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ANTIPARASITIC PRENYLATED ISOFLAVONOIDS FROM SEEDS OF *MILLETTIA GRIFFONIANA*[1]

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ABSTRACT. Two new prenylated isoflavonoids, namely 7-methoxyebenosin and griffonianone E along with the known calopogonium isoflavone B and 7,2'-dimethoxy-4',5'-methylenedioxy isoflavone were isolated from the seeds of *Millettia griffoniana*. Their structures were assigned on the basis of spectroscopic data. The new compounds exhibit moderate trypanocidal and anti-plasmodial activities.

KEY WORDS: *Millettia griffoniana*, Fabaceae, Papilionoideae, Isoflavones, Trypanocidal, Anti-plasmodial

INTRODUCTION

The genus *Millettia* (Fabaceae) includes about 150 subtropical species. Phytochemical research has revealed the presence of alkaloids [1, 2], furanonaphthoquinone [3] and isoflavonoids [4], which are the main constituents of the genus. Previously, Yankep *et al.* have reported the isolation and structural elucidation of a chalcone and several isoflavonoids including griffonianone A, B and C [5-7] from the root bark of *Millettia griffoniana* (Bail). Some of them, e.g., griffonianone D possess anti-inflammatory properties [3]. We have now investigated the seeds and herein, we report the structures, trypanocidal and anti-plasmodial activities of two new prenylated isoflavonoids designated as 7-methoxyebenosin (**1**) and griffonianone E (**2**) along with two known isoflavones, calopogonium isoflavone B (**3**) [8] and 7,2′-dimethoxy-4',5'-methylenedioxy isoflavone (**4**) [9]. The known compounds were identified by comparison of their spectroscopic data with the literature and by co-TLC with authentic samples.

RESULTS AND DISCUSSION

Compound **1** was obtained as a white powder, m.p.: 129-130 °C from acetone. The molecular formula $C_{22}H_{22}O_4$ was determined by HREIMS (*m/z* 350.1023, M^+). The isoflavone nature was deduced from its IR (ν_{max} 1637 cm^{-1}), UV (λ_{max} 249 and 295 nm), ^1H NMR (7.99, *s*, H-2) and ^{13}C NMR (152.7, C-2) spectra [10]. The ^{13}C NMR (Table 1) along with the DEPT, revealed the presence of four methyls, one methylene, eight methines and nine quaternary carbons including a carbonyl carbon (δ 176.4). The ^1H NMR also revealed the presence of two methoxyl groups (δ 3.86 and 3.92) and that of a dimethylallyl group [δ 5.22: 1H, *t*, *J* = 7.1 Hz, H-2″; δ 3.57: 2H, *d*, *J*

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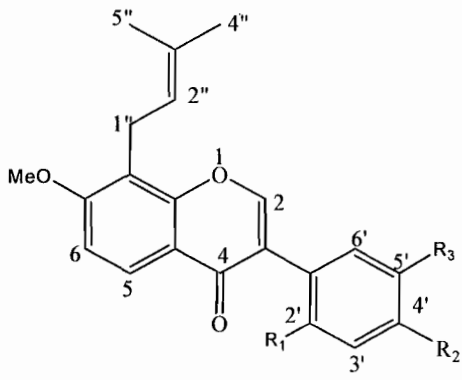
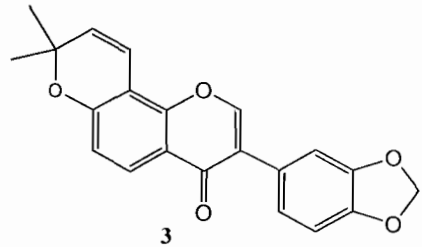
[1]Part 14 of the series *Millettia* of Cameroon. For part 13, see reference [3].

= 7.1 Hz, H-1″; and δ 1.72: 6H, *s*, H-4″ and H-5″]. The presence of one deshielded *ortho*-coupled doublet at δ 8.19 and 7.02, (J = 8.7 Hz), indicated an isoflavone nucleus unsubstituted at C-5 and C-6 positions.



	R_1	R_2	R_3
1	H	OMe	H
2	OMe	O--CH_2--O	



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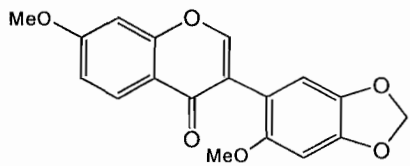

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Table 1. ^{1}H and ^{13}C-MMR assignments of 7-methoxyebenosin (**1**) and griffonianone-E (**2**).

Position	**1**		**2**	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
2	152.7	7.99 (*s*)	154.3	7.96 (*s*)
3	124.4	-	121.4[A]	-
4	176.4	-	176.3	-
5	125.6	8.19 (*d*, 8.7)	125.6	8.18 (*d*, 8.9)
6	109.5	7.02 (*d*, 8.7)	109.4	7.04 (*d*, 8.9)
7	160.9	-	160.9	-
8	117.6	-	117.7	-
9	155.1	-	155.2	-
10	118.5	-	118.9	-
1′	124.4	-	120.4[A]	-
2′	130.5	7.54 (*d*, 8.5)	153.1	-
3′	114.3	6.99 (*d*, 8.5)	111.6	6.85 (*s*)
4′	159.5	-	148.4	-
5′	113.9	6.86 (*d*, 8.5)	141.2	-
6′	130.5	7.54 (*d*, 8.5)	95.9	6.64 (*s*)
1″	22.5	3.57 (*d*, 7.1)	22.5	3.57 (*d*, 6.7)
2″	122.3	5.22 (*t*, 7.1)	122.5	5.24 (*t*, 6.7)
3″	130.1	-	132.4	-
4″	25.7	1.72 (*s*)	26,1	1.71 (*s*)
5″	17.8	1.85 (*s*)	18.2	1.84 (*s*)
O-CH_2-O	-	-	101.7	5.97 (*s*)
OCH_3	56.5	3.98 (*s*)	56.5	3.98 (*s*)
OCH_3	51.7	3.86 (*s*)	57.3	3.76 (*s*)

[a]Values within a column with the same subscript may be interchanged.

An AA′XX′ spin coupled system at δ 6.99 and 7.54 (2H each, *d*, *J* = 8.5 Hz), indicated that ring B is substituted at 4′ position. Location of one of the methoxyl groups at 4′ position came from the observation of the RDA fragment ion at *m/z* 132 in the MS. The second methoxyl and the dimethyl allyl groups are, therefore, located in ring A, and this was confirmed by the presence of a fragment ion at *m/z* 218 in the mass spectrum. Further proof for the location of the methoxyl and dimethylallyl group at 7- and 8-positions, respectively, came from the HMBC experiment (Figure 1a), which revealed among others, correlations between the oxygenated carbon at δ 160.9 (C-7) and the two methylene protons (H-1″) on the one hand and with the aromatic proton (H-5) on the other. These data are consistent with 7,4′-dimethoxy-8-(3″,3″-dimethylallyl)isoflavone for the structure of **1**, which is the first 8-prenyldaidzein derivative [11] from *M. griffoniana*, and for which the trivial name 7-methoxyebenosin [12] is suggested.

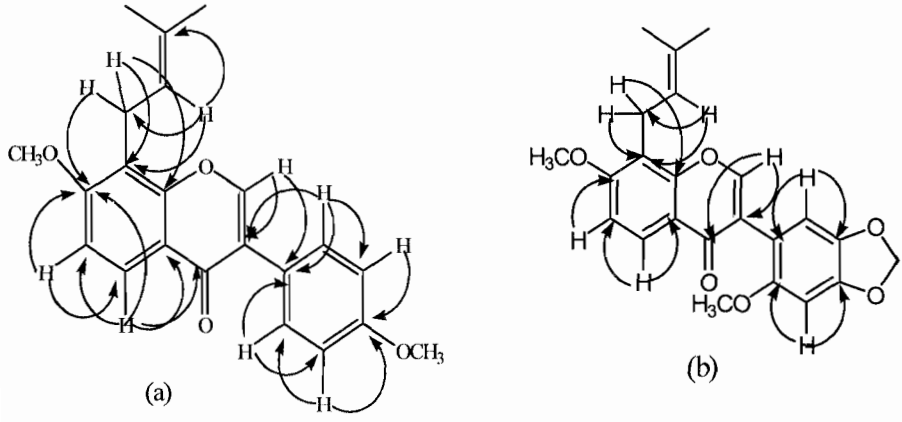

Figure 1. Selected HMBC correlations for 7-methoxyebenosin (**1**) (a) and griffonianone E (**2**) (b).

Compound **2** was obtained as a white powder, m.p.: 130-131 °C from acetone. It gave a negative $FeCl_3$ test and the molecular formula $C_{23}H_{22}O_6$ was determined by HREIMS (*m/z* 394.2134). The presence in its ^{1}H NMR spectrum of a singlet at δ 7.96 and a pair of deshielded *ortho*-coupled doublet at δ 8.18 and 7.04 (*J* = 8.9 Hz) indicated an isoflavone nucleus with no substitution at the C-5 and C-6 positions. The ^{13}C NMR (Table 1) along with the DEPT spectra, revealed the presence of four methyls, two methylenes, six methines and eleven quaternary carbons, including a carbonyl at δ 176.3. A one-proton triplet at δ 5.24 (*J* = 6.7 Hz), a two protons doublet at δ 3.57 (*J* = 6.7 Hz) and two methyl signals at δ 1.71 and 1.84 suggested clearly the presence of a 3,3-dimethyl allyl group [11]. The ^{1}H NMR spectrum also showed the presence of two methoxyl groups at δ 3.76 and 3.98 and a methylenedioxy substituent at 5.99. The placement of the dimethylallyl moiety and one of the methoxyl groups on ring A and the methylenedioxy with the other methoxyl group on ring B was possible from observation of fragment ions at *m/z* 218 and 176 in the mass spectrum. This confirms the position of the dimethylallyl moiety at position 8. The two *para* protons appearing at δ 6.64 and 6.85, coupled with the observation of a fragment ion at *m/z* 176 allowed the placement of the methylenedioxy group at 4′ and 5′ positions and the other methoxyl group at position 2′. Additional evidence in support of the above substitution pattern came from the HMBC spectrum (Figure 1b), which

revealed, among others, correlations between proton at δ 6.85 (H-3′) and carbons at δ 148.4 and 153.1 (C-4′ and C-2′), and proton at δ 6.64 (H-6') and carbons at δ 120.4 (C-1′) and δ 141.2 (C-5′). The structure of griffonianone E is, therefore, 7,2′-dimethoxy-4′,5′-methylenedioxy-8-(3″,3″-dimethylallyl)isoflavone **(2)**.

The two new compounds were tested *in vitro* for activity against three major strains of trypanosome, Lab 110 EATRO, a drug sensitive strain of *Trypanosoma brucei brucei* (a veterinary pathogen), KETRI 243 an uncloned clinical isolate of *Trypanosoma brucei rhodesiense,* and KETRI 243-As-10-3, a pentamidine and melarsol-resistant clone of *Trypanosoma brucei rhodesiense.* The trypanocidal activities determined as IC_{50} values are given in Table 2 showing that compound **2** exhibited significant inhibitory activity against the three trypanosome strains.

The results of the anti-plasmodial tests for the isolated compounds are summarised in Table 3. All the compounds have moderate activities with the strongest effect found for grifonnianone E **(2)**.

Table 2. Antitrypanosomal activities of *Millettia griffoniana* isoflavonoids.

Compounds	IC_{50} (μg/mL)		
	Lab 110 EATRO *T.b. brucei*	KETRI *T.b. rhodesiense* KETRI isolates	
		243	243 as 10.3
7-Methoxyebenosin **(1)**	125	-	-
Griffonianone E **(2)**	8.65	6.35	4.26
Melarsoprol	0.002	0.05	0.005
Pentamidine	0.0006	0.0005	0.004

Table 3. Antiplasmodial activities of compounds from *Millettia griffoniana.*

Compounds	*Plasmodium falciparum* clones (IC_{50}, μg/mL)	
	D-6	W-2
Calopogoniumisoflavone B **(3)**	38.68	36.08
7-Methoxyebenosin **(1)**	36.70	28.73
Griffonianone E **(2)**	28.57	28.29
7,2′-Dimethoxy-4',5'-methylenedioxy isoflavone **(4)**	48.77	34.48
Chloroquine	0.028	-

EXPERIMENTAL

General. M.ps. uncorr.; UV: MeOH; IR: KBr disk; EIMS: direct inlet 70 eV; ^{1}H and ^{13}C NMR (Bruker DMX 300, $CDC1_3$) 300 and 75 MHz, respectively, with the residual solvent signals as internal reference; CC: Si gel $60F_{254}$ (230-400 mesh, Merck) and 60 F_{254} (Merck) with hexane containing increasing amounts of EtOAc as eluent. Spots were viewed by UV illumination at 254 and 366 nm.

Plant material. The seeds of *Millettia griffoniana* Baill were collected in November 2001 at Onguesse, in the central province of Cameroon. A voucher specimen (N°. 32315/SRF/HNC) identified by Mr Nana has been deposited at National Herbarium, Yaounde, Cameroon.

Extraction and isolation. The dried and powdered seeds of *M. griffoniana* (2 kg) were extracted with n-hexane (24 h, 3 x 20 L) at room temperature yielding 460 g of residue. A portion of this residue (200 g) was subjected to column chromatography on silica gel (600 g) and eluted with hexane containing increasing amounts of ethyl acetate. A total of 27 fractions of *ca* 500 mL each were collected and combined on the basis of their TLC analysis leading to two main series A and B. Fractions 3 to 4 eluted with hexane – EtOAc (95:5) gave series A (10 g). This series was chromatographed over silica gel (160 g). A total of 46 fractions of ca 200 mL each were collected. Fractions 27 – 46 eluted with hexane – EtOAc (80:20) afforded an oily residue, which was further purified by column chromatography over silica gel to give compounds **3** (5 g) and **1** (900 mg). Series B was chromatographed over silica gel (200 g). A total of 28 fractions of ca 200 mL each were collected. Combined fractions 18-24 was further purified by column chromatography to afford compounds **(2)** (500 mg) and **(4)** (600 mg).

7-Methoxyebenosin **(1)**

White powder from acetone, m.p: 129-130 °C. UV (MeOH): λ_{max} nm: 249 and 295. IR ν_{max} cm^{-1}: 2950, 1665, 1637, 1503, 1475, 1450, 1410, 1368, 1300, 1275, 1245, 1224, 983, 863, 810 and 725. ^{1}H and ^{13}C-NMR (see Table 1). EIMS: *m/z* (rel. int.): 351($[M+H]^+$, 100), 336 (41), 319 (22), 218 (44), 132(10), 187(15). Molecular formula $C_{22}H_{22}O_4$, (*m/z* 350.1023, M^+, calc. 350.1518).

Griffonianone E **(2)**

White powder, m.p.: 130-131°C from acetone. UV (MeOH): λ_{max} nm: 249 and 295. IR ν_{max} cm^{-1}: 2950, 1660, 1630, 1505, 1470, 1450, 1410, 1368, 1300, 1275, 1245, 1224, 983, 863, 810 and 724. ^{1}H and ^{13}C NMR (see Table 1). EIMS: *m/z* (rel. int.): 394 $[M]^+$, 100), 363 (26), 354(15), 218(22), 187 (18), 176(10). Molecular formula $C_{23}H_{22}O_6$, (m/z 394.2134, M^+, calc 394.1416).

Biological assays

Trypanocidal tests

Bloodstream form trypanosomes were cultured in modified IMDM with 20% horse serum at 37° [13, 14]. Drug studies were done in duplicate in 24 well plates (1ml/well) with final inhibitor concentrations of 0.1, 1, 10, 25, 100, and 125 µg/mL. Wells were inoculated with 10^5 trypanosomes and one half the volume of each well was changed daily. After 48 hours, the parasite number was determined in a model Z1 Coulter counter and IC_{50} values were calculated from semi-log plots.

Compounds were dissolved in 100% dimethylsulfoxide and diluted so that the solvent concentration was kept below the non-inhibitory concentration of 0.3%. Strains used were: *Trypanosoma brucei brucei* Lab 110 EATRO; and Kenya Trypanosomiasis Research Institute (KETRI) isolates *Trypanosoma brucei rhodesiense* 243 and 243 AS 10-3. The latter is a melarsen oxide and pentamidine resistant clone of a clinical isolate.

Anti-plasmodial tests

The *in vitro* anti-plasmodial assays were performed by using a modification of semi-automated microdilution technique [15]. Two *plasmodium falciparum* malaria parasite clones designated Indochina (W-2) and Sierra Leone (D-6) were utilised in susceptibility tests. The W-2 clone is

resistant to chloroquine, pyrimethanine, sulfadoxine, and quinine, while the D-6 clone is resistant to mefloquine. The tested calopogoniumisoflavone B(3), 7-methoxyebenosin (**1**), griffonianone E (**2**) and 7,2′-dimethoxy-4',5'-methylenedioxy isoflavone (**4**) were each dissolved in DMSO and serially diluted using malarial growth medium. Drug-induced reduction uptake of tritiated hypoxanthine was used as index of inhibition of parasite growth.

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