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Investigation of Bioactive Compounds on Relict Endemic *Ajuga relicta* P. H. Davis (Lamiaceae) from Turkey

Nazan Comlekcioglu [1,*], **Yusuf Ziya Kocabas** [2], **Ashabil Aygan** [1]

[1] Kahramanmaras Sutcu Imam University, Faculty of Science and Letters, Department of Biology, Kahramanmaras, Turkey
[2] Kahramanmaras Sutcu Imam University, Turkoglu Vocational High School, Medicinal Plants Department, Kahramanmaras, Turkey

Abstract: Species belonging to genus *Ajuga* (Lamiaceae) have been used to treat many diseases in traditional medicine. The plants of the genus *Ajuga* have been reported to have antifungal, antibacterial, antimycobacterial, antihypertensive, antiplasmodial, hypoglycaemic, and larvae and insect activity. *Ajuga relicta* is a relict endemic plant which grows only in Kahramanmaras. The total phenolic contents of the extracts have been quantified with Folin Ciocalteu colorimetric method, and the antioxidant activities of the extracts have been tested with DPPH, and FRAP. Antimicrobial activities of plant extracts were determined by the well-diffusion method against seven bacteria and four yeasts. Besides, the fatty acid composition was determined in GC-MS. As a result of GC-MS analysis of the oil obtained from the *A. relicta* extracts, 21 different fatty acids were identified. The highest contents of these fatty acids were palmitic acid (29.50%), oleic acid (23.51%), stearic acid (9.13%) and linoleic acid (7.18%). Total phenolic value of plant extract was 11.94 mg/g, total flavonoid amount 2.28 mg/g, FRAP value 43.53 µg/g and DPPH value 1.63 mg/g. Antimicrobial activity experiment on a total of 11 microorganisms (seven bacteria and four yeast) showed that *Ajuga* extracts inhibited the growth of tested microorganisms except *Enterobacter cloacae*, *Candida glabrata* and *Saccharomyces cerevisiae*. *A. relicta* was found to have high bioactive content and antimicrobial activity. The plant extracts are rich in constant fatty acids and similar to olive oil (palmitic-oleic-linoleic), which is well known for its health benefits in terms of major fatty acids.

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1. INTRODUCTION

Plants are generally unique sources of new drugs that play an important role in the treatment of human diseases. Infectious diseases are one of the most important problems the communities face all over the world. Because of the adverse side effects of synthetic drugs and the emergence of antibiotic-resistant bacteria, new natural compounds with broad activity against bacterial strains are required. Plant secondary metabolites are excellent candidates for developing new phytopharmaceuticals with various biological activities. However, herbal

*CONTACT: Nazan Comlekcioglu ✉ noktem80@gmail.com Kahramanmaras Sutcu Imam University, Faculty of Science and Letters, Department of Biology, Kahramanmaras, Turkey

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medicines have not been scientifically researched enough. It is often applied on the basis of knowledge and experimental observations of traditional healers [1]. In this context, new investigations on medicinal plants or herbal products, which traditionally used but not scientifically researched, will greatly, contribute to the development of new herbal medicines or herbal formulations [2].

Species belonging to Lamiaceae family are known for their biological activities and especially their antioxidative properties. Lamiaceae is represented by 236 genera and 7200 species worldwide [3]. Lamiaceae is the third largest plant family in Turkey, 46 genera and 580 species are represented. Of these species, 260 are endemic and the endemism rate is approximately 45% [4, 5]. The *Ajuga* genus, a member of the Lamiaceae family, has more than 300 species of annual and perennial herbaceous flowering plants distributed in the temperate regions of Asia, Europe, Australia, North America and Africa [6]. *Ajuga* has 13 species (6 endemic) and 10 subtypes (1 endemic) in Turkey [4].

Several species of the genus *Ajuga* (Lamiaceae) are used in African and Asian folk medicine. These plants are used as folk remedies in the world for effective antihelminthic, tightening, antifungal, and anti-inflammatory agents as well as rheumatism, fever, toothache, dysentery, malaria, hypertension, diabetes and gastrointestinal disorders [7]. In addition to these features, this plant is used against eczema, tonic, menstrual diuretic, wounds and as an antidote against the bites of venomous animals in Turkey [8]. This study was done with *A. relicta*, which is known very little about it. The plant was first collected in 1907 from Kahramanmaraş Ahırdağı. But the plant is no longer seen in Ahırdağı. It is estimated that *A. relicta* has disappeared as a result of the elimination of moist habitats around 1830 m and heavy grazing [9]. *A. relicta*, an herbaceous perennial plant, is an endemic species. It is a plant belonging to the old glacial period and it is only grown in the world in Kahramanmaraş, Çimendağı/Yavşan Plateau. Recently, the demand for *Ajuga* species, which is considered to be an ornamental plant, has increased significantly in addition to its medical and pharmacological properties. Although the genus *Ajuga* has been widely studied, there is very little literature about *A. relicta*. There is no study about *A. relicta* in the literature other than systematic, steroids and terpenoids, morpho-anatomical study and antioxidant activity [5, 10-12].

The aim of this study was to investigate the antioxidant and antimicrobial activities, total phenol and flavonoid content of aerial parts of endemic *A. relicta*, whose taxa are in restricted areas and exhausted over the years. Fatty acid content of plant extracts was also investigated by using GC-MS. Although the antioxidant activity and total phenolic content of *A. relicta* has been previously investigated, but total flavonoid content, antimicrobial activity, fatty acid content has been investigated for the first time.

2. MATERIAL and METHODS

2.1. Plant materials

The plants used in this study were collected from openings in the forested areas of Çimendağı-Yavşan Plateau at an altitude of approximately 1500 m, on 20 July 2015 (Figure 1). Plants were identified according to Flora of Turkey [4]. A voucher specimen was deposited in the Herbarium of the KSU [YZK-980].

2.2. Sample Preparation and Extraction

The plants have been dried for a week (in a room temperature) and then powdered by grinding in a Waring blender. The extraction was performed by using Soxhelet apparatus at 60 °C for 6 hours with the addition of methanol (100 ml) on to 10 g of the plant material. After elimination of the solvent in a vacuum rotary evaporator at 40 °C, the extract was stored at -20 °C for further analysis [13]. Total phenolics, total flavonoids, antioxidant activity and

antimicrobial activity were analyzed using these obtained extracts. Total phenolics, total flavonoids, antioxidant activity and antimicrobial activity were also analyzed on this extracts.

Fig 1. Appearance of *A. relicta* in nature (a), flower (b).

2.3. Determination of ash and protein content

The ash content was analyzed according to the European standard method UNIEN 14775 [14]. Protein content of the samples was assayed by using AOAC method [15]. All experiments were done in triplicate.

2.4. Antioxidant Assay

2.4.1. *Determination of Total Phenolic and Flavonoid Content*

Total phenolic contents of the fractions were determined using the Folin–Ciocalteau colorimetric method [16]. The total flavonoid content in leaf extracts was determined spectrophotometrically [17]. All experiments were performed in triplicate.

2.4.2. *DPPH and FRAP Analysis*

Scavenging free radical potentials were analyzed using 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) [18]. Ascorbic acid was used as positive control. The results were indicated as IC50 value which is the concentration of sample required to scavenge 50% of DPPH free radicals. The FRAP assay was carried out according to Benzie and Strain [19]. All experiments were done in triplicate.

2.5. Antimicrobial Assay

The antimicrobial activities were researched using the well-diffusion method. The test microorganisms were *Enterobacter cloacae* ATCC 13047D, *Escherichia coli* ATCC 39628, *Klebsiella pneumonia*, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 6538P, *Sarcina lutea* ATCC 9341NA, *Candida albicans*,

Candida parapsilosis, *Candida glabrata* and *Saccharomyces cerevisiae*. Mueller Hinton agar plates were cultured with standardized inoculums (10^8 cfu/ml) of each bacterial strain and also Sabouraud dextrose agar were cultured with each of yeast strains (2.1 x 10^3 cfu/ml) [20]. Extracts (50 µl) were added into wells and the plates were incubated at 37 ^oC for 16-18 h. After incubation, the diameter of inhibition zones was measured by a compass. DMSO was used as solvent control since it was used as a solvent for extraction. The plant extracts showing antimicrobial activity were then tested to determine the MIC values in a microwell plate [20].

2.6. Determination of Fatty Acid Content

Total 0.1 g of plant extract was mixed with 1 ml of KOH solution prepared with 2 N methanol and then vortexed for 2 min. After 15 minutes, 10 ml of hexane was added and the mixture was stirred well. After centrifugation at 7000 rpm for 10 min, 1 microliter of the upper phase was injected into the GC-MS device [21]. GC-MS analyses were quantified using a Shimadzu 2025 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a Shimadzu AOC-20i automatic sampler (Shimadzu, Kyoto, Japan). The condition of GC analysis was as follows: flame ionization detector (FID) 250 °C; column TR-CN100, 60 m × 0.25 mm × 0.20 mm (Teknokroma); carrier gas He with a flow rate of 1.5 mL/min. Fatty acid peaks were identified against the chromatogram of a mixed fatty acid methyl ester standard (37 Comp. FAME Mix 10 mg/mL in CH_2Cl_2; Supelco, USA). The injector and detector temperatures were kept at 250 °C. The column oven temperature was programmed at 80 °C for 2 min initially, then 5 °C/min up to 140 °C (maintained for 2 min at 140 ^oC), and then 3 °C/min up to 240 °C (maintained for 5 min at 240 ^oC). The injection and detector temperatures were set at 240 and 250 °C, respectively. The fatty acids were expressed as percentage of the total fatty acids, calculated with peak areas.

3. RESULT and DISCUSSION

3.1. Protein, ash, fatty oil content and fatty acid composition of *A. relicta*

The results of protein, ash and oil content in leaves of *A. relicta* ranged to 6.36%, 8.64% and 4.53%, respectively. Fatty acid composition in leaves of *A. relicta* is given in Table 1 and GC MS chromatogram is given in Figure 2.

21 fatty acids were found as a result of fatty acid analysis of *Ajuga* plant extract (Table 1). The major components in oil were palmitic acid (29.50 %), oleic acid (23.51 %), stearic acid (9.13 %), and linoleic acids (7.18 %). The researchers indicated that the need for saturated fats for energy, hormone production, cellular membranes and organs [22]. Total 50% of the fatty acids of *Ajuga* are saturated fats. Some saturated fatty acids are also necessary for essential signalling and stabilization processes in the body. Saturated fatty acids that play an important role in these processes are known as palmitic acid, myristic acid and lauric acid [23]. *A. relicta*, which contains all three fatty acids, has palmitic acid predominantly. The most commonly found and produced fatty acids in animal fats are palmitic, stearic and oleic acids [24, 25]. Additionally, C16:0, C18:0, and *cis*-9 C18:1 are typically the most abundant FA found in commercial fat supplements commonly fed to dairy cows. The increase in the the level of palmitic and oleic acid gave better results in terms of energy quality [25]. These two fatty acids are also the major fatty acids in *Ajuga* oil. Essential fatty acids that animals cannot produce and must take from outside are linoleic acid (LA) (omega-6), arachidonic acid (AA) (omega-6), gamma linolenic acid (GLA) (omega-6), alpha linolenic acid (LNA) (omega-3), eicosapentaenoic acid (EPA) (omega-3) and docosahexaenoic acid. (DHA) (omega-3). According to our result, *A. relicta* contains all of these fatty acids in different proportions.

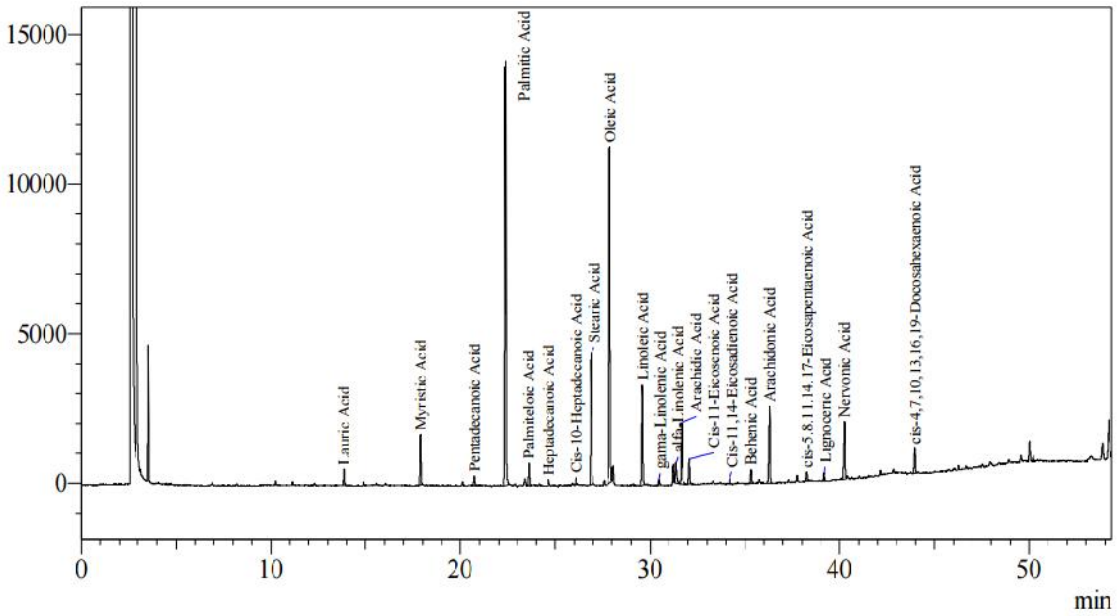

Figure 2. GC-MS chromatogram analysis of *A. relicta* extract

Table 1. Fatty acid compositions (%) of the *A. relicta*.

No	Carbon Number	Fatty Acids	Content (%)
1	C12:0	Lauric Acid	0.89 ± 0.02
2	C14:0	Myristic Acid	3.27 ± 0.01
3	C15:0	Pentadecanoic Acid	0.68 ± 0.01
4	**C16:0**	**Palmitic Acid**	**29.50 ± 0.03**
5	C17:0	Heptadecanoic Acid	0.38 ± 0.01
6	**C18:0**	**Stearic Acid**	**9.13 ± 0.01**
7	C20:0	Arachidic Acid	4.73 ± 0.01
8	C21:0	Behenic Acid	0.92 ± 0.00
9	C24:0	Lignoceric Acid	0.50 ± 0.00
10	C16:1	Palmitoleic Acid	1.60 ± 0.01
11	C17:1	Cis-10-Heptadecanoic Acid	0.48 ± 0.00
12	**C18:1**	**Oleic Acid**	**23.51 ± 0.01**
13	C20:1	Cis-11-Eicosenoic Acid	1.86 ± 0.00
14	C24:1	Nervonic Acid	4.46 ± 0.01
15	**C18:2**	**Linoleic Acid**	**7.18 ± 0.01**
16	C20:2	Cis-11,14-Eicosadienoic Acid	0.38 ± 0.00
17	C18:3	Gamma-Linolenic Acid	0.49 ± 0.00
18	C18:3	Alfa-Linolenic Acid	1.90 ± 0.00
19	C20:4	Arachidonic Acid	5.66 ± 0.01
20	C20:5	cis-5,8,11,14,17-Eicosapentaenoic Acid	0.69 ± 0.00
21	C22:6	cis-4,7,10,13,16,19-Docosahexaenoic Acid	1.79 ± 0.00
		SFA	50.0
		MUFA	31.91
		PUFA	18.09

3.2. Total phenolic, flavonoid contents and antioxidant activity of *A. relicta*

One of the most important points to be considered when working on the biological activities of plants is to elucidate the phytochemical composition. Herbal extracts consist of a mixture of very different phytochemicals. There is a strong relationship between phytochemical content and pharmacological potential of plants. One of the most studied and important phytochemical components of plants are phenolic compounds [26]. Various studies have shown that phenolic compounds are common in *Ajuga* species and may contribute to their antioxidant activity [2]. In this study, the total phenolic and flavonoid amounts of *A. relicta* extracts were determined in order to establish a relationship between the phenolic components and the biological activities of extracts. Phenolic, flavonoid and antioxidant activity properties were studied in *Ajuga iva* plant and methanolic extract showed the highest performance against hexane, chloroform and water extracts [27]. The researchers concluded that methanol is the most suitable solvent for the extraction of phenolic compounds and determination of antioxidant activity. Therefore, methanol was preferred as extraction solvent in this study. The results of antioxidant activity, total phenolic and flavonoid contents in *A. relicta* species are listed in Table 2.

Table 2. Total phenolic and flavonoid contents with antioxidant activity in the extracts of *A. relicta*

Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg QE/g)	IC_{50} of DPPH% (mg dw/ml)	FRAP (µg AAE/g)
11.94 ± 0.66	2.28 ± 0.64	1.63 ± 0.13	43.53 ± 0.48

The total phenolic content of *A. relicta*'s methanolic extracts was 11.94 mg GAE/g dw and the total flavonoid content was 2.28 mg QE/g dw. In the literature, the total phenolic content and IC_{50} value of the methanolic extract of *A. relicta* reported as 34.6 mg GAE/g and 0.205 mg/ml, respectively [12]. Although plants were collected from the same location and in the same year, we have obtained a significant decrease compared to Sönmez and Köse [12] in terms of phenolic content and IC50 value. The decrease may be due to seasonal differences and vegetative maturity. Collection time of the plant in their study [12] was at the beginning of June while our collection time was at the end of July. Since the June is flowering stage, while the July is seeding stage of plant. The total phenolic content of *A. relicta* was found to be lower than the methanolic extract of *A. laxmannii* (56.76 mg GAE/g dw) [2]. The total phenolic content in flower portions of *A. reptans* and aerial fragments of *A. chamaecistus* subsp. *scoparia* were 20.86 mg GAE/g dw and 20.32 mg GAE/g dw, respectively [28, 29]. The total phenolic content of *A. relicta* was found be higher than *A. reptans* and *A. chamaecistus* subsp. *scoparia*. In previous studies, Toiu et al. [28] found a TFC value of 12.38 ± 0.22 mg RE/g dw for a methanol extract of *A. reptans* flowers. The flavonoid content of *A. laxmannii* was 36.14 ± 0.53 mg RE/g dw; water, ethyl acetate, methanol and acetone extract of *A. chamaepitys* L. Schreb TFC value 9.32 ± 0.33, 91.76 ± 0.81, 63.87 ± 0.66, and 61.77 ± 0.51 mg RE/g dw, respectively [2, 30].

Numerous test systems have been developed to determine the antioxidant activity of plant extracts. Each test system evaluates the antioxidant activity of the test material from a different perspective [31]. The best way to measure the antioxidant activity of a plant extract is to combine two or more complementary test systems. In this study, antioxidant activity of *A. relicta* extracts was evaluated using radical scavenging and reducing power analysis. DPPH analysis was used to determine the radical scavenging activity of the extract and FRAP analysis was used to determine the activity of reducing power (Table 2). According to the data presented in the table, the clearance activity of the extract on DPPH radicals was calculated as IC_{50} value

and was determined to be 1.63 mg dw/ml. In FRAP analysis, the FRAP activity of *Ajuga* extract was obtained as 43.53 µg AAE/g.

Phenolic compounds are considered to be functional bioactive compounds. According to the results of many studies, these compounds are also the main compounds that contribute to the antioxidant activities of plants [32]. The high antioxidant activity of *A. relicta* extracts can be explained by the high phenolic compound contents.

3.3. Antimicrobial activity of *A. relicta*

Most of the therapeutic agents used in disease treatment are obtained from plant sources. The number of plant species that are under investigation to reveal their therapeutic potential, have been increasing day by day [26]. Despite all these efforts, there are many plant species that have not yet been investigated for their biological and/or pharmacological potential. *A. relicta*, which is the subject of this study, is one of these plants. According to the results of our literature review, no data is available in the literature concerning the antimicrobial activity of *A. relicta*.

The antimicrobial effect of the methanolic extract of *A. relicta* was examined against seven bacteria and four yeasts. The results showed that the methanolic extracts of *A. relicta* have significant inhibitory activity against all tested bacteria except *E. cloaca*. The inhibition zones of the methanolic extract of *A. relicta*, which were obtained against all test bacteria, were in the range of 10-12 mm and 9 mm for tested fungi (Table 3). When the MIC values were examined, it was seen that *A. relicta* extract had higher inhibitory effect on *E. coli* than other bacteria. The highest inhibitory activity was determined against *E. coli*. On the other hand, the weakest inhibitory activity was determined against *E. faecalis, K. pneumonia* and *S. lutea*.

Table 3. The antimicrobial activity of *A. relicta* against test microorganisms

Test bacteria	Inhibition Zone (mm)	Gentamicin (mm)	MIC (mg/ml)
Bacillus subtilis ATCC6633	10	21	12.5
*E. Cloaca**	-	16	NT
*Enterococcus faecalis**	12	26	25
Escherichia coli 309628	12	24	6.25
*Klebsiella pneumonia**	11	28	25
*Staphylococcus aureus**	10	25	12.5
Sarcina lutea ATCC 9341NA	12	28	25
Test fungus		Nystatine (mm)	
*Candida albicans**	9	18	1.562
*Candida glabrata**	-	18	NT
*Candida parapsilosis**	9	18	12.5
Saccharomyces cerevisia	-	24	NT

*Clinical isolate, NT: Not tested, -: No inhibition zone

Among the 4 yeast strains, *C. parapsilosis and C. albicans* was inhibited by *A. relicta* extracts, while *Candida glabrata* and *Saccharomyces cerevisiae* were not affected. It could be an important property having an inhibitory and non-inhibitory activity against pathogenic and non-pathogenic strains, respectively, for food and pharmaceuticals. In terms of extraction method, although various the most promising results were obtained with the activity of *A. relicta* against *C. albicans* (MIC: 1.562 mg/ml). Around the world, so many plants were screened by many researchers with different methods against different microorganisms. Here in this study, the methanolic extract of *A. relicta* obtained was tested against common

microorganisms. As a result, these extracts seem to be reasonably effective against test organisms including clinical isolates. Plant phenolic compounds are known to be responsible for a variety of biological properties, including antimicrobial properties [27]. Therefore, it is thought that the antimicrobial activity of *A. relicta* extracts is related to the phenolic and flavonoid compounds of the plant.

4. CONCLUSION

In this study, phenolic and flavonoid content, antioxidant, antimicrobial activity and fatty acids of *A. relicta*, which is endemic and a relict species were evaluated. Other species belonging to the genus *Ajuga* spreads in 5 continents around the world and used for medical purposes among the population. *A. relicta* is a forgotten plant because of its naturally spread in a very narrow environment and also narrowing with various pressures. With this study, *A. relicta* was found to have high bioactive content and antimicrobial activity, as well as other members of the genus. It has been found that plant extracts show profile rich in constant fatty acids and show a profile similar to olive oil (palmitic-oleic-linoleic), which is well known for its health benefits in terms of major fatty acids.

Orcid

Nazan Çömlekcioğlu https://orcid.org/0000-0001-7729-5271
Yusuf Ziya Kocabaş https://orcid.org/0000-0003-2831-8910
Ashabil Aygan https://orcid.org/0000-0003-4936-9872

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