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LC-MS/MS and GC-MS analyses of three endemic *Astragalus* species from Anatolia towards their total phenolic-flavonoid contents and biological activities

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Abstract

Present work aims to determine the chemical profile and some biological activity of three endemic *Astragalus* species from Anatolia. The chemical contents of *Astragalus leporinus* var. *hirsutus*, *Astragalus distinctissimus* and *Astragalus schizopterus* were characterised by LC-MS/MS and GC-MS. In terms of biological activity; the antioxidant, anticholinesterase, antimicrobial and cytotoxic activities were determined. Additionally, the antioxidant properties of the major components were also determined and compared to the antioxidant capacities of these extracts. The most abundant flavonoids in these *Astragalus* species were determined as rutin (1028.276-13351.76 µg/g extract) and hesperidin (1604.348-9695.435 µg/g extract). A high amount of quinic acid (111302.774 µg/g extract) was detected in *A. schizopterus* methanol extract. Palmitic acid (C16:0) was found to be the major compound in *A. leporinus* var. *hirsutus* (32.9%), *A. distinctissimus* (32.5%), and *A.schizopterus* (23.4%). *A. schizopterus* methanol extract exhibited the highest antioxidant effect in lipid peroxidation (19.62±0.29), DPPH free (54.61±0.38) and ABTS cation radicals scavenging activity (22.01±0.07), and CUPRAC assays. Among all of the extracts, only *A. leporinus* var. *hirsutus* petroleum ether extract showed moderate inhibitory activity against acetyl- and butyryl-cholinesterase enzymes. The methanol extracts of the plants exhibited moderate activity against *C. albicans*. *A. leporinus* var. *hirsutus* methanol extract indicated the most viability against L929 fibroblast cells, and the highest cytotoxic effect against A549 cells. In consideration of our findings , these *Astragalus* species used as animal feed could be a source of naturally, biologically active agents that can be used in food and pharmaceutical industry.

Key words: *Astragalus*, LC-MS/MS, antioxidant, anticholinesterase, antimicrobial

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Anadolu'daki üç endemik Astragalus türünün toplam fenolik-flavonoid içerikleri ve biyolojik aktivitelerine yönelik LC-MS / MS ve GC-MS analizleri

Özet

Bu çalışmada, Anadolu'da yetişen üç endemik *Astragalus* türünün kimyasal içeriği ile bazı biyolojik aktivitelerinin belirlenmesi hedeflenmiştir. *Astragalus leporinus* var. *hirsutus*, *Astragalus distinctissimus* ve *Astragalus schizopterus* türlerinin kimyasal içeriği LC-MS/MS ve GC-MS ile karakterize edilmiştir. Biyolojik aktivite açısından; ekstrelerin antioksidan, antikolinesteraz, antimikrobiyal ve sitotoksik aktiviteleri tespit edilmiştir. Ek olarak, ekstrelerin ana bileşenlerinin de antioksidan özellikleri belirlenerek elde edilen sonuçlar ekstrelerin antioksidan kapasiteleri ile

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karşılaştırılmıştır. Çalışılan *Astragalus* türlerinde en bol bulunan flavonoidin rutin (1028,276-13351,76 µg/g ekstre) ve hesperidin (1604,348-9695,435 µg/g ekstre) olduğu, ayrıca *A. schizopterus* metanol ekstresinde de yüksek miktarda kuinik asit (111302,774 µg/g ekstre) bulunduğu belirlenmiştir. Palmitik asitin (C16:0), *A. leporinus* var. *hirsutus* (%32,9), *A. distinctissimus* (%32,5) ve *A.schizopterus* (%23,4)'da başlıca bileşik olduğu saptanmıştır. *A. schizopterus* metanol ekstresi lipit peroksidasyon (19,62±0,29), DPPH serbest radikal (54,61±0,38) , ABTS katyon radikali süpürücü aktivitesi (22,01±0,07), ve CUPRAC yönteminde en yüksek aktiviteyi göstermiştir. Çalışılan tüm ekstrelerden sadece *A. leporinus* var. *hirsutus* petrol eteri ekstresi orta derecede asetil- ve bütiril- kolinesteraz enzim inhibisyonu sergilemiştir.. Bitkilerin metanol ekstreleri *C. albicans*'a karşı orta derecede antimikrobiyal aktivite ortaya koymuşlardır. *A. leporinus* var. *hirsutus* metanol ekstresi, L929 fibroblast hücrelerine karşı en fazla canlılığı ve A549 hücrelerine karşı en yüksek sitotoksik etkiyi göstermiştir. Çalışmdan elde ettiğimiz bulgular ışığında, hayvan yemi olarak kullanılan bu *Astragalus* türleri, gıda ve ilaç endüstrisinde kullanılabilen doğal, biyolojik olarak aktif maddeler kaynağı olabilir.

Anahtar kelimeler: *Astragalus*, LC-MS/MS, antioksidan, antikolinesteraz, antimikrobiyal

1. Introduction

The genus *Astragalus* L. (Fabaceae), with about 2500–3000 species, is the largest genus of flowering plants in the world. It comprises about 439 taxa, 309 of them are endemic, and it is classified into 63 sections in Turkey (Chamberlain and Matthews 1970; Ozhatay and Kultur 2006). *Astragalus* species have been used in the pharmaceutical, food, loom, paper and cosmetic industries (Golmohammadi, 2013). Some of them has been used to obtain the gum tragacanth (Calis and Sticher, 1996; Yılmaz et al., 2013). In Anatolia, some Turkish *Astragalus* species are known as "geven" and they are generally consumed as animal feed in Turkey (Baytop, 1984).

Phytochemical investigations have shown up the main component of Turkish *Astragalus* species as cycloartane-type triterpene glycosides, a series of oleanane- and triterpenoidal saponins. (Horo et al. 2012; Savran et al. 2012). These compounds have potent pharmacological traits such as being cytotoxic (Krasteva et al. 2008), anti-protozoal (Ozipek et al., 2005), antiviral (Gariboldi et al., 1995), wound healing (Sevimli-Gur et al., 2011), immunostimulating and adjuvant effects (Nalbantsoy et al., 2011).

Astragalosides I-VII obtained from the roots of *Astragalus* species (Calis et al., 1997) have been commonly used as dietary supplement to enhance the immune system, additive in foods and beverages in European, American and Asian countries (Chu et al., 2010). Because of these features, *Astragalus* species have an important potential in the field of health care and food industry (Verotta and Sebakhy, 2001).

Recently, there have been quite a lot of studies related to the biological properties and quantitation of natural phenolic compounds (Ertas et al. 2014a; Awad et al. 2014; Abdelhady et al., 2015). The facts that there isn't any detailed study on phenolic constituents of Turkish A*stragalus* species. The lack of the research on endemic *Astragalus leporinus* Boiss. var. *hirsutus* (Post) Chamberlain, *A. distinctissimus* Eig and *A. schizopterus* Boiss. species have led us to this investigation. Thus, in this study, we tried to determine their total phenolic and flavonoid contents together with their fatty acid compositions. The antioxidant, anticholinesterase, antimicrobial and cytotoxic activities of these species were also investigated. Twenty-four phenolic compounds (flavonoids, flavonoid glycosides, phenolic acids, phenolic aldehyde, coumarin) and three non-phenolic organic acids in the methanol extracts of these *Astragalus* species were analyzed by LC-MS/MS, and chemical composition-activity relationships were evaluated together with chemotaxonomical aspects.

2. Materials and methods

1.1. Plant material

The aerial parts and roots (whole plants) of *Astragalus leporinus* Boiss. var. *hirsutus* (Post) Chamberlain, *A. distinctissimus* Eig and *A. schizopterus* Boiss. were collected from southeastern Turkey (Kahramanmaras) in May, August and May 2012, respectively, and identified by S. Demirci. Voucher specimens were deposited in the Herbarium of Istanbul University, Faculty of Pharmacy (*Astragalus leporinus* var. *hirsutus* ISTE 97142, *A. distinctissimus* ISTE 98035, *A. schizopterus* ISTE 97141).

1.2. Preparation of plant extracts for LC-MS/MS, biological activities and GC-MS

Plant marerials were dried at shadow and powdered.Ten grams of each plant materials were extracted three times with Methanol (50 mL each) at room temperature for 24 hours. Afterwards, the extracts obtained were combined, filtered and evaporated under low pressure. Dry filtrates were reconstituted in methanol at a concentration of 250 mg/L and passed through the 0.2 µm PTFE filter for LC-MS/MS.

Then,100 g plant materials macerated three times with petroleum ether (250 mL), acetone (250 mL), methanol (250 mL) and water (250 mL) at 25 ºC for 24 hours. After filtration, the solvent was evaporated under vacuum. The

yields of the petroleum ether extracts were calculated as *A. leporinus* var. *hirsutus* petroleum ether extract (ALP) 0.8%, *A. distinctissimus* petroleum ether extract (ADP) 0.6%, *A. schizopterus* petroleum ether extract (ASP) 0.60%, the acetone extracts as ALA 1.3%, ADA 0.7%, ASA 0.80%, the methanol extracts as ALM 9.1%, ADM 5.2%, ASM 7.2%, and the water extracts as ALW 2.8%, ADW 2.3%, ASW 3.1% (w/w).

Esterification of the petroleum ether extracts, and GC-MS procedure described by Ertas et al. (2014a; 2014b) were applied. Thermo Scientific Polaris Q GC-MS/MS was used.

1.3. *Identification and quantitation of phenolic compounds*

LC-MS/MS analyses of the phenolic compounds were performed by using a Nexera model Shimadzu UHPLC coupled to a tandem MS instrument. The liquid chromatograph was equipped with LC-30AD binary pumps, DGU-20A3R degasser, CTO-10ASvp column oven and SIL-30AC autosampler. The chromatographic seperation was performed on a C18 reversed-phase Inertsil ODS-4 (150 mm × 4.6 mm, 3μm) analytical column. The column temperature was fixed at 40°C. The elution gradient consisted of mobile phase A (water, 5mM ammonium formate and 0.1% formic acid) and mobil phase B (methanol, 5mM ammonium formate and 0.1% formic acid). The gradient program with the following proportions of solvent B was applied t (min), %B: (0, 40), (20, 90), (23.99, 90), (24, 40), (29, 40). The solvent flow rate was maintained at 0.5 mL/min and injection volume was settled as 4 μL.

Subsequent to several combinations of trials, a gradient of methanol (5mM ammonium formate and 0.1% formic acid) and water (5mM ammonium formate and 0.1% formic acid) system was concluded to be the best mobile phase solution for LC-MS/MS analyses. For rich ionization and the seperation of the molecules, the mentioned mobile phase was proved to be the best of all. ESI source was chosen instead of APCI (Atmospheric Pressure Chemical Ionization) and APPI (Atmospheric Pressure Photoionization) sources as the phenolic compounds were small and relatively polar molecules. Tandem mass spectrometry was decided to be used for the current study since this system is commonly used for its fragmented ion stability (Ertas et al. 2014a; Ertas et al. 2015). The working conditions were determined as interface temperature; 350°C, DL temperature; 250°C, heat block temperature; 400°C, nebulizing gas flow (Nitrogen); 3 L/min and drying gas flow (Nitrogen); 15 L/min.

Detailed information on method validation parameters and Estimation of uncertainty can be provided from previous manuscripts of our study group (Ertas et al. 2014a; Ertas et al. 2015).

1.4. Biological activities of the extracts

In terms of biological activity, the total phenolic and flavonoid contents, antioxidant, anticholinesterase, antimicrobial and cytotoxic activity were determined. The total amount of phenolic and flavonoid contents of extracts was calculated according to Slinkard and Singleton (1977) and Moreno et al. (2000) using following equations and stated as pyrocatechol and quercetin equivalents, respectively.

Absorbance = 0.0123 pyrocatechol (μg) + 0.0349 (R2 = 0.9910)

Absorbance = 0.1701 quercetin (μg) – 0.7078 (R2 = 0.9939)

Antioxidant activity was determined according to the relevant literature by using four different methods, including β-Carotene linoleic acid test system, DPPH free radical scavenging activity, ABTS cation radical decolonization and cupric reducing antioxidant capacity (CUPRAC) assay (Blois, 1958; Miller, 1971; Re et al., 1999; Apak et al., 2004).

Anticholinesterase activity of the extracts was assessed according to the literature (Ellman et al. 1961) based on acetyl- and butyryl-cholinesterase enzyme inhibitory effect detected spectrophotometrically.

Streptococcus pyogenes ATCC19615, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC10231 were used for evaluating the antimicrobial activity. The disc diffusion method (NCCLS, 1997) was established for this purpose. The minimum inhibitory concentration (MIC) of active extracts was also determined (NCCLS, 2009).

Cytotoxic activity was determined by MTT assay performed in accordance with ISO 10993-5 standards. A549 and L929 fibroblast cells, which were stored in liquid nitrogen tank were centrifuged after dissolution. Later, these cells were placed on a 96-well plate after addition of 3 mL (DMEM 10% + fetal bovine serum 100%+ containing 1% antibiotics), and incubated under 5% CO2 and at 37°C. When the cells reached a sufficient growth, they were discharged with trypsin-EDTA solution, and passaging process was continued. The MTT assay is sensitive for cell proliferation measurement that 3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide (MTT) tetrazolium salt is used. MTT is reduced to insoluble formazan dye in water by mitochondrial enzymes associated with metabolic activity. MTT reduction is primarily associated with the glycolytic activity in the cells and depends on the presence of NADH (nicotine amide adenine dinucleotide) and NADPH (nicotinamide adenine dinucleotide phosphate). In the reactions of mitochondria of healthy cells or early stages of apoptotic cells, colored formazan crystals constitute with the degradation of the tetrazolium ring which is found in MTT solution by dehydrogenase enzymes in cell mitochondria. The color change which observed in living cells gives the absorbance values in Elisa reader.

L929 fibroblast and A549 (human lung cancer) cells were cultivated on 96-well plate (10x103 cells/well). Cells were incubated for 24 hours. Later, previously prepared plant extracts at different concentrations (0-12.5µg/mL - 25µg/mL - 50µg/mL - 100µg/mL) was applied onto the cells and then incubated for 24 hours. The samples were studied 5 times. As a positive control, the medium was only applied onto cells. After 24 hours, waste in each well was discarded and 100 µL of medium and 20 µL of MTT solution were added. After 3.5 h incubation at 37 °C, 150 µL of MTT solvent was added to the wells, and cells were incubated for extra 15 minutes. In order to determine cell viability, absorbance values of plates were recorded by ELISA reader at 570 nm. According to the absorbance values of control group, percentage cell viability was calculated.

1.5. Statistical analysis

The results of the antioxidant and anticholinesterase activity assays were represented as means ± SD. The results were evaluated using an unpaired *t*-test and ANOVA variance analysis with the NCSS statistical computer package. The differences were considered statistically significant at $p < 0.05$.

3. Results

1.1. Quantitative analysis of phenolic and flavonoid compounds by LC-MS/MS

Since the research of fruits and vegetables have revealed the protective effect of phenolic acids on oxidative damage diseases such as coronary heart disease, stroke, cancers, cardiovascular diseases and inflammation, phenolic acids have been a remarkable research topic for researchers. Because of their powerful antioxidant properties not only the researchers but also the food manufacturers deal with the phenolic compounds. (Awad et al., 2014; Ertas et al., 2014a). There are some studies about the use of liquid chromatography electrospray ionization tandem mass spectrometry for determining the phenolic compounds quantitatively (Yunfei et al., 2008; Ertas et al., 2014a). In this way, a valid method was developed for the analyses of twenty-four phenolic compounds and three non-phenolic organic acids in the methanol extracts of *A. leporinus* var. *hirsutus*, *A. distinctissimus* and *A. schizopterus*. The specific MRM (Multiple reaction monitoring) fragmentation reactions was selected in order to monitor the aforesaid twenty-seven compounds. Twenty-four phenolic compounds and three non-phenolic organic acids were monitored by the transition from the specific deprotonated molecular ions to the corresponding fragment ions. Molecular ions, fragments observed in MS/MS, related collision energies for these fragments and the quantified results for three *Astragalus* species were presented in Table 1.

Our results showed that the most abundant flavonoids in the three studied *Astragalus* species were rutin (1028.276-13351.76 µg/g extract), hesperidin (1604.348-9695.435 µg/g extract) and hyperoside (228.3135-1992.697 µg/g extract) (Table 1 and Figure 1). ASM extract was found to be the richest extract in terms of myricetin (110.769 µg/g extract), quercetin (75.94 µg/g extract), naringenin (76.0115 µg/g extract), luteolin (86.6995 µg/g extract), kaempferol (123.662 µg/g extract) and apigenin (30.5195 µg/g extract) as well as rutin (13351.76 µg/g extract), hesperedin (9695.435 µg/g extract) and hyperoside (1992.697 µg/g extract) (Table 1 and Figure 1). Furthermore, the highest amount of phenolic acids such as chlorogenic (400.3195 µg/g extract), protocatechuic (1607.988 µg/g extract), p-coumaric (475.859 µg/g extract) and rosmarinic acids (751.17 µg/g extract) was determined in ASM extract. On the other hand, ADM and ALM extracts possessed the highest quantity of 4-OH benzoic acid (1799.27 µg/g extract) and salicylic acid (1696.10 µg/g extract), and tr-caffeic acid (144.7665 µg/g extract), respectively.

When the analysis results were investigated in terms of the non-phenolic organic acids, ALM extract was found to be the richest extract in terms of malic (7802.659 µg/g extract) and tr-aconitic acids (972.159 µg/g extract). Indeed, quinic acid (111302.774 µg/g extract) content of ASM extract was probably the most distinctive result of this study. Because quinic acid is known to be a versatile chiral starting material for the synthesis of new pharmaceutical compounds. To treat influenza A and B strains, a medicine called “Tamiflu” has been developed via this way (Kim et al. 1997). In the previous studies, HPLC-DAD and HPLC-MS/MS techniques were used to detect several phenolic and flavonoid compounds such as rutin, quercetin, rosmarinic acid, kaempferol, calycosin, ononin, formononetin and some derivatives of them in *Astragalus* species (Qi et al., 2008; Yunfei et al., 2008; Montoro et al., 2012; Zhang et al., 2013). As far as we know, this research is the first on detecting naringenin, vanillin, hesperidin, myricetin and protocatechuic, quinic, tr-aconitic and 4-OH benzoic acids in *Astragalus* species.

In the flora of Turkey, the genus *Astragalus* is classified into 63 sections (Chamberlain and Matthews, 1970). *A. leporinus* var. *hirsutus* is grouped into the *Myobroma* Bunge, *A. distinctissimus* into the *Dasyphyllium* Bunge, and *A. schizopterus* into the *Proselius* Bunge sections. Our results indicated that the *Myobroma*, *Dasyphyllium* and *Proselius* sections contain different amount of phenolic compounds and non-phenolic organic acids. According to our analysis results, quinic acid content might be used as a chemotaxonomical marker for *A. schizopterus*. In addition, quercetin, naringenin, luteolin, and kaempferol is encountered in *A. schizopterus* methanol extract.

Table 1. Analytical parameters of LC-MS/MS method, and identification and quantification of phenolic compounds in ALM, ADM and ASM

No	Analyte	Parent ion (m/z)[a]	MS²(CE)[b]	Ionization Mode	RT[c]	R²,[d]	RSD%[e]	Linearity Range (mg/L)	LOD/LOQ (µg/L)[f]	Recovery (%)	U[g]	Quantification (µg analyte/g extract)[h]		
												ALM	ADM	ASM
1	Quinic acid	190,95	85 (22),93 (22)	Neg	3.32	0.9927	0.0388	250-10000	22.3 / 74.5	103.3	4.8	**4814.49±23.23**	**5094.77±244.51**	**111302.77±5342.49**
2	Malic acid	133,05	115 (14),71 (17)	Neg	3.54	0.9975	0.1214	250-10000	19.2 / 64.1	101.4	5.3	**7802.65±413.50**	**7531.38±399.14**	**5286.27±280.16**
3	tr-Aconitic acid	172,85	85 (12),129 (9)	Neg	4.13	0.9933	0.3908	250-10000	15.6 / 51.9	102.8	4.9	972.15±47.62	461.00±22.58	400.83±19.60
4	Gallic acid	169,05	125 (14),79 (25)	Neg	4.29	0.9901	0.4734	25-1000	4.8 / 15.9	102.3	5.1	24.25±1.22	28.18±1.43	290.63±14.79
5	Chlorogenic acid	353	191 (17)	Neg	5.43	0.9932	0.1882	250-10000	7.3 / 24.3	99.7	4.9	173.95±8.47	306.45±14.99	400.31±19.60
6	Protocatechuic acid	152,95	109 (16),108 (26)	Neg	5.63	0.9991	0.5958	100-4000	25.8 / 85.9	100.2	5.1	267.26±13.61	122.04±6.22	**1607.98±81.95**
7	Tannic acid	182,95	124 (22),78 (34)	Neg	6.46	0.9955	0.9075	100-4000	10.2 / 34.2	97.8	5.1	115.50±5.86	50.38±2.55	104.79±5.30
8	tr-Caffeic acid	178,95	135 (15),134 (24),89 (31)	Neg	7.37	0.9942	1.0080	25-1000	4.4 / 14.7	98.6	5.2	144.76±7.48	25.67±1.30	94.80±4.88
9	Vanillin	151,05	136 (17),92 (21)	Neg	8.77	0.9995	0.4094	250-10000	10.1 / 33.7	99.2	4.9	57.34±2.79	77.06±3.77	132.21±6.46
10	p-Coumaric acid	162,95	119 (15),93 (31)	Neg	9.53	0.9909	1.1358	100-4000	15.2 / 50.8	98.4	5.1	460.36±23.46	392.87±19.99	475.85±24.22
11	Rosmarinic acid	358,9	161 (17),133 (42)	Neg	9.57	0.9992	0.5220	250-10000	10.4 / 34.8	101.7	4.9	392.09±19.20	235.44±11.51	751.17±36.79
12	Rutin	609,1	300 (37), 271 (51), 301 (38)	Neg	10.18	0.9971	0.8146	250-10000	17.0 / 56.6	102.2	5.0	**7389.81±369.45**	**1028.27±51.40**	**13351.76±667.55**
13	Hesperidin	611,1	303 (24),465 (12)	Poz	9.69	0.9973	0.1363	250-10000	21.6 / 71.9	100.2	4.9	**9441.78±462.61**	**1604.34±78.59**	**9695.43±475.06**
14	Hyperoside	463,1	300 (27),301 (26)	Neg	10.43	0.9949	0.2135	100-4000	12.4 / 41.4	98.5	4.9	932.24±45.66	228.31±11.17	**1992.69±97.60**
15	4-OH Benzoic acid	136,95	93 (17),65 (27)	Neg	11.72	0.9925	1.4013	25-1000	3.0 / 10.0	106.2	5.2	184.86±9.56	**1799.27±93.54**	**1033.00±53.71**
16	Salicylic acid	136,95	93 (16),65 (31),75 (30)	Neg	11.72	0.9904	0.6619	25-1000	4 / 13.3	106.2	5.0	170.09±8.50	**1696.10±84.80**	**1003.78±50.15**
17	Myricetin	317	179 (19),151(23),137 (26)	Neg	11.94	0.9991	2.8247	100-4000	9.9 / 32.9	106.0	5.9	N.D.[i]	D.[j]	110.76±6.49
18	Fisetin	284,95	135 (22),121 (27)	Neg	12.61	0.9988	2.4262	100-4000	10.7 / 35.6	96.9	5.5	D.	N.D.	N.D.
19	Coumarin	146,95	103 (17),91 (26),77 (27)	Poz	12.52	0.9924	0.4203	100-4000	9.1 / 30.4	104.4	4.9	D	D.	D.
20	Quercetin	300,9	179 (19),151 (21),121 (28)	Neg	14.48	0.9995	4.3149	25-1000	2.0 / 6.8	98.9	7.1	N.D.	N.D.	75.94±5.32
21	Naringenin	270,95	151 (18),119 (24),107 (26)	Neg	14.66	0.9956	2.0200	25-1000	2.6 / 8.8	97.0	5.5	17.92±0.93	D.	76.01±4.18
22	Hesperetin	300,95	164 (25),136 (33),108 (42)	Neg	15.29	0.9961	1.0164	25-1000	3.3/ 11.0	102.4	5.3	N.D.	N.D.	N.D.
23	Luteolin	284,95	217 (25),199 (28),175 (29)	Neg	15.43	0.9992	3.9487	25-1000	5.8 / 19.4	105.4	6.9	39.37±2.69	N.D.	86.69±5.93
24	Kaempferol	284,95	217 (29),133 (32),151 (23)	Neg	15.43	0.9917	0.5885	25-1000	2.0 / 6.6	99.1	5.2	N.D.	N.D.	123.66±6.39
25	Apigenin	268,95	151 (25),117 (35)	Neg	17.31	0.9954	0.6782	25-1000	0.1 / 0.3	98.9	5.3	22.89±1.16	N.D.	30.51±1.59
26	Rhamnetin	314,95	165 (23),121 (28),300 (22)	Neg	18.94	0.9994	2.5678	25-1000	0.2 / 0.7	100.8	6.1	16.40±0.97	N.D.	N.D.
27	Chrysin	253	143 (29),119 (32),107 (26)	Neg	21.18	0.9965	1.5530	25-1000	0.05 / 0.17	102.2	5.3	5.60±0.29	8.02±0.42	6.18±0.31

[a]Parent ion (*m/z):* Molecular ions of the standard compounds (mass to charge ratio), [b]MS²(CE): MRM fragments for the related molecular ions (CE refers to related collision energies of the fragment ions), [c]RT: Retention time, [d]R²: coefficient of determination, [e]R²: coefficient of determination, [f]LOD/LOQ (µg/L): Limit of deteection/Limit of quantification, [g]U (%): Percent relative uncertainty at 95% confidence level (k=2), [h]Values in µg/g (w/w) of plant extract, [i]N.D: not detected, [j]D: peak observed, concentration is lower than the LOQ but higher than the LOD, ALM: *Astragalus leporinus* var. *hirsutus* methanol extract , ADM: *A. distinctissimus* methanol extract , ASM: *A. schizopterus* methanol extract

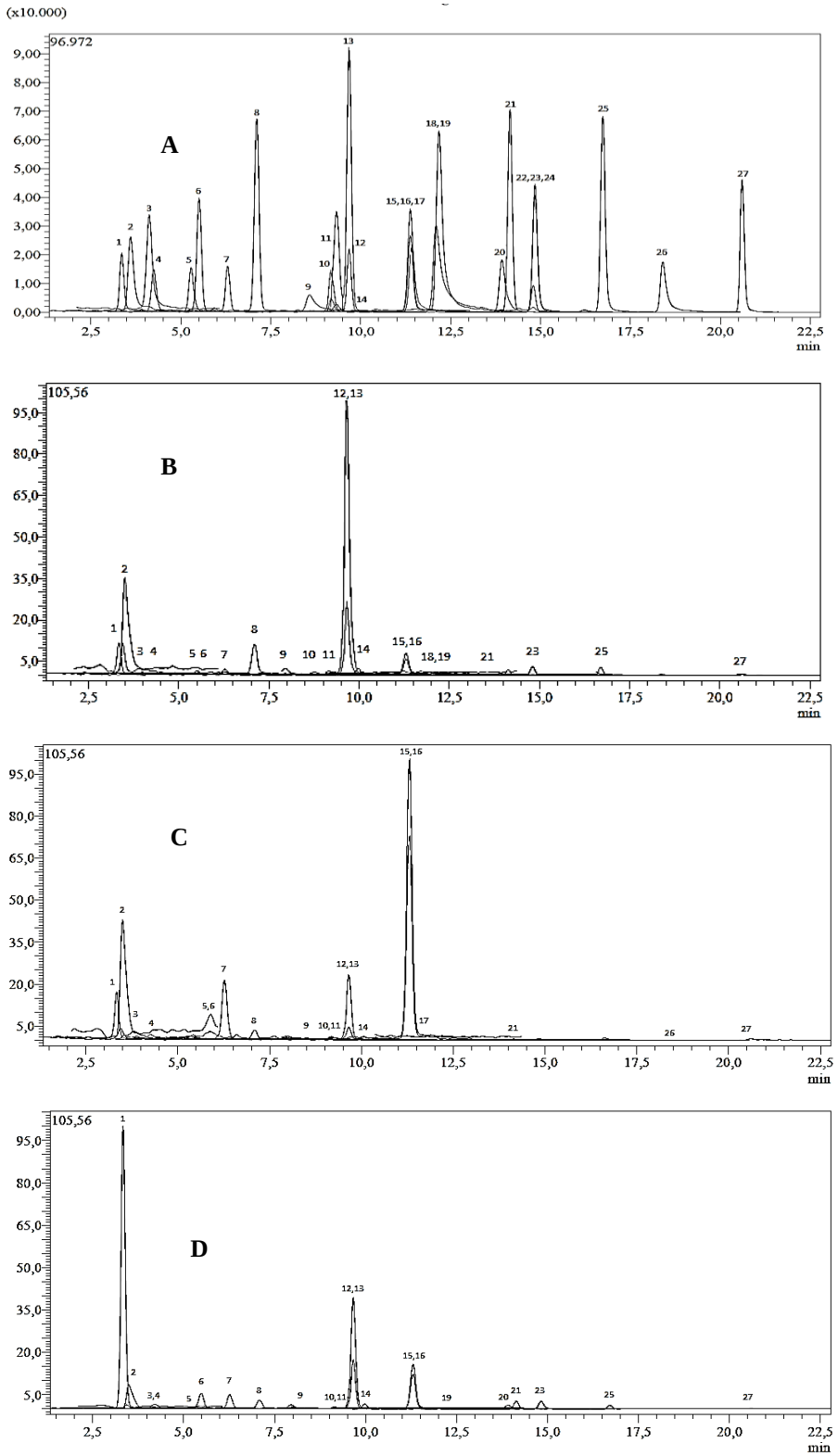

Figure 1. LC-MS/MS chromatograms of A: 250 µg/L of standard mix, B: AL methanol extract, C: AD methanol extract, D: AS methanol extract. 1: Quinic acid, 2: Malic acid, 3: tr-Aconitic acid, 4: Gallic acid, 5: Chlorogenic acid, 6: Protocatechuic acid, 7: Tannic acid, 8: tr-Caffeic acid, 9: Vanillin, 10: p-Coumaric acid, 11: Rosmarinic acid, 12: Rutin, 13: Hesperidin, 14: Hyperoside, 15: 4-OH Benzoic acid, 16: Salicylic acid, 17: Myricetin, 18: Fisetin,19: Coumarin, 20: Quercetin, 21: Naringenin, 22: Hesperetin, 23: Luteolin, 24: Kaempferol, 25: Apigenin, 26: Rhamnetin, 27: Chrysin.

1.1. Fatty acid compositions by GC-MS

The fatty acid compositions of the petroleum ether extracts were determined by GC-MS analysis. As shown in Table 2, eleven components were identified, constituting 99.7% of the petroleum ether extract of *A. leporinus* var. *hirsutus*, and the main constituents were identified as palmitic acid (C16:0) (32.9%), arachidic acid (C20:0) (13.6%) and linoleic acid (C18:2 omega-6) (12.7%). Eleven components were identified, constituting 99.8% of *A. distinctissimus* petroleum ether extract in which palmitic acid (32.5%), linolenic acid (C18:3 omega-3) (17.5%) and linoleic acid (15.9%) were found as major components. Fourteen components were identified, constituting 99.8% of the petroleum ether extract of *A. schizopterus*, and the main constituents were behenic acid (C22:0) (24.5%), palmitic acid (23.4%), arachidic acid (14.0%), stearic acid (C18:0) (8.9%), and oleic acid (C 18:1 omega-9) (8.8%). This is the first report on the fatty acid compositions of these three endemic *Astragalus* species. Palmitic, linoleic and arachidic acids could be chemotaxonomically important for the *Myobroma* section, palmitic, linoleic and linolenic acids for the *Dasyphyllium* section, arachidic and behenic acids for *Proselius* section.

Table 2. GC-MS analysis of ALP, ADP and ASP

Rt (min)[a]	Constituents[b]	Composition (%)		
		ALP	**ADP**	**ASP**
12.00	Lauric acid	-	2.5	0.3
12.75	Nonanedioic acid	-	-	0.3
14.39	10-Undecenoic acid	2.5	-	1.4
18.60	Myristic acid	2.1	2.7	-
24.94	Palmitoleic acid	2.1	-	0.4
25.27	Palmitic acid	32.9	32.5	23.4
28.47	Margaric acid	-	-	0.3
29.75	Phytol	-	-	1.5
30.64	Linoleic acid	12.7	15.9	6.7
30.77	Oleic acid	9.2	7.8	8.8
30.86	Linolenic acid	5.7	17.5	8.3
31.54	Stearic acid	8.9	7.6	8.9
36.23	Nonacosanol	-	6.7	-
37.38	Arachidic acid	13.6	1.3	14.0
38.19	6-Hexadecenoic acid	-	3.8	-
39.36	Docosane	0.8	-	1.0
43.82	Behenic acid	9.2	1.5	24.5
	Total	99.7	99.8	99.8

[a]Retention time (as minute), [b]A nonpolar Phenomenex DB-5 fused silica colum, ALP: *Astragalus leporinus* var. *hirsutus* petroleum ether extract, ADP: *A. distinctissimus* petroleum ether extract, ASP: *A. schizopterus* petroleum ether extract.

There are some studies on fatty acid compositions of *Astragalus* species from Turkey in literature. Bagci (2006) reported that the main constituents of the fatty acids in *A. echinops* Aucher ex. Boiss., *A. subrobustos* Boiss., *A. jodostachys* Boiss. & Buhse., *A. falcatus* Lam., *A. fraxinifolius* DC. were linolenic acid (23–41%), linoleic acid (23–37%), and oleic acid (8–19%). Adiguzel et al. (2006) reported that the major constituents of the fatty acids were identified as *cis*-9-octadecenoic acid (39.23%) for *A. coadunatus*, 25:0 N alcohol for *A. kurdicus* Boiss., palmitic acid (24.66%) for *A. lagurus* Willd, palmitic acid (21.62%) for *A. christianus* L., 1-docosanol (57.85%) for *A. cicer* L. and palmitic acid (32.60%) for *A. atrocorpus* Champ & Matthews. In Bagci's report (2006), the amount of unsaturated fatty acids was found to be higher than the amount of saturated fatty acids in studied *Astragalus* species. However, our results were in accordance with those of Adiguzel et al. (2006); the amount of saturated fatty acids was found to be higher than that of unsaturated fatty acids.

1.2. Antioxidant activity and total phenolic-flavonoid content

The antioxidant activity of the petroleum ether (ALP, ADP and ASP), acetone (ALA, ADA and ASA), methanol (ALM, ADM and ASM) and water (ALW, ADW and ASW) extracts prepared from the whole plants of *A. leporinus* var. *hirsutus*, *A. distinctissimus* and *A. schizopterus*, respectively, was carried out using *β*-carotene bleaching, DPPH free radical scavenging, cupric reducing antioxidant capacity and ABTS cation radical decolorisation assays (Table 3). The total phenolic and flavonoid contents of these extracts were also investigated. Total phenolic and flavonoid amounts in the crude extracts were expressed as pyrocatechol and quercetin equivalents, respectively ($y = 0.0123$ pyrocatechol (μg) + 0.0349 ($R^2 = 0.9910$), and $y = 0.1701$ quercetin (μg) – 0.7078 ($R^2 = 0.9939$)). The phenolic and flavonoid components of the ASM extract were identified to be the richest. The amount of total flavonoid from ASM was about 50 μg/mg according to the quercetin standard substance. The phenolic components were found to be higher than flavonoid components. The results were shown in Table 3.

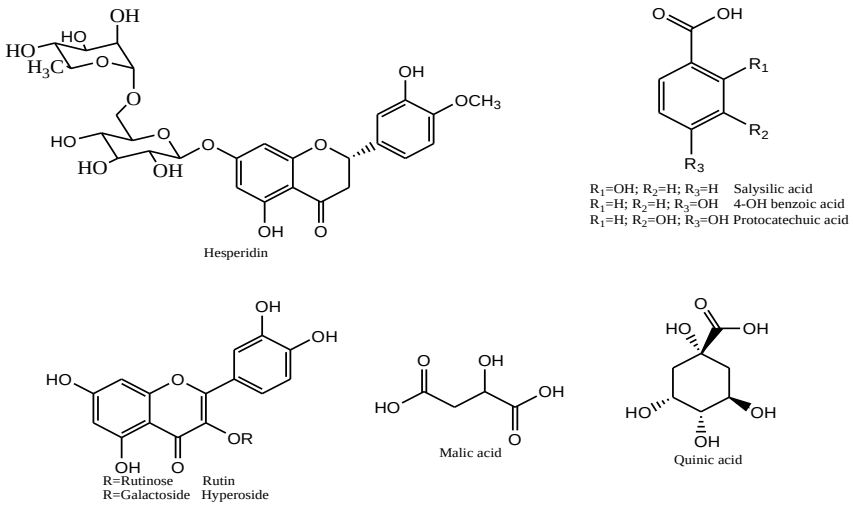

Figure 2. Quantified major compounds from *Astragalus* species

In this study, antioxidant and radical scavenging activities of various extracts of three endemic *Astragalus* species were compared to BHT and α-tocopherol. These comparisons were performed by using various in vitro bioanalytical methods such as; *β*-carotene bleaching, DPPH free radical scavenging, cupric reducing antioxidant capacity and ABTS cation radical decolorisation assays. As it is known from several studies, the abilities of antioxidants that affect diseases are relative to their power to lower DNA damage, mutation, carcinogenesis and bacterial growth. Antioxidant property is a marker for biologicaly active compounds (Roginsky and Lissi, 2005).

Lipid peroxidation includes a series of chain reactions caused free radicals and related to cellular damages. Antioxidants serve a function in inhibition of lipid peroxidation or defence against biological damages caused by free radicals (Dargel, 1992). Besides, the electron donating capacity of bioactive components reflect their reduction power and related to their antioxidant activity. It is a widespread phenomenon that free radical chain reaction is the common mechanism for lipid peroxidation. Radical scavenging substances can directly react with and dispel peroxide radicals in order to terminate chain reactions and develop the quality and stability of food products (Soares et al., 1997). To determine the antioxidant chromogens assays attributed to the use of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals are amongst the most widely used spectrophotometric methods and free radicals can directly react with antioxidants. Also, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging methods are commonly used to determine the antioxidant activities of compounds as they are simple, fast, sensitive and repeatable (Ozcelik et al., 2003). Furthermore, antioxidants could be reductants and inactive oxidants. . On the other hand, another low cost, fast and selective method applied in this study is Cuprac method being founded on the reduction of Cu^{2+} to $Cu^{+\cdot}$ Also, this method can apply to various antioxidants without making a distinction of structure or hyrophilicity (Apak et al., 2004).

While ASA and ASM extracts showed high inhibition of lipid peroxidation (IC_{50}: 26.38±0.74 and 19.62±0.29 μg/mL, respectively) in β-carotene bleaching method, ALA, ADA and ASP extracts exhibited moderate inhibition (IC_{50}: 98.32±0.31, 88.21±1.18 and 61.93±0.82 μg/mL, respectively) (Table 3). ASA and ASM extracts indicated moderate activity in DPPH free radical scavenging activity (IC_{50}: 65.89±0.21 and 54.61±0.38 μg/mL, respectively) (Table 3).

Our results were found to be parallel to the literature. According to Adiguzel et al. (2009), the methanol extracts of the aerial parts of the *Astragalus* species showed slight free radical sweeping effect with 50% inhibition between the concentrations 68.8 and 400.4 μg/mL. However, hexane/dichloromethane extracts of the aerial parts of the *Astragalus* species showed no free radical sweeping effect. While methanolic extracts of the roots of *A. microcephalus* Willd., *A. macrocephalus* Willd., *A. erinaceus* Fisch. & Mey. ex Fischer, *A. psoraloides* Lam. ve *A. argyroides* Becker ex Stapf. were slight antioxidants, their non-polar extracts were highly active in DPPH test. Their IC_{50} values were detected as 35.2 μg/mL, 21.0 μg/mL, 22.0 μg/mL, 20.3 μg/mL ve 38.0 μg/mL respectively. Among the studied *Astragalus* species, *A. psoraloides* Lam. extracts showed the strongest inhibitory effect in β-carotene-linoleic acid system.

ALM, ALW, ADM, ADW and ASA extracts indicated moderate activity (IC_{50}: 65.37±0.44, 48.82±0.35, 54.71±0.09, 64.01±0.17 and 71.09±0.63 μg/mL, respectively) in ABTS cation radical scavenging assay (Table 3). However, ASM and ASW extracts exhibited good effects in ABTS cation radical scavenging assay (IC_{50}: 22.01±0.07 and 32.91±0.80 μg/mL, respectively). ASM extract exhibited higher activity (91.22% inhibition) at 100 μg/mL than α-tocopherol (89.14%) and BHT (88.34%), which were used as standards in the ABTS cation radical scavenging assay. The other tested five extracts showed weak or no activity in ABTS cation radical scavenging assay. Our results of ABTS cation radical scavenging assay were parallel to the literature. According to Luo and Fan's (2011) report, the

polar methanolic extracts of the four studied *Astragalus* species showed good cation radical scavenging activity. ASM extract and α-tocopherol exhibited the same activity (22.35±0.12 and 22.94±0.17 $A_{0.5}$ value, respectively) in CUPRAC (Table 3). The other tested extracts showed weak or no activity in CUPRAC. According to the literature search, this is the first study of the cupric reducing antioxidant capacity of the *Astragalus* species.

Table 3. Antioxidant and anticholinesterase activities[a], and total phenolic-flavonoid contents[a] of AL, AD and AS extracts, BHT, α-TOC and galanthamine

Sample	Inhibition % against AChE	Inhibition % against BChE	Phenolic content (μg PEs/mg extract)[d]	Flavonoid content (μg QEs/mg extract) [e]	IC_{50} (μg/mL) Lipid Peroxidation	IC_{50} (μg/mL) DPPH Free Radical	IC_{50} (μg/mL) ABTS Cation Radical	$A_{0.5}$ (μg/mL) CUPRAC
ALP	46.96± 4.06[a]	66.15± 4.08[a]	-	-	113.21± 0.94[a]	>200[a]	156.82± 1.92[a]	NA[c]
ALA	NA	37.50± 2.46[b]	-	-	98.32± 0.31[b]	127.39± 0.49[b]	109.62±1.28[b]	NA
ALM	NA	19.78± 1.24[c]	82.68± 0.12[a]	15.88±0.33[a]	127.43±0.82[c]	102.62±0.97[c]	65.37±0.44[c]	50.06±0.21
ALW	NA	NA	-	-	>200[d]	>200[a]	48.82±0.35[d]	NA
ADP	17.79± 0.81[b]	41.36± 3.31[b]	-	-	112.63±0.11[a]	>200[a]	183.61±1.39[e]	NA
ADA	NA	19.71± 1.15[c]	-	-	88.21±1.18[e]	142.82±1.93[d]	89.81±0.43[f]	NA
ADM	NA	NA	83.73± 2.81[a]	16.51±0.10[b]	138.41±1.03[f]	101.21±0.27[c]	54.71±0.09[g]	NA
ADW	NA	NA	-	-	>200[d]	>200[a]	64.01±0.17[h]	NA
ASP	13.21± 0.21[c]	46.01± 0.45[d]	-	-	61.93±0.82[g]	129.89±0.17[b]	119.02±0.77[i]	44.22±0.36
ASA	12.11± 0.34[c]	32.13± 0.32[e]	-	-	26.38±0.74[h]	65.89±0.21[e]	71.09±0.63[j]	NA
ASM	NA	17.12± 0.87[c]	89.12± 2.13[b]	50.12 ± 0.07[c]	19.62±0.29[i]	54.61±0.38[f]	22.01±0.07[k]	22.35±0.12
ASW	NA	NA	-	-	111.61±0.49[a]	113.92±0.72[g]	32.91±0.80[l]	NA
Galanth.[b]	85.11 ± 0.69[d]	82.51±0.48[f]	-	-	-	-	-	-
α-TOC[b]	-	-	-	-	15.21±0.09[j]	17.41±0.39[h]	9.54±0.09[m]	22.94±0.17
BHT[b]	-	-	-	-	9.48±0.12[k]	49.91±0.17[i]	10.31±0.14[n]	7.81±0.28

[a]Values expressed are means ± SEM of three parallel measurements ($p<0.05$), [b]Standard drug, [c]NA: Not active, [d]PEs, pyrocatechol equivalents ($y = 0.0123 x + 0.0349$ $R^2 = 0.9910$), [e]QEs, quercetin equivalents ($y = 0.1701 x – 0.7078$ $R^2 = 0.9939$)

In general, the result of antioxidant activity tests showed that among the twelve different extract ASM exhibited strongest activity followed by ADM and ALM. High antioxidant activity is mainly due to the phenolic compounds in the extracts. Significant correlations were observed between the total phenolic contents and antioxidant activities of the methanolic extracts (Table 5). The high antioxidant activity of ASM may mainly stem from gallic, chlorogenic, protocatechuic and rosmarinic acid contents in this extract. These compounds had potent antioxidant activity (Table 4) and the highest amounts were found in ASM (Table 1). The results showed that there was a strong negative correlation between antioxidant activity and these phenolic acids in the extracts (Table 6). Moreover, some flavonoids such as rutin, hesperidin, myricetin, hyperoside, quercetin, luteolin, kaempferol and apigenin found in ASM may contributed to its high antioxidant activity (Table 4 and Table 6).

1.3. Anticholinesterase activity

The extracts exhibited enzyme inhibitory activity at various values indicating weak activity except ALP extract which recorded 46.96± 4.06 and 66.15± 4.08 inhibition ratio against acetyl- and butyryl-cholinesterase, respectively. Also, ADP and ASP extracts showed moderate enzyme inhibitory activity against butyryl-cholinesterase with 41.36± 3.31 and 46.01± 0.45 inhibion ratio, respectively. (Table 3). So,we can say that petroleum ether extract more active than other extract in term of cholinesterase enzyme inhibitory activity. Previously, Zengin et. al. (2016) have investigated the enzyme inhibitory effects of ethyl acetate, methanol, and aqueous extracts from *Astragalus lagurus* against cholinesterase, tyrosinase, α-amylase and α-glucosidase and observed the higher enzyme inhibitory effects of ethyl acetate compared to methanol and aqueous extracts. According to Teyeb et. al.(2011), among the *Astragalus gombiformis* extracts the ethyl acetate aerial part extract was found to be the most active extract in terms of anticholinesterase activity with an IC_{50} of 110μg/ml.

Table 4. Antioxidant activity of the main compounds in the methanol extracts and standard compounds

Samples	IC_{50} (µg/mL)		
	Lipid Peroxidation	DPPH Free Radical	ABTS Cation Radical
Quinic acid	>200	>200	>200
Malic acid	>200	>200	>200
tr-Aconitic acid	>200	>200	>200
Gallic acid	48.41±0.12	7.52±0.28	<<1
Chlorogenic acid	>200	6.33±0.09	3.58±0.05
Protocatechuic acid	48.02±0.21	10.23±1.07	1.82±0.04
Tannic acid	9.14±0.06	5.61±0.30	<<1
Caffeic acid	26.15±1.29	16.82±0.58	2.41±0.08
p-coumaric acid	>200	>200	<1
Rosmarinic acid	12.12±0.02	1.21±0.06	1.70±0.07
Rutin	18.54±0.03	24.18±0.86	2.48±0.06
Hesperidin	>200	>200	5.02±0.03
Hyperoside	>200	5.12±0.31	2.81±0.12
4-Hydroxy benzoic acid	>200	>200	24.38±0.12
Salycilic acid	105.62±0.72	>200	>200
Myricetin	8.92±0.35	9.47±0.12	<1
Quercetin	5.17±0.71	2.79±0.14	1.03±0.09
Luteolin	9.63±0.03	11.83±0.09	3.03±0.03
Kaempferol	8.04±0.06	82.09±0.82	2.82±0.02
Apigenin	5.63±0.08	>200	3.03±0.08
α-TOC	15.54±0.12	18.76±0.41	8.06±0.08
BHT	10.35±0.67	48.86±0.50	10.67±0.11

Values are means ± S.D., n = 3, $p < 0.05$, significantly different with Student's *t*-test

Table 5. Correlation coefficients (r^2) for relationships between phenolic contents and antioxidant activities (IC_{50} value) of methanol extracts

	Phenolic content	Lipid Peroxidation	DPPH Free Radical
Lipid Peroxidation	-0.97222		
DPPH Free Radical	-0.99195	0.994033	
ABTS Cation Radical	-0.99634	0.948644	0.97749

Table 6. Correlation coefficients (r^2) for relationships between quantification of the compounds in methanol extracts and antioxidant activities (IC_{50} value) of methanol extracts

Quantification	Lipid Peroxidation	DPPH Free Radical	ABTS Cation Radical
Gallic acid	-0.99534	-0.99992	-0.97473
Chlorogenic acid	-0.76124	-0.82744	-0.92729
Protocatechuic acid	-0.99999	-0.99347	-0.94704
Tannic acid	-0.43799	-0.33732	-0.13111
Rosmarinic acid	-0.97654	-0.94723	-0.85828
Rutin	-0.89668	-0.84304	-0.71059
Hesperidin	-0.5931	-0.50174	-0.30794
Hyperoside	-0.94806	-0.9077	-0.79874

1.4. Antimicrobial activity

The antimicrobial activity of *A. leporinus* var. *hirsutus*, *A. distinctissimus* and *A. schizopterus* extracts against different microorganisms were assessed according to the inhibition zone diameter. The results of the active extracts were given in Table 7. The methanol extracts showed weak activity (inhibition zone < 12 mm) against bacteria, and moderate activity (inhibition zone < 20-12 mm) against yeast. ADA extract was active against *S. aureus*, *S. pyogenes* and *C. albicans*. On the other hand, ADA extract was found to be not active against gram negative bacteria. The other tested extracts showed no antimicrobial activity. The highest activities were recorded by methanol extracts against *C. albicans* with 15 mm inhibition zone diameter, and the lowest MIC value was recorded by ALM extract against *C. albicans* (20 µg/mL). Adiguzel *et al*. (2009) reported that the methanol and hexane extracts of thirteen *Astragalus* species have no antimicrobial activity against 40 microorganisms including 24 bacteria, 15 fungi and a yeast species, in the current study the antimicrobial screening revealed that the methanol and acetone extracts had inhibitory effect towards bacteria and yeast. According to Pistelli et al. (2002), several extracts of *A. verrucosus* (ethyl acetate, butanol, ethanol) had strong antimicrobial activity against *Aspergillus* and *Botrytis* species and hexane extract had inhibitory effect against *S. aureus*.

1.5. Cytotoxicity activity

MTT assay was used to determine cell viability. Only the medium was applied for control group. Figure 3 shows viability of L929 fibroblast cells and Figure 4 cell viability of A549. In the control group there were no toxicity. At low concentration (12.5 µg/mL), the highest viability was found in ALM extract applied L929 fibroblast cells; however, in the same concentration (12.5 µg/mL), ALM extract applied to A549 cells were found to possess more cytotoxic effect than the other methanol extracts. Briefly, *Astragalus* species contain oleanane- and cycloartane-type glycosides and triterpenoidal saponins as principal constituents besides phenolic compounds. There are few studies on their phenolic contents (Zhang et al. 2013; Qi et al. 2008; Yunfei et al. 2008; Montoro et al. 2012). Thus, the phenolic contents of three endemic *Astragalus* species might be a valuable data in this field.

Table 7. Zones of growth inhibition (mm) and MIC values of AL, AD, and AS extracts compared to positive controls

		Microorganisms				
		Gram positive		***Gram negative***		***Yeast***
		S. aureus	***S.pyogenes***	***E.coli***	***P. aeruginosa***	***C. albicans***
ALA	**[a]DD**	9±0.4	11±0.3	10±0.1	10±0.3	9±0.3
	MIC	250±0.1	1000±0.4	1250±0.2	80±0.2	1000±0.5
ALM	**[a]DD**	10±0.6	10±0.3	11±0.3	10±0.4	15±0.2
	MIC	45±0.5	75±0.5	50±0.1	40±0.7	20±0.1
ADA	**[a]DD**	9±0.2	11±0.2	-	-	12±0.3
	MIC	50±0.2	250±0.3	-	-	100±0.2
ADM	**[a]DD**	10±0.5	11±0.3	11±0.2	10±0.4	15±0.1
	MIC	55±0.3	260±0.2	265±0.7	80±0.3	30±0.4
ASM	**[a]DD**	10±0.5	11±0.2	10±0.4	9±0.5	15±0.2
	MIC	65±0.6	300±0.4	280±0.3	95±0.4	30±0.5
Positive controls	**[b]DD**	35±0.2	19±0.2	20±0.1	-	30±0.3
	MIC	1.95±0.3	7.815±0.1	7.815±0.4	-	3.125±0.2

-: Not active, [a]DD: Inhibition zone in diameter (mm) around the discs (6 mm) impregnated with 30 mg/mL of plant extracts, [b]DD: Inhibition zone in diameter (mm) of positive controls that are ampicillin for bacteria and fluconazole for yeast. Minimum inhibitory concentration (MIC) values are given as µg/mL

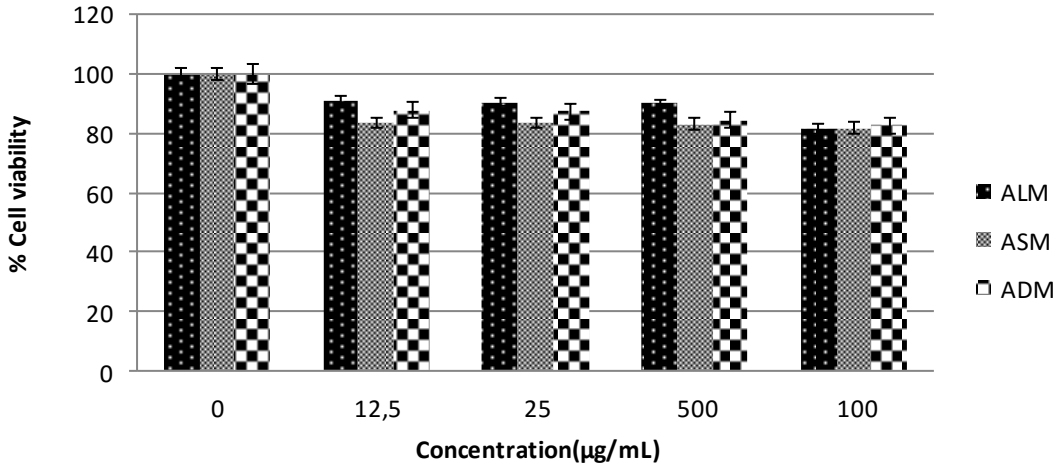

Figure 3. L929 Cytotoxic activity of AL, AD and AS methanol extracts

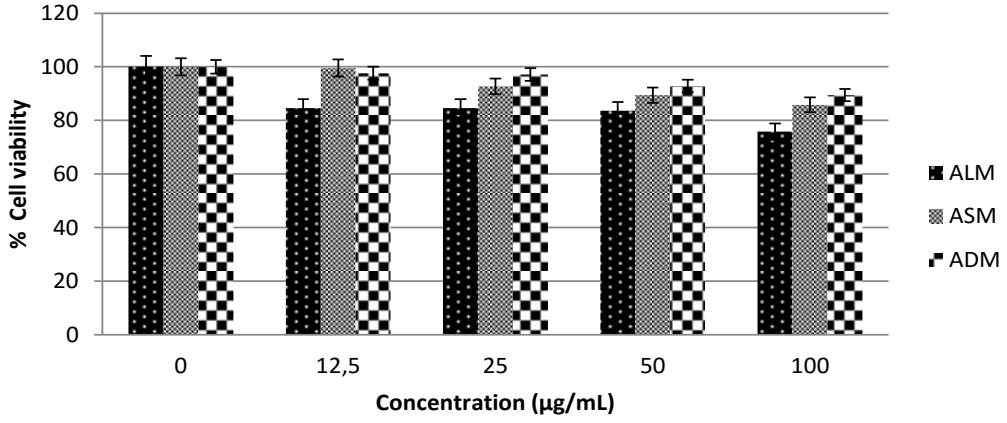

Figure 4. A549 Cytotoxic activity of AL, AD and AS methanol extracts

This study indicates that the different type and quantity of phenolic compounds in three endemic *Astragalus* species grouped into different sections could be of chemotaxonomic importance, along with their fatty acid profiles. Further phytochemical studies on other *Astragalus* species grown in Turkey are needed to confirm this assumption.

The present study showed that *A. schizopterus* methanol extract had strong antioxidant activity in *β*-carotene-linoleic acid test system, DPPH free radical and ABTS cation radical scavenging activities. The antioxidant capacity of *A. schizopterus* methanol extract was the highest among three *Astragalus* species, and that result was in accordance with the total phenolic and flavonoid contents. The reason why the methanolic extract of *A. schizopterus* was the most active of all the twelve extracts tested for four antioxidants methods used, could be related to its high phenolic acids and flavonoid contents. According to our study, rutin and hesperidin were found to be the most abundant flavonoid in three endemic *Astragalus* species. Moreover, naringenin, vanillin, hesperidin, myricetin and protocatechuic, quinic, tr-aconitic and 4-OH benzoic acids were detected for the first time in *Astragalus* species. The high total phenolic and flavonoid content of the methanol extract of *A. schizopterus* showed parallelism to the LC-MS/MS results. In addition, the methanol extract of *A. schizopterus* contained a high level of quinic acid.

The high antioxidant capacity of *A.schizopterus* methanol extract being quite rich with respect to flavonoid content might be related to its high total flavonoid content.

As a consequence, *A. schizopterus* methanol extract which possessed high amounts of quinic acid and total flavonoid content with strong antioxidant capacity could be phytochemically investigated to find their active secondary metabolites.

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