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ORIGINAL ARTICLE

Phytochemical Characterization of Phenolic Compounds by LC-MS/MS and Biological Activities of *Ajuga reptans* L., *Ajuga salicifolia* (L.) Schreber and *Ajuga genevensis* L. from Turkey

Türkiye'den *Ajuga reptans* L., *Ajuga salicifolia* (L.) Schreber ve *Ajuga genevensis* L.'nin LC-MS/MS ile Fenolik Bileşiklerinin Fitokimyasal Karakterizasyonu ve Biyolojik Aktiviteleri

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ABSTRACT

Objectives: In this study, it was aimed to characterize the phenolic contents of *Ajuga reptans* L., *Ajuga salicifolia* (L.) Schreber and *Ajuga genevensis* L. and to investigate their *in vitro* antioxidant and antimicrobial activities.

Materials and Methods: Air dried aerial parts of *A. reptans* L., *A. salicifolia* (L.) Schreber, and *A. genevensis* L. collected from Turkey were extracted with methanol (70%), and the phenolic composition of the crude extracts was analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) method. To determine the total phenolic content the Folin-Ciocalteu method was used. The radical scavenging activities of the extracts were evaluated by the photometric 1,1-diphenyl-2-picrylhydrazyl radical, and trolox equivalent antioxidant capacity assays (TEAC). Furthermore, *Ajuga* sp. extracts were tested against *Escherichia coli* NRRL B3008, *Staphylococcus areus* ATCC 6538, *Salmonella thyphimurium* ATCC 13311, *Bacillus cereus* NRRL B-3711, *Candida albicans* ATCC 90028, *Candida tropicalis* ATCC 1369, and *Candida parapsilosis* ATCC 22019 using the *in vitro* broth dilution assay.

Results: The LC-MS/MS analyses identified 19 compounds. The amount of total phenolics ranged from 30.0 to 42.2 mg gallic acid equivalent/g in all extracts. According to the results of TEAC assay, the tested extracts were found to have relatively high activity at 1.2-1.5 mM concentrations. *Ajuga* sp. extracts inhibited all tested microorganisms; however, *C. albicans, C. tropicalis,* and *C. parapsilosis* exhibited relatively more susceptibility (minimum inhibitory concentration: 156.25 µg/mL) compared to the bacteria tested.

Conclusion: The antioxidant activities of all extracts were determined for the first time by the TEAK method, and the *in vitro* antimicrobial activity of *A. salicifolia* was investigated for the first time against selected strains.

Key words: *Ajuga reptans, Ajuga salicifolia, Ajuga genevensis*, LC-MS/MS, antioxidant activity, antimicrobial activity

ÖZ

Amaç: Bu çalışmada Türkiye'de yetişen *Ajuga reptans* L., *Ajuga salicifolia* (L.) Schreber ve *Ajuga genevensis* L.'nin fenolik içeriklerinin karakterizasyonu, *in vitro* antioksidan ve antimikrobiyal aktivitelerinin araştırılması amaçlanmıştır.

Gereç ve Yöntemler: *A. reptans* L., *A. salicifolia* (L.) Schreber ve *A. genevensis* L'nin toprak üstü kısımları metanol (%70) ile ekstre edilmiş ve liyofilize edilerek ardından sıvı kromatografi tandem kütle/kütle spektrometre (LC-MS/MS) ile karakterizasyonları yapılmıştır. Toplam fenolik madde miktarları Folin-Ciocaltaeu yöntemi ile belirlenmiştir Ekstrelerin radikal süpürücü etkileri 1,1-difenil-2-pikrilhidrazil ve troloks eşdeğeri antioksidan

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kapasite (TEAK) yöntemleri kullanılarak değerlendirilmiştir. Ayrıca, *Ajuga* sp. ekstrelerinin antimikrobiyal aktiviteleri, *Escherichia coli* NRRL B3008, *Staphylococcus areus* ATCC 6538, *Salmonella thyphimurium* ATCC 13311, *Bacillus cereus* NRRL B-3711, *Candida albicans* ATCC 90028, *Candida tropicalis* ATCC 1369 ve *Candida parapsilosis* ATCC 22019'a karşı *in vitro* mikrodilüsyon yöntemiyle çalışılmıştır.

Bulgular: LC-MS/MS analizleriyle 19 fenolik bileşik tanımlanmıştır. Tüm ekstrelerde toplam fenol miktarı 30,0-42,2 mg gallik asit eşdeğeri/g arasında bulunmuştur. TEAK antioksidan aktivite sonucunda ekstreler (1,2-1,5 mM) konsantrasyonlarda nispeten yüksek aktivite göstermiştir. *Ajuga* sp. ekstreleri, test edilen tüm mikroorganizmalara karşı antimikrobiyal aktivite göstermiştir. Ancak ekstreler, test edilen bakterilere kıyasla *C. albicans, C. tropicalis* ve *C. parapsilosis* suşlarına karşı nispeten daha fazla etkili (minimal inhibisyon konsantrasyonu: 156,25 µg/mL) bulunmuştur.

Sonuç: TEAK yöntemi ile tüm ekstrelerin ilk defa antioksidan aktiviteleri belirlenmiştir ve *A. salicifolia*'nın *in vitro* antimikrobiyal aktivitesi seçilen suşlara karşı ilk kez incelenmiştir.

Anahtar kelimeler: *Ajuga reptans, Ajuga salicifolia, Ajuga genevensis*, LC-MS/MS, antioksidan aktivite, antimikrobiyal aktivite

INTRODUCTION

The Lamiaceae family includes more than 245 genera and 7886 species distributed worldwide.[1] *Ajuga* L. is a genus of annual and perennial herbaceous flowering plants in the Lamiaceae family, with most species native to Asia, Africa, and Europe. *Ajuga* is represented by 14 species and 27 taxa in Turkey.[2]

Ajuga has a long history of use for wound healing preparation, and although little used today, it is well known in Anatolia as "Mayasil otu". Some *Ajuga* species are widely consumed as diuretic, diaphoretic, astringent, antipyretic, and tonic in Turkish traditional medicine.[3]

Ajuga sp. plants are reported for their *in vitro* antimalarial,[4] antimicrobial,[5,6] antioxidant,[5] anti-inflammatory,[7] lipoxygenase, acetylcholinesterase, and butyrylcholinesterase inhibition,[8] antipyretic,[9] and antiproliferative[10] activities.

Phytochemical constituents diterpenoids, such as phenylethanoid glycosides, sterols, phytoecdysteroids, flavonoids, and iridoids were reported as the main active compounds in *Ajuga* L. species.[11]

Ajuga salicifolia sterol glycosides were isolated and tested for antimicrobial and cytotoxic activity.[12] Iridoid, ionone, and phenylethanoid glycosides from the same group were also reported for this species.[13]

Phytochemical profile of Romanian *Ajuga genevensis* L. and *A. reptans reptans* were recently reported.[6] A summary of phytochemical investigations on *A. salicifolia*, *A. reptans,* and *A. genevensis* species are listed in Table 1.[6-36] *In vitro* antioxidant and antimicrobial activity of different extracts of Romanian *A. genevensis* L. and *A. reptans* were recently reported.[6] Previous antimicrobial activity results of *A. reptans*, *A. genevensis* and *A. salicifolia* are listed in Table 2.[37-40]

Table 1. Literature data on phytochemical profile for *Ajuga* species

Species	Compounds	References
Ajuga reptans	Iridoid glycoside (ajureptaside)	19
Ajuga reptans	Iridoid glucosides (ajureptaside A-D)	24
Ajuga salicifolia	Iridoid, ionone and phenylethanoid glycosides (8-O-acetylharpagide corchoionoside C, leonosides A)	13
Ajuga genevensis	Neo-clerodane diterpenoids (ajugavensins A-C)	18
Ajuga salicifolia	Clerodane diterpene (ajugachin a derivative)	20
Ajuga reptans	Neo-clerodane diterpenes (ajugatansins)	21
Ajuga salicifolia	Sterol glycosides (ajugasalicioside A-E)	12
Ajuga reptans	Phytoecdysteroids (28-Epi-sengosterone)	36
Ajuga salicifolia	Stigmastane sterols (ajugasalicigenin)	22
Ajuga reptans	Anthocyanins (cyanidin)	25
Ajuga reptans	Anthocyanins (delphinidin)	26
Ajuga reptans	Anthocyanins (cyanidin and delphinidin glucosides)	27
Ajuga genevensis *Ajuga reptans*	Hydroxycinnamic acids (caffeic acid, chlorogenic acid), flavonoids (apigenin and luteolin-7-O-glucoside)	17
Ajuga genevensis	Hydroxycinnamic acids (caffeic acid, *p*-coumaric acid, ferulic acid), flavonoids (hyperoside, isoquercitrin, rutin, quercitrin, luteolin, apigenin)	6
Ajuga reptans	Hydroxycinnamic acids (*p*-coumaric acid, ferulic acid), flavonoids (isoquercitrin, rutin, quercitrin, luteolin, apigenin)	6

Table 2. Literature survey of antimicrobial activity for *Ajuga salicifolia, Ajuga genevensis, and Ajuga reptans*

Componuds/species	Microorganisms	MIC (mg/mL) inhibition zone (mm)	References
Ajugasalicioside A, B, C, D, E compounds from *A. salicifolia*	*B. cereus* ATCC 10702, *S. epidermidis* ATCC 12228 *S. aureus* ATCC 25923 *M. luteus* ATCC 99431 *P. aeruginosa* ATCC 27853 *C. albicans* ATCC 2579	No activity was found	12
MeOH extract of *A. reptans* MeOH extract of *A. reptans*	*F. oxysporum, F. verticillioides* *P. brevicompactum, P. expansum* *A. flavus, A. fumigatus* *F. oxysporum, F. verticillioides* *P. brevicompactum*	Range of 2.65 mm-31.65mm	37
Water extract from aerial parts of *A. genevensis*	*S. aureus* 209, *S. aureus* (Makarov) *S. aureus* Type, *S. epidermidis* Wood 46, *E. coli* 675, *S. gallinarum*, *P. vulgaris* *B. subtilis* L2, *B. anthracoides* 96	Range of 7 mm-15 mm	38
MeOH extract from *A. reptans*	*B. subtilis* ATCC 6633 *E. coli* ATCC 25922	8.5 mm-10.00 mm	39
Water, MeOH and EtOH extracts from aerial parts of *A. reptans*	*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. typhimurium* ATCC 14028, *S. marcescens* ATCC 8100 *P. vulgaris* ATCC 13315, *E. cloacae* ATCC 23355, *K. pneumoniae* ATTC 13883, *S. pyogenes* ATTC 19615 *S. aureus* ATTC 25923, *S. epidermidis* ATCC 12228	7.0 mm-11.7 mm	40
MeOH and EtOH extracts from flowers of *A. genevensis*	*S. aureus* ATCC 49444, *P. aeruginosa* ATCC 27853, *L.monocytogenes* ATCC 19114, *E. coli* ATCC 25922 *S. typhimurium* ATCC 14028	MIC value of 1.56-6.25	29
MeOH and EtOH extracts from flowers of *A. reptans*	*S. aureus* ATCC 49444, *P. aeruginosa* ATCC 27853, *L. monocytogenes* ATCC 19114, *E. coli* ATCC 25922 *S. typhimurium* ATCC 14028	MIC value of 1.56-6.25	28
EtOH, PE and Chl. extracts from aerial parts of *A. genevensis*	*A. flavus* ATCC 9643, *A. niger* ATCC 6275, *C. albicans* ATCC 10231, *C. parapsilosis* ATCC 22019, *P. funiculosum* ATCC 56755, *A. flavus* ATCC 9643	MIC value of 0.05-0.1	6
EtOH, PE and Chl. extracts from aerial parts of *A. reptans*	*A. niger* ATCC 6275 *C. albicans* ATCC 10231 *C. parapsilosis* ATCC 22019 *P. funiculosum* ATCC 56755	MIC value of 0.05-0.025	6

A. genevensis was used in traditional Austrian medicine and consumed as medicinal tea in treating respiratory tract disorders,[14] and *in vitro* anticancer activity studies were reported from Europe, Asia, and America.[15]

A. reptans grows natively in Europe and have bluish-purple flowers colored with anthocyanin pigments.[11] It was used in Mediterranean traditional medicine for cardiovascular complications and skin disorders[16] and in traditional Austrian medicine as a medicinal tea for the treatment of respiratory tract disorders.[14] A previous study showed that *A. reptans* L. is used due to the anti-inflammatory effects of its polyphenols, its wound healing properties, and antidiarrhea, antiulcerogenic, and hepatoprotective effects due to the presence of iridoids.[17]

In the present study, 70% methanol extract of aerial parts of *A. reptans* L., *A. salicifolia* (L.) Schreber, and *A. genevensis* L. from Turkey were evaluated for their phytochemical profiles, total phenol, and total flavonoid contents, as well as their *in vitro* antioxidant and antimicrobial activities. LC-MS/MS techniques

were used for phytochemical analyses. *In vitro* 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, and trolox equivalent antioxidant capacity (TEAC) assays were performed. Additionally, antimicrobial properties of *Ajuga* extracts were assessed against microbial strains of *Escherichia coli* NRRL B3008, *Staphylococcus areus* ATCC 6538, *Salmonella thyphimurium* ATCC 13311, *Bacillus cereus* NRRL B-3711, *Candida albicans* ATCC 90028, *Candida tropicalis* ATCC 1369, and *Candida parapsilosis* ATCC 22019.

To the best of our knowledge, this is the first study to perform the TEAC antioxidant activity for all extracts and the *in vitro* antimicrobial activity of *A. salicifolia*.

MATERIALS AND METHODS

Chemicals

Antimicrobial standards, Mueller Hinton Broth, and RPMI-1640 medium were purchased from Sigma-Aldrich Chemical Co (Sigma-Aldrich Corp., St. Louis, MO). All chemicals and solvents used were of analytical grade.

Plant materials

A. reptans L.: [A1(E) Kırklareli: İğneada, Fidanlık kavşağı, 350 m, N 41 52' 25.3'' E 27 56' 11'', 21 ıv 2009], *A. salicifolia* (L.) Schreber: (B3, Eskişehir: Çağlan köyü, 1000 m, K 390 39' 971'' D 300 31' 185'', 31 vi 2010), and *A. genevensis* L. [A1(E) Kırklareli: Dereköy yolu, 449 m, K 410 50' 6.13 D 270 18' 3.18'', 22 ıv 2009] were collected and identified by Dr. Y.B Köse, and herbarium materials were deposited in the Herbarium of Anadolu University, Faculty of Pharmacy under herbarium code YBK1560, YBK1575, and YBK 1561, respectively.

Preparation of extracts

The aerial parts of the plants were dried in the shade at room temperature and ground to powder in a mechanical grinder. Each species (1 g) was extracted with methanol (70%, 100 mL) for 24 h, three times a day. After filtration, the solvents were evaporated under vacuo.

Phytochemical analysis by liquid chromatography with tandem mass spectrometry (LC-MS/MS)

The phytochemical analyses were performed using LC-MS/MS techniques.[5]

Determination of phenolic compounds

The total phenols contained in the extracts were calculated using the Folin-Ciocalteu method equivalent to gallic acid (GA).[30] A sample solution (100 µL) and Folin-Ciocalteu reagent (500 µL) were added to a 10 mL scale vessel containing 6 mL of distilled water. After 1 min, 1.5 mL of 20% aqueous Na_2CO_3 was added and completed with water to reach 10 mL. The reagent-free of extracts was used as the control. After incubation at 25ºC for 2 h, the absorbance was read at 760 nm and compared with the GA calibration curve. The total amount of phenolic was calculated as equivalent to GA. Three parallel experiments were performed, and the results were reported as mean values.

Biological activity

DPPH radical scavenging assay

The DPPH radical scavenging activity was performed according to Kumarasamy et al.[31] For this purpose, 100 µL of methanol and samples were transferred to the first column of 96-well microtiter plates. A 10-well dilution was made in an equal amount of MeOH via a multi-channel pipette and stirred in the vortex for 5 min. The DPPH stock solution was prepared by dissolving 2 mg of DPPH• in 25 mL of MeOH, and solution was added to each well and left in a dark place for 30 min. Butylated hydroxy toluene (BHT) and GA at the same concentration were used as positive controls, and ultraviolet (UV) absorbance was measured at room temperature using a Biotek microplate spectrophotometer at 517 nm.

The following equations using 50% inhibition concentration (IC_{50}) (equation 1) and percentage (%) inhibition values (equation 2) were calculated as follows:

$$IC_{50} = [(A_0-A_1)/A_0] \times 100 \quad \text{equation (1)}$$

$$\text{Percentage Inhibition} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] x100 \quad \text{equation (2)}$$

TEAC assay

Experiments were performed as declared by Papandreou et al.[32] sweeping $ABTS^{\bullet+}$ (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) radical and vitamin E. It is based on the comparison of water-soluble analog with trolox. The mixture of 7 mM $ABTS^{\bullet+}$ and 2.5 mM sodium persulfate was kept in the dark for 12-16 h, resulting in the formation of blue-colored radicals. A sample (10 µL) and $ABTS^{\bullet+}$ solution (990 µL) were mixed, and absorbance was measured at 734 nm per minute intervals for 30 min. To find out the TEAC activity results, the $ABTS^{\bullet+}$ radical was plotted using Trolox's 2.5-2-1.5-1-0.5-0.1 (mM) concentrations, according to the % inhibition values. For quantification, a Trolox calibration curve was used where all experiments were repeated in triplicates.

Antimicrobial activity

Antimicrobial activity testing was performed according to the guidelines of broth microdilution methods.[33-35] Standard strains, *E. coli* NRRL B3008, *S. areus* ATCC 6538, *S. thyphimurium* ATCC 13311, *B. cereus* NRRL B-3711, *C. tropicalis* ATCC 1369, *C. parapsilosis* ATCC 22019, and *C. albicans* ATCC 90028, as well as antimicrobial standards, such as ampicillin, tetracycline, ketoconazole, and oxiconazole, were used in this study. Methanol extracts were prepared at 1250-2.44 µg/mL concentrations and dissolved in dimethylsulfoxide and initial test solutions. Serial dilutions were prepared at 64-0.125 µg/mL for ampicillin, tetracycline, and ketoconazole. All experiments were evaluated in triplicates, and mean values were reported.

Statistical analysis

Data obtained from antioxidant and total phenolic content experiments were expressed as mean standard error. IC_{50} values were estimated using a non-linear regression algorithm.

RESULTS AND DISCUSSION

LC-MS/MS analysis of the extracts

Screening of the extracts by LC-MS/MS enabled the identification of phenolic acids, such as coumaroyl glucoside, flavonoids, and phenylethanoid glycosides. Figures 1-3 show the 280 nm UV chromatograms of *A. reptans*, *A. genevensis* and *A. salicifolia*, respectively. The compounds detected from *Ajuga* sp. methanol extracts are listed in Table 3.

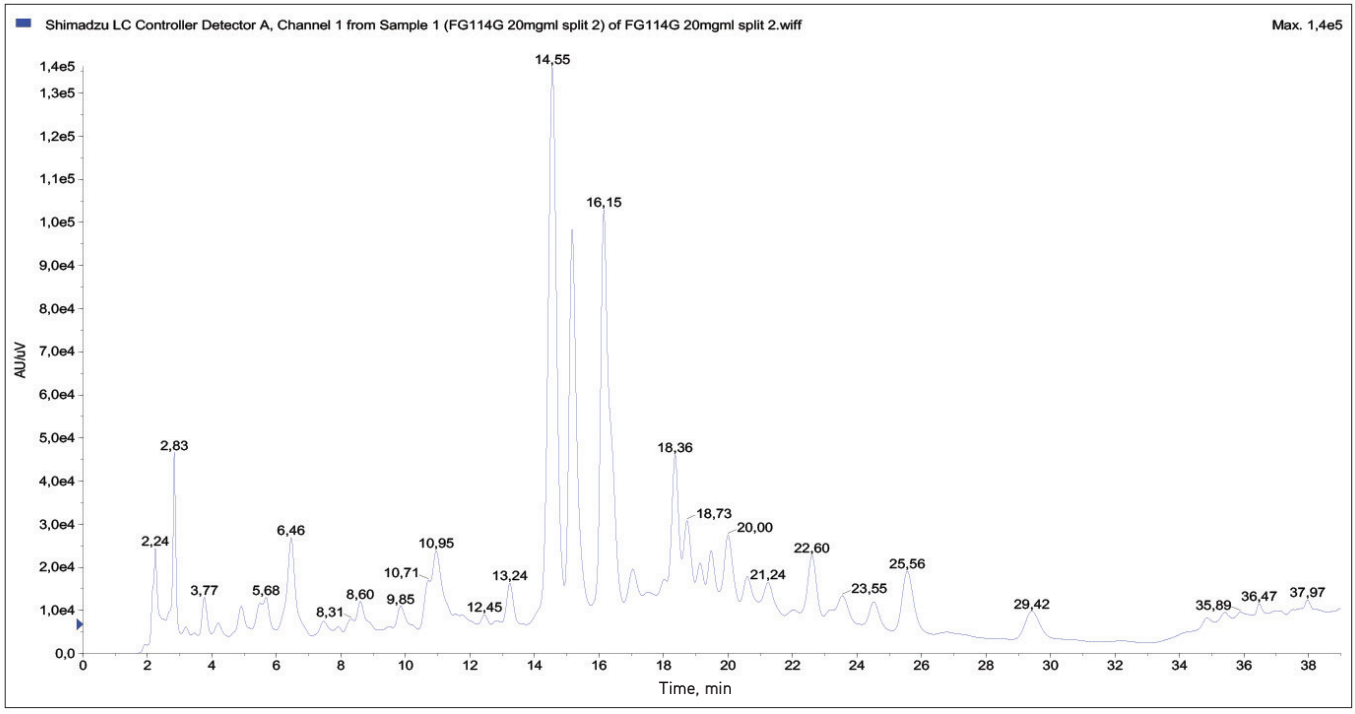

Figure 1. HPLC-UV chromatogram (280 nm) of *A. reptans*

HPLC: High performance liquid chromatography, UV: Ultraviolet

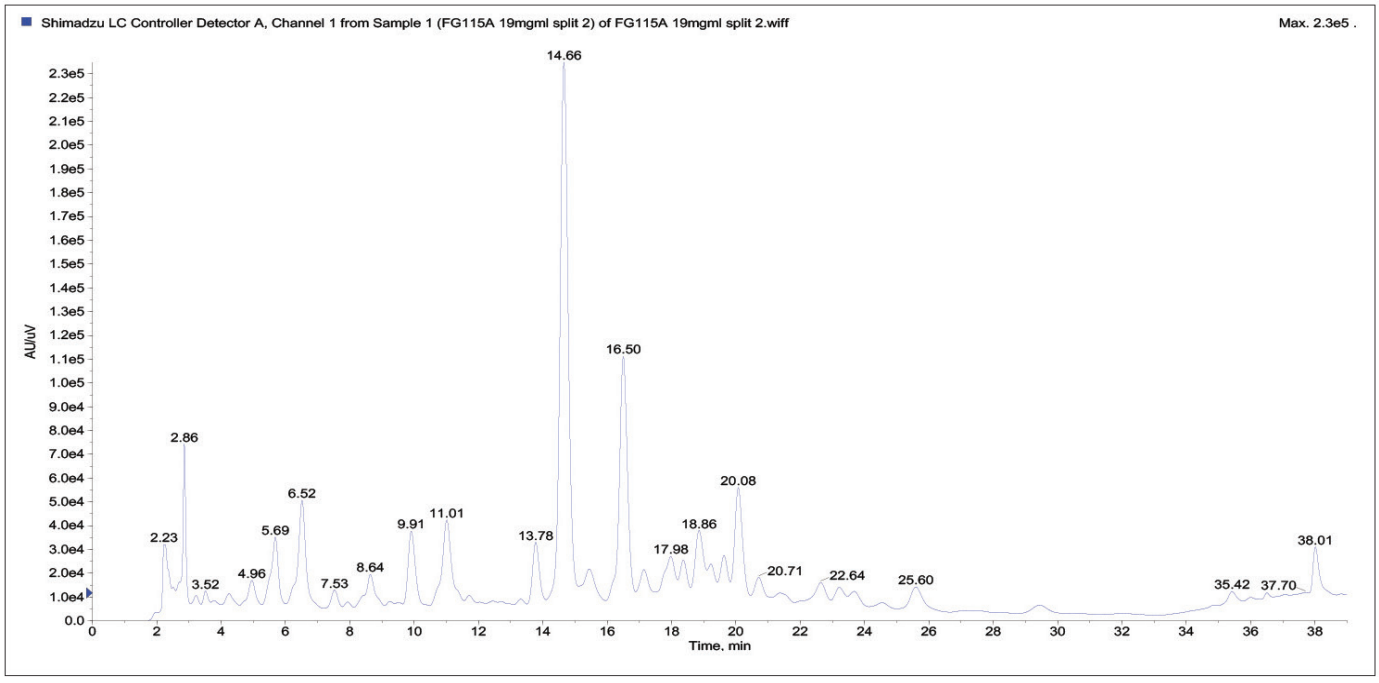

Figure 2. HPLC-UV chromatogram (280 nm) of *A. genevensis*

HPLC: High performance liquid chromatography, UV: Ultraviolet

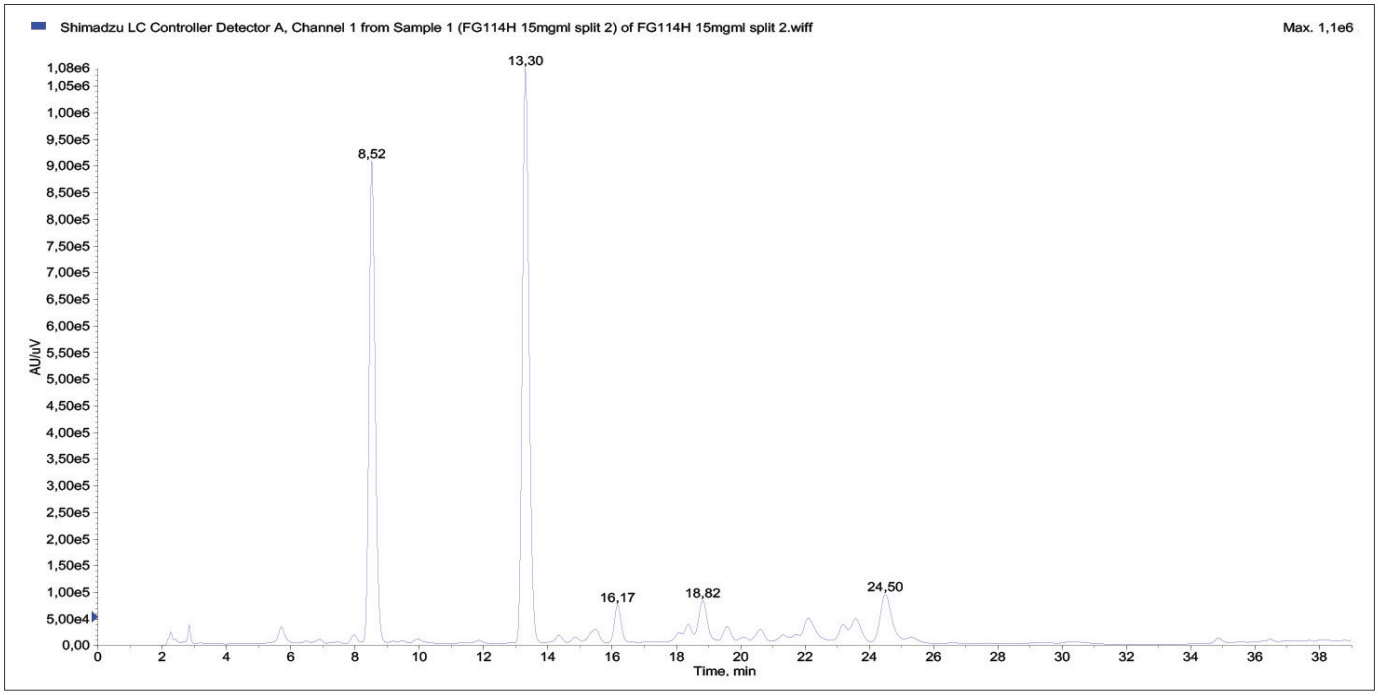

Figure 3. HPLC-UV chromatogram (280 nm) of *A. salicifolia*
HPLC: High performance liquid chromatography, UV: Ultraviolet

Table 3. Phytochemical composition of *A. reptans*, *A. salicifolia* and *A. genevensis* extracts

No	Compound	Rt	$[M-H]^-$	Fragments	Plant	Reference
1	Caffeoyl glucose	6.4	341	179, 161, 133	R	[41]
2	Coumaroyl glucoside	8.5	325	163, 119	S (major), G	[41,42]
3	Caffec acid	11.2	179	135	G	-
4	Coumaroyl glucoside isomer	13.3	325	163, 119	S (major), G	[41,42]
5	Luteolin derivative	14.6	487	285, 133, 117	R (major), G (major)	[43]
6	Apigenin-C-hexoside-C-pentoside	14.7	563	443, 383, 353	G	[44]
7	Echinocoside	15.4	785	623, 461, 161	R (major)	[44]
8	Forsythoside B	15.6	755	593, 461, 161	S	[45]
9	Forsythoside A	16.3	623	461, 315, 161	R (major), G (major)	[45]
10	Cistanoside A	18.5	799	623, 461, 175, 193	S, R	[45]
11	Leonosides A	18.9	769	593, 461, 315, 193, 175	S	-
12	Quercetin glucuronide	19.0	477	301, 227, 133	G	[47]
13	Verbascoside	19.2	623	461,315, 161	R	-
14	Leucoseptoside A	19.6	637	461, 175	R, S	[45]
15	Luteolin glucuronide	20,1	461	285	R, G (major)	[43]
16	Luteolin glucoside	21.6	447	285	S	[46]
17	Luteolin	35.4	285	175,133	G	[6,29]
18	Apigenin	37.9	269	149,117	G	[29]

R: *A. reptans*, S: *A. salicifolia*, G: *A. genevensis*

As a result, the only substance commonly identified in all *Ajuga* species was forsythoside A and luteolin glucuronide.

The coumaroyl glucose and its glucoside isomer (Figure 4, 5) were determined for *A. salicifolia* extract. The echinacoside (Figure 6) was detected in *A. reptans* extract. The LC-MS/MS sprectrum of luteoline derivative (Figure 7), forsytoside A (Figure 8), and luteoline glucuronide (Figure 9) were observed in both *A. reptans* and *A. genevensis* extracts.

The phenolic acids as caffeic acid and flavonoids; apigenin-C-hexoside-C-pentoside, quercetin glucuronide, luteolin, and apigenin were identified only for *A. genevensis.* Furthermore, the phenylethanoid glycosides forsythoside B and leonosides A were identified only for *A. salicifolia*. The phytochemical research on *Ajuga* species focus on the isolation of flavonoids, caffeic and chlorogenic acid type derivates, phenylethanoid glycosides, phytoecdysteroids, iridoids, and diterpenes.[12,13,17-24] Some anthocyanins, delphinidin, and cyanidin 3-*O*-sophoroside-5-*O*-glucosides, were acylated with *p*-coumaric acid, while ferulic acid and malonic acid were isolated from the flowers of cell cultures of *A. reptans.*[25-27]

Total phenolic amounts of the extracts

The amount of total phenolics ranged from 30.0 to 42.2 mg GA equivalent (GAE)/g of the extracts. The phenolic amounts equivalent to GA in all three methanol extracts are shown in Table 4. The highest total phenolic level was found in the methanol extract of *A. reptans.* In previous studies, the total phenolic content of methanol extracts of *A. reptans* and *A. genevensis* has been evaluated to be 20.86±0.53 mg RE/g dw[28] and 22.63±0.61 mg GAE/g.[29]

DPPH radical scavenging activity

DPPH radical scavenging activity results are presented in Table 4. The positive control, BHT with IC_{50} value of 0.06 mg/mL, was found as the most potent antioxidant. The highest radical scavenging activity were obtained for *A. salicifolia* (IC_{50}: 0.28±0.01 mg/mL) and *A. reptans* (IC_{50}: 0.30±0.01 mg/mL) extracts. A correlation was also found between radical scavenging capacity and total phenol content. Previous studies showed the antioxidant activity of the methanol extract of *A. genevensis* flowers as IC_{50}: 72.08±6.02 µg/mL[29] and *A. reptans* as IC_{50}: 83.16±5.21.[28]

However, there have been no reports on the antioxidant activity of *A. salicifolia.* This study is the first to determine the antioxidant activity of *A. salicifolia*.

TEAC assay

The results obtained for the evaluation of the antioxidant activity using TEAC assay are presented in Table 4. $ABTS^{\bullet+}$ radical sweeping impact results are in parallel with the results of the DPPH radical scavenging effect. Extracts from all three plants show $ABTS^{\bullet+}$ radical scavenging activity at 1% concentrations, but these effects are not as high as the BHT used as the standard.

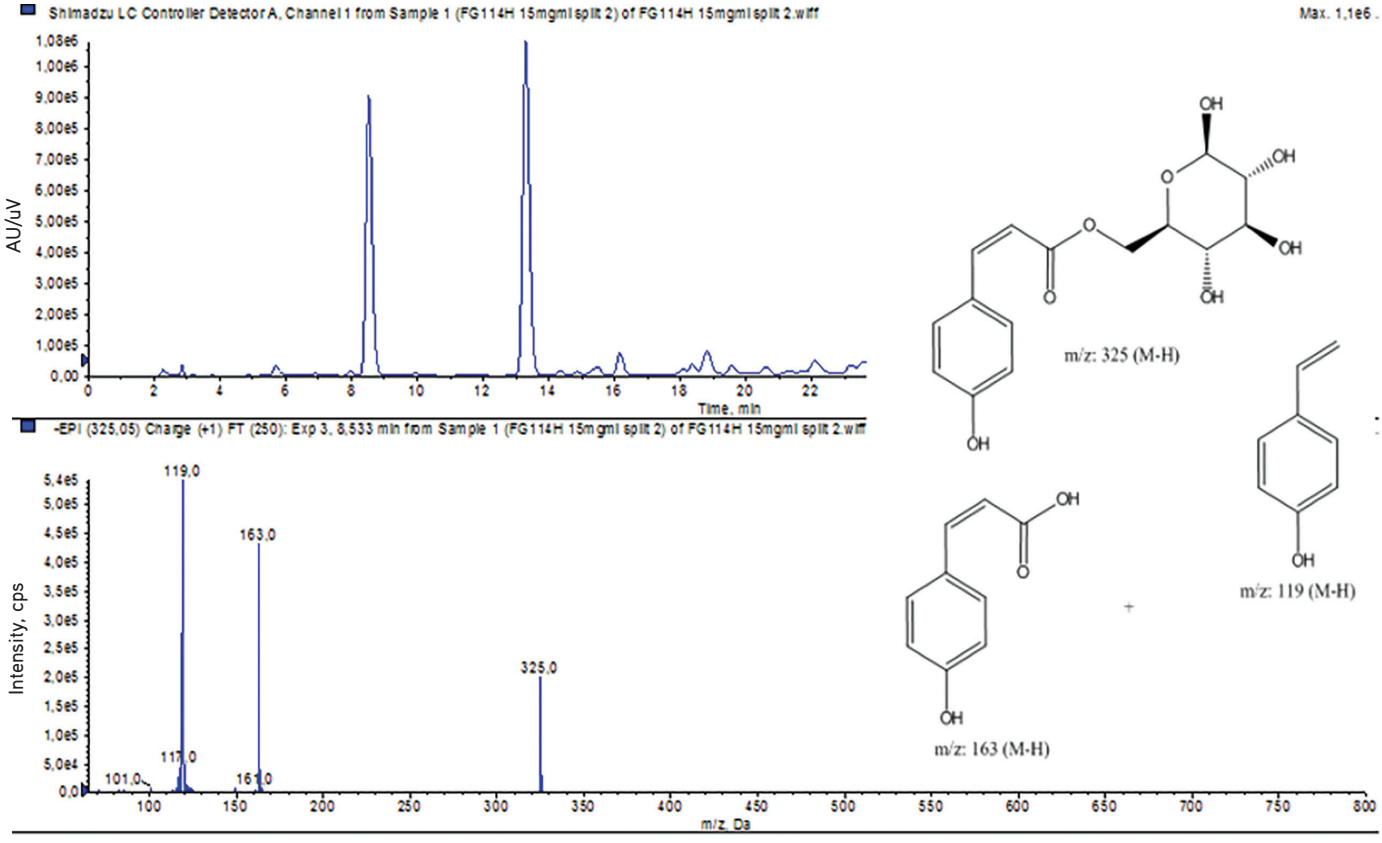

Figure 4. LC-MS/MS sprectrum of in coumaroyl glucose (2) in *A. salicifolia* extract

LC-MS/MS: Liquid chromatography with tandem mass spectrometry

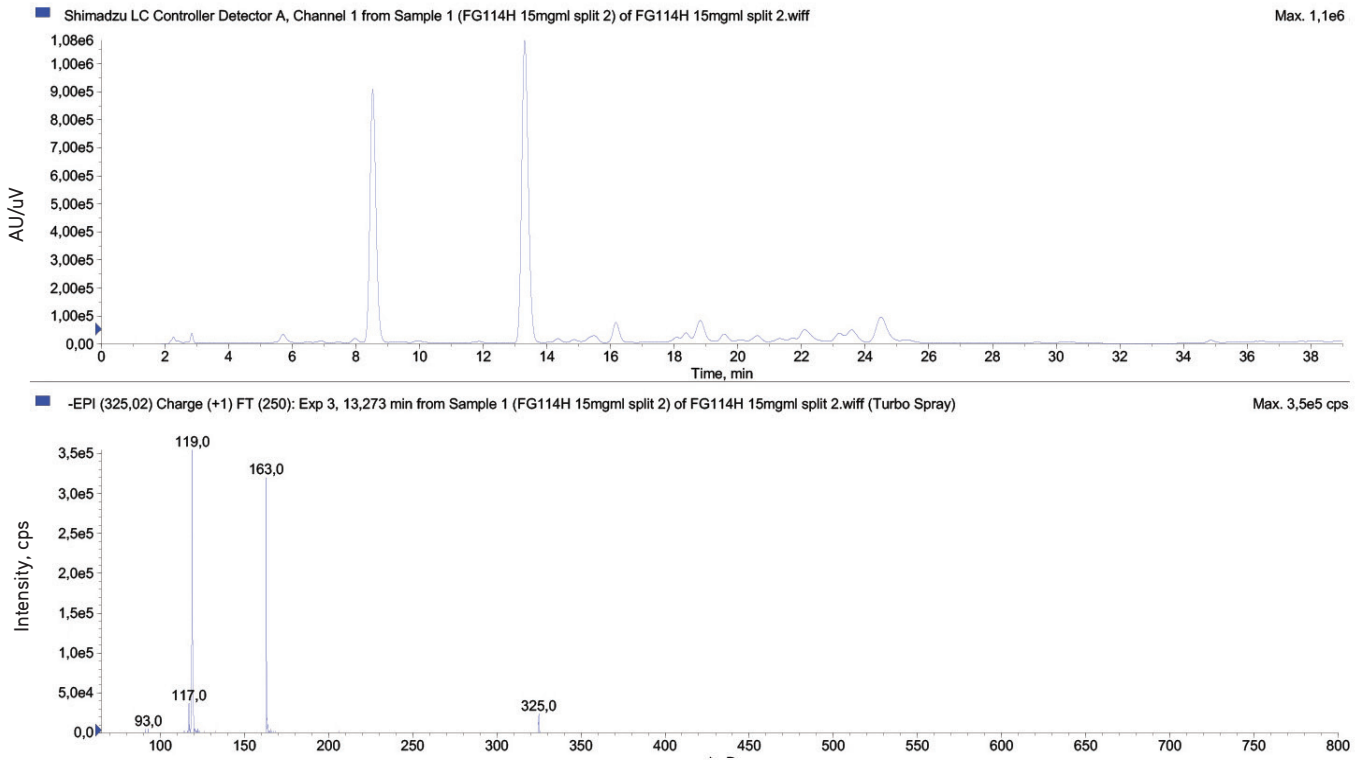

Figure 5. LC-MS/MS sprectrum of in coumaroyl glucoside isomer (4) in *A. salicifolia* extract
LC-MS/MS: Liquid chromatography with tandem mass spectrometry

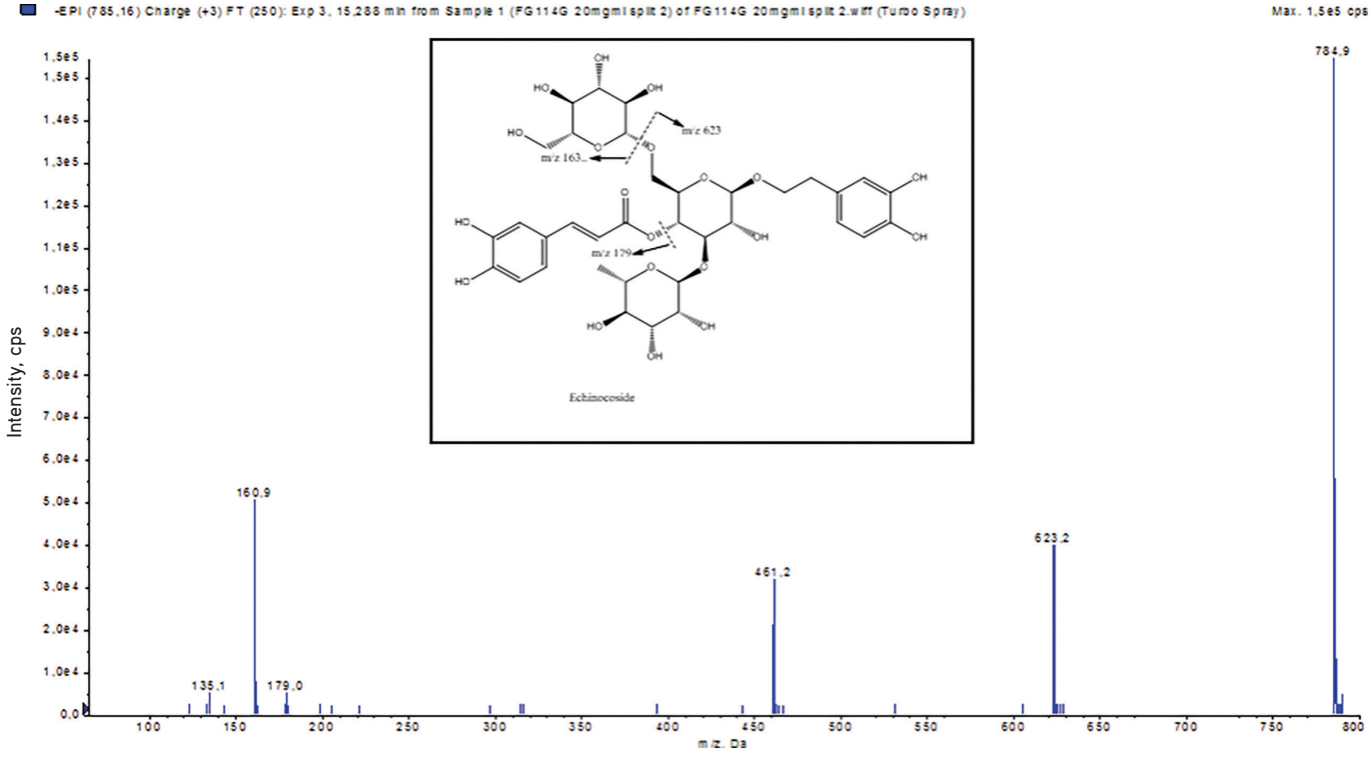

Figure 6. LC-MS/MS sprectrum of echinacoside (7) in *A. reptans* extract
LC-MS/MS: Liquid chromatography with tandem mass spectrometry

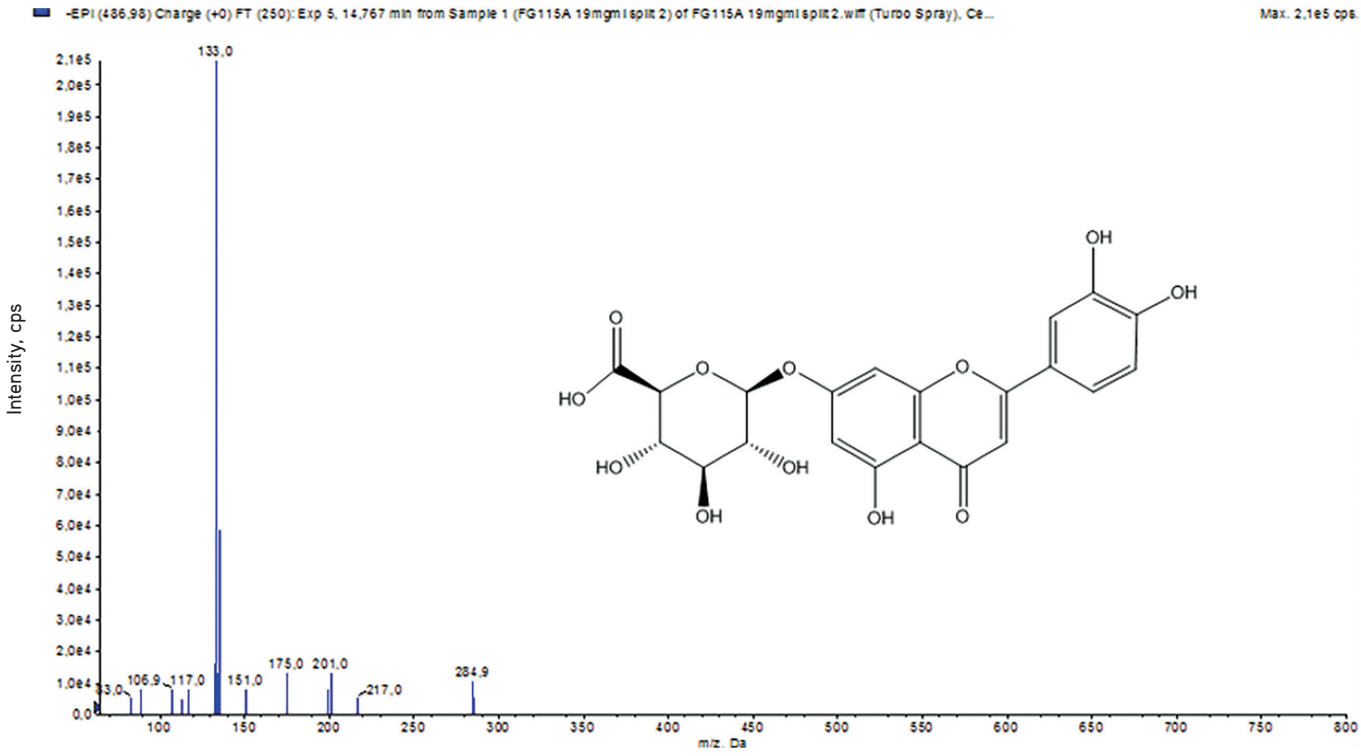

Figure 7. LC-MS/MS sprectrum of luteoline derivative (5) in *A. reptans* and *A. genevensis* extracts
LC-MS/MS: Liquid chromatography with tandem mass spectrometry

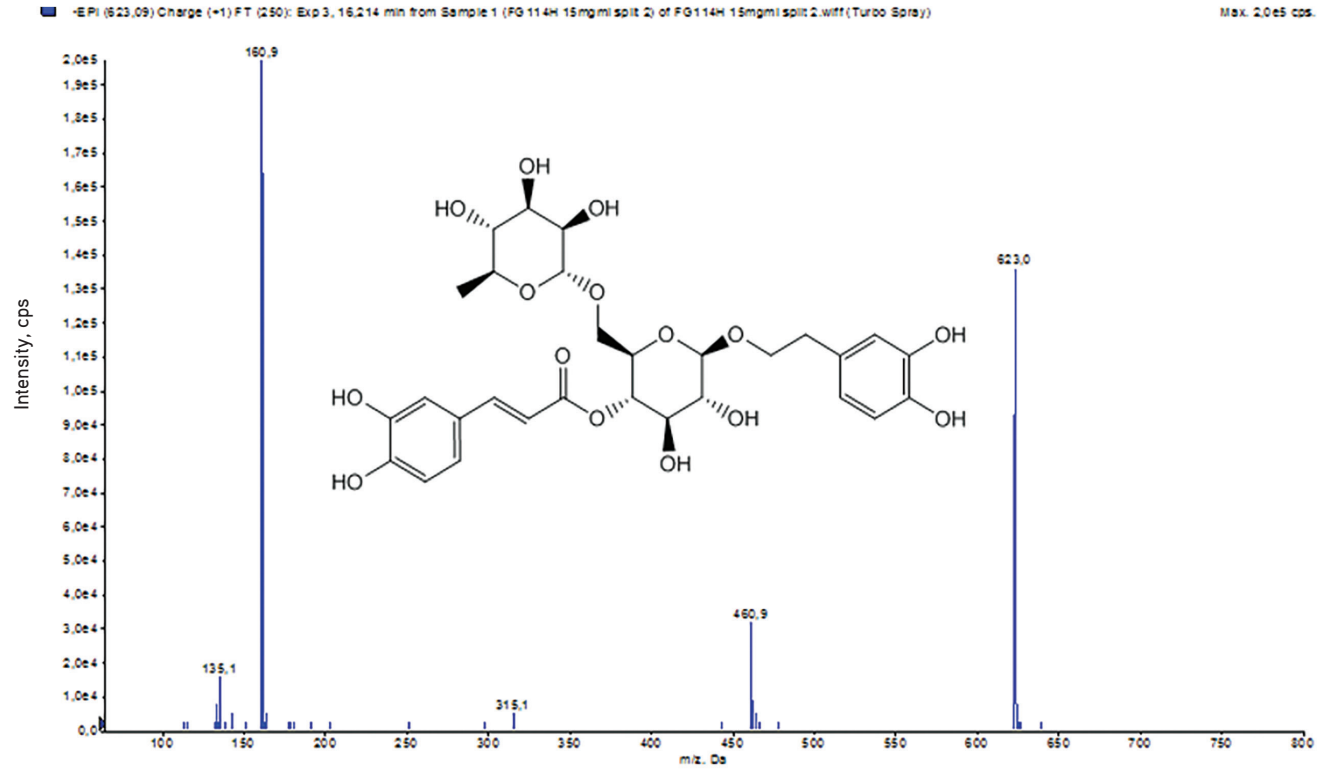

Figure 8. LC-MS/MS sprectrum of forsytoside A (9) in *A. reptans* and *A. genevensis* extracts
LC-MS/MS: Liquid chromatography with tandem mass spectrometry

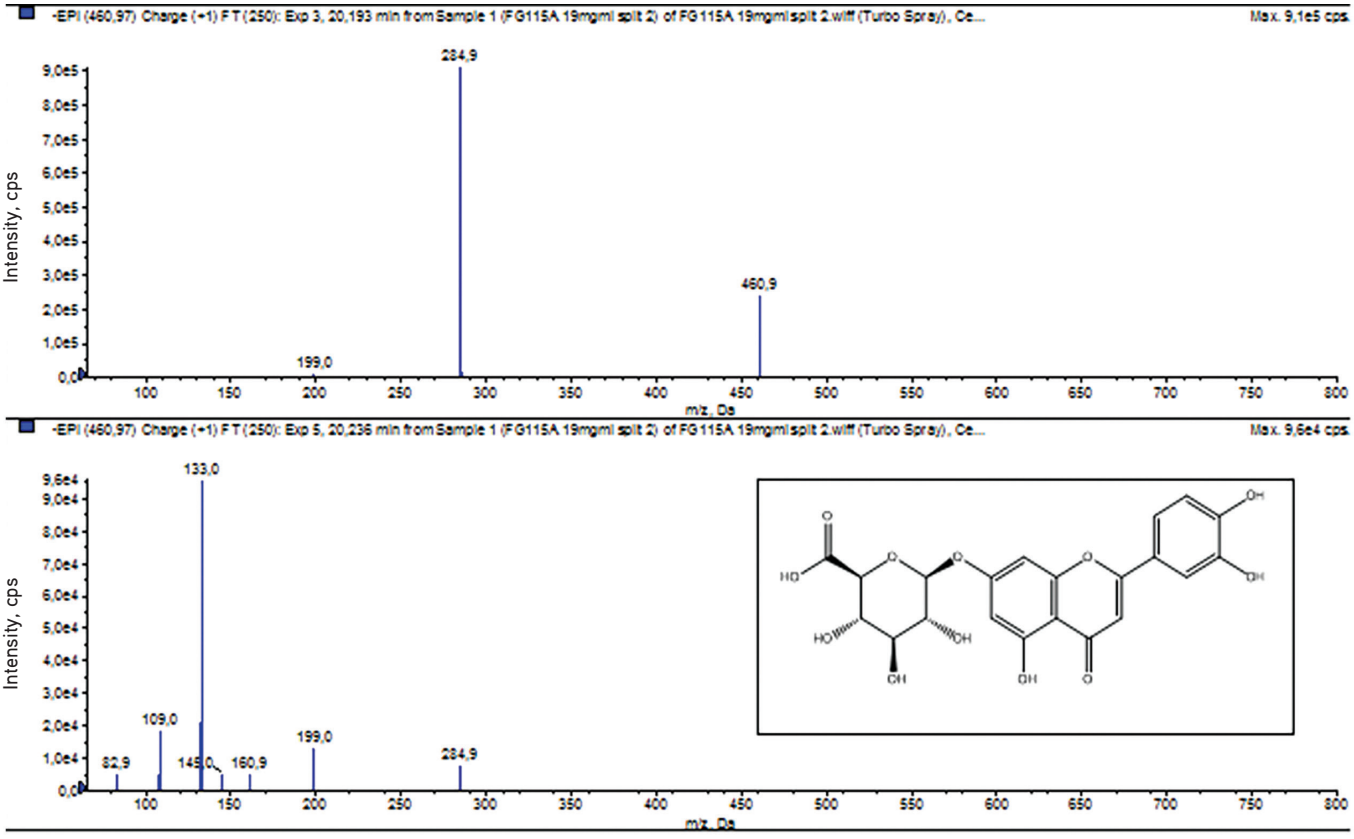

Figure 9. LC-MS/MS sprectrum of luteoline glucuronide (15) in *A. reptans* and *A. genevensis* extracts
LC-MS/MS: Liquid chromatography with tandem mass spectrometry

Table 4. Total phenolic contents and antioxidant activities of the extracts

Extract	Total phenolic content (mg GAE/g extract)*	DPPH activity (IC_{50}, mg/mL)	TEAC (mM)
A. reptans	42.0±0.01	0.30±0.01	1.4±0.02
A. salicifolia	38.0±0.00	0.28±0.01	1.5±0.02
A. genevensis	30.0±0.01	0.44±0.02	1.2±0.04
BHT	-	0.06±0.00	1.9±0.03

*Total phenolic content was expressed as GAE, each value in the table is represented as the mean ± SD (n=3). TEAC: Trolox equivalent antioxidant activity, GAE: Gallic acid equivalent, BHT: Butylated hydroxy toluene, DPPH: 1,1-diphenyl-2-picrylhydrazyl, SD: Standard deviation, IC: Inhibition concentration

There have been no reports on the antioxidant activity performed by a TEAC assay. Antioxidant activity was performed for the first time in this study for *A. reptans* L., *A. salicifolia* (L.) Schreber, and *A. genevensis* L. species found in Turkey.

Antimicrobial activity

The seven different strains tested in this study are presented in Table 5. *Ajuga* L. extracts showed antimicrobial effect against all microorganisms tested and were more effective against yeasts than bacteria [minimum inhibitory concentration (MIC): 156.25 µg/mL]. The MIC was 312.5 µg/mL for *E. coli* NRRL B-3008, *S. areus* ATCC 6538, *S. thyphimurium* ATCC 13311, and *B. cereus* NRRL B-3711 for methanol *Ajuga* extracts. As a result, the antimicrobial activity was observed in *Ajuga* extracts, especially against *Candida* strains.

Data from previous studies on the antimicrobial activity of *A. reptans*, *A. genevensis,* and *A. salicifolia* are listed in Table 2. To the best of our knowledge, this is the first report on the antimicrobial evaluation for methanol extract of *A. salicifolia,* which was more effective against yeast than bacteria.

CONCLUSION

We disclose the phytochemical profiles of *A. reptans*, *A. salicifolia,* and *A. genevensis* collected from Turkey.

Table 5. Minimum inhibitory concentrations (µg/mL)

Microorganisms	Extracts			Standard antimicrobials			
	A. genevensis	*A. reptans*	*A. salicifolia*	KT	OXC	AMP	TCY
Escherichia coli NRRL B-3008	312.5	312.5	312.5	-	-	4	16
Staphylococcus aureus ATCC 6538	312.5	312.5	312.5	-	-	2	8
Salmonella typhimurium ATCC 13311	312.5	312.5	312.5	-	-	2	8
Bacillus cereus NRRL B-3711	312.5	312.5	312.5	-	-	0.5	4
Candida albicans ATCC 90028	156.25	156.25	156.25	4	1	-	-
Candida tropicalis ATCC 1369	156.25	156.25	156.25	2	0.5	-	-
Candida parapsilosis ATCC 22019	156.25	156.25	156.25	2	2	-	-

KT: Ketoconazole, OXC: Oxiconazole, AMP: Ampicillin, TCY: Tetracycline, (-): Not tested

The extracts were found to contain valuable metabolites; phenolics acids, coumaroyl glucoside, flavonoids, and phenylethanoid glycosides. The phytochemicals could be employed as potential chemotaxonomic markers because different phytochemicals were observed between the three *Ajuga* species.

The scope of this study included the biological potential of methanol extracts of *A. reptans*, *A. salicifolia,* and *A. genevensis* evaluated for the first time against some pathogenic strains.

Ajuga species may be considered a valuable natural source against *Candida* infections and candidal resistance. However, further *in vitro* and *in vivo* experiments using different alternative *Candida* and fungal species are required to validate these screening results.

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