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Original Article

In vitro antitubercular activity of extract and constituents from the stem bark of *Disthemonanthus benthamianus*

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ABSTRACT

A new *C*-glycosylflavone, apigenin 7-methyl ether 6-*C*-[β-xylopyranosyl-(1→3)-β-glucopyranoside] named distemonanthoside was isolated from the stem bark of *Distemonanthus benthamianus* Baill., Fabaceae, along with six known compounds, sitosterol 3-*O*-β-D-glucopyranoside, 4-methoxygallic acid, syringic acid, quercetin, 6″-*O*-acetylvitexin, quercetin 3-*O*-β-D-glucopyranoside. The structures of those compounds and others were determined through spectral analyses. Compounds distemonanthoside, sitosterol 3-*O*-β-D-glucopyranoside, 4-methoxygallic acid and quercetin were tested against a clinical isolate strain of *Mycobacterium tuberculosis* AC 45; they exhibited good to moderate antitubercular activities with MIC values ranged from 31.25 to 125 μg/ml.

Introduction

Tuberculosis (TB) is a chronic contagious disease caused by several species of *Mycobacterium*. Due to the fact that there is a doubt nowadays on the efficiency of current antibiotics for the treatment of tuberculosis, micro-organisms developed resistance inducing an increase of the number of patients with the disease in worldwide (WHO, 2016). This increasing of MDR-TB incidence has led to an urgent need for the discovery of new plant natural products that may potentially eradicate TB. Several *in vitro* growth inhibition of different strains of *M. tuberculosis* by plant extracts have been reported (Okunade et al., 2004; Copp and Norrie Pearce, 2007; Gautam et al., 2007; Mc Gaw et al., 2008). The Cameroonian medicinal plant *Distemonanthus benthamianus* Baill., Fabaceae, is a large rainforest tree widely distributed in Africa, especially in equatorial region. This essence is highly appreciated industrially for heavy construction and some countries use to export it as "Movingi". In Mayombé region (Congo), traditional healers employ the stem bark in the treatment of several diseases as: parasitic, dermatitis, furuncles, aces and chancres. In the Chaillu region (Congo), that plant is used to cure bronchitis affections and children fever (Bouquet, 1969). In previous works carried on *D. benthamianus*, mainly methoxylated flavonols and flavones were isolated (King et al., 1952; King et al., 1954; Malan and Roux, 1979; Happi and Mpondo, 1994); this paper describes the isolation and structure elucidation of constituents from stem bark of *D. benthamianus*. The evaluation of antitubercular activities of compounds distemonanthoside (**1**), sitosterol 3-*O*-β-D-glucopyranoside, 4-methoxygallic acid (**2**) and quercetin against resistant strain of *M. tuberculosis* was also examined.

Material and methods

General procedures

Melting points were uncorrected and were measured on a Mettler Toledo instrument. IR spectra were recorded on an Alpha FT-IR Spectrometer from Bruker, while 1D and 2D NMR spectra were

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obtained on a Bruker DRX 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C spectra) spectrometer (Bruker, Rheinstetten, Germany) with chemical shifts reported in δ (ppm) using TMS (δ_H) as an internal standard. The HR-ESI-MS was obtained on LTQ-FT instrument (Thermo Scientific). UPLC–MS was measured by a Shimadzu UPLC-MS system. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. Silica gel 60 (230–400 mesh E. Merck, Darmstadt, Germany) was employed for column chromatography, the solvent mixing systems for elution were mainly CH_2Cl_2/MeOH with increasing polarity each.

Plant material

Stem bark of *Distemonanthus benthamianus* Baill., Fabaceae, were collected at Eséka (Koumoul) near Yaoundé (3° 38′ 60.00″ N, 10° 46′ 0.00″ E) in the Centre Region of Cameroon in March 2014 and identified by Victor Nana. A voucher specimen (No. 45488 HCN) was deposited at the National Herbarium in Yaoundé, Cameroon.

Extraction and isolation

Dried and powdered stem bark of *D. benthamianus* (254 g) were extracted for 48 h with MeOH (3 × 1l) at room temperature. After filtration and evaporation of solvent, the crude MeOH extract (16 g) was subjected to CC (150 × 3 cm) [(SiO_2), eluting with a gradient solvent system (CH_2Cl_2/MeOH)] giving four main fractions: I (1.9 g), II (3.8 g), III (3.6 g) and IV (6.7 g). Fractions (100 ml) were collected and grouped on the basis of TLC analysis. Fraction II (3.8 g) was submitted to CC (SiO_2, 100 × 1 cm) using solvent system CH_2Cl_2/MeOH (50/1) to give sitosterol 3-*O*-β-D-glucopyranoside (65 mg). Fraction III (3.6 g) was submitted to CC (SiO_2, 100 × 1 cm) using solvent system CH_2Cl_2/MeOH (60/1 to 5/1) to give four sub-fractions (IIIa, IIIb, IIIc and IIId). Sub-fraction IIIc (1 g) was chromatographed (SiO_2, 50 × 1 cm) using CH_2Cl_2/MeOH (40/1–15/1) to afford compound **2** (480 mg) and compound **3** (3 mg). Sub-fraction IIId (0.65 g) was subjected to a silica gel column in gradient elution mixture solvent composed of CH_2Cl_2/MeOH (25/1–5/1) to afford quercetin (8 mg) and quercetin 3-*O*-β-D-glucopyranoside (11 mg). Using the same process, fraction IV (6.7 g) gave three sub-fractions (IVa, IVb and Vc). Sub-fraction IVa (0.98 g) was further chromatographed on a silica gel column (100 × 1 cm) using CH_2Cl_2/MeOH (10/1) to afford compound **4** (4 mg). Sub-fraction IVb (2.8 g) was purified by repeated CC on silica gel (100 × 1 cm) with the solvent system CH_2Cl_2/MeOH (10/1–1/1) to provide compound **1** (28 mg).

*Structural characterization of distemonanthoside (**1**)*

Yellow solid; $[\alpha]_D^{25} = -54°$ (*c* 0.05, MeOH); m.p. 285–287 °C; $IR_{\upsilon max}^{KBr}$ cm^{-1}: 3267, 2923, 2853, 1595, 1512, 1226, 1159; TLC, R_f: 0.28 (CH_2Cl_2/MeOH: 90/10); ESI-MS *m/z*: ESI-MS: 577.4 $[M-H]^{-\bullet}$, LC–MS: *m/z* 579 $[M+H]^+$ and ESI-MS: *m/z* 601.5 [M+Na] + (Calcd for $C_{27}H_{30}O_{14}Na$ = 601.5); ^{1}H NMR (500 MHz, DMSO-d_6), δ_H: 8.09 (2H, d, *J* = 8.8 Hz, H-2′ and H-6′), 6.96 (2H, d, *J* = 8.8 Hz, H-3′ and 5′), 6.82 (1H, s, H-3), 6.44 (1H, s, H-8), 4.81 (1H, d, *J* = 10.0 Hz, H-1″), 4.01[b] (1H, H-3″), 3.86 (3H, s, –OCH_3), 3.82 (1H, d, *J* = 7.0 Hz, H-1‴), 3.72 (1H, dd, *J* = 11.3; 2.4 Hz, H-6″), 3.42[b] (1H, H-6″), 3.39[b] (1H, H-2″), 3.36[b] (1H, H-4″), 3.21[b] (1H, H-5″), 2.83 (1H, dd, *J* = 11.5; 4.0 Hz, H-5‴), 2.81[b] (1H, H-3‴), 2.78[b] (1H, H-2‴), 2.39[b] (1H, H-5‴); ^{13}C NMR (125 MHz, DMSO-d_6): δ_C: 182.1 (C-4), 164.1 (C-2), 163.4 (C-7), 161.4 (C-9), 161.2 (C-4′), 155.5 (C-5), 128.9 (C-2′ and C-6′) 121.3 (C-1′), 115.8 (C-3′ and C-5′), 105.3 (C-1‴), 104.7 (C-6), 104.1 (C-10), 102.3 (C-3), 94.8 (C-8), 81.7 (C-5″), 80.4 (C-3″), 78.1 (C-2″), 76.4 (C-3‴), 75.6 (C-2‴), 73.5 (C-1″), 71.3 (C-4″), 69.2 (C-4‴), 65.3 (C-5‴), 60.8 (C-6″), 56.5 (–OCH_3).

[b]Signal patterns are unclear due to overlap.

Antitubercular activity

MIC values were determined for the extract against *M. tuberculosis* strain AC 45 (clinical isolate obtained from Sangmelima district's Hospital in South Region of Cameroon) employing the microplate Alamar Blue assay, using Rifampicin as reference. The 96 wells plate received 100 μl of Middlebrook 7H9 medium supplemented with 10% OADC (oleic acid, albumin, dextrose, catalase) 2% glycerol and 0.05% v/v of tween 80. Broth and serial dilution of compounds were made directly on the plate with drug concentrations of 0.244 to 250 μg/ml. Plates were covered and sealed with parafilm and incubated at 37 °C for 14 days. Then, 40 μl Alamar Blue solution was added to the plate and incubated for 24 h. A blue colour in the well was interpreted as no bacterial growth and pink colour was scored as growth. The MIC was defined as the lowest drug concentration, which prevented colour change from blue to pink. The result of antitubercular activity depicted in Table 1. The MIC and MBC were determined according to the guidelines of CLSI (2011). Each experiment was performed at least twice according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2011).

*Acid hydrolysis of **1***

Compound **1** (8 mg) was dissolved in7% H_2SO_4 (0.5 ml) and heated on an aqueous bath at 100 °C for 4 h. The reaction mixture was diluted with H_2O and extracted with CH_2Cl_2. The CH_2Cl_2 layer was evaporated to dryness and purified by preparative TLC over silica gel with CH_2Cl_2–MeOH (5/1) as eluent. Apigenin-7-methyl ether 6-*C*-glucoside (3 mg) was isolated and identified through direct comparison with authentic samples (TLC, MP, and IR). The neutralized and lyophilized aqueuous hydrosylates of the aqueous solution gave only xylose. GC-MS (Column: 5% phenyl and 95% methyl silicone on ultra 2, 0.2 × 46 m, column temp.: 250 °C, carrier gas: He 0.8 ml/min, sample: trimethylsilyl derivatives: t_R (min) xylose (19.29 for **1**).

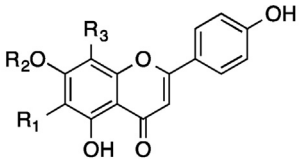
Table 1
MIC and MBC values of the methanol extract and the isolated compounds against clinical isolate strain of *Mycobacterium tuberculosis* (AC 45).

Plant species/compounds	MIC[a] (μg/ml)	MIC[a] (μM)	MBC[b] (μg/ml)	MBC/MIC
D. benthamianus	1250	nd[c]	2500	2
1	125	216.3	125	1
Sitosterol 3-*O*-β-D-glucopyranoside	62.5	108.5	125	2
2	31.25	169.8	125	4
Quercetin	62.5	207.0	125	2
RMP	0.976	nd[c]	7.8125	8

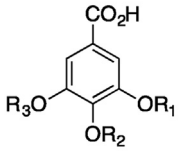
RMP, Rifampicin.
[a] MIC, Minimum Inhibitory Concentration.
[b] MBC, Minimum Bactericidal Concentration.
[c] nd, Not determined.

Results and discussion

The detailed investigation of methanol extract of the stem bark of *D. benthamianus* led to the isolation of seven compounds. Six of them were identified as the known sitosterol 3-*O*-β-D-glucopyranoside (Ngono Bikobo et al., 2014), 4-methoxygallic acid (**2**) (Ouyang et al., 2007), syringic acid (**3**) (Bayiha Ba Njock et al., 2011), quercetin (Güvenalp and Demirezer, 2005), 6″-*O*-acetylvitexin (**4**) (Bayiha Ba Njock et al., 2011), quercetin 3-*O*-β-D-glucopyranoside (Murai et al., 2014). The structures of these compounds were elucidated by NMR spectroscopy analysis, including 1D and 2D techniques and also by comparing experimental data with respective literature data



1 R_1=β-xylopyranosyl-(1→3)-β-glucopyranosyl; R_2=CH_3; R_3=H
4 R_1=R_2=H; R_3=6"-*O*-acetylglucopyranosyl



2 R_1=R_2=OH; R_3=CH_3
3 R_1=R_2=CH_3; R_3=OH

Compound **1** was obtained as yellow amorphous powder, $[\alpha]_D^{25} = -54^\circ$ (c = 0.05, MeOH). Its molecular formula, $C_{27}H_{30}O_{14}$ was established by negative-ion HR-ESI-MS (Fig. S4). The spectrum displayed the deprotonated molecule peak $[M-H]^-$ at m/z = 577.4 in agreement with the above formula (calcd, 577.44). The IR spectrum of **1** showed absorption bands characteristic of hydroxyl groups (3219 cm^{-1}), conjugated carbonyl groups (1652 cm^{-1}) and aromatic rings (1603 and 1572 cm^{-1}). UV spectral properties of **1** showed absorption maxima at λ_{max} 340 nm and 268 nm in MeOH, characteristic for a substituted flavone (Mabry et al., 1970). In addition, acid hydrolysis of **1** gave apigenin 7-methyl ether 6-*C*-glucoside and β-xylose which were identified by TLC analysis and comparison with authentic samples (GC; t_R 19.29 min). In the ^{1}H NMR spectrum (Table 2) the set of *ortho*-coupled AA′BB′ type protons at δ_H 8.04 (2H, d, J = 8.8 Hz) and 6.96 (2H, d, J = 8.8 Hz), was respectively assigned to H-2′/6′ and H-3′/5′ protons of the B-ring of the molecule, while an isolated aromatic proton appeared at δ_H 6.44 (s, H-8) from A ring. The spectrum also revealed the presence of a methoxyl group at δ_H 3.86 and two signals assignable to anomeric sugar protons, which were identified to be an inner β-glucopyranose and a terminal β-pyranose structure of xylose. This was strengthened by the observation in ^{13}C NMR and DEPT spectra of eleven carbon signals (Table 2) among which two are anomeric carbon signals at δ_C 73.5 and 105.3, seven methine carbon signals, two oxymethylene carbons at δ_C 60.8 and 65.3. Since the anomeric protons of glucose and xylose at δ_H 4.81 and 3.82 exhibited large coupling constants (J = 10.0 and 7.0 Hz), the sugars were considered of the β-pyranose type. The HMBC spectrum of compound **1** revealed correlations of the anomeric proton at δ_H 4.81 (H-1″) and carbons at δ_C 161.3 (C-7), 155.6 (C-5) 104.7 (C-6) and 81.7 (C-5″) (Fig. 1), indicating the C-C bond between the inner β-glucopyranosyl moiety and the aglycone at 6-position. In addition, H-1‴ at δ_H 3.82 correlates to both C-3″ (δ_C 80.4) and C-5‴ (δ_C 65.3) revealing that the β-xylopyranosyl moiety was linked to C-3″ at δ_C 80.4, showing that glucose and xylose are linked through a 1→3 type. This was strengthened by the NOESY crosspeaks of the protons H-3″ (δ_H 4.01) with H-1‴ (δ_H 3.82) confirming the aforementioned bonding. The attachment of a methoxyl group to the 7-position was shown by the observation of the crosspeaks at δ_H 3.86 (3H, s, OMe) and δ_C 164.1 (C-7) in the long-range HMBC spectrum. Moreover the NOESY (Fig. 1) experiment confirmed this position through the correlation between H-8 (δ_H 6.44) and the methoxyl proton signals at δ_H 3.86. The complete assignment of all proton and carbon resonances was achieved after careful analysis of COSY, HSQC and HMBC techniques.

Table 2
^{1}H and ^{13}C NMR spectroscopic data of compound 1 (500 and 125 MHz in DMSO-d_6) δ in ppm.

Position	δ_C	DEPT	δ_H (J in Hz)	HMBC (C→H)
Apignenin				
2	164.1	C		H-C(3); H-C(2′)
3	102.3	CH	6.82 (s)	
4	182.1	C		H-C(3)
5	155.5	C		H-C(1″)
6	104.7	C		H-C(1″)
7	163.4	C		H-C(1″);CH_3O-
8	94.8	CH	6.44 (s)	
9	161.4	C		H-C(8)
10	104.1	C		H-C(3)
1′	121.3	C		H-C(3); H-C(2′, 6′)
2′	128.9	CH	8.04 (d, 8.8)	H-C(3′, 5′)
3′	115.8	CH	6.96 (d, 8.8)	H-C(2′, 6′)
4′	161.2	C		H-C(2′); H-C(5′)
5′	115.8	CH	6.96 (d, 8.6)	H-C(2′, 6′)
6′	128.9	CH	8.04 (d, 8.6)	H-C(3′, 5′)
7-OCH_3	56.5	CH_3	3.86 (s)	
Inner glucose				
1″	73.5	CH	4.81 (d, 10)	H-C(2″); H-C(5″)
2″	78.1	CH	3.39[a]	H-C(1″)
3″	80.4	CH	4.01[a]	H-C(1″)
4″	71.3	CH	3.36[a]	H-C(2″); H-C(5″)
5″	81.7	CH	3.21[a]	H-C(1″); H-C(6″)
6″	60.8	CH_2	3.72 (dd, 11.3; 2.4) 3.42[a]	H-C(5″)
Terminal xylose				
1‴	105.3	CH	3.82 (d, 7.0)	H-C(2‴); H-C(5‴)
2‴	75.6	CH	2.78[a]	H-C(1‴)
3‴	76.4	CH	2.81[a]	H-C(1‴); H-C(5‴)
4‴	69.2	CH	2.89[a]	H-C(5‴)
5‴	65.3	CH_2	2.83 (dd, 11.5; 4.0) 2.39[a]	H-C(1‴); H-C(4‴)

[a] Signal patterns are unclear due to overlap.

Some significant HMBC correlations are shown in Fig. 1 and in Table 2. Compound **1** is closely related to the previously reported swertisin 2″-*O*-arabinoside from the tall bearded *iris* (Takayuki et al., 2012); meanwhile, differences occur in the sequence of sugar moieties and this is exemplified by the values of the retention times of xylose [which is close to reported data (Liu et al., 2009)]. This assertion is also strengthened by the upper chemical shift values of protons of the xylose moieties compared to those of arabinose (Gu et al., 2011).

Accordingly, **1** was defined as apigenin 7-methyl ether 6-*C*-[β-xylopyranosyl-(1→3)-β-glucopyranoside] named distemonanthoside. To the best of our knowledge, this is the first report of the isolation of this compound and others (phenolic acids and sterols) from *D. benthamianus*.

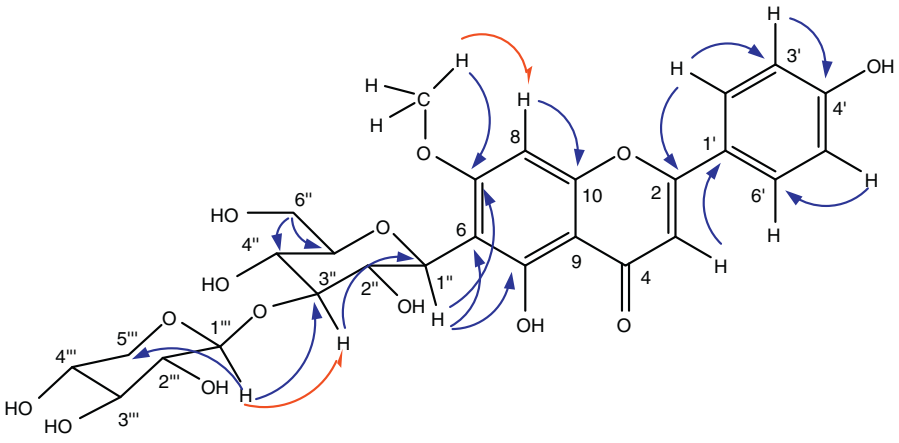


Fig. 1. Selected HMBC ( ) and NOESY ( ) correlations of compound **1**.

According to Cantrell et al. (2001) isolated compounds that exhibit a MIC of 64 μg/ml or lower are considered promising. For crude extracts, the MIC should be equal to or lower than 125 μg/ml (Gu et al., 2004). Thus, the values of 125, 62.5, 31.25 and 62.5 μg/ml for **1**, sitosterol 3-*O*-β-D-glucopyranoside, 4-methoxygallic acid and quercetin, respectively obtained here, are as good as a promising isolated compounds except for compound **1** (Table 1). According to Gu et al. the methanol extract of *D. benthamianus* showed poor inhibitory activity against *M. tuberculosis*, exhibiting a MIC and MBC of 1250 and 2500 μg/ml respectively, suggesting the low lipophilicity of its constituents (more polar compounds) when they act mutually in synergy. According to Peterson and Shanholtzer (1992) bacteriostatic activity has been defined as a ratio of MBC to MIC of >4. Thus, all tested compounds exhibited bactericidal activity. The results of the present study are in accordance with previous report regarding the values of MIC of isolated compounds (Gu et al., 2004; Jiménez-Alleranes et al., 2007).

Conclusion

The species *D. benthamianus*, is known as abundant sources of flavonoids. Compounds **1**, sitosterol 3-*O*-β-D-glucopyranoside and 4-methoxygallic acid were isolated for the first time from this species. The bioactivity study of the isolated compounds indicated that three compounds (sitosterol 3-*O*-β-D-glucopyranoside, 4-methoxygallic acid and quercetin) exhibited interesting antitubercular activity.

Authors' contributions

JNE (PhD student) contributed running the laboratory work, and drafted the paper; EFMN, PHBD and MAN contributed to biological studies, running the laboratory work, analysis of the data and drafted the paper; NMN contributed to analysis of the data and drafted the paper; UK, ATA, DEP and CB contributed to critical reading of the manuscript; AAZ and DSNB contributed in collecting plant samples, supervised the laboratory work, did the NMR investigations and revised the paper. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2017.09.006.

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