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Research Article

Antiproliferative activity of extract from *in vitro* callus cultures of *Astragalus vesicarius* ssp. *carniolicus* (A. Kern.) Chater

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Abstract

Five isoflavonoids, i.e. 5-hydroxy-7-methoxy-2', 5'-dihydroxyisoflavone (AV4), 5, 7-dihydroxy-4'-methoxyisoflavone (AV6), 7-methoxy-5-hydroxy-4'-methoxy-2'-hydroxyisoflavone (AV7), 8-pregnyl genistein (AV9), 5,7-dihydroxy-8-pregnyl-4'-methoxy-2'-hydroxyisoflavone (AV10) and one coumarochromone – sophorophenolone (AV8) were isolated from EtOAc of *in vitro* callus cultures of *Astragalus vesicarius* ssp. *carniolicus*, after enzymatic hydrolysis with *β*-glucosidase. Their structures were tentatively elucidated by spectroscopic mean (^{1}H NMR and HR-ESI-MS spectra). Antiproliferative activity of EtOAc extract and isolated aglycones against chemosensitive human promyelocyte cell line HL-60 and its multidrug-resistant variant HL-60/Dox was assessed *in vitro*. Despite the strong activity of EtOAc (IC_{50} 8.8 μg/mL (HL-6, 72 h) to 11.8 μg/mL (HL-60/Dox, 72 h)), prenylated compound AV9 showed also antiproliferative activity – 36.1 μg/mL (HL-60 and HL-60/Dox, 72 h).

Keywords

Astragalus vesicarius ssp. *carniolicus*, *in vitro* callus cultures, antiproliferative activity

Introduction

Astragalus L. Fabaceae (Leguminosae) is the largest genus among angiosperms. It consist of about 3000 species distributed on all continents except Australia. The center of origin and biodiversity of plants of the genus *Astragalus* is Eurasia, and in particular, the mountainous parts of Southwest Asia. It is found mainly in Southwest and Central Asia (1500 species), the Sino-Himalayan region, North America (500 species), the Andes in South America (150 species) and Europe (133 species) (Podlech 2008; Benchadi et al. 2013). The Bulgarian flora is represented by 31 species, including local endemics, nine of which are included in the Red Book of Bulgaria. Endemic plants are an emblematic symbol of the Bulgarian flora and one of the most sensitive and vulnerable units in the natural ecosystems of the country (Anchev 2011). Recently, the interest in the chemical composition of species of the genus *Astragalus* has increased significantly and various flavonoids, amino acids, saponins, alkaloids, polysaccharides, sterols has been established (Xiaoxia et al. 2014). Phytochemical studies on Astragalus species have been conducted due to their effects as immunostimulants or as anticancer agents (Ionkova et al. 2014; Krasteva et al. 2016). In particular, the chemical composition of the dried roots of *Astragalus* spp. protects the heart, brain, kidneys, intestines, liver and lungs from

various diseases associated with oxidative stress (Hong et al. 1992; Shahzad et al. 2016). Various isolated components of *Astragalus* spp. show significant antiproliferative activity. The highest activity against T-cell leukemia (SKW-3) cells was registered for rhamnocitrin 4'-D-galactopyranoside isolated from *Astragalus hamosus* (Krasteva et al. 2008). Flavonoids from the seeds of *Astragalus complanatus* inhibited the growth of human hepatocarcinoma (SMMC-7721 and HepG2) cells *in vitro* (Hu et al. 2009).

In this context, as alternative methods for production of secondary metabolites appear plant cell and tissue culture techniques. Some of the advantages of the *in vitro* techniques are the propagation of the plants in aseptic controlled conditions and their large-scale production in a year-round system without seasonal constraints (Isah et al. 2018). The plant cell techniques provide some high efficiency methods for isolation and extraction of the secondary metabolites within a short time compared from the wild plant populations and the simplicity of the methods from *in vitro* produced tissues makes it suitable for commercial application (Kolewe et al. 2008). Apart these advantages, there are metabolites that can be produced by *in vitro* cultures but generally not found in the intact plants (Pavlov et al. 2005).

Thus, the focus of the present study is to determine the antiproliferative activity of EtOAc extract and isolated aglycones from *in vitro* callus cultures of *A. vesicarius* ssp. *carniolicus*.

Materials and methods

General experimental procedures

Solvents were obtained from Fischer Chemicals (Loughborough, UK) and were at least of analytical grade, whereas solvents used for semi-preparative HPLC analysis, i.e. ACN and MeOH were HPLC grade and were purchased from Fischer Scientific (Loughborough, UK). Fischer Scientific (Loughborough, UK) supplied formic acid (FA). Deuterated solvents as MeOD (99.8 atom % D) were purchased from Sigma-Aldrich (Germany). H_2O was prepared by a Milli-Q system, Millipore (Bedford, MA, USA) and filtered through 0.22 µm membrane filter. Series of chromatographic separation were performed with Diaion HP-20 (Supelco, USA) and Silica gel 60–200 µm (Merck – Millipore, Germany) column chromatography (CC). HPLC system Young Lin 9100 (Hogye – dong, Anyang, Korea) consisting of vacuum degasser, YL9110 quaternary pump, YL 9160 PDA photodiode detector, hand injector and YL Clarity software was used to perform semi-preparative column chromatography and isolation of pure compounds. ^{1}H NMR spectra were recorded on a Bruker AVII+ 600 spectrometer (Bruker, Karlsruhe, Germany), operating at a proton NMR frequency of 600.13 MHz. LC-MS analysis of pure compounds were performed at Thermo Scientific Q Exactive plusquadrupole – Orbitrap mass spectrometer used in ultra-high resolution mode (70 000, at *m/z* 200) coupled with a UPLC Dionex Ultimate 3 000 RSLC system equipped with a RP-18 Kinetex column (2.10 mm × 100 mm, 2.6 µm, Phenomenex (Corporation, Torrence, CA, USA). MS grade solvents ACN and H_2O were used (Fischer). Gradient elution (1.4 min 10% ACN; 7 min 40% ACN; 10 min 100% ACN) of filtered and degassed ACN/H_2O solution of FA 0.1% (v/v); column temperature 30 °C; a flow rate of about 300 µl/min was used during the analysis. The operating conditions of the HR-ESI source ionization device were: 3.5 kV voltage and 320 °C capillary temperature, 25 units of carrier gas flow and 5 units of dry gas flow. All other detector parameters were set in such a way as to obtain the most intense signal from $[M+H]^+$. Nitrogen was used to atomize the samples. All data were recorded and processed using Xcalibur software, version 2.0 (Thermo Fisher).

Plant material

Callus cultures from *A. vesicarius* ssp. *carniolicus* were successfully established and maintained in our lab (Zarev et al. 2019).

Extraction and isolation

The air-dried powdered plant materials from callus cultures of *A. vesicarius* spp *carniolicus* (18 g) were exhaustively extracted with 80% MeOH under reflux (3 × 50 mL). The extract was filtered and concentrated under reduced pressure and the residue was applied to CC over Diaion HP-20 eluted with 500 mL MeOH (0–100%). Derived fractions were concentrated under reduced pressure and the pH of suspended residue was adjusted to 5.0. After adding *β*-glucosidase, each of the fractions was incubated at 35 °C for 12 h. Liquid/liquid parturition derived EtOAc fractions which were subjected to silica gel CC, eluted with CH_2Cl_2/EtOAc (6:1, 5:1, 3:1, 2:1, 1:1). Fractions 6:1 and 5:1 were subjected to CC over Diaion eluted with MeOH (50%, 80% and 100%). Semi-preparative HPLC analysis was performed with HPLC grade ACN in H_2O, each containing 0.1% FA with RP-18 Ascentis column (25 cm × 4.6 mm, 10 µm) at a flow rate of 4 mL/min. The mobile phase gradient as follows: 0 to 20 min – 40% ACN, 25 to 35 min – 45%, 35 to 36 min – 55%, 36 to 39 min – 100%, until 40 min back to 40% ACN. HPLC analyses conducted at UV detection of 260 nm resulted with isolation of six compounds – AV4, AV6, AV7, AV8, AV9 and AV10.

Biological evaluation

The cell survival of all six pure compounds and EtOAc extract were tested against chemosensitive human promyelocyte cell line HL-60 and its multidrug-resistant variant HL-60/Dox. Concentration ranged from 0.001 µg/mL to 100 µg/mL.

Table 1. 1H NMR data for isolated compounds.

Position	AV4	AV6	AV7	AV8	AV9	AV10
2	1H, δ 8.10, s	1H, δ 8.12, s	1H, δ 8.20, s		1H, δ 8.15, s	1H, δ 8.22, s
6	1H, δ 6.60, d (2.29)	1H, δ 6.59, d (2.38)	1H, δ 6.59, d (2.24)	1H, 6.76, d (2.24)	1H, 6.28, s	1H, δ 6.31, s
8	1H, δ 6.43, d (2.36)	1H, δ 6.40, d (2.20)	1H, δ 6.41, d (2.36)	1H, δ 6.50, d (2.25)		
2'		1H, δ 7.42, dt (2.96, 8.74)			1H, δ 7.38, dt (2.93, 8.62)	
3'	1H, δ 7.09, d (8.20)	1H, δ 6.88, dt (2.93, 8.67)	1H, δ 6.88, d (8.12)	1H, δ 7.07, d (2.00)	1H, δ 6.85, dt (2.98, 8.66)	1H, δ 6.88, d (8.00)
4'	1H, δ 6.40, dd (2.43, 8.51)		1H, δ 7.01, dd (2.04, 8.08)			1H, δ 7.01, dd (1.63, 8.13)
5'		1H, δ 6.88, dt (2.93, 8.67)		1H, δ 6.96, dd (2.10, 8.40)	1H, δ 6.85, dt (2.98, 8.66)	
6'	1H, δ 6.41, d (2.07)	1H, δ 7.42, dt (2.96, 8.74)	1H, δ 7.21, (2.05)	1H, δ 7.85, d (8.34)	1H, δ 7.38, dt (2.93, 8.62)	1H, δ 7.20, d (2.29)
1"					2H, δ 3.41, (7.05)	2H, δ 4.14, dd (4.16, 10.96); 4.05, dd (6.19, 10.61)
2"					1H, δ 5.20, t	1H, δ 5.20, t
4"					3H, δ 1.67, s	3H, δ 1.66, s
5"					3H, δ 1.80, s	3H, δ 1.80, s
OCH_3 (3H)	3H, δ 3.92, s	3H, δ 3.92, s	3H, δ 3.92, s	3H, δ 3.94, s		3H, δ 3.93, s
OCH_3 (3H)			3H, δ 3.92, s			

[a] Spectra were recorded at 600 MHz. Solvent: MeOD

Results and discussion

Identification of isolated compounds

Based on the ^{1}H NMR data (Table 1), HR-ESI-MS spectra and UV spectrum (Suppl. material 1: Fig. S1) tentatively were identified five derivatives of isoflavones and one coumarochromone. Compound AV4 was isolated as a white powder (0.5 mg) and a protonated molecular ion with *m/z* 301.0700 $[M+H]^+$ was observed in HR-ESI-MS analysis, corresponding to a molecular formula of $C_{16}H_{13}O_6$ (calcd for *m/z* 301.0707) (Suppl. material 1: Fig. S2). The UV spectrum of the compound AV4 shows two maxima at 260 nm and 290 nm (Suppl. material 1: Fig. S1). Observing a single proton δ_H 8.10 (s, 1H, H-2) and UV maximum of 260 nm, we assume the structure to isoflavonoids (Fig. 1). The presence of two protons with the following chemical shifts and coupling constants δ_H 6.60 (d, *J* 2.29 Hz, 1H, H-5) and δ_H 6.43 (d, *J* 2.36 Hz, 1H, H-7) is associated with C-5 and C-7 substitution in ring B of compound AV4. A singlet at δ_H 3.92 (s, 3H) is characteristic for methoxy group in the molecule. Considering the coupling constants of the remaining protons in the aromatic region of the spectrum, it is visible the presence of two vicinal protons at δ_H 7.09, (d, *J* 8.20 Hz, 1H, H-3') and 6.40, (dd, *J* 2.43, 8.51 Hz, 1H, H-4'). The proton at H-4' is a doublet of doublets with a low value of the spin-spin constant, which is marker for H-atom at meta position δ_H 6.41 (d, 2.07, 1H, H-6') (Moco et al. 2006). Therefore, the structure of compound AV4 was established as 5-hydroxy-7-methoxy-2', 5'-dihydroxyisoflavone (Suppl. material 1: Fig. S3 and Suppl. material 1: Fig. S4).

Compound AV6 was isolated as a white powder (0.1 mg) and a protonated molecular ion with *m/z* 285.0752 $[M+H]^+$ was observed in HR-ESI-MS analysis, which corresponded to a molecular formula $C_{17}H_{14}O_6$ (calcd for *m/z* 285.0758) (Suppl. material 1: Fig. S5). The UV spectrum of the compound showed three maxima at 202 nm, 262 nm and 294 nm (Suppl. material 1: Fig. S1). Similarly, to compound AV4 in the ^{1}H NMR spectrum of compound AV6, a single proton at δ_H 8.12 (s, 1H, H-2) proved the isoflavonoid skeleton (Fig. 1). The

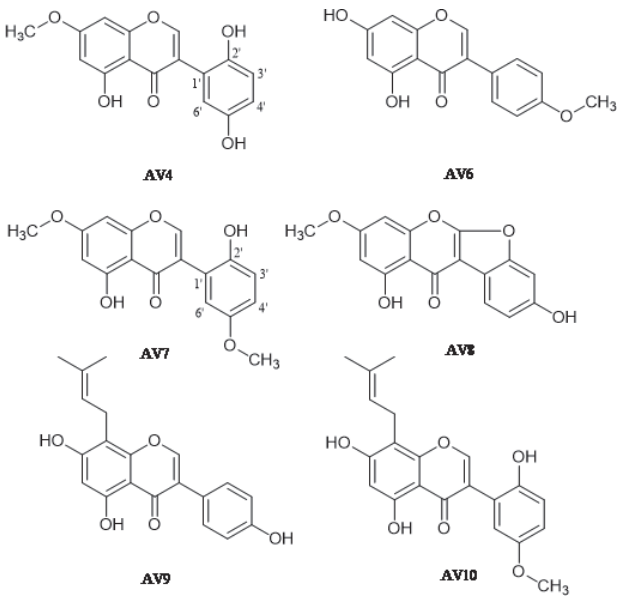

Figure 1. Structures of isolated aglycones from *in vitro* callus cultures of *A. vesicarius*.

arrangement of the substituents in ring A is also identical to that of compound AV4, which is confirmed by the presence of two protons with low values of the spin-spin constant located in the aromatic region – δ_H 6.59 (d, *J* 2.38, 1H, H-5) and δ_H 6.40 (d, *J* 2.20, 1H, H-7) (Suppl. material 1: Fig. S6 and Suppl. material 1: Fig. S7). The presence of two doublet doublets at δ_H 7.42 (dt, *J* 2.96, 8.74, 2H, H-2' and H-6') and δ_H 6.88 (dt, *J* 2.93, 8.67, 2H, H-3' and H-5') suggests replacement of ring B at 4' position. A singlet at δ_H 3.92 (s, 3H) is characteristic of a methoxy group in the molecule. Thus, compound AV6 was defined as 5, 7-dihydroxy-4'-methoxyisoflavone.

Compound AV7 was isolated as a white powder (0.2 mg) and a protonated molecular ion with *m/z* 315.0855 $[M+H]^+$ was observed in HR-ESI-MS analysis, which corresponds to a molecular formula $C_{17}H_{15}O_6$ (calcd for *m/z* 315.0863) (Suppl. material 1: Fig. S8). The UV spectrum of the compound showed two maxima at 262 nm and 294 nm (Suppl. material 1: Fig. S1). The chemical shifts in the ^{1}H NMR spectrum are similar to

those for compound AV4, as well as coupling constants (Table 1). The difference between both compounds is the presence of an additional methoxy group in AV7 δ_H 3.93 (s, 3H). Thus, compound AV7 is defined as 7-methoxy-5-hydroxy-4'-methoxy-2'-hydroxyisoflavone (Suppl. material 1: Fig. S9 and Suppl. material 1: Fig. S10).

Compound AV8 was isolated as a white powder (0.4 mg) and a protonated molecular ion with *m/z* 299.0545 $[M+H]^+$ was observed in HR-ESI-MS analysis, which corresponds to a molecular formula $C_{16}H_{11}O_6$ (calcd for *m/z* 299.0550) (Suppl. material 1: Fig. S11). The UV spectrum of the compound showed three maxima at 256 nm, 282 nm and 338 nm (Suppl. material 1: Fig. S1). In the MS/MS spectra of compound AV8 was observed increase of a RDB value with one unit, suggesting the presence of one more heterocycle in the structure when compared to the compounds described above. The lack of a characteristic singlet for a proton atom at H-2 or H-3 position is another evidence of the formation of an additional heterocycle in the molecule. Like compound AV4, the location of the substituents in ring A is determined on the basis of the chemical shifts and coupling constants – δ_H 6.76 (d, *J* 2.24, 1H, H-6), δ_H 6.50 (d, *J* 2.25, 1H, H-8). Protons from the aromatic region and their spin-spin constants are characteristic of substitution in 4' position δ_H 7.07 (d, *J* 2.00, 1H, H-3'), δ_H 6.96 (dd *J* 2.10, 8.40, 1H, H-5'), δ_H 7.85 (d, *J* 8.34, 1H, H-6'). Signal at δ_H 3.94 (s, 3H) also indicates the presence of a methoxy group in the molecule, thus the compound AV8 is defined as sophorophenolone (Suppl. material 1: Fig. S12 and Suppl. material 1: Fig. S13) (Tang et al. 2002).

Compound AV9 was isolated as a white powder (0.3 mg) and a protonated molecular ion with *m/z* 339.1221 $[M+H]^+$ was observed in HR-ESI-MS analysis, which corresponds to a molecular formula $C_{20}H_{19}O_5$ (calcd for *m/z* 339.1227) (Suppl. material 1: Fig. S14). The UV spectrum of the compound shows two maxima at 261 nm and 333 nm (Suppl. material 1: Fig. S1). In the ^{1}H NMR spectrum of compound AV9, similarly to the series of described compounds a single proton at δ_H 8.15 (s, 1H, H-2) was observed. The ^{1}H NMR spectrum of compound AV9 is similar to that of compound AV6, with no signal for a methoxy group and proton H-8 in the molecule. Chemical shifts for protons at δ_H 3.41 (d, *J* 7.05, 2H, H-1"), δ_H 5.20 (t, 1H, H-2"), δ_H 1.67 (s, 3H, H-4"), δ_H 1.80 (s, 3H, H-5") are characteristic for a prenylated substituent. Thus, compound AV9 was defined as 8-pregnyl genistein (Suppl. material 1: Fig. S15).

Substance AV10 was isolated as a white powder (0.4 mg) and a protonated molecular ion with *m/z* 369.1329 $[M+H]^+$ was observed in HR-ESI-MS analysis, which corresponds to a molecular formula $C_{21}H_{21}O_6$ (calcd for *m/z* 369.1338) (Suppl. material 1: Fig. S16). The UV spectrum of the compound shows two maxima at 267 nm and 347 nm (Suppl. material 1: Fig. S1). Similarly to the above described compound, in the ^{1}H NMR spectrum of compound AV10, a single proton at δ_H 8.22 (s, 1H, H-2) was observed as well as signals for prenylation at C-8. The chemical shifts of the protons from the aromatic region showed a substitution in ring B identical to that in AV7. Thus, compound AV10 was defined as 5, 7-dihydroxy-8-pregnyl-4'-methoxy-2'-hydroxyisoflavone (Suppl. material 1: Fig. S17).

Screening for antiproliferative activity

EtOAc extract from callus of *A. vesicarius* ssp. *carniolicus* showed the highest antiproliferative activity. IC_{50} values were in the range of 8.8 μg/mL (HL-6, 72 h) to 11.8 μg/mL (HL-60/Dox, 72 h) (Table 2). Based on the results from the treated cell lines HL-60 and HL-60/Dox, it could be concluded that compound AV9 showed highest antiproliferative activity – 36.1 μg/mL (HL-60 and HL-60/Dox, 72 h), which may be due to prenylation of the molecule. Compound AV4 possess also strong antiproliferative activity within the range of 35.2 μg/mL (HL-60/Dox, 72 h) to 38.9 μg/mL (HL-60, 72 h). Nevertheless, compound AV8 had low antiproliferative activity. IC_{50} was in the range of 78.0 μg/mL on HL-60 treated cells to 63.0 μg/mL on HL-60/Dox cell line (Suppl. material 1: Fig. S18 and Suppl. material 1: Fig. S19).

Table 2. *In vitro* cytotoxicity (mean IC_{50} values, [μg/ml] ± SD) of the tested pure compounds and EtOAc fraction on the chemosensitive human promyelocyte cell line (HL-60) and its multidrug-resistant variant (HL-60/Dox).

Compounds	Cell lines	
	HL-60	HL-60/Dox
AV-4	38.9 ± 3.8	35.2 ± 2.7
AV-6	41.4 ± 1.4	42.4 ± 3.5
AV-7	64.1 ± 5.2	41.8 ± 2.2
AV-8	78.0 ± 8.2	63.0 ± 8.9
AV-9	36.1 ± 2.3	36.l ± 3.4
AV-10	56.3 ± 1.9	56.8 ± 8.5
EtOAc extract	8.8 ± 0.4	11.8 ± 1.7

Conclusion

The results herein provide additional phytochemical and biological data concerning the flavonoids isolated from *Astragalus vesicarius* ssp. *carniolicus*. Five isoflavonoids and one coumarochromone aglycones were characterized. Their antiproliferative activity was evaluated against chemosensitive human promyelocyte cell line HL-60 and its multidrug-resistant variant HL-60/Dox in MTT test. Some of those aglycones as well as the output for isolation EtOAc fraction displayed strong antiproliferative activity, especially prenylated derivatives such as compound AV9. Therefore, *in vitro* cultures of plant cells represent a promising future for the production of many valuable compounds.

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Supplementary material 1

Figure S1–S19

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