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Phytochemical characterization of phenolics by LC-MS/MS and biological evaluation of *Ajuga orientalis* from Turkey

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Abstract

The aim of this study was to reveal the phytochemical constituents, antioxidant and antimicrobial activity of *Ajuga orientalis*. According to the antimicrobial results, the methanol extract of *A. orientalis* showed a MIC value of 312.5 µg/mL against the tested pathogenic bacterial strains. Anticandidal activity of extract was found as 156.3 µg/mL both against *Candida albicans* and *C. parapsilosis* strains. Whereas the extract was more effective against *C. tropicalis* with the MIC value of 78.1 µg/mL. The *in vitro* DPPH radical scavenging activity of the extract was determined as IC_{50}=0.4 ± 0.02 mg/mL whereas the standard BHT IC_{50} was 0.01 ± 0.00 mg/mL. Trolox Equivalent Antioxidant Capacity (TEAC) of extract was determined 1.3 mM TEAC, while BHT, 1.9 mM TEAC.

Introduction

Ajuga L. is one of the largest genera of the *Lamiaceae* family with 301 species all over the world. In flora of Turkey, *Ajuga* is represented by 13 species and 23 taxa (Güner et al., 2012). *Ajuga* has some traditional therapeutic uses such as diuretic, antipyretic, tonic, diaphoretic and astringent in Turkey (Baytop, 1984). *Ajuga* species are used in traditional medicine all over the world against some illness such as gout, rheumatism, malaria, asthma and gastrointestinal diseases and have antibacterial, antitumor (Ben Jannet et al., 2006; Chen et al., 1996; Israili and Lyoussi, 2009, Setif, 2011), neuroprotective effects (Guo et al., 2011), anti-inflammatory and antioxidant (Makni et al., 2013; Turkoglu et al., 2010) activities among others biological effects.

Ajuga genus contains many important bioactive compounds like anthocyanins, diterpenoids, sterols, ionones, iridoids, phenylethanol and flavonoid glycosides (Maria et al, 1997, Chen et al., 1996; Shimomura et al., 1987; Terahara et al., 2001; Akbay et al., 2003).

Essential oil composition of *A. orientalis* was investigated and main compounds were germacren D (24.7%), *β*-cubaben (18.3%) and *β*-caryophyllene (16.9%) (Sajjadi and Ghanadi, 2004).

Even though several bioactive compounds have been well studied on some *Ajuga* species, there is not enough data on phytochemical and biological activities for *A. orientalis*. To the best of our knowledge, this is the first report on determination of the major phenolic compounds, antioxidant activity and antimicrobial properties of the methanol extract of *A. orientalis*.

Materials and Methods

Chemicals

All antimicrobial agents, Mueller Hinton II broth and RPMI medium were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO). Solvents were used analytical grade.

Plant material and preparation of the extract

A. orientalis was collected during flowering stage from Bursa, Turkey [A2 (A) Bursa: Bursa, Uludağ,1773 m, 19.6.2008, K 400 06′ 23.3′′ D 290 08′ 11.1′′].

Microbial strains

The antimicrobial activities of the extract were tested against a panel of microbial strains, including: *Escherichia coli* NRRL B3008, *Staphylococcus aureus* ATCC 6538, *Salmonella typhimurium* ATCC 13311, *Bacillus cereus* NRRL B-3711, *Candida albicans* ATCC 90028, *C. tropicalis* ATCC 1369 and *C. parapsilosis* ATCC 22019.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC values of the strains were determined by broth microdilution methods (CSLI, 2006, 2008). The methanol extract of *A. orientalis* was examined against a panel of seven different human pathogenic bacteria and *Candida* strains using the microdilution method according to CSLI method compared with standard antimicrobial agents. Methanol extract of *A. orientalis* was studied between [1250-2.44 µg/mL] for MIC and the antimicrobial standard agents ampicillin, tetracycline, ketoconazole (64-0.125 µg/mL) and oxiconazole (16-0.03 µg/mL) were prepared in dimethyl sulfoxide (DMSO) and water.

The extract (100 µL) was added to wells of row A, while the remaining wells in rows B to H received 50 µL of cation adjusted Mueller-Hinton Broth-2 (MHB-2). Bacterial suspensions were grown overnight in double strength broth and were standardized to 10^5 cfu/mL for bacteria. Each bacterial suspension (50 µL) was added to the appropriate well. All *Candida* strains were inocula -ted on Patoto Dextrose Agar (PDA) prior the experiments at 35°C. After incubation grown microorganisms were inoculated sterile saline %0.85. And then standardized using a turbitometer (Biosan) (McFarland No: 0.5) to 5 x 10^3 cfu per well in RPMI medium under sterile conditions. Serial dilution series were prepared in 100 µL RPMI medium with an equal amount of the test samples. After serial dilution 100 µL each microorganism suspension was pipetted into each well and incubated at 35°C for 24 hours. Positive growth controls (to assess the presence of turbidity) were performed in wells not containing antimicrobial agents. In addition, negative growth control (medium) was applied in 96-well plate. After incubation at 35°C for 24 hours the first well without turbidity was determined as the MIC (µg/mL).

Antioxidant activity (total phenolics)

Total phenols were estimated as gallic acid equivalents (GAE), expressed as mg gallic acid/g extract (Singleton Vernon et al., 1999).

TEAC assay (Trolox Equivalent Antioxidant Capacity)

This test measures the ability of a compound to scavenge the ABTS (2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulfonate) radical in comparison to antioxidant activity of trolox which is a water-soluble form of vitamin E, used as a standard. The blue–green ABTS radical was formed through the reaction of 7 mM ABTS with 2.5 mM sodium persulfate ($Na_2S_2O_8$) in the dark at room temperature for 12–16 hours before use. ABTS solution was diluted with ethanol to a final absorbance of 0.7–0.8 at 734 nm. 10 µL portion of the sample was added to 990 µL of ABTS solution, and the reduction in absorbance was measured 1 min after addition of trolox up to 40 min after addition of the extract. The stock solution of trolox (2.5 mM) was prepared in ethanol. Absorbance was measured on a UV/spectrophotometer (Papandreou et al., 2006).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

Serial dilutions were prepared with the stock solutions (4 mg/mL) of the extract to get the half concentration of previous one. Diluted solutions were added with DPPH•(equal amounts). After 30 min UV absorbance was recorded at 517 nm. The experiment was performed in triplicate for extract and positive standard control, BHT (butylated hyroxytoluene). The average of the absorption was noted for each concentration. The percentage inhibition was calculated using Eq 1. The IC_{50} value, which is the concentration of the test material that inhibits 50% of the free radical concentration, was calculated as mg/mL using Sigma Plot statistical program (Kumarasamy et al., 2007).

Percentage Inhibition =

$$\left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] x100 \qquad \text{Equation 1}$$

Phenolic compound determination

Experiments were performed with a Shimadzu 20A HPLC system coupled to an Applied Biosystems 3200 Q -Trap LC- MS/MS instrument equipped with an ESI ion source used in the negative ionization mode. Separations were performed on an ODS 150 x 4.6 mm, i.d., 3 µm particle sizes, octadecyl silica gel analytical column operating at 40°C at a flow rate of 1 mL/min.

Powdered dried herbal parts of *A. orientalis* were macerated with methanol 70% at 25°C for 24 hours. After evaporation of the methanol part, the aqueous part freeze-dried and the dry extract was used in all experiments.

Results and Discussion

An antioxidant activity generally occurs a hydrogen donating procedure. Phenolic acids and flavonoids are well known hydrogen donating agents due to their high PKa values. Determination of the total phenol com-

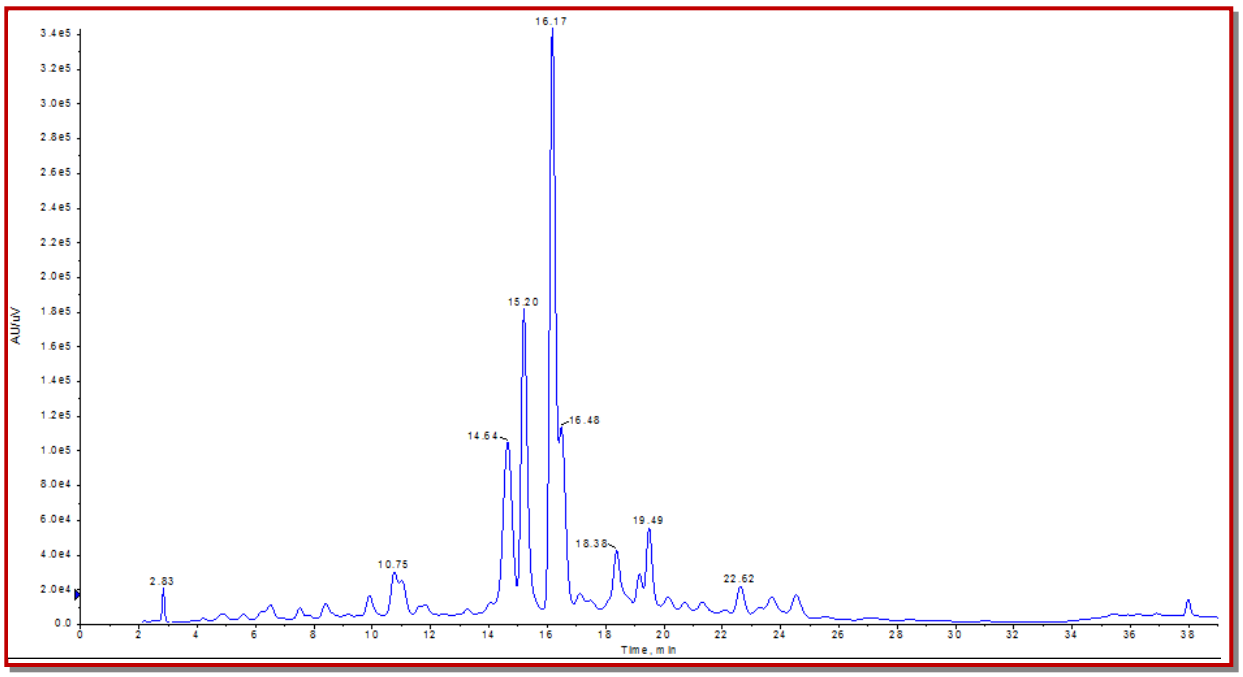

Figure 1: *Ajuga orientalis* LC chromatogram at 320 nm

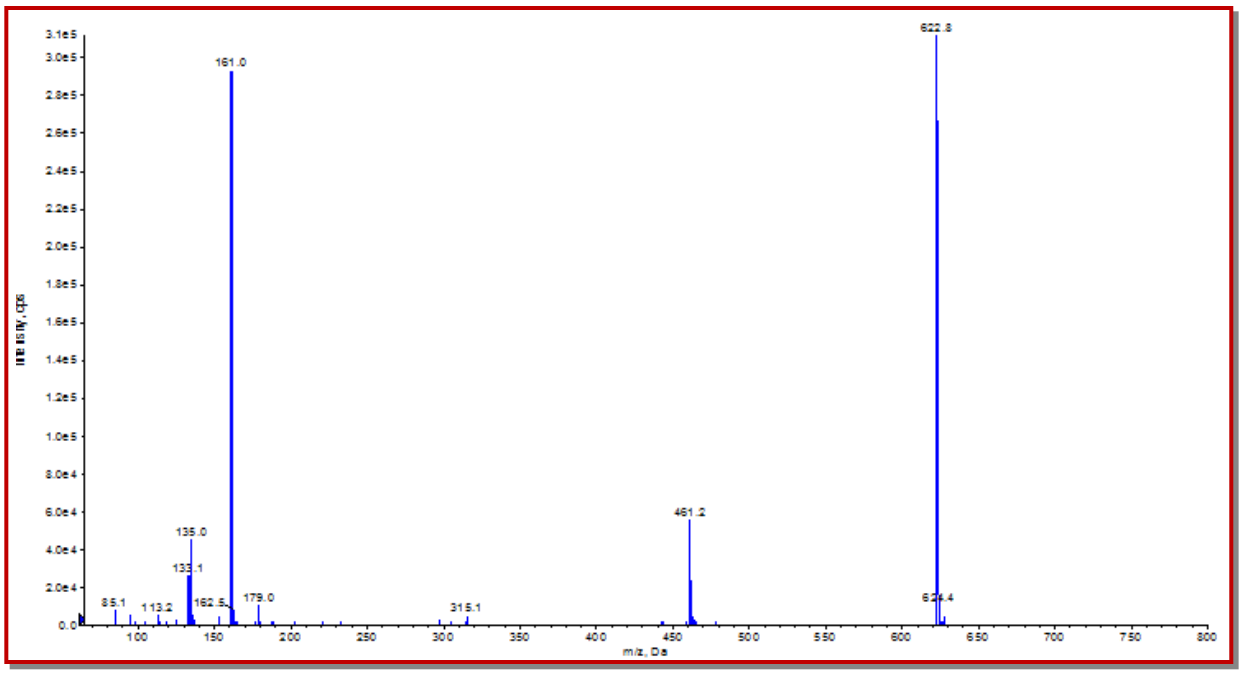

Figure 2: Mass spectrum of compound 5: verbascoside

pounds provides preliminary information about antioxi -dant activity results. The more total phenolic compounds means the more antioxidant activity.

According to results, extract of *A. orientalis* showed similar amount of gallic acid equivalent total phenol content in 1 g methanol extract. *A. orientalis*: 32 mg GAE/g ext.

According to the identification of phenolic profile by LC-MS/MS (Table I) 9 compounds were determined (Figure 1).

Compound 1 Rt =2.8 co-eluted with [M-H]-molecular ions at m/z 195 (galactonic acid or gluconic acid) and m/z 341 (disaccharide like sucrose). High absorbance at 280 nm must be related to gradient changing of mobile phase. Product ions of m/z 341 similar with caffeoyl glucose fragments (m/z 179, m/z 161) but it is not

Table I

Compounds determined with LC-MS/MS

No.	Compound	Rt	(M-H)	Fragments	Reference
1	Galactonic acid	2.8	195	177, 159, 129, 11	Nist 14
2	Ascorbic acid	9.8	175	115	Nist 14
3	Luteolin glucuronide derivative	14.6	513	487, 461, 285, 179, 135	(Cvetkovikj et al., 2013)
4	Echinacoside	15.4	785	623, 461, 161	(Mitreski et al., 2014)
5	Verbascoside	16.2	623	461, 315, 161	(Mitreski et al., 2014)
6	Forsythoside A	16.5	623	461, 315, 161	(Guo et al., 2007; Mitreski et al., 2014)
7	Castanoside A	18.5	799	623, 461	(Mitreski et al., 2014)
8	4"-O-methylisoscutellarein 7-O-allosyl(1-2)glucoside	19.2	623	461, 315, 161, 135	(Petreska et al., 2011)
9	Leucoseptoside A	19.6	637	461, 175	(Mitreski et al., 2014)

possible to determine a caffeoyl glucose molecule at this retention time. Other fragments of galactonic acid can be seen in Table I.

Compound 2 was identified as ascorbic acid according to Nist 14 MS/MS library and comparison with authentic standard's fragmentation patterns.

Compound 3 Rt = 14.6 displayed $[M-H]^-$ ion at m/z 513 and a base peak ion at m/z 461 further fragmentation showed several ions at m/z 487, 461, 285 (most probably a luteolin), 179 and 135. Following fragmentation of m/z 461, it was found as similar with luteolin glucuronide. However, compound 6 was exhibited 26 amu higher than luteolin glucuronide so this compound was labeled as luteolin glucuronide derivative.

Compound 4 Rt = 15.4 displayed $[M-H]^-$ ion at m/z 785 yielded a base peak ion at m/z 623 due to the loss of a hexose (or caffeoyl) moiety, the same loss of base peak ion was led to form ion at m/z 461. Other fragmentation corresponding with cinnamic acids, m/z 179 and 161, was also observed. The compound was identified as echinacoside by compression to compound previously reported (Petreska et al., 2011).

Two compound, compound 5 (Figure 2) and 6 were co-eluted at Rt = 16.2 and Rt = 16.5 with a same deprotonated molecular ion at m/z 623 which showed same fragmentation pattern. Both of the spectrums yielded m/z 461 owing to loss of a caffeoyl moiety (-162) and m/z 315 due to the loss of a rhamnose unit (-146). Caffeoyl part of the compounds were also observed at m/z 179 and further fragmentation at m/z 161 and 135. Compound 5 at RT 16.2 was determined as verbascoside whereas compound 6 at Rt = 16.5 was identified as fosythoside A using similar mass spectrum and chromatographic separation of previously reported data (Guo et al., 2007; Mitreski et al., 2014).

Compound 7 Rt = 18.5 showed $[M-H]^-$ ion at m/z 799 its MS/MS spectrum yielded two main fragments at m/z 623 (loss of a feruloyl, -176) and 461 (loss of a caffeoyl moity). This compound was identified as castanoside A according to previous data (Mitreski et al., 2014).

Compound 8 showed MS fragmentation characteristic of verbascoside and forsythoside A. Pseudomolecular ion at m/z 623 and its MS^2 experiment yielded in several ions at m/z 461, 315, 161 and 135. According to previously published data (Petreska et al., 2012) the compound, eluted after verbascoside and forsythoside A, with the same molecular weight is 4"-O-methylisoscutellarein 7-O-allosyl(1-2)glucoside. These data sugges-ted that compound could be identified tentatively as 4"-O-methylisoscutellarein 7-O-allosyl(1-2)glucoside.

Compound 9 showed $[M-H]^-$ molecular ion at m/z 637 in MS^2 spectrum a ferruloyl break at m/z 461 and the ferruloyl part at m/z 175 was also observed. This compound was labeled as leucoptoside A according to literature data (Mitreski et al., 2014; Petreska et al., 2011).

DPPH•is a radical that gives maximum absorption at 517 nm in alcoholic solutions because of its intense purple color. The color of radical converts pale yellow if neutralized via an antioxidant compound. DPPH radical was used for determination of the radical scavenging antioxidant potential of *A. orientalis* extract. The extract radical scavenging activity was not effective as a positive control standard BHT. IC_{50} value of the extract was 0.4 ± 0.02 mg/mL and the positive control of BHT 0.01 ± 0.00 mg/mL.

The calibration curve of trolox that is water soluble analog of vitamin E is used in the quantification. A radical form of ABTS is used as an indicator for evaluation of the antioxidant activities. This radical occurs after treatment of the non-radical ABTS salt with potassium persulfate after 16 hours. Alcoholic solution of radical gives maximum absorption at 734 nm owing to its green color. Green color of the radical converts to colorless and the absorption reduces if it encounters with antioxidant compound. In this test high TEAC

Table II

Minimum Inhibitory Concentrations (MIC, µg/mL)

Microorganisms	*Ajuga orientalis* extract	Ketoconazole	Oxiconazole	Ampiciline	Tetracycline
Escherichiacoli NRRL B-3008	312.5	-	-	4	4
Staphylococcus aureus ATCC 6538	312.5	-	-	2	2
Salmonella typhimurium ATCC 13311	312.5	-	-	2	2
Bacillus cereus NRRL B-3711	312.5	-	-	0.5	0.5
Candida albicans ATCC 90028	156.25	1	4	-	-
Candida tropicalis ATCC 1369	78.12	0.5	2	-	-
Candida parapsilosis ATCC 22019	156.25	2	2	-	-

values shows high antioxidant activity results. ABTS $^{\bullet+}$ radical scavenging and DPPH radical scavenging effect showed a high correlation with each other. The extract at 1% concentration, showed ABTS $^{\bullet+}$ radical scavenging effect. But the extract was not found as effective as standard BHT. TEAC values were found 1.3 mM for extract and 1.9 mM for BHT.

Oxidative attack of free radicals is the reason for many chronic diseases. Natural antioxidants such as flavonoids and phenolic acids help to protect human body from free radicals (Koolen et al., 2013). Antioxidant activity of this plant can be used as natural radical scavenger but not as good like as synthetic standard antioxidants. Determined phenolic compounds are the first reason of the antioxidant activities. Hydroxyl rich compounds is well known free radical scavengers. Obtained total phenolic compounds amount can be low for this plant material using this extraction procedure. However, high amount of hydroxyl groups of molecules that we determined showed moderate activity.

Discovering new antimicrobial agents are also important because of antimicrobial resistance of some microorganism. Synthetic antimicrobial agents are used in several years and today some of them are ineffective and researchers are focused on to investigate new natural antimicrobial agents.

Additionally phytochemical studies of the extract, *in vitro* antimicrobial activity was evaluated seven different strains in this study (Table II) using microdilution methods. *A. orientalis* extract exhibited antimicrobial action against all tested microorganisms, being more effective against yeasts than bacteria. The MIC was found at 312.5 µg/mL for *Escherichia coli, Staphylococcus aureus*, *Salmonella typhimurium* and *Bacillus cereus.* MIC value of 156 µg/mL for *C. albicans* and *C. parapsilosis* for the extract. Our results showed that *C. tropicalis* was the most sensitive strain for the extract (MIC=78.12 µg/mL).

There have been some reports for antimicrobial activities belong to *A. orientalis*. Ali-Shtayeh et al (2013) found that extract of *A. orientalis* inhibited the growth of some acne-inducing bacteria and aerobic bacteria (Ali-Shtayeh et al 2013). *A. orientalis* was also evaluated antifungal activity against *Phytophthora capsici* which is a pathogen fungus (Kıran et al, 2010).

As a result, the antimicrobial activity was determined the first time for *A. orientalis* extract, especially against *Candida* species. The extract can be used as an alternative natural prevention of *Candida* infections and candidal resistance.

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