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Secondary Metabolites from *Astragalus lycius* and Their Cytotoxic Activities

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Dedicated to Prof. Dr. Wilhelm Fleischhacker on account of his 85th Birthday.

Eight known secondary metabolites were isolated from the methanolic extract of the whole plant of *Astragalus lycius* Boiss. They were identified as 5,5'-dihydroxy-3'-methoxy-isoflavone-7-*O*-*β*-D-glucoside (**1**), genistin (**2**), sissotrin (**3**), 5,4'-dimethoxy-isoflavone-7-*O*-*β*-D-glucopyranoside (**4**), (7*S*,8*R*)-5-methoxydehydrodiconiferyl alcohol-4-*O*-*β*-D-glucopyranoside (**5**), 4-*O*-lariciresinol-glucoside (**6**), 2-phenylethyl-*β*-D-glucopyranoside (**7**) and *β*-sitosterol-3-*O*-*β*-D-glucopyranoside (**8**) by spectroscopic methods including ^{1}H- and ^{13}C-NMR and HR-MS experiments, and by comparison with literature values. Compounds **1-7** are reported for the first time from *Astragalus* taxa. All of the compounds were tested for their cytotoxic activities against a number of cancer cell lines. Among them, only **6** exhibited significant activity against human colon carcinoma (HT-29) at 2.69 μM concentration.

Keywords: *Astragalus lycius*, Isoflavone, Lignan, Cytotoxic activity, Cancer, Chemotaxanomy.

Leguminosae is a family of flowering plants comprising about 269 genera and 5100 species [1]. *Astragalus* L. is represented by 62 sections, 447 species in the Flora of Turkey, 50% of which are endemic [1, 2]. In Turkish folk medicine, while the aqueous extracts of some *Astragalus* species are used to treat leukemia besides wound healing properties [3a,b] in the East part of Anatolia, gums of some *Astragalus* species used to cure sore throat, stomach aches and some cancers [4]. Decoctions prepared from the roots are also used in the treatment of diabetes and jaundice [5]. It has been reported that a series of triterpene saponins both oleanane and cycloartane types together with flavonoids were isolated in previous studies performed on *Astragalus* species [6a,b,c,d].

As part of our studies on the chemical constituents of Turkish *Astragalus* species, we carried out a study on an endemic species, namely *A. lycius* Boiss. (Leguminosae). This is the first phytochemical study reported from the section Onobrychium, describing isolation and structure determination of eight known secondary metabolites from the methanolic extract of the whole plant of *A. lycius*. The isolates were identified by ^{1}H- and ^{13}C-NMR as well as ESI-MS and HR-MS analyses. Seven of the isolated compounds are reported for the first time from *Astragalus* taxa.

The methanolic extract of the air-dried and powdered plant material of *A. lycius* was separated by a combination of VLC (vacuum liquid chromatography) on stationary and reverse phases, CC (column chromatography) on silica gel and Sephadex LH-20 yielding eight glycosides (**1**-**8**). All isolated compounds were in monodesmosidic form possessing the same sugar moiety, β-glucose. Furthermore, compounds **1-4** were isoflavonoid glycosides with different substitution patterns on the A and B rings (Figure 1).

The HR-MS spectra of **1** showed major ion peaks in the positive and negative modes at *m/z* 463.1278 $[M+H]^+$ and 497.0490 $[M+Cl]^-$ respectively in accordance with the molecular formula $C_{22}H_{22}O_{11}$. The NMR data of **1** were consistent with the presence of an isoflavonoid type glycoside with a glucose moiety at C-7 position. All NMR data presented in Table 1 were superimposable with those reported by Vitor et al. [7]; therefore, the structure of **1** was identified as 5,5'-dihydroxy-3'-methoxy-isoflavone-7-*O*-*β*-D-glucopyranoside**.**

The MS spectrum of **2** (*m/z* 467.0409 $[M+Cl]^-$) revealed the molecular formula $C_{21}H_{20}O_{10}$, 30 amu less than **1** implying loss of a methoxy group ($C_{21}H_{20}O_{10}$). A glycosidation with same sugar residue at C-7 was evident from the NMR data (Table 1), superimposable with those reported for **1** [7]. Thus, compound **2** was established as genistin. In a similar fashion by using MS analyses and 1D NMR data, structures of compounds **3** and **4** were determined as sissotrin [7] and 5,4'-dimethoxy-isoflavone-7-*O*-*β*-D-glucopyranoside [8]. The molecular formula of **5** was determined as $C_{26}H_{32}O_{11}$ based on the MS analyses (*m/z* 555.1200, $[M+Cl]^-$). A comparison of **5**'s NMR data (Table 1) with those reported by Machida et al. [9] led to the identification of **5** as (7*S*,8*R*)-5-methoxydehydro-diconiferyl alcohol-4-*O*-*β*-D-glucopyranoside. The NMR data of **6** (Table 1) were superimposable with those reported for 4-*O*-lariciresinol-glucoside [10]**.** The structure of **7** was readily deduced from the NMR data as 2-phenylethyl-*β*-D-glucopyranoside, a simple phenyl ethanoyl glycoside [11a,b]. All compounds (**1**-**7**) are reported for the first time from *Astragalus* taxa. Additionally, a phytosterol (**8**) was isolated from CH_2Cl_2 extract and identified as *β*-Sitosterol-3-*O*-*β*-D-glucopyranoside, a common metabolite encountered in plant kingdom, based on NMR data in comparison with those reported in the literature [12a,b].

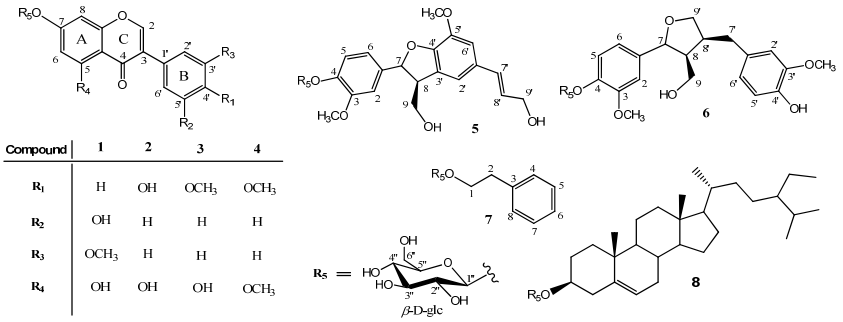

Figure 1: Formula of compounds **1-8**

Table 1: ^{13}C-NMR data for compounds **1-7** (150 MHz, DMSO-d_6).

	1	**2**	**3**	**4**	**5**	**6**	**7**
1	-	-	-	-	135.8	135.1	69.9
2	155.2	154.9	155.5	154.8	115.8	113.2	36.1
3	122.9	122.7	122.9	122.3	149.7	149.2	139.2
4	180.9	180.8	181.1	180.3	118.4	145.9	129.4
5	162.1	162.0	163.8	164.9	144.2	115.9	128.7
6	100.1	100.1	100.3	99.5	118.5	118.2	126.5
7	163.4	162.9	162.3	162.7	87.3	82.1	128.7
8	95.1	95.0	96.3	94.4	53.7	52.9	129.4
9	157.6	157.6	157.8	157.1	62.1	59.1	-
10	106.6	106.6	106.8	106.1	-	-	-
1′	123.6	123.5	123.4	122.6	131.1	138.1	-
2′	116.8	130.6	130.8	136.7	110.8	115.5	-
3′	148.2	115.6	114.5	113.7	146.7	147.9	-
4′	112.5	155.4	159.9	159.2	147.5	145.1	-
5′	146.6	115.3	114.5	113.7	129.7	121.1	-
6′	120.4	130.6	130.8	136.7	115.4	132.1	-
7′	-	-	-	-	133.7	32.6	-
8′	-	-	-	-	128.6	42.4	-
9′	-	-	-	-	63.4	72.4	-
OCH$_3$	56.2	-	55.8	55.2	56.2	56.1	-
OCH$_3$	-	-	-	57.8	56.2	56.2	-
1″	100.4	100.4	100.6	99.2	100.5	100.6	103.3
2″	73.5	73.6	73.8	73.8	73.7	73.7	73.9
3″	76.8	76.9	77.3	76.1	77.3	77.3	77.2
4″	70.8	70.1	70.3	70.0	70.1	70.2	70.6
5″	77.6	77.7	77.9	76.2	77.5	77.5	77.4
6″	61.1	61.1	61.3	64.0	61.2	61.2	61.6

Compounds **1**−**7** were evaluated for their cytotoxic activity against three cancer cell lines (PC3, HT-29 and MDA-MB-231) and a transformed cell line (HEK 293) using the MTT method. None of the isolates displayed cytotoxicity toward the prostate cancer cell line PC-3. The isoflavone metabolites (**1**-**4**) and the dehydrodiconiferyl alcohol derivative (**5**) exhibited modest cytotoxicity against HT-29 and MDA-MB-231 with IC_{50} values ranging from 29.76 to 73.01 μM. Against the colon carcinoma HT-29, the activity of 4-*O*-lariciresinol-glucoside (**6**) with an IC_{50} value of 2.69 μM was noteworthy. Lariciresinol and its derivatives were shown to be cytotoxic agents against a series of cancer cell lines, viz. P-388, SK- MEL 2, SGC-7901 and SMMC-7721 [13a,b,c]. 4-*O*-lariciresinol-glucoside's cytotoxicity was reported by Kim et al. against the melanoma cell line SK-MEL 2 (IC_{50} = 13.46 μM), whereas the compound was found to be inactive versus HCT-15, a colon carcinoma cell line (IC_{50} = >30 μM) [13c].

Cytotoxicity of lignans can be associated with different mechanisms such as topoisomerase II inhibition, anti-tubulin, oestregonic and anti-angiogenic effects. Since compound **6** exhibits a selective and potent cytotoxicity compared to the positive control doxorubicin, further studies are warranted to investigate its toxicity against different cancer cell lines as well as its mechanism of action.

Saponins, polysaccharides and flavonoids, are the principle active constituents of *Astragalus* [14]. It also includes components such as

Table 2: IC_{50} values (μM) of *A. lycius* compounds (**1-7**) and doxorubicin as positive control.

Compound	IC_{50} (μM)			
	PC-3	HT-29	MDA-MB-231	HEK 293
1	>100	29.76 ± 2.5	35.37 ± 1.6	>100
2	86.13 ± 3.9	55.30 ± 1.8	46.82 ± 3.1	>100
3	>100	47.24 ± 1.9	>100	>100
4	>100	41.28 ± 1.7	73.01 ± 3.8	60.85 ± 2.3
5	>100	39.07 ± 1.7	88.12 ± 4.9	>100
6	>100	2.69 ± 0.2	>100	>100
7	>100	97.29 ± 2.1	>100	>100
Doxorubicin	9.16 ± 0.8	7.35 ± 0.9	11.08 ± 1.4	1.58 ± 0.3

β-sitosterol, anthraquinones, alkaloids, amino acids and metallic elements.

Consequently, as part of our ongoing works on the Turkish *Astragalus* species, we investigated the glycosidic contents of *A. lycius*. We have shown that *A. lycius*, which belongs to Section Onobrychium, contains only phenolic and flavonoid type glycosides. Based on earlier phytochemical studies, 31 out of 447 Turkish *Astragalus* species, from 17 different sections, have been investigated for their secondary metabolite contents. Most of the studied sections, namely Sect. Pterophorus (*A. brachypterus* [15a], *A. baibutensis* [15b], *A. trojanus* [15c], *A. ptilodes* var. *cariensis* [15d], *A. wiedemannianus* [15e], *A. tmoleus* var. *tmoleus* [15f]), Sect. Rhacophorus (*A. microcephalus* [16a], *A. zahlbruckneri* [16b], *A. cephalotes* var. *brevicalyx* [16c], *A. amblolepis* [16d], *A. pycnocephalus* var. *pycnocephalus* [16e], *A. erinaceus* [16f], *A. prusianus* [16g], *A. schottianus* [16h] and *A. plumosus* [6a]), Sect. Macrophyllium (*A. oleifolius* [17a]), Sect. Proselius (*A. campylosema* ssp. *campylosema* [17b], *A. elongates* [17c]), Sect. Stereocalyx (*A. stereocalyx* [17d]), Sect. Christiana (*A. gilvus* [17e] and *A. melanophrurius* [3]), Sect. Halicacabus (*A. halicacabus* [17f]), Sect. Hypoglottis (*A. cicer* [15d]) and Sect. Adiaspastus (*A. aureus* [18]), provided cycloartane glycosides, whereas cycloartane and oleanane type saponins were encountered together in a few sections, Sect. Chronopus (*A. hareftae* [6b] and *A. icmadophilus* [6c]), Sect. Melanocercis (*A. angustifolius* [19a]), Sect. Eustales (*A. flavescens* [19b]) and just one species of Sect. Rhacophorus (*A. pycnocephalus* [16e]). The sect. Malacothrix (*A. tauricolus* [20] were provided oleanane type saponins wholly.

A phytochemical study on *A. lycius* from Sect. Onobrychium resulted in the isolation of eight secondary metabolites. Interestingly, no cycloartane or oleanane type triterpene glycoside, the main constituents of *Astragalus* spp., was isolated. This peculiar feature characterizes a very limited group of *Astragalus* spp. such as Sect. Hymenostegis (*A. lagurus* [21a]) and Sect. Vulneraria (*A. vulneraria* [21b]; *A. onobrychis* [21c]).

Our results clearly indicate that the presence of cycloartane type saponins is not the case for all *Astragalus* spp. Since *A. lycius* is

only phytochemically studied species of the Sect. Onobrychis including 31 species, it is not rational to place the section amongst cycloartane deficient members of the genus. Further studies are warranted for the Sect. Onobrychis to clarify its chemotaxonomic significance in the genus *Astragalus*.

Experimental

Plant Material: *A. lycius* Boiss. was collected from Cevizli Village, Muradiye from altitude of 1754 m, Van, East Anatolia, Turkey in June 2010. Samples of the plant material were identified by Fevzi. Özgökçe (Yüzüncü Yıl University, Faculty of Science & Art, Department of Biology, Van, Turkey). Voucher specimen has been deposited in the Herbarium of Yüzüncü Yıl University, Van, Turkey (F 13 739).

Extraction and Isolation: Air-dried and powdered plant material of *A. lycius* (whole plant; 655 g) was first extracted with *n*-hexane (1 x 4 L) under reflux at 68 ºC and then with MeOH (3 x 4 L) under reflux at 64 ºC for 6 hours (each). After filtration and evaporation procedures, *n*-hexane (2.9 g) and MeOH (55.75 g) extracts were obtained. The MeOH extract (55.75 g) was dissolved in water (500 mL) and partitioned with *n*-hexane (3 x 250 mL), CH_2Cl_2 (3 x 250 ml) and *n*-BuOH (4 x 250 mL), respectively, then *n*-BuOH phase (9.2 g) subjected to vacuum liquid chromatography (VLC), using reverse-phase material (Lichroprep RP-18, 25-40 mm, 130 g). Elution was performed with water (750 mL), H_2O:MeOH (7:3, 1350 mL; 5:5, 1350 mL; 4:6, 1050 mL) and MeOH (1050 mL) to afford 3 main fractions (Fr.1-3). Main fraction (Fr.1, 4.85 g) was subjected to open column chromatography by using Sephadex LH-20, eluted with MeOH to give 2 sub-fractions (Fr.1.A, Fr.1.B). The sub-fraction Fr.1.A (2.35 g) was chromatographed on medium pressure liquid chromatography (MPLC) by using RP-18 as an adsorbent with the solvent system H_2O:MeOH (9:1), which was increased step wise to H_2O:MeOH (6:4) to give 3 sub-fractions. The sub-fraction Fr.1A.1 (274 mg) was applied to Sephadex LH-20, eluted with MeOH to give a sub-fraction (Fr.1.A.1.1, 25 mg) that was further applied to Si gel (Merck. 7734, 18 g) column chromatography. Elution was carried out under isocratic condition with CH_2Cl_2:MeOH (95:5) to give **7** (3.3 mg). The sub-fraction Fr.1.A.2 (300 mg) was applied to Sephadex LH-20, eluted with MeOH to give a sub-fraction (Fr1.A.2.1, 90 mg) that was further applied to Si gel (Merck. 7734, 32 g) column chromatography. Elution was carried out with solvent system CH_2Cl_2:MeOH (96:4) to give **6** (9 mg) and **5** (4.6 mg). Fr.1.A.3 (210 mg) was subjected to open column chromatography by using Sephadex LH-20, eluted with MeOH to give **2** (6.3 mg). The sub-fraction Fr.1.B (650 mg) was subjected to Si gel (Merck. 7734, 80 g) open column chromatography, developed with CH_2Cl_2:MeOH:H_2O (90:10:1) to afford **1** (50 mg).

The CH_2Cl_2 phase (5.5 g) was subjected to Si gel column chromatography, eluting with CH_2Cl_2:MeOH (95:5) to give four main fractions (Fr.A-Fr.D). The main fraction (Fr.A; 280 mg) was precipitated using MeOH. The precipitate (180 mg) was subjected to Si gel column chromatography. Elution was carried out with CH_2Cl_2: MeOH (95:5) yielding **8** (60 mg). The same method was applied to the main fraction (Fr.B; 170 mg) that precipitated using MeOH. The obtained precipitate (72 mg) was subjected to Si gel column chromatography with solvent system CH_2Cl_2:MeOH (95:5) to give **4** (17 mg). The main fraction (Fr.C; 400 mg) was applied to Si gel column chromatography with solvent system CH_2Cl_2:MeOH (95:5) to give **3** (45 mg).

Cell Culture and in vitro Cytotoxicity Assay: Cytotoxicity of *A. lycius* compounds were determined using the general procedure based on cell viability with a modified MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide)] assay [22], which measures the activity of mitochondrial reductase of viable cells colorimetrically. The test was carried out with three cancerogenic cell lines [PC3 (human prostate carcinoma), (ATCC catalog no: CRL-1435TM)], [HT-29 (human colon carcinoma) (ATCC catalog no: HTB-38TM)], [MDA-MB-231 (human breast carcinoma) (ATCC catalog no: HTB-26TM)] and a transformed cell line [HEK 293 (Human Embryonic Kidney 293) (ATCC catalog no: CRL-1573TM)].

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