

LC-HRMS profiling of phytochemicals, antidiabetic, anticholinergic and antioxidant activities of evaporated ethanol extract of *Astragalus brachycalyx* Fischer

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Abstract: *Astragalus* is a perennial plant that has existed for about 2500-3000 years and consists of more than 250 taxonomic parts. Twenty species of *Astragalus* are endemic to Turkey, as well as the richest genus with 425 taxa. The roots of *Astragalus* species are used in folk medicine as hepatoprotective, antioxidant, antibacterial, antihypertensive, antidiabetic and diuretic. Also, it is used to treat diabetes mellitus, leukemia, nephritis and uterine cancer. It is known that in Anatolia, *Astragalus* roots are traditionally used against leukemia and wound healing. For the purpose, the measuring of antioxidant activity of evaporated ethanol extract of *Astragalus brachycalyx* FISCHER (EEAB), some bioanalytic methods including DPPH[•] and ABTS^{•+} scavenging effects, ferric ions (Fe³⁺) and cupric ions (Cu²⁺) reducing abilities, and metal (Fe²⁺) chelating activity were realized. α -Tocopherol, ascorbic acid, and BHT were used as the standard antioxidants. On the other hand, some phenolic compounds, which responsible for antioxidant activities of EEAB was determined by liquid chromatography-high resolution mass spectrometry (LC-HRMS). At the similar concentration, EEAB exhibited efficient antioxidant effects when compared to standard compounds. Additionally, EEAB showed IC₅₀ values of 1.985 μ g/mL toward acetylcholinesterase (AChE), 0.620 μ g/mL on α -glycosidase and 0.306 μ g/mL against α -amylase enzymes.

Keywords: *Astragalus brachycalyx*; phenolic compounds; antioxidant activity; acetylcholinesterase; α -glycosidase; α -amylase. © 2021 ACG Publications. All rights reserved.

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1. Introduction

Astragalus L., which belongs to the Fabaceae family, is locally called as “geven”. *Astragalus* L. is a perennial herb that has existed for about 2500-3000 years and consists of more than 250 taxonomic parts [1]. Twenty species of *Astragalus* L. are endemic to Turkey, as well as the richest genus with 425 taxa [2]. Plants of this genus are used in scientific or folk medicine in many countries [3]. It is known that the roots of *Astragalus* species are used in folk medicine as hepatoprotective, antioxidant, antibacterial, antihypertensive, antidiabetic and diuretic. It is also used for treatment of diabetes mellitus, leukemia, uterine and nephritis cancers. In Anatolia, *Astragalus* roots are traditionally used for treatment of wound healing and leukemia [2]. These biological activities depend on the presence of various secondary metabolites of this plant [4]. In particular, phenolics, alkaloids, flavonoids, anthraquinones and saponins are thought to be the main chemicals responsible for its biological activities [5]. It has been reported that some bioactive compounds from *Astragalus* species had immunostimulant and anticancer activities [6].

Changing living conditions, environmental pollution, industrial wastes, sun rays, exhaust gases, heavy metals, cigarettes and various chemicals have become elements that today's people cannot escape [7,8]. All this causes the formation of reactive oxygen species (ROS) and free radicals, which have hazardous effects for living organisms [9-11]. Despite the increasing number of free radicals, the protective effect of antioxidant metabolism can often be insufficient [12-14]. Oxidative stress plays a crucial role in many diseases including cancer, Alzheimer's disease (AD) and type-2 diabetes mellitus (T2DM) [15-17]. On the other hand, antioxidants delay the formation of degenerative diseases due to free radicals and ROS formation by reducing oxidative stress in the body. These chemicals also had protective effect of body against metabolic disorders or damages caused by free radicals and ROS [18-20]. Recently, the use of synthetic antioxidants like BHA and BHT has been restricted due to their undesired effects like carcinogenic effect. So, the studies on natural antioxidants are of great importance [21,22].

Today, many plants are used both as medicine among the public in terms of the effective substances they contain and medical studies are carried out on them [23]. The fact that natural antioxidant vitamins such as β -carotene, α -tocopherol, ascorbic acid are abundant among the active components that plants contain, makes these products of natural origin very important [24-26]. For this purpose, great importance has been recently attached for evaluation of medicinal plants as antioxidant sources in many related industrial fields, especially in medicine, food and cosmetics [27,28]. In addition, the use of drugs derived from medicinal plants and plant-based products is increasing significantly [15,29-31].

This study aimed to determine the antidiabetic, anticholinergic, antioxidant and radical scavenging activities of the evaporated ethanol extract (EEAB) of aerial parts of *Astragalus brachycalyx*. For this purpose, potassium ferricyanide reducing, Fe^{3+} -TPTZ reduction (FRAP), copper ions (Cu^{2+}) reducing capacity (CUPRAC), DPPH \cdot and ABTS \cdot^+ scavenging and metal chelating experiments were used. Total phenolic contents in EEAB were quantitatively determined by liquid chromatography and high resolution mass spectrometry (LC-HRMS), in addition, total phenolic and flavonoid contents were determined. Another aim of this study was identified the possible inhibitory effects of EEAB toward acetylcholinesterase, α -amylase and α -glucosidase enzymes. It is aimed that our study will be a reference for future studies.

2. Materials and Methods

2.1. Chemicals

The compounds, which are used for determination of antioxidant activity including 2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), neocuproine (2,9-dimethyl-1,10-phenanthroline), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), α -tocopherol and ascorbic acid, standard phenolics compounds for LC-HRMS were obtained from Sigma (Sigma-Aldrich GmbH,

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Steinheim, Germany). The other compounds used were of analytical grade and purchased from either Sigma-Aldrich or Merck Millipore.

2.2. Plant Material

Astragalus brachycalyx FISCHER was collected from Bitlis in July 2019 (Location: 38°24'21.2"N 42°06'04.2"E, MP16561 code). This *Astragalus* species was identified by botanist Dr. Süleyman Mesut PINAR, Department of Nutrition and Dietetics, Faculty of Health Sciences, Van Yüzüncü Yıl University. Plant samples were deposited at Herbarium of the Biology Department (VANF), Faculty of Science, Van Yüzüncü Yıl University, Van, Turkey.

2.3. Evaporated Ethanolic Extract (EEAB)

The extraction procedure was applied as described formerly [32,33]. To prepare the EEAB, first 50 g of the plant material was ground to powder and soaked in 0.5 L ethanol then the solvent was evaporated using a rotary evaporator (Heidolph Hei-VAP HL, Germany) [34]. Plant extract which obtained in dry form is stored at -20° C until used.

2.4. Total Phenolics and Flavonoids Contents

To determine the total phenolic contents of EEAB, a previous method was used [35]. Gallic acid (GA) was used as standard phenolic. Total phenolic content in EEAB was determined as GA equivalent (GAE) [36]. The total flavonoids in EEAB carried out as described previously [37]. Quercetin (Q) was used as standard flavonoid. The quantity of total flavonoid was determined as microgram Q equivalent (QE) from the equation obtained from the standard quercetin plot.

2.5. Reducing Ability Assays

The Fe^{3+} reducing ability of EEAB was carry out by the $\text{Fe}^{3+}(\text{CN})_6$ reducing procedure [38-42]. For determination of copper ions (Cu^{2+}) reduction capacity of EEAB, the method of Apak et al. was applied with some modification [43,44]. The FRAP reducing method was realized based on spectrophotometric measurement of reduction of TPTZ- Fe^{3+} complex under acidic conditions [45-47].

2.6. Metal Chelating Ability

Metal chelators prevent oxidation by reducing the activity of metals such as iron after complex formation. Metal chelating capacity of EEAB was measured according to the disruption of Fe^{2+} -ferrozine complex with some modifications [48,49].

2.7. Radical Scavenging Ability

DPPH \cdot scavenging ability of EEAB was determined according to Blois method [50] as given previously [51,52]. Different concentrations (25-75 $\mu\text{g}/\mu\text{L}$) of EEAB were completed with ethanol and 1 mL DPPH \cdot in test tubes and their absorbances were measured at 517 nm. Another radical scavenging assay is ABTS $^{+\cdot}$ scavenging effect and realized according to the previous study [53]. This method was used to define the ABTS $^{+\cdot}$ scavenging efficacies of EEAB.

2.8. Anticholinergic and Antidiabetic Assays

The inhibition ability of EEAB on acetylcholinesterase (AChE) from Electric eel was realized according to previous studies [54-58]. To determine the α -glycosidase inhibition of EEAB, the method, which described in previous researches was applied [58-60]. The α -amylase inhibition efficacy of EEAB was estimated according to previous studies [61,62]. The IC_{50} was calculated from activity (%) versus of EEAB concentration plots [63-66].

2.9. LC-HRMS Analysis

Determination of phenolic content of EEAB was made according to LC-HRMS analysis method [67-69]. LC-HRMS experiments were performed on a Thermo ORBITRAP Q-EXACTIVE mass spectrometry equipped with a Troyasil C18 column (150 x 3 mm i.d., 3 μ m particle size) [70]. The finest mobile phase was determined in acidified methanol and water gradient by HPLC method [67,70-72]. The identification of phenolic compounds was made by comparing the retention times of the standard compounds (in the range of purity 95%-99% see section chemicals) and HRMS data of Bezmialem Vakıf University, Drug Application and Research Centre Library (ILMER). Dihydrocapsaicin (95% purity) used as an internal standard for LC-HRMS measurements for reduce to repeatability problem of caused by external effects, such as ionization repeatability, in mass spectrometry measurements. 100 mg/L dihydrocapsaicin (97%, Sigma-Aldrich) solution was used as an internal standard (IS). The mass parameter of the target compounds is given in previous studies [16,68,69].

3. Results and Discussion

In recent years, there has been a significant increase in studies investigating the biological activities of crude extracts or isolated pure compounds from plants. Natural products had a great importance in the drug development process for cancer and infectious diseases. Many species have been investigated in terms of their antioxidant activities and some species have been determined to have an important biological potential in these subjects [73-75]. Many *Astragalus* species have been examined for their antioxidant, antiviral, anticancer, cytotoxic, immunostimulant, analgesic, and anti-inflammatory effects. *Astragalus* species have been reported to contain bioflavonoids, amino acids, triterpene glycosides, flavonoids, isoflavonoids, and saponins [76]. The antioxidant potential of *Astragalus brachycalyx* has been investigated in order to determine its health benefits and to be a step for further studies. For this intention, three metal reduction methods (FRAP, CUPRAC and Fe³⁺ reduction) and two radical removing assays (DPPH and ABTS scavenging) and Fe²⁺ chelating ability were applied for the EEAB. In this study, a combination of selected bioanalytical antioxidant methods was also performed to extract complementary information about antioxidant mechanisms. The antioxidant profile of ethanol extract obtained from aerial parts of *Astragalus brachycalyx*, as characterized using Fe³⁺ reducing, CUPRAC and Fe³⁺-TPTZ reducing (FRAP) assays, shown in Table 1 and Figure 1 .

Table 1. The reducing power of the EEAB and standards by Fe³⁺ reducing (120 μ g/mL) and Fe³⁺-TPTZ reducing (150 μ g/mL) and Cu²⁺ reducing (30 μ g/mL) methods

Antioxidants	Fe ³⁺ reducing		Cu ²⁺ reducing		Fe ³⁺ -TPTZ reducing	
	λ_{700}	r ²	λ_{450}	r ²	λ_{593}	r ²
Ascorbic acid	1.520 \pm 0.028	0.9970	1.069 \pm 0.007	0.9722	1.624 \pm 0.015	0.9930
α -Tocopherol	0.990 \pm 0.007	0.9942	0.785 \pm 0.061	0.9986	0.755 \pm 0.075	0.9867
BHT	1.269 \pm 0.005	0.9880	1.561 \pm 0.089	0.9978	0.909 \pm 0.006	0.9874
EEAB	0.849 \pm 0.001	0.9460	0.598 \pm 0.003	0.9996	0.521 \pm 0.108	0.9808

Ferric reducing power is an important test for determination of antioxidant activity. It is widely used for investigation of antioxidant activities of natural compounds and extracts. This test can show the donation of electrons or hydrogen. In the reaction system, Fe³⁺ in potassium ferricyanide is reduced to Fe²⁺ with the addition of antioxidant substance and the resulting Prussian blue color which is observed at 700 nm [77-79]. The reducing ability of aerial parts of the EEAB and positive controls was found to be descending order of Ascorbic acid (1.520 \pm 0.028, r²:0.9970) > BHT (1.269 \pm 0.005, r²:0.9880) > α -Tocopherol (0.990 \pm 0.007, r²:0.9942) > EEAB (0.849 \pm 0.001, r²:0.9460) (Table 1 and Figure 1a). The results demonstrated that *Astragalus brachycalyx* has a high Fe³⁺ reduction capacity and electron donor

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ability to neutralize free radicals and ROS. However, this reducing ability was lower than that of standard compounds. In a conducted previous study, reducing power of *Astragalus diptherites* and *Astragalus gymnaepecias* shoot parts at 150 µg/mL concentrations in the methanol extracts are as follows 0.120 and 0.100 µg/mL, respectively [80]. It is seen that these results are compatible with the results of the current study.

The CUPRAC test is based on the absorbance measuring at 450 nm of a stable complex formed between neocuproine and copper ions. A higher absorbance exhibits higher reducing ability of antioxidants [81-83]. Cu²⁺ ions reducing ability of 30 µg/mL concentrations of positive controls and EEAB are shown in (Table 1 and Figure 1b). Cu²⁺ ions reducing ability of EEAB and standards at the concentration of 30 µg/mL were found as order of BHT (1.561±0.089, r²: 0.9978) > Ascorbic acid (1.069±0.007, r²: 0.9722) > α-Tocopherol (0.785±0.061, r²: 0.9986) > EEAB (0.598 ± 0.003, r²: 0.9996). According to Haşimi et al. the related results of methanol extracts of *Astragalus leporinus* and *Astragalus schizopterus* plants were determined as follows, 50.06±0.21 µg/mL and 22.35±0.12 µg/mL, respectively [84]. In another study, the Cu²⁺ reducing results of ethyl acetate extract of *Astragalus armatus* was determined as 50.60±0.9 µg/mL [85]. The results of the current study were found to be parallel to the literature.

The last reducing method studied in this regard is the Fe³⁺-TPTZ reducing ability. This is one of the reduction power methods of Fe³⁺ to Fe²⁺ and as a result of it a color change occurs, which has an absorption at 593 nm [86-88]. The reducing power of EEAB and standards decreased in the following order of Ascorbic acid (1.624 ±0.015, r²:0.9930) > BHT (0.909±0.006, r²:0.9874) > α-Tocopherol (0.755±0.075, r²:0.9867) > EEAB (0.521±0.108, r²:0.9808) (Table 1 and Figure 1c). High absorbance values on the basis of this method indicate that the Fe³⁺-TPTZ complex has strong reduction ability [89]. As a result, EEAB was found to have a strong FRAP reduction ability. When the literature on similar studies was searched, it was seen that there were few studies. In a recent conducted study, the FRAP assay result of the methanol extract of the *Astragalus glaucacanthus* plant was determined as 0.289 mmol Fe²⁺ per g extract [90]. In all reduction methods, EEAB exhibited effective reducing abilities and these activities close to the standards including ascorbic acid, α-Tocopherol and BHT.

The DPPH scavenging method is frequently used for evaluation *in vitro* antioxidant ability of plant extracts as it is a reliable, rapid and reproducible test. The obtained results are commonly expressed as IC₅₀ values. Lower IC₅₀ values indicate stronger antiradical activity [91-93]. As a result of our literature review, we did not find a publication that studied the DPPH or ABTS radical scavenging activity of *A. brachycalyx*, but a few studies using different species of *Astragalus* genus are given below. The IC₅₀ values of DPPH scavenging for EEAB and standards were determined in following order of Ascorbic acid (16.116±0.003, r²: 0.9566) > α-Tocopherol (23.1±0.032, r²: 0.9825) > BHT (31.500±0.011, r²: 0.9754) > EEAB (115.5 ± 0.030, r²: 0.9769) (Table 2 and Figure 2a). Ascorbic acid was found as most effective DPPH radical scavenging effect when considering other samples. In a previous study [94], DPPH radical removing in 70% ethanol extract of *A. glycyphyllosin* flowers; it was found to be 35.64 µmol/g plant. In a recent conducted study, the IC₅₀ result of DPPH radical removing in the methanol extract of *Astragalus squarrosus* was determined as 1220 mg/L [95]. When we evaluated all the results together with the current study, it was determined that the highest IC₅₀ values were obtained from *Astragalus* species according to the standards. It is seen that the EEAB extract does not have a very strong DPPH[•] removal activity, which is compatible with the literature.

EEAB showed high scavenging activity against ABTS radicals. The IC₅₀ values of ABTS^{•+} scavenging for EEAB and standards were found in descending order of α-Tocopherol (15.400±0.003, r²: 0.9866) > EEAB (16.116±0.069, r²: 0.9732) > Ascorbic acid (23.10±0.001, r²:0.9998) > BHT (26.654±0.008, r²:0.9717) (Table 2 and Figure 2b). It was determined that IC₅₀ values of ABTS^{•+} removing of methanol extracts of *Astragalus leporinus*, *Astragalus distinctissimus*, *Astragalus schizopterus* plants as follows, 65.37±0.44 µg/mL, 54.71±0.09 µg/mL, 22.01±0.07 µg/mL, respectively [84]. In another study, ABTS^{•+} removing result in ethyl acetate extract of *Astragalus armatus* plant was determined as 11.30±0.09 µg/mL [85]. In our study, the IC₅₀ values obtained for ABTS^{•+} removing was similar to the above studies. Moreover, it was observed that *A. brachycalyx*, the subject of our study, had a very strong ABTS^{•+} scavenging activity.

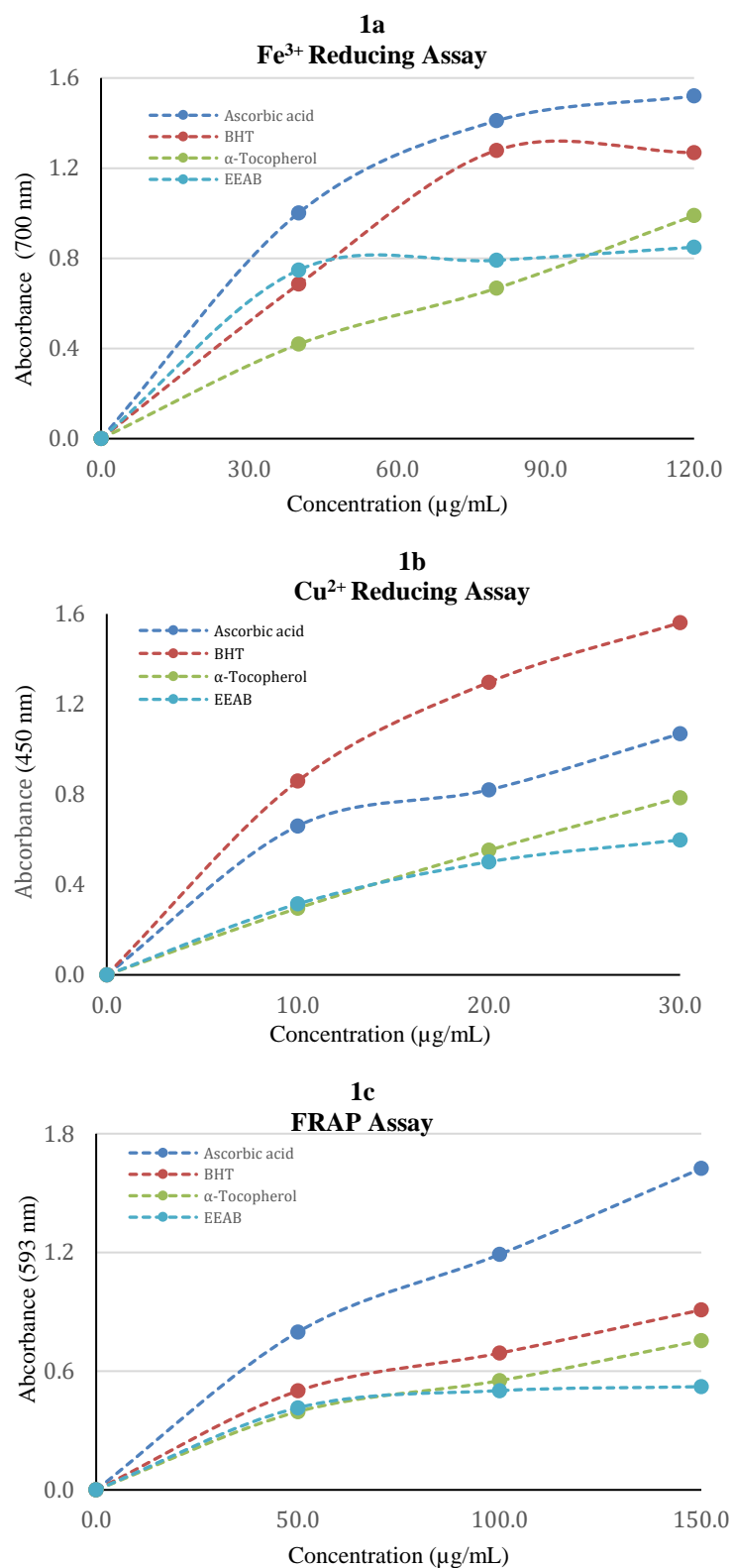


Figure 1. The reducing ability of the EEAB and standards by Fe³⁺ reducing (120 µg/mL) (a) and Fe³⁺-TPTZ reducing (FRAP) (150 µg/mL) (b) and Cu²⁺ reducing (30 µg/mL) (c) methods

LC-HRMS profile of *Astragalus brachycalyx* extracts and their biological activities**Table 2.** IC₅₀ values (µg/mL) of EEAB and standards for the DPPH and ABTS radicals scavenging and metal chelating activities (EEAB: Evaporated ethanol extract of *Astragalus brachycalyx* FISCHER)

Compounds	DPPH• scavenging		ABTS ^{•+} scavenging		Metal chelating	
	IC ₅₀ *	r ²	IC ₅₀ *	r ²	IC ₅₀ *	r ²
α-Tocopherol	23.1 ± 0.032	0.9825	15.400 ± 0.003	0.9866	330.0 ± 0.017	0.9109
Ascorbic acid	16.116 ± 0.003	0.9566	23.10 ± 0.001	0.9998	99.0 ± 0.036	0.9985
BHT	31.500 ± 0.011	0.9754	26.654 ± 0.008	0.9717	14.745 ± 0.056	0.9646
EEAB	115.5 ± 0.030	0.9769	16.116 ± 0.069	0.9732	5.095 ± 0.043	0.9261

Transition metals in biological fluids cause radical degradation by Fenton reaction. Metal chelating is secondary antioxidants, which responsible for metals chelation [85]. When metal chelating activity was evaluated for *Astragalus brachycalyx* and standards, it was found that EEAB and ascorbic acid had IC₅₀ value of 5.095 µg/mL and 99.0 µg/mL, respectively (Table 2 and Figure 2c). EEAB was found the most effective chelating activity. The IC₅₀ values of metal chelating activity of EEAB and standards decreased in following order of EEAB (5.095±0.043, r²: 0.9261) > BHT (14.745±0.056, r²:0.9646) > Ascorbic acid (99.0±0.036, r²: 0.9985) > α-Tocopherol (330.0±0.017, r²: 0.9109). In a prior conducted study, the metal chelating activity was found to be 17.4±0.3 at 100 µg/mL in the ethyl acetate extract of *Astragalus armatus* [82]. In another study, the metal chelating capacity of flowers and stems of *Astragalus glycyphyllos* were respectively; 10.09 µmol/g plant; 6.34 µmol/g plant. Also, the metal chelating capacity for the extracts were obtained from the leaves and flowers of the *Astragalus cicer* was found as 9.94 and 9.90 µmol/g, respectively [94]. In a previous conducted study, metal chelating activity of methanol extracts of *Astragalus diphtherites* and *Astragalus gymnaopecias* at 25 µg/mL were found as follows: 4.1±0.1, 22.0±0.1 µg/mL, respectively [80]. When all these results were evaluated, *Astragalus brachycalyx* has a quite effective metal chelating ability.

The total phenolic and flavonoid contents in EEAB was found to be 23.182 µg GAE and 4.672 µg QE, respectively. There was found a positive correlation between total phenolics/flavonoids and antioxidant activity. Biologically active substances such as phenolics and flavonoids are the main components of plants with antioxidant activity [96]. Also, flavonoids are known to be potent inhibitors of several enzymes including xanthine oxidase, cyclooxygenase, and lipoxygenase [97,98]. In a study, the total phenolics and flavonoids in the methanol extract of *Astragalus squarrosus* were found as 23.3 mg/g and 26.0 mg/g, respectively [92]. In another study, the total phenolics and flavonoids in the methanol extracts of the stem parts of *Astragalus diphtherites* and *Astragalus gymnaopecias* were found as 76.1±0.9, 54.66±2.3 µg GAE/mg and 39.31±0.2, 36.81±0.3 µg QE/mg [80]. In this study, it was observed that the results obtained for the total phenolic and flavonoid amounts were compatible with the above studies.

The antioxidant ability of medicinal plants is due to their phenolic and flavonoids contents [99,100]. Phenolic compounds act as hydrogen donors, reducing agents, metal chelators and singlet oxygen quenchers. In this way, they can terminate the oxidation mechanism [101]. In this way, the plants can provide significant protection against oxidative stress and free radical damage. It has been determined that flavonoids, as a main phenolic class, have anti-inflammatory, antiallergic, antiviral and anticarcinogenic properties [102,103]. The phenolic content in EEAB was investigated by LC-HRMS analysis and the quantification and identification of thirty-seven phenols were performed in this study (Table 3). The main compound identified in 1 mg of EEAB is fumaric acid (7229.15 mg/kg) which is a natural organic acid. It finds implementation in nearly every field of industrial chemistry, hispidulin (5797.94 mg/kg) is a medicinal natural compound, which shows strong anticancer properties and luteolin (722.48 mg/kg) that is a natural flavonoid, and it presents in many plant species extensively. Luteolin shows many biological effects such as anti-inflammation, anti-allergy and anticancer and can biochemically act as an antioxidant or a pro-oxidant [104]. When the literature on the subject was searched, no study investigating the phenolic compound content of *Astragalus brachycalyx* was found, but it was determined that there were studies conducted on different *Astragalus* species.

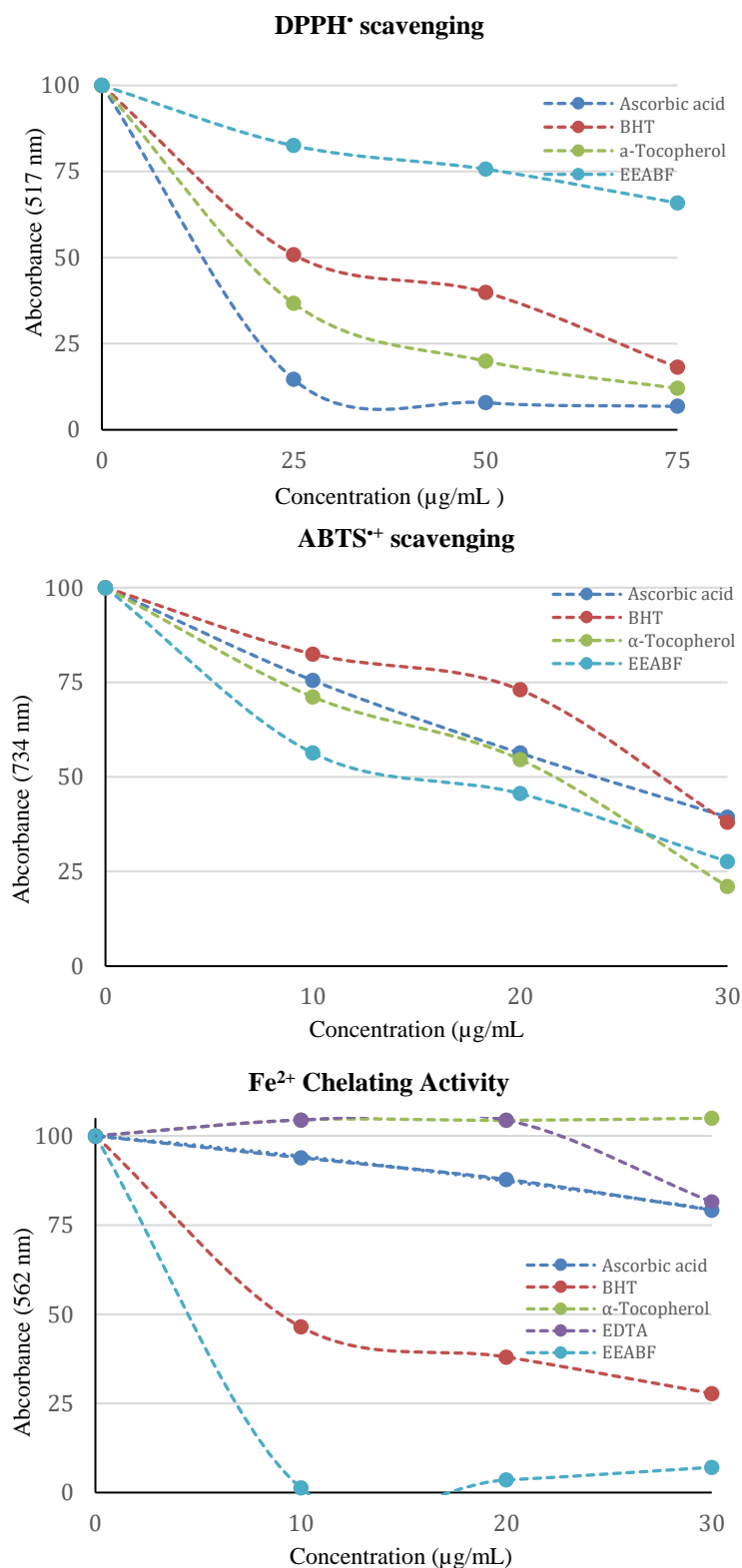


Figure 2. DPPH and ABTS radical scavenging activities and metal binding ability of EEAB and standards

In another recent study in this context, methanolic extracts of *A. leporinus*, *A. distinctissimus* and *A. schizopterus* were analyzed by LC-MS/MS. They reported that the most abundant phenolic compound in all three *Astragalus* species was rutin (1028.276-13351.76 µg/g extract) [84]. In a recent study, it was determined that the plentiful phenolic compound was quercetin (353.11 µg/g extract) [105].

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In another study carried out recently, methanol extract of *Astragalus gymmolobus* plant was examined in HPLC-DAD system and rutin (50.48 mg g⁻¹ dry extract) [106]. Also, the high amount of phenolic content determined in previous studies is similar to the current study results.

Table 4. The quantity (mg/kg extract) of phenolics in EEAB determined by LC-HRMS

Compounds	Quantity (mg/kg)	U (%)
Ascorbic acid	223.14	3.94
(-)-Epigallocatechin gallate	2.25	3.76
Chlorogenic acid	160.89	3.58
Fumaric acid	7229.15	2.88
(-)-Epicatechin	<LOD	3.17
Verbascoside	303.51	2.93
Orientin	<LOD	3.67
Caffeic acid	19.88	3.74
(+)- <i>Trans</i> taxifolin	2.27	3.35
Luteolin-7-rutinoside	26.09	3.06
Vanillic acid	<LOD	3.49
Naringin	<LOD	4.20
Luteolin 7-glucoside	<LOD	4.14
Hesperidin	<LOD	3.79
Rutin	179.93	3.07
Syringic acid	258.59	3.71
Rosmarinic acid	30.03	3.77
Hyperoside	9.81	3.46
Dihydrokaempferol	<LOD	2.86
Apigenin 7-glucoside	9.29	3.59
Ellagic acid	<LOD	4.20
Quercitrin	<LOD	3.78
Nepetin-7-glucoside	<LOD	3.07
Quercetin	8.76	2.95
Salicylic acid	121.44	1.89
Naringenin	280.73	4.20
Luteolin	722.48	3.42
Nepetin	24.17	2.19
Apigenin	585.36	2.87
Hispidulin	5797.94	3.41
Isosakuranetin	<LOD	3.98
Penduletin	<LOD	3.20
Caffeic acid phenethyl ester	1.11	3.13
Chrysin	11.60	3.24
Acacetin	149.81	3.98
Emodin	<LOD	4.27
Hederagenin	<LOD	1.80

Alzheimer's disease (AD) occurs at the molecular level through protein misfolding and aggregation, oxidative stress, mitochondrial abnormalities and neuroinflammatory processes, leading to progressive dementia, memory loss and other cognitive impairments [107,108]. Acetylcholine (ACh), which hydrolyzed by acetylcholinesterase (AChE), is a crucial neurotransmitter in the regulation of learning and memory processes. So, cholinesterase inhibitors increase the level of acetylcholine [109,110]. AD is associated with low acetylcholine levels. Therefore, the cholinergic system is under serious

investigation as it represents an important solution for the treatment of AD. Tacrine, donepezil and galantamine are FDA approved drugs, which improve AD symptoms as clinical AChE inhibitors [111]. Although current AChE inhibitors have documented beneficial effects on cognition and behavior, they have moderate benefits and serious side effects [112]. Therefore, effective therapeutic molecules are being investigated for permanent treatment of AD [113]. In our study, the results of AChE inhibition linked to AD were evaluated. It was determined that EEAB effectively inhibited AChE with an IC_{50} value of 1.985 $\mu\text{g/mL}$ (r^2 : 0.9838). Tacrine was used as positive control for AChE inhibition with an IC_{50} value of 0.124 μM against AChE [49,114]. Although EEAB showed a high inhibition of AChE, this amount of inhibition was found to be very low compared to Tacrine. According to the literature review, while there no study for investigating the cholinesterase inhibition effects of *Astragalus brachycalyx*. It was determined that there were studies on different *Astragalus* species. In a recent conducted study, ethyl acetate extract of *Astragalus armatus* exhibited weak inhibitory effect against AChE [85]. Also, it was found that AChE inhibition effects of ether extract of *Astragalus leporinus*, *Astragalus distinctissimus*, *Astragalus schizopterus* plants were determined as follows, 46.96 ± 4.06 , 54.71 ± 0.09 and $22.01 \pm 0.07\%$, respectively [84].

Table 5. IC_{50} values ($\mu\text{g/mL}$) of EEAB against α -glycosidase, α -amylase and acetylcholinesterase

Enzymes	EEAB		Standards
	IC_{50} ($\mu\text{g/mL}$)	r^2	IC_{50} (μM)
α -Glycosidase ^a	0.620	0.9526	22.80
α -Amylase ^a	0.306	0.9935	10.01
Acetylcholinesterase ^b	1.985	0.9838	0.124

^a Acarbose was used as positive control for α -glycosidase and α -amylase enzymes and taken from reference 107.

^b Tacrine was used as positive control for acetylcholinesterase enzyme and taken from reference of 46.

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder manifested by increased blood glucose and insulin resistance. It leads to cardiovascular, nephropathy, retinopathy and neuropathy diseases and ultimately causes serious damage to vessels and nerves [115]. α -Amylase and α -glycosidase enzymes act as important hydrolytic enzymes in carbohydrate catabolism. It is known that the inhibition of bot enzymes helps lower blood sugar levels [116]. α -Glycosidase and α -amylase enzymes was conducted to determine the antidiabetic properties of the plants and herbs. The IC_{50} values for α -glycosidase were measured as 0.620 $\mu\text{g/mL}$ (r^2 :0.9526) for EEAB and 22.80 μM for acarbose [107]. In addition to, the IC_{50} values for α -amylase were measured as 0.306 $\mu\text{g/mL}$ (r^2 : 0.9935) for EEAB (Table 5). When the results were evaluated, it was seen that EEAB has quite high affinity to α -amylase and α -glycosidase enzymes. Also, EEAB was more effective than that of acarbose, a standard inhibitor. These results were found to be compatible with our study. Since literature data on AChE, α -glycosidase and α -amylase inhibitory properties are not available for *Astragalus brachycalyx*. The present study provides a reference for it for the first time.

This study provides important information about the antioxidant capability, phenolic and flavonoid contents of EEAB, as well as its antidiabetic and anti-Alzheimer properties. It was determined that the ethanol extract of EEAB has a value close to the standard antioxidants. Also, *Astragalus brachycalyx* was found to have a high content of total phenolic and flavonoids. It was determined by LC-HRMS analysis that fumaric acid, hispidulin and luteolin are major phenolic compounds of EEAB. Also, EEAB showed a high inhibitory effect against AChE, α -glycosidase and α -amylase enzymes which means have effective anti-AD and antidiabetic activities. Based on these results, it was determined that EEAB, which is the subject of the research, has a natural antioxidant potential that can be used in food and drug applications, and it is thought that the study may be a basis for further phytochemical research.

4. Conclusions

Evaluation of the biological screening including antioxidant effectiveness of EEAB had a great importance. In this context, reducing ability methods were used to provide information on single electron transfer, while radical scavenging methods were used as indicator radicals that can be neutralized by reduction via electron transfers as well as quenching via hydrogen atom transfer. mechanism. The EEAB was analyzed for its antioxidant activities and bioactivities including inhibitory properties of some metabolic enzymes associated with some global diseases. In addition, a positive correlation was found between the total phenolic or flavonoid contents of EEAB and its biological ability. Also, when the chromatographic results were evaluated, it was seen that the main biological activities of the EEAB were hispidulin, luteolin and apigenin. Ethanol was observed as an effective solvent for the extraction procedure of phenolics with effective antioxidant and enzyme inhibition abilities. Nowadays, enzyme inhibition had crucial importance for controlling overactive enzyme has become a key target to treated many chronic diseases including cancer, AD and diabetes.

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