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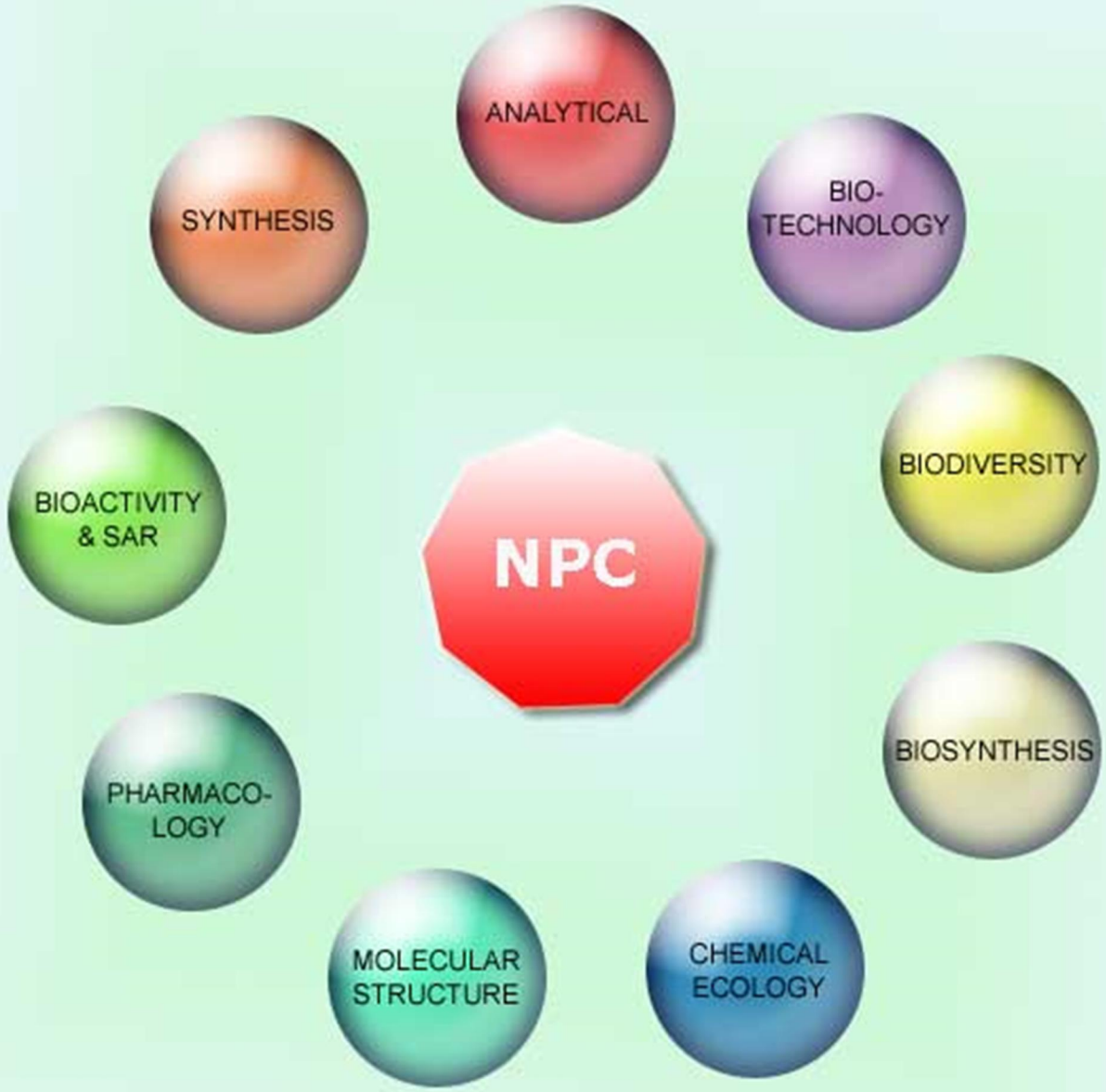

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New Flavonol Glycoside from the Leaves of *Ventilago africana*

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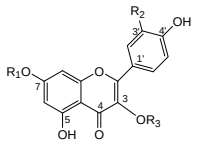
A new flavonol diglycoside, rhamnocitrin-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside, was isolated from the leaves of *Ventilago Africana*, in addition to 11 known flavonoids. Their structures were determined by spectroscopic methods including 1D- and 2D-NMR, and HR-ESI-MS analysis. The isolated compounds were evaluated for their antioxidant activity by using DPPH radical-scavenging assay. Compounds *4, 7-9* have discrete to good antioxidant potential with EC_{50} values ranging from 20.9 to 40.4 μM, compared with ascorbic acid (EC_{50} 60 μM) used as positive control.

Keywords: *Ventilago africana*, Rhamnaceae, Flavonoids; DPPH free radical scavenging activity.

Ventilago africana Exell is a liane with a thick, soft, woody stem belonging to the family Rhamnaceae [1]. It is distributed in evergreen forests, especially marshy sites and mangroves, from Guinea-Bissau to Nigeria, and across the Congo basin to Uganda and Angola [2]. *V. africana* has been used in traditional medicine for the treatment of *dysmenorrhea* and as a febrifuge [2]. Previous chemical studies of the genus *Ventilago* have shown the presence of a variety of anthraquinones, naphthoquinones, quinones, and benzisochromanquinones [3]. In the course of our search for interesting bioactive substances in nature, the 80% aqueous methanol extract of the leaves of *V. africana* was investigated. As a result, a new flavonol diglycoside was identified, together with eleven known flavonoids. Free radical scavenging activities of the isolated compounds are also described in this paper.

The *n*-BuOH-soluble fraction of the 80% MeOH extract of the leaves of *V. africana* was separated by a combination of chromatographic methods to obtain a new flavonol diglycoside **1**, in addition to eleven known compounds (**2-12**) (Figure 1). Compounds **2-12** were elucidated as kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**2**) [4], kaempferol-3-*O*-(2,6-di-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside (**3**) [5], kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (**4**) [6], rhamnocitrin-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**5**) [6], rhamnocitrin-3-*O*-(2,6-di-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside (**6**) [7], quercetin (**7**) [8], quercetin-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**8**) [9], quercetin-3-*O*-(2,6-di-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside (**9**) [9], rhamnetin-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**10**) [10], isorhamnetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**11**) [10], and rhamnazin-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**12**) [11]. To the best of our knowledge, this is the first report of flavonoids in the genus *Ventilago*.

Compound **1** was isolated as an amorphous, yellow powder, and its molecular formula was determined to be $C_{28}H_{32}O_{15}$ by HR-ESI-MS



	R_1	R_2	R_3
1:	CH_3	H	rha-(1→6)-gal-
2:	H	H	rha-(1→6)-glc-
3:	H	H	rha-(1→2)-[rha-(1→6)]-glc-
4:	H	H	rha-(1→6)-gal-
5:	CH_3	H	rha-(1→6)-glc-
6:	CH_3	H	rha-(1→2)-[rha-(1→6)]-glc-
7:	H	OH	H
8:	H	OH	rha-(1→6)-glc-
9:	H	OH	rha-(1→2)-[rha-(1→6)]-glc-
10:	CH_3	OH	rha-(1→6)-glc-
11:	H	OCH_3	rha-(1→6)-glc-
12:	CH_3	OCH_3	rha-(1→6)-glc-

Figure 1: Chemical structure of compounds **1-12**, isolated from *V. africana*.

from the ion at *m/z* 631.1636 $[M + Na]^+$ ($C_{28}H_{32}O_{15}Na$, calcd 631.1639). The UV spectrum revealed absorption bands at 266 and 346 nm, suggesting a flavonol skeleton. The ^1H and ^{13}C NMR spectra of **1** comprised resonances corresponding to aromatic and glycosidic protons and carbons, and an OCH_3 (δ_C 56.5; δ_H 3.92). Four aromatic doublets corresponded to H-6 [δ_H 6.38 (1H, J = 1.1Hz)], *meta*-coupled with H-8 [δ_H 6.65 (1H, J = 1.1 Hz)] on the A ring, and to H-2′, H-6′ [δ_H 8.14 (2H, J = 8.0 Hz)], *ortho*-coupled with H-3′,H-5′ [δ_H 6.92 (2H, J = 8.0 Hz)] on the B ring. Full identification of the aglycone was finally achieved by 2D-NMR spectroscopy. The HMBC correlation between δ_H 3.92 (OCH_3) and δc 167.5 (C-7) placed the OCH_3 at C-7 of the aglycone, which led to the rhamnocitrin structure (7-methoxy 3,5,4'-trihydroxy-flavone) [7]. Furthermore, two anomeric proton resonances corresponding to *O*-linked sugars were displayed in the ^1H NMR spectrum as two doublets at $\delta_{H\text{-}1''}$ 5.10 (d, J = 7.8 Hz) and $\delta_{H\text{-}1'''}$ 4.54, J = 1.2 Hz. Based on the results of acid hydrolysis of the flavonoids mixture, the magnitudes of their $J_{1,2}$ coupling constants and the analysis of 1D and 2D-NMR data, the sugar units were elucidated as β-D-galactopyranose ($\delta_{H\text{-}1''}$ 5.10; $\delta_{C\text{-}1''}$ 105.3) and α-L-rhamnopyranose ($\delta_{H\text{-}1'''}$ 4.54; $\delta_{C\text{-}1'''}$ 101.9) [12]. The β-D-gal was characterized by the

large coupling constants $J_{H-1,H-2}$ and $J_{H-2,H-3}$ (> 7.8 Hz) and the small coupling constant $J_{H-3,H-4}$ (3.3 Hz), whereas the α-L-rha was characterized by the small coupling constants $J_{H-1,H-2}$ (1.2 Hz) and its methyl group at δ_{H-6} 1.20 (d, J = 6.0 Hz) and δ_{C-6} 18.0. The glycosidic linkage was established by the HMBC correlations between $\delta_{H-1''}$ 5.10 and δ_C 135.8 (C-3) and between $\delta_{H-1'''}$ 4.54 and $\delta_{C-6''}$ 67.5, indicating that the galactose unit was located at C-3 of the aglycone and the rhamnose unit was attached to C-6 of galactose. Thus, **1** was characterized as rhamnocitrin-3-*O*-α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranoside.

In order to assess the antioxidative properties of **1-12**, the radical scavenging activity of these compounds on DPPH was measured. According to the results, summarized in Table 1, compounds *4, 7, 8, and 9* exhibited a good antioxidant potential (EC_{50} values ranging from 20.9 to 40.4 μM) compared with ascorbic acid, which was used as a positive control (EC_{50} 60 μM) (Table 1).

Compounds **7-10** were found to be active, which indicated that the di-OH substitution at 3′ and 4′ of the B-ring is particularly important to the antiradical activity of a flavonoid. Compounds **2** and **4** shared a common aglycone mono-OH substituted in the B-ring. The only difference was the nature of the disaccharide chain linked at C-3, rha-(1→6)-glc- (**2**) and rha-(1→6)-gal- (**4**), suggesting that the galactose might contribute to the antioxidant activity.

Table 1: DPPH radical scavenging activity of compounds **1-12**.

Compds	EC_{50} ± S.D. (μM)	Compds	EC_{50} ± S.D. (μM)
1	-[a]	**8**	40.2 ± 0.7
2	-[a]	**9**	36.5 ± 0.6
3	-[a]	**10**	65.5 ± 1.9
4	40.4 ± 0.9	**11**	-[a]
5	-[a]	**12**	-[a]
6	-[a]	**Ascorbic acid**[b]	60.2 ± 1.2
7	20.9 ± 0.5		

[a] 50% inhibition not achieved at the concentration of 200 μg/mL. [b] Used as a positive control.

Comparison of the antioxidant activity of compounds **1** with **4** and **8** with **10** indicated that the methoxy group at C-7 in **1** or **10** decreased activity. The free 3′-OH was also found to be important for antioxidant activity by comparison of the antioxidant activity of **8** (3′-OH) with **11** (3′-OCH_3). The same result was found by comparison of activity of **10** and **12**. Thus, the hindrance effect (methoxy groups at C-7 and/or C-3′), the nature of the saccharide chain linked at C-3, and the B ring OH substitution (4′-OH or 3′,4′-di-OH) in the flavonoid structures are determinant in the scavenging of free radicals.

Experimental

General: NMR, Bruker Avance DRX III 500; HR-ESI-MS, Micromass Q-TOF micro instrument; Optical rotations, Perkin-Elmer 341 polarimeter; UV, Shimadzu UV-2450 spectrophotometer in MeOH; TLC, pre-coated silica-gel 60 F_{254}; CC, Kieselgel 60 (63-200 mesh) or LiChroprep RP-18 (40-63 μm); Flash chromatography, Grace Reveleris system with dual UV and ELSD detection equipped with a 12g RP-C_{18} column. The mobile phase was water and methanol with a flow rate of 30 mL/min and the effluents were monitored at 205, 225, 250, and 360 nm. HPLC was performed on a Dionex apparatus equipped with an ASI-100 autosampler, an Ultimate 3000 pump, a diode array detector UVD 340S and a Chromeleon software. RP-C_{18} column (Phenomenex 250x15 mm, Luna 5μ) was used for semi preparative HPLC with a binary gradient eluent (H_2O (pH 2.4 with TFA); MeCN) and a flow rate of 6 mL/min; the chromatogram was monitored at 205, 225, 250, and 350 nm. Absorbance values in the DPPH free radical scavenging assay were read on a Fluostar omega microplate reader (BMG labtech).

Plant material: The leaves of *V. africana* were collected in Adiopodoumé, Ivory Coast, in January 2011. A voucher specimen (Aké-Assi-21254) has been deposited in the herbarium of the National Center of Floristics of FHB University of Cocody (Ivory Coast).

Extraction and isolation: The powdered dry leaves of *V. africana* (800 g) were percolated with 14 L of 80% MeOH. After methanol evaporation and concentration to 2 L, the aqueous residue was successively partitioned with light petroleum, CH_2Cl_2, and *n*-BuOH. The *n*-BuOH extract (43.8 g) was subjected to Diaion HP20 macroporous adsorbent resin CC using a MeOH-H_2O gradient (25, 50, 75, and 100% MeOH) to yield fractions I-IV, successively. Fraction II (9.7 g) was subjected to RP-C_{18} CC with a gradient system of MeOH-H_2O (25-45% MeOH) to afford 12 fractions (B_1-B_{12}). Further purification of B_4 (0.42 g) (eluted with MeOH-H_2O, 3:7) by RP-C_{18} chromatography using a MeOH-H_2O gradient (6:4-7:3) and then by semi-prep HPLC (14% MeCN) afforded compounds **9** (*rt* 10.9 min, 9 mg), **8** (*rt* 11.9 min, 10 mg), and **3** (*rt* 14.0 min, 19 mg). Further purification of B_7 (0.61 g) (eluted with MeOH-H_2O, 35:65) by RP-C_{18} chromatography using a MeOH-H_2O gradient (5:5-6:4) and then by semi-prep HPLC using a binary gradient (16-20% MeCN in 30 min) yielded compounds **4** (*rt* 16.5 min, 5 mg), **2** (*rt* 17.5 min, 26 mg), and **11** (*rt* 20.4 min, 4 mg). Fraction III (12 g) was subjected to silica gel VLC eluted successively with $CHCl_3$-MeOH-H_2O (95:5:0, 9:1:0, 85:15:0, 8:2:0, 75:25:0, 7:3:0.5) to yield fractions C_1-C_6. Fraction C_2 (0.28 g) was further separated by RP-C_{18} CC eluted with a gradient of MeOH:H_2O (4:6-8:2) to afford 16 mg of **13** and 33 mg of **7**. Fraction C_4 (0.84 g) was further purified by RP-C_{18} flash chromatography using a binary gradient (30-80% MeOH in 60 min) and then by semi-prep HPLC (27-32% MeCN in 50 min) to afford compound **5** (*rt* 12.5 min, 11 mg). Fraction C_6 (1.1 g) was further purified by RP-C_{18} VLC and then by semi-prep HPLC (24-26% MeCN in 50 min) to yield compounds **6** (*rt* 11.1 min, 12 mg) and **12** (*rt* 13.3 min, 8 mg) or isocratic elution with 14% MeCN to give compounds **10** (*rt* 13.8 min, 10 mg) and **1** (*rt* 21.3 min, 11 mg).

Rhamnocitrin-3-*O*-α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranoside (1)

$[\alpha]_D$: -27.2 (*c* 0.13, MeOH).

UV/Vis λ_{max} (MeOH) nm (Abs.): 266 (0.58), 348 (0.50).

^{1}H NMR (500 MHz, MeOH-d_4): 1.20 (1H, d, J = 6.0 Hz, H-6'''), 3.30 (1H, t, J = 9.4 Hz, H-4'''), 3.42 (1H, dd, J = 10.3-6.1 Hz, H-6''), 3.51 (1H, dd, J = 9.4-3.2 Hz, H-3'''), 3.54 (1H, m, H-5'''), 3.56 (1H, dd, J = 9.0-3.3 Hz, H-3''), 3.60 (1H, dd, J = 3.2-1.2 Hz, H-2'''), 3.64 (1H, brdt, J = 6.1 Hz, H-5''), 3.74 (1H, dd, J = 10.3-5.5 Hz, H-6''), 3.79 (1H, d, J = 3.2 Hz, H-4''), 3.82 (1H, dd, J = 9.0-7.8 Hz, H-2''), 3.92 (3H, s, 7-OMe), 4.54 (1H, d, J = 1.2 Hz, H-1'''), 5.10 (1H, d, J = 7.8 Hz, H-1''), 6.38 (1H, s, H-6), 6.65 (1H, s, H-8), 6.92 (2H, d, J = 8.0 Hz, H-3',5'), 8.14 (2H, d, J = 8.0 Hz, H-2',6').

^{13}C NMR (125 MHz MeOH-d_4): 18.0 (CH_3, C-6'''), 56.5 (7-OCH_3), 67.5 (CH_2, C-6''), 69.7 (CH, C-5'''), 70.2 (CH, C-4''), 72.1 (CH, C-2'''), 72.3 (CH, C-3'''), 73.0 (CH, C-2''), 73.9 (CH, C-4'''), 75.0 (CH, C-3''), 75.4 (CH, C-5''), 93.3 (CH, C-8), 99.2 (CH, C-6), 101.9 (CH, C-1'''), 105.3 (CH, C-1''), 106.5 (C, C-10), 116.2 (CH, C-3',5'), 122.6 (C, C-1'), 132.6 (CH, C-2',6'), 135.8 (C, C-3), 158.5 (C, C-9), 159.7 (C, C-2), 162.0 (C, C-4'), 163.8 (C, C-5), 167.5 (C, C-7), 179.8 (C, C-4).

HR-ESI-MS: *m/z* [M + Na^+] calcd for $C_{28}H_{32}O_{15}Na$: 631.1639; found: 631.1636.

Acid hydrolysis: Acid hydrolysis of the *n*-BuOH soluble fraction was realized as previously described [13]. Briefly, 1 g was refluxed with 50 mL of 2M HCl for 4 h. After extraction with ethyl acetate (3 x 30 mL), the aqueous layer was evaporated to furnish the monosaccharide residue (300 mg). Three sugars were identified as D-glucose, D-galactose and L-rhamnose by comparison with authentic samples on TLC and by measurement of the optical rotation of each purified sugar.

DPPH free radical scavenging assay: The scavenging activity of isolated compounds against DPPH was investigated by spectrophotometric methodology, as previously described [13]. Briefly, 5 µL of either the standard or sample solutions (dissolved in DMSO) was mixed with 95 µL of DPPH solution (158 µM, dissolved in absolute EtOH). After mixing gently and incubating for 30 min at 37°C, the optical density was measured at λ 515 nm. The percentage of absorbance inhibition at λ 515 nm was calculated using the following equation: % inhibition $[(Ab_{control} - Ab_{sample})/Ab_{control}] \times 100$. DPPH solution in EtOH was used as a control. The curve of the % scavenging activity against the concentration of sample was prepared by the MSExcel based program to obtain the EC_{50}. All the tests were conducted in triplicate. The experimental data were expressed as mean ± standard deviation.

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