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ORIGINAL PAPER

Sage (*Salvia pilifera*): determination of its polyphenol contents, anticholinergic, antidiabetic and antioxidant activities

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Abstract

In this work, we determined for the first time the *Salvia pilifera* Montbret & Aucher ex Bentham as an important source for natural products with antioxidant and antidiabetic potentials. In this context, methanol (MESP) and water (WESP) extracts were prepared from aerial parts of *S. pilifera*. Also, it was evaluated for antioxidant profile by eight distinguishes bioanalytical methods and inhibition effects against enzymes linked to different diseases, namely butyrylcholinesterase (BChE), acetylcholinesterase (AChE), α-glycosidase and α-amylase. Also, the polyphenolic compositions of MESP and WESP were evaluated by high performance liquid chromatography and tandem mass spectrometry (LC–MS/MS). Fourteen phenolics were identified in the evaporated MESP and thirteen phenolic compounds were identified in the lyophilized WESP. Also, we performed the antioxidant properties of both extracts. In order to estimate the capacity of MESP and WESP to act as antioxidants, 1,1-diphenyl-2-picryl-hydrazyl radicals ($DPPH^{\cdot}$), 2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radicals ($ABTS^{\cdot+}$) and *N*,*N*-dimethyl-*p*-phenylenediamine radicals ($DMPD^{\cdot+}$), scavenging activities, ferric ions (Fe^{3+}), Fe^{3+}-TPTZ and cupric ions (Cu^{2+}) reducing assays were studied. MESP and WESP were found as potent effective $DPPH^{\cdot}$ (IC_{50}: 7.05 and 8.56 µg/mL), $ABTS^{\cdot+}$ (IC_{50}: 3.52 and 4.76 µg/mL) and $DMPD^{\cdot+}$ (IC_{50}: 28.92 and 30.95 µg/mL) scavenging effects. On the other hand, MESP and WESP showed the potent inhibition effects against AChE (IC_{50}: 94.93 and 138.61 µg/mL), BChE (IC_{50}: 60.05 and 99.13 µg/mL), α-glycosidase (IC_{50}: 23.28 and 36.47 µg/mL) and α-amylase (IC_{50}: 46.21 and 97.67 µg/mL) enzymes. This study will be an innovative and guider for further studies for antioxidant properties for industrial or medicinal plants.

Keywords *Salvia pilifera* · Antioxidant activity · Polyphenol content · Enzyme inhibition · LC–MS/MS

Introduction

Plants are potential sources of materials for the improving of novel products for food, cosmetic and pharmaceutical industries [1, 2]. Recently, biologically active compounds of plant have proved and desired as potent natural agents. Medicinal plants had a crucial role in basic healthcare and cosmetic industry in many developing and developed countries [3]. It was estimated that Turkey flora to includes approximately 11,000 taxa and close to 1300 plants were used in traditional folk medicine in Anatolia [4]. Medicinal plants have a considerable content of bioactive compounds and had specific biochemical and organoleptic properties, allowing different usage in food, pharmaceutical and cosmetic industries [5, 6]. On the other hand, secondary metabolites of plants have been extensively used in folk medicine because of well-established potential biological properties [7]. Biologically active secondary metabolites including phenolics have been isolated and used for treatment of some health disorders. Recently, there are growing demands and many studies for validation of the usage of herbal medicines for the treatment of many diseases in Anatolia [6].

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Turkey is considered as an important region and gene-center for the Lamiaceae family. It was reported that this family had 45 genera, 546 species and a total of 731 taxa in Turkey. Also, the high diversity of the Salvia genus and phytochemical richness generate great interest for discovering new biological active compounds from these plants [8]. The local name of this plant is "etekli şalba" in Turkish. *Salvia* L., the largest genus in the Lamiaceae family, contains about 1000 species worldwide. It is represented in Turkey contains 86 species including *Salvia pilifera*. Salvia species, usually named as "sage" are among the important aromatic spice and medicinal plants used worldwide [9, 10].

Reactive oxygen species (ROS) occur in living organisms during normal cellular metabolism and can be harmful decisive biomolecules including lipids, carbohydrates, nucleic acids, and proteins [11–13]. Also, ROS, which have been implicated in many diseases, are produced in the all-living organisms as primary immune defense [14, 15]. Recently, oxidative stress and ROS had been accepted as an important environmental risk for different types of chronic disorders such as cancer, immunodeficiency syndrome, age-related pathologies, cardiovascular diseases, arteriosclerosis, diabetes, and obesity [16, 17]. Antioxidants are the compounds that can delay, inhibit, or prevent the oxidation of these biomolecules. They include phenols and polyphenols are very effective agents, which reduced or neutralized undesired and hazardous effects of ROS and oxidative stress [18]. In terms of pharmaceutical products, they can easily scavenge ROS and free radicals and increase lipid peroxidation time of pharmaceutical and food products during storage processes [19, 20]. An antioxidant was described as any molecules in low concentrations when compared to an oxidisable substance. Also, they significantly delays or inhibits the oxidation of substrates [21, 22]. Therefore, attention has recently turned to the research for effective antioxidants including phenolic compounds from natural and accessible resources [23, 24]. Plants include a lot biological active phytochemicals such as phenols, polyphenols that possess structural features, which had antioxidant activities [25, 26]. On the other hand, according to the toxicologists and nutritionists, the side effects of some synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which used in food processing, have been documented. However, the use of these compounds has been restricted by legislation due to doubts over their toxic and carcinogenic effects in living organisms [27–29]. Hence, these phytochemicals from plants have been intensely investigated for their possible antioxidant activities. Many works had been performed on antioxidants and crude plant extracts [30]. Hence, there is a growing interest in natural and safer antioxidants for food and pharmaceutical applications, and a growing trend in consumer preferences towards natural antioxidants from natural sources including medicinal plants [31, 32]. The antioxidant properties of the phenolic compounds in medicinal plants make them an important product for preventing human health against degenerative diseases such as cancer, hypercholesterolemia, diabetes, arteriosclerosis, or cardiovascular diseases [33].

Alzheimer's disease (AD) affects mostly the aged people and above resulting in impaired behaviour and memory. This neurological disorder clinically involves the growing degeneration of brain tissue that is influenced by the deficit in acetylcholine (ACh) [34, 35]. Acetylcholinesterase (AChE), as a major element of the cholinergic system in the central and peripheral nervous system, is able to convert ACh to acetate and choline (Ch) [36–38]. It was reported that the reduction of ACh and BCh levels in hippocampus and cortex in the brain is the most remarkable biochemical change in AD patients. As a result of this, one of the treatment approaches for AD is inhibition of both cholinesterase enzymes that break down ACh and BCh [39]. Because of serious side effects of the available AChE, there is the need to search for newer effective and safe AChE to treat neurodegenerative damages. AChE inhibitors (AChEIs) are known as anti-cholinesterases and inhibit cholinesterase enzymes [40]. AChEIs had a common usage in medicine, especially for the treatment of AD. They have been used in clinical trials, including natural substances. Phenolic compounds had been also recognized as AChEIs and promising lead compounds for AD [41].

Diabetes mellitus (DM) is a chronic and metabolic disease, which participated by abnormal plasma blood levels and disorders such as neuropathy, retinopathy and cardiovascular diseases. The inhibition of digestion enzymes that can hydrolyse the polysaccharides into monosaccharide units is one of the most important therapeutic ways for treatment of DM [42, 43]. α-Amylase and α-glycosidase enzymes, which released from small intestine cells hydrolyses oligosaccharide and polysaccharide molecules to monosaccharide units, such as glucose and fructose [42, 43]. Digestive enzymes inhibitors can decrease the absorption of dietary carbohydrates and suppressed postprandial hyperglycaemia and T2DM. Thus, these digestive enzymes inhibitors are endowed with sugar molecule such as compete and moieties with the oligosaccharides for binding to the active site of digestive enzymes. Thus, they effectively reduce the postprandial polysaccharide units including glucose in T2DM [44, 45].

In this study, we realized the ferric (Fe^{3+}) and cupric ions (Cu^{2+}) ions reducing antioxidant power, $ABTS^{\cdot+}$ scavenging, $DPPH^{\cdot}$ scavenging, and $DMPD^{\cdot+}$ scavenging activities of MESP and WESP. These biochemical methods are used for measurement of antioxidant profiles of foods, plants and pharmacological materials. Also, this study was aimed to clarify the polyphenol contents in both extract by LC–MS/MS. Furthermore; another significant

goal of this study is to demonstrate the inhibition effects MESP and WESP against some metabolic enzymes including BChE, AChE, α-glycosidase and α-amylase enzymes linked to global and common health diseases.

Materials and methods

Chemicals

N,*N*-dimethyl-*p*-phenylenediamine (DMPD), neocuproine (2,9-dimethyl-1,10-phenanthroline), 2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\cdot}$), α-tocopherol, trolox and curcumin were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). The other chemicals are used for analytical grade and obtained from either Sigma-Aldrich or Merck.

Plant material

Plant samples (*Salvia pilifera* Montbret & Aucher ex Bentham) were collected in the Gerger-Adiyaman provinces of Turkey in May (25) of 2018 (coordinates: (1) UTM, X: 509964-Y: 4212288, Alt: 1032 m, and (2) UTM, X: 509884-Y: 4212113 Alt: 1054 m). The taxonomical classification was confirmed by the taxonomist Dr. Ahmet Zafer TEL and a voucher specimen (voucher code: 1385) is kept in the herbarium of Adiyaman University, Herbarium of Faculty of Arts and Sciences (ADYUHER). *S. pilifera*'s feature of species: Perennial herb to c. 90 cm. Stems ascending-erect from creeping rhizome, glabrous below, glandular-villous above. Leaves pinnatisect with a broad ovate-oblong terminal segment, to 8 × 4 cm, and 1–2 pairs of smaller lateral segments, submembranous, adpressed pilose; petiole to c. 5 cm. Verticillasters 2–8-flowered, distant. Bracts c. 20 × 10 mm, long acuminate. Pedicels c. 8 mm, erecto-patent. Calyx infundibular, c. 15 mm, to 20 mm in fruit, densely glandular-villous or glandular-pilose; upper lip tridentate, median tooth larger, longer than lower. Corolla blue-purple with white markings, 25–30 mm; tube straight, slightly pilose within; upper lip ± straight. Stamens A. Nutlets broadly elliptic, c. 4.5 × 3 mm. Fl. 5–6. Limestone slopes, in Quercus scrub, among bushes, macchie, 200–2300 m. Endemic. Ir.-Tur. Element [46]. Plant material was dried for 3 days at room temperature protected from direct sun light, powdered in a mechanic grinder and stored at − 20 °C until used in the dark until processing.

Preparation of MESP and WESP

For determination of the methanol extraction of *S. pilifera* (MESP), a 20 g plant sample was powdered and mixed with methanol (400 mL). Then, the extracted sample was filtered through Whatman No. 1 paper and evaporated using a rotary evaporator at 40 °C [47]. The residue was re-extracted under the same conditions until extraction solvents became colorless. MESP was placed in a plastic bottle and then stored at − 20 °C until used.

On the other hand, water extraction of *S. pilifera* (WESP) was carried out according the previous method [48]. For this purpose, 20 g of dried aerial parts of *S. pilifera* was powdered, mixed with boiling water (400 mL) and stirred by magnetic stirrer for 30 min. Then the water extract was filtered over cheesecloth and Whatman paper (No. 1). The residue was frozen in a freezer at − 84 °C (Sanyo, Japan) and lyophilized in a lyophilizator (5 mm-Hg, − 50 °C, Labconco, Freezone, Japan). WESP was placed in a plastic bottle and then stored at − 20 °C until used.

Determination of total phenolic content

Quantification of the phenolic compounds in both extracts of *S. pilifera* was performed according to the colorimetric method of Folin–Ciocalteau described by Singleton and Rossi [49] with some modifications [50]. The appropriate dilutions sample solution (0.5 mL) was mixed with diluted Folin–Ciocalteu reactive (1.0 mL). Then the solution was vigorously mixed and neutralized with Na_2CO_3 (0.5 mL, 1%). After 2 h incubation in dark at room temperature, the absorbance of samples was recorded at 725 nm. The content of total phenolic quantity was calculated as milligrams of gallic acid equivalents (GAE) per gram of extract.

Determination of total flavonoids contents

The total flavonoid content in MESP and WESP was done using the aluminum chloride colorimetric method [51]. Briefly, both extract solutions (0.5 mL) were mixed with ethanol (1.5 mL, 95%) and mixed to the same volume of aluminum chloride (1.5 mL, 10%) and potassium acetate solution (0.5 mL 1.0 M) and of distilled water (2.3 mL). After 30 min incubation at room temperature, the absorbance of the mixture was spectrophotometrically measured at 415 nm. The total flavonoid content was calculated as milligrams of quercetin equivalents (QE) per gram of extract.

LC–MS/MS analysis

A quantity of (100 mg) of MESP and WESP was dissolved in 5 mL of ethanol–water (50:50 v/v) in a flask, from which 1 mL was transferred into another 5 mL of flask. Then,

100 µL of curcumin was added and diluted to the volume with ethanol–water (50:50 v/v). From the final solution, an aliquot (1.5 mL) was added to a capped autosampler vial and 10 µL sample was injected to LC. The samples in the autosampler were kept at 15 °C during the experiment. Also, chromatographic conditions, instruments and optimization of LC–MS–MS procedure and HPLC methods, linearity, recovery, repeatability and precision, limits of the quantification (LOD), limits of the quantification (LOQ), identification of uncertainty sources, and identification of standard uncertainties were performed according to our previous studies (Tables 1 and 2) [52].

Reducing ability assays

Fe^{3+}-reducing effects of both MESP and WESP was determined by the direct reduction of $Fe^{3+}(CN^{-})_6$ to $Fe^{2+}(CN^{-})_6$ according to the procedure of Oyaizu [53] as described previously [54]. Briefly, different concentrations of MESP and WESP (10–30 µg/mL) in distilled water (0.75 mL) were mixed same volume of phosphate buffer (1.25 mL 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] solution (1.25 mL, 1%). The mixture was incubated at 50 °C for 20 min and acidified with trichloroacetic acid (1.25 mL, 10%). Finally, an aliquot of $FeCl_3$ solution (0.5 mL, 0.1%) was transferred and the absorbance was measured at 700 nm [55].

Cupric ions (Cu^{2+}) reducing ability of MESP and WESP was realized according to the method of Apak et al. [56] as described in detail [57]. To this end, same volumes of $CuCl_2$ solution (0.25 mL, 10 mM), ethanolic solution of neocuproine (0.25 mL, 7.5 mM) and CH_3COONH_4 buffer solution (0.25 mL, 1.0 M) were added to a test tube and mixed with MESP and WESP solution (10–50 µg/mL). Total mixture volumes were completed to 2 mL with distilled and deionized water. Then, test tubes were closed and detained at room temperature. Finally, their absorbances were spectrophotometrically measured at 450 nm. In this assay, increased absorbance of values indicates increased Cu^{2+} reduction ability [58].

The ferric (Fe^{3+}) reducing antioxidant power (FRAP) method is relied on reduction of Fe^{3+}-TPTZ in acidic contexts. Increased absorbance of blue color of ferrous form of complex (Fe^{2+}-TPTZ) is spectrophotometrically determined at 593 nm. Briefly, 2.25 mL of TPTZ solution (10 mM), which freshly prepared in HCl (40 mM), added to acetate

Table 1 Validation and uncertainty parameters for antioxidant phenolic acids

No.	Compounds	Linear regression equation	r^2	LOD (mg/L)	LOQ (mg/L)	RSD (%)
1	Quercitrin	y = 0.0290x + 0.0058	0.9918	0.001	0.002	4.28
2	Gallic acid	y = 0.0569x + 0.0177	0.9912	0.002	0.008	4.85
3	Epigallocatechin	y = 0.0227x + 0.0191	0.9566	0.211	0.704	0.21
4	Epicatechin	y = 0.1594x + 0.0205	0.9581	0.002	0.007	4.78
5	Cyanidin-3-*O*-glycoside	y = 0.3840x + 0.0809	0.9762	0.019	0.064	6.57
6	Cyanidin chloride	y = 0.2613x + 0.0035	0.9059	0.006	0.021	6.39
7	Catechin	y = 0.9045x + 0.0061	0.9099	0.008	0.027	0.3
8	Ascorbic acid	y = 0.4892x + 0.0090	0.9788	0.199	0.665	9.42
9	Apigenin	y = 0.1780x + 0.0850	0.9961	0.15	0.501	4.01
10	Caffeic acid	y = 0.3300x + 0.0036	0.9924	0.028	0.093	8.04
11	Chlorogenic acid	y = 0.2620x + 0.0674	0.998	0.445	1.483	5.45
12	Ellagic acid	y = 0.0244x + 0.0048	0.9951	0.02	0.068	0.11
13	Kaempferol	y = 0.0230x + 0.0116	0.9841	0.002	0.008	5.47
14	Salvigenin	y = 0.0355x + 0.8620	0.9912	0.036	0.119	5.21
15	Fumaric acid	y = 0.0569x + 0.0177	0.9912	0.003	0.01	5.44
16	Pyrogallol	y = 0.0438x + 0.0073	0.9803	0.001	0.002	5.47
17	*t*-Ferulic acid	y = 0.0655x + 0.0266	0.9925	0.047	0.158	5.21
18	Luteolin	y = 0.2120x + 0.0699	0.9937	0.062	0.207	0.16
19	Isorhamnetin	y = 0.0739x + 0.5100	0.9608	0.088	0.294	3.67
20	Quercetagetin-3,6-dimethylether	y = 0.0181x + 0.0202	0.9924	0.022	0.074	0.1
21	Rosmarinic acid	y = 0.1960x + 0.0043	0.9982	0.022	0.072	3.73
22	Luteolin-7-*O*-glycoside	y = 0.1350x + 0.0246	0.9957	0.022	0.072	8.56
23	Luteolin-5-*O*-glycoside	y = 0.2300x + 0.0413	0.9926	0.01	0.034	1.12
24	Kaempferol-3-*O*-rutinoside	y = 0.1080x + 0.0135	0.9977	0.014	0.045	8.15
25	Rutin	y = 0.0232x + 0.0008	0.9969	0.01	0.034	7.9

Table 2 LC–MS/MS parameters of selected compounds and amount of antioxidants in WESP and MESP in mg/kg concentration (WESP: lyophilized water extract of *S. pilifera*, MESP: evaporated methanolic extract of *S. pilifera*)

No.	Compounds	Parent ion	Daughter ion	Collision energy (V)	Phenolics (mg/kg)[Ψ,§]	
					MESP	WESP
1	Quercitrin	471.9	309.9	16	nd	nd
2	Gallic acid	168.6	124	13	1.33 ± 0.09	0.5 ± 0.03
3	Epigallocatechin	305	125	18	nd	nd
4	Epicatechin	289	245	14	6.84 ± 0.69	21.26 ± 2.16
5	Cyanidin-3-*O*-Glu	449	287	16	nd	nd
6	Cyanidin chloride	611.5	287	28	7.27 ± 0.49	nd
7	Catechin	289	245	15	nd	4.86 ± 0.33
8	Ascorbic acid	175	114.6	12	nd	nd
9	Apigenin	269	151	22	7.89 ± 0.64	24.09 ± 1.94
10	Caffeic acid	179	135	10	9.35 ± 1.85	12.38 ± 2.45
11	Chlorogenic acid	353	191	14	7.97 ± 1.1	1.37 ± 0.19
12	Ellagic acid	301	150.5	10	nd	nd
13	Kaempferol	287	152.3	30	nd	3.63 ± 0.26
14	Salvigenin	329	295.8	15	95.11 ± 6.47	1.78 ± 0.12
15	Fumaric acid	115	71	8	182.41 ± 12.65	175.21 ± 12.15
16	Pyrogallol	125	80	16	nd	nd
17	*t*-Ferulic acid	193	133	15	2.51 ± 0.18	13.79 ± 0.96
18	Luteolin	285	132	30	7.94 ± 2.04	2.35 ± 0.6
19	Isorhamnetin	315	300	15	3.88 ± 0.34	nd
20	Quercetagetin-3,6-dimethylether	345.1	329.5	16	21.19 ± 3.97	2.56 ± 0.48
21	Rosmarinic acid	359.2	160.5	15	2.05 ± 0.16	nd
22	Luteolin-7-*O*-glucoside	447	284.5	14	nd	nd
23	Luteolin-5-*O*-Glucoside	447	289.5	20	nd	nd
24	Kaempferol-3-*O*-rutinoside	593	284.4	18	2.41 ± 0.22	2.71 ± 0.24
25	Rutin	609	301	16	nd	nd
26	Curcumin[a]	369.3	176.9	20	–	–

nd not determined. These values are below the limits of the quantification

[a]It was used for internal standard

buffer (2.5 mL, pH 3.6, 0.3 M), and $FeCl_3$ solution in water (2.25 mL, 20 mM). Then, MESP and WESP (10–30 µg/mL) were dissolved in buffer solution (5 mL) and mixture incubated at 37 °C for 30 min. Finally, the absorbance of the samples was measured [59].

Chelating activity on ferrous ions (Fe^{2+})

Fe^{2+} chelating ability of MESP and WESP was done according to the method of Dinis et al. [60] with minor modification [61]. Fe^{2+}-binding ability of MESP and WESP was recorded at 522 nm [62]. Briefly, to an aliquot of $FeCl_2$ (0.1 mL, 0.6 mM) was added to 0.4 mL MESP and WESP solution (10–30 mg/mL). The disrupting of ferrozine-Fe^{2+} complex formation as (%) was calculated by following formula: chelated ferrous ions (%) = (1 − As/Ac) × 100. Where Ac is the absorbance value of control and As is the absorbance value in the presence of MESP and WESP. The control sample contains only $FeCl_2$ and ferrozine reagent [63].

Radicals scavenging activities

The $DPPH^{\cdot}$ scavenging effects MESP and WESP were determined according to the method of Blois [64], as described in a previous study [65]. The N-centered $DPPH^{\cdot}$ is frequently used for measurement of radical scavenging capacity of plant extracts and pure antioxidants. The $DPPH^{\cdot}$ shows maximum absorbance at 517 nm; however, its absorption reduces by an antioxidant agent. For this aim, an aliquot of $DPPH^{\cdot}$ in ethanol (0.5 mL, 0.1 mM) was transferred to sample solution (1.5 mL) in ethanol (10–30 µg/mL) and incubated in dark for 30 min. Finally, the absorbance samples were recorded at 517 nm against blank samples lacking scavenging compounds. Analyses were achieved in triplicate.

$ABTS^{\cdot+}$ scavenging method is based on the ability of method of Gulcin [66]. The ABTS solution (2.0 mM) in water with oxidizing agent of potassium persulfate (2.3 mM) yielded the ABTS cation radical ($ABTS^{\cdot+}$), which was soluble in both aqueous and organic solvents. It was diluted with phosphate buffer (0.1 mM, pH 7.4) to adjust inquired absorbance (0.700 ± 0.025) at 734 nm. Finally, MESP and WESP solution (3.0 mL) at various concentrations (10–30 µg/mL) was interacted to $ABTS^{\cdot+}$ (1.0 mL) and the remaining absorbance was spectrophotometrically recorded at 734 nm.

$DMPD^{\cdot+}$ scavenging effects of MESP and WESP were realized according to the method of described by Gülçin [67]. For this purpose, an aliquot of $DMPD^{\cdot+}$ solution (1 mL, 0.1 M) was added to acetate buffer (100 mL, pH 5.25, 0.1 M) containing different concentration of MESP and WESP (10–30 µg/mL). Then, 0.2 mL of ferric chloride ($FeCl_3$, 50 mM) was added to this mixture. Then, absorbance of this solution was spectrophotometrically measured at 505 nm.

The radical scavenging capacities (RSC) of MESP and WESP was calculated as mM in the reaction mixture, and determined from the calibration curve by linear regression. All radicals ($DPPH^{\cdot}$, $DMPD^{\cdot+}$ and $ABTS^{\cdot+}$) scavenging effects (RSC) were calculated as following: $RSC\ (\%) = (1 - As/Ac) \times 100$. Where Ac is the absorbance at the indicated wavelength of the control reaction and As is the absorbance of the indicated wavelength of the tested samples. The half maximal scavenging concentration of sample (IC_{50}) was determined from the graph plotted inhibition percentage against MESP and WESP concentrations (µg/mL) [68].

Enzymes inhibition studies

AChE/BChE inhibition studies

AChE and BChE inhibitory activities of MESP and WESP were determined by modified spectrophotometric method of Ellman et al. [69] as described previously [70]. Electric eel AChE and equine serum BChE (E.C.3.1.1.8, Sigma) were used as the enzyme sources. Acetylthiocholine iodide/butyrylcholine iodide (AChI/BChI) were used as substrate for both cholinergic reactions. In brief, an aliquot (100 µL) of Tris/HCl buffer (1.0 M, pH 8.0) and different concentration of MESP and WESP solutions (10–30 µg/mL) were added to 50 µL of AChE/BChE enzymes solution (5.32×10^{-3} EU). The solutions were incubated at 20 °C for 10 min. An aliquot (50 µL, 0.5 mM) of DTNB (5,5′-dithio-bis(2-nitro-benzoic) acid) and AChI/BChI were added to incubated mixture and enzymatic reactions were initiated. AChE/BChE activities were spectrophotometrically determined at 412 nm [71].

α-Glycosidase inhibition studies

α-Glycosidase inhibition effect of MESP and WESP was evaluated according to the method of Tao et al. [72] as detailed described [73]. Firstly, phosphate buffer (pH 7.4, 75 µL) was mixed with of 5 µL of the sample and α-glycosidase enzyme solution (20 µL), which prepared in phosphate buffer (pH 7.4, 0.15 U/mL). After preincubation 50 µL of *p*-nitrophenyl-D-glycopyranoside (*p*-NPG) in phosphate buffer (pH 7.4, 5 mM) was added and solution was re-incubated at physiological temperature (37 °C). The absorbance o mixtures were recorded at 405 nm.

α-Amylase activity

The α-amylase inhibition effects of MESP and WESP were performed according to the procedure of Xiao et al. [74]. Briefly, 1 g starch dissolved in NaOH solution (40 mL, 0.4 M) and heated at 80 °C during 30 min. After cooling, pH was set to 6.9 and total volume complete 100 mL using deionized water. Then, 35 µL of starch solution, 35 µL of phosphate buffer (pH 6.9) and 5 µL MESP and WESP solutions were mixed and were pre-incubated at 35 °C for half hour. Finally, 20 µL enzyme solution was added was incubated for 30 min again. The reaction was finished by HCl addition (50 µL, 0.1 M). The absorbances of incubated mixture were measured at 580 nm.

Statistical analysis

The experimental results were performed in triplicate. The data were recorded as mean ± standard deviation and analyzed. One-way analysis of variance ANOVA was realized by following the procedures. Significant differences between means were determined by Duncan's Multiple Range tests. $p < 0.01$ was regarded as very significant and $p < 0.05$ as significant.

Results and discussion

Phenolic composition

Phenolic compounds are plant metabolites widely spread throughout the plant kingdom [75]. Phenolic compounds are likely to contribute to the biological activity including antioxidant activities of MESP and WESP. It is well known that the amount of extracted phenolics depends on some parameters including the extraction time, pH, temperature; solvent used, and varies with the polarity [76]. Also, the plant organ used for the extraction is very important because of different patterns of secondary metabolites' accumulation between the plant organs [77]. For this purpose, water, ethanol, and

acetone are considered effective solvents for the extraction of polyphenols, however, different solvents including ethanol and ethyl acetate can also extract those compounds [78]. MESP and WESP had the highest contents of total phenolics as 36.35 and 44.72 gallic acid equivalents (GAE) per gram extract, respectively. The high levels of phenolic compounds in any extract indicate its high antioxidant capability. On the other hand flavonoids are the most common group in medicinal plants. The content of total flavonoids in MESP and WESP was determined spectrophotometrically as 68.70 and 78.83 quercetin equivalents (QE), respectively. The results clearly showed that there is a positive correlation between the total flavonoid content in MESP and WESP and antioxidant activity.

It was reported that the chemical composition of phytochemicals varies in the same Salvia species but growing in different geographic regions [79]. However, LC–MS/MS was employed to generate chromatographic profiles of the both Salvia extracts for the identification and quantification of 25 phenolic compounds as tabulated in Table 2. However, *S. pilifera* has not been investigated up to date for its bioactivity screening or phytochemical content. According to the LC–MS/MS experiments, MESP is rich with regard to phenolic contents including Salvigenin (95.11 ± 6.47 mg/kg extract) as an active biocompound isolated from Salvia species [80], quercetagetin-3,6-dimethylether (21.19 ± 3.97 mg/kg extract) and caffeic acid (9.35 ± 1.85 mg/kg extract). However, apigenin (24.09 ± 1.94 mg/kg extract), which found in many plants as natural product belonging to the flavone class that is the aglycone of several naturally occurring glycosides, epicatechin (21.26 ± 2.16 mg/kg extract) as the most represented flavanol, and ferulic acid (13.79 ± 0.96 mg/kg extract), which is one of the most abundant phenolic acids found in plants, and in many staple foods, such as fruits, vegetables, cereals, and coffee [81] were found as the most plentiful phenolics in WESP. Additionally, fumaric acid was found the most abundant organic acids in MESP (182.41 ± 12.65 mg/kg extract) and WESP (175.21 ± 12.15 mg/kg extract, Table 2). These phenolic components could be responsible for the antioxidant effects of MESP and WESP. Plant phenols are the major antioxidant groups of compounds that possess a large spectrum of biological activities. They are multifunctional properties and can act as reactive oxygen species and singlet oxygen scavengers, reducing agents, hydrogen atom donators [82]. Also phenolics, especially flavonoids are capable of metal chelating and reducing capacities.

Antioxidant results

Antioxidant activity of some Salvia species including *S. amplexicaulis*, *S. aytachii*, *S. cadmica*, *S. cassia*, *S. cerino-pruinosa*, *S. ekimiana*, *S. eriophora*, *S. freyniana*, *S. marashica*, *S. nutans*, *S. pilifera*, *S. potentillifolia*, *S. sericeo-tomentosa*, *S. sericeo-tomentosa*, *S. suffruticosa*, and *S. tchiatcheffii* extracts was tested using DPPH radical scavenging activity, metal chelating capacity, and FRAP assay. Also, their phenolic acid contents were analyzed by HPLC and their total flavonoid content was determined as quercetin equivalent. Their extracts were screened against four metabolic enzymes; AChE, BChE, tyrosinase and lipooxygenase [9]. Reducing power potential of the *S. pilifera* extracts was examined by using three different test systems named as CUPRAC, FRAP and Fe^{3+} reducing abilities. An antioxidant molecule or plant extracts can be reductants and inactivate oxidant agents and ROS. This method can easily measured reducing of $Fe[(CN^{-})_6]_3$ to $Fe[(CN^{-})_6]_2$. Ferric ions (Fe^{3+}) addition to MESP and WESP leads to occurring of the Perl's Prussian blue complex of $Fe_4[Fe(CN^{-})_6]_3$, which had a maximum absorbance at 700 nm. Thus, the yellow color of the test mixture changes to various shades of green and blue depending on the reducing power of antioxidant extracts [83]. In this context, MESP and WESP had potent reducing effects by using the potassium ferricyanide reduction and cupric ions (Cu^{2+}) and Fe^{3+}-TPTZ reducing methods. For measurement of the reductive abilities of MESP and WESP, Fe^{3+}–Fe^{2+} transformation was determined in the presence of MESP and WESP according to the method of Oyaizu [53]. As can seen in Table 3, MESP (r^2: 0.9470) and WESP (r^2: 0.9878) showed powerful Fe^{3+} reducing profile ($p < 0.01$). The Fe^{3+} reducing power of MESP, WESP and standard antioxidants including BHA, BHT, α-tocopherol and trolox decreased in following orders: BHA (2.404 ± 0.13, r^2: 0.9945) ≥ BHT (2.307 ± 0.09, r^2: 0.9622) Trolox (2.177 ± 0.07, r^2: 0.9736) > MESP (1.762 ± 0.008, r^2: 0.9470) ≥ α-tocopherol (1.644 ± 0.17, r^2: 0.9118) ≈ WESP (1.636 ± 0.018, r^2: 0.9878). An increase in absorbance indicates an increased reducing capacity due to an increase in the formation of the complex (Fig. 1a). The results demonstrated that both MESP and WESP had potent Fe^{3+} reducing ability and electron donor properties for neutralizing free radicals and reactive oxygen species by forming stable products. Antioxidant activity including reducing ability of Salvia species, which has been extensively studied by different in vivo and in vitro methods, has been generally attributed to their high phenolic contents, which is in agreement with our present data [84].

Cupric ions (Cu^{2+}) reducing abilities of MESP and WESP are shown in Table 3, and a correlation was observed between the Cu^{2+} reducing effect and MESP (r^2: 0.9508) and WESP (r^2: 0.9736) and sample concentrations. Also, these results found to be concentration-dependent (10–50 µg/mL). However, the highest absorbance of reducing power was displayed by MESP (1.609 ± 0.012). Cu^{2+} ions reducing power of MESP and WESP and standards at the 30 µg/mL concentration were as follows (Fig. 1b):

Table 3 Reducing ability by Fe^{3+}–Fe^{2+} and Fe^{3+}-TPTZ transformation methods and Cu^{2+} reducing ability by Cuprac method of WESP, MESP and standard compounds such as BHA, BHT, α-tocopherol and trolox at the same concentration (30 µg/mL; *WESP* lyophilized water extract of *S. pilifera*, *MESP* evaporated methanolic extract of *S. pilifera*, *BHA* butylated hydroxyanisole, *BHT* butylated hydroxytoluene)

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
BHA	2.404 ± 0.13	0.9622	2.398 ± 0.020	0.9588	2.733 ± 0.017	0.9629
BHT	2.307 ± 0.09	0.9902	2.568 ± 0.011	0.9362	2.809 ± 0.012	0.9788
α-Tocopherol	1.644 ± 0.17	0.9118	1.371 ± 0.09	0.9277	2.327 ± 0.001	0.9998
Trolox	2.177 ± 0.07	0.9736	1.282 ± 0.08	0.9811	2.432 ± 0.015	0.9611
WESP	1.636 ± 0.018	0.9878	1.115 ± 0.006	0.9736	1.676 ± 0.007	0.9477
MESP	1.762 ± 0.008	0.9470	1.211 ± 0.013	0.9508	1.722 ± 0.008	0.9292

BHT (2.568 ± 0.011, r^2: 0.9362) > BHA (2.398 ± 0.020, r^2: 0.9588) > α-Tocopherol (1.371 ± 0.09, r^2: 0.9277) > Trolox (1.282 ± 0.08, r^2: 0.9811) ≥ MESP (1.211 ± 0.013, r^2: 0.9508) > WESP (1.115 ± 0.006, r^2: 0.9736). Cuprac assay is based on Cu^{2+}–Cu^{+} reducing by antioxidants and simultaneously cost-effective, stable, rapid, suitable and selective for a different antioxidant regardless of hydrophobicity or chemical type [85].

According to the results determined from the FRAP assay (Table 3 and Fig. 1c) the reducing power of MESP and WESP and standards decreased in the following order: BHT (2.809 ± 0.012, r^2: 0.9788) > BHA (2.733 ± 0.017, r^2: 0.9629) > Trolox (2.432 ± 0.015, r^2: 0.9611) > α-Tocopherol (2.327 ± 0.001, r^2: 0.9998) > MESP (1.722 ± 0.008, r^2: 0.9292) > WESP (1.676 ± 0.007, r^2: 0.9477). In this method, higher absorbance values indicate higher reducing ability of indicated complex. The FRAP method is performed at acidic medium (pH 3.6) to maintain iron solubility. Results of FRAP assay are determined by measuring the increased absorbance at 593 nm and relating [86]. Some research had shown that *Salvia* species efficacy is comparable to that of synthetic preservatives and can thus be used in as a natural preservative. Consequently, several *Salvia* spp. have potential to be as antioxidants [87, 88].

$DPPH^{\cdot}$, $ABTS^{\cdot+}$ and $DMPD^{\cdot+}$ radical scavenging assays are among the most putative and spectrophotometric methods to determine of the radical scavenging and antioxidant capacities of beverages, foods, and plants [89]. The electron or hydrogen atom donation capacity, of MESP and WESP was computed from the bleaching property of the purple-colored ethanol solution of $DPPH^{\cdot}$. This radical scavenging ability is the most used and oldest method for determining of radical scavenging and antioxidant activities. In radical scavenging assay, the antioxidant compounds and plant extracts were able to reduce the stable radical $DPPH^{\cdot}$ to the yellow-colored and non-radical form of DPPH-H [53]. As seen in Table 4 and Fig. 1d, a significant decrease ($p < 0.01$) in the concentration of DPPH radical due to the scavenging ability of MESP and WESP and the positive standard compounds including α-tocopherol, trolox, BHA and BHT was found. When an antioxidant component or plant extracts reacts with DPPH radicals, they can donate hydrogen and consequently reduced $DPPH^{\cdot}$. The color changes were spectrophotometrically measured at 517 nm. IC_{50} values DPPH radical scavenging were fond as 7.05 µg/mL (r^2: 0.9774) for MESP, 8.56 µg/mL (r^2: 0.9803) for WESP, 10.66 µg/mL (r^2: 0.9508) for BHA, 11.01 µg/mL (r^2: 0.9810) for BHT, 15.37 µg/mL (r^2: 0.9684) for α-Tocopherol and 9.83 µg/mL (r^2: 0.9927) for Trolox. A lower EC_{50} value indicates a higher DPPH free radical scavenging profile.

ABTS radical scavenging can applicable for plant extracts including both hydrophilic and lipophilic compounds. This assay is based on the inhibition of the absorbance of the radical cation $ABTS^{\cdot+}$ that has a characteristic wavelength showing absorption at 734 nm. $ABTS^{\cdot+}$ scavenging assay can be used in a large spectrum of pH range. This is allowed to study the effect of pH on antioxidant mechanisms for food extracts or components [90]. Both tested MESP and WESP in this study exhibited effective radical scavenging activity against ABTS radicals ($p > 0.001$). As seen in Table 4, MESP and WESP effective scavenged ABTS radicals in a concentration-dependent manner (10–50 µg/mL) and these differences were found statistically significant ($p < 0.001$). EC_{50} values for MESP and WESP in this assay were calculated as 3.52 µg/mL (r^2: 0.9990) and 4.76 µg/mL (r^2: 0.9381). On the other hand, EC_{50} values were found as for 8.07 µg/mL (r^2: 0.9720) for BHA, 7.16 µg/mL (r^2: 0.9955) for BHT, 10.12 µg/mL (r^2: 0.9429) for α-Tocopherol and 6.28 µg/mL (r^2: 0.9112) for Trolox (Fig. 1e). The $ABTS^{\cdot+}$ scavenging effect of MESP and WESP and standards decreased in the following order: MESP > WESP > Trolox ≥ BHT ≥ BHA > α-Tocopherol.

In the study, the last evaluated radical scavenging assay is $DMPD^{\cdot+}$ scavenging activity. The $DMPD^{\cdot+}$ scavenging method guarantees a very stable endpoint like $ABTS^{\cdot+}$ scavenging assay [91]. As shown in Table 4, MESP and WESP

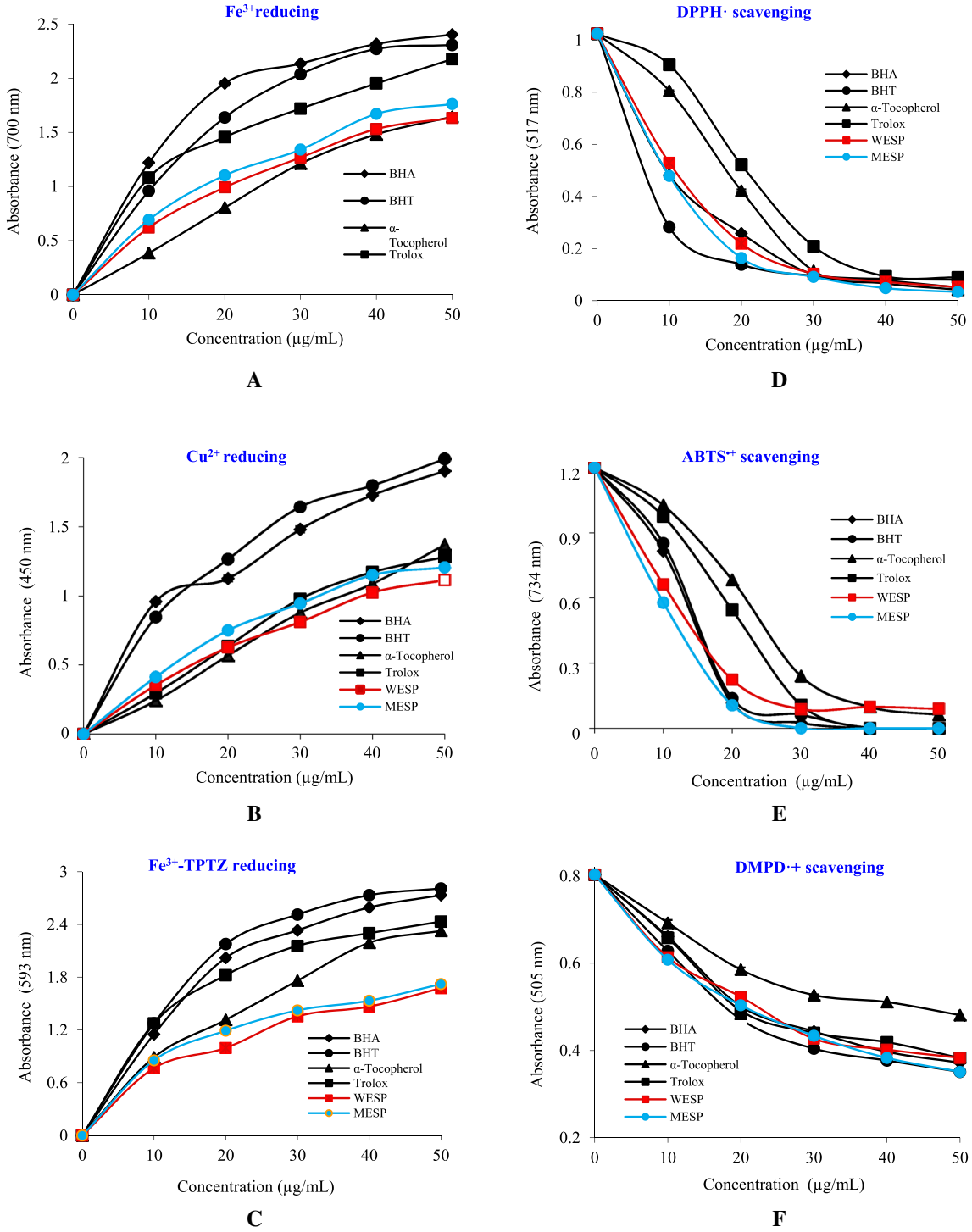

Fig. 1 Antioxidant activity of lyophilized water (WESP) and evaporated methanolic (MESP) extracts of *S. pilifera*. **a** Fe^{3+}–Fe^{2+} reducing ability assay, **b** Cu^{2+} reducing ability by Cuprac assay, **c** Fe^{3+}-TPTZ^{+} reducing ability assay, **d** 1,1-diphenyl-2-picryl-hydrazyl radicals (DPPH$^{\cdot}$) scavenging assay, **e** 2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radicals (ABTS$^{\cdot+}$) scavenging assay, **f** *N*,*N*-dimethyl-*p*-phenylenediamine radicals (DMPD$^{\cdot+}$) scavenging assay

were an effective DMPD$^{\cdot+}$ scavenging in a concentration-dependent manner (10–50 µg/mL). EC_{50} values of MESP and WESP were calculated as 28.92 µg/mL (r^2: 0.9309) and 30.95 µg/mL (r^2: 0.9722). On the other hand, EC_{50} values were found as for 28.17 µg/mL (r^2: 0.9618) for BHA, 33.81 µg/mL (r^2: 0.9082) for BHT, 40.62 µg/mL (r^2: 0.9593)

Table 4 Determination of half maximal concentrations (IC_{50}, µg/mL) of *Salvia pililfera* and standards for 1,1-diphenyl-2-picryl-hydrazyl free radical ($DPPH^{\cdot}$) scavenging, 2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid ($ABTS^{\cdot+}$) scavenging, *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride radical ($DMPD^{\cdot+}$) scavenging, activities of WESP, MESP and standard compounds such as BHA, BHT, α-tocopherol and trolox

Compounds	$DPPH^{\cdot}$ scavenging		$ABTS^{\cdot+}$scavenging		$DMPD^{\cdot+}$scavenging	
	IC_{50}	r^2	IC_{50}	r^2	IC_{50}	r^2
BHA	10.66	0.9508	8.07	0.9720	28.17	0.9618
BHT	11.01	0.9810	7.16	0.9955	33.81	0.9082
α-Tocopherol	15.37	0.9684	10.12	0.9429	40.62	0.9593
Trolox	9.83	0.9927	6.28	0.9112	31.18	0.9887
WESP	8.56	0.9803	4.76	0.9381	30.95	0.9722
MESP	7.05	0.9774	3.52	0.9990	28.92	0.9309

for α-Tocopherol and 31.18 µg/mL (r^2: 0.9887) for Trolox (Fig. 1f). There is a significant decrease ($p < 0.05$) in the concentration of $DMPD^{\cdot+}$ scavenging effects due to the scavenging capacity at all MESP and WESP concentrations. The results shown that both MESP and WESP can easily transfer hydrogen atoms to $DMPD^{\cdot+}$ and quench the color and produce a decoloration of the solution.

Enzymes inhibition results

Enzyme inhibitory activity of *S. pilifera* extracts was determined using α-amylase, α-glycosidase, acetylcholinesterase (AChE), and butyrylcholinesterase (BChE) enzymes. Considering the fact that MESP and WESP are found as effective AChE, BChE, α-amylase and α-glycosidase inhibition effects. Enzyme inhibitions are one of the most interesting and studied therapeutic tools for the cosmetic and pharmaceutical industries. They are clinically used drugs for managing of some health problems, such as obesity, diabetes and AD [92]. It was reported that reported there is side effects including gastrointestinal disturbances and hepatotoxicity of synthetic inhibitors. However, there are great interests for finding innovative natural inhibitors without side effects when compared to synthetic ones [1, 93]. The inhibition data are summarized in Table 5. For evaluation of the effect of MESP and WESP on the indicated metabolic enzymes, the following results had been delineated. Both cholinergic enzymes inhibition properties were determined according to the procedure of Ellman et al. [69]. MESP and WESP had IC_{50} values 94.93 µg/mL (r^2: 0.9753) and 138.61 µg/mL (r^2: 0.9891) for AChE, respectively. These values were calculated as 69.05 µg/mL (r^2: 0.9333) and 99.13 µg/mL (r^2: 0.9797) for BChE, respectively. On the other hand, tacrine as standard inhibitors for both cholinergic enzymes had Ki values of 0.124 µM (r^2: 0.9804) and 0.101 µM (r^2: 0.9698) toward AChE and BChE, respectively. The evaluated MESP and WESP showed effective inhibition against both AChE and BChE enzymes, but MESP showed more inhibition effect against both cholinergic enzymes than that of WESP. AChE hydrolysis acetylcholine (ACh) to choline and acetate. The ACh levels decreases with the ageing process, which results in the progression of neurological disorders, as for example AD. The AChE inhibition increases the levels of ACh, thus AChE inhibition were considered as useful therapeutic approach to treat neurological disorders including AD [94]. As can seen in Table 4, as observed in antioxidant effects, the MESP and WESP had the effective AChE and BChE inhibition capacities. We speculated that the major phenolics identified in MESP and WESP act as AChE inhibitors. It is known that phenolic compounds had cholinergic enzymes inhibitors [95]. Also, it was reported that some Salvia species had neurobiological effects [9] and were used in European folk medicine for their memory-enhancing property [96].

Table 5 The enzyme inhibition results (IC_{50} values; µg/mL) of WESP and MESP against α-glycosidase, α-amylase, acetylcholinesterase, and butyrylcholinesterase enzymes

Enzymes	WESP		MESP		Standards	
	IC_{50}	r^2	IC_{50}	r^2	IC_{50}	r^2
α-Glycosidase[a]	36.47	0.9809	23.28	0.9818	22.80	0.9922
α-Amylase[a]	97.67	0.9856	46.21	0.9505	10.01	0.9424
Acetylcholinesterase[b]	138.61	0.9891	94.93	0.9753	0.124	0.9804
Butyrylcholinesterase[b]	99.13	0.9797	69.05	0.9333	0.101	0.9698

[a]Acarbose was used as positive control for α-glycosidase and α-amylase enzymes

[b]Tacrine was used as positive control for acetylcholinesterase and butyrylcholinesterase enzymes

Digestive enzymes of α-amylase and α-glycosidase are critical glycoside hydrolases involved in carbohydrate digestion. Bothe enzymes are present on cells lining the intestine and hydrolysis polysaccharide to monosaccharide units for absorbing through the intestine. In order to reduce body weight and control blood glucose level, specific inhibitors can inhibit the activities of both digestive enzymes. Plant-based foods take place among the relatively safe sources of inhibitory compounds [97]. MESP and WESP exhibited IC_{50} values of 23.28 µg/mL (r^2: 0.9818) and 36.47 µg/mL (r^2: 0.9809) for α-glycosidase, 46.21 µg/mL (r^2: 0.9505) and 97.67 µg/mL (r^2: 0.9856) for α-amylase, respectively (Table 5). The obtained results showed that MESP and WESP had more affinity for α-amylase than that of α-glycosidase enzyme. The inhibition of both carbohydrate-hydrolysing enzymes can reduce the postprandial blood glucose levels. Also, they can be crucial therapeutic strategy for managing of hyperglycaemia linked to diabetes. Also both MESP and WESP had more α-glycosidase inhibition profiles than that of acarbose (IC_{50}: 22.800 mM) as standard α-glycosidase inhibitor [72]. The inhibition of both digestive enzyme had great importance due treating and preventing diabetes, postprandial glucose amounts pand hyperglycemia.

Conclusions

The evaluation of bioactivity and phytochemistry screening of this Salvia species is important. So far there is no report on bioactivity and phytochemistry of *S. pilifera* and it might only be speculated that its high enzymes inhibitory effect could be related to its rich phenolic acid contents, which considered being the major contributors to a wide range of its antioxidant properties. MESP and WESP as natural sources of phenolic compounds were evaluated for their biological assays like antioxidant activity and AChE, BChE, α-glycosidase and α-amylase inhibition properties. MESP and WESP were found to be effective antioxidant activities in several in vitro assays including ferric ions (Fe^{3+}) and cupric ions (Cu^{2+}) reducing ability, $DPPH^{\cdot}$, $ABTS^{\cdot+}$ and $DMPD^{\cdot+}$ radical scavenging activities. In addition, both extracts were found as powerful antioxidant activity and inhibition effects of indicated metabolic enzymes. Also, ethanol was efficient for extraction of phenolic compounds with inhibitory capacity of the α-glycosidase, α-amylase AChE and BChE enzymes. Overall, this work suggests that *S. pilifera* may be a promising potential source of benefit compounds for treatment of some diseases such as postural tachycardia syndrome, myasthenia gravis, diabetes, neurodegenerative diseases including AD.

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Compliance with ethical standards

Conflict of interest The authors report no conflict of interests.

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