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# New antioxidant flavonoids from the aerial parts of *Secamone afzelii*

Abdulmagid Alabdul Magid<sup>\*a</sup>, Philomène Akoua Yao-Kouassi<sup>b</sup>, Diane Patricia Apie Gossan<sup>ab</sup>, Claire Mairot<sup>a</sup>, Laurence Voutquenne-Nazabadioko<sup>a</sup>

<sup>a</sup>ICMR-UMR CNRS 7312, Groupe Isolement et Structure, Campus Sciences, Bât. 18, BP 1039, 51687 Reims Cedex 2, France

<sup>b</sup>Laboratoire de Chimie Organique et Substances Naturelles, 22 BP 582 Abidjan 22, Université Félix-Houphouët Boigny d'Abidjan-Cocody, Côte d'Ivoire

\*Corresponding author. Tel.: +33-3-26-91-82-08; fax: +33-3-26-91-35-96. E-mail address: [abdulmagid.alabdulmagid@univ-reims.fr](mailto:abdulmagid.alabdulmagid@univ-reims.fr) (A. Abdul Magid)

## Abstract

A bioassay-guided fractionation of petroleum ether, EtOAc and *n*-BuOH soluble parts of the 80% hydromethanol extract was performed to investigate the antioxidant activity of *Secamone afzelii* aerial parts using DPPH free radical scavenging assay. The results revealed that EtOAc and *n*-BuOH soluble parts have moderate to good DPPH radical scavenging activity (EC<sub>50</sub> = 139.3 and 30.5 µg/mL, respectively). Therefore, from the most active fractions of EtOAc and *n*-BuOH soluble parts were isolated two new flavonoid diglycosides quercetin-3-*O*-β-D-

apiofuranosyl-(1→2)-α-L-rhamnopyranoside and genkwanin-8-*C*-β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside in addition to nine known compounds (2-10). Their structures were elucidated on the basis of spectroscopic data including 1D- and 2D-NMR and ESI-MS. The ability of the isolated compounds to scavenge the DPPH was evaluated. The new compound **1**, quercitrin (**3**) and rutin (**6**) have antioxidant potential with EC<sub>50</sub> values ranging from 8.4 to 13.6 µg/mL, compared to the standard ascorbic acid (EC<sub>50</sub> 7.4 µg/mL).

**Keywords:** *Secamone afzelii*; Asclepiadaceae, Flavonoids; DPPH free radical scavenging activity.

## 1. Introduction

Antioxidants are compounds that inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reaction due to oxidative stress [1]. Antioxidants were known as free radical scavengers, reducing agents, chelators of pro-oxidant metals, or as quenchers of singlet oxygen. Described as an imbalance between free radicals and the body's ability to detoxify these molecules or repair the resulting damage, oxidative stress causes extensive damage to biological molecules such as DNA, lipids and proteins [2, 3]. Thus, it is the cause of several diseases including cancer, cataracts, amyotrophic lateral sclerosis, accelerated aging, the acute respiratory distress syndrome and pulmonary oedema [4, 5]. In order to provide a solution to this problem, we propose to contribute to the search for new antioxidants from indigenous natural sources [6-8]. In our continued search for new bioactive compounds from Ivory Coast medicinal plants [8], we investigated *Secamone afzelii* (Roem. & Schult.) K. Schum, a creeping woody climber belonging to the family Apocynaceae [9]. *S. afzelii* is used in traditional medicine for stomach problems, diabetes, colic, dysentery and also for kidney problems [10]. The decoction of the entire plant is prescribed for cough, catarrhal conditions and as galactagogue. For the treatment of gonorrhoea, the whole plant is crushed with fresh palm nuts and oil [9]. Previous studies have shown that *S. afzelli* has antioxidant, anti-inflammatory and antimicrobial properties [11-13]. A phytochemical screening of the methanol extract of the leaves and the stems of *S. afzelii*

showed the presence of high amount of flavonoids, but none, to the best of our knowledge, have reported their structural elucidation [14]. Lack of chemical scientific data about the flavonoids contents of *S. afzelii* and the antioxidant property of this plant, prompted us to make this study. A bioassay-guided fractionation of the hydromethanol extract using DPPH free radical scavenging assay was used to investigate the antioxidant activity and isolate flavonoids from the most active fractions.

## 2. Results and discussion

The 80% hydromethanol extract of the aerial parts of *S. afzelii* was concentrated and partitioned successively with petroleum ether (PE), ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH), followed by concentrating. The crude hydromethanol extract, the PE soluble part, EtOAc soluble part and *n*-BuOH soluble part were tested for their radical scavenging activity by DPPH assay. The results indicated that the *n*-BuOH soluble part has significant activity with IC<sub>50</sub> value of 30.5 µg/mL, whereas the hydromethanol extract and EtOAc soluble part exhibited a moderate activity with IC<sub>50</sub> value of 150.5 and 139.3 µg/mL, respectively. In order to isolate the compounds involved in this antioxidant action, a bioassay-guided fractionation strategy was applied throughout the separation procedure.

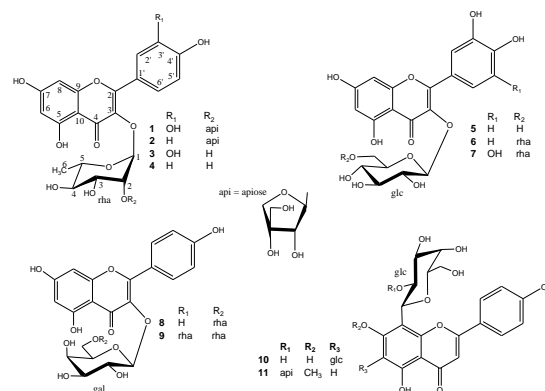
Four fractions (from B1 to B4) were obtained from the *n*-BuOH soluble part by vacuum liquid chromatography (VLC) over RP-18. The fraction B2 exhibited the highest DPPH radical scavenging activity with an EC<sub>50</sub> of 30.3 µg/mL (Table 1). Accordingly, fraction B2 was subjected

to column chromatography (CC) fractionation over silica gel and the resulting subfractions were tested for their DPPH radical scavenging activity. The active subfractions were further purified by semi-preparative HPLC affording two new compounds (**1** and **11**) in addition to kaempferol-3-*O*- $\beta$ -D-apiofuranosyl-(1 $\rightarrow$ 2)- $\alpha$ -L-rhamnopyranoside (**2**) [15], rutin (**6**) [16], myricetin 3-*O*- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside (**7**) [17], kaempferol-3-*O*- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-galactopyranoside (**8**) [18], mauritianin (**9**) [19], and vicenin-2 (**10**) [20] (Figure 1).

The EtOAc fraction was also fractionated by VLC over RP-18 affording fractions A1-A4. The most active fraction (A3) was subjected to VLC over silica gel and the active fractions were purified by semi-preparative HPLC affording quercitrin (**3**) [21], afzelin (**4**) [21], and isoquercitrin (**5**) [22] (Figure 1). The structural assignments of these compounds were made by HR-ESI-MS, 1D and 2D-NMR analysis.

Compound **1**,  $[\alpha]_D^{20}$  -50.2, was isolated as a yellow amorphous powder. The positive HR-ESI-MS showed a molecular ion peak at  $m/z$  603.1329  $[M + Na]^+$  (calcd for  $C_{26}H_{28}O_{15}Na$ , 603.1323) enabling us to determine the molecular formula  $C_{26}H_{28}O_{15}$ . The UV spectrum displayed maximum absorption bands of a flavonol skeleton at  $\lambda_{max}$  257 and 357 nm [23]. The  $^1H$  and  $^{13}C$  NMR spectra of **1** comprised resonances corresponding to aromatic and glycosidic protons and carbons. The A-ring of the flavonol was represented by two *meta*-coupled resonances at  $\delta_H$  6.23 (d,  $J = 2.1$  Hz,  $\delta_C$  99.8) and  $\delta_H$  6.39 (d,  $J = 2.1$  Hz,  $\delta_C$  94.8), assigned to H-6 and H-8, respectively (Table 2). The  $^1H$  and COSY NMR spectra of **1** exhibited in aglycone region an ABX system at  $\delta$  7.35 (1H, d,  $J = 2.1$  Hz, H-2'), 7.32 (1H, dd,  $J = 8.3$  and 2.1 Hz, H-6') and 6.95 (1H, d,  $J = 8.3$  Hz, H-5'), due to a 3',4'-disubstituted B-ring. Complete assignment of the remaining resonances of the aglycone in the  $^{13}C$  NMR spectrum of **1** was achieved by analysis of the HSQC and HMBC data, which confirmed the presence of quercetin (3,5,7,3',4'-pentahydroxy-flavone). A full list of the corresponding assignments is given in Table 2 [17]. The sugar part of **1** consisted of two residues as evidenced by  $^1H$  and  $^{13}C$  NMR spectra which displayed two anomeric protons at  $\delta_H$  5.43 ( $J = 1.6$  Hz) and 5.15 ( $J = 2.6$  Hz), whose carbon resonances were assigned by HSQC- $J_{mod}$  experiments at  $\delta_C$  102.1 and 112.2, respectively. The first sugar unit with its anomeric proton resonating at  $\delta_H$  5.43 was identified as  $\alpha$ -L-rhamnopyranose (rha), characterized by the small coupling constants  $J_{H-1,H-2}$  (1.6 Hz) and its methyl group at  $\delta_{H-6}$  1.10 (d,  $J = 6.2$  Hz) and  $\delta_{C-6}$  18.0 as summarized in Table 2. The second sugar residue showed NMR signals for two methines and two methylenes groups, in addition to a quaternary carbon, in agreement with an apiofuranose moiety [24]. The deshielded chemical shifts of the anomeric signals at  $\delta_H$  5.15 (d,  $J = 2.7$  Hz) and  $\delta_C$  112.1 and the HMBC correlations observed between anomeric proton H-1 with its C-3 and C-4 indicate that this sugar residue is a  $\beta$ -erythroapiofuranose (api). The

most commonly D- configuration for apiofuranose and L- for rhamnopyranose were assumed on the basis of the result of the acid hydrolysis of flavonoids mixture. A correlation between H-1-rha and  $\delta_C$  136.2 in the HMBC spectrum of **1** defined C-3 of quercetin as the site of *O*-glycosylation, whereas the correlations from H-1-api to the downfield-shifted C-2-rha ( $\delta_C$  79.4) indicated that the api residue was 2-*O*-linked to rha. Therefore, the structure of compound **1** was determined as quercetin-3-*O*- $\beta$ -D-apiofuranosyl-(1 $\rightarrow$ 2)- $\alpha$ -L-rhamnopyranoside.



**Fig.1.** Chemical structures of flavonoids **1-11**, isolated from *Secamone afzelii*.

Compound **11** was obtained as yellow powder and exhibited UV absorptions at 262 and 336 nm characteristic of a flavone [23]. The HR-ESI-MS spectrum displayed a molecular ion peak  $[M + Na]^+$  at  $m/z$  601.1528, (calcd for  $C_{27}H_{30}O_{14}Na$ , 601.1533) in agreement with a molecular formula of  $C_{27}H_{30}O_{14}$ . The  $^1H$  and  $^{13}C$  NMR spectra of **11** comprised resonances corresponding to aromatic and glycosidic protons and carbons, and a methoxy group ( $\delta_C$  57.0;  $\delta_H$  3.96). The  $^1H$  NMR spectrum of **11** (Table 2) indicated signals of two set of doublets at  $\delta$  8.03 (2H, d,  $J = 8.4$  Hz) and 6.98 (2H, d,  $J = 8.4$  Hz), due to the protons H-2', 6' and H-3', 5' of a 4'-hydroxyphenyl moiety in B-ring, and two singlets at  $\delta$  6.64 and 6.62, due to the protons at C-3 and C-6 in rings C and A of a flavone skeleton, respectively. Full identification of the aglycone was finally achieved by 2D-NMR spectroscopy. The HMBC correlation between  $\delta_H$  3.96 (OCH<sub>3</sub>) and  $\delta_C$  165.1 (C-7) placed the OCH<sub>3</sub> at C-7 of the aglycone, which led to the genkwanin structure (7-methoxy-3,5,4'-trihydroxyflavone) [25]. The HMBC cross peaks from H-3 ( $\delta_H$  6.64) to C-1' ( $\delta_C$  123.6), C-2 ( $\delta_C$  167.0), C-4 ( $\delta_C$  184.4), and C-10 ( $\delta_C$  106.0), and from H-6 ( $\delta_H$  6.62) to C-5 ( $\delta_C$  163.8), C-7 ( $\delta_C$  165.1), C-8 ( $\delta_C$  106.6), and C-10 confirmed that the two singlet protons were at C-3 and C-6, respectively. Two anomeric proton resonances corresponding to an *O*-linked and a *C*-linked sugars were present in the  $^1H$  NMR spectrum at  $\delta_H$  5.02 (1H, d,  $J = 9.2$  Hz,  $\delta_C$  73.5) and 5.14 (1H, br s,  $\delta_C$  111.1). Based on the results of the acid hydrolysis of flavonoids mixture, the chemical shift values, multiplicities and  $J$ -values, the magnitudes of their  $J_{1,2}$  coupling constants and the analysis of 2D NMR data, the two sugar residues were elucidated as a  $\beta$ -D-



HPLC with binary gradient eluent (H<sub>2</sub>O (pH 2.4 with TFA); MeCN) and a flow rate of 4 mL/min; the chromatogram was monitored at 205, 225, 250, and 350 nm. Absorbance (A) values in the DPPH free radical scavenging assay were read on a Fluostar omega microplate reader (BMG labtech). UV spectra were recorded on Shimadzu UV-2450 spectrophotometer in MeOH.

### 3.2. Plant material

The aerial parts of *S.afzelii* were collected from Cocody-Abidjan, Ivory Coast, in December 2010. The plant was identified by Pr. Laurent AKE-ASSI of FHB University and a voucher specimen (No Aké-Assi 21253) has been deposited in the herbarium of the National Center of Floristic of FHB University of Cocody (Ivory Coast).

### 3.3. Extraction and isolation

The powdered dry aerial part of *S. afzelii* (1 Kg) were macerated with 14 L of 80% aqueous MeOH and further refluxed for 3 h. After filtration, this extract was concentrated under reduced pressure using rotatory evaporator to 2 L and was successively extracted with petroleum ether (PE), ethyl acetate, and *n*-butanol (each 3 x 1 L). After evaporation of the solvents, 10.8 g of PE soluble part, 7.5 g of EtOAc soluble part and 18.7 g of *n*-BuOH soluble part were obtained.

The *n*-BuOH soluble part was subjected to VLC over RP-18 (9 x 5 cm) eluted successively with 25, 50, 75, and 100% MeOH in H<sub>2</sub>O, to give four fractions (B1-B4, respectively). Fraction B2 (2.9 g) was applied to a silica gel CC (2.5 x 20 cm) eluted with a gradient of CHCl<sub>3</sub>:MeOH (9:1→7:3) to afford 67 fractions, each 40 mL. Frs [41-43] eluted with CHCl<sub>3</sub>:MeOH (7:3) contain 36 mg of compound **9**. Frs [34-40] (120 mg), eluted with CHCl<sub>3</sub>:MeOH (75:25), were purified by semi-prep HPLC using a gradient of MeCN:H<sub>2</sub>O (from 13 to 30%; 22 min) to afford compounds **10** (*rt* 9.1 min, 9 mg), **11** (*rt* 10.2 min, 8 mg), **8** (*rt* 13.6 min, 10 mg), and **2** (*rt* 16.3 min, 24 mg). Frs [48-51] (90 mg), eluted with CHCl<sub>3</sub>:MeOH (7:3), were purified by semi-prep HPLC using a gradient of MeCN:H<sub>2</sub>O (from 10 to 25%; 20 min) to afford compounds **7** (*rt* 7.2 min, 7 mg), **1** (*rt* 8.9 min, 5 mg), and **6** (*rt* 9.3 min, 10 mg).

The EtOAc extract was subjected to VLC over RP-18 (9 x 5 cm) eluted successively with 25, 50, 75, and 100% MeOH in H<sub>2</sub>O, to give four fractions (A1-A4, respectively). Fraction A3 (1.8 g) was applied to a silica gel CC (2 x 16 cm) eluted with a gradient of CHCl<sub>3</sub>:MeOH (10:0→7:3) to afford 60 fractions, each 30 mL. Frs [20-25] (83 mg), eluted with CHCl<sub>3</sub>:MeOH (8:2) were purified by semi-prep HPLC using a gradient of MeCN:H<sub>2</sub>O (from 18 to 30%; 20 min) to yield compounds **3** (*rt* 7.5 min, 11 mg), **5** (*rt* 10 min, 8 mg), and **4** (*rt* 13.5 min, 9 mg).

### 3.4. Acid hydrolysis

A part of fractions A<sub>3</sub> and B<sub>2</sub> (200 mg each) was refluxed (90 °C) with 50 mL of 2M TFA for 4 h. After extraction with ethyl acetate (3 x 15 mL), the aqueous layer was evaporated to furnish the monosaccharides residue (200 mg). Four sugars were identified as apiose, glucose, galactose and rhamnose by comparison with authentic samples on TLC in MeCOEt:*iso*-PrOH:acetone:H<sub>2</sub>O (20:10:7:6) [29]. A part of the monosaccharides residue (50 mg) was subjected to a preparative TLC using the same solvent. The optical rotation of each purified sugar was measured to reveal L-rhamnose, D-glucose, D-galactose, and D-apsiose [29].

### 3.5. Quercetin-3-*O*-β-D-apiofuranosyl-(1→2)-α-L-rhamnopyranoside (**I**)

Yellow amorphous powder,  $[\alpha]^{20}_D$  -50.2 (*c* 0.1, MeOH); UV (MeOH)  $\lambda_{max}$  nm (log  $\epsilon$ ): 257 (4.34), 357 (4.25); <sup>1</sup>H and <sup>13</sup>C NMR (MeOH-*d*<sub>4</sub>) spectroscopic data, see Table 2; HR-ESI-MS *m/z*: 603.1329 [M + Na]<sup>+</sup> (calcd for C<sub>26</sub>H<sub>28</sub>O<sub>15</sub>Na, 603.1323).

### 3.6. Genkwanin-8-*C*-β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside (**II**)

Yellow amorphous powder,  $[\alpha]^{20}_D$  -45.6 (*c* 0.1, MeOH); UV (MeOH)  $\lambda_{max}$  nm (log  $\epsilon$ ): 272 (4.15), 332 (4.24); <sup>1</sup>H and <sup>13</sup>C NMR (MeOH-*d*<sub>4</sub>) spectroscopic data, see Table 2; HR-ESI-MS *m/z*: 601.1528 [M + Na]<sup>+</sup> (calcd for C<sub>27</sub>H<sub>30</sub>O<sub>14</sub>Na, 601.1533).

### 3.7. DPPH free radical scavenging assay

All tested compounds **1-11** showed on HPLC purity of more than 95%. The free radical scavenging activity of the extracts and isolated compounds against DPPH was investigated by spectrophotometric methodology, as previously described [8]. Briefly, 5 μL of 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  $\lambda$  515 nm using a Fluostar omega microplate reader (BMG labtech). The percentage of absorbance inhibition at  $\lambda$  515 nm was calculated using the following equation: % inhibition  $[(A_{control} - A_{sample})/A_{control}] \times 100$ . DPPH solution in EtOH was used as a control. The curve of the % scavenging activity against the concentration of sample was prepared by an MSEXcel based program to obtain the EC<sub>50</sub> (concentration required to obtain a 50% antioxidant effect). All the tests were conducted in triplicate. The experimental data were expressed as mean  $\pm$  standard deviation.

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