ORIGINAL ARTICLE

A C G publications

Rec. Agric. Food. Chem. 3:2 (2023) 36-44

records of agricultural and food chemistry

Determination of Antiaging and Antidiabetes Effects of *Astragalus leporinus* Boiss. Var. *hirsutus* (Post) Chamberlain, *A. distinctissimus* Eig and *A. Schizopterus* Boiss. Three Endemic Species Growing in Anatolia

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(Received November 02, 2023; Revised December 19, 2023; Accepted December 19, 2023)

Abstract: *Astragalus* L. is the largest flowering genus of the Fabaceae family and is represented by approximately 3000 species worldwide. This study aims to determine the anti-aging and anti-diabetes effects of *Astragalus leporinus*, *A. distinctissimus*, and *A. schizopterus*, three endemic species in Anatolia. The anti-aging effects against elastase and collagenase enzymes and the anti-diabetic effects against α-glucosidase and α-amylase enzymes were determined. Some triterpene contents were also determined by GC-MS. Ursolic acid (85.86 mg/g extract) and oleanolic acid (49.11 mg/g extract) were detected in the acetone extract of *A. schizopterus* species, and only (2.10 mg/g extract) oleanolic acid was detected in the ethanol extract. α-Amyrin (5.46 mg/g extract), oleanolic acid (6.09 mg/g extract), and ursolic acid (8.95 mg/g extract) were identified in the acetone extract of *A. distinctissimus* species. In terms of anti-aging, *A. leporinus* var. *hirsutus* ethanol extract has shown the highest activity in elastase and collagenase inhibition activity methods (inhibition %: 17.26±0.18 and 11.47±0.15, respectively), standard reference substances (oleanolic acid and epicatechin gallate, inhibition %: 43.80±0.76, 84.08±0.49, respectively). When it is evaluated in terms of anti-diabetic, it was determined that acetone extracts of *A. schizopterus* and *A. distinctissimus* species inhibited α-glucosidase at a higher level than acorbose, which was used as the standard reference substance, at all concentrations. In addition, it has also been observed that both acetone and ethanol extracts of the three species studied showed moderate inhibitory activity against the α-amylase enzyme. When the results obtained were evaluated, it is possible to state that *A. schizopterus* and *A. distinctissimus* species used as animal feed should be subjected to more detailed studies to be used for the pharmaceutical industry, as they are rich in oleanolic and ursolic acids and show anti-diabetic.

Keywords: *Astragalus*; GC-MS; oleanolic acid; ursolic acid; anti-aging; anti-diabetic.

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The article was published by ACG Publications
https://www.acgpubs.org/journal/records-of-agricultural-and-food-chemistry July-Decembber 2023 EISSN:2792-0763
DOI: http://doi.org/10.25135/rfac.19.2311.2951
Available online: December 28, 2023

1. Introduction

It is known that medicinal and aromatic plants have been used for purposes such as medicine, food, and treatment since ancient times [1-4]. As a result of archaeological investigations, it has been stated that according to ancient data, human beings used plants to meet their basic nutritional needs and solve health problems [5-8]. For this reason, many herbal products continue to be grown from ancient times to the present day. Approximately 40% of the drugs used in the 20th century were of plant origin, and this rate is increasing due to the expansion of the areas of use of plants used for medicinal purposes today. According to World Health Organization (WHO) data, the average number of plant species used for treatment is around 20,000 [3].

Astragalus L. is the largest flowering genus of the Fabaceae (legume) family and has approximately 3000 species worldwide, 309 of which are endemic [9, 10]. Species of the genus *Astragalus* are used in many areas. These areas can be listed as food, medicine, cosmetics, and textiles [9, 11]. *Astragalus* plants are annual and perennial, stemmed and herbaceous plants or small shrubs up to 150-200 cm. When it was looked at the general distribution of the *Astragalus* genus around the world, it is spread across Asia, Europe, South and North America [12, 13]. Plants belonging to the *Astragalus* genus are popularly called geven [14]. When the flora of Turkey is examined, it is seen that there are approximately 400 *Astragalus* (geven) species [15, 16]. The roots of the *Astragalus* species are used against diabetes, kidney diseases, and some types of cancer. They are also known to be used for treatment against leukemia in the Southeastern region of Turkey. Chemical analysis studies of *Astragalus* species show that the main components of the plant species are saponins and polysaccharides [17, 18].

Saponins, one of the two main chemical components detected in *Astragalus* species, have lower cholesterol, counteract depressive effects, and support the immune system, while semi-synthetic glycosides strengthen the heart and also provide benefits in terms of toxicity [17, 19-21]. It has also been stated that *Astragalus* species contain flavonoids, phenylpropanoids, alkaloids, and steroids, which are secondary metabolites, and the presence of these species has anti-inflammatory, immune system regulators, antioxidant and anti-diabetic effects [22].

Saponins, widely found as secondary metabolites in plants, are chemicals that play an important role in plants' defense systems [23, 24]. Saponins are polar molecules consisting of a triterpene or steroid (aglycone) structure, and those with a triterpenic structure are structures that contain carbohydrate groups in their structure [25].

Ursolic acid (3β-hidroxy-urs-12-en-28-oic acid) and oleanolic acid (3β-hidroxy-olea-12-en-28-oic acid), which are from the triterpenoid class, are five-ring pentacyclic compounds and are saponin-derived secondary metabolites widely found in plants [26, 27].

In literature studies, it was determined that ursolic and oleanolic acids have medical effects in many areas. These areas can be listed as anti-inflammatory, anti-tumor, anti-hyperlipidemic, antioxidant, antimicrobial, anti-ulcer, hypoglycemic, and anti-aging [27-29]. Studies have shown that oleanolic and ursolic acids inhibit the elastase enzyme, delaying the deterioration of the skin's elastic structure and aging [30].

In the literature review regarding these three species in their study, Hasimi et al. [9], three endemic species in Anatolia, *Astragalus leporinus* var. *hirsutus*, *Astragalus distinctissimus*, and *Astragalus schizopterus*, were characterized by LC-MS/MS and GC-MS. Additionally, these three species' antioxidant, anticholinesterase, antimicrobial, and cytotoxic effects were determined. According to the results, the highest phenolic compound content in *Astragalus* species was found to be routine (1028.27-13351.76 µg/g extract) and hesperidin (1604.34-9695.43 µg/g extract). A high amount of quinic acid (111302.77 µg/g extract) was detected in the methanol extract of *A. schizopterus* species. The main component of these three species was palmitic acid, *A. leporinus* var. *hirsutus* (32.90%), *A. distinctissimus* (32.50%), and *A. schizopterus* (23.40%). It was also found to exhibit high amounts of antioxidant activity (lipid peroxidation (IC_{50}: 19.62±0.29 µg/mL), DPPH free (IC_{50}: 54.61±0.38 µg/mL), ABTS cation radical scavenging activity (IC_{50}: 22.01±0.07 µg/mL) and CUPRAC ($A_{0.5}$: 22.35±0.12 µg/mL). In addition, it was determined that the methanol extracts of the species showed moderate activity against *C. albicans*, *A. leporinus* var. *hirsutus* methanol extract, showed the highest viability against L929 fibroblast cells, and the highest cytotoxic effect against A549 cells.

In their study, Sarikurkcu et al. [31] evaluated the phytochemical compositions, antioxidant, tyrosinase, and α-amylase activities of methanol extracts of *Astragalus gymnolobus* Fisch., *Astragalus leporinus* Boiss. var. *hirsutus* (Post) D. F. Chamb., and *A. onobrychis* species. According to the results of Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS) analysis, it was determined that hesperidin and hyperoside were found in high amounts in the extracts of the species. It was determined that there is a strong correlation between the phytochemical compositions and antioxidant activities of these *Astragalus* species. It was found that *A. leporinus* var. *hirsutus* species exhibited the highest α-amylase inhibition activity (4.06 mg/mL, 298.54 mg ACE/g extract).

Due to the lack of studies in the literature review to determine the anti-aging and anti-diabetic effects of *Astragalus* species, we decided to conduct this study. In this study, endemic species *A. leporinus* var. *hirsutus*, *A. distinctissimus*, and *A. schizopterus* were analyzed for the α-amyrin and moronic, oleanonic, oleanolic, betulinic, ursolic and ursonic acids using by GC-MS. Anti-elastase and anti-collagenase enzyme activities of these endemic species were measured as anti-aging. α-glucosidase and α-amylase enzyme inhibition activities were measured as anti-diabetic effects.

2. Materials and Methods

2.1. Plant Material

A. leporinus Boiss. var. *hirsutus* (Post) Chamberlain, *A. distinctissimus* Eig and *A. schizopterus* Boiss. species were collected and described by S. Demirci from Southeastern Turkey (Kahramanmaraş) in May and August 2012, respectively. Herbarium samples were left to Istanbul University Faculty of Pharmacy Herbarium (ISTE 97142, ISTE 98035, and ISTE 97141).

2.2. Extraction and GC-MS Analysis

The aerial parts of the plant samples were dried in the shade before extraction. Dried plant samples were crushed using a grinder and weighed on a precision scale (10 g) to prepare alcohol extracts. It was then placed in a beaker (50 mL) of pure acetone, and ethanol (separately) was added to cover it completely and left for 8 hours. After 8 hours, it was kept in an ultrasonic water bath for 30 min at room temperature and then filtered. This process was repeated three times, and then acetone and ethanol were removed from the total filtrate with the help of an evaporator. The extracts were placed in tared tubes, weighed, and stored at +4ºC until enzyme analysis. These procedures were performed for all samples. Stock solutions were prepared from crude extracts at 4000 µg/mL concentrations, and dilution was performed according to the studied method [32].

2.3. GC-MS for Triterpenoid Content

Analyzes were conducted using (Agilent Technologies, USA) model 5977B mass spectrometer (MS) device together with 7890A Model GC-FID. Triterpene contents of the samples were determined using a model 5977B mass spectrometer (MS) device combined with an Agilent Technologies, USA (120 min at 70°C) brand 7890A Model GC-FID derivatized with N,O–bis (trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane. Samples and standards were studied in the same way. First of all, 100 µL of sample/standard solution was taken into a glass vial, and the solvents were evaporated until dry. Then, 100 µL of BSTFA+TMCS (99:1) was added to the samples whose solvent was evaporated. To optimize the derivatization process, the samples were placed at different temperatures (Room temperature, 40°C, 50°C, 60°C, 70°C, 80°C, 90°C, 100°C and 110°C) and different time periods (5 min, 10 min, 15 min, 20 min, 30 min, 45 min, 60 min, 90 min, 120 min, 180 min 24 h) and the appropriate temperature (70°C) and time interval (120 min) were determined. Chromatographic separation was done with a nonpolar HP-5MS column (30 m x 0.25 mm x 0.25 µm film thickness). The GC oven temperature started from 200°C and was increased to 300°C at a rate of 10°C/min and kept constant at this temperature (300°C) for 15 min. Then, the temperature was increased to 310°C at a rate of 5°C/min and kept constant at this temperature for 2 min. Helium gas (0.8 mL/min) at constant flow was used as the carrier gas. Injection block and transfer line temperatures were set at

300°C. Injections were made in splitless mode. The injection volume was taken as 2.0 µL. The mass spectrometer (EI/MS) was set at 70 eV ionization energy. The temperature of the ion source was set at 230°C. Mass spectrometry (MS) data were obtained in full scan mode with the scan range set to *m/z* 50-650 atomic mass units (amu) [33, 34].

2.4. Enzyme Activities

2.4.1. Anti-aging Activity

Elastase and collagenase inhibition activity methods were used to determine the anti-aging effects of plant species. Determination of elastase inhibition activity was determined by making some modifications to the method developed by Kraunsoe et al. [35]. Collagenase inhibition activity determination was determined by making some changes in the method developed by Thiring et al. [32, 33, 36, 37].

2.4.2. Anti-diabetic Activity

α-Glucosidase and *α*-amylase inhibition activity methods were used to determine the anti-diabetic effects of plant species. *α*-Glucosidase inhibitory activity was determined by modifying the method developed by Lazarova et al. [38]. *α*-Amylase inhibitor activity was performed by modifying the Caraway Somogyi iodine/potassium iodide (I/KI) method developed by Lazarova et al. [38].

3. Results and Discussion

3.1. Triterpenoid Content

Tritepene contents of the species were determined with an Agilent brand 7890A Model GC-FID gas chromatograph combined with an Agilent 5977B model mass spectrometer (MS) device at Dicle University Faculty of Pharmacy. Acetone and ethanol extracts of the species were analyzed by GC-MS for the quantitative analysis of moronic, *α*-amyrin, oleanonic, oleanolic, betulinic, ursolic, and ursonic acid compounds, which are especially commonly found in natural products (Table 1).

Table 1. Triterpene contents of *Astragalus* species by GC-MS (mg/g extract).

Compound	RT [a]	Mother ion-*m/z* (%) [b]	RSD% [c]	*A. leporinus* var. *hirsutus*		*A. schizopterus*		*A. distinctissimus*	
				Acettone	Ethanol	Acetone	Ethanol	Acetone	Ethanol
α-Amyrin	17.99	498 (2.5)	0.025	*ND*	*ND*	*ND*	*ND*	5.46	*ND*
Moronic Acid	20.71	527 (21.1)	0.029	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*
Oleanonic Acid	20.96	527 (12.3)	0.023	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*
Oleanolic Acid	21.55	601 (2.3)	0.026	*ND*	*ND*	49.11	2.10	6.09	*ND*
Betulinic Acid	21.90	601 (4.9)	0.019	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*
Ursolic Acid	22.55	601 (2.3)	0.015	*ND*	*ND*	85.86	ND	8.95	*ND*
Ursonic Acid	22.91	527 (9.5)	0.028	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*

[a] RT: Retention time.
[b] Mother ion relative intensity (*m/z*): Molecular ions of the standard compounds (*m/z* ratio).
ND: Not detected
[c] RSD: Relative standard deviation

To facilitate the analysis of these compounds by the GC-MS method, N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) derivatization agent containing 1% trimethylchlorosilane was used. *α*-Amyrin was detected only in the acetone extract of *A. distinctissimus* species (5.46 mg/g extract). Oleanolic acid was detected in acetone and ethanol extracts of *A. schizopterus* species and acetone extract of *A. distinctissimus* species (49.11, 2.10, and 6.09 mg/g extract, respectively). Ursolic acid was detected in acetone extracts of *A. schizopterus* and *A. distinctissimus* species (85.86 and 8.95 mg/g

extract, respectively). Other components were absent or below the lower limit of determination in acetone and ethanol extracts of the three species (Figure 1).

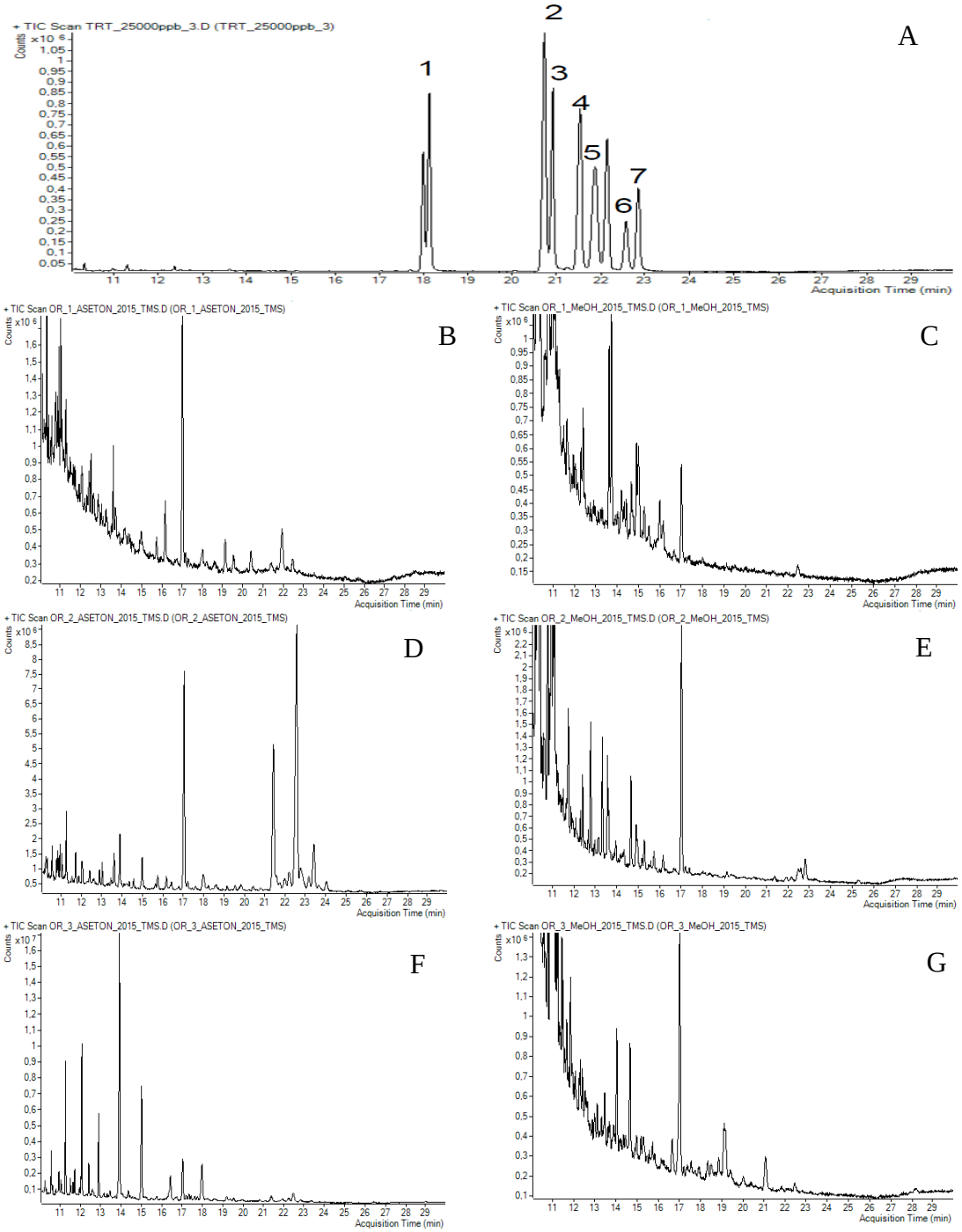

Figure 1. GC-MS chromatograms, **A:** TIC chromatogram of standards in the GC-MS method, **1:** *α*-Amyrin, **2:** Moronic acid **3:** Oleanonic acid **4:** Oleanolic acid, **5:** Betulinic acid, **6:** Ursolic acid, **7:** Ursonic acid, **B** and **C:** GC-MS chromatogram of *A. leporinus* var. *hirsutus* acetone and ethanol extracts, **D** and **E:** GC-MS chromatogram of *A. schizopterus* acetone and ethanol extracts, **F** and **G:** GC-MS chromatogram of *A. distinctissimus* acetone and ethanol extracts.

In their study, Yigitkan et al. [33] examined the roots and aerial parts of the *Thymus pubescens* species in terms of GC-MS and triterpene ingredients. They stated that there are oleanolic and ursolic acids (92785.96 and 63373.32 µg/g extract, respectively) in the ethanol extract of the aerial parts of the

species, and *α*-amyrin, oleanolic, betulinic and ursolic acid (419.565, 14735.70, 1509.79 and 12085.24 µg/g extract, respectively) in the root part of the species.

3.2. Enzyme Inhibition Activity Results

In terms of anti-aging, it found that both acetone and ethanol extracts of *A. leporinus* var. *hirsutus*, *A. schizopterus,* and *A. distinctissimus* species exhibited moderate inhibitory activity, (inhibition %: 13.72±0.49, 17.26±0.18, 9.79±0.17, 13.84±0.15, 12.88±0.26, 5.21±0.02, and oleanolic acid: 43.80±0.76, respectively) against the elastase enzyme. Regarding collagenase enzyme inhibition activity, it determined that *A. leporinus* var. *hirsutus* and *A. distinctissimus* ethanol extracts exhibited low (inhibition %: 11.47±0.15, 8.16±0.05, respectively) and epicatechin gallate (inhibition %: 84.08±0.49) enzyme inhibition activity. It was determined that other extracts did not show collagenase enzyme inhibition activity (Table 2).

Table 2. Anti-aging enzymatic activities of *Astragalus* species [a]

Samples	Solvent	Elastase (inhibition %, at 100 µg/mL)	Collagenase (inhibition %, at 100 µg/mL)
A. leporinus var. *hirsutus*	Acetone	13.72±0.49	*NA*
	Ethanol	17.26±0.18	11.47±0.15
A. schizopterus	Acetone	9.79±0.17	*NA*
	Ethanol	13.84±0.15	*NA*
A. distinctissimus	Acetone	12.88±0.26	*NA*
	Ethanol	5.21±0.02	8.16±0.05
Oleanolic acid [b]		43.80±0.76	-
Epicatechine gallate [b]		-	84.08±0.49

[a] Values are given as means and standard deviations of 3 parallel measurements
[b] Standard compounds
NA: Not active

When it evaluates in terms of anti-diabetic properties, acetone extracts of *A. schizopterus* and *A. distinctissimus* species inhibit *α*-glucosidase at high levels at concentrations of 12.5, 50, and 200 µg/mL (inhibition %: 60.69±1.73, 84.98±1.81, 85.93±1.15 and 67.80±1.06, 76.91±1.15, 86.28±1.73, respectively) and acarbose (inhibition %: 6.32±0.12, 18.20±0.13, 67.74±0.53, respectively). It was found that ethanol extracts of *A. schizopterus* and *A. distinctissimus* species exhibited moderate *α*-glucosidase inhibitory activity. Additionally, it was determined that acetone and ethanol extracts of the three studied species exhibited moderate *α*-amylase inhibitory activity (Table 3).

Table 3. Anti-diabetic enzymatic activities of *Astragalus* species [a]

Samples	Solvent	*α*-Glucosidase inhibitory activity (Inhibition %)			*α*-Amylase inhibitory activity (Inhibition %)		
		12.5 µg/mL	50 µg/mL	200 µg/mL	25 µg/mL	100 µg/mL	400 µg/mL
A. leporinus var. *hirsutus*	Acetone	*NA*	4.80±0.07	26.97±0.69	0.89±0.18	2.10±0.19	33.84±0.12
	Ethanol	*NA*	*NA*	*NA*	1.86±0.03	4.98±0.28	16.56±0.15
A. schizopterus	Acetone	60.69±1.73	84.98±1.81	85.93±1.15	*NA*	4.29±0.17	12.65±0.39
	Ethanol	1.72±0.06	9.28±0.18	21.30±0.54	3.20±0.04	6.77±0.21	17.08±0.19
A. distinctissimus	Acetone	67.80±1.06	76.91±1.15	86.28±1.73	1.83±0.01	3.73±0.22	19.23±0.58
	Ethanol	3.97±0.09	8.95±0.32	17.76±0.14	3.51±0.07	4.01±0.13	32.43±0.15
Acarbose [b]		6.32±0.12	18.20±0.13	67.74±0.53	6.26±0.13	27.86±0.64	87.15±1.14

[a] Values are given as means and standard error meaning of three parallel measurements
[b] Standard compound
NA: Not active

In their study, Kızıltas et al. [39] evaluated the anti-diabetic *α*-glucosidase and *α*-amylase enzyme inhibition activity of *A. brachycalyx* species, and IC_{50} (0.62 µg/mL, 0.30 µg/mL, acarbose: 22.80 µg/mL ve 10.01 µg/mL, respectively) values were found.

In their study, Kocyigit et al. [40] evaluated the *α*-glucosidase enzyme inhibition activity of *A. smokeii* species in terms of anti-diabetic properties and compared it with acarbose (0.48 and 22.8 µg/mL, respectively) as a positive control.

In their study, Ghaffari et al. [41] measured the α-glucosidase enzyme inhibition activity of dichloromethane and methanol extracts of the *A. creticus* species in terms of anti-diabetic properties and compared them against acarbose (inhibitions, 91.28±0.85%, 86.32±0.74% and 65.73±1.93%, respectively).

It can be said that this study shows parallelism with the anti-diabetic α-glucosidase enzyme inhibition activity studies conducted on the genus *Astragalus* in the literature review. In this study, it was determined that only acetone extracts of *A. schizopterus* and *A. distinctissimus* had high α-glucosidase enzyme inhibition activity.

4. Conclusion

In this study, the effects of acetone and ethanol extracts prepared from the aerial parts of *A. leporinus* var. *hirsutus, A. schizopterus,* and *A. distinctissimus* plants on anti-aging (elastase and collagenase enzyme inhibition activities) and anti-diabetic (α-glycosidase and α-amylase enzyme inhibition activities) were determined. Additionally, some triterpene contents were determined by GC-MS.

The research results showed that a very high amount of ursolic acid (85.86 mg/g extract) was detected in the acetone extract of *A. schizopterus* species. Furthermore, it was broadly determined that acetone extracts of *A. schizopterus* and *A. distinctissimus* species exhibit a high degree of inhibitory activity of α-glucosidase. Gathering the above points together, it is reasonable to conclude that acetone extracts of *A. schizopterus* and *A. distinctissimus* species should be subjected to more detailed studies to be used in pharmaceutical industries due to their ursolic and oleanolic acid content and anti-diabetic potential.

Conflict of Interest

The authors declared no potential conflicts of interest concerning the research, authorship, and publication of this article.

Acknowledgment

The authors thank Dr. Serpil Demirci Kayıran (Çukurova University Faculty of Pharmacy, Pharmacy Vocational Sciences, and Pharmaceutical Botany Department) for supporting plant identification in this study. This research was supported by Dicle University Scientific Research Projects Coordination Unit Coordinatorship (Project Numbers: ECZACILIK.22.001 and ECZACILIK.23.002).

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