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Isolation and Quantification of Flavonoids from *Allium saxatile Bieb.* Growing in Georgia

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ABSTRACT

Background: This study investigated the phytochemical composition of *Allium saxatile Bieb.*, a plant species belonging to the Alliaceae family.
Objectives: The objectives of this research were to investigate the phytochemical composition of *Allium saxatile* isolate and identify the chemical structure of the active constituents.
Methods: The aerial parts of the plant were extracted using 80% ethanol and then fractionated using Diaion HP-20 column chromatography to obtain four enriched fractions from the plant. Previous research has demonstrated that the 50%-methanolic fraction exhibits antioxidant activity, and this biologically active fraction was subsequently purified using column chromatography. The chemical structure of the compounds was determined using spectroscopic techniques, including 1D and 2D NMR spectroscopy and mass spectrometry.
Results: The investigation led to the isolation of two flavonoids, namely kaempferol-3-O-glucoside (Astragalin) and kaempferol 3-O-neohesperidoside, from the 50%-methanolic fraction of *A. saxatile*. This is the first report of the isolation and identification of astragalin and kaempferol 3-O-neohesperidoside from A. saxatile.
Conclusions: Two flavonoids, astragalin and kaempferol 3-O-neohesperidoside, have been isolated from *A. saxatile* for the first time in Georgia, contributing to the phytochemical profile of this species. Additionally, a sensitive and reproducible HPLC method has been developed and validated for the simultaneous quantification of astragalin in the crude extract of *A. saxatile*.
Keywords: Alliaceae; *Allium saxatile*; Flavonoids; Kaempferol 3-O-glucoside; Kaempferol 3-O-neohesperidoside; quantification.

INTRODUCTION

Genus *Allium L.*, consisting of various species, has been cultivated worldwide for centuries. In Georgia, 38 species of Allium are recognized, with five species being endemic to the Caucasus region and three endemic to Georgia itself.[1] This genus is known to produce secondary metabolites such as phenolic acids and their derivatives, flavonoids, and flavonoid polymers, which have been linked to various health benefits, such as free radical scavenging activity, cardiovascular disease prevention, anti-inflammatory, antimicrobial, etc.[2] The different species of Allium have demonstrated pharmacological activity, including cytotoxicity, antioxidant, antibacterial, anti-inflammatory, and other properties.[3–6] In Georgian traditional medicine, Allium species are widely used as antifungal, antiseptic, and antibacterial remedies.[7,8] Antioxidant activity has been found in several species of the genus, including *A. cepa, A. sativum, A. schoenoprasum*, and others.[9] Recent research has identified *A. saxatile Bieb*. as another species with antioxidant activity, with IC50 of 40.13 µg/mL.[10]

Our study reports the isolation of two previously unreported flavonoids from *A. saxatile* and the development of an analytical method for their quantification in the crude extract.

METHODS

Chemicals and reagents

The following adsorbents were used for purification: Diaion-HP20 (Mitsubishi, Japan) and silica gel 60 (0.04-0.063 mm) (Merck KGaA, Darmstadt, Germany). For the mobile phase, Dichloromethane, methanol, and ethyl acetate were obtained from VWR Chemicals (France).

Plant material

The aerial parts of *Allium saxatile Bieb*. were collected in Javakheti, the region of Georgia. The plant was collected and identified by Dr. Tsiala Gviniashvili of the Institute of Botany at Ilia State University. The voucher specimen had been deposited at the same institute (# TBI1034088).

Extraction and fractionation

The entire plant was dried and milled into particles of 1 mm. The powdered plant was extracted with 80% EtOH using an ultrasonic water bath at 50°C. The extract was dried with a rotary evaporator.

The dried extract of the plant was subjected to Diaion HP-20 column chromatography. The mobile phase was H_2O-MeOH

in gradient conditions and EtOAc to give four enriched fractions: *A. saxatile* F1, *A. saxatile* F2, *A. saxatile* F3, and *A. saxatile* F4.

Isolation

Isolation of compounds was carried out using column chromatography. The stationary phase was silica gel 60 (0.04-0.063 mm) in a dichloromethane/MeOH/water (24:14:3 V/V/V) system.

Nuclear Magnetic Resonance (NMR)

The ^{1}H and ^{13}C NMR spectra of the obtained compounds were recorded using a Bruker AVANCE NEO 500 MHz NMR spectrometer equipped with a cryoprobe, where deuterated methanol was used as the deuterated solvent.

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Mass spectrometry

Samples for mass spectrometry were prepared in acetonitrile at a concentration of 100 µg/mL. The experiments were performed on an Acquity ultra-high-performance liquid chromatography (Waters) system equipped with an ACQUITY UPLC BEH C18 column (2.1 mm x 50 mm, 1.7 µm particle size; Waters). The UPLC is coupled to a Synapt XS HDMS Q-TOF mass spectrometer with an ESI source (Waters). The mobile phase consisted of a mixture of water and acetonitrile, each containing 0.1% formic acid. The LC was performed at a flow rate of 0.4 mL/min, with the following gradient conditions: 5% acetonitrile from 0 to 1 minute, 5–100% acetonitrile from 1 to 28 minutes, and 100% acetonitrile from 28 to 30 minutes. The samples were analyzed using a UPLC-HDMSe workflow with a mass range from 50 to 2000 Da and a collision energy ramp from 20 to 70 V. Data analysis was performed using MassLynx V4.2.

Chromatographic instrument and conditions

An Agilent Technologies Model 1260 Infinity liquid chromatography system was used for HPLC analyses, which was equipped with a vacuum degasser, a binary pump, an autosampler, and a photodiode array detector (DAD). The system was operated using ChemStation software. The separation was achieved using an Eclipse plus C18 (4.6 x 250 mm; 5 µm) column, with a mobile phase composed of water and acetonitrile (77:23, v/v) at a flow rate of 0.8 mL/min. The injection volume was 10 µL, and all separations were performed at 25°C. UV spectra were recorded in the detection range of 200-400 nm for all peaks, with quantification carried out at a single wavelength of 320 nm for astragalin.

Preparation of standard solution

The standard solution of astragalin was prepared in 50% methanol to a final concentration of 1.0 mg/mL. A series of working solutions of astragalin (n=5) was ready to obtain various concentration levels (0.06-1.0 mg/mL). The appropriate volume of astragalin solution was transferred into a 10.0 mL volumetric flask, and the volume was adjusted to 10.0 mL with the mobile phase. All prepared standard solutions were filtered through a 0.45 µm membrane filter (Millipore, ref HVPL04700) before HPLC analyses.

Preparation of sample solution

10.0 mg of the crude extract of *A. saxatile* was weighed and dissolved in 50% methanol to make a final volume of 100.0 mL in a 100 mL volumetric flask. Then, 2 mL of this solution was filtered through a 0.45 µm Millipore filter into an HPLC vial.

Validation and assay

The HPLC method developed for analyzing the crude extract of *A. saxatile* was validated in accordance with the ICH guidelines. The linearity of the method was determined for astragalin using five different concentrations, and calibration curves were constructed. The concentration range was 0.06 to 1.0 mg/mL for astragalin. Calibration curves were constructed by plotting peak areas against concentrations, and the linearity was assessed using least squares regression to calculate the slope, y-intercept, and coefficient of correlation (R2). The limits of detection and quantification were determined based on signal-to-noise ratios of 3:1 and 10:1, respectively. The precision of the method was evaluated for both intra-day and inter-day variability, and the accuracy of the method was assessed using a recovery test. Intra-day precision was calculated from the analysis of six sample solutions prepared independently on the same day. Inter-day precision was evaluated by repeating the same procedure on three other days; the standard deviation and the RSD (Relative Standard Deviation) values were calculated for each day. The recovery test is a method used to evaluate the accuracy of an analytical method. In this case, known quantities of astragalin standard solutions were added to the real samples (crude extract of *A. saxatile*) at three different concentration levels: 50%, 100%, and 150%. The spiked samples were then analyzed in triplicate

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using the HPLC method previously described. The recovery was calculated as the ratio of the measured concentration of the spiked sample to the expected concentration, expressed as a percentage of the predicted concentration.

RESULTS

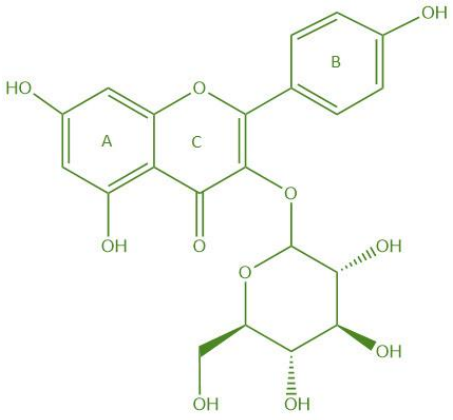
Isolation of compounds

500 g of aerial part of *A. saxatile* was extracted with 80% ethanol, and the yield of crude extract was 35.7 g for *A. saxatile*, resulting in 7.5% of plant material. The obtained crude extract was subjected to fractionation using column chromatography. Open-column chromatography was utilized for the fractionation of the crude extract, employing Diaion HP-20 (Mitsubishi, Japan) as the stationary phase. The mobile phase consisted of water-methanol mixtures (100:0, 50:50, 0:100) and 100% ethyl acetate. The biological activities of the obtained fractions were evaluated, and research has shown that, among the obtained fractions, *A. saxatile* F2, obtained with 50% MeOH, exhibited antioxidant activity with an IC_{50} value of 2.93 µg/mL.[10] This fraction was purified with column chromatography (Stationary phase: Silica gel 60 (0,04-0,063 mm), Mobile phase: Chloroform-methanol-water _60:10:1 V/V/V; 50:10:1 V/V/V; 45:12:1 V/V/V; 40:12:1 V/V/V) to obtain compound 1 (16 mg) and compound 2 (11 mg).

Characterization of the isolated compounds

Compound 1 was isolated as a yellow, amorphous powder with a molecular weight of 447.0992 m/z [M-H]-, corresponding to the molecular formula C21H20O11 (Fig. 1).

FIGURE 1. Chemical structure of compound 1



^{1}H NMR (500 MHz, CD_3OD, δ, ppm, J/Hz): 8.06 (2H, d, J = A_2B_2, H-2′,6′), 6.89 (2H, d, J = A_2B_2, H-3′,5′), 6.41 (1H, s, H-8), 6.21 (1H, s, H-6), 5.25 (1H, d, J = 7.5, H-1″), 3.71 (2H, m, CH_2), 3.44 (1H, m, H-2″), 3.43 (1H, m, H-3″), 3.31 (1H, m, H-4″), 3.2 (1H, m, H-5″), 3.79 (1H, m, H-6″).

^{13}C NMR (125 MHz, CD_3OD, δ, ppm): 157.7 (C-2), 134.4 (C-3), 178.15 (C-4), 161.7 (C-5), 98.48 (C-6), 164.6 (C-7), 93.34 (C-8), 157.12 (C-9), 104.35 (C-10), 121.39 (C-1′), 130.88 (C-2′,6′), 114.68 (C-3′,5′), 160.18 (C-4′), 102.63 (C-1″), 74.33 (C-2″), 76.63 (C-3″), 69.95 (C-4″), 77.03 (C-5″), 61.21 (C-6″).

^{1}H NMR spectrum has revealed an aromatic A2B2 spin system for B ring at δH 8.06 (2H, H-2′,6′) and 6.89 (2H, H-3′, 5′) and aromatic proton signals for A ring at δH 6.41 (1H, s, H-8) and 6.21 (1H, s, H-6). These data suggest that this compound represents a kaempferol derivative. Proton and NMR data are coherent with those of a kaempferol aglycon, which can be found in the literature.[11] Proton signals of four CH in the δH 3.2-3.44 range (4H, m, H-2″, -3″, -4″, -5″), CH2 at δH 3.71 and anomeric CH at δH 5.25, along with a coupling constant of 7.5 Hz, indicate the presence of a β-glucose moiety. This is further confirmed by the ^{13}C spectrum, which shows signals at δC 102.63 (C-1″), 74.33 (C-2″), 76.63 (C-3″), 69.95 (C-4″), 77.03 (C-5″), and 61.21 (C-6″).[12] The position of the glucose moiety at C-3 is confirmed by the existence of an HMBC correlation between H-1" and C-3.

According to the results of TLC, nuclear magnetic resonance, and mass spectrometry, the structure of compound one was determined to be kaempferol 3-O-glucoside (Astragalin) (Fig.1).

The NMR data of the sample were compared to those in the literature. A comparison of the data showed that the NMR data are consistent with the ^{13}C-NMR data of astragalin in the literature. The identity of astragalin was also confirmed by TLC using different mobile phases and a reference standard of astragalin. Results have determined the identity of compound one as Astragalin (Fig.1). This compound was also isolated from other Allium species, such as *A. ursinum, A. victorialis, A. ampeloprasum, A. paradoxum*.[12,14–16] Astragalin is also isolated from the plants of different families, such as *Convolvulaceae Ebenaceae, Rosaceae, Eucommiaceae,* etc.[17] *Cuscuta chinensis* has an extremely high content of kaempferol 3-O-glucoside (29–34% of the total phenolics) among all species.[18]

Compound 2 was isolated from the 50% methanolic fraction of *A. saxatile* as a yellow amorphous powder with a molecular weight of 594.5181 m/z $[M–H]^-$ and 617.1475 m/z $[M+Na]^+$, corresponding to the molecular formula $C_{27}H_{30}O_{15}$ (theoretical isotopic mass: 594.1584 m/z) (Fig.2).

Obtained signals at δH 5.74 (d, J = 7.5 Hz) and 5.22 (d, J = 1.3 Hz) revealed the presence of two sugar moieties that present, in one case, the β-configuration and, in the other case, the α-configuration. Complete assignments of each sugar proton system were achieved by considering TOCSY and ^{1}H–^{1}H COSY spectra. The presence of a glucose unit was confirmed by the large vicinal couplings among ring protons, characteristic of their trans-diaxial orientation.

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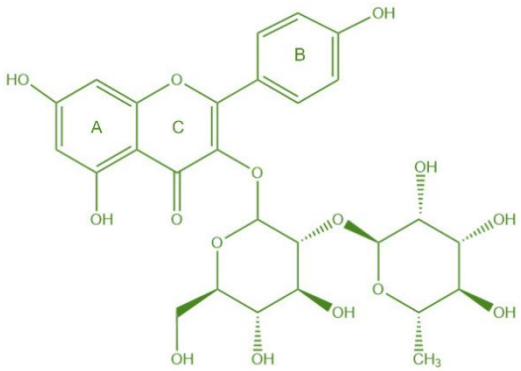
FIGURE 2. Chemical structure of compound 2



^{1}H NMR (500 MHz, CD3OD, δ, ppm, J/Hz): 8.04 (2H, d, J = 8.9, H-2′,6′), 6.89 (2H, d, J = 8.9, H-3′,5′), 6.38 (1H, d, J = 8.9, H-8), 6.18 (1H, d, J = 8.9, H-6), 5.74 (1H, d, J = 7.5, H-1″), 5.22 (1H, d, J = 1.3, H-1‴), 4.00 (1H, m, H-2‴), 4.00 (1H, m, H-5‴), 3.80 (1H, m, H-3‴), 3.59 (1H, m, H-2″), 3.59 (1H, m, H-3″), 3.59 (1H, m, H-6″), 3.37 (1H, m, H-4‴), 3.31 (1H, m, H-4″), 3.31 (1H, m, H-5″), 0.99 (3H, s, H-6‴).

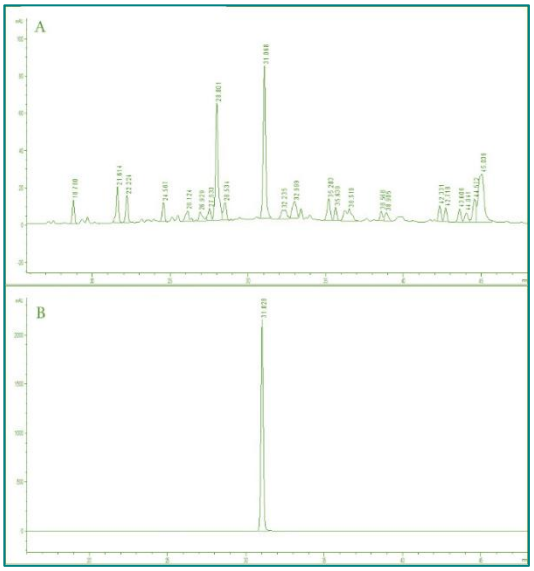
^{13}C NMR (125 MHz, CD3OD, δ, ppm): 157.09 (C, C-2), 133.03 (C, C-3), 178.00 (C, C-4), 161.84 (C, C-5), 98.27 (CH, C-6, δH = 6.18), 164.27 (C, C-7), 93.14 (CH, C-8, δH = 6.38), 157.04 (C, C-9), 104.57 (C, C-10), 121.71 (C, C-1′), 130.74 (CH, C-2′, 6′, δH = 8.04), 114.67 (CH, C-3′, 5′, δH = 6.89), 159.93 (C, C-4′), 101.22 (CH2, C-1″, δH = 5.22), 78.65 (C, C-2″), 77.54 (C, C-3″), 70.42 (CH2, C-4″, δH = 3.31), 76.99 (C, C-5″), 61.22 (CH2, C-6″, δH = 3.59), 70.99 (C, C-2‴), 70.88 (C, C-3‴), 78.64 (C, C-4‴), 68.51 (C, C-5‴), 16.12 (CH3, C-6‴, δH = 0.99).

The ^{1}H NMR spectrum showed two coupled doublets with a very small J-value, typical of the two meta-related H-6 and H-8 protons of the A ring of the flavonoid unit and an A2B2 system (d 8.04 and 6.89, J = 8.9 Hz) that is due to the B ring of the same flavonoid unit. This led to the formation of kaempferol aglycon, as seen in compound 1. The proton and carbon data showed, this time, the presence of two sugar moieties: one corresponding to glucose and the other to rhamnose. The position of the sugar moieties is confirmed by the existence of HMBC correlations between H-1" (glucose) and C-3 and between H-1'" (rhamnose) and C-2" (glucose). Then, the glucose unit, which was directly connected to kaempferol, was linked through position 2 to rhamnose. The obtained NMR data were correlated with the data given in the literature.[19] The combination of NMR data and the results of MS analysis helped to elucidate the structure of compound two as kaempferol 3-O-neohesperidoside (Fig.2). NMR data of the sample were compared to data in the literature to confirm its structure.[13]

Method development

To quantify the isolated compounds in a high-throughput manner, an HPLC method was developed. Astragalin was found to be the dominant compound in A. saxatile *Bieb*. The F2 fraction was chosen as the chemical marker for quantification. The HPLC separation conditions were optimized to achieve satisfactory resolution. The best separation of the crude extracts of *A. saxatile* was achieved using a reverse-phase column, Eclipse Plus C18 (4.6 x 250 mm, 5 µm). The mobile phase was examined to achieve optimal resolution, and it was found that satisfactory separation was achieved with a mobile phase consisting of acetonitrile and water in a 23:77 (v/v) ratio. The retention time of astragalin was observed to be 31.0 min, and measurement at 320 nm provided sufficient sensitivity and a satisfactory chromatographic baseline. Under the optimized conditions, a baseline separation was achieved within 50 min, with a symmetrical, sharp, and well-resolved peak for astragalin. The chromatogram indicated complete baseline separation of astragalin in the crude extract of *A. saxatile* (Fig.3).

FIGURE 3. HPLC chromatogram of A-Crude extract of *A. saxatile* and *B-Astragalin*



Method validation

A quantitative method for analyzing the crude extract of *A. saxatile* was successfully developed and validated in accordance with the ICH guidelines for the validation of analytical methods. The calibration curves for the crude extract of *A. saxatile* were found to be linear within the tested concentration range, with a correlation coefficient of 0.9996 (Fig. 4). The method exhibited good precision, as evidenced by the inter-day % RSD of 3.34% for *A. saxatile*. The limit of detection was 0.00012 mg/mL for the crude extract of *A. saxatile*, and the limit of quantification was 0.00024 mg/mL.

Quantification of astragalin

This validated HPLC method was successfully applied for the quantification of astragalin in the crude extract of *A. saxatile*, using external calibration. The concentration of astragalin in the crude extract of *A. saxatile* is 0.87%.

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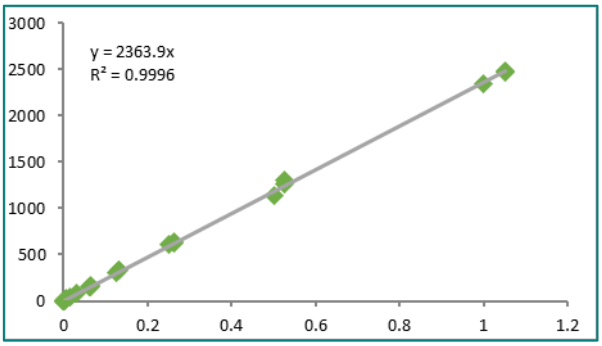
FIGURE 4. Calibration curve of Astragalin



DISCUSSION

The discovery of astragalin and kaempferol 3-O-neohesperidoside in *A. saxatile* significantly expands the known phytochemical profile of this species, positioning it as a valuable source of biologically active flavonoids. Astragalin is widely recognized for its potent antioxidant activity, as evidenced in this study, with an IC50 value of 2.93 µg/mL. This aligns with previous research demonstrating its role in scavenging free radicals and protecting cells from oxidative stress.[21] Moreover, astragalin's anti-inflammatory effects, achieved through the inhibition of the NF-κB signaling pathway, highlight its therapeutic potential in managing chronic inflammatory diseases, including rheumatoid arthritis and inflammatory bowel disease. The presence of kaempferol 3-O-neohesperidoside in *A. saxatile* is particularly noteworthy, as it has only been reported in a few plant species, including *Allium ursinum*[23] and *Paris verticillate*.[24] The rarity of this compound, coupled with its established pharmacological properties, enhances the medicinal value of *A. saxatile*. The discovery of these compounds also reinforces the growing recognition of Allium species as a rich source of secondary metabolites with diverse biological activities. While other Allium species, such as *A. ursinum*,[14] *A. victorialis*,[15] *A. ampeloprasum*,[16] and *A. paradoxum*[12] have been known for their flavonoid content, *A. saxatile* emerges as a novel source, underscoring the potential of lesser-studied Allium species in phytochemical and pharmacological research. Our study has revealed *A. saxatile* as a new source of astragalin and kaempferol 3-O-neohesperidoside.

CONCLUSIONS

Two flavonoids, astragalin, and kaempferol 3-O-neohesperidoside, have been isolated from *A. saxatile* for the first time in Georgia, contributing to the phytochemical profile of this species. Additionally, a sensitive and reproducible HPLC method has been developed and validated for the simultaneous quantification of astragalin in the crude extract of *A. saxatile*. The technique demonstrates excellent precision, sensitivity, and linearity, making it a valuable tool for future phytochemical studies and quality control.

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