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Analysis of Phenolic Compounds by LC-HRMS and Determination of Antioxidant and Enzyme Inhibitory Properties of *Verbascum speciousum* Schrad

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Abstract: Studies have shown an inverse correlation between age-related illnesses like coronary heart disease and cancer and fruit and vegetable intake. Given the probable health benefits of natural antioxidants from plants, research on them has increased. Verbascum L is a large genus of Scrophulariaceae family and 323 species distributed worldwide. Since *Verbascum* L. species are plants that are grown in many regions of Turkiye and are used in folk medicine, it is important to evaluate the biological activity of these species. In this study antioxidant properties of *Verbascum speciosum* Schrad. were investigated. The antioxidant capacities of water and ethanol-based extracts obtained from air parts were evaluated with Fe^{3+} reducing, CUPRAC, FRAP, DPPH$^{\bullet}$, and $ABTS^{\bullet+}$ scavenging antioxidant methods. Extracts were also examined to determine their AChE, α-glycosidase and α-amylase enzyme inhibitions. This investigation could be a basis for further phytochemical investigations of *Verbascum speciosum* Schrad.

Keywords: *Verbascum speciosum;* antioxidant activity; reducing power; radical scavenging; enzyme inhibition.

1. Introduction

Oxidation is essential to fuel the biological processes of living organisms. However, it can cause uncontrolled production of oxygen-induced free radicals [1]. Free radicals are highly unstable, reactive, short-lived atoms or molecules with unpaired electrons. Therefore, they tend to attack and bind with other molecules to gain a more stable form [2,3]. During the functioning of aerobic metabolism, it is inevitable for cells to produce free radicals and especially reactive oxygen species (ROS) [4]. The

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balance between ROS formation and reduction is controlled by the antioxidant system [5-7]. But, exposure to UV light, smoking, and other environmental pollutants and cell metabolism disorders also increase the body's free radical level and a defect in the cell's redox balance becomes [8,9]. Increased ROS attacks membranes, enzymes, lipids, carbohydrates, proteins, and DNA causing various disorders and diseases [10]. There are studies confirming the role of free radicals in the generation of diseases such as atherosclerosis, cancer, cardiovascular diseases, and brain dysfunction [11]. Antioxidants consist of some enzymes, vitamins E, β-carotene, or organic substances such as phenols and amines, which can stop radical chain reactions and counter the harmful effects of oxidation in tissues [12,13]. Antioxidants delay or completely inhibit the oxidation process of unstable molecules. In this way, antioxidants are molecules that can prevent the oxidation of biomolecules caused by free radicals and related cell damage [14]. Insufficient intake and production of antioxidants can cause oxidative stress in case of excessive formation of free radicals [15,16]. Natural antioxidants are created in living cell metabolism to maintain the oxidation-reduction balance [17]. Synthetic antioxidants are often used nowadays [1]. Recently, synthetic antioxidants are suspected to have carcinogenic effects and therefore their use has been limited, so there is significant attention on the discovery of natural antioxidants that can be used as substitutes [18-20]. Plants constitute an important source of active compounds and thus can show strong antioxidant effects. The fact that plants contain these active compounds that create physiological effects in the human body adds medicinal value to them [21,22]. Medicinal plants are recognized as a resource for the prevention of many diseases [23]. They attract the attention of researchers in the therapy of some diseases such as metabolic inflammations and cancer. Some phytochemicals are the subject of cancer therapy research. Many phenolic and flavonoid compounds are frequently used in drug development processes [24-26]. Studies have determined that phenolic compounds in plants reduce oxidative damage and prevent from free radicals [27]. Therefore, the commercial importance of plants containing these secondary compounds is increasing [28-30]. We believe *Verbascum speciosum* (*V. speciosum*) is one of those medicinal plants.

The genus *V. speciosum* belongs to the family Scrophulariaceae and includes 360 species distributed worldwide [31]. It is represented by 132 additional hybrids and 257 species in Turkiye among them, 202 species are endemic [32]. *Verbascum* L. genus is known as mullein. It has been reported that drugs prepared from different parts of *Verbascum* L. are used for skin, lung, and intestinal diseases, cough, headaches, and chills in Europe, Asia, Africa, and North America [33]. Traditionally, all parts of *Verbascum* L. species are used in Turkiye for the treatment of eczema and other skin disorders and for drying wounds [34]. *V. speciosum* is one of the well-known species of Verbascum L. It has been reported to be used traditionally for different diseases including hemorrhoids and coughs [33].

In the present study, ethanol extract of aerial parts of *V. speciosum* and lyophilized water extract of aerial part of *V. speciosum* (WEVS) were evaluated by several bioanalytical antioxidant methods including Fe^{3+}-TPTZ reducing capacity (FRAP), Fe^{3+} reducing, Cu^{2+} reducing ability (CUPRAC), DPPH and ABTS radical scavenging. Furthermore, total phenol and flavonoid contents were also investigated. Quantitative determination of phenolic substances was made by LC-HRMS. EEVS and WEVS were examined to determine their α-amylase, acetylcholinesterase (AChE), and α-glycosidase enzyme inhibitions.

2. Materials and Methods

2.1. Chemicals

All chemicals for antioxidant activity end enzyme inhibition such as α-tocopherol, neocuproine (2,9-dimethyl-1,10-phenanthroline), BHT (butylated hydroxytoluene), DPPH, ascorbic acid, ABTS, 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), ascorbic acid, trichloroacetic acid (TCA), α-tocopherol were obtained from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany). The standard phenolics for LC-HRMS including ascorbic acid (≥99%), chlorogenic acid (≥95%), fumaric acid (≥99%), caffeic acid (≥98%), vanillic acid (≥97%), naringin (≥90%), rutin (≥94%), syringic acid (≥95%), rosmarinic acid (≥96%), p-coumaric acid (≥98%), quercetin (≥95%), salicylic acid (≥98%), naringenin (≥95%), luteolin (95%), emodin (90%) and chrysin (≥96%) were purchased from Sigma-

Aldrich, (-)-epigallocatechin (>97%), (-)-epigallocatechin gallate (>97%), orientin (>97%), (+)-trans-taxifolin (>97%), luteolin 7-glucoside (>97%), hyperoside (>97%), ellagic acid (>97%), quercitrin (>97%), apigenin (>97%), hispidulin (>97%), acacetin (>97%) and hederagenin (>97%) were purchased from TRC Canada, verbascoside (86.31%) was obtained from HWI Analytik GMBH. Luteolin-7-rutinoside (>97%) was purchased from Carbosynth limited. Hesperidin (≥98%) was purchased from J&K, dihydrokaempferol (>97%), nepetin-7-glucoside (>97%), isosakuranetin (>97%), and penduletin (>97%) was purchased from Phytolab, apigenin 7-glucoside (>97%) was purchased from EDQM CS. Myricetin (>95%) was purchased from Carl Roth GmbH+Co, nepetin (98%) was purchased from Supelco and caffeic acid phenethyl ester (≥97%) was purchased from European Pharmacopoeia.

2.2. Plant Materials

Verbascum speciousum Schrad. was collected from (A9) Kars: Sarıkamış, Soğuksu in June 2019 (Location: 40°18'19"K, 42°39'32"D, 2070 m. It was identified by botanist Dr. Süleyman Mesut PINAR, Van Yüzüncü Yıl University, Faculty of Science, Biology Department. The voucher specimen (voucher code: VANF 164145) is deposited at Van Yüzüncü Yıl University, Faculty of Science, Herbarium of the Biology Department (VANF), Van, Turkey.

2.3. Plant Extraction

To prepare WEVS, 40 g aerial parts *V. speciousum* were taken and shade-dried. It is powdered and boiled for 15 minutes with 500 mL distilled water, then, it is lyophilized in a lyophilizer (Labconco, Freezone 1L) at -50°C at 5 mm-Hg a pressure setting and stored at –20 °C [35,36]. The preparation of the EEVS was first started by pulverizing *V. speciosum* (25 g). The ground plant material was soaked with 0.5 L of ethanol, then the ethanol was evaporated via a rotary evaporator (Heidolph Hei-VAP HL, Germany) [37,38].

2.4. Radical Scavenging Assays

For evaluating the DPPH radical scavenging activity of EEVS and WEVS prepared and 0.5 mL of DPPH radical solution (0.1 mM) was transferred to each sample [39]. After 30 minutes of incubation, then, the absorbance was measured at 517 nm [40,41]. To determine the $ABTS^{\bullet+}$ scavenging activity of EEVS and WEVS the method in a previous study was used [42]. First, 2.45 mM persulfate solution was transferred to 2 mM ABTS solution to generate ABTS radicals. The absorbance of the $ABTS^{+\bullet}$ radical control solution was adjusted to 0.750 ± 0.025 nm at 734 nm with a phosphate buffer of 0.1 M and pH 7.4. Then 1 mL of $ABTS^{+\bullet}$ solution was added to different extract quantities and after 30 min incubation absorbances were recorded at 734 nm [43].

2.5. Reducing Ability

The Fe^{3+} reducing capability of EEVS and WEVS was measured by a method that can measure the reducing ability of an extract by direct reduction of $Fe[(CN)_6]_3$ to $Fe[(CN)_6]_2$ [44]. As a result, the Perl-Prussian blue complex, which possesses absorbance at 700 nm, leads to the formation of $Fe_4[Fe(CN^-)_6]_3$ [45]. To determine the CUPRAC of EEVS and WEVS, a previous method was realized with some changes [46]. The FRAP assay is based on the reduction of the TPTZ-Fe^{3+} complex [47].

2.6. Total Phenolics and Flavonoids

Quantification of phenolic compounds present in EEVS and WEVS was performed as described in a prior study [48]. The total amount of flavonoids found in EEVS and WEVS was carried out as described previously [49].

2.7. Enzyme Inhibition Assays

The enzyme inhibitory properties of EEVS were measured in accordance with previous studies [50,51]; Firstly, the AChE enzyme inhibitory property of EEVS was measured using electric eel as the source of AChE enzyme [52] and then, the inhibitory effect of EEVS on α-glycosidase and α-amylase was realized [53-55]. The IC_{50} value is the concentration of extracts causing 50% inhibition. It was obtained from activity (%) against extract plots [56-58].

2.8. LC-HRMS Analysis

The phenolic contents of EEVS and WEVS were made according to the LC-HRMS analysis method [59]. LC-HRMS experiments were realized on a Thermo ORBITRAP Q-EXACTIVE mass spectrometry equipped with a Troyasil C18 column (3 µm particle size, 150 x 3 mm i.d.) [60]. Acidified methanol and water gradient by HPLC assay was determined as the finest mobile phase [61,62]. The identification of the compounds was made by comparing the retention times of the standards (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 was used as an internal standard (IS) for LC-HRMS analysis for the reduction of repeatability problems caused by external effects including ionization repeatability, in mass spectrometry measurements. Dihydrocapsaicin solution (100 mg/L) was used as an IS. The mass values of the target phenolics are summarized in Table 4. Method Validation data and LC-HRMS chromatograms of the extracts were given as Table S1, Figure S1, and Figure S2 in Supporting Information.

2.9. Statistical Analyses

The results of the experiments were determined by averaging the triplicate analyses. For statistical analysis one-way analysis of variance (ANOVA) test was used. The differences between groups were made with Duncan's correction. The significance level was taken as $p<0.05$; $p<0.001$ was very significant and SPSS statistical software version 19.0 (SPSS Inc, Chicago, III, USA) package was used for analysis.

3. Results and Discussion

It is important to choose the most appropriate method when determining the antioxidant capacity of plants. In this study, two different free radical scavenging methods ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$) were used to determine the antioxidant ability of the extracts [63,64]. The basis of the DPPH method is based on the DPPH· scavenging percentage of antioxidants in the plant extract. ABTS assay is based on the percentage of antioxidants in the plant extract to scavenge $ABTS^{\bullet+}$ radicals [65,66]. $DPPH^{\bullet}$ scavenging activities of EEVS and WEVS and standard antioxidant compounds were investigated. The IC_{50} values EEVS and WEVS and standard antioxidants demonstrated in the following order: Ascorbic acid (16.12±0.003, r^2: 0.9566) < α-Tocopherol (23.10±0.032, r^2:0.9825) < EEVS (24.70±0.009, r^2: 0.9878) <BHT (31.50±0.011, r^2: 0.9754) < WEVS (173.25±0.029, r^2: 0.9704) (Table 1 and Figure 1). The lower IC_{50} values suggest an effective scavenged $DPPH^{\bullet}$ scavenging activity [67]. Ascorbic acid was found to be the compound with the most powerful $DPPH^{\bullet}$ scavenging activity. EEVS was found to have a free radical scavenging ability close to standard compounds. DPPH radical scavenging activity of the methanol extract of *V. speciosum* was determined in a study and the IC_{50} value was found as 32.35 µg/mL [68]. In another study, $DPPH^{\bullet}$ scavenging of water extract of *V. speciosum* aerial parts was found to be 48.27 ± 4.12 µg/mL [33]. In the current study, it was determined that especially EEVS exhibited a strong $DPPH^{\bullet}$ scavenging activity. When all these results were interpreted, it was observed that the IC_{50} value levels were low in all studies. According to $ABTS^{\bullet+}$ scavenging activity methods, a stable form of the radical is produced in the experiment and forms blue-green $ABTS^{\bullet+}$ by reacting with an antioxidant, and decolorization specifies the rate of $ABTS^{\bullet+}$ inhibition [45,69]. The IC_{50} values of $ABTS^{\bullet+}$ removing for EEVS, WEVS, and standards were determined in the following order: α-

Tocopherol (15.400 ± 0.003, r^2: 0,9866) < EEVS (19.8±0.017, r^2: 0.9956) < Ascorbic acid (23.10±0.001, r^2: 0.9998) < BHT (26.654±0.008, r^2: 0,9717) < WEVS (34.65±0.010, r^2: 0.9969) (Table 1 and Figure 1). It was found that EEVS has an efficient $ABTS^{\bullet+}$ scavenging activity close to standard compounds. In a study conducted with *Verbascum euphraticum* and *Verbascum oocarpum*, $ABTS^{\bullet+}$ scavenging activity of methanol extract was found as 90.52 ± 0.05 and 90.62 ± 0.06 µg/mL, respectively [34]. In the present study, the IC_{50} amounts obtained for $ABTS^{\bullet+}$ scavenging activity were found to be more effective when compared with the different species mentioned above.

Table 1. DPPH radical and ABTS radical scavenging capability of the EEVS, WEVS, and standard compounds

Antioxidants	DPPH· scavenging		$ABTS^{\bullet+}$ scavenging	
	IC_{50}	r^2	IC_{50}	r^2
Ascorbic acid	16.116 ± 0.003	0.9566	23.10 ± 0.001	0.9998
α-Tocopherol	23.1 ± 0.032	0.9825	15.400 ± 0.003	0.9866
BHT	31.500 ± 0.011	0.9754	26.654 ± 0.008	0.9717
EEVS	24.75±0.009	0.9878	19.8±0.017	0.9956
WEVS	173.25±0.029	0.9704	34.65±0.010	0.9969

The antioxidant profile of extracts of *V. speciosum* was characterized using the ferric ions (Fe^{3+}) reducing, CUPRAC, and FRAP assays [70], shown in Table 2 and Figure 2. Reduction capacity is an important factor in determining whether a molecule has antioxidant activity [71,72]. The first method used was to reduce Fe^{3+} to Fe^{2+} in $Fe[(CN)_6]^{3+}$ solution, which is one of the common methods. The reaction system is based on the reduction of Fe^{3+} in potassium ferricyanide to Fe^{2+} with the addition of an antioxidant can be recorded at 700 nm [73,74]. The concentrations of positive controls EEVS and WEVS were increased steadily (µg/mL). The reducing power of EEVS, WEVS, and standard antioxidants were as follows: Ascorbic acid (1.520±0.028, r^2: 0.9970) > BHT (1.269±0.005, r^2: 0.9880) > EEVS (1.177±0.018, r^2: 0.9805) > α-Tocopherol (0.990±0.007, r^2: 0.9942) > WEVS (0.409±0.005, r^2: 0.9964) (Table 2 and Figure 2a). According to the results, it was determined that EEVS has a strong ability to reduce ferric ions (Fe^{3+}) ($p<0.001$) (Table 2).

In the CUPRAC test, the absorbance measurement of the stable complex formed between copper ions and neocuproine is observed at 450 nm. High absorbance values indicate high reducing ability [75,76]. The CUPRAC of 30 µg/mL of EEVS, WEVS, and standards are demonstrated in Table 2 and Figure 2b. The CUPRAC of EEVS, WEVS, and standards were as following order: BHT (1.561±0.089, r^2: 0.9978) > Ascorbic acid (1.069±0.007, r^2: 0.9722) > α-Tocopherol (0.785±0.061, r^2: 0.9986) > EEVS (0.744±0.016, r^2: 0.9858) > WEVS (0.236±0.023, r^2: 0.9536) ($p < 0.001$, Table 2). According to the results, the reducing power of EEAC, Cu^{2+} ions was high, although not as strong as standard compounds.

The FRAP method is based on measuring the power of a sample with antioxidant properties to reduce oxidant ferric iron to ferrous form [77,78]. The FRAP of EEVS, WEVS, and standard antioxidants decreased as follows: Ascorbic acid (1.624±0.015, r^2: 0.9930) > BHT (0.909±0.006, r^2: 0.9874) > α-Tocopherol (0.755±0.075, r^2: 0.9867) > EEVS (0.584±0.002, r^2: 0.9288) > WEVS (0.361±0.005, r^2: 0.9567) (Table 2 and Figure 2c). According to the method, higher absorbances represent the higher reduction capability of the Fe^{3+}-TPTZ. EEVS and WEVS demonstrated an effective FRAP reduction ability although not as strong as standard compounds ($p<0.001$, Table 2). In a study, the FRAP of water extract from *V. speciosum* was found to be 754.41±10.39 µg/mL [33]. In conclusion, it was found that extracts of *V. speciosum* showed a value close to the standard compounds in all antioxidant activity tests.

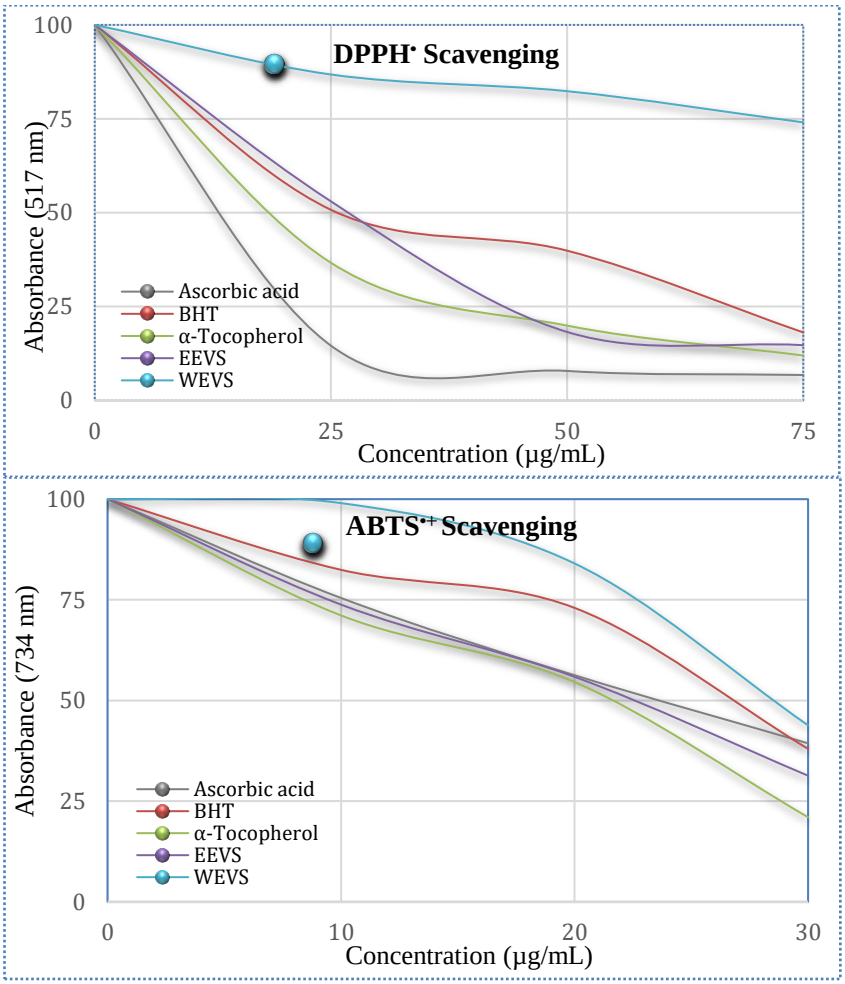

Figure 1. Radical removing activity of EEVS and WEVS. **A.** DPPH radical removing activity. **B.** ABTS radical removing activity (10-30 µg/mL)

Table 2. The reducing ability of the EEVS, WEVS, and standards by Fe^{3+} (120 µg/mL), Cu^{2+} (30 µg/mL) and Fe^{3+}-TPTZ (150 µg/mL) reducing ability

Antioxidants	Fe^{3+}-Fe^{2+} reducing		Cu^{2+}-Cu^{+} reducing		Fe^{3+}-TPTZ reducing	
	λ_{700}	r^2	λ_{450}	r^2	λ_{593}	r^2
α-Tocopherol[a]	1.520 ± 0.028[b,c,d,e]	0.9970	1.069 ± 0.007[b,d,e]	0.9722	1.624 ± 0.015[b,c,d,e]	0.9930
Ascorbic acid[b]	0.990 ± 0.007**e**	0.9942	0.785 ± 0.061[d,e]	0.9986	0.755 ± 0.075[d,e]	0.9867
BHT[c]	1.269 ± 0.005[b,d,e]	0.9880	1.561 ± 0.089[a,b,d,e]	0.9978	0.909 ± 0.006[b,d,e]	0.9874
EEVS[d]	1.177±0.018[b,e]	0.9805	0.744±0.016[e]	0.9858	0.584±0.002[e]	0.9288
WEVS[e]	0.409±0.005	0.9964	0.236±0.023	0.9536	0.361±0.005	0.9567

*Different letters in the same column were significantly different ($p < 0.05$). *a*: α-Tocopherol, *b*: Ascorbic acid, *c*: BHT, *d*: EEVS, *e*: WEVS.

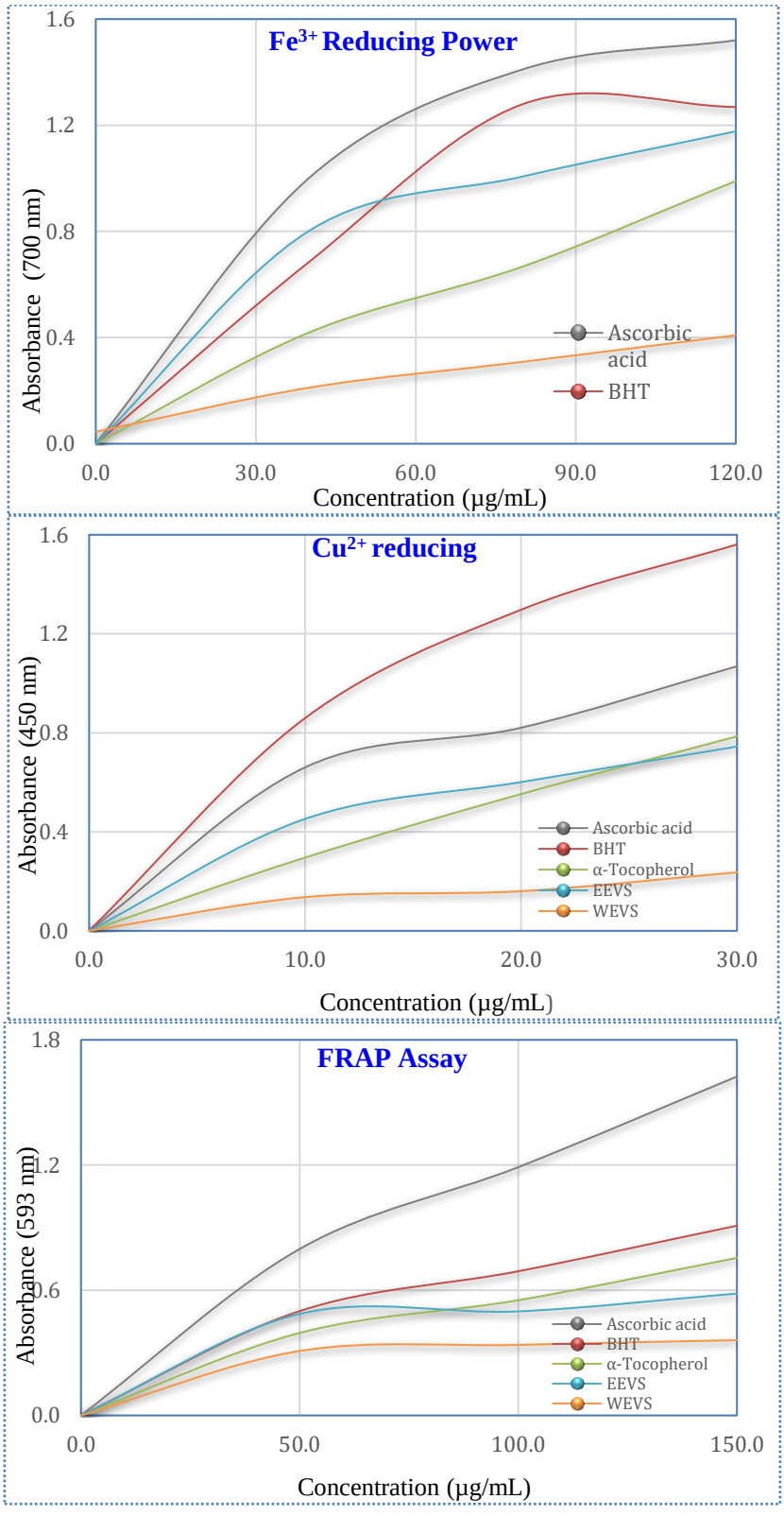

Figure 2. The reducing abilities of EEVS, WEVS and standard compounds.

The antioxidant effects of plants are due to the secondary metabolites such as phenolic and flavonoid compounds they contain [79]. Phenolic compounds are among the main plant secondary metabolites [80]. It was determined that the foods consumed rich in phenolic have a protective effect against cardiovascular diseases and cancer [81]. Phenolics and flavonoids have anticancer, anti-allergic, antibacterial, and anti-inflammatory properties and are free radical scavengers. Flavonoids are an important class of secondary metabolites found in plants. Numerous phenolic hydroxyl groups attached to the ring structures of flavonoids give them antioxidant activity [82]. Due to their strong free radical scavenging properties, flavonoids show antioxidant activities such as metal chelation and reduction [83].

The total phenolic contents of EEVS and WEVS were found to be 24.545±4.45 µg GAE and 16.364±0.01 µg GAE, respectively. The total flavonoid contents of EEVS and WEVS were found to be 5.328±0.75 µg QE and 16.750±0.69 µg QE, respectively. It was found a positive correlation between total phenolics and flavonoids in EEVS and WEVS and antioxidant abilities. In a study, the methanol extract of *Verbascum speciosum* was investigated and the total phenol and flavonoid contents were found as 82.00±6.43 mg/g GAE and 30.79±0.50 (mg/g) rutin equivalent, respectively [84]. In a study conducted in 2020, total phenolic and flavonoid contents in the *V. speciosum*' methanol extract were found to be 45.01±1.44 mg GAE and 14.26±1.81 mg QE, respectively [33]. *Verbascum euphraticum* and *Verbascum oocarpum* species were examined in a study and their total phenolic contents were respectively; 50.29±0.29 (mg GAE/g) and 61.23±1.74 (mg GAE/g) and total flavonoid contents were found as 28.47±0.25 (mg RE/g) and 49.97±0.32 (mg RE/g), respectively [34]. The results obtained in the previous studies mentioned above were found to be sometimes higher and sometimes lower than our results. The reason for this is thought to be due to differences in the ecological and soil structure of the region where the plant is grown, analysis methods, solvents, and extraction conditions.

Table 3. The total phenolics and flavonoids in EEVS and WEVS

Extracts	**Total phenolics** (µg/mL extract)	**Total flavonoid** (µg/mL extract)
EEVS	24.545±4.45	5.328±0.75
WEVS	16.364±0.01	16.750±0.69

Various iridoids, phenyletanoids, phenylpropanoids, saponins, and flavonoids were determined as secondary metabolites in *Verbascum* species [85]. In this study, the phenolic compound contents of EEVS and WEVS were determined by the LC-HRMS technique and thirty-eight polyphenols were quantified and identified (Table 4). Verbascoside (13740.0 mg/kg), p-coumaric acid (10983.89 mg/kg), and fumaric acid (2221.61 mg/kg) are the main polyphenols identified in 1 mg of EEVS while verbascoside (4390.71 mg/kg), rutin (561.82 mg/kg) and apigenin (561.54 mg/kg) are the main polyphenols in 1 mg of WEVS. It was determined that the ethanolic extracts showed the highest compound content from both extracts. Among the 38 compounds, 17 molecules were found in both EEVS and WEVS, and verbascoside was most abundant in the EEVS and WEVS. Verbascoside is a phenylethanoid glycoside and has been isolated from many different plant species, *Verbascum sinuatum* being one of them. Verbascoside has been the subject of many studies due to its pharmacological effects such as antioxidant, anti-inflammatory, neuroprotective, and anticancer effects. Polyphenols, which are secondary plant metabolites, contain thousands of structural variations from simple phenolics to tannins [86-88]. The main dietary polyphenols are phenolic acids, flavonoids, tannins, catechins, lignans, stilbenes, and anthocyanidin phytochemicals. It has been reported that more than 8,000 of the many dietary polyphenols are of plant origin. Studies have shown that polyphenolics in the diet are helpful in preventing diseases [89,90]. Polyphenols have antioxidant properties, so they can significantly lower the risk of developing many chronic diseases, particularly cancer, cardiovascular disease, inflammatory diseases, diabetes, kidney failure, and neurodegenerative disorders [81,82]. Plants have been used for a long time because of the natural products they contain such as antioxidants [93]. Plants have also been used for medicinal applications from time immemorial. Since the level of side effects, they show is low, their use in the pharmaceutical and food industry is increasing today [94]. One of the reasons for this

increase is that thanks to scientific developments, the presence of phytochemical compounds exhibiting pharmacological properties of medicinal plants can be detected so that the therapeutic properties of these plants can be verified [95].

Table 4. The quantity of phenolics (mg/kg extract) in EEAC and WEAC by LC-HRMS

Compounds	**EEVS**	**WEVS**
Ascorbic acid	68.30	302.93
(-)-Epigallocatechin	<LOD	<LOD
(-)-Epigallocatechin gallate	<LOD	-
Chlorogenic acid	31.25	86.95
Fumaric acid	2221.61	-
(-)-Epicatechin	<LOD	-
(-)-Epicatechin gallate	<LOD	-
Verbascoside	13740.00	4390.71
Orientin	0.42	<LOD
Caffeic acid	33.65	302.76
(+)-t*rans* taxifolin	0.02	-
Luteolin-7-rutinoside	261.72	99.70
Naringin	<LOD	<LOD
Luteolin 7-glucoside	1305.78	191.67
p-Coumaric acid	10983.89	-
Hesperidin	<LOD	-
Rutin	805.58	561.82
Syringic acid	<LOD	-
Rosmarinic acid	10.20	13.08
Hyperoside	590.93	83.68
Dihydrokaempferol	0.05	-
Apigenin 7-glucoside	26.51	6.15
Ellagic acid	<LOD	-
Quercitrin	38.93	<LOD
Myricetin	0.02	-
Quercetin	2.63	1.86
Salicylic acid	6.72	5.53
Naringenin	3.09	<LOD
Luteolin	409.17	371.33
Nepetin	0.65	-
Apigenin	77.97	561.54
Hispidulin	188.79	56.17
Isosakuranetin	<LOD	<LOD
Penduletin	3.98	-
Caffeic asit phenethyl ester	0.14	-
Chrysin	1.32	4.74
Acacetin	3.42	2.82
Hederagenin	<LOD	-

Alzheimer's disease (AD) is a common and irreversible age-related neurodegenerative disease and is the most common cause of dementia [98]. Memory loss, daily life disorders, neuropsychiatric symptoms, and other behavioral disorders are among the clinical symptoms of AD [99]. Decreased acetylcholine (ACh) levels are among the etiological features of AD. The enzyme acetylcholinesterase (AChE) hydrolyzes ACh to acetate and choline [100]. By using AChE inhibitors, inhibition of ACh

hydrolysis can be achieved, increasing neurotransmitter levels in synapses and improving cholinergic and cognitive neurotransmission capacity [101]. In the current study, the results of AD-related cholinesterase inhibition were evaluated and a high anti-AChE activity measured with the ethanol acetate extract of *V. speciosum* (IC_{50}: 1.516 µg/mL, r^2: 0.9719) (Table 5). Tacrine was used with an IC_{50} value of 0.124 µM against AChE (Table 5) [19]. In a previous study, *Verbascum euphraticum* and *Verbascum oocarpum* species were examined for their AChE inhibition, the results were 1.93±0.21 (mg Galantamine/g) and 0.81±0.16 (mg Galantamine/g), respectively [34].

Table 5. The half-maximal enzyme inhibition values (IC_{50}, µg/mL) of EEVS against α- glycosidase, acetylcholinesterase, and α-amylase

Enzymes	**EEVS**		**Standards**
	IC_{50} (µg/mL)	**r^2**	**IC_{50}** (µM)
α-Glycosidase [a]	1.120	0.9441	22.80
α-Amylase [a]	0.859	0.9811	10.01
Acetylcholinesterase [b]	1.516	0.9719	0.124

[a]Acarbose was used as a standard for α-glycosidase and α-amylase enzymes and taken from the reference of [96] and [97], respectively.
[b]Tacrine was used as a standard for acetylcholinesterase enzyme and taken from our previous study [41].

Diabetes mellitus (DM) is a main cause of mortality and morbidity worldwide. DM is a chronic disease that includes markers of hyperglycemia and insulin deficiency. WHO reported that deaths from diabetes mellitus will double between 2005-2030 [102]. α-Glycosidase is a crucial enzyme, which catalyzes the final step in the digestive mechanism of carbohydrates. α-Glycosidase inhibitors inhibit enzyme activity and prevent the rapid breakdown of sugar units, thus keeping the blood sugar level under control. Therefore, α-glucosidase inhibitors are used clinically to treat diabetes, but most of these inhibitors are synthetic drugs with some harmful reactions [103]. Investigation of the inhibitory effects of dietary polyphenols on α-glucosidases is of great interest [104]. α-Amylase is an enzyme that helps break down large, insoluble molecules such as starch into soluble ones. α-Amylase inhibitors delay the breakdown of carbohydrates and prevent the sudden increase of postprandial glucose in the blood [105]. The α-amylase enzyme is under investigation specifically to treat diabetes and hyperlipidemia. Inhibitory effects of polyphenols for α-amylases have been widely reported [106].

The determination of the inhibition of antidiabetic enzymes of α-amylase and α-glycosidase enzymes was evaluated for the determination of antidiabetic profiles of *V. speciosum*. The IC_{50} values of α-glycosidase were 1.120 µg/mL (r^2: 0.9441) for EEAC and 22.80 µM for acarbose (Table 5). Also, the IC_{50}s of α-amylase were 0.859 µg/mL (r^2: 0.9811) for EEVS and 10.01 µM for acarbose, respectively (Table 5) [97]. According to the results, it was shown that EEVS has a very high affinity towards enzymes including α-glycosidase and α-amylase. It was more effective than the standard inhibitor acarbose. In a previous study, *Verbascum euphraticum* was examined for its α-glycosidase inhibition, the result was 0.65 ± 0.01 (mmol Acarbose equivalent/g), and also at the same research *Verbascum euphraticum* and *Verbascum oocarpum* examined for their α-amylase inhibition and the results were as 0.31±0.02 (mmol Acarbose E/g) and 0.31±0.01 (mmol Acarbose equivalent/ g), respectively [34].

4. Conclusions

In this study, data on the phytochemical bioactivity and properties of *V. speciosum* extracts were evaluated for their antioxidant ability, phenolics, and flavonoids, as well as their anti-Alzheimer and anti-diabetic properties. The ethanol extract of the aerial part of *V. speciosum* was found to have a value close to the standard antioxidants, also, a high content of total flavonoids and phenolics. In addition, it was also evaluated that there was a statistically significant difference between the averages of the groups. It was determined by LC-HRMS for the main phenolic compound in both extracts was verbascoside.

Also, *V. speciosum* showed high inhibition effects against AChE, α-glycosidase, and α-amylase enzymes. EEAC has higher antioxidant activity and inhibition effect compared to WEAC as it takes both hydrophobic and hydrophilic components. Values obtained from this study have shown that *V. speciosum* as a source of potential bioactive components is a candidate for use in drug design processes for the treatment of DM and AD, due to its potent pharmacological activity.

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