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Isolation and Identification of Steroid and Flavonoid Glycosides from the Flowers of *Allium gramineum*

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ABSTRACT
The isolation and identification of β-sitosterol 3-*O*-β-glucopyranoside, quercetin 3-*O*-β-glucopyranoside, isorhamnetin 3,4'-di-*O*-β-glucopyranoside and isorhamnetin 3,7-di-*O*-β-glucopyranoside from the flowers of *Allium gramineum* that grows in Georgia. The structures of isolated compounds have been determined by 1H and 13C nuclear magnetic resonance (NMR).

Keywords: *Allium gramineum*, Alliaceae, Steroid glycoside, Flavonoid glycoside, Structure determination..

INTRODUCTION

Over the past 10 years, researchers have focused increasing attention on extraction, identification, fractionation and isolation of phenolic compounds in plants. They have great abundance in our diet, and they have a probable role in the prevention of various diseases associated with oxidative stress, such as cancer as well as cardiovascular and neurodegenerative diseases. [1-4] Flavonoids represent a large group of phenolic compounds found in plants, these compounds demonstrable human health related benefits including antioxidant status, antiviral, anti-inflammatory and anti-cancer properties. [5-10]

Allium species are rich sources of steroid and flavonoid glycosides, among which quercetin and isorhamnetin glucosides are the major components. They are known for their antioxidant and free radical scavenging potencialities, their antiallergic, anti-inflammatory and cytotoxic activities. [11-14] A study using the prostate cancer cell line LNCaP (an androgen-dependent tumor) showed that β-sitosterol-3-O-β-D-glucoside decreased cancer cell growth by 24% and induced apoptosis (programmed cell death) fourfold, with little side effect. Also, β-sitosterol-3-O-β-D-glucoside characterized by antioxidant and free radical scavenging activities. [15-16]

Allium gramineum (family Alliaceae) is an indigenous Allium species from Caucasus and, along with other species, is widely used in Georgian traditional medicine as an antifungal, antiseptic and antibacterial

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remedy. [17] Phytochemical and biological studies of Allium species growing in Georgia have been previously reported and showed promising results. [18-21] This prompted use to continue in-depth the phytochemical study of the flowers of *Allium gramineum* growing in Georgia. This paper describes the isolation and identification of β-sitosterol 3-*O*- β-glucopyranoside, quercetin 3-*O*-β-glucopyranoside, isorhamnetin 3, 4'-di-*O*-β-glucopyranoside and isorhamnetin 3, 7-di-*O*-β-glucopyranoside from the flowers of *Allium gramineum*. The structures of isolated compounds described for the first time in *Allium gramineum,* were determined by 1H and 13C nuclear magnetic resonance (NMR).

MATERIALS AND METHODS

General experimental

For column chromatography, silica gel 60 (40-63μm, Merck) and Diaion HP-20 resin (Mitsubishi) were used. TLC analysis were performed on Silica gel 60 F254 plates (Merck) and eluted with CH_2Cl_2- MeOH-H_2O (55:12:1, v/v/v). Spots were detected by spraying the plates with vanillin-sulphuric acid (in EtOH) reagent, followed by heating at 110°C. Preparative HPLC was a Varian PrepStar 218 coupled with a DAD (diode-array detector) detector set at 350 nm and equipped with a fraction collector (440LC) on a C-18 column, using a binary solvent system with a flow rate of 30 mL/min: solvent A: trifluoroacetic acid 0.05% in water and solvent B: acetonitrile. The Purity of isolated compounds was estimated on Agilent 1100 HPLC with DAD (diode-array detector) detection (working wavelength : 350 nm) using Hypersil ODS (C-18) columns (58μm, 4.6 × 250 mm) with the same binary solvent system as described above, injection volume 10μL with a flow rate of 1 mL/min. The isolated compounds were identified by NMR. 1H and 13C NMR spectra were recorded on a Bruker Avance DRX 500 instrument equipped with a cryoprobe. 1H and 13C NMR chemical shifts in ppm were referenced to the residual solvent (DMSO) signals or to TMS as internal standard (1H at 500 MHz and 13C at 125 MHz). 2D experiments were performed using standard Bruker microprograms.

Plant material

The whole plants of *Allium gramineum* were collected in the Didgori region of Georgia in June 2014, and identified by Dr. J. Aneli (Iovel Kutateladze Institute of Farmacochemistry, Tbilisi State Medical University). A voucher specimen (Flowers #133, 134 TBPN) was deposited at the herbarium of Iovel Kutateladze Institute of Farmacochemistry, Tbilisi State Medical University.

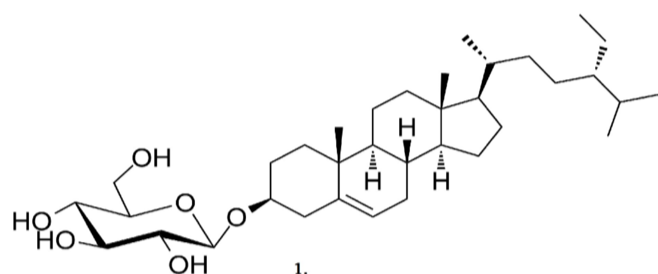
Extraction and isolation

Dried and powdered flowers of *Allium gramineum* (100 g) were extracted twice with hot EtOH-H_2O (8:2 v/v 3 x 500 ml) to give the total extract (Ext.-1) After evaporation of the solvent, the residue Ext.-1 (30.8 g) was chromatographied over Diaion HP-20, using H_2O-MeOH as eluent in gradient conditions (100:0; 50:50; 0:100 v/v each 2 L) and finally EtOAc (1 L) to give four enriched fractions (F-1; F-2; F-3; F-4). After removing the solvents, the fraction F-1 (1.3 g) which was collected in H_2O 100% was subjected to silica gel CC and eluted with CH_2Cl_2-MeOH–H_2O (55:12:1, v/v/v) to give: compound 1 (23 mg). The fraction F-2 (1.7 g) which was collected in H_2O-MeOH 50% was purified by Preparative HPLC coupled with a DAD detector set at 350 nm, using a binary solvent system: A: trifluoroacetic acid 0.05% in water and solvent B: acetonitrile (0 to 1 min 0% B, 100%A; 1 to 45 min 3%B, 97%A; 45 to 55 min 40%B, 60%A, 55 to 56 min 60%B, 40%A, 56 to 66 min 60%B, 40%A, 66 to 67 min 0%B, 100%A and 67 to 82 min 0%B, 100%A) to give: compound 2 (21 mg) compound 3 (16 mg) compound 4 (14 mg).

RESULTS AND DISCUSSION

A 80% EtOH extract of the flowers of *Allium gramineum* was subjected to successive fractionations to provide one steroid and flavonoid glycosides. Comparison of the ^{1}H- and ^{13}C-NMR spectral data with those reported in the literature led to the identification of the structures of compounds 1, 2, 3, 4 (Figure 1), described for the first time in *Allium gramineum.*

Compound 1 was identified as β-sitosterol 3-*O*-β-glucopyranoside by comparison with literature. [22] Compound 2 was obtained as an amorphous yellow powder. The APT spectrum indicated the presence of one methylene, ten methines and ten quaternary carbons, including one carbonyle.



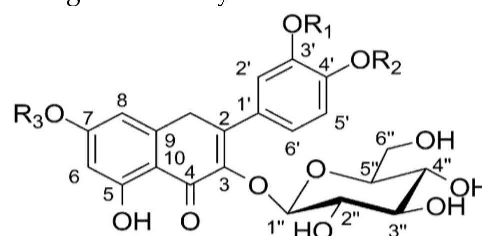


Fig. 1: Chemical structures of compound 1, 2, 3, 4.

The COSY spectrum indicated that aromatic methines H-5' and H-6' were vicinal. The HMBC spectrum suggested that the aromatic ring was completed with oxygenated C-4' and H-2' in *meta* of the doublet doublet H-6'; and C-1' and oxygenated C-3' in *meta* of the doublet H-5'. Proton H-6' also exhibited a HMBC correlation with the downshifted C-2, suggesting it as the attachment point of the aromatic ring. Furthermore, another aromatic system was determined from *meta* coupling doublets H-6 and H-8. Both protons showed HMBC correlations with oxygenated C-7, and quaternary C-10, while H-6 correlated with oxygenated C-5 and H-8 with oxygenated C-9. These information, along with the presence of a relatively upshifted ketone, suggested a flavonol moiety with oxygenated groups in positions 3, 5, 7, 3' and 4', corresponding to a quercetin scaffold. Of these positions, only C-3 showed a HMBC correlation with H-1'', the others being free phenol groups. Along with the downshifted H-1'' signal in the anomeric region, a hexose moiety was inferred from the presence of the methylene and four remaining methines in the glycosidic region of the 1D NMR spectra. Proton H-1'' showed a HMBC correlation with C-5'', which suggested a pyranosyl conformation. Comparison with literature confirmed that 2 was quercetin 3-*O*-β-glucopyranoside, also known as isoquercetin. The large coupling constant of 7.6 Hz for proton H-1'' confirmed that the sugar moiety was the β anomer of glucose. [23]

Compound 3, isolated as an amorphous yellow powder, had an APT spectrum similar to that of 2, to the exception of a downshift of C-3' and the presence of one methyl, one more methylene and five additional methines. The methyl group chemical shift corresponded to a methoxy group, whose protons showed a HMBC correlation with C-3', indicating that the flavone was an isorhamnetin scaffold. The other additional signals were attributed to a second hexose moiety from their chemical shifts. As in 2, the anomeric proton H-1'' correlated with C-3, while the other anomeric signal H-1''' correlated with C-4'. Both H-1'' and H-1''' correlated with their 5 counterpart, indicating pyranoses, and exhibited a large coupling constant corresponding to their β anomers. [23] Furthermore, the chemical shifts of both hexoses were closely similar to those of the glucosyl of 2, suggesting that both were glucose residues. Thus, 3 were identified as isorhamnetin 3, 4'-di-*O*-β-glucopyranoside. Since no ^{13}C NMR reference could be found for this compound, data were compared with the spectrum of the equivalent quercetin diglucoside. [24] The two molecules showed a very similar NMR behavior except for positions 2', 3' and 4', which are slightly downshifted in compound 3 owing to the 3' methoxyl.

Compound 4, obtained as an amorphous yellow powder, showed an APT spectrum closely resembling that of 3. Examination of the HMBC correlations indicated that C-3 was again substituted with a glucoside, but that the second hexose moiety was in position 7 of the flavone core. Both anomeric protons again exhibited a large coupling constant. Thus, the structure of 4 was determined as being isorhamnetin 3, 7-di-*O*-β-glucopyranoside. In absence of retrievable reference for the ^{13}C NMR spectrum of this flavonoid, the molecule was compared to its analogue quercetin 3, 7-di-*O*-β-glucopyranoside [25], with good agreement except for C-2′ and C-3′, near the methoxy substituent.

Interestingly, compounds 2 and 3 have already been reported in *Allium cepa* and *A. ascalonicum*. [26-27]

Isorhamnetin 3, 4'-O-β-diglucopyranoside (3). ^1H NMR (500 MHz, DMSO-*d6*) δ: 6.22 (d, *J* = 2.1, H-6), 6.47 (d, *J* = 2.1, H-8), 7.97 (d, *J* = 2.0, H-2'), 7.23 (d, *J* = 8.9, H-5'), 7.54 (dd, *J* = 8.9, 2.0, H-6'), 5.58 (d, *J* = 7.3, H-1''), 3.21 (m, H-2''), 3.10 (m, H-3'', H-4''), 3.23 (m, H-5''), 3.59 (d, *J* = 11.7, H-6''a), 3.39 (dd, *J* = 11.7, 5.7, H-6''b), 5.06 (d, *J* = 7.6, H-1'''), 3.29 (m, H-2''', H-5'''), 3.36 (m, H-3'''), 3.18 (m, H-4''') 3.67 (d, *J* = 11.7, H-6'''a), 3.46 (dd, *J* = 11.7, 5.5, H-6'''b), 3.84 (s, 3'-OCH_3); ^{13}C NMR (125 MHz, DMSO-*d6*; APT) δ: 155.7 (C-2), 133.4 (C-3), 177.5 (C-4), 161.2 (C-5), 98.8 (C-6), 164.5 (C-7), 93.8 (C-8), 156.5 (C-9), 104.1 (C-10), 123.6 (C-1'), 113.5 (C-2'), 148.1 (C-3'), 148.5 (C-4'), 114.5 (C-5'), 121.4 (C-6'), 100.7 (C-1''), 74.3 (C-2''), 77.5 (C-3''), 69.8 (C-4''), 76.5 (C-5''), 60.6 (C-6''), 99.5 (C-1'''), 73.1 (C-2'''), 77.1 (C-3'''), 69.6 (C-4'''), 76.8 (C-5'''), 60.6 (C-6'''), 55.7 (3′-OCH_3).

Isorhamnetin 3, 7-O-β-diglucopyranoside (4). ^1H NMR (500 MHz, DMSO-*d6*) δ: 6.45 (d, *J* = 2.1, H-6), 6.81 (d, *J* = 2.1, H-8), 7.95 (d, *J* = 2.0, H-2'), 6.93 (d, *J* = 8.5, H-5'), 7.53 (d, *J* = 8.5, 2.0, H-6'), 5.58 (d, *J* = 7.3, H-1''), 3.23 (m, H-2''), 3.10 (m, H-3'', H-4''), 3.27 (m, H-5'', H-2''', H-5'''), 3.58 (d, *J* = 11.2, H-6''a), 3.38 (dd, *J* = 11.2, 4.3, H-6''b), 5.08 (d, *J* = 7.3, H-1'''), 3.44 (m, H-3''', H-6'''b), 3.15 (m, H-4'''), 3.69 (d, *J* = 9.5, H-6'''a), 3.84 (s, 3'-OCH_3); ^{13}C NMR (125 MHz, DMSO-*d6*; APT) δ: 156.9 (C-2), 133.3 (C-3), 177.6 (C-4), 160.9 (C-5), 99.4 (C-6), 162.9 (C-7), 94.6 (C-8), 156.0 (C-9), 105.7 (C-10), 120.9 (C-1'), 113.5 (C-2'), 146.9 (C-3'), 149.6 (C-4'), 115.2 (C-5'), 122.2 (C-6'), 100.7 (C-1''), 74.3 (C-2''), 77.5 (C-3''), 69.8 (C-4''), 76.5 (C-5''), 60.6 (C-6''), 99.7 (C-1'''), 73.1 (C-2'''), 77.2 (C-3'''), 69.6 (C-4'''), 76.4 (C-5'''), 60.6 (C-6'''), 55.7 (3'-OCH_3).

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