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International Journal of Pharmaceutical Sciences and Drug Research
2015; 7(1): 78-82

Research Article

ISSN: 0975-248X
CODEN (USA): IJPSPP

Identification of Cytotoxic and Antioxidant Compounds from *Allium gramineum* Flowers

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ABSTRACT

The present study evaluates the *in vitro* anticancer, antiplasmodial and antioxidant activity of the ethanolic crude extract from the flowers of *Allium gramineum* growing in Georgia and of one flavonol and two steroidal glycosides which were isolated from this plant. The flowers were extracted with ethanol and this total extract was subjected to successive bioguided fractionations to provide glycosides 1-3. Their structures were elucidated on the basis of NMR and ESI-MS spectrometric data in comparison with the existing literature and have been identified as: isorhamnetin-3-O-β-D-glucopyranoside (1), diosgenin-3-O-α-rhamnopyranosyl-(1→2)-β-D-diosgenin -3-O-α-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)-]-β-D-glucopyranoside (Deltonine) (3). The ethanolic extract has been shown to strongly inhibit the growth of breast adenocarcinoma cell lines, with an IC_{50} of 4.5 ± 0.7μg/mL for MDAMB-231 and 4.8 ± 0.9μg/mL for MCF-7 cells. The cytotoxic activity was related to 2 and 3 which exhibited potent cytotoxicity, with an IC_{50} of ± 3μM. Concerning antiplasmodial activities, only weak activities were observed using the ethanolic extract and the two saponins. The flavonoid was almost inactive. Finally, the radical-scavenging activity of the ethanolic extract was tested in presence of $ABTS^{•+}$ solution. A decrease of the absorbance intensity was observed, with an IC_{50} value of 22.1 ± 0.6μg/mLwhile trolox, used as Standard drug, showed a pronounced activity (IC_{50} = 12.7±0.5μM). The glycoside 1 showed the lowest IC_{50} value of 20.1 ± 0.8μM while both 2 and 3 exhibited very weak radical scavenging activity.

Keywords: *Allium gramineum*, Alliaceae, Steroidal saponine, Flavonoid, Structure determination, pharmacological activities.

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Received: 03 November, 2014; **Accepted:** 19 November, 2014

INTRODUCTION

The genus *Allium* (family Alliaceae) includes up to 800 species in the world flora. Among them, some 70 grow in the Caucasus region and 35 species are described in Georgia. [1] Garlic and onions are among the oldest cultivated herbs and their use is well documented, dating back to 3200-2800 B.C. This plant, as food

flavoring, is known worldwide and has been particularly used in folk medicine since ancient times. *Allium* species contain high amounts of secondary metabolites and they exhibit different pharmacological activities. [2-3] A large part of the literature in the recent years is devoted to the general evaluation of their antibacterial [4], antifungal [5], antioxidant [6] and cytotoxic properties. [7]

The antioxidant compounds of plants, particularly flavonoids and other glycosides have been reported to inhibit the propagation of free radical reactions, to protect the human body from disease. [8] The importance of reactive oxygen species (ROS) and free radicals in cellular damage and the ageing process has attracted increasing attention over the last 20 years. [9]

Allium gramineum 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 remedy. [10]

Phytochemical and biological studies of *Allium leucanthum,* another Caucasian endemic species, have been previously reported and showed promising results. [11-12] This prompted us to undertake for the first time the phytochemical and biological study of *Allium gramineum* flowers.

MATERIALS AND METHODS

Cell lines, chemicals and biochemicals

The human breast cancer cell lines MDA-MB-231 (HTB-26, ATCC) and MCF-7 (HTB-22 ATCC) were grown in Dublecco's Modified Eagle's Medium (DMEM) and Eagle's Minimum Essential Medium (EMEM) supplemented with 10% of fetal bovine serum 1% L-glutamine and non-essentials amino acid for EMEM. All the cells were grown at 37°C in a humidified atmosphere and 5% CO_2. Spectra were recorded on a Bruker Avance II 500 instrument equipped with a cryoprobe. ^1H and ^{13}C NMR chemical shifts in ppm were referenced to the residual solvent (CD_3OD; DMSO) signals or to TMS as internal standard. High resolution electrospray ionization mass spectrum was conducted in positive mode on an Applied Biosystems/MDS Sciex QSTAR XL QqTOF MS system. After harvesting, the plants were directly dried with a Microwave irradiation (Pr KS–22E, 850 W, 2450 MHz) apparatus. For column chromatography, silica gel 60 (40-63μm, Merck) and Diaion HP20 resin (Mitsubishi) were used. TLC analysis of saponins was performed on Silica gel 60 F254 plates (Merck). Compound 1 (flavonoid) was detected by spraying the plates with 2-aminoethyl diphenylborate and compound 2, 3 (saponine) by vanillin-sulphuric acid (in EtOH) reagent, followed by heating at 110°C. The cytotoxic activity was monitored by a Labsystems Multiskan MS. 2,2′-Azinobis-(3-ethylbenzohiazoline-6-sulphonic acid (ABTS) was from Fluka (Bornem, Belgium), dimethylsulfoxide (DMSO) and sodium persulfate ($Na_2S_2O_8$) were both purchased from Sigma-Aldrich (Bornem, Belgium) as well as standard drugs: Doxorubicune, Trolox and Artemisinin. The radical scavenging activities of all samples were monitored on a UV-visible spectrophotometer (HP 8453, Waldbronn-Germany). The antiplasmodial activity was monitored by a multiwell scanner (Stat Fax 2100, Awareness Technology Inc).

Plant material

The whole plants of *Allium gramineum* were collected in the Didgori, region of Georgia in June 2011, 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 solvent (34.2 g). The residue Ext.-1 was suspended in MeOH (100 mL) and triturated with acetone (1000 mL) to give sediment (Ext.-2) and acetone soluble extract (Ext.-3). After evaporation of the solvent, the aceton layer Ext.-3 (1.7 g) was subjected to silica gel CC and eluted with CH_2Cl_2-MeOH–H_2O (50:8:1, v/v/v) to give compound 1 (125 mg). The sediment Ext.-2 (19.8 g) was chromatographed 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-3 (2.2 g) which was collected in MeOH 100% was subjected to silica gel CC and eluted with CH_2Cl_2-MeOH–H_2O (45:8:1, v/v/v) to give: compound 2 (53 mg) and compound 3 (210 mg) (Fig. 1).

Physico-chemical characterization of compounds

Compound 1 was isolated as yellow needles, ESI-MS (negative ion mode) *m/z* = 477.1038 $[M-H]^-$, corresponding to the empirical molecular formula of $C_{22}H_{21}O_{12}$, Based on MS and NMR analysis and literature data, compound 1 was determined as isorhamnetin-3-*O*-β-D-glucopyranoside, which had been previously isolated from *Eupatorium tinifolium.* [13]

Compound 2 white amorphous powder, ESI-MS (positive ion mode) *m/z* = 723, 4320 $[M+H]^+$, corresponding to the empirical molecular formula of $C_{39}H_{63}O_{12}$. Based on MS and NMR analysis and literature data, compound 2 was determined to be diosgenin-3-0-α-rhamnopyranosyl-(1→2)-β-D-glucopyranoside. [14] Compound 3 white amorphous powder, ESI-MS (positive ion mode) *m/z* = 885.4848 $[M+H]^+$, corresponding to the empirical molecular formula of $C_{45}H_{72}O_{17}$. Based on MS and NMR analysis and literature data, compound **3** was determined to be diosgenin-3-0-α-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)-]-β-D-glucopyranoside. [14-15]

Copies of the original spectra are obtainable from the coresponding author.

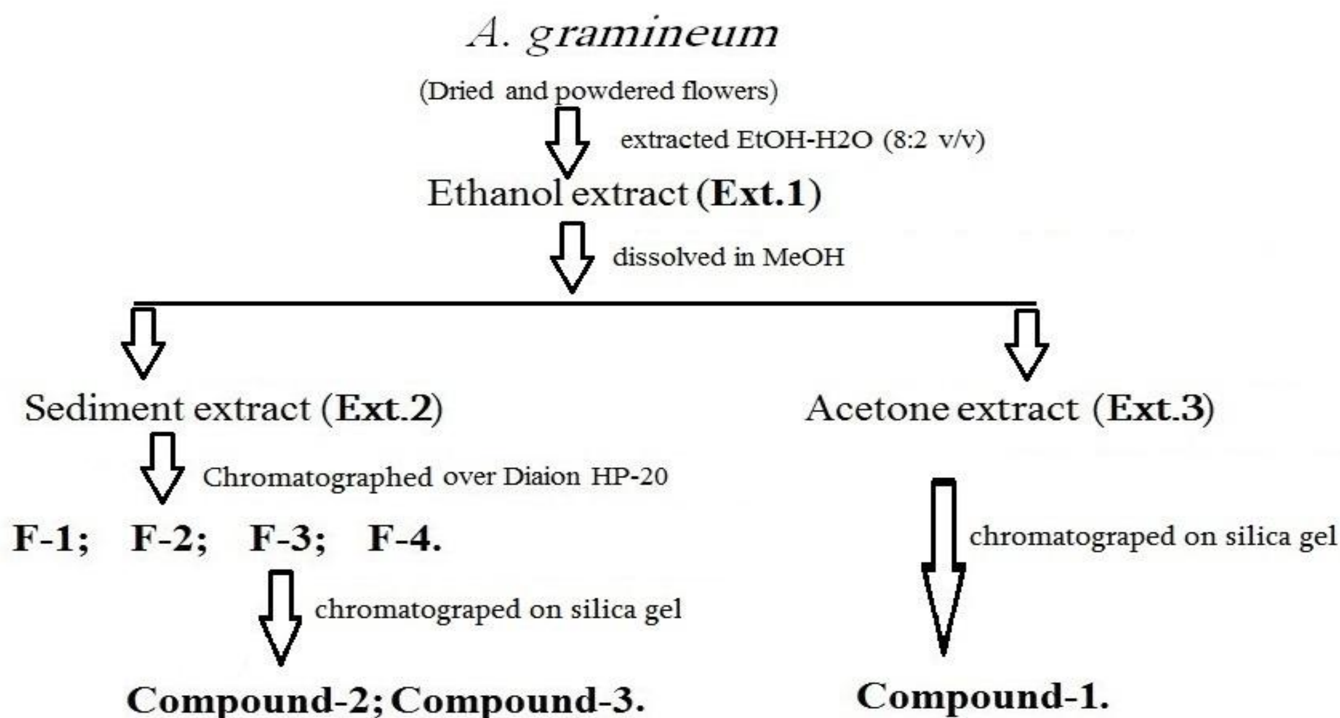

Fig. 1: Isolation of various extract/fractions/compounds from the flowers of *A. Gramineu*

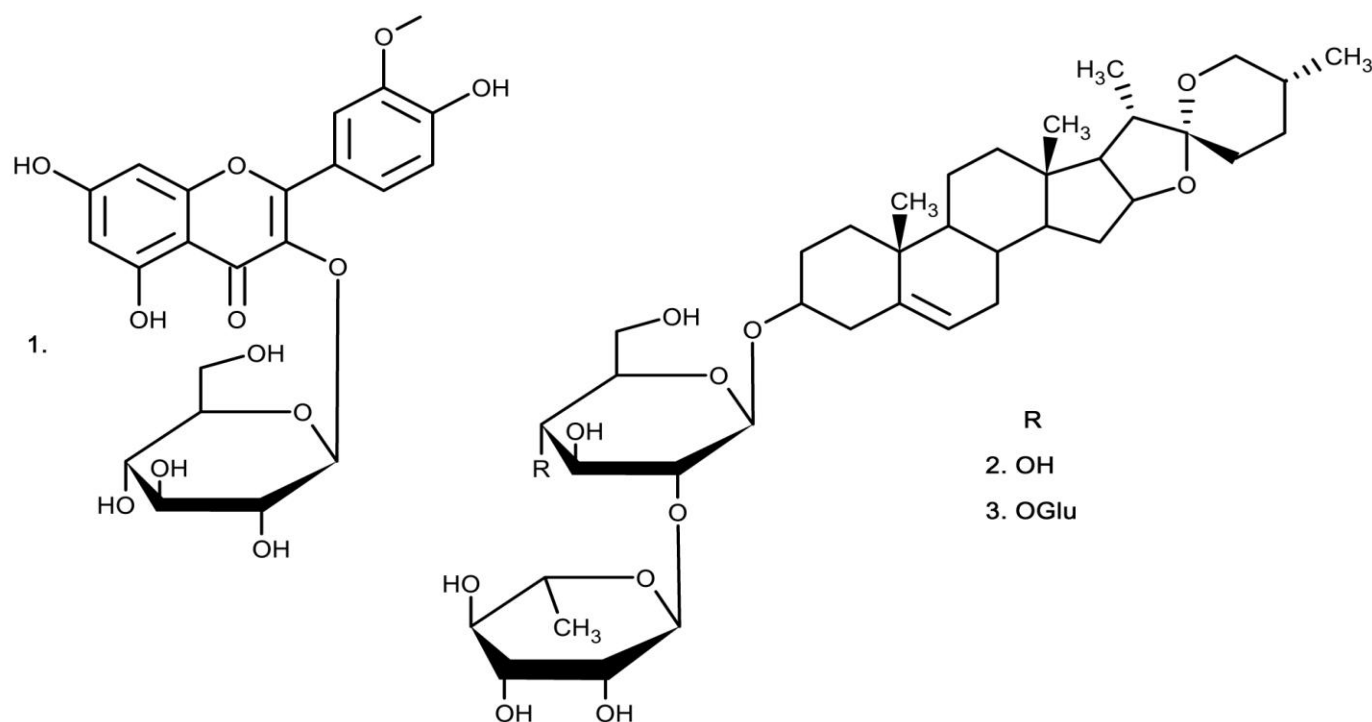

Fig. 2: Chemical structures of compounds 1-3.

Table 1: ***In vitro*** **cytotoxic, antiplasmodial and antioxidant activity of the extract, compounds 1-3 of** ***A. gramineum*** **and standard drugs.**

	cytotoxic		cytotoxic		antiplasmodial		antioxidant	
	IC-50 MDAMB-231± S.D.		IC-50 MCF-7 ± S.D.		IC-50 3D7 ± S.D.		IC-50 ABTS$^{\cdot+}$ ± S.D.	
	μM	μg/ml	μM	μg/ml	μM	μg/ml	μM	μg/ml
Ext.-1	-	4.5 ± 0.7	-	4.8 ± 0.9	-	45.5 ± 8.9	-	22.1 ± 0.6
Compound 1	38.1 ± 7.8	18.2 ± 3.7	21.0 ± 3.9	10.1 ± 1.8	> 50	> 100	20.1 ± 0.8	9.8 ± 0.4
Compound 2	2.9 ± 0.7	2.1 ± 0.5	4.4 ± 1.4	3.2 ± 1.0	41.4 ± 6.3	29.9 ± 4.5	> 100	> 100
Compound 3	2.7 ± 0.5	2.4 ± 0.4	2.8 ± 0.8	2.5 ± 0.7	27.1 ± 5.2	24.2 ± 6.4	> 100	> 100
Doxorubicin	5.2 ± 0.9	2.8 ± 0.4	4.8 ± 0.8	4.2 ± 0.7	n.d.	n.d.	n.d.	n.d.
Artemisinin	n.d.	n.d.	n.d.	n.d.	0.015 ± 0.008	0.0042 ± 0.002	n.d.	n.d.
Trolox	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	12.7 ± 0.5	3.2 ± 0.1

* n.d.= not done

Acid hydrolysis
Each compound (10 mg) was dissolved in 2 N HCl (3 ml) and stirred at 100°C for 2 h. The reaction mixture was extracted with chloroform and the aqueous phase was neutralized with *N,N*-dioctylmethylamine (10% in CHCl3) and was chromatographed on silica gel eluted with $CHCl_3$-MeOH (9:1 v/v) to give: for 1 Isoramnetin, for 2 and 3 Diosgenin. The sugar fractions were analyzed on TLC by comparison with standard sugars in a CH_2Cl_2-MeOH-H_2O (50:25:5 v/v/v) solvent system, and further developed using an orthophosphoric acid solution of naphtoresorsinol 5% in EtOH, followed by heating at 110°C. The sugar fractions were identified as, glucose for 1, glucose and rhamnose for 2 and 3, respectively.

Cancer cells viability analysis
Cell viability was determined using the cell proliferation reagent WST-1 according to the manufacturer's instructions (Roche, Basel, Switzerland). All analyzed cells were seeded to obtain 50% of confluence after 24 h of incubation in 96-well plates, 5 × 103 cells per well and then treated for 24h with serial dilution extract (0 - 7.5 µg/mL) and compounds 1-3 (0 - 7.5µM, respectively). Cells were then incubated with WST-1 reagent for 4 h. After this incubation period, the formazan dye formed was quantified by dual wavelength spectrophotometry at 450 and 690 nm. The measured absorbance directly correlates to the number of viable cells. The experiment was performend in triplicate for each sample.

Antiplasmodial assays
The culture of *Plasmodium falciparum* strains and the antiplasmodial assay were carried out as previously described by Frederich et al., 2001. [16] Both strains were obtained from Prof Grellier (Museum National d'Histoire Naturelle, Paris, France). The extract and compound **1-3** were evaluated *in vitro* for their activity against the chloroquine-sensitive strain of *Plasmodium falciparum* (3D7). [16-17] Artemisinin (98%, Sigma–Aldrich) was used as standard (IC_{50} 0.00425 ± 0.008 µM). The results were expressed as the mean IC50 (the concentration of a drug that reduced the level of parasitaemia to 50%). All tests were performed in triplicate.

Radical scavenging activity on ABTS
The ABTS free radical-scavenging activity of the extract and compounds 1-3 was determined according to the method described by Re *et al.* [18] The radical cation $ABTS^{•+}$ was generated by persulfate oxidation of ABTS. A mixture of ABTS (0.7 mM) and sodium persulfate (2.45 mM) was realized and kept overnight at room temperature in the dark to form a radical cation $ABTS^{•+}$, for a further use. Then, the solution was diluted 200 times with methanol and 1.980 mL was transferred into a 5-mL tube at which 20µL of the tested samples were added at the final concentrations of 5 to 40µg/mL (extract) and 5 to 40µM (compounds 1-3), respectively. The resulting solution was mixed using a vortex and stored at room temperature in the dark for 1 h. The radical scavenging activity of each compound was evaluated by measuring the absorbance decrease of the radical cation $ABTS^{•+}$ at fixed wavelength located at 734 nm. All tests were performed in triplicate.

RESULTS AND DISCUSSION
A 80% EtOH extract of the flowers of *Allium gramineum* was subjected to successive bioguided fractionations to provide one flavonol glycoside and two spirostane-type glycosides. Comparison of the NMR and MS data with literature values led to the identification of the structures of compounds 1-3 as isorhamnetin-3-O-β-Dglucopyranoside (1) [12], diosgenin-3-O-α-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (Prosapogenin A of dioscin**)** (2) [13], diosgenin-3-O-α-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)-]-β-D-glucopyranoside (Deltonine) (3). [13-14] Particularly, the sugars branching was confirmed by means of HMBC analysis. The structures of componds 1-3 are shown in Figure 2. The two spirostane glycosides were recently described in the whole plant of *Allium schoenoprasum* [19], and previously in *Dioscorea* species. [20]

The extracts, the enriched fractions (data not shown) and the isolated compounds (Table 1) were evaluated for their *in vitro* anticancer, antioxidant and antiplasmodial activity. The 80% EtOH extract **(Ext.-1)** of the flowers of *Allium gramineum* has been shown to strongly inhibit the growth of breast adenocarcinoma cell lines, with an IC_{50} of 4.5 ± 0.7µg/mL for MDAMB-231 and 4.8 ± 0.9µg/mL for MCF-7 cells. The cytotoxic activity was related to the saponins which exhibited a potent cytotoxicity, with an IC_{50} around 3µM. These activities confirmed the results previously reported on human colon carcinoma cells, and the hypothesis that the interglycosidic linkages of the oligosaccharidic chain can modulate the biological effect, as hypothesized previously by Rezgui *et al.* [21]

The crude extract and isolated compounds were also evaluated for their antiplasmodial and antioxidant activities. Concerning antiplasmodial activities, only weak activities were shown by the total extract, fractions and the two saponins. The flavonoid was almost inactive. The scavenging effect of extract and isolated compounds 1-3 was then evaluated in comparison to trolox used as standard drug. In the absence of the tested sample or in presence of vehicle (DMSO), no scavenging effect was observed. On the contrary, when the extracts (Ext.-1, Ext.-2 and Ext.-3) were added to the $ABTS^{•+}$ solution, a decrease of the absorbance intensity was observed, with following IC_{50} values of 22.1 ± 0.6; 54.4 ± 2.8; 20.3 ± 1.4µg/mL, respectively, while trolox showed a pronounced activity (IC_{50} = 12.7 ± 0.5µM). When the isolated compounds 1 - 3 were evaluated, a strong scavenging effect was obtained with compound 1 with the lowest IC_{50} values of 20.1 ± 0.8µM while both 2 and 3 exhibited very weak radical scavenging activity as the IC_{50} values were higher (>100µM). Overall, among the tested

samples only compound 1, isolated from acetone extract **(Ext.-3)** showed strong antioxidant activity against the radical cation, ABTS^{+}, even though it remains less pronounced than the standard drug trolox, and even if it is in the same range as its cytotoxic activity.

In conclusion, this study of *A. gramineum* resulted for the first time in the identification, isolation, characterisation and biological evaluation of one flavonol and two saponins from this plant. The first one exhibits moderate antioxydant activity and the two saponins exhibit potent cytotoxic and weak antiplasmodial activities.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. J. Aneli for identification of *A. gramineum*, Olivia Jansen, Ewa Cieckiewicz, Naïma Maloujahmoum for technical help. This research has been partially supported by the program Shota Rustaveli National Science Foundation "President Research Grants for Young Scientists 2012" (Grant Agreement Nr. 12/30) and by the Belgian Fund for Scientific Reserch (Grant Nr. T.0190.13). A.B is a Senior Research Associate from the Belgian Fund for Scientific Reserch.

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Source of Support: Nil, Conflict of Interest: None declared.