

Jgerenaia G.^{1,2}, Gviniashvili Ts.³, Frederich M.², Mskhiladze L.¹

DOSAGE OF FUROSTANOL AND FLAVONOID GLYCOSIDES OF PLANTS OF THE ALLIUM GENUS, GROWING IN GEORGIA

TSMU, DEPARTMENT OF PHARMACOGNOSY¹, LABORATORY OF PHARMACOGNOSY, CENTER OF INTERDISCIPLINARY RESEARCH ON MEDICINES (CIRM), FACULTY OF MEDICINE. UNIVERSITY OF LIÈGE, LIÈGE BELGIUM²; INSTITUTE OF BOTANY, ILIA STATE UNIVERSITY, TBILISI, GEORGIA³

Introduction. The genus *Allium* belongs to family Alliaceae. The plants of the genus *Allium* are very important herbaceous plants. This genus involves up to 1233 species, growing especially in the northern hemisphere.[1] Among them, 70 species grow in the Caucasus region and 35 species are described in Georgia, among which 7 species are endemic for the Caucasus region and 5 species are endemic to Georgia [2]. Garlic (*Allium sativum* L.), onion (*Allium cepa* L.), shallot (*Allium ascalonicum* L.), chive (*Allium schoenoprasum* L.), and leek (*Allium porrum* L.), as the common members of the *Allium* genus, have been historically used as edible vegetable, medicinal, and ornamental plants for a long time[3]. Furthermore, they are vital to human development and life and have significant roles in agriculture, breeding, medicine, and the food industry. For instance, garlic and onion have long been used in folk medicine to treat tumors, cholera, dysentery, intestinal worms, and migraines as well as fevers, bites, and headaches[4]. According to the ancient Georgian books of traditional medicine “Karabadi” and “Iadigar-Daudi” plants of *Allium* species were widely used in folk medicine as antifungal, antiseptic and antibacterial remedies[5], [6]. Different types of secondary metabolites, including as flavonoids, anthocyanins, saponins, phenolic acids, amino acids, glutamyl peptides, minor organic acids, fatty acids, steroids, vitamins, and nucleosides, have been discovered in the major *Allium* species. Among these, saponins and flavonoids have also been shown to have substantial activity[7]. Flavonoids play a variety of biological activities in plants, animals, and bacteria. Flavonoids protect plants from different biotic and abiotic stresses and act as unique UV filters[8]. Many studies have suggested that flavonoids exhibit biological activities, including anti-allergenic, antiviral, anti-inflammatory and vasodilating actions. However, most interest has been devoted to the antioxidant activity of flavonoids which is due to their ability to reduce free radical formation and to scavenge free radicals[9]. Steroidal saponins and saponins have been identified so far in over 40

different *Allium* species. Several studies describe the pharmacological effects of steroidal saponins. Promising antifungal, cytotoxic, anti-inflammatory, antithrombotic, and hypocholesterolemic actions were demonstrated by several of them. Most significantly, these substances serve as substrates for the synthesis of steroid hormones and medications[10]. Thin Layer Chromatography (TLC) is frequently used to obtain and characterize secondary metabolites that is present in herbal medicines. The TLC method can be used to analyze both the active compound in the plant and the quality of medicinal plant. Because it is an easy, inexpensive, and quick way to do qualitative analysis, TLC with UV lamp detection and color reagents is widely used[11]. Spectrophotometry methods are practical, simple, and very less expensive, making them preferable for specification testing. Because of the weak absorbance of saponins, a colorimetric determination is used for their evaluation[12]. The aim of this research was to investigate, for the first time, total flavonoid content, total ash of plant material and to elaborate method for quantification of total furostanolic saponin content of the plant genus *Allium*: *Allium saxatile* M. Bieb, Sect. *Oreiprason* F. Hern and *Allium ponticum* Misch, Sect. *Allium*, growing in Georgia.

Materials and Methods

Plant material.

The objects of the research were plants *Allium saxatile* and *Allium ponticum* growing in Georgia. The whole plants of *A. saxatile* and *A. ponticum* were collected, respectively, in Racha and Javakheti, regions of Georgia[13].

Extraction and fractionation.

Extraction and fractionation was performed according to previously published article[13]. Quantification of furostanolic glycosides in plant material and crude extract of *A. saxatile* and *A. ponticum*.

Preparation of reference solution.

Furostanolic glycosides were quantified using UV/VIS spectrophotometry (UV/VIS spectrophotometer model: Nanolytik NanoSpec 2). A calibration curve was elaborated using Protodioscin standard. 2.5 mg/ml, 5 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml and 25 mg/ml solution of Protodioscin was prepared using the mixture of water and PDAB (para-Dimethylaminobenzaldehyde) with 1:1 proportion. Obtained solutions were diluted 100x times to obtain final 25 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml and 250 µg/ml concentrations. Prepared solutions were heated to 57.5-58.5 °C for 2 hours. After 2 hours the solution was cooled down to room temperature and absorbance was measured at wavelength 518 nm. Mixture of water and PDAB (para-Dimethyl-amino benzaldehyde) with 1:1 proportion heated for 2 hours was used as a compensation solution. All the solvents and reference standards used in this research were obtained from, Sigma-Aldrich.

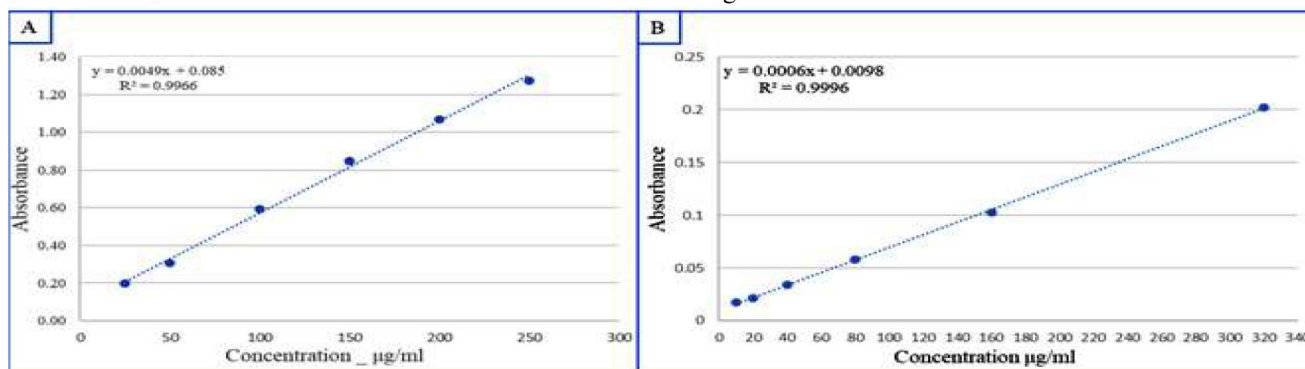


Figure 1. A-Calibration curve of Protodioscin; B- Calibration curve of Quercetin

Plant material preparation.

2.5 g of each plant was put in flask, 50 ml 80% methanol was added and was heated for 1 hour. After 1 hour, solution was cooled down to room temperature and 10 ml of solution was transferred to 50 ml volumetric flask and volume was filled with 80% methanol (solution "A"). 5 ml of solution A was transferred to 25 ml volumetric flask and 5 ml of PDAB (para-Dimethylaminobenzaldehyde) was added (solution "B"). Prepared mixtures were heated to 57.5-58.5 °C for 2 hours. After 2 hours the solution was cooled down to room temperature and absorbance was measured at 518 nm. Solution A and 4N HCl methanolic solution was used as a reference.

Crude extract solution preparation.

0.25 g of crude extract was dissolved in 50 ml 80% methanol using ultrasonic water bath. 10 ml of solution was transferred to 50 ml volumetric flask and volume was filled with 80% methanol (solution "A"). 5 ml of solution A was transferred to 25 ml volumetric flask and 5 ml of PDAB (para-Dimethylaminobenzaldehyde) was added (solution "B"). Prepared mixtures were heated to 57.5-58.5 °C for 2 hours. After 2 hours the solution was cooled down to room temperature and absorbance was measured at 518 nm. Solution A and 4N HCl methanolic solution was used as a reference.

The content of furostanol glycosides (X, %) was calculated by the formula:

$$X = \frac{C * 50 * 10 * 100 * 100}{K * a * (100 - b)}$$

C – content of furostanolic glycosides according to calibration curve, g;

a – mass of material aliquot, g;

b - moisture content in raw material and crude extract, %.

K - Correction coefficient on acid titer-0.98;

TLC of furostanolic saponins

Furostanol glycosides were identified in caltrop herb by thin-layer chromatography (TLC).

Sample preparation: samples were prepared with 50 mg/ml concentration, dissolved in methanol.

Plate: Silica gel F₂₅₄ Merck, size 20*20 cm on glass substrate.

Mobile phase: Mixture of solvents: Dichloromethane: methanol: water (30:12:2).

After sample application the plate was dried on air for about 5–10 minutes and put in a chamber.

When the solvent reached the plate front, the TLC chromatogram was taken out, dried on air and spots were observed after sprinkling with p-dimethylamine benzaldehyde solution and then heating at 105°C.

Sample volume: Experimental solution 50 µl.

Developer reagent: p-dimethylamine benzaldehyde solution (0.5 g p-dimethylamine benzaldehyde was dissolved in 25 ml concentrated hydrochloric acid and 25 ml methanol), heating was done at 105°C.

TLC of flavonoids

Presence of flavonoids was evaluated using TLC.

Sample preparation: samples were prepared with 50 mg/ml concentration, dissolved in methanol.

Plate: Silica gel F₂₅₄ Merck, size 20*20 cm on Aluminum substrate.

Mobile phase: Mixture of solvents: Ethyl acetate: Formic acid: Acetic acid: water (100:11:11:26)

After sample application the plate was dried on air for about 5–10 minutes and put in a chamber.

When the solvent reached the plate front, the TLC chromatogram was taken out, dried on air and spots were observed after sprinkling with 2-aminoethyl Diphenylborinate and observed under visible light and after 366 nm wavelength.

Total flavonoid content quantification

The total flavonoid content of total extract and fractions were quantified as described in the article [14]. An aliquot of 0.5 ml of solutions with 1 mg/ml concentrations and Quercetin standard solution with different concentration (10-320 µg/ml) were mixed with 2 ml distilled water and 1.5 ml 5%-NaNO₂ after 5 minutes, 10% AlCl₃ was added and after 6 minutes 1M NaOH was mixed. Absorbance was measured at 510 nm wavelength and the outcome data were expressed as mg/g of Quercetin equivalents in milligrams per gram (mg QE/g) of dry extract. All experiments were performed in triplicate.

Determination of ash

About 1 g of each plant were accurately weighed in a previously ignited and tared crucible. The material was spread in an even layer and ignited it by gradually increasing the heat to 500 °C until it is white, indicating the absence of carbon. The crucible was cooled in desiccator and weighed. The content of total ash was calculated in % of air-dried plant material [15]. Quantification was performed in triplicate.

Results and discussion



Figure 2. A-TLC of furostanolic saponins: 1-Crude extract of *A. ponticum*; 2-Crude extract of *A. Saxatile*; B-TLC of flavonoids under 366 nm wavelength

Steroidal compounds isolated from *Allium* plants mainly include steroidal saponins and sterols. Steroidal saponins are a kind of important chemical component in *Allium* plants and mainly exist in bulbs. More than 100 steroidal saponins have been isolated from species of *Allium* plants. They include approximately 58 spirostanol saponins, 32 furostanol saponins, and 11 cholestane (open-chain) saponins. The sugar residues in the saponins of *Allium* spp. are mainly composed of linear or branched glucose (Glc), rhamnose, galactose (Gal), xylose, and arabinose units[10]. *Allium* saponins are mostly mono- or bidesmosides, however a tridesmodic cholestane glycoside has been reported in the bulbs of *A. macleanii*[16]. From *Allium* species Tigogenin, Diosgenin, Neotigogenin, α -Chlorogenin, Agigenin, Eruboside B and many other saponins were isolated[17], [18].

The TLC analysis of crude extract of *A. ponticum* and *A. saxatile* revealed existence of furostanolic glycosides as pink spots, after revelation with para-Dimethylaminobenzaldehyde solution (Ehrlich's reactive) (Figure 2.A). This result shows that *A. saxatile* crude extract contains more furostanolic saponins than *A. ponticum*.

The calibration curve of for the quantification with spectrophotometry method presented a coefficient of determination of $R^2 = 0.9966$, this indicates that there is significant correlation between the different concentrations of Protodioscine (ig/ml) and absorbance (Figure 1.A).

Table N1
Quantification of furostanolic glycoside content

	Plant material - %		Crude extract - %	
	<i>A. saxatile</i>	<i>A. ponticum</i>	<i>A. saxatile</i>	<i>A. ponticum</i>
Content of furostanolic glycosides (%)	0.69	0.152	15,16	4,7
Moisture content (%)	7.21	7.77	12,8	8,2

Quantification of the sum of saponins in plant material and crude extract of both plants have shown that, *A. saxatile* contains 0.69% of furostanolic saponins, and *A. ponticum* 0.15%. after extraction with 80% ethanol, showed that total saponin content quantified using Protodioscine calibration curve has increased to 15.16% for *A. saxatile*, and 4.7% for *A. ponticum*. Same result was observed also after TLC analysis of crude extracts of both plants. Overall, we can conclude that *A. saxatile* is rich with furostanolic saponins, this can explain better biological activity of extract and fractions obtained from *A. saxatile*.

Total ash content of plant materials was determined by dry ashing. Quantification showed that total ash content of *Allium ponticum* was 4.9% and for *Allium saxatile* was 8.09%.

Table N2
Total flavonoid content of total extract and fraction

Sample	TFC (QE/g)
A.S. total extr.	11.07 \pm 7.05
A.S.F2	21.49 \pm 10.79
A.S.F3	9.61 \pm 4.25
A.P. total extr.	13.08 \pm 6.05
A.P.F2	11.49 \pm 7.19
A.P.F3	16.28 \pm 6.16

Flavonoids are secondary metabolites with antioxidant activity, the potency of which depends on the number and position of free OH groups[9]. Flavonoid compounds are widely distributed in plants. They mainly include flavonoids, flavonols, flavones, flavanones, isoflavonoids and anthocyanidins[19]. *Allium* species are rich with flavonoids, for example: Kaempferol, Quercetin, Myricetin, Isorhamnetin, Quercetin-7,42 -diglucoside etc[18].

Quantitative determination of flavonoid content in total extract and fractions of plants *A. saxatile* and *A. ponticum* was performed using aluminum chloride in a colorimetric method. The results were derived from the calibration curve ($y = 0.0006x + 0.0098$, $R^2 = 0.9996$. Figure 1.B) of Quercetin (10-320 ig/ml). and exposed in quercetin equivalents (QE) per gram dry extract weight (Table 2). According to the results we see that, total flavonoid content for crude extract of *A. saxatile*, is 11.07 QE/g and highest concentration TFC among the fractions was observed in fraction A.S.F2, obtained with 50% methanol_21.49 QE/g, in case of crude extract of *A. ponticum*, total flavonoid content was 13.08 QE/g, studying the fractions showed that TFC concentration is higher in A.P.F3 fraction, which is obtained with 100% methanol_16.28 QE/g.

Flavonoid content of both plants was observed using TLC analysis (Figure 2.B), existence of flavonoids was revealed after spraying with 2-aminoethyl Diphenylborinate solution, as yellow-green spots. Comparing the TLC results of fractions we see that for *A. Saxatile*, fraction A.S.F2 is rich with flavonoids, in case of *A. ponticum* fraction A.P.F3 is richer. Same results were found after determination with UV spectrophotometer.

Conclusions

In this research, secondary metabolites, from the plants *A. saxatile* and *A. ponticum*, such as furostanolic saponins and flavonoids were characterized using TLC. Furostanolic saponins were identified as pink spots under visible light and flavonoids as a yellow - green spots under 365 nm wavelength. Total flavonoid content for both plants and extracts and quantification of furostanolic saponins was done using UV-vis spectrophotometry. The quantity of furostanolic saponins were determined using Protodioscine as a standard solution and flavonoids using Quercetin. Research methodology for identification and quantification of furostanolic glycosides in *Allium saxatile* and *Allium ponticum* were proposed for first time and will be used for standardization of described plants.

References:

- [1] Registry-Migration.Gbif.Org, "GBIF Backbone Taxonomy." GBIF Secretariat, 2021. doi: 10.15468/390MEI.
- [2] R. Gagnidze, *Vascular Plants of Georgia a nomenclatural Checklist*, Institute of Botany. Tbilisi, Republic of Georgia, 2005.
- [3] R. Kamenetsky and H. D. Rabinowitch, "The Genus *Allium*: A Developmental and Horticultural Analysis," in *Horticultural Reviews*, John Wiley & Sons, Ltd, 2006, pp. 329–378. doi: 10.1002/9780470767986.ch7.
- [4] M. Corzo-Martínez, N. Corzo, and M. Villamiel, "Biological properties of onions and garlic," *Trends Food Sci. Technol.*, vol. 18, no. 12, pp. 609–625, Dec. 2007, doi: 10.1016/j.tifs.2007.07.011.
- [5] Z. Panaskerteli-Tsitsishvili, *Karabardini*. Tbilisi, Republic of Georgia: Sabchota Sakartvelo, 1978.
- [6] D. Bagrationi, *Iadigar Daudi*, Edition of Tbilisi University. Republic of Georgia, 1993.

[7] Ž. Fredotovi  and J. Puizina, "EDIBLE ALLIUM SPECIES: CHEMICAL COMPOSITION, BIOLOGICAL ACTIVITY AND HEALTH EFFECTS," *Ital. J. Food Sci.*, vol. 31, no. 1, 2019, doi: 10.14674/IJFS-1221.

[8] A. Takahashi and T. Ohnishi, "The significance of the study about the biological effects of solar ultraviolet radiation using the Exposed Facility on the International Space Station," *Uchu Seibutsu Kagaku*, vol. 18, no. 4, pp. 255–260, Dec. 2004, doi: 10.2187/bss.18.255.

[9] A. N. Panche, A. D. Diwan, and S. R. Chandra, "Flavonoids: an overview," *J. Nutr. Sci.*, vol. 5, p. e47, ed 2016, doi: 10.1017/jns.2016.41.

[10] D. Sobolewska, K. Michalska, I. Podolak, and K. Grabowska, "Steroidal saponins from the genus *Allium*," *Phytochem. Rev.*, vol. 15, no. 1, pp. 1–35, Feb. 2016, doi: 10.1007/s11101-014-9381-1.

[11] A. Syarifah, R. Retnowati, and Soebiantoro, "Characterization of Secondary Metabolites Profile of Flavonoid from Salam Leaves (*Eugenia polyantha*) Using TLC and UV Spectrophotometry," *Pharm. Sci. Res.*, vol. 6, no. 3, Dec. 2019, doi: 10.7454/psr.v6i3.4219.

[12] R. Singh, R. Sharma, P. Soren, G. Mal, and B. Singh, "Extraction and detection of saponin-enriched fractions from different plants of North-western Himalayas, India," *J. Pharmacogn. Phytochem.*, vol. 8, no. 3, pp. 3817–3820, 2019.

[13] G. Jgerenaia *et al.*, "PHARMACOLOGICAL ASSESSMENT OF CONSTITUENTS OF SPECIES ALLIUM SAXATILE AND ALLIUM PONTICUM GROWING IN GEORGIA," *Exp. Clin. Med. Ga.*, Jun. 2022, doi: 10.52340/jecm.2022.06.04.

[14] S. Aryal, M. K. Baniya, K. Danekhu, P. Kunwar, R. Gurung, and N. Koirala, "Total Phenolic Content, Flavonoid Content and Antioxidant Potential of Wild Vegetables from Western Nepal," *Plants Basel Switz.*, vol. 8, no. 4, p. E96, Apr. 2019, doi: 10.3390/plants8040096.

[15] World Health Organization, "Quality control methods for herbal materials," World Health Organization, 2011. Accessed: Oct. 12, 2022. [Online]. Available: <https://apps.who.int/iris/handle/10665/44479>

[16] I. T. M. Y, S. Y, N. A. S. Y, and N. H, "Steroidal glycosides from *Allium macleanii* and *A. senescens*, and their inhibitory activity on tumour promoter-induced phospholipid metabolism of HeLa cells," *Phytochemistry*, vol. 40, no. 2, Sep. 1995, doi: 10.1016/0031-9422(95)00223-t.

[17] L. Mskhiladze *et al.*, "Cytotoxic Steroidal Saponins from the Flowers of *Allium leucanthum*," *Molecules*, vol. 13, no. 12, pp. 2925–2934, Nov. 2008, doi: 10.3390/molecules13122925.

[18] A. Alam *et al.*, "Allium vegetables: Traditional uses, phytoconstituents, and beneficial effects in inflammation and cancer," *Crit. Rev. Food Sci. Nutr.*, vol. 0, no. 0, pp. 1–35, Feb. 2022, doi: 10.1080/10408398.2022.2036094.

[19] A. Wach, K. Pyrzyńska, and M. Biesaga, "Quercetin content in some food and herbal samples," *Food Chem.*, vol. 100, no. 2, pp. 699–704, Jan. 2007, doi: 10.1016/j.foodchem.2005.10.028.

SUMMARY

Jgerenaia G.^{1,2}, Gviniashvili Ts.³, Frederich M.², Mskhiladze L.¹

DOSAGE OF FUROSTANOL AND FLAVONOID GLYCOSIDES OF PLANTS OF THE ALLIUM GENUS, GROWING IN GEORGIA

TSMU, DEPARTMENT OF PHARMACOGNOSY¹, LABORATORY OF PHARMACOGNOSY, CENTER OF INTERDISCIPLINARY RESEARCH ON MEDICINES (CIRM), FACULTY OF MEDICINE. UNIVERSITY OF OF LI GE, LI GE BELGIUM²; INSTITUTE OF BOTANY, ILIA STATE UNIVERSITY, TBILISI, GEORGIA³

This study describes the identification and quantification of flavonoids and furostanolic glycosides in plants of the genus *Allium*: *Allium saxatile* and *Allium ponticum*, growing in Georgia. Flavonoids and Furostanol glycosides were identified by thin-layer chromatography (TLC) and quantified by UV-vis spectrophotometry. The furostanol glycosides were visualized on the TLC plate as pink spots after treatment with para-Dimethylaminobenzaldehyde solution, flavonoids were identified as yellow-green spots under 366 nm wavelength, after spraying with a 10 g/L solution of diphenylboric acid aminoethyl ester R in methanol R. Furostanolic glycoside were quantified by UV-Visible spectrophotometry to the amount of at least 0.15% for *A. ponticum* plant material, and at least 0.6% for *A. saxatile*. Total ash value for *A. ponticum* was 4.9%, for *A. saxatile* 8.1%. During this research, the total flavonoid content (TFC) of both plants crude extract and fractions was determined, and the TFC of crude extract of *A. saxatile* was 11.07 QE/g, among the fractions, the highest TFC value was found in A.S.F2 with a value of 21.49 QE/g. In case of the crude extract of *A. ponticum*, the TFC was 13.08 QE/g for the crude extract, and the fraction with the greatest TFC value was found to be A.P.F3 with a value of 16.28 QE/g. The data obtained from this research on the identification and quantification of flavonoid and furostanol glycosides, ash value and moisture content will be used for standardization and quality assessment of raw material.