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The major phenolic compound of the roots and leaves of *Rafnia amplexicaulis* (Fabaceae), a liquorice substitute and traditional tea used in Cape Herbal Medicine

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ABSTRACT

The roots of *Rafnia amplexicaulis* have been used as a substitute for liquorice root and the leaves as a traditional medicinal tea in Cape (Khoi-San and Cape Dutch) Herbal Medicine. All published ethnobotanical data are presented, showing that two other species, *Rafnia acuminata* and *Rafnia angustifolia*, have also been used as a source of tea. The major compound of the roots and leaves of *Rafnia amplexicaulis*, responsible for the intense bittersweet taste, was isolated and identified as 8-glucopyranosyl-4,5,7-trihydroxyisoflavone (genistein 8-*C*-β-D-glucoside). The structure was determined on the basis of NMR and MS data, as well as X-ray crystallographic analysis. *R. amplexicaulis* and related species are a newly discovered source of this isoflavone (a compound with demonstrated antioxidant and radioprotective effects) that may be at least partly responsible for the traditional uses in treating asthma, influenza, back problems, infertility, catarrh and wasting. *Rafnia* species may therefore have potential value as new ingredients of herbal teas and functional foods.

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1. Introduction

Rafnia amplexicaulis (L.) Thunb. is a woody shrub of the legume family (Fabaceae, subfamily Papilionoideae) endemic to the Cape fynbos region of South Africa (Campbell, 1998; Campbell and Van Wyk, 2001). Vernacular names in Afrikaans include *soethoutbossie* ("liquorice bush" or literally "bush with sweet-tasting wood") and *veldtee* ("veld tea") (Smith, 1966). Tea made from leaves is used in Cape Herbal Medicine (Van Wyk, 2008) for treating asthma, influenza, back problems and infertility, while roots are traditionally used as a substitute for liquorice or taken in the form of a decoction to treat catarrh (nasal congestion with mucous) and phthisis (wasting of a body) (Pappe, 1847, 1868; Watt and Breyer-Brandwijk, 1962; Anonymous, 1998). The importance of this plant in traditional medicine is evident from the fact that it appears in full colour on the front cover of a publication (leaflet) on herbal remedies by the Montagu Museum (Anonymous, 1998).

Only one other species of Cape legumes of the tribe Crotalarieae (Boatwright et al., 2008) has been extensively researched in terms of its flavonoids, namely rooibos tea, *Aspalathus linearis* (Burm. f.) Dahlg. This commercial product and close relative of *Rafnia* is known to accumulate dihydrochalcones, of which aspalathin is the main phenolic compound (Joubert and De Beer, 2011). From a chemosystematic point of view, similar or structurally related compounds were therefore expected to occur in *Rafnia* species.

The aim of this paper is not only to summarise all recorded ethnobotanical uses of *Rafnia* species but also to make a first contribution to the phenolic chemistry of the genus. We present here the identity and structural elucidation of the main compound in the roots and leaves that is responsible for the bittersweet taste and perhaps also for the health benefits traditionally associated with these plants.

2. Materials and methods

2.1. Plant materials and extracts

Fresh leaf and root samples of *R. amplexicaulis* for large-scale extraction were collected on the farm Kleinplaas near Citrusdal, South Africa. The air-dried and finely ground materials were extracted for four hours in methanol and filtered. The dried extracts were dissolved in methanol for thin layer chromatography analysis or were dissolved in methanol/water (50:50, v/v) and filtered through a 55 μm C18 (100 mg/1 ml) solid phase extraction column (SPE, from Phenomenex) before HPLC analysis, before and after acid hydrolysis (in 4 N HCl at

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95 °C for 5 min, 15 min, 30 min and 60 min). All solvent systems below are given in v/v ratios.

2.2. Chromatography and other methods

Thin layer chromatography (TLC) was performed on pre-coated silica plates (Sil G-25 UV_{254}, 0.25 mm layer thickness, Macherey-Nagel). The solvent systems were ethyl acetate:methanol:water (100:16.5:13.5) (De Nysschen et al., 1998) and chloroform:acetic acid:methanol:water (64:32:12:8) (Wagner and Bladt, 1996). The plates were inspected under UV light and then sprayed with anisaldehyde–sulphuric acid reagent (Wagner and Bladt, 1996) and heated at 100 °C for 5 to 10 min. The main compound was isolated by column chromatography using silica gel 60 under gravitational force and ethyl acetate:methanol:water (100:16.5:13.5) as eluent. The yields of the main compound were 490 mg from 40.11 g of leaves (i.e. 12.2 mg per g dry weight) and 340 mg from 20.32 g of root bark (i.e. 16.7 mg per g dry weight).

High pressure liquid chromatography (HPLC) was done on a Beckman System Gold system with a programmable Solvent Module 126 and a Beckman Diode Array Detector Module 168, using a Luna 5 μm C18 (2) 100 Å column (250 × 4.6 mm). The dried sample extracts were dissolved in a methanol:water (50:50) solution and filtered through a 55 μm C18 (100 mg/1 ml) solid phase extraction column (SPE) before loading onto the HPLC. The flow rate was 1 ml/min and the absorbance changes were read at 275 nm (channel A) and 365 nm (channel B). The mobile phase was (A) 1% acetic acid in water and (B) methanol. The solvent system was a linear gradient from 30% B and 70% A to 60% B and 40% A over 24 min followed by a linear gradient to 100% B and 0% A over 1 min. This was held for 2 min, dropped to 0% B and 100% A over 2 min, held for 2 min and then back to the initial conditions (Campbell, 1998).

For liquid chromatography–mass spectrometry (LC–MS) analysis, the samples were reconstituted in 50% acetonitrile, 49% water and 1% formic acid in varying volumes to yield a final concentration of exactly 500 mg original dry leaf weight in 10 ml solvent. The samples were diluted a further 10 times and a 3 μl injection volume was used. The analyses were performed on a Waters (Milford, MA, USA) Synapt G2 quadrupole time of flight mass spectrometer coupled to a Waters Acquity ultra performance liquid chromatograph (UPLC) fitted with an Acquity photo diode array (PDA) detector. Separation was achieved on a Waters UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm particle size) with 0.1% formic acid as mobile phase A and acetonitrile as mobile phase B. The flow rate was 0.35 ml per min. The gradient that was employed started for the first 30 s at 100% solvent A followed by a concave gradient (curve 7 setting on Waters Masslynx software) to 100% B over the next 16.5 min. The column was washed for 1 min in 100% B, followed by re-equilibration to the starting conditions for 2 min. Electrospray ionization was applied in the positive mode at a capillary voltage of 2.5 kV, cone voltage of 25 V, desolvation temperature of 275 °C and desolvation gas setting of 650 L/h. The rest of the MS settings were optimized for best sensitivity. The instrument was calibrated with sodium formate and leucine enkaphelin was used as lock mass for accurate mass determinations. The MS acquisition method consisted of a low energy function at a trap voltage of 6 V and a high energy function where the trap collision energy was ramped from 15 to 60 V to generate fragmentation data (MS^E).

2.3. Pentacetylation of genistein 8-C-glucoside (*1*)

All the solvents used were freshly distilled. The reaction was monitored by TLC on aluminum-backed Merck silica gel $60F_{254}$ plates using an ascending technique. The plates were visualized by spraying with a 1:1 solution of 5% *p*-anisaldehyde in ethanol and 10% sulphuric acid in ethanol followed by baking at 150 °C. Gravity column chromatography was done on Merck silica gel 60 (70–230 mesh). Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded as deuteriochloroform solutions using tetramethylsilane as an internal standard on a Varian Gemini (300 MHz) spectrometer. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded on the same instrument at 75 MHz using tetramethylsilane as an internal standard. The chemical shifts are reported in ppm.

Genistein 8-*C*-glucoside (**1**). Mp 198–200 °C; $[\alpha]_D$ (c 0.1 g/ml, CH_3OH at 25 °C) +47; IR (neat cm^{-1}) 3285, 1650, 1549, 1513, 1367, 1316, 1259, 1202, 1175, 1068, 1010, 882, 836. The spectra (including the NMR spectrum) are provided as Supporting information.

Synthesis of (2*R*,3*R*,4*R*,5*S*,6*S*)-2-(acetoxymethyl)-6-(3-(4-acetoxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-8-yl)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (**2**): The major compound (**1**) which was isolated from the crude extract (1 g) was dispersed in pyridine (5 ml) and treated with acetic anhydride (2 ml) and a catalytic amount of 4-dimethylaminopyridine (DMAP). After stirring overnight at room temperature, the reaction was quenched by addition of water and the aqueous phase was extracted several times with ethyl acetate. The combined organic phases were then washed with ice-cold 1N aq HCl solution, followed by water and then dried over $MgSO_4$. After filtration, the solvent was evaporated in vacuo and the residue product purified by column chromatography on silica gel using a mixture of toluene and acetone (9:1) as eluent. The product was crystallized from a mixture of toluene and hexane to provide white crystals of the title compound (**2**). Mp 106–108 °C; $[\alpha]_D$ (*c* 0.1 g/ml, CH_3CN at 25 °C) +13; IR (neat cm^{-1}) 1737, 1647, 1570, 1509, 1355, 1315, 1195, 1015, 911, 881, 848, 679. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 13.00 (s, 1H, OH), 8.23 (bs, 1H, OH), 7.93 (s, 1H, Ar), 7.50 (d, J = 8.7 Hz, 2H, Ar), 7.14 (d, J = 8.7 Hz, 2H, Ar), 6.31 (s, 1H, H-2′), 5.57–5.16 (m, 4H, H-3, H-4, H-5 and H-6), 4.30 (dd, J = 3.8 and 12.5 Hz, 1H, $AcOCH_AH_B$-), 4.16 (dd, J = 2.4 and 12.5 Hz, 1H, $AcOCH_AH_B$-), 3.91 (ddd, J = 2.3, 3.8 and 9.6 Hz, 1H, H-2), 2.29 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.05 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.72 (s, 3H, OAc); ^{13}C{^{1}H} NMR ($CDCl_3$, 75 MHz) δ 180.2, 170.6, 170.3, 169.4, 168.8, 163.0, 162.6, 152.4, 150.8, 129.9, 127.8, 123.2, 121.8, 105.4, 99.5, 76.4, 73.6, 72.8, 70.2, 67.8, 61.6, 21.1, 20.6, 20.5 (x 2), 20.2.

2.4. Crystal structure report

A colourless, plate-like specimen of $C_{31}.5H_{30}O_{15}$, approximate dimensions 0.43 mm × 0.40 mm × 0.06 mm, was used for the X-ray crystallographic analysis. The X-ray intensity data were measured on a Bruker D8 Venture Photon. The frames were integrated with the Bruker SAINT software package using a narrow-frame algorithm. The integration of the data using a triclinic unit cell yielded a total of 13,533 reflections to a maximum angle of 25.50, of which 3309 were independent completeness = 99.8%, Rint = 2.46%, and 3086 were greater than 2σ. Data were corrected for absorption effects using the multi-scan method (SADABS). The calculated minimum and maximum transmission coefficients (based on crystal size) are 0.9939 and 0.9576. The structure was solved and refined using SHELXS and SHELXL Software Packages. The final anisotropic full-matrix least-squares refinement on F2 with 463 variables converged at R1 = 4.30%, for the observed data and wR2 = 12.12% for all data. The goodness-of-fit was 1.073. The largest peak in the final difference electron density synthesis was 0.451 e-/Å3 and the largest hole was 0.275 e-/Å3. The crystal structure has been deposited at the Cambridge Crystallographic Data Centre, with CCDC 1058513 as the assigned deposition number.

3. Results

3.1. Ethnobotanical data

Full details of all published ethnobotanical data on *Rafnia* species and their uses are presented in Table 1.

The leaves of three species are used to brew tea. Tea made from *R. amplexicaulis* leaves (Fig. 1) has been used for treating asthma,

Table 1
All published ethnobotanical data on *Rafnia* species.

Ethnobotanical uses	Reference
Rafnia acuminata (E. Mey.)	
A strong decoction or tea made from the leaves was used as a powerful diuretic and in the treatment of dropsies.	Pappe (1850, 1857, 1868)
The common name is *vascobossie*.	Kling (1923)
The common name is *soethoutbossie*.	Smith (1966)
Rafnia amplexicaulis (L.) Thunb.	
Soethoutbossie root has a sweet taste, whence the vernacular name which was first recorded by Drége (c.1828). The root was formerly used in decoctions like "Liquorice root". Also known as "veldtee".	Drége (c.1828) cited in Smith (1966)
Zoethoutboschje colonis. The colonial name is *Zoethoutboschje. Liquiritiam sapit, eique substitui solet*, translated has a liquorice taste and is used as a substitute (for liquorice).	Meyer (1836)
The roots taste like liquorice, whence its name of *Zoethout-boschje*. They are used in the form of decoction, as a demulcent in catarrh and phthisis, and are a good substitute for the liquorice itself.	Pappe (1847, 1850, 1857, 1868)
Pappe records that the root of *R. amplexicaulis* Thunb. (*Vascoa amplexicaulis* DC.), *Soethoutbossie*, tastes like liquorice, and that a decoction of it was taken as a demulcent in catarrh and phthisis.	Watt and Breyer-Brandwijk (1932, 1962)
Boesmantee/veldtee is used for asthma, influenza, back problems and for fertility.	Anonymous (1998)
A tannin-rich tea is brewed from various *Rafnia* species.	Van Wyk and Gericke (2000)
Rafnia angulata Thunb. subsp. *angulata*	
A dark black to brown tea, rich in tannins, can be brewed from *R. angulata*.	Van Wyk and Gericke (2000)

influenza, back problems and infertility. The roots (Fig. 1) were used as a substitute for liquorice or in the form of a decoction to treat catarrh (nasal congestion with mucous) and phthisis (wasting of the body). The tea made from *R. acuminata* leaves was used as a powerful diuretic and in the treatment of dropsies (oedema).

3.2. Major phenolic compound in leaves and roots

The major compound in the roots and leaves of *A. amplexicaulis* was identified as 8-glucopyranosyl-4,5,7-trihydroxyisoflavone (= genistein 8-*C*-β-D-glucoside) (**1** in Fig. 2) on the basis of NMR, MS and X-ray analysis. Small amounts of the isolated compound were tasted and gave a strong bittersweet taste, similar to tea made from the roots and leaves. Several minor isoflavonoids are also present in the leaves but rarely in more than trace amounts. Impure small amounts of a second compound were isolated from the roots and tentatively identified as a derivative of genistein 8-*C*-glucoside having an additional hydroxyl group in the aromatic ring. HPLC screening showed that genistein 8-*C*-β-D-glucoside is also a major compound in all other species of *Rafnia* studied (see HPLC results in Campbell (1998)). These included *R. angulata* Thunb. subsp. *angulata*, *R. triflora* Thunb., *R. amplexicaulis* and *R. schlechteriana* Schinz.

4. Discussion

R. amplexicaulis is not only a traditional herbal tea and liquorice substitute but is also used to treat a wide range of ailments including nasal congestion, influenza, asthma, back problems, wasting and infertility. Tea made from *R. acuminata* leaves is considered to have diuretic activity. This diversity of uses may indicate potential value as general tonic and/or adaptogen. The intense bittersweet, liquorice-like taste suggests the use of *Rafnia* leaves as a flavour masking agent in health tea blends and herbal teas.

Nothing is as yet known about the flavonoids of *Rafnia* species except for a preliminary HPLC survey (Campbell, 1998) that tentatively identified the main compounds (on the basis of the UV spectrum only) as isoflavones. Information on the chemical compounds in the genus *Rafnia* in general is limited to one report (Van Wyk and Verdoorn, 1989) of trace amounts of quinolizidine alkaloids in leaves. Apart from the large genus *Crotalaria*, there is as yet no published information on any phenolic compounds in *Rafnia* or any of the other 14 genera of the tribe Crotalarieae. A notable exception is rooibos tea – *A. linearis* (Burm. f.) Dahlg. – a well-known Cape herbal tea and close relative of *Rafnia*. This plant and its commercial products have been extensively researched in terms of phenolic constituents. Aspalathin, a dihydrochalcone, is the major phenolic compound in the leaves of the red type (Rocklands type) of *A. linearis* that is used for commercial production (Joubert and De Beer, 2011). It is curious that rooibos tea does not accumulate any isoflavones. Other phenolic compounds present in rooibos tea include nothofagin, a dihydrochalcone similar to aspalathin, flavones (orientin, isoorientin, vitexin, isovitexin, luteolin, chrysoeriol), flavanones (dihydro-orientin, dihydro-isoorientin, hemiphlorin) and flavonols (quercetin, hyperoside, isoquercitrin, rutin) (Joubert and De Beer, 2011).

Isoflavone *C*-glycosides are relatively rare although genistein- and orobol-8-*C*-glycosides have been extracted from the bark of *Dalbergia nitidula* Welw. ex Bak. (Van Heerden et al., 1980; Williams and Harborne, 1989). Genistein 8-*C*-glucoside has also been isolated from two other sources in the legume family: the roots and stems of *Pueraria lobata* (Willd.) Ohwi (Kinjo et al., 1987) and most parts of *Lupinus luteus* L. (Watanabe et al., 1993; Combined Chemical Dictionary, 2013). The known biological activities of orobol- and genistein 8-*C*-glycosides differ from those of isoflavone aglycones, which show activities analogous to

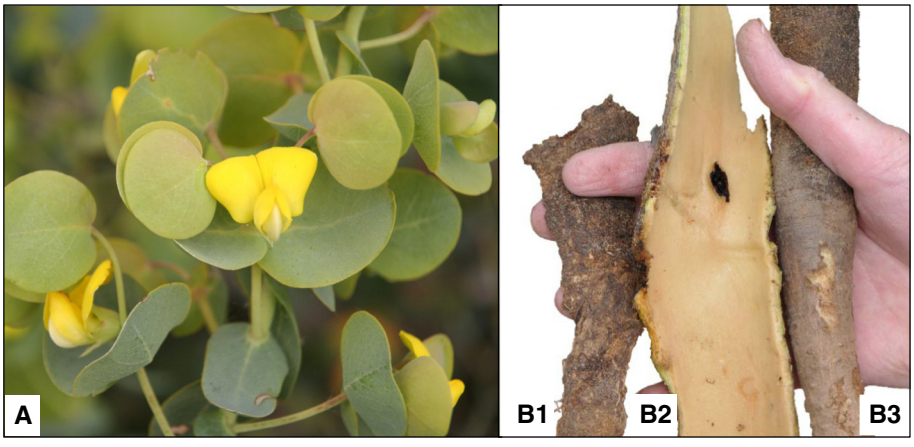

Fig. 1. *Rafnia amplexicaulis*: A, leaves and flowers; B1, root bark (outer surface); B2 root bark (inner surface); B3, root.

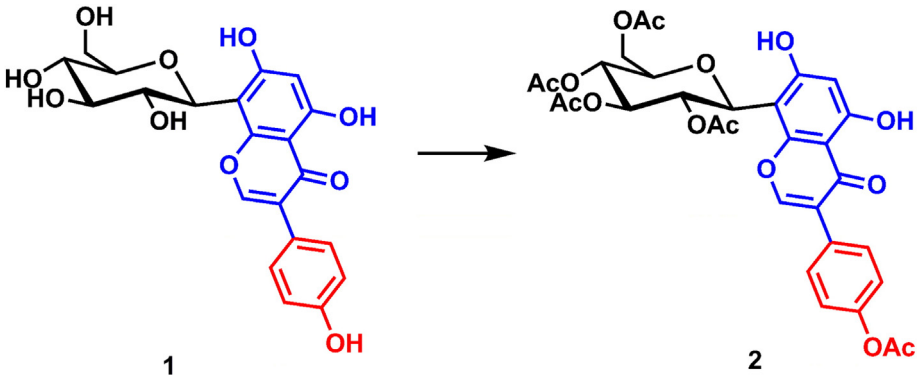

Fig. 2. Chemical structures of genistein 8-*C*-β-D-glucoside (**1**) and (2*R*,3*R*,4*R*,5*S*,6*S*)-2-(acetoxymethyl)-6-(3-(4-acetoxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-8-yl)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (**2**). Reagents and reaction conditions: acetic anhydride, 4-dimethylaminopyridine and pyridine, at room temperature, overnight.

estrogen, the female hormone (Sato et al., 2006). Genistein-8-*C*-glycoside inhibits HOCl-induced damage to human erythrocytes (Zavodnik et al., 2000) and was shown to have antioxidant activity as well as protective effects against gamma-irradiation in rats (Zavodnik, 2003). Antioxidant effects were confirmed in vitro (Rucinska et al., 2007; Rucinska and Gabryelak, 2009). These results suggest that *R. amplexicaulis* may be suitable for commercial development as a new health drink or phytomedicine with antioxidant effects and other potential benefits.

Attempts to establish the chemical structure of the major compound of the extract were made after the LC–MS spectrum indicated that the product was indeed constituted of one compound. However, the proton NMR spectrum of the compound could only indicate the presence of a 1,4 di-substituted aromatic ring with clarity while assignments of the other signals were difficult. Thus, the compound was acetylated to provide the pentacetylated derivative (**2**) in the hope of simplifying the assignment of the signals as shown in Fig. 2. Accordingly, the structure of **2** was established using NMR and X-ray crystal structure. Among others, the appearance of an unprotected (unacetylated) OH signal at δ_H 13.0 which is stabilized by a hydrogen bonding with the carbonyl oxygen as well as the resonance of two singlet signals at δ_H 7.93 and 6.31 suggest the presence of the isoflavone core. In agreement to the literature report (Sato et al., 2006), the appearance of signals at δ_c180.2, 163.0, 150.8, 105.4 and which correspond to the C-4, C-7, C-2, and C-8, respectively, confirmed the presence of the isoflavone core. However, due to the overlap of the anomeric proton signal of the sugar moiety with the other signals, the anomeric configuration of the aryl-*C*-glucoside bond could not be confirmed. Therefore, crystals of the product were grown from a toluene and hexane solvent system and the absolute structure and anomeric configuration were determined using X-ray crystallography (Fig. 3). By inference from the X-ray structure of product **2** in conjunction with the $[M + H]^+$ of 433.1124 ($C_{21}C_{21}O_{10}$) obtained from LC–MS data, which is consistent with the theoretical mass ($[M + H]^+ = 433.1135$, error -2.5 ppm), the absolute structure of the major compound proved to be genistein 8-*C*-glucoside (**1** in Fig. 2). The MS^E data (fragment ions: *m/z*: 415, 397, 379, 367, 337, 313, 283) show strong ions at *m/z* 313 and 283 which correspond to a neutral loss of 120 amu and 150 amu, indicating a cross-ring cleavage of a hexoside moiety which is indicative of the presence of a C-glycoside rather than a flavonoid *O*-glycoside (Abad-Garcia et al., 2009). The fragment ions also agree with that published by Rauter et al. (2005).

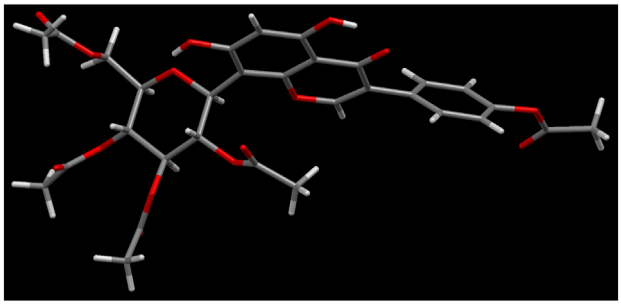

Fig. 3. The X-ray crystal structure of (2*R*,3*R*,4*R*,5*S*,6*S*)-2-(acetoxymethyl)-6-(3-(4-acetoxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-8-yl)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (**2**).

Collecting enough sample of the minor compound for structural elucidation proved to be difficult. The LC–MS spectrum of this minor compound, however, displayed a signal at 449.1091 which is 16 amu units higher than the 433.1135 $[M + H]^+$ of genistein 8-*C*-glucoside (**1**). The 16 amu difference indicates that the minor compound possesses one hydroxyl group more than the genistein 8-*C*-glucoside (**1**) does with an elemental composition of C21H21O11for the $[M + H]^+$. This molar mass difference coupled with the UV spectrum which confirmed the presence of an isoflavone moiety suggest that the minor compound is a derivative of genistein 8-*C*-glucoside (**1**) having an additional hydroxyl functional group at one of its aromatic rings. The MS^E data (fragment ions: *m/z*: 431, 413, 395, 383, 353, 329, 299, 283) show strong ions at *m/z* 329 and 299 which correspond to a neutral loss of 120 amu and 150 amu and correspond to the cross-ring cleavage of a hexoside moiety which is indicative of the presence of a *C*-glycoside rather than a flavonoid *O*-glycoside (Abad-Garcia et al., 2009). The fragment ions and accurate mass correspond to those of luteolin-6-*C*-glucoside (isoorientin) and luteolin-8-*C*-glucoside (orientin) in rooibos tea by Beelders et al. (2012); the retention time and the UV spectrum, however does not correspond to either of these compounds. The difference in UV indicates that this compound is possibly also an isoflavone.

5. Conclusions

The absolute structure of the major compound isolated from the roots and leaves of *R. amplexicaulis* is 8-glucopyranosyl-4,5,7-trihydroxyisoflavone (**1**). The structure was confirmed by the X-ray crystal structure of the pentacetylated derivative (**2**), which is the crystalline acetylated derivative of (**1**). The identification of genistein 8-*C*-glucoside as the main phenolic compound in *Rafnia* is a first report for the genus and its relatives. It is responsible for the intense bittersweet taste of the roots and their use as a liquorice substitute. A chemotaxonomic survey of isoflavonoids in the tribe Crotalarieae may therefore yield interesting results, especially since isoflavones are apparently not found in the related *A. linearis*. A detailed comparison between *Pueraria lobata* (a well-known traditional Chinese medicine) and *R. amplexicaulis* is another potentially valuable line of investigation that may lead to new and commercially relevant pharmacological data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.sajb.2015.05.014.

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