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-glucopyranoside (Prosapogenin A of dioscin) (2), 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 IC₅₀ of 4.5 ± 0.7 μg/mL for MDAMB-231 and 4.8 ± 0.9 μg/mL for MCF-7 cells. The cytotoxic activity was related to 2 and 3 which exhibited potent cytotoxicity, with an IC₅₀ of ± 3 μM. 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 IC₅₀ value of 22.1 ± 0.6 μg/mL while trolox, used as Standard drug, showed a pronounced activity (IC₅₀ = 12.7 ± 0.5 μM). The glycoside 1 showed the lowest IC₅₀ value of 20.1 ± 0.8 μM while both 2 and 3 exhibited very weak radical scavenging activity.

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

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 Dulbecco’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% CO₂. Spectra were recorded on a Bruker Avance II 500 instrument equipped with a cryoprobe. ¹H and ¹³C NMR chemical shifts in ppm were referenced to the residual solvent (CDCl₃; 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 sediment and air atmosphere and 5% C₀₂. The human breast cancer cell lines MDA-MB-231 (HTB-26, ATCC) and MCF-7 ATCC) were grown supplemented with 10% of fetal bovine serum 1% L-glutamine and non-essentials amino acid for EMEM.

**A. gramineum**

(Dried and powdered flowers)

- Ethanol extract (Ext.1)
  - extracted EtOH-H2O (8:2 v/v)
  - dissolved in MeOH

- Sediment extract (Ext.2)
  - Chromatographed over Diaion HP-20

- Acetone extract (Ext.3)
  - chromatographed on silica gel

**F-1; F-2; F-3; F-4.**

**Compound-2; Compound-3.**

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

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

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

<table>
<thead>
<tr>
<th></th>
<th>cytotoxic IC-50 MDAMB-231 ± S.D.</th>
<th>cytotoxic IC-50 MCF-7 ± S.D.</th>
<th>antiplasmodial IC-50 3D7 ± S.D.</th>
<th>antioxidant IC-50 ABTS**⁺⁺ ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pM</td>
<td>pg/ml</td>
<td>pM</td>
<td>pg/ml</td>
</tr>
<tr>
<td>Ext-1</td>
<td>4.5 ± 0.7</td>
<td>-</td>
<td>4.8 ± 0.9</td>
<td>-</td>
</tr>
<tr>
<td>Compound 1</td>
<td>38.1 ± 7.8</td>
<td>18.2 ± 3.7</td>
<td>4.0 ± 1.8</td>
<td>10.1 ± 1.8</td>
</tr>
<tr>
<td>Compound 2</td>
<td>2.9 ± 0.7</td>
<td>2.1 ± 0.5</td>
<td>4.4 ± 1.4</td>
<td>3.2 ± 1.0</td>
</tr>
<tr>
<td>Compound 3</td>
<td>2.7 ± 0.5</td>
<td>2.4 ± 0.4</td>
<td>2.8 ± 0.8</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>5.2 ± 0.9</td>
<td>2.8 ± 0.4</td>
<td>4.8 ± 0.8</td>
<td>4.2 ± 0.7</td>
</tr>
<tr>
<td>Artemisinin</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Trolox</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* 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-diocetylthiophenolamine (10% in CHCl3) and was chromatographed on silica gel eluted with CHCl3-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 CH2Cl2-MeOH-H2O (50:25:5 v/v/v) solvent system, and further developed using an orthophosphoric acid solution of naphthoresorcinol 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 24 h 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 performed in triplicate for each sample.

Antiplasmodial assays
The culture of Plasmodium falciparum strains and the antiparasomal assay were carried out as previously described by Frederich et al., 2001. Both strains were obtained from Prof Grellet (Musée 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). Artemisinin (98%, Sigma-Aldrich) was used as standard (IC50 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., 1995. 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-D-glucopyranoside (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 compounds 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 IC50 of 4.5 ± 0.7μg/mL for MDA-MB-231 and 4.8 ± 0.9μg/mL for MCF-7 cells. The cytotoxic activity was related to the saponins, which induced a potent cytotoxicity, with an IC50 around 3μM. These activities confirmed the results previously reported on human colon carcinoma cells, and the hypothesis that the interferglycosidic 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 IC50 values of 22.1 ± 0.6; 54.4 ± 2.8; 20.3 ± 1.4μg/mL, respectively, while trolox showed a pronounced activity (IC50 = 12.7 ± 0.5μM). When the isolated compounds 1-3 were evaluated, a strong scavenging effect was obtained with compound 1 with the lowest IC50 values of 20.1 ± 0.8μM while both 2 and 3 exhibited very weak radical scavenging activity as the IC50 values were higher (>100μM). Overall, among the tested compounds, spirostane glycosides 1 and 3 were the most active.
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 antioxidant activity and the two saponins exhibit potent cytotoxic and weak antiplasmodial activities.

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