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A comparative study of phenolic profiles and biological activities of *Allium sphaerocephalon* L. subsp. *sphaerocephalon* L. and *Allium sphaerocephalon* L. subsp. *trachypus* (Boiss. Et Spruner) K. Richter

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ABSTRACT: *Allium sphaerocephalon* subsp. *sphaerocephalon*, the common name is known as "Round-Headed Leek", is an edible plant in different countries. In the present study, bulb, stem and flower extracts of *A. sphaerocephalon* subsp. *sphaerocephalon* were compared in terms of phenolic profile and enzyme inhibitory activity, with *A. sphaerocephalon* subsp. *trachypus* which is not an edible species. Total phenolic and flavonoid contents of extracts were determined by spectrophotometrically, and bulb parts of both plants were found to be rich in phenolics. Twenty-four selected phenolic compounds were investigated qualitatively and quantitatively in the extracts by LC-MS-MS. Gallic acid (458.4-1246.4 µg/g extract) as phenolic acid and 3-*O*-methyl quercetin (46.6-136.2 µg/g extract) as a flavonoid were the major compounds in all extracts. In addition, enzyme (acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and tyrosinase) inhibitory potentials of all samples were performed using a 96-well microplate reader. Flower parts (IC_{50}, 11.49 µg/mL for AChE) and bulb parts of (IC_{50}, 16.62 µg/mL for BuChE, 65.94 µg/mL for tyrosinase) *A. sphaerocephalon* subsp. *sphaerocephalon* were the most potent samples. Also, the results of all analyses were subjected to principal component analysis, to gain insight into the relationship between data of assays.

KEYWORDS: *Allium sphaerocephalon*; phenolics; cholinesterases; tyrosinase.

1. INTRODUCTION

Phenolic compounds are one of the most significant groups of secondary metabolites due to their biological effects and widely distributed in plants. Biogenetically, these metabolites consist of the shikimic acid pathway where, mainly, phenylpropanoids are formed and malonate/acetate pathway, in which the main products are the simple phenol [1]. Also, they divide into subgroups according to their chemical structure where has, as a common typical, the existence of at least one aromatic ring hydroxyl-substituted. Primarily, this group is based on two main subgroups; (i) flavonoids, including flavones, flavonols, flavanones, flavan-3-ols, anthocyanidins, isoflavonoids and; (ii) nonflavonoids, including simple phenols, phenolic acids (hydroxybenzoic acids and hydroxycin-namic acids), tannins and lignans [2]. Plant phenolics show free radical scavenging, reducing agents and metal chelating activities with nonspecific mechanisms depending on the presence of the phenyl rings and hydroxyl groups. The redox characteristics of these compounds enable them to exhibit strong antioxidant activity. In addition, depending on the chemical properties of active phenolics, by specific mechanisms, they interact with transcription factors, receptors, and enzymes such as α-amylase and α glucosidase, angiotensin I-converting enzyme, lipase, pro-inflammatory enzymes, cholinesterases, and tyrosinase [3]. Enzymes are essential protein molecules due to their catalytic role in several physiological processes and are the target of 47% of small molecule drugs marketed [4].

Alzheimer's disease (AH) is a progressive neurodegenerative disorder, the etiology of which is unknown, characterized by impairment of cognitive functions such as learning, memory, language skills. Different hypotheses related to the etiology of the AD have been proposed, and according to the "cholinergic hypothesis", the degeneration of cholinergic neurons in the basal forebrain, as well as the loss of cholinergic neurotransmission in the cerebral cortex and other areas, have a significant effect to the degradation in

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cognitive functions seen in patients [5, 6]. Thus, cholinesterase inhibitors are currently major drugs of the symptomatic treatment of AD. Among them donepezil and galantamine (an Amaryllidaceae alkaloid) more selective for acetylcholinesterase (AChE), others are effective both on AChE and butyrylcholinesterase (BuChE) enzymes [7].

Tyrosinase is a multifunctional, glycosylated, and copper-containing oxidase, which catalyzes the first two steps in mammalian melanogenesis by the hydroxylation of tyrosine and the oxidation of 3,4-dihydroxyphenylalanine. The accumulation of abnormal amounts of melanin might cause skin disorders such as vitiligo, hyperpigmentation, and melanoma [8, 9]. Also, tyrosinase catalyzes the formation of the neuromelanin and the dopaquinone which causes dopaminergic neuron loss. Neuromelanin interacts with the α-synuclein protein, which is thought to be responsible for familial Parkinson's disease, and it also renders neurons in the substantia nigra pars compacta more susceptible to toxic effects [10, 11]. Therefore, tyrosinase inhibitors have become increasingly important in the medicinal, and cosmetic industries. In addition, in the food industry, inhibition of tyrosinase is important because it causes enzymatic browning in fruits and vegetables [12].

Allium species belong to the Amaryllidaceae family and are represented with about 900 species. Several of these species have been used as vegetables, spices, and natural therapeutics since ancient times [13]. In our ongoing studies on *Allium* species, it has been determined that the different parts of these species are rich in phenolic compounds and also have the potential to inhibit cholinesterase and tyrosinase enzymes [13-15]. *A. sphaerocephalon* subsp. *sphaerocephalon*, the common name is known as "Round-Headed Leek", is an edible plant in different countries. The whole plant is used as a condiment and as an onion substitute. Also, different parts of plant (bulb, stem, leaves) are consumed as raw or cooked in Spain, Ukraine, the Lake Baikal region of Siberia and Italy [16]. In contrast, there is no report that *A. sphaerocephalon* subsp. *trachypus* is edible.

The aim of present study is the comparison of bulb, stem and flower parts of these two subspecies in terms of phenolic profile and also inhibitory activity against AChE, BuChE and tyrosinase, for the first time, to the best of our knowledge.

2. RESULTS AND DISCUSSION

2.1. Phenolic profile

Determination and quantification of total phenolic contents (TPCs), total flavonoid contents (TFCs), and selected phenolic compounds were carried out for the phenolic profiling of bulb, stem, and flower extracts of *A. sphaerocephalon* subsp. *sphaerocephalon* and *A. sphaerocephalon* subsp. *trachypus*. Identification (ID) of samples, and also the results of TPCs and TFCs which were indicated as gallic acid and quercetin equivalents, respectively were shown in Table 1. TFCs of samples, varied from 1.09 mg QE/g for AT-H to 2.07 mg QE/g for AT-F, were assigned according to the $AlCl_3$ method. Additionally, the Folin Ciocalteu method was used for the determination of TPCs that varied from 11.4 mg GAE/g for AT-F to 34.1 mg GAE/g for AS-B. Compared with the AT extracts, the AS extracts were observed to contain higher levels of TPCs. Also, bulb parts of both plants had higher TPCs than the other parts. Phenolic compounds are thought to be synthesized in plants with different purposes such as for pollination, for protection from insects, oxidizing agents, and ultraviolet radiation. Environmental factors and/or physiological factors are most likely responsible for these differences in the phenolic contents of the plants [1, 17]. In the previously reported TPC studies on different parts of the *Allium* species, stem parts of *A. pallens* [13], bulbs of *A. stylosum* [14], *A. subhirsutum* [15] and *A. roseum* var. *odoratissimum* [18], aerial parts of *A. nigrum* [15] and *A. orientale*, leaves of *A. ursinum* [19] were found to be richer than other parts.

Table 1. Sample ID and TPC and TFC of extracts.

Plant	Part used	Sample ID	TPC (mg GAE[a]/g extract)	TFC (mg QE[b]/g extract)
Allium sphaerocephalon subsp. *sphaerocephalon*	Flower	AS-F	14.83 ± 0.93	1.56 ± 0.81
	Stem	AS-H	21.39 ± 1.44	1.35 ± 0.64
	Bulb	AS-B	34.16 ± 1.87	1.93 ± 0.27
Allium sphaerocephalon subsp. *trachypus*	Flower	AT-F	11.44 ± 0.69	2.07 ± 0.55
	Stem	AT-H	15.38 ± 0.72	1.09 ± 0.62
	Bulb	AT-B	19.71 ± 1.56	1.23 ± 0.78

a: gallic acid equivalents, b: quercetin equivalents.

In order to determine which compound may contribute more to the biological activity of the samples, the quantification of twenty-four selected phenolic compounds in different parts of both plants was carried out by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). The results of analysis were given as µg/g extract and showed in Table 2. Compared to AT samples, AS samples had more major phenolics. Six of the analyzed compounds in AS-F (3-hydroxybenzoic acid, gallic acid, vanillic acid, 3-hydroxyflavone, galangin, morin) and in AS-B (benzoic acid, 4-hydroxybenzoic acid, *p*-coumaric acid, ferulic acid, fisetin, 3-*O*-methylquercetin) were found to be the most dominant phenolics while AS-S (catechol, naringenin, genistein, epicatechin) had four major compounds. Among the detected phenolic compounds generally, phenolic acids were the major compounds and also, gallic acid (458.4-1246.4 µg/g) as phenolic acid and 3-*O*-methyl quercetin (46.6-136.2 µg/g) as a flavonoid had the highest values in all samples. Similarly, in the studies of quantification by LC on *Allium* species, phenolic acids were the major compounds and also, gallic acid in *A. stylosum* [14], and in *A. pallens* [13], 3- hydroxybenzoic acid in *A. nigrum* [15], *p*-coumaric acid in *A. subhirsutum* [15], protocatechuic acid in *A. hookeri* [20], 3,4-dihidroksi benzoic acid in *A. reuterianum* [21] were the most dominant phenolic compounds in these species.

Table 2. Concentrations of phenolic compounds (µg g-1 of extract) of different parts of *Allium* species.

Compound	AS-F	AS-S	AS-B	AT-F	AT-S	AT-B
Catechol	103.3 ± 2.01	233.3 ± 1.59	80.0 ± 1.62	T	36.7 ± 2.72	103.3 ± 2.51
Benzoic acid	345.6 ± 1.86	335.4 ± 0.97	360.2 ± 0.77	140.0 ± 2.83	184.2 ± 0.39	165.6 ± 0.89
3-Hydroxybenzoic acid	672.8 ± 3.14	641.6 ± 2.65	601.7 ± 1.98	274.2 ± 3.19	247.5 ± 1.85	223.8 ± 1.39
4-Hydroxybenzoic acid	491.6 ± 0.92	533.5 ± 2.41	536.6 ± 1.33	341.0 ± 0.92	143.4 ± 0.96	369.7 ± 2.44
Gallic acid	1246.4 ± 2.63	1104.4 ± 3.77	889.2 ± 3.02	458.4 ± 1.37	626.4 ± 2.43	952.8 ± 3.17
Vanillic acid	93.6 ± 0.65	17.1 ± 0.62	33.1 ± 2.79	20.3 ± 1.98	34.4 ± 2.71	22.4 ± 2.56
Syringic acid	28.5 ± 1.84	51.0 ± 2.43	36.5 ± 1.52	59.0 ± 2.71	42.5 ± 3.08	32.5 ± 0.74
p-Coumaric acid	458.2 ± 2.64	531.6 ± 3.79	567.3 ± 0.95	158.6 ± 2.79	106.1 ± 1.65	116.8 ± 1.36
Ferulic acid	19.2 ± 0.48	18.7 ± 2.18	21.4 ± 0.72	19.5 ± 0.58	17.6 ± 1.99	18.7 ± 0.91
3-Hydroxyflavone	5.5 ± 0.39	4.5 ± 0.77	5.1 ± 0.38	1.3 ± 0.47	1.4 ± 0.22	1.0 ± 0.22
Kaempferol	60.8 ± 1.85	30.4 ± 1.34	33.6 ± 1.44	68.0 ± 1.39	38.4 ± 1.93	53.6 ± 1.54
Galangin	63.3 ± 1.44	57.5 ± 1.82	62.2 ± 0.71	29.2 ± 1.77	43.2 ± 1.18	3.0 ± 1.29
Fisetin	5.9 ± 0.36	2.8 ± 0.97	6.4 ± 2.38	6.2 ± 0.85	4.7 ± 0.77	6.0 ± 0.73
Morin	2.3 ± 0.75	1.4 ± 0.31	1.5 ± 1.77	T	2.1 ± 0.93	T
Quercetin	6.4 ± 1.88	6.3 ± 0.72	10.1 ± 3.82	11.8 ± 2.74	10.9 ± 2.47	T
3-*O*-methylquercetin	73.0 ± 1.46	68.4 ± 0.53	136.2 ± 0.51	80.7 ± 1.53	46.6 ± 1.98	62.4 ± 3.11
Isorhamnetin	2.5 ± 0.33	3.7 ± 0.49	2.8 ± 0.36	4.9 ± 0.97	3.9 ± 0.64	3.0 ± 1.24
Myricetin	T	T	T	T	T	T
Hesperidin	1.2 ± 0.37	2.5 ± 0.33	T	T	T	3.2 ± 1.56
Naringenin	11.0 ± 2.58	11.9 ± 2.64	11.8 ± 0.74	6.1 ± 0.72	8.4 ± 2.03	5.5 ± 0.82
Genistein	22.3 ± 1.55	30.7 ± 1.88	35.7 ± 1.92	20.9 ± 1.85	27.3 ± 0.88	12.4 ± 1.79
(+)-Catechin	T	T	T	T	T	1.2 ± 0.82
(−)-Epicatechin	4.8 ± 0.91	9.2 ± 0.39	4.6 ± 0.96	4.5 ± 1.36	3.8 ± 0.95	5.8 ± 2.75
(−)-Epigallocatechin gallate	10.2 ± 1.43	6.3 ± 1.86	12.9 ± 3.05	17.4 ± 2.51	6.1 ± 0.42	9.1 ± 0.63

T:Trace amounts were detected in the sample.

2.2. Biological activity

The AChE and BuChE inhibition activities of methanol extracts were performed by Ellman's method and galanthamine was used as the positive control. IC_{50} values of galanthamine were 0.106 µg/mL and 1.04 µg/mL in anti-AChE and anti-BuChE assays, respectively. The results, given in Table 3, varied from 11.49 to 223.86 µg/mL for AChE inhibitory activity and also from 16.33 to 116.52 µg/mL for BuChE inhibitory activity. All samples showed moderate cholinesterase inhibitory activity but the significant values of IC_{50} were observed in AS-F (11.49 µg/mL for anti-AChE) and in AS-B (16.62 µg/mL for anti-BuChE). Some flavonoids (kaempferol, galangin 3-*O*-methylquercetin, genistein) detected with higher concentrations in LC-MS/MS analysis, may have participated in its anticholinesterase activity since they are known from the literature for such inhibitory effects [22-24]. In the literature, detailed anti-AChE activity research on twenty-three *Allium*

species was reported by Hadacova et al., all species displayed activity and *A. obliquum* was the most potent [25]. Additionally, in our previous comparative studies on different parts of *Allium* species; aerial parts of *A. nigrum* [15], bulbs of *A. subhirsutum* [15], leaves of *A. stylosum* [14], stem parts of *A. pallens* [13] showed significant cholinesterase inhibitory activity.

Kojic acid was used as a positive control for the determination of the anti-tyrosinase activity of samples and its IC_{50} value was found to be 7.9 µg/mL. The extracts had low activity when compared to reference compound. Results were indicated in Table 3, the activity was not observed in AT-F, and AS-B was the most active sample (IC_{50}, 65.94 µg/mL). Compounds (4-hydroxybenzoic acid, vanillic acid, and kaempferol) that had anti-tyrosinase activity may well contribute to the activity of extracts [26]. Also, catechol and *p*-coumaric acid, with a similar chemical structure to L-tyrosine, affect as substrate molecules [27, 28].

Table 3. Enzyme inhibitory properties of the samples.

Sample	AChE Inhibition (IC_{50} µg/mL)	BuChE Inhibition (IC_{50} µg/mL)	Tyrosinase Inhibition (IC_{50} µg/mL)
AS-F	11.49 ± 0.16	116.52 ± 1.03	179.42 ± 0.15
AS-H	149.15 ± 0.88	95.17 ± 0.59	204.71 ± 1.30
AS-B	59.81 ± 0.42	16.62 ± 0.22	65.94 ± 0.63
AT-F	76.19 ± 0.38	19.33 ± 0.65	NA
AT-H	223.86 ± 1.63	89.41 ± 0.78	262.50 ± 0.97
AT-B	168.75 ± 0.79	72.18 ± 0.34	315.88 ± 0.83
Galanthamine (std)	0.106 ± 0.01	1.04 ± 0.01	-
Kojic acid (std)	-	-	7.9 ± 0.02

NA: not active

3. CONCLUSION

Apart from the cultural forms of the *Allium* species, the wild species are also widely consumed in different countries. In this study, the bulb, stem and flower parts of the two subspecies of the *Allium sphaerocephalon*, one edible and the other non-edible, were investigated in terms of phenolic profile and enzyme inhibitory activities, for the first time. The edible species, *Allium sphaerocephalon* L. subsp. *sphaerocephalon* was found to be richer in phenolic content and stronger in biological activity than the other species. The results of this research revealed that *Allium sphaerocephalon* L. subsp. *sphaerocephalon* could be a natural source for phenolic compounds that we found in high concentrations. Also, different parts of this species had a potency for further *in vivo* investigations related to enzyme inhibitory activities.

4. MATERIALS AND METHODS

4.1. Chemicals

Reference standards used in the LC-MS/MS analysis, AChE (from *Electrophorus electricus*), BuChE (from equine serum), Acetylthiocholine/Butyrylthiocholine iodide, DTNB (Ellman's reagent) [5,5′-dithio-bis-(2-nitrobenzoic acid)], tyrosinase (from mushroom), galanthamine, L-dopa, kojic acid used for enzyme inhibitory activities, were all purchased from Sigma Aldrich (USA).

4.2. Plant material

Allium sphaerocephalon L. subsp. *sphaerocephalon* L. was collected in June 2018 from Bozdağ/Ödemiş (İzmir/Turkey) and *Allium sphaerocephalon* L. subsp. *trachypus* (Boiss. Et Spruner) K. Richter was collected in July 2018 from Spil/Manisa (İzmir/Turkey). The plants were identified by Hasan Yıldırım. Voucher specimens (No: 1612 and 1625, respectively) have been deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ege University.

4.3. Extraction of samples

Bulb, stem, and flower parts of both plants were extracted with methanol in order to use in phenolic profiling and enzyme inhibitory activity experiments. One gram of air-dried, powdered plant materials and 20 mL methanol were added in the falcon tubes. The extraction was achieved 3 times under continuous shaking in the rotator. Then the centrifugation at 4000 rpm for 10 min, was carried out to obtain the

supernatant. After evaporating the liquid phase by a rotary (Buchi) under reduced pressure, extracts were kept at 4°C until used for assays.

4.4. Total phenolic content (TPC)

The determination of total phenolic contents (TPCs) of all samples was evaluated spectrophotometrically using a modified Folin-Ciocalteu method [29]. The process of the method was described in our previous study [16], gallic acid was used as the standard compound and the results were expressed as gallic acid equivalents (mg GAE/g extract) based on the standard curve created.

4.5. Total flavonoid content (TFC)

The assigning of total flavonoid contents (TFCs) of samples was carried out by the $AlCl_3$ method with slight modifications [16, 18]. Quercetin was used as standard compound and the results were given as quercetin equivalents (mg QE/g extract) based on a standard curve created.

4.6. Determination of phenolic compounds by LC-ESI-MS/MS

Twenty-four phenolic compounds were determined and quantified in the methanol extracts of different parts of both plants using a TSQ Quantum™ Access MAX Triple Quadrupole Mass Spectrometer (Thermo Scientific™, USA). Chromatographic separation of phenolics was performed using a GL Sciences ODS C18 column (150 mm × 4.6 mm × 5 µm) with a gradient mobile phase. LC-MS grade water (solvent A) with 0.1% formic acid and also methanol (solvent B) with 0.1% formic acid as follows: 5 to 20 % in 1.5 min, 20 to 30 % from 1.5 to 3 min, 30 to 50 % from 3 to 4.5 min, 50 to 70 % from 4.5 to 6.5 min, 70 to 80 % from 6.5 to 8 min, 80 to 90 % from 8 to 12.5 min and 90 to 95 % from 12.5 to 15 min, at a flow rate of 1.0 mL/min. Analytical parameters and chemical properties of analyzed compounds were given in Tables 4 and 5. The identification of the phenolics in the extracts was determined by comparing their retention times and MS/MS fragments with reference standards. An external standard method was performed, and the results are expressed as µg per gram of extracts.

Table 4. Analytical parameters of LC-MS/MS analysis.

Compound	Ranges (µg/mL)	Linear equation	R^2	LOD (ng/mL)	LOQ (ng/mL)
1	1-500	y = 36.082x – 148.22	0.9975	31	102
2	1-500	y = 60.105x – 217.8	0.9984	19	63
3	1-1000	y = 6.2718x + 36.061	0.9996	24	79
4	1-1000	y = 13.267x + 195.64	0.9963	18	55
5	1-1500	y = 1.94x + 65	0.9959	14	47
6	1-100	y = 3.0818x + 3.6932	0.9993	22	69
7	1-100	y = 1.6575x + 5.4167	0.9939	12	38
8	1-1000	y = 18.309x + 1104.2	0.9923	7	25
9	1-100	y = 1.5051x – 0.9492	0.9985	11	39
10	1-10	y = 5666.7x + 55.542	0.9966	9	31
11	1-100	y = 0.9916x + 0,.9701	0.9971	18	62
12	1-100	y = 274.98x + 355.61	0.9975	25	81
13	1-10	y = 2.7177x – 1.25	0.9962	17	46
14	1-10	y = 36.898x + 4.4167	0.9995	8	27
15	1-20	y = 34.139x + 12.935	0.9972	6	19
16	1-500	y = 11.842x + 23.284	0.9991	12	32
17	1-10	y = 196x + 16	0.9954	21	66
18	-	-	-	15	49
19	1-10	y = 110.77x + 22	0.9989	11	35
20	1-20	y = 685.75x – 141.67	0.9972	14	42
21	1-100	y = 19.636x + 5.8064	0.9984	28	95
22	-	-	-	16	44
23	1-10	y = 80.919x – 26.5	0.9931	20	65
24	1-20	y = 1.9946x – 0.6667	0.9947	19	59

Table 5. Analytical parameters of LC-MS/MS analysis.

	No	Compound Name	Molecular Formula	Collision energy (V)	Precursor ion [m/z]	Product ion [m/z]
Simple Phenol	1	Catechol	$C_6H_6O_2$	27	109.22	108.10
	2	Benzoic acid	$C_7H_6O_2$	15	121.18	77.23
Phenolic Acid	3	3-Hydroxybenzoic acid	$C_7H_6O_3$	15	137.18	93.18
	4	4-Hydroxybenzoic acid	$C_7H_6O_3$	15	137.15	93.15
	5	Gallic acid	$C_7H_6O_5$	15	169.07	125.02
	6	Vanillic acid	$C_8H_8O_4$	7	167.10	152.6
	7	Syringic acid	$C_9H_{10}O_5$	15	197.11	182.18
Hydroxycinnamic Acid	8	*p*-Coumaric acid	$C_9H_8O_3$	13	163.01	119.08
	9	Ferulic acid	$C_{10}H_{10}O_4$	15	193.04	134.10
Flavone	10	3-Hydroxyflavone	$C_{15}H_{10}O_3$	34	239.10	165.08
Flavonol	11	Kaempferol	$C_{15}H_{10}O_6$	35	285.10	238.95
	12	Galangin	$C_{15}H_{10}O_5$	35	271.20	153.11
	13	Fisetin	$C_{15}H_{10}O_6$	30	285.10	135.01
	14	Morin	$C_{15}H_{10}O_7$	23	301.22	150.94
	15	Quercetin	$C_{15}H_{10}O_7$	26	301.20	150.94
	16	3-*O*-methylquercetin	$C_{16}H_{12}O_7$	20	315.10	270.98
	17	Isorhamnetin	$C_{16}H_{12}O_7$	23	315.09	300.02
	18	Myricetin	$C_{15}H_{10}O_8$	25	317.05	191.00
Flavanone	19	Hesperidin	$C_{28}H_{34}O_{15}$	20	609.40	300.88
	20	Naringenin	$C_{15}H_{12}O_5$	22	271.05	151.01
Isoflavone	21	Genistein	$C_{15}H_{10}O_5$	30	271.09	153.11
Flavan-3-ol	22	(+)-Catechin	$C_{15}H_{14}O_6$	22	289.10	203.07
	23	(-)-Epicatechin	$C_{15}H_{14}O_6$	15	291.22	139.10
	24	(-)-Epigallocatechin gallate	$C_{22}H_{18}O_{11}$	15	459.18	139.10

4.7. Cholinesterase inhibitory activity

AChE and BuChE inhibitory activity of the extracts were applied according to Ellman's method [30] with described modifications, using microplate reader (Varioskan Flash Multimode Reader, Thermo Scientific, USA). Dilutions were made at concentrations of 0.001-1000 µg/mL using 0.05 M phosphate buffer. 0.25 U / mL enzyme was added to the samples in a microplate and incubated for 30 minutes before mixing with the substrate. Subsequently, 0.24 mM acetylthiocholine iodide or butyrylthiocholine iodide solution as the substrate and 0.2 mM Ellman reagent (5,5-dithiobis-2-nitrobenzoic acid) (DTNB) were added. Finally, measurements were made at 405 nm by a 96-well microplate reader.

4.8. Tyrosinase inhibitory activity

The antityrosinase activity of samples was performed by the dopachrome method with a 96-well microplate reader [31]. 1/15 M potassium phosphate buffer (pH 6.8) containing 5 % DMSO were used to prepare in 7 different concentrations of methanol extract (1000, 750, 500, 250, 100, 10, 1 µg/mL) and tyrosinase enzyme solution (46 U/mL). The extracts and the enzyme added to the microplate and incubated for 10 minutes at 25 °C. After mixing with 2.5 mM L-Dopa substrate solution, incubated again at 25 °C for 20 minutes. Then, the absorbance was measured at 475 nm.

IC_{50} values of extracts for enzyme inhibitory assays were calculated using GraphPad Prism V5.0 software based on the results from three independent experiments.

4.9. Statistical analysis

Bioactivity and chemical analyses were performed in triplicate and the averages and standard deviations were calculated using Excel (Office 2016). The statistical analysis of the results was conducted by analysis of variance (one-way ANOVA) using SPSS 25 software that was used to determine differences among means and the differences were considered as significant with $P<0.05$.

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