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

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A B S T R A C T

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-ethylbenzothiazoline-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-ethylbenzothiazoline-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; Israfil and Lyossui, 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 antihelmintic, antifungal, antifebrile, antitumor, antimicrobial, wound healing, and diuretic agents (Cocqy et al., 2011; Eddouks et al., 2007; Israfil and Lyossui, 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 Lyossui, 2002), anti-inflammatory (Gautam et al., 2011), analobic, anti-arthritis, analgesic, antipyretic, antibacterial, hepatoprotective, antifungal, cardiotoxic, antioxidant (Israfil and Lyossui, 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-
teroids compounds which have insect antifeedant and moulding hormone activities (Camps and Coll, 1993). Phytochemical studies on this genus led to the isolation of many bioactive compounds such as diterpenoids (Coll and Tardón, 2008), strols, triterpenoids (Ni et al., 2015), iridoids, phenylethanoid glycosides (Abay et al., 2003), anthocyanins (Terahara et al., 2001), and phytocoumarins (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. (Moizzaffarian, 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 Cleveenger 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 × 0.32 mm i.d., film thickness 0.25 μm). 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 μL in split mode (1:100). Essential oil was diluted in n-hexane (1/100, v/v) and 0.5 μL 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 (C₅–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 × 4.6 mm length, 5 μm particle size, Agilent, Santa Clara, CA, USA). The eluates were detected at 278 nm. The phenolic compounds of the ethanol and water extracts were determined using a previously modified method (Sarikurku 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, vanilin, p-coumaric acid, ferulic acid, sinapinic acid, benzolic acid, o-coumaric acid, rutin, hesperidin, rosmaric acid, eriodictiol, cinnamic acid, quercetin, luteolin, kaemferol, 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 μg 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 (Singleton 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-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS) (Zengin et al., 2014) and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Sarikurku, 2013) scavenging methods.

2.9. Reducing power

The cupric ion reducing activity (CUPRAC) of the essential oil and extracts were determined according to a stablished 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...
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 galantine (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 (carbose 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% v/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 μg/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 mortality percentage was calculated. LC50 values were calculated and expressed as average value of three independent experiments ± SD.

2.13. Statistical analysis

All the measurements were carried out in triplicates. The results were expressed as mean ± 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 α = 0.05. These analyses 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.](image)
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 ethanol 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 ethanol 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 ethanol 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, leoniside, and myristinoside. In another study, phenolic components such as acteoside, chrysoeriol 7-oglucoptaroynoside, and apigenin 7-orthanomypyransoside were purified from the methanolic extract of the aerial parts of A. chamaepitys (L.) Schrebl (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 processes. 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

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

<table>
<thead>
<tr>
<th>No.</th>
<th>Phenolic compounds</th>
<th>Extracts</th>
<th>Linear range (ppm)</th>
<th>r²</th>
<th>LOD (ppm)</th>
<th>LOQ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>Ethanol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Gallic acid</td>
<td>95 ± 4</td>
<td>nd</td>
<td>0.20–25.0</td>
<td>0.9993</td>
<td>0.075</td>
</tr>
<tr>
<td>2</td>
<td>Protocatechuic acid</td>
<td>nd&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nd</td>
<td>0.20–25.0</td>
<td>0.9991</td>
<td>0.086</td>
</tr>
<tr>
<td>3</td>
<td>(+)-Catechin</td>
<td>nd</td>
<td>nd</td>
<td>0.90–113</td>
<td>0.9988</td>
<td>0.172</td>
</tr>
<tr>
<td>4</td>
<td>p-Hydroxybenzoic acid</td>
<td>nd</td>
<td>22 ± 0.3</td>
<td>0.20–25.0</td>
<td>0.9994</td>
<td>0.007</td>
</tr>
<tr>
<td>5</td>
<td>Chlorogenic acid</td>
<td>10 ± 1</td>
<td>nd</td>
<td>0.35–45.0</td>
<td>0.9888</td>
<td>0.080</td>
</tr>
<tr>
<td>6</td>
<td>Caffeic acid</td>
<td>16 ± 1</td>
<td>32 ± 2</td>
<td>0.16–21.0</td>
<td>0.9993</td>
<td>0.054</td>
</tr>
<tr>
<td>7</td>
<td>(−)-Epicatechin</td>
<td>nd</td>
<td>nd</td>
<td>0.50–66.0</td>
<td>0.9990</td>
<td>0.170</td>
</tr>
<tr>
<td>8</td>
<td>Syringic acid</td>
<td>nd</td>
<td>6 ± 0.1</td>
<td>0.05–12.0</td>
<td>0.9995</td>
<td>0.030</td>
</tr>
<tr>
<td>9</td>
<td>Vanillin</td>
<td>nd</td>
<td>15 ± 0.1</td>
<td>0.08–10.0</td>
<td>0.9995</td>
<td>0.020</td>
</tr>
<tr>
<td>10</td>
<td>p-coumaric acid</td>
<td>692 ± 42</td>
<td>574 ± 18</td>
<td>0.04–6.0</td>
<td>0.9966</td>
<td>0.066</td>
</tr>
<tr>
<td>11</td>
<td>Ferulic acid</td>
<td>nd</td>
<td>106 ± 3</td>
<td>0.12–17.0</td>
<td>0.9993</td>
<td>0.004</td>
</tr>
<tr>
<td>12</td>
<td>Sinapic acid</td>
<td>37 ± 1</td>
<td>47 ± 1</td>
<td>0.12–17.0</td>
<td>0.9993</td>
<td>0.017</td>
</tr>
<tr>
<td>13</td>
<td>Benzoic acid</td>
<td>nd</td>
<td>nd</td>
<td>0.85–55.0</td>
<td>0.9998</td>
<td>0.111</td>
</tr>
<tr>
<td>14</td>
<td>o-Coumaric acid</td>
<td>11 ± 0.1</td>
<td>9 ± 0.1</td>
<td>0.24–32.0</td>
<td>0.9988</td>
<td>0.023</td>
</tr>
<tr>
<td>15</td>
<td>Rutin</td>
<td>30 ± 1</td>
<td>44 ± 1</td>
<td>0.40–56.0</td>
<td>0.9989</td>
<td>1.113</td>
</tr>
<tr>
<td>16</td>
<td>Hesperidin</td>
<td>26 ± 0.6</td>
<td>20 ± 0.6</td>
<td>0.43–55.0</td>
<td>0.9992</td>
<td>1.080</td>
</tr>
<tr>
<td>17</td>
<td>Rosmarinic acid</td>
<td>nd</td>
<td>nd</td>
<td>0.02–7.0</td>
<td>0.9998</td>
<td>0.148</td>
</tr>
<tr>
<td>18</td>
<td>Erinolein</td>
<td>nd</td>
<td>nd</td>
<td>0.33–28.0</td>
<td>0.9999</td>
<td>0.030</td>
</tr>
<tr>
<td>19</td>
<td>trans-Cinnamic acid</td>
<td>5 ± 0.5</td>
<td>3 ± 0.1</td>
<td>0.02–7.0</td>
<td>0.9998</td>
<td>0.148</td>
</tr>
<tr>
<td>20</td>
<td>Quercetin</td>
<td>nd</td>
<td>nd</td>
<td>0.40–55.0</td>
<td>0.9999</td>
<td>0.013</td>
</tr>
<tr>
<td>21</td>
<td>Luteolin</td>
<td>30 ± 3</td>
<td>28 ± 3</td>
<td>0.13–17.0</td>
<td>0.9999</td>
<td>0.020</td>
</tr>
<tr>
<td>22</td>
<td>Kaempferol</td>
<td>nd</td>
<td>nd</td>
<td>0.05–15.0</td>
<td>0.9996</td>
<td>0.021</td>
</tr>
<tr>
<td>23</td>
<td>Apigenin</td>
<td>24 ± 1</td>
<td>42 ± 1</td>
<td>0.17–11.0</td>
<td>0.9997</td>
<td>0.034</td>
</tr>
</tbody>
</table>

<sup>a</sup> Not detected.
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 µg/mL). Essential oil of this plant showed IC$_{50}$ value of 7146 µg/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

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total bioactive compounds</th>
<th>Total antioxidant activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total phenolic (mg GAEs/g extract)</td>
<td>Total flavonoid (mg REs/g extract)</td>
</tr>
<tr>
<td>Essential oil</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Water extract</td>
<td>18.94 ± 0.13</td>
<td>9.14 ± 0.09</td>
</tr>
<tr>
<td>Ethanol extract</td>
<td>20.32 ± 0.39</td>
<td>11.61 ± 0.16</td>
</tr>
</tbody>
</table>

* Values expressed are mean ± SD of three parallel measurements.

* GAEs: gallic acid equivalents.
* REs: rutin equivalents.
* TEs: trolox equivalents.

3.8. Enzyme inhibitory activity

Today's 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 cholinesterase 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 monoterpenic 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 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).

Table 4

<table>
<thead>
<tr>
<th>Samples</th>
<th>Radical scavenging activity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPPH radical scavenging (mg TEs/g oil or extract)</td>
<td>ABTS radical scavenging (mg TEs/g oil or extract)</td>
</tr>
<tr>
<td>Essential oil</td>
<td>6.31 ± 0.83</td>
<td>191.01 ± 0.33</td>
</tr>
<tr>
<td>Water extract</td>
<td>20.23 ± 0.60</td>
<td>73.47 ± 1.04</td>
</tr>
<tr>
<td>Ethanol extract</td>
<td>22.69 ± 1.30</td>
<td>53.87 ± 2.11</td>
</tr>
</tbody>
</table>

* TEs: trolox equivalents.
* Values expressed are mean ± SD of three parallel measurements.

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 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).

Table 5

<table>
<thead>
<tr>
<th>Samples</th>
<th>Reducing power activity</th>
<th>Metal chelating activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CUPRAC (mg TEs/sample)⁴</td>
<td>FRAP (mg TEs/sample)⁴</td>
</tr>
<tr>
<td>Essential oil</td>
<td>329.64 ± 4.40</td>
<td>97.75 ± 1.72</td>
</tr>
<tr>
<td>Water extract</td>
<td>59.57 ± 0.71</td>
<td>43.19 ± 1.04</td>
</tr>
<tr>
<td>Ethanol extract</td>
<td>61.67 ± 2.68</td>
<td>39.41 ± 2.13</td>
</tr>
</tbody>
</table>

* TEs: trolox equivalents.
* EDTAEs: EDTA equivalents.
* Values expressed are mean ± SD of three parallel measurements.
anticholinesterase activity of ferulic acid and p-coumaric acid in the literature (Szewagier 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 alkaldoids (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 Parvini, 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; Aghbari 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. Artemisia salina larvae toxicity

None of the extracts and essential oil exhibited toxicity in applied concentration range (312–1000 μg/mL) against A. salina. So, LC50 values were not reached, while podophyllotoxin as a standard drug showed the LC50 of 35.2 μg/mL. Generally, natural agents with the LD50 values more than 1000 μg/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.

Acknowledgment

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References
