



Ajuga chamaecistus subsp. *scoparia* (Boiss.) Rech.f.: A new source of phytochemicals for antidiabetic, skin-care, and neuroprotective uses



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ABSTRACT

The genus *Ajuga* is used traditionally as food and medicine. The aim of this study was to investigate the antidiabetic, anti-Alzheimer's diseases, skin-protective, and antioxidant activities of essential oil and extracts of *Ajuga chamaecistus* subsp. *scoparia* (Boiss.) Rech.f., together with the identification of their chemical composition. The 1,1-diphenyl-2-picrylhydrazyl radical and 2,2-azino-bis (3-ethylbenzothiazolone-6-sulfonic acid) radical cation scavenging activity, metal chelating activity, reducing power, content of total bioactive compounds, general toxicity, and enzyme inhibitory potential of essential oil, ethanol extract, and water decoction of the plant were evaluated. Phenolics profile of the extracts was analyzed using the reverse-phase high-performance liquid chromatography with diode array detector and the chemical composition of the essential oil was determined using the gas chromatography and gas chromatography–mass spectroscopy techniques. The essential oil could significantly inhibit the activity of α -glucosidase (4.3 mmol Acarbose equivalents/g), α -amylase (2.8 mmol Acarbose equivalents/g), acetylcholinesterase (1.96 mg gallic acid equivalents/g), butyrylcholinesterase (2.2 mg gallic acid equivalents/g), and tyrosinase (36 mg kojic acid equivalents/g) while ethanol and water extracts showed moderate enzyme inhibitory activity. Furthermore, essential oil had the strongest antioxidant capacity in the 1,1-diphenyl-2-picrylhydrazyl scavenging, 2,2-azino-bis (3-ethylbenzothiazolone-6-sulfonic acid) scavenging, cupric reducing antioxidant capacity, ferric reducing antioxidant power, and metal chelating assays. Spathulenol (18.0%), thymol (15.1%), octen-3-ol (14.3%), and linalool oxide (11.2%) were identified as major constituents in the essential oil. Quantitative high-performance liquid chromatography analysis indicated that *p*-coumaric acid, gallic acid, and ferulic acid are the main phenolic compounds in this plant. The results suggested that *Ajuga chamaecistus* could be useful for several applications as functional foods, cosmetics, and pharmaceuticals.

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1. Introduction

Ajuga L. is a member of Lamiaceae family comprising about 300 taxa (including subspecies and varieties) distributed in Asia, Africa, Australia, North America, and Europe (Atay et al., 2016; Israili and Lyoussi, 2009). This genus is represented by 6 species in Iran (Hassanzadeh et al., 2011). Some *Ajuga* species are used in folk medicine for the treatment of analgesia, diabetes, inflammation, malaria, high blood pressure, toothache, fever, and as anthelmintic,

antifungal, antifebrile, antitumor, antimicrobial, wound healing, and diuretic agents (Cocquyt et al., 2011; Eddouks et al., 2007; Israili and Lyoussi, 2009). Also, several species belonging to the genus *Ajuga* are traditionally used in Iran for management of joint pains, jaundice, and gout (Khanavi et al., 2014). Many studies have been carried out on *Ajuga* species indicating pharmacological activities such as antimalarial (Atay et al., 2016; Kuria et al., 2001), hypoglycemic (El Hilaly and Lyoussi, 2002), anti-inflammatory (Gautam et al., 2011), anabolic, anti-arthritis, analgesic, antipyretic, antibacterial, hepatoprotective, antifungal, cardiotonic, antioxidant (Israili and Lyoussi, 2009), and pain killer properties (Ono et al., 2008). Also, *Ajuga* species have been shown to have insecticidal activities. This property is due to clerodane diterpenoids and phytoecdys-

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teroids compounds which have insect antifeedant and moulting hormone activities (Camps and Coll, 1993). Phytochemical studies on this genus led to the isolation of many bioactive compounds such as diterpenoids (Coll and Tandrón, 2008), strolls, triterpenoids (Ni et al., 2015), iridoids, phenylethanoid glycosides (Akbar et al., 2003), anthocyanins (Terahara et al., 2001), and phytoecdysteroids (Castro et al., 2008). Essential oils of *Ajuga* species mainly are rich in α -pinene, β -pinene, linalool, germacrene D, and β -caryophyllene (Baser et al., 2001; Delazar et al., 2012).

Ajuga chamaecistus contains several subspecies, including *Ajuga chamaecistus* subsp. *scoparia* (Boiss.) Rech.f. (Mozaffarian, 1996), which is endemic to Iranian flora. *Ajuga chamaecistus* is a valuable forage for animal feeding in Iran. Although some subspecies of this plant have been studied for their essential oil composition, cytotoxicity, antinociceptive, and antimicrobial activities (Khanavi et al., 2014; Mohammadhosseini et al., 2011; Moshefi et al., 2014; Sadati et al., 2012), there is no information about the phytochemical and functional properties of *A. chamaecistus* subsp. *scoparia* (Boiss.) Rech.f. in the literature. So, at the present work, the chemical composition of the essential oil, ethanolic extract, and water decoction of this plant were examined. Also, biological activities of *A. chamaecistus* such as antioxidant, reducing power, metal chelating, antidiabetic, skin-protective, and anti-Alzheimer's disease properties were evaluated.

2. Materials and methods

2.1. Plant material

The aerial parts of *A. chamaecistus* subsp. *scoparia* (Boiss.) Rech.f. were collected in May 2015 at their full flowering stage from Taleqan, Alborz province, Iran. The plant was identified by Mr. Shahram Bahadori, taxonomist at the herbarium of Urmia University of Medical Sciences, Urmia, Iran. A voucher specimen was deposited for the plant (USPH-1120).

2.2. Preparation of extracts

The powdered aerial parts of *A. chamaecistus* (50 g) were extracted using ethanol (500 mL) by shaking at room temperature in 3 days. The solvent of the extract was removed using rotary evaporator at 40 °C to afford crude ethanol (6.5 g) extract. For preparation of the aqueous extract, 50 g of the plant material was suspended in distilled water (500 mL). The mixture was boiled for 10 min and the decoction was centrifuged and filtered using Whatman filter paper. Afterward, the obtained solution was lyophilized.

2.3. Isolation of essential oil

The essential oil was isolated by hydrodistillation using a Clevenger type apparatus in 3 h. The obtained oil was dried using anhydrous sodium sulfate and stored at 4 °C in dark until analysis.

2.4. GC and GC/MS analysis

The essential oil analysis was performed using an Agilent instrument (model 7890A) equipped with a flame ionization detector (FID) and a DB-5 capillary column (30 m \times 0.32 mm i.d., film thickness 0.25 μ m). Helium was used as carrier gas (1.1 mL/min, in constant linear velocity mode). Temperatures of the injector and detector were set at 240 and 250 °C, respectively. The oven temperature was programmed from 35 to 180 °C at the rate of 4 °C/min, then raised to 250 °C at 17 °C/min and held at this temperature for 10 min. The injection volume was 1 μ L in split mode (1:100). Essential oil was diluted in *n*-hexane (1/100, v/v) and 0.5 μ L was injected manually. The GC–MS analysis was carried out using a Thermoquest

Finnigan instrument (model Trace GC, Trace MS) equipped with fused silica capillary DB-5 column. The temperature program of column was the same as GC–FID. Spectra were obtained in the electron ionization (EI) mode. Identification of individual compounds was carried out by calculating of their retention indices (RI) using *n*-alkanes (C₆–C₂₄) under the same GC condition. The constituents of the oil were identified by comparison of their retention indices and mass spectra with those published in the literature and by using NIST, Wiley and Adams Mass Spectral libraries.

2.5. Phenolic compounds profiling by RP–HPLC–DAD

Phenolic metabolites were analyzed using RP–HPLC–DAD (Shimadzu Scientific Instruments, Kyoto, Japan). Separation procedure was carried out at 30 °C on Eclipse XDB C-18 reversed-phase column (250 mm \times 4.6 mm length, 5 μ m particle size, Agilent, Santa Clara, CA, USA). The eluates were detected at 278 nm. The phenolic compounds of the ethanolic and water extracts were determined using a previously modified method (Sarikurkcu et al., 2014). Twenty three standard phenolic compounds were used for the analysis including gallic acid, protocatechuic acid, catechin, *p*-hydroxybenzoic acid, chlorogenic acid, caffeic acid, epicatechin, syringic acid, vanillin, *p*-coumaric acid, ferulic acid, sinapinic acid, benzoic acid, *o*-coumaric acid, rutin, hesperidin, rosmarinic acid, eriodictiol, cinnamic acid, quercetin, luteolin, kamferol, and apigenin. Retention times, UV spectra, and comparison with commercial standard compounds, were used for the characterization of phenolic compounds. The results were expressed as μ g per gram of dry extracts using external calibration curves.

2.6. Quantitative analysis of bioactive metabolites

The total phenolic contents were determined using Folin–Ciocalteu method with some modifications (Slinkard and Singleton, 1977). The results were expressed as equivalents of gallic acid (GAEs/g dry extract). The total flavonoid contents were determined using a previously published method (Zengin et al., 2015) and the results were expressed as equivalents of rutin (REs/g dry extract).

2.7. Total antioxidant activity

Total antioxidant activity of the essential oil and extracts of *A. chamaecistus* was determined using phosphomolybdenum method (Zengin et al., 2014) with some modifications.

2.8. Radical scavenging activity

Radical scavenging activity of *A. chamaecistus* was evaluated using 2,2-azino-bis (3-ethylbenzothiazolone-6-sulfonic acid) radical cation (ABTS) (Zengin et al., 2014) and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Sarikurkcu, 2013) scavenging methods.

2.9. Reducing power

The cupric ion reducing activity (CUPRAC) of the essential oil and extracts were determined according to a established method (Apak et al., 2006). Also, the ferric reducing antioxidant power (FRAP) assay was carried out as previously described (Zengin et al., 2015) with slight modifications. The results of both the CUPRAC and FRAP assays were expressed as equivalents of trolox (mg TE/g sample).

2.10. Metal chelating activity on ferrous ions

The metal chelating activity of *A. chamaecistus* on ferrous ions was determined using a previously described method (Aktumsek

Table 1
Chemical composition of the essential oil of *Ajuga chamaecistus* subsp. *scoparia* (Boiss.) Rech.f.

No.	Compound	Percentage	RT	RI	Identification method
1	Octen-3-ol	14.3	5.15	965	RI, MS
2	1,8-Cineol	6.9	6.25	1018	RI, MS
3	Linalool oxide	11.2	7.24	1062	RI, MS
4	Dihydromyrcenol	9.5	7.62	1079	RI, MS
5	Linalool	8.8	7.82	1090	RI, MS
6	Thymol	15.1	12.8	1282	RI, MS
7	Carvacrol	10.8	13.04	1293	RI, MS
8	Spathulenol	18.0	19.87	1564	RI, MS
Alkanol		14.3			
Oxygenated monoterpenes		62.3			
Oxygenated sesquiterpenes		18.0			
Total identified		94.6			

et al., 2013). The metal chelating activity was expressed as equivalents of EDTA (mg EDTAE/g sample).

2.11. Enzyme inhibition assays

Cholinesterase inhibitory activities, including acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were evaluated using Ellman method and the results were expressed as equivalents of galantamine (mg GALAE/g sample). Determination of α -amylase, α -glucosidase, and tyrosinase inhibitory activities were carried out using a previously described method (Zengin, 2016). The results of inhibitory activities were expressed as equivalents of standard drugs (acarbose for α -amylase and α -glucosidase inhibition assays as mmol ACAEs/g sample and kojic acid for tyrosinase inhibition assay as mg KAEs/g sample).

2.12. Acute toxicity test

The acute toxicity of the plant samples was evaluated using Brine shrimp lethality assay (Bahadori et al., 2015). *Artemia salina* larva were used as the test organism. Cysts of *A. salina* were hatched in artificial sea water (3.8% w/v salt) in 48 h with incubation at 28 °C. The extracts and EO samples were dissolved in DMSO. Ten larvae were counted and added to the two fold serially diluted test solutions (concentration range was 31.2–1000 μ g/mL). An untreated control (DMSO) without sample and a positive control (podophyllo-toxin) were also assayed. Test tubes were incubated for 48 h at 28 °C and then the number of immobile larvae was counted and the mor-

tality percentage was calculated. LC₅₀ values were calculated and expressed as average value of three independent experiments \pm SD.

2.13. Statistical analysis

All the measurements were carried out in triplicates. The results were expressed as mean \pm standard deviation (SD). The differences between the different samples were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with $\alpha=0.05$. These analysis were performed by SPSS v. 16.0 software.

3. Results and discussion

3.1. Essential oil composition

The yield of essential oil obtained from aerial parts of *A. chamaecistus* subsp. *scoparia* (Boiss.) Rech.f. was 0.3% v/w. Chemical constituents of the EO are shown in Table 1 and their structural formula are presented in Fig. 1. The EO was characterized by the presence of 8 volatile compounds, representing 94.6% of the total oil. Spathulenol (18.0%), thymol (15.1%), octen-3-ol (14.3%), and linalool oxide (11.2%) were identified as major components. All of the identified compounds are oxygenated volatiles. Oxygenated monoterpenes were the most abundant class of identified compounds. The essential oil composition of *A. chamaecistus* subsp. *scoparia* (Boiss.) Rech.f. has not been investigated up to now. The results show that the EO of *A. chamaecistus* contains pharmacologically and commercially important metabolites. A literature review showed that other varieties and subspecies of *A. chamaecistus* have been studied for their EO composition. Main compounds of *A. chamaecistus* Ging. subsp. *tomentella* Rech. f. were reported as thymol (34.4%), *exo*-fenchol (15.5%), β -pinene (8.26%), and octen-3-ol (5.9%) (Ardekani et al., 2010). In another study, *p*-cymene (34.5%), β -pinene (18%), α -phellandrene (17.8%), and α -pinene (15.2%) were determined as the major constituents of *A. chamaecistus* Ging. (Mohammadhosseini et al., 2011). Observed differences in the oil yields and chemical compositions between different varieties could be associated with their different climatic and geographical conditions, and also genetic factors.

3.2. Phenolic composition

Phenolic compounds are known for their beneficial effects on human health. Polyphenols have been shown to have important

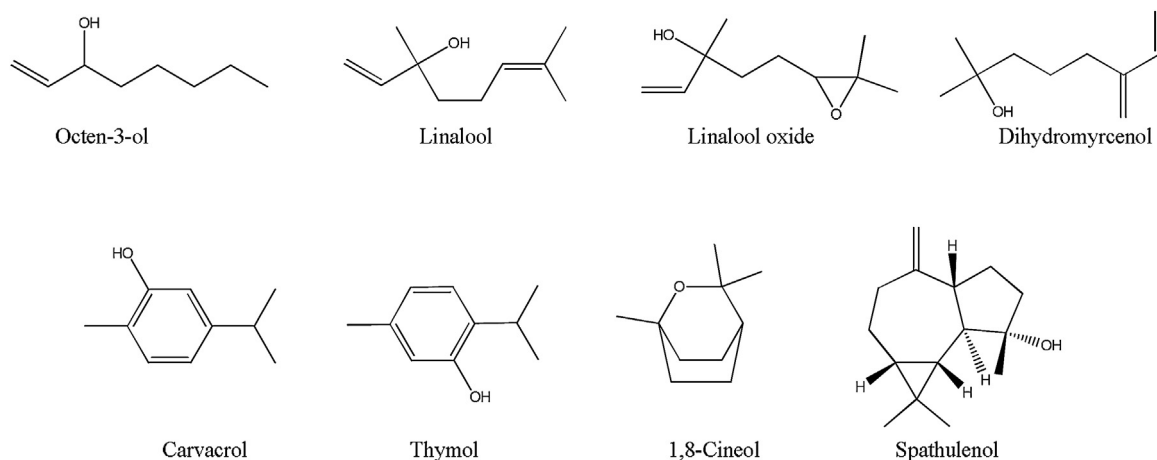


Fig. 1. Volatile compounds identified in the essential oil of *Ajuga chamaecistus*.

Table 2
Quantitative results for determination of phenolic components in the water and ethanol extracts of *A. chamaecistus*.

No.	Phenolic compounds	Extracts		Analytical characteristics			
		Water	Ethanol	Linear range (ppm)	r ²	LOD (ppm)	LOQ (ppm)
1	Gallic acid	95 ± 4	nd	0.20–25.0	0.9993	0.075	0.227
2	Protocatechuic acid	nd ^a	nd	0.20–25.0	0.9991	0.086	0.260
3	(+)-Catechin	nd	nd	0.90–113	0.9988	0.172	0.522
4	<i>p</i> -Hydroxybenzoic acid	nd	22 ± 0.3	0.20–25.0	0.9994	0.007	0.020
5	Chlorogenic acid	10 ± 1	nd	0.35–45.0	0.9988	0.080	0.241
6	Caffeic acid	16 ± 1	32 ± 2	0.16–21.0	0.9993	0.054	0.162
7	(–)-Epicatechin	nd	nd	0.50–66.0	0.9990	0.170	0.514
8	Syringic acid	nd	6 ± 0.1	0.05–12.0	0.9995	0.030	0.090
9	Vanillin	nd	15 ± 0.1	0.08–10.0	0.9995	0.020	0.060
10	<i>p</i> -coumaric acid	692 ± 42	574 ± 18	0.04–6.0	0.9996	0.066	0.199
11	Ferulic acid	nd	106 ± 3	0.12–17.0	0.9993	0.004	0.011
12	Sinapic acid	37 ± 1	47 ± 1	0.12–17.0	0.9993	0.017	0.053
13	Benzoic acid	nd	nd	0.85–55.0	0.9998	0.111	0.335
14	<i>o</i> -Coumaric acid	11 ± 0.1	9 ± 0.1	0.24–32.0	0.9988	0.023	0.069
15	Rutin	30 ± 1	44 ± 1	0.40–56.0	0.9989	1.113	3.373
16	Hesperidin	26 ± 0.6	20 ± 0.6	0.43–55.0	0.9992	1.080	3.280
17	Rosmarinic acid	nd	nd	0.02–7.0	0.9998	0.148	0.447
18	Eriodictyol	nd	nd	0.33–21.0	0.9998	0.140	0.410
19	<i>trans</i> -Cinnamic acid	5 ± 0.5	3 ± 0.1	0.02–7.0	0.9998	0.148	0.447
20	Quercetin	nd	nd	0.40–55.0	0.9999	0.013	0.040
21	Luteolin	30 ± 3	28 ± 3	0.13–17.0	0.9999	0.020	0.060
22	Kaempferol	nd	nd	0.05–15.0	0.9996	0.021	0.062
23	Apigenin	24 ± 1	42 ± 1	0.17–11.0	0.9997	0.034	0.104

^a Not detected.

role in the treatment of several disorders such as atherosclerosis, tumors, diabetes, Parkinson's disease, and Alzheimer's disease. The identification and quantification analysis of phenolic compounds of the water decoction and ethanolic extract of *A. chamaecistus* were carried out using a RP-HPLC–DAD system. Analytical characteristics like quantification linearity, limit of detection, and limit of quantification were performed to validate the used HPLC method. These data together with phenolics profile of the extracts are listed in Table 2. Twenty three standard phenolic compounds were used in analysis and 11 and 13 phenolics were detected in the water decoction and ethanolic extract of *A. chamaecistus*, respectively (Fig. 2). *p*-Coumaric acid was the most abundant phenolic component in both of the extracts, followed by gallic acid in water extract and ferulic acid in ethanolic extract. Detection and quantification of phenolic compounds of *A. chamaecistus* extracts have not been previously reported in the literature. *n*-Butanolic fraction of the 80% methanolic extract from aerial parts of *Ajuga chamaecistus* ssp. *tomentella* has been phytochemically analyzed and led to the isolation and identification of 3 phenolic compounds, including lavandulifolioside, leonoside, and martynoside. In another study, phenolic components such as acteoside, chrysoeriol 7-*o*-glucopyranoside, and apigenin 7-*o*-rhamnopyranoside were purified from the methanolic extract of the aerial parts of *A. chamaepitys* (L.) Schreb (Delazar et al., 2012). According to the obtained results at the present and previous studies, it seems that *Ajuga* species are rich in bioactive phenolic metabolites and could be the subject of phytochemical studies for the isolation of polyphenolics as natural antioxidants and functional foods.

3.3. Quantification of bioactive compounds

Total phenolics (TPC) and flavonoid contents (TFC) of the water and ethanol extracts are presented in Table 3. The ethanol extract contained higher TPC (20.3 mg GAEs/g extract) and TFC (11.6 mg REs/g extract) than water decoction (18.9 mg GAEs/g and 9.1 mg REs/g, respectively). In comparison with similar works, total phenolics and flavonoid contents of this subspecies of *Ajuga chamaecistus* are higher than the ethanol extract of *Ajuga iva* (16.5 mg GAEs/g and 1.1 mg QEs/g, respectively) (Makni et al., 2013). Total phenolics

and flavonoid contents in this work are lower than those reported for the ethyl acetate extract of *A. chamaepitys* (57.0 mg GAEs/g and 91.7 mg REs/g, respectively) (Jakovljević et al., 2015).

3.4. Total antioxidant activity

Total antioxidant activities of the extracts and EO were determined using the phosphomolybdenum method (Table 3). The EO of *A. chamaecistus* exhibited the strongest total antioxidant activity (8.8 mmol TE/g oil). The phosphomolybdenum activities of tested extracts showed good correlation with their TPC and TFC. In this regard, total antioxidant capacities obtained from the phosphomolybdenum assay could be described by the presence of flavonoids and phenolic compounds.

3.5. Radical scavenging activity

During the last decades, it has been demonstrated that oxidative stress plays a vital role in a wide range of human diseases. Oxidative stress is defined as the imbalance between the generation of oxidants like reactive oxygen and nitrogen species (ROS/RNS) and endogenous antioxidants defenses. However, the antioxidant compounds are characterized by the ability of reaction with free radicals and consequently, slowing and prevention of oxidation process. The search for new antioxidant agents, especially from natural resources has given rise because most common synthetic antioxidants such as BHT and BHA are suspected to be harmful to human health. Hydrogen atom transfer, single electron transfer, and metal chelating are three main mechanisms which the antioxidants counteract the oxidative process.

The DPPH and ABTS radical scavenging assays are reliable and commonly used methods for evaluation of the radical scavenging activity. These measurements are based on the reduction of radical species by electron-transferring or hydrogen-donating radical scavengers. As shown in Table 4, the EO showed the highest DPPH (6.3 mg TE/g oil) and ABTS (191 mg TE/g oil) radical scavenging activities. Antiradical activity of *A. chamaecistus* was investigated for the first time. In comparison with reported works, *A. chamaecistus* has promising antiradical capacity. For example, different

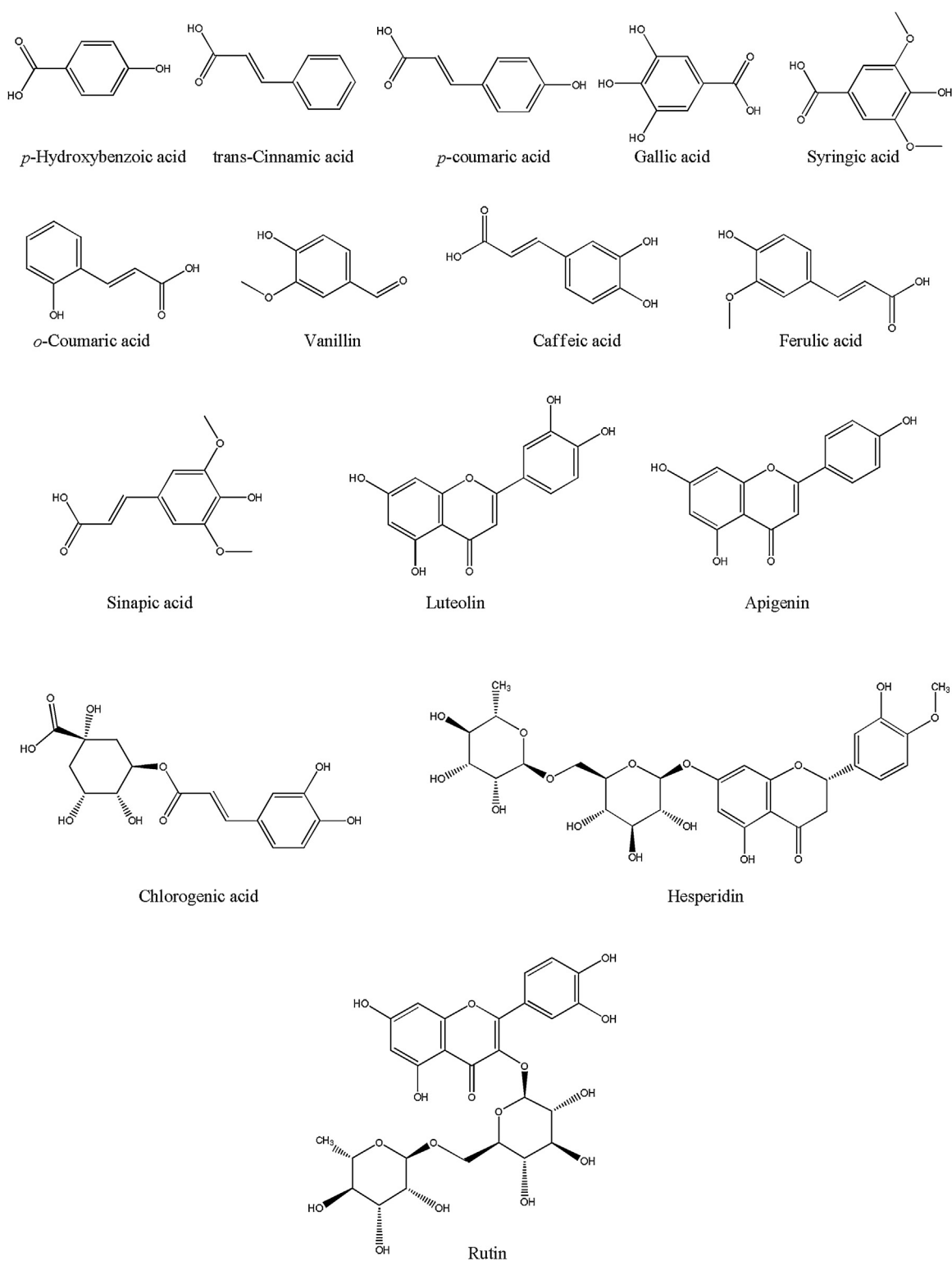


Fig. 2. Phenolic compounds identified in the extracts of *Ajuga chamaecistus*.

extracts of *A. chamaepitys* have been screened for their antioxidant activities by the DPPH assay (Jakovljević et al., 2015). The acetone extract indicated the highest antioxidant activity (SC_{50} value = 330.52 $\mu\text{g/mL}$). Essential oil of this plant showed IC_{50} value of 7146 $\mu\text{g/mL}$ (Mitić et al., 2011).

3.6. Reducing power

Antioxidant activity and reducing power are highly related. Reducing agents have shown to apply their antioxidant action with breaking free radical chain reactions by donating a hydro-

Table 3
Total bioactive compounds and total antioxidant activity of *A. chamaecistus*.

Samples	Total bioactive compounds		Total antioxidant activity
	Total phenolic (mg GAEs/g extract) ^a	Total flavonoid (mg REs/g extract) ^b	Phosphomolybdenum (mmol TEs/g oil or extract) ^c
Essential oil	–	–	8.80 ± 0.33 ^d
Water extract	18.94 ± 0.13	9.14 ± 0.09	0.56 ± 0.01
Ethanol extract	20.32 ± 0.39	11.61 ± 0.16	1.18 ± 0.04

^a GAEs: gallic acid equivalents.

^b REs: rutin equivalents.

^c TEs: trolox equivalents.

^d Values expressed are means ± SD of three parallel measurements.

Table 4
Radical scavenging activity of the essential oil and extracts of *A. chamaecistus*.

Samples	Radical scavenging activity	
	DPPH radical (mg TEs/g oil or extract) ^a	ABTS radical cation (mg TEs/g oil or extract) ^a
Essential oil	6.31 ± 0.83 ^b	191.01 ± 0.33
Water extract	20.23 ± 0.60	73.47 ± 1.04
Ethanol extract	22.69 ± 1.30	53.87 ± 2.11

^a TEs: trolox equivalents.

^b Values expressed are means ± SD of three parallel measurements.

gen atom and thus inhibition of oxidative damages. At the present work, reducing power of the EO and extracts of *A. chamaecistus* were investigated by their ability for transformation of Cu²⁺ to Cu⁺ (CUPRAC assay) and Fe³⁺ to Fe²⁺ (FRAP assay). The results are expressed as trolox equivalents per gram of oil or extract (TEs/g oil or extract) and are shown in Table 5. Similar to radical scavenging activity, the EO exhibited great reducing power activity in both CUPRAC (329 mg TEs/g oil) and FRAP (97 mg TEs/g oil) assays. This reducing power is higher than other essential oils, reported in the literature. For example, EOs of 2 subspecies of *Origanum vulgare* showed CUPRAC and FRAP activities as 46–222 and 17–133 mg TEs/g oil, respectively (Sarikurcu et al., 2015).

3.7. Metal chelating activity

Fe²⁺ ions have an important role as catalyst in the formation of hydroxyl radicals by the Fenton reaction. Accordingly, these ions could initiate lipid peroxidation which leads to health damages. Metal chelating is one of the most important mechanisms of the antioxidant action for neutralizing oxidative process. In this study, the ferrous ion chelating activity of *A. chamaecistus* was investigated via ferrozine assay (Table 5). The Fe²⁺ chelating potential of EO (17 mg EDTAEs/g oil) was about 3 fold greater than water decoction and ethanol extract (5.2 and 6.9 mg EDTAEs/g extract, respectively).

Forasmuch as hydrogen and electron donating compounds could exert antioxidant activities, it seems that phenolic compounds in essential oil and extracts of *A. chamaecistus* are responsible for its radical scavenging, reducing power, and metal

Table 5
Reducing power and metal chelating activity of the essential oil and extracts of *A. chamaecistus*.

Samples	Reducing power activity		Metal chelating activity
	CUPRAC (mg TEs/g sample) ^a	FRAP (mg TEs/g sample) ^a	Chelating effect (mg EDTAEs/g sample) ^b
Essential oil	329.64 ± 4.40 ^c	97.75 ± 1.72	17.01 ± 0.38
Water extract	59.57 ± 0.71	43.19 ± 1.04	5.25 ± 0.21
Ethanol extract	61.67 ± 2.68	39.41 ± 2.13	6.95 ± 0.99

^a TEs: trolox equivalents.

^b EDTAEs: EDTA equivalents.

^c Values expressed are means ± SD of three parallel measurements.

chelating properties. For example major constituents of essential oil in this study such as thymol, linalool, carvacrol, spathulenol, and 1,8-cineol are known as strong antioxidant agents (Mastelic et al., 2008). Also, phenolic acids and flavonoids are well-known for their strong antioxidant properties (Zhang and Tsao, 2016).

3.8. Enzyme inhibitory activity

Today, evaluation of enzyme inhibitory activity of natural products is considered as one of the most important strategies to find effective compounds for treatment of many illnesses such as Alzheimer's diseases (AD), inflammation, skin disorders, and diabetes mellitus (DM). For example, the α-glucosidase and α-amylase inhibitors are used for the treatment of metabolic disorders like DM. Also, acetylcholinesterase and butyrylcholinesterase are key enzymes in discovery of neuroprotective agents. In this regard, enzyme inhibitory activities of the EO, water decoction, and ethanol extract of *A. chamaecistus* were examined against cholinesterases, α-glucosidase, α-amylase, and tyrosinase *in vitro*. The results of enzyme inhibition assays are summarized in Table 6.

3.8.1. Anticholinesterase activity

Alzheimer's disease (AD) is a common neurodegenerative disorder in the elderly (Kaufmann et al., 2011). Inhibition of cholinesterases activity is the most used strategy for treatment of AD (Bahadori et al., 2016). In this work, the EO exhibited stronger anticholinesterase activity than the water and ethanol extracts. The water decoction and ethanol extract showed moderate inhibitory effects against cholinesterases (Table 6). Thymol as one of the main compounds in the EO is known for its anticholinesterase activity (Duke, 2007). 1,8-cineol has been reported as potent acetylcholinesterase inhibitor (Miyazawa et al., 1998). Also, there are several reports on strong cholinesterases inhibitory activity of carvacrol (Can Baser, 2008; Jukic et al., 2007). Carvacrol is one of the main abundant volatile compounds in *A. chamaecistus*. So, this observation may suggest that oxygenated monoterpene compounds are the major responsible agents for anti-Alzheimer's disease activities of essential oil components. Also, phenolic acids could be considered as anticholinesterase compounds in the extracts. There are several evidences for the strong

Table 6
Enzyme inhibitory activity of the extracts and essential oil of *A. chamaecistus*.

Samples	Neuroprotective effects		Anti-diabetic effects		Skin-care effects
	AChE Inhibition (mg GALAEs/g sample) ^a	BChE Inhibition (mg GALAEs/g sample) ^a	α -amylase inhibition (mmol ACEs/g sample) ^b	α -glucosidase inhibition (mmol ACEs/g sample) ^b	Tyrosinase inhibition (mg KAEs/g sample) ^c
Essential oil	1.96 ± 0.11 ^d	2.24 ± 0.01	2.81 ± 0.01	4.34 ± 0.01	36.07 ± 0.10
Water extract	0.15 ± 0.04	na ^e	0.25 ± 0.01	1.48 ± 0.19	na
Ethanol extract	1.80 ± 0.02	1.18 ± 0.40	0.56 ± 0.01	1.46 ± 0.17	na

^a GALAEs: galantamine equivalents. na: not active.

^b ACEs: acarbose equivalents.

^c KAEs: kojic acid equivalents.

^d Values expressed are means ± SD of three parallel measurements.

^e Not active.

anticholinesterase activity of ferulic acid and *p*-coumaric acid in the literature (Szwajgier and Borowiec, 2012). It has been observed that ferulic acid is a competitive inhibitor of acetylcholinesterase (Kumar et al., 2009).

3.8.2. Antidiabetic activity

There are several types of natural and synthetic compounds with α -glucosidase and α -amylase inhibitory properties, including flavonoids, terpenoids, benzimidazoles, and alkaloids (Dinparast et al., 2016). These are key enzymes involved in the breakdown of carbohydrates and intestinal absorption, respectively (Ceylan et al., 2015). Acarbose, metformin, and miglitol are commercial enzyme inhibitors for the clinical treatment of Type 2 diabetes. However, these drugs have several side effects. Hence, there is an increasing interest to find out alternative natural sources as α -amylase and α -glucosidase inhibitors with comparatively more activity and less side effects. There are no scientific information on the antidiabetic potential of *A. chamaecistus*. Therefore, we aimed to investigate the α -amylase and α -glucosidase inhibitory effects of its extracts and essential oil. In this work, the EO showed the highest α -glucosidase and α -amylase inhibitory activity (Table 6). It has been indicated that thymol has strong effects on treatment of type 2 diabetes *in vivo* (Saravanan and Pari, 2015, 2016). In a recently published study, the methanol extract of *A. chamaecistus* has been evaluated for its antidiabetic activity and exhibited higher α -glucosidase and lower α -amylase inhibitory activity in comparison with our results (Eskandani et al., 2016). The ethanol extract showed equal or higher antidiabetic activity than the water extract. Phenolic compounds from medicinal plants have been reported to be effective α -glucosidase and α -amylase inhibitors (Arun et al., 2015; Asghari et al., 2015; Emmanouil Apostolidis and Kwon, 2006; Moradi-Afrapoli et al., 2012). Accordingly, enzyme inhibitory potential of studied extracts could be due to their phenolic phytochemicals. Our finding are in agreement with traditional uses of *Ajuga* species in treatment of diabetes (El Hilaly and Lyoussi, 2002).

3.8.3. Tyrosinase inhibitory activity

Tyrosinase is a copper-containing enzyme that catalyzes melanin biosynthesis. Melanin plays an important role in protection of skin from ionizing radiations like UV. Common tyrosinase inhibitors such as kojic acid and arbutin have some negative effects on human health. So, discovery of safer natural inhibitors is warranted. At the present study, tyrosinase inhibitory activity of *A. chamaecistus* was evaluated *in vitro*. The water and ethanolic extracts were inactive against tyrosinase in tested concentrations but the essential oil indicated excellent tyrosinase inhibitory activity. With the exception of linalool, thymol, and carvacrol, none of the main constituents of *A. chamaecistus* oil have been previously reported to exert antityrosinase activity. There are some evidences for strong tyrosinase inhibitory activity of linalool rich essential oils in the literature (Sarikurkcu et al., 2015; Souza et al., 2012). Also,

thymol and carvacrol have been reported to exert antityrosinase effects (Loizzo et al., 2012).

According to the results, *Ajuga chamaecistus* subsp. *scoparia* (Boiss.) Rech.f. has a great potential for pharmaceutical uses to treat several disorders like AD, DM, and skin disorders. To our knowledge, this is the first study on the enzyme inhibitory activity of this plant.

3.9. *Artemia salina* larvae toxicity

None of the extracts and essential oil exhibited toxicity in applied concentration range (31.2–1000 μ g/mL) against *A. salina*. So, LC₅₀ values were not reached, while podophyllotoxin as a standard drug showed the LC₅₀ of 35.2 μ g/mL. Generally, natural agents with the LD₅₀ values more than 1000 μ g/mL are regarded as non-toxic (Valizadeh et al., 2015).

4. Conclusions

The results indicate that essential oil and extracts of *Ajuga chamaecistus* subsp. *scoparia* (Boiss.) Rech.f. could inhibit key enzymes involved in diabetes, Alzheimer's, and skin disorders. They can also act as radical scavengers. Moreover, their high reducing power and metal chelating activity confirmed the ability of these natural agents as potent antioxidants. The GC and GC/MS analysis of essential oil and HPLC analysis of the extracts showed the presence of important biologically active compounds in this plant. According to the results, *Ajuga chamaecistus* subsp. *scoparia* (Boiss.) Rech.f. could be considered as a valuable source of natural products for possible uses as dietary supplements, cosmetics, and pharmaceuticals. Moreover, the EO of *A. chamaecistus* could be used in the food industries for its flavoring and preservative properties. Due to the lack of information in the literature, this study should be extended to individual constituents of the essential oil and the extracts of *A. chamaecistus* to explore their activity order and mechanism of action in enzyme inhibitory assays in the near future.

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References

- Akbay, P., Çaltı, İ., Heilmann, J., Sticher, O., 2003. Ionone, iridoid and phenylethanoid glycosides from *Ajuga salicifolia*. Z. Naturforsch. C 58, 177–180.
- Aktumsek, A., Zengin, G., Guler, G.O., Cakmak, Y.S., Duran, A., 2013. Antioxidant potentials and anticholinesterase activities of methanolic and aqueous extracts of three endemic *Centaurea* L. species. Food Chem. Toxicol. 55, 290–296.
- Apak, R., Güçlü, K., Özyürek, M., Esin Karademir, S., Erçağ, E., 2006. The cupric ion reducing antioxidant capacity and polyphenolic content of some herbal teas. Int. J. Food Sci. Nutr. 57, 292–304.

- Ardekani, M.S., Khanavi, M., Taheri, P., Samadi, N., Safaripour, E., Salimpour, F., 2010. The essential oil composition of *Ajuga chamaecistus* Ging: subsp. *tomentella* Rech. f. J. Essent. Oil Bear Pl. 13, 45–51.
- Arun, K., Chandran, J., Dhanya, R., Krishna, P., Jayamurthy, P., Nisha, P., 2015. A comparative evaluation of antioxidant and antidiabetic potential of peel from young and matured potato. Food Biosci. 9, 36–46.
- Asghari, B., Salehi, P., Sonboli, A., Ebrahimi, S.N., 2015. Flavonoids from *Salvia chloroleuca* with α -amylase and α -glucosidase inhibitory effect. Iran. J. Pharm. Res.: IJPR 14, 609.
- Atay, I., Kirmizibekmez, H., Kaiser, M., Akaydin, G., Yesilada, E., Tasdemir, D., 2016. Evaluation of in vitro antiprotozoal activity of *Ajuga laxmannii* and its secondary metabolites. Pharm. Biol., 1–7.
- Bahadori, M.B., Valizadeh, H., Asghari, B., Dinparast, L., Farimani, M.M., Bahadori, S., 2015. Chemical composition and antimicrobial, cytotoxicity, antioxidant and enzyme inhibitory activities of *Salvia spinosa* L. J. Funct. Foods 18, 727–736.
- Bahadori, M.B., Dinparast, L., Valizadeh, H., Farimani, M.M., Ebrahimi, S.N., 2016. Bioactive constituents from roots of *Salvia syriaca* L.: acetylcholinesterase inhibitory activity and molecular docking studies. S. Afr. J. Bot. 106, 1–4.
- Baser, K., Kurkuoglu, M., Erdemgil, F., 2001. The essential oil of *Ajuga bombycina* from Turkey. Chem. Nat. Compd. 37, 242–244.
- Camps, F., Coll, J., 1993. Insect allelochemicals from *Ajuga* plants. Phytochemistry 32, 1361–1370.
- Can Baser, K., 2008. Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils. Curr. Pharm. Des. 14, 3106–3119.
- Castro, A., Coll, J., Tandrón, Y.A., Pant, A.K., Mathela, C.S., 2008. Phytoecdysteroids from *Ajuga macrosperma* var. *breviflora* roots. J. Nat. Prod. 71, 1294–1296.
- Ceylan, R., Zengin, G., Uysal, S., Ilhan, V., Aktumsek, A., Kandemir, A., Anwar, F., 2015. GC–MS analysis and in vitro antioxidant and enzyme inhibitory activities of essential oil from aerial parts of endemic *Thymus spathulifolius* Hausskn. et Velen. J. Enzyme Inhib. Med. Chem., 1–8.
- Cocquyt, K., Cos, P., Herdewijn, P., Maes, L., Van den Steen, P., Laekeman, G., 2011. *Ajuga remota* Benth.: from ethnopharmacology to phytomedicine perspective in the treatment of malaria. Phytomedicine 18, 1229–1237.
- Coll, J., Tandrón, Y.A., 2008. neo-Clerodane diterpenoids from *Ajuga*: structural elucidation and biological activity. Phytochem. Rev. 7, 25–49.
- Delazar, A., Delnavazi, M.-R., Yassa, N., Parkhideh, S., Delazar, N., Nahar, L., Sarker, S.D., 2012. Essential oil composition and isolation of free radical-scavenging phenolic glycosides from the aerial parts of *Ajuga chamaepitys* growing in Iran. Rev. Bras. Farmacogn. 22, 399–305.
- Dinparast, L., Valizadeh, H., Bahadori, M.B., Soltani, S., Asghari, B., Rashidi, M.-R., 2016. Design, synthesis, α -glucosidase inhibitory activity, molecular docking and QSAR studies of benzimidazole derivatives. J. Mol. Struct. 1114, 84–94.
- Duke, J.A., 2007. The garden pharmacy: the evidence for lemon balm. Alternat. Complement. Ther. 13, 173–177.
- Eddouks, M., Ouahidi, M., Farid, O., Moufid, A., Khalidi, A., Lemhadri, A., 2007. The use of medicinal plants in the treatment of diabetes in Morocco. Phytotherapie 5, 194–203.
- El Hilaly, J., Lyoussi, B., 2002. Hypoglycaemic effect of the lyophilised aqueous extract of *Ajuga iva* in normal and streptozotocin diabetic rats. J. Ethnopharmacol. 80, 109–113.
- Emmanouil Apostolidis, M., Kwon, Y.-I., 2006. Potential of cranberry-based herbal synergies for diabetes and hypertension management. Asia. Pac. J. Clin. Nutr. 15, 433–441.
- Eskandani, M., Bahadori, M.B., Zengin, G., Dinparast, L., Bahadori, S., 2016. Novel natural agents from Lamiaceae family: an evaluation on toxicity and enzyme inhibitory potential linked to diabetes mellitus. Curr. Bioact. Compd. 12, 34–38.
- Gautam, R., Jachak, S.M., Saklani, A., 2011. Anti-inflammatory effect of *Ajuga bracteosa* Wall Ex Benth: mediated through cyclooxygenase (COX) inhibition. J. Ethnopharmacol. 133, 928–930.
- Hassanzadeh, M., Emami, S., Asili, J., Najaran, Z.T., 2011. Review of the essential oil composition of Iranian *Lamiaceae*. J. Essent. Oil Res. 23, 35–74.
- Israili, Z.H., Lyoussi, B., 2009. Ethnopharmacology of the plants of genus *Ajuga*. Pak. J. Pharm. Sci. 22, 425–462.
- Jakovljević, D.Z., Vasić, S.M., Stanković, M.S., Čomić, L.R., Topuzović, M.D., 2015. Secondary metabolite content and in vitro biological effects of *Ajuga chamaepitys* (L.) Schreb. subsp. *chamaepitys*. Arch. Biol. Sci., 95.
- Jukic, M., Politeo, O., Maksimovic, M., Milos, M., Milos, M., 2007. In vitro acetylcholinesterase inhibitory properties of thymol, carvacrol and their derivatives thymoquinone and thymohydroquinone. Phytother. Res. 21, 259–261.
- Kaufmann, D., Dogra, A.K., Wink, M., 2011. Myrtenal inhibits acetylcholinesterase, a known Alzheimer target. J. Pharm. Pharmacol. 63, 1368–1371.
- Khanavi, M., Davoodipour, A.M., Sadati, S.N., Ardekani, M.R.S., Sharifzadeh, M., 2014. Antinociceptive effect of some extracts from *Ajuga chamaecistus* Ging ssp. *tomentella* (Boiss.) Rech. f. aerial parts. Daru J. Pharm. Sci. 22, 1.
- Kumar, P., Singh, V., Singh, D., 2009. Kinetics of enzyme inhibition by active molluscicidal agents ferulic acid, umbelliferone, eugenol and limonene in the nervous tissue of snail *Lymnaea acuminata*. Phytother. Res. 23, 172–177.
- Kuria, K., De Coster, S., Muriuki, G., Masengo, W., Kibwage, I., Hoogmartens, J., Laekeman, G., 2001. Antimalarial activity of *Ajuga remota* Benth (Labiatae) and *Caesalpinia volkensii* Harms (Caesalpinaceae): in vitro confirmation of ethnopharmacological use. J. Ethnopharmacol. 74, 141–148.
- Loizzo, M., Tundis, R., Menichini, F., 2012. Natural and synthetic tyrosinase inhibitors as antibrowning agents: an update. Compr. Rev. Food Sci. Food Saf. 11, 378–398.
- Makni, M., Haddar, A., Kriaa, W., Zeghal, N., 2013. Antioxidant, free radical scavenging, and antimicrobial activities of *Ajuga iva* leaf extracts. Int. J. Food Prop. 16, 756–765.
- Mastelic, J., Jerkovic, I., Blažević, M., Poljak-Blaži, S., Borovčić, I., Ivančić-Bacić, N., Smrečki, V., Žarković, K., Brčić-Kostić, D., Vikić-Topić, M., 2008. Comparative study on the antioxidant and biological activities of carvacrol, thymol, and eugenol derivatives. J. Agric. Food Chem. 56, 3989–3996.
- Mitić, V.D., Stankov-Jovanović, V.P., Jovanović, O.P., Palić, I.R., Djordjević, A.S., Stojanović, G.S., 2011. Composition and antioxidant activity of hydrodistilled essential oil of Serbian *Ajuga chamaepitys* (L.) Schreber ssp. *chia* (Schreber) Arcangelii. J. Essent. Oil Res. 23, 70–74.
- Miyazawa, M., Watanabe, H., Umamoto, K., Kameoka, H., 1998. Inhibition of acetylcholinesterase activity by essential oils of *Mentha* species. J. Agric. Food Chem. 46, 3431–3434.
- Mohammadhosseini, M., Pazoki, A., Zamani, H.A., Akhlaghi, H., 2011. Chemical composition of the essential oil from aerial parts of *Ajuga chamaecistus* Ging: subsp. *scopria* in brackish regions of Iran. J. Essent. Oil Bear. Pl. 14, 101–105.
- Moradi-Afrapoli, F., Asghari, B., Saeidnia, S., Ajani, Y., Mirjani, M., Malmir, M., Bazaz, R.D., Hadjiakhoondi, A., Salehi, P., Hamburger, M., 2012. In vitro α -glucosidase inhibitory activity of phenolic constituents from aerial parts of *Polygonum hyrcanicum*. Daru J. Pharm. Sci. 20, 1.
- Moshefi, M.H., Mehrahani, M., Moeini, M., Saffari, F., 2014. Study of antibacterial effects of different fractions of leaves extract of *Ajuga Chamaecistus* Ging subsp. *scoparia* (Boiss) Rech. f. and bioautography of effective fraction. J. Kerman Univ. Med. Sci. 21, 313–320.
- Mozaffarian, V., 1996. A Dictionary of Iranian Plant Names. Farhang Moaser Publisher, Tehran.
- Ni, B., Dong, X., Fu, J., Yin, X., Lin, L., Xia, Z., Zhao, Y., Xue, D., Yang, C., Ni, J., 2015. Phytochemical and biological properties of *Ajuga decumbens* (Labiatae): a review. Trop. J. Pharm. Res. 14, 1525–1536.
- Ono, Y., Fukaya, Y., Imai, S., Yamakuni, T., 2008. Beneficial effects of *Ajuga decumbens* on osteoporosis and arthritis. Biol. Pharm. Bull. 31, 1199–1204.
- Sadati, N., Jenett-Siems, K., Siems, K., Ardekani, M.R.S., Hadjiakhoondi, A., Akbarzadeh, T., Ostad, S.N., Khanavi, M., 2012. Major constituents and cytotoxic effects of *Ajuga chamaecistus* ssp. *tomentella*. Z. Naturforsch. C 67, 275–281.
- Saravanan, S., Pari, L., 2015. Role of thymol on hyperglycemia and hyperlipidemia in high fat diet-induced type 2 diabetic C57BL/6J mice. Eur. J. Pharmacol. 761, 279–287.
- Saravanan, S., Pari, L., 2016. Protective effect of thymol on high fat diet induced diabetic nephropathy in C57BL/6J mice. Chem.-Biol. Interact. 245, 1–11.
- Sarikurkcü, C., Uren, M.C., Tepe, B., Cengiz, M., Kocak, M.S., 2014. Phenolic content: enzyme inhibitory and antioxidative activity potentials of *Phlomis nissolii* and *P. pungens* var. *pungens*. Ind. Crops Prod. 62, 333–340.
- Sarikurkcü, C., Zengin, G., Oskay, M., Uysal, S., Ceylan, R., Aktumsek, A., 2015. Composition antioxidant, antimicrobial and enzyme inhibition activities of two *Origanum vulgare* subspecies (subsp. *vulgare* and subsp. *hirtum*) essential oils. Ind. Crops Prod. 70, 178–184.
- Sarikurkcü, C., 2013. Antioxidant activities of solvent extracts from endemic *Cyclamen mirabile* Hildebr. tubers and leaves. Afr. J. Biotechnol. 10, 831–839.
- Slinkard, K., Singleton, V.L., 1977. Total phenol analysis: automation and comparison with manual methods. Am. J. Enol. Vitic. 28, 49–55.
- Souza, P.M., Elias, S.T., Simeoni, L.A., de Paula, J.E., Gomes, S.M., Guerra, E.N.S., Fonseca, Y.M., Silva, E.C., Silveira, P.O., Magalhaes, D.m., 2012. Plants from Brazilian Cerrado with potent tyrosinase inhibitory activity. PLoS One 7, e48589.
- Szwajgier, D., Borowiec, K., 2012. Phenolic acids from malt are efficient acetylcholinesterase and butyrylcholinesterase inhibitors. J. Inst. Brew. 118, 40–48.
- Terahara, N., Callebaut, A., Ohba, R., Nagata, T., Ohnishi-Kameyama, M., Suzuki, M., 2001. Acylated anthocyanidin 3-sophoroside-5-glucosides from *Ajuga reptans* flowers and the corresponding cell cultures. Phytochemistry 58, 493–500.
- Valizadeh, H., Sonboli, A., Kordi, F.M., Dehghan, H., Bahadori, M.B., 2015. Cytotoxicity, antioxidant activity and phenolic content of eight fern species from North of Iran. Pharm. Sci. 21, 18.
- Zengin, G., Sarikurkcü, C., Aktumsek, A., Ceylan, R., Ceylan, O., 2014. A comprehensive study on phytochemical characterization of *Haplophyllum myrtifolium* Boiss. endemic to Turkey and its inhibitory potential against key enzymes involved in Alzheimer, skin diseases and type II diabetes. Ind. Crops Prod. 53, 244–251.
- Zengin, G., Locatelli, M., Ceylan, R., Aktumsek, A., 2015. Anthraquinone profile, antioxidant and enzyme inhibitory effect of root extracts of eight Asphodeline taxa from Turkey: can Asphodeline roots be considered as a new source of natural compounds? J. Enzyme Inhib. Med. Chem. 1–6.
- Zengin, G., 2016. A study on in vitro enzyme inhibitory properties of *Asphodeline anatolica*: new sources of natural inhibitors for public health problems. Ind. Crops Prod. 83, 39–43.
- Zhang, H., Tsao, R., 2016. Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. Curr. Opin. Food Sci. 8, 33–42.