.22829; ^{1}H and ^{13}C NMR (500 and 125 MHz, CD_3OD) data, see Table 1; (+)-HRESIMS *m/z* 863.1801 $[M+H]^+$ (calcd for $C_{45}H_{35}O_{18}$, 863.1823).

3.5. Allylation and acetylation of betulinic acid

Betulinic acid (**9**, 50 mg) was dissolved in 12 ml of acetone, 10 ml of allyl bromide and 1 g of potassium hydroxide were added. The stirred solution was heated at 70 °C under reflux for 4 h. The yellow powder of **9a** was obtained by filtration under reduce pressure and washed with ethyl acetate (Barron et al., 1996).

Betulinic acid (**9**, 50 mg) was dissolved in 5 ml of pyridine, 15 ml of acetic anhydride was added, the stirred solution was heated at 100 °C for **3** hours. The solution was then cooled to 5 °C for 3 days. White powder of **9b** was removed from the solution by filtration under reduced pressure and washed with ethyl acetate and MeOH (Urban et al., 2004).

Allyl 3β -Hydroxylup-20(29)-en-28-oate (**9a**): ESI-MS *m/z* 497 $[M+H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.93 (1H, ddt, *J* = 17.2, 10.4, 5.7 Hz, H-2′), 5.34 (1H, dd, *J* = 17.2, 1.5 Hz, H-3′a), 5.23 (1H, dd, *J* = 10.4, 1.4 Hz, H-3′b), 4.73 (1H, d, *J* = 2.4 Hz, H-29b), 4.60 (1H, d, *J* = 1.4 Hz, H-29a),4.56 (2H, m, H-1′), 3.18 (1H, *dd*, *J* = 11.4, 4.8 Hz, H-3), 3.01 (1H, *td*, *J* = 11.1, 4.6 Hz, H-19), 1.68 (3H, s, H-30), 0.96 (6H, s, H-23, H-27), 0.91 (3H, s, H-26), 0.81 (3H, s, H-25), 0.75 (3H, s, H-24). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 175.9 (C-28), 150.7 (C-20), 132.7 (C-2′), 118.3 (C-3′), 109.7 (C-29), 79.1 (C-3), 64.7 (C-1′), 56.7 (C-17), 55.5 (C-5), 50.7 (C-9), 49.6 (C-18), 47.1 (C-19), 42.5 (C-14), 40.9 (C-8), 39.1 (C-4), 38.9 (C-1), 38.4 (C-13), 37.4 (C-10), 37.2 (C-22), 34.5 (C-7), 32.3 (C-16), 30.8 (C-15), 29.8 (C-21), 28.1 (C-23), 27.6 (C-2), 25.7 (C-12), 21.0 (C-11), 19.5 (C-30), 18.4 (C-6), 16.3 (C-25), 16.1 (C-26), 15.5 (C-24), 14.9 (C-27) (Gauthier et al., 2006).

Anhydride of 3β-acetoxylup-20-(29)-en-28-oic acid and acetic acid (**9b**): ESI-MS *m/z* 563 $[M + Na]^+$; ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.74 (1H, d, *J* = 2.2 Hz, H-29a), 4.62 (1H, d, *J* = 1.6 Hz, H-29b), 2.97 (1H, td, *J* = 11.0, 4.6 Hz, H-19), 2.23 (3H, s, 28 – COOAc), 2.04 (3H, s, 3-OAc), 1.68 (3H, s, H-30), 0.96 (3H, s, H-23), 0.95 (3H, s, H-27), 0.84 (3H, s, H-26), 0.84 (3H, s, H-25), 0.83 (3H, s, H-24).

3.6. Cytotoxicity assay

Cytotoxic activity screening of the isolated compounds was done as described in previous reports (Awantu et al., 2011). The KB-3-1 cell was cultivated as a monolayer in DMEM (Dulbecco's modified Eagle medium) with glucose (4.5 g.L^{-1}), L-glutamine, sodium pyruvate and phenol red, supplemented with 10 % (KB-3-1) and foetal bovine serum (FBS). The cells were maintained at 37 °C and 5.3 % CO_2-humidified air. On the day before the test, the cells (70 % confluence) were detached with trypsin-ethylenediamine tetraacetic acid (EDTA) solution (0.05 %; 0.02 % PBS) and placed in sterile 96-well plates in a density of 10 000 cells in 100 μL medium per well. The dilution series of the compounds were prepared from stock solutions in DMSO of concentrations of 10 mM. The stock solutions were diluted with culture medium (10 % FBS [KB-3-1]) at least 50 times. Some culture medium was added to the wells to adjust its volume to the wanted dilution factor. The diluted solution from stock solution was added to the wells. Each concentration was tested in six replicates. Dilution series were prepared by pipetting liquid from well to well. The control contained the same concentration of DMSO as the first dilution. After incubation for 72 h at 37 ° C and 5.3 % CO_2-humidified air, 30 μL of an aqueous resazurin solution (175 μM) was added to each well. The cells were incubated at the same conditions for 6 h. Subsequently, the fluorescence was measured. The excitation was recorded at a wavelength of 530 nm, whereas the emission was recorded at a wavelength of 588 nm. The IC_{50} values were calculated as a sigmoidal dose response curve using GRAPHPAD PRISM 4.03.

3.7. Antioxidant assay

The DPPH (1,1-diphenyl-2-picrylhydrazyl) assay was used to evaluate the free radical scavenging activity of the compounds (Molyneux, 2004). Briefly, the compounds were dissolved in 10 % DMSO and diluted at different concentrations of 250 to 1 μg/mL. Then, 500 μL of a 4 % (w/v) solution of DPPH radical in methanol was mixed with 500 μL of samples under investigation. The mixture was incubated for 30 min in the dark at room temperature. The scavenging capacity was determined spectrophotometrically by monitoring the decrease in absorbance at 517 nm against a blank. The percentage of antioxidant activity was calculated

As antioxidant activity (%) = $[(A_{control} - A_{sample}) / A_{control}] \times 100$. BHA (Butylhydroxyanisole) and BHT (Butylatedhydroxytoluene) (Sigma, USA) were used as positive controls. A_{sample} was the absorbance of the sample and $A_{control}$ was the absorbance of the blank.

3.8. Acetyl cholinesterase inhibitory potential assay

AChE inhibitory activity was measured according to a slightly modified Ellman's spectrophotometric method. Electric eel AChE was used, while acetylthiocholine iodide (ATCI) was used as substrate of the reaction. Hydrolysis of ATCI was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion at 405 nm as a result of the

reaction of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) with thiocholines, released by the cholinesterase in a 96 well microplate reader (Thermo Scientific/Varioskan Flash, Germany) (Ellman et al., 1961). 100 μL of Tris buffer at 50 mM (pH 8.0), 30 μL of sample (100 μg/mL) and 5 μL of AChE (0.5 U/mL) were incubated for 10 min at 25 °C. Then, 27 μL of DTNB (3 mM) and 23 μL of ATCI (1.16 mM) were added. Percentage of inhibition of AChE was determined by comparing of sample reaction relative rates to control (10 % DMSO in Tris buffer) using the following formula:

AChE inhibition (%) = 1 - (δA_{sample} / $\delta A_{control}$) x 100.

δA_{sample} = Sample absorbance at zero time - Sample absorbance at the end of reaction.

$\delta A_{control}$ = Control absorbance at zero time - Control absorbance at the end of reaction. Tacrine (99 %) was used as the reference AChE inhibitor

3.9. In vitro antibacteria assay

The two Gram positive bacteria *Salmonella typhi* (clinical isolate) and *Staphylococcus aureus* ATCC 25923 and the three Gram negative bacteria: *Streptococcus pneumoniae* ATCC 491619, *Salmonella enterica* NR 13555, and *Pseudomonas aeruginosa* HM 801 were tested for their susceptibility to extracts/compounds. The tests were performed in duplicate, following the method by Eloff (1998). In a 96-well microplate, 100 μL of sterile culture broth (MHB) were introduced. Then 100 μL of each stock sample solution (2000 μg / mL) were added to the first wells and then distributed to all other wells, with concentrations ranging from 3.8–500 μg / mL and < 0.15 to 500 μg / mL for ciprofloxacin. Then 100 μL of liquid culture medium (MHB) inoculated with the test organism (2×10^6 CFU / mL) were introduced into the wells in order to obtain a final concentration of 10^6 CFU / mL. Ciprofloxacin was used as reference. The negative controls consisted of wells containing only the culture medium, and wells containing a mixture of culture broth and test organism. The culture microplates were covered and incubated at 37 °C (18 h). Twenty microliters (20 μL) of resazurin were then introduced into both and then incubated again at 37 °C for 24 h (Mativandlela et al., 2006).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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