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Investigation of Biological and Chemical Effects of Extracts from *Arum rupicola* Boiss. var. *rupicola*

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Abstract

The present study aimed to evaluate the fatty acid and phenolic profiles, and antioxidant and antibacterial activities of *A. rupicola*. For this purpose, For this purpose, the chemical content of the methanol extract was first determined using the HPLC-TOF/MS method. Thereafter, five extracts having different polarities (hexane-hexane, hexane-chloroform, methanol-hexane, methanol-chloroform and methanol-butanol) were obtained using the partitioning method, and the chemical contents of these extracts were determined by the GC-MS method. According to these data, various fatty acids and phenolic contents were observed. Furthermore, the antioxidant studies were performed with total phenolic compounds and ABTS, while antibacterial studies were performed with the microdilution test and the disc diffusion test using three bacteria, one gram-positive and two gram-negative bacteria. The antioxidant and antibacterial tests revealed that *A. rupicola* is both an antioxidant and an antibacterial plant. According to the obtained results, the microdilution test was shown to be more effective than the disc diffusion test. On the other hand, the inhibition percent vs. extract concentration graph showed that the MH was the most effective inhibitor. In this study, it was revealed that the methanol extract and five extracts from *A. rupicola* had a variety of phenolic compositions and fatty acids, respectively, and these five extracts also possess antioxidant effects and antibacterial activities.

1. Introduction

There has been a strong relationship between humans and plants throughout history. Today, this occurs in a variety of forms, in particular, use of plants for nutrition, fiber, drugs, and energy for humans and animals. Plants are a very important part of our lives, religion, and wealth, as well as our nourishment [1]. Plants and humans are a part of the whole which is survival for lives. They cannot be concerned to be a differ part of each other.

Arum L. is a part of the Araceae family in the flowering plants. It includes 29 species and covers a wide area of the world, from North Africa, Europe, and west and central Asia to China [2-4]. *Arum* genus plants contain many phytochemicals such as polyphenols, alkaloids, glycosides, proanthocyanidins, 2-heptanone, indoles, p-cresol, (E)-caryophyllene, monoterpenes, two sesquiterpenes, and lectin [5]. In Turkey, *Arum* genus includes a total of 18 taxa, 14 species, and 4 varieties, and its cosmopolitan species cover Turkey [6-8]. Palestinian folkloric medicine used *Arum* genus leaves in the treatment of cancer, cough, worms, constipation, hemorrhoids and urinary tract infections

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[9-11]. It is known only for the treatment of hemorrhoids in Turkey [12].

The isolation of pure compounds from natural products, namely plants, is very important. However, this cannot be easy and need to more time for studding of natural products. After the extraction process, a couple separation techniques come. One of the separation techniques is the solvent partitioning method. This method usually involves the use of two insoluble solvents in a separating funnel. Natural products are dispersed in solvents depending on the solvents different partition coefficients. This technique is very effective as a first step in separating large amounts of compounds from the crude extract [13].

Some extracts from the plant and its metabolites biologically exhibited antibacterial [14], antimicrobial [15], antifungal [16], and antioxidant activities [17]. The bioactive components that block pathogens and have low toxicity to host cells should be considered for designing new antimicrobial drugs [18]. These compounds exist in many parts of plants such as stems, roots, leaves, barks, flowers, fruits, and seeds [19]. Alkaloids, tannins, flavonoids, phenolics [20], and fatty acids [21-23] are medicinally the most important among these compounds.

Several subspecies of *Arum* species have been studied in the past, including *'Arum elongatum* Steven' and *'Arum rupicola* Boiss. var. *virescens'*. These studies focused on the different chemical and medical properties of isolates of the *Arum* subspecies. In a survey by Özok and Güneş, the antidiabetic effect of *A. rupicola* was investigated. For this purpose, the dried form of *Arum rupicola* Boiss. var. *virescens* was extracted in water, and then its antidiabetic activity was observed in vivo [24]. In another study by Alan, the antimicrobial and antioxidant effects of ethanol and purified water extracts of the *Arum elongatum* Steven plant and their role in preventing DNA damage were investigated [25]. In this study, the antimicrobial and antioxidant effects of "*'Arum rupicola* Boiss. var. *rupicola'*" extracts obtained with different solvents and their combinations were studied for the first time. For the purpose, five extracts (hexane-hexane (HH), hexane-chloroform (HC), methanol-hexane (MH), methanol-chloroform (MC), and methanol-butanol (MB)) from *A. rupicola* collected from mountains of the Gevas district were obtained using the partition method with polar and nonpolar solvents. The antibacterial activity of these extracts was studied on *Staphylococcus aureus* and *Bacillus subtilis*, gram-positive bacterial strains, and *Escherichia coli*, gram-negative bacterial strain. Also, the fatty acid contents of these extracts were detected by GC-MS analysis.

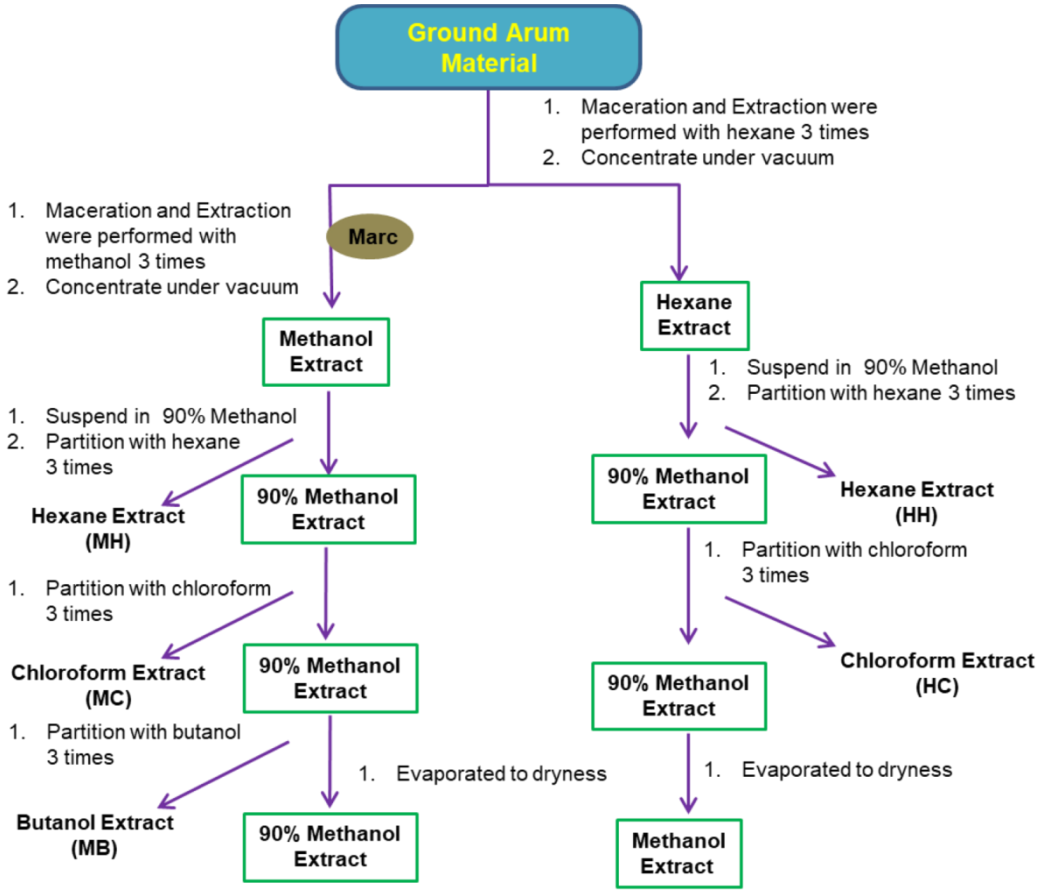
2. Material and Methods

2.1. Material

In brief, all organic solvents, tryptic soy broth (TSB) and tryptic soy agar (TSA) were obtained from Merck. *Arum rupicola* Boiss. var. *rupicola* (*A. rupicola)* was collected from mountains of the Gevas district (Van province, Turkey) in May 2019. It was identified by Dr. Suleyman Mesut Pinar and then updated by Dr. Mehmet Fırat in a previous study [26].

2.2. Extraction protocol

Extractions of *A. rupicola* were prepared as presented in Scheme 1. This procedure was performed with minor modification and configuration [13]. Five extracts were obtained, and these were coded as hexane-hexane (HH), hexane-chloroform (HC), methanol-hexane (MH), methanol-chloroform (MC), and methanol-butanol (MB).



Scheme 1. The extraction and sub-fraction protocol for *A. rupicola*

2.3. HPLC-TOF/MS analysis for phenolic content of the *A. rupicola* methanol extract

Phenolic components data for polar extracts from *A. rupicola* were detected by HPLC-TOF/MS (Agilent 1260 infinity LC, 6210 TOF-MS with column Zorbax SB-C18) analysis, as described in the literature [27]. For this, the methanol extract obtained from the extraction protocol was used. The spectrum obtained from this method is given in Figure 1.

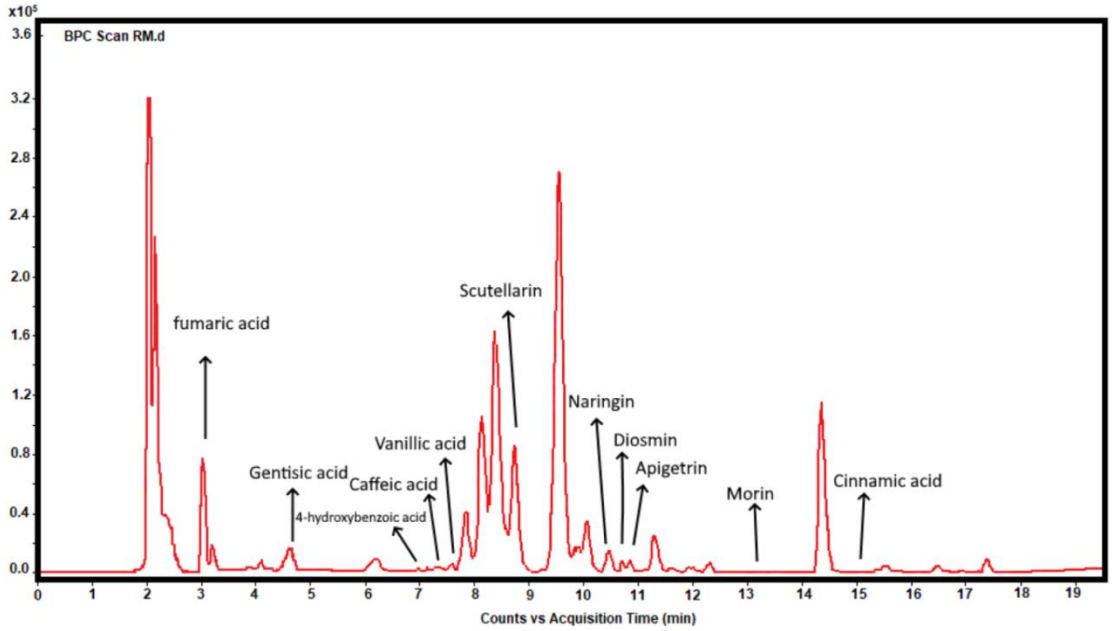


Figure 1. HPLC-TOF analysis spectrum for methanol extract of *A. rupicola*

2.4. GC-MS analysis for fatty acid profiling

The fatty acid content of the *A. rupicola* extracts was determined using GC-MS (Thermo ITQ 900 with column Thermo TR-5ms SQC) analysis. For this purpose, the five extracts were firstly removed from their solvent and then 20-30 mg of these extracts was dissolved in 3 ml of 2M KOH solution in methanol. This mixture was combined with 3 ml of hexane and these were vigorously shaken using a vortex for 20 minutes. After all experimental processes, two different phases were obtained. These were esterified and hexane solutions were added. The hexane solution was carefully extracted from the lower phase and added into the vial to determine fatty acid content [27]. The GC-MS spectrum for the MB extract is presented in Figure 2.

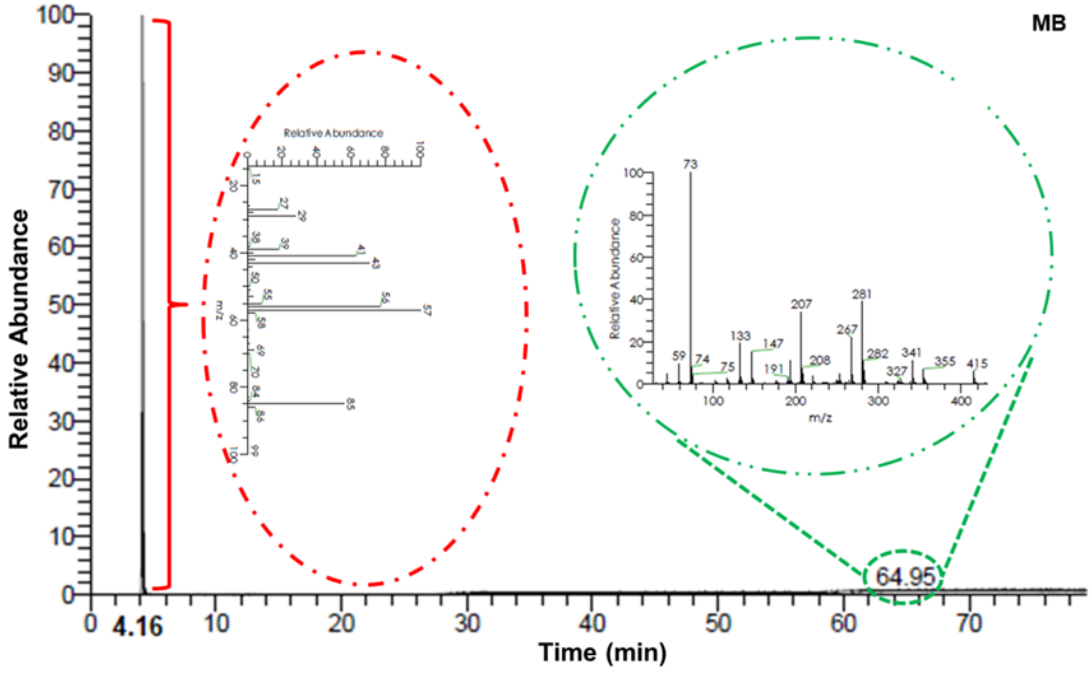


Figure 2. The GC-MS spectrum for the MB extract

2.5. Trolox equivalent antioxidant capacity (TEAC)

$ABTS^{\bullet+}$, a radical cation and a reagent, is obtained from oxidation of the ABTS reactive with persulfate to determine the antioxidant activity of different materials. This assay was carried out according to the literature [28-30] and was used for the activity of extracts from *A. rupicola*. In brief, to obtain the $ABTS^{\bullet+}$ solution, the ABTS solution was firstly prepared in water at 7 mM concentration, and then potassium persulfate with final concentration of 2.45 mM was created by using the ABTS stock solution. This mixture was placed in the dark at room temperature for 16 h to complete the reaction. For the antioxidant capacity study of *A. rupicola* extracts, the spectrophotometric value of the $ABTS^{\bullet+}$ solution should be in the range of 0.700 ± 0.02 (A_s) at $\lambda = 734$ nm before starting measurements. For this, dilution was performed using ethanol. Different concentrations (from 12.5 mL to 100 mL) of *Arum* extracts were mixed with 2.0 mL ABTS•+ solution and the spectrophotometric values were read in the 6th minute (A_f). These values were applied to the following equation and the radical scavenging activities were found.

$$inhibition\% = \left[{\left(A_s - A_f\right)}/{A_s} \right] \times 100 \qquad (1)$$

The Trolox graph was drawn by using the concentration of Trolox vs inhibition%. The TEAC value for *Arum* extracts at different concentrations was calculated from this Trolox standard graph.

2.6. Determination of total phenolic contents for *Arum* extracts

This assay was carried out according to Velioglu et al. and used the Folin-Ciocalteu reagent as standard. All extracts were prepared in DMSO at a concentration of 1 mg/mL. For this, 100 µL concentration of the extracts and 0.75 mL of Folin-Ciocalteu reagent diluted 10 times using deionized water were mixed and vortexed in a test tube. The mixture was incubated at room temperature for 5 min. A triple mixture was obtained to adding 0.75 mL of sodium carbonate (6% w/v) and then vortexed gently. After these steps, the mixture was incubated at room temperature for 90 min, and then spectrophotometric data were obtained at 725 nm using an UV−Vis spectrophotometer (T80+ UV/VIS Spectrometer, PG Ins. Ltd.). A variety of concentrations (0.1−0.01 mg/mL) of gallic acid as standard were used to draw the standard calibration graph. The total phenolic content was mean gallic acid equivalent in milligram per 100 g of each extract. The phenolic content analysis of each extract was performed in triplicate [31-32].

2.7. Antibacterial activities of the extracts

To determine the antibacterial activities of extracts from *A. rupicola*, all extracts were dissolved in DMSO (dimethyl sulfoxide) and these concentrations were prepared to 1 mg/mL. The gram-negative bacteria strain used was *Escherichia coli* (ATCC-8739) and two gram-positive bacteria strains of *Bacillus subtilis* (DSM-347) and *Staphylococcus aureus* (ATCC-6538) were used. The antibacterial activities of extracts were evaluated by the disc diffusion test and micro-dilution assay. These were performed as mentioned in the literature [33]. Firstly, bacteria were inoculated on TSB medium independently and grown overnight at 37 ℃. For the disc diffusion test, 10 µL of bacterial culture was spread on the surface of agar plates separately. Then, 30 µL of extract was dropped on agar plates individually and finally incubated at 37 ℃ overnight. For the micro-dilution assay, 100 µL of bacterial culture was added to a tube containing 20 ml of TSB liquid medium. Then, 30 µL of extract was added to this tube. Finally, tubes were incubated at 37 ℃ overnight. These tubes were spectrophotometrically assayed at 600 nm and compared to control tubes that only contained bacteria.

3. Results and Discussion

3.1. Phenolic contents in the plant

Phenolic content of the methanol extract of *A. rupicola* was examined with HPLC-TOF/MS analysis. According to this analysis, 11 types of phenolic compounds were found and these were fumaric acid, scutellarin, gentisic acid, naringin, 4-hydroxybenzoic acid, diosmin, caffeic acid, apigenin, vanillic acid, morin and cinnamic acid, given in Table 1. Among these, fumaric acid had the highest amount, followed by scutellarin and diosmin. In a previous study about phenolic contents in the plant, the phenolic content of ethanol and pure water extracts of *A. elongatum* were investigated with HPLC analysis [25]. Caffeic acid, vanillic acid, and cinnamic acid were the same, while the other components were different in this paper. As a result, *A. rupicola* contains more phenolic compounds. Some of them were found with developed instrumental techniques, HPLC-TOF/MS analyses, and methods.

Table 1. Phenolic compounds in methanol extract

Phenolic	Retention time	Results mg phenolic/kg plant
Fumaric acid	**3.29**	**277.2**
Gentisic acid	**4.50**	**1.13**
4-hydroxybenzoic acid	**6.96**	**13.6**
Caffeic acid	**7.45**	**1.12**
Vanillic acid	**7.87**	**1.14**
Scutellarin	**9.73**	**19.46**
Naringin	**10.50**	**0.7**
Diosmin	**10.62**	**17.16**
Apigenin	**10.79**	**2.75**
Morin	**13.01**	**1.69**
Cinnamic acid	**15.16**	**1.44**

3.2. Fatty acid contents in the plant

Fatty acids in five different extracts from *A. rupicola* were detected using GC-MS analysis and these are listed in Table 2. There are many different studies about the fatty acid contents of *A. rupicola* and other *Arum* varieties. The fatty acids in these were analyzed by GC-MS in seeds of *A. maculatum* [34], diethyl ether fraction of *A. palaestinum* [35], methanol extract of *A. dioscoridis* [36], acetone extract of *A. cyrinaicum* [37], methanolic and acetone extracts of *A. dioscoridis* [38], and sub-fractions of hexane extract from *A. rupicola* [26]. In this study, the fatty acids in *A. rupicola* were researched and traced more widely compared to the previous studies.

Table 2: Fatty acid content for each extract from *A. rupicola*

Sample Code	Fatty acid[a]	RT*	Area %	Molecular Formula	Molecular Weight
HH	Butane, 2,2,3-trimethyl-	4.47	19.27	C7H16	100
	2-tert-Butyl-4-isopropyl-5-methylphenol	13.61	0.11	C14H22O	206
	Dimethoxyglycerol docosyl ether	17.31	0.02	C27H56O5	460
	Spirost-8-en-11-one,3-hydroxy, (3á,5à,14á,20á,22á,25R)	20.12	0.01	C27H40O4	428
	2-Pentadecanone, 6,10,14-trimethyl-	20.85	0.10	C18H36O	268
	Hexadecanoic acid, methyl ester	24.04	0.98	C17H34O2	270
	Octadecanal,2-Bromo-	29.30	79.52	C18H35BrO	346
HC	Hexane, 2-methyl-	4.18	21.59	C7H16	100
	1,1,3,3,5,5,7,7,9,9,11, 11-dodecamethylhexasiloxane	75.51	78.41	C12H38O5Si6	430
MH	Hexane, 2-methyl-	4.31	92.41	C7H16	100
	Pregnane-3,11,20,21-tetrol, cyclic 20,21-(butylboronate), (3à,5á,11á,20R)-	23.66	0.13	C25H43BO4	418
	Stigmast-5-en-3-ol, (3á)-	40.08	7.46	C29H50O	414
MB	Butane, 2,2,3-trimethyl-	4.16	21.81	C7H16	100
	1,1,3,3,5,5,7,7,9,9,11,11-dodecamethylhexasiloxane	67.29	78.19	C12H38O5Si6	430
MC	Cyclopentane, methyl-	4.0	0.22	C6H12	84
	1,1,3,3,5,5,7,7,9,9,11, 11,13,13,15,15-hexadecamethyloctasiloxane	74.36	99.78	C16H50O7Si8	578

[a] Names of fatty acids were taken from Wiley GC-MS library
*Retention Time

3.3. The evaluation of the phenolic amount and antioxidant activity

As summarized in Table 3, the total phenolic content and the antioxidant activity of five extracts from *A. rupicola* generally increased when the polarity of these extracts increased. Also, the highest antioxidant activity and total phenolic content in the extracts was for MB. All results are found in Table 3. In a study performed by Alan (2018), although the antioxidant activities of only ethanolic and pure water extracts from *A. elongatum* were measured by ABTS, but the total phenolic content of both extracts was not researched. In this paper, both the antioxidant activities and the total phenolic content of five different extracts, which were both polar and apolar phases from *A. rupicola,* were investigated. In other studies with various species from this plant family, the total phenolic content and the antioxidant activity were researched; for example, only polar phase extracts of *A. maculatum* [5], ethanol, methanol, acetone, and water extracts of *A. dioscoridis* [39], and methanol extracts of *A. dioscoridis*, *A. elongatum*, *A. hygrophilum* and *A. palaestinum* [40].

Table 3. Antioxidant activity for five extracts of *A. rupicola* by ABTS assay

	Extracts				
	HH	**HC**	**MH**	**MB**	**MC**
(mM/mg)[a]	**0.51±0,02**	**0.52±0,02**	**1,27±0,01**	**4,87±0,03**	**3,54±0,04**
(mg/ml)[b]	**0.0227**	**0.0038**	**0.1415**	**1.1347**	**0.3119**

[a]Trolox equivalent
[b]Gallic acid equivalent

3.4. Inhibition results for plant extracts

Inhibition% vs. concentration graphs were drawn for the five extracts from *A. rupicola*. These are given in Figure 3. Of these extracts, the concentrations of HH, HC, and MH were 0.25 mg/mL while the other concentrations of the others, MC and MB, were 0.5 and 0.1 mg/mL, respectively. This graph was very detailed in the previous study [40]. That study drew the graph for a single extract, ethanol, of *A. elongatum* but this study drew the graph for five extracts of *A. rupicola*. Also, the values for inhibition% of HH, HC, MH, MC, and MB were found using Trolox equivalent as 61, 65, 75, 66, and 66%, respectively. As understood from both the graph and these values, MH had the biggest value. In the other studies, the value of inhibition% for extracts of pure water and ethanol from *A. elongatum* were 96.63 and 94.15, respectively [25]. Both values were higher than the ones in this paper and these were obtained using BHA and BHT standard compounds. However, the value of inhibition% for methanol extract from *A. elongatum* was between 50-60% using Trolox standard compounds [40]. This value is lower than the ones in this study.

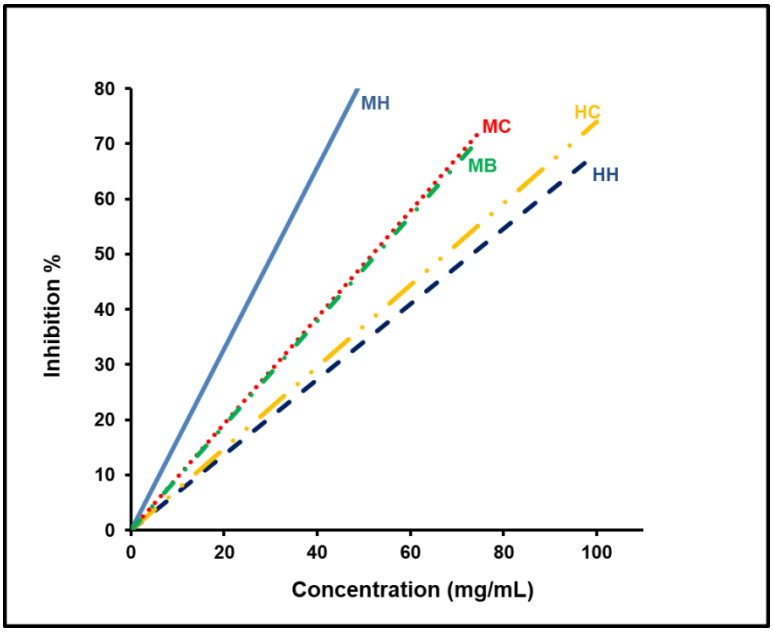


Figure 3. Relation between inhibition determined by ABTS method for five extracts of *A. rupicola*

3.5. Antimicrobial tests for plant extracts

The antimicrobial effects of the five extracts from *A. rupicola* were determined using both disc diffusion and micro-dilution assay methods. According to the disc diffusion assay, these extracts affected gram-negative bacterial strains more the gram-positive bacterial strains. The results of both antibacterial methods are given in Figure 4. Four of the five extracts except MC inhibited *E. coli* while only HH extract inhibited *B. subtilis*. The zone diameters calculated for *E. coli* using HH, HC, MH, and MB were 10.0, 10.0, 13.0, and 10.0 mm, respectively, while the value for *B. subtilis* using HH was about 6 mm. The results of the micro-dilution assay were better than the disc diffusion assay, according to the data obtained. These results are summarized in Table 4. For these microorganisms, only *S. aureus* was inhibited by all extracts. Alan investigated the antimicrobial effects of ethanol and pure water extracts obtained from *A. elongatum*. *E. coli* was inhibited only by the ethanol extract, while both extracts inhibited *S. aureus*, and, *B. subtilus* [25]. The values obtained for both extracts are similar to this paper as shown by the diameters. Both extract and the assay variety are discussed more extensively in this study. Moreover, in some studies about the different species of *Arum*, the antimicrobial activity of a variety of extracts from *A. maculatum* [5] and *A. hygrophilum* [41] were examined using both assays and disc diffusion, respectively.

Table 4. Antimicrobial activity for five extracts from *A. rupicola*

Extracts	Disc Diffusion Assay (mm)			Micro-Dilution Assay (SR %)		
	E. coli	***S. aureus***	***B. subtilis***	***E. coli***	***S. aureus***	***B. subtilis***
HH	**10.0**	**0.0**	**6.0**	**83.25**	**66.88**	**100.0**
HC	**10.0**	**0.0**	**0.0**	**100.0**	**100.0**	**100.0**
MH	**13.0**	**0.0**	**0.0**	**100.0**	**43.41**	**68.50**
MC	**0.0**	**0.0**	**0.0**	**99.69**	**68.41**	**81.76**
MB	**10.0**	**0.0**	**0.0**	**71.37**	**90.04**	**86.32**

SR % **:** survival rate percentage of microorganism

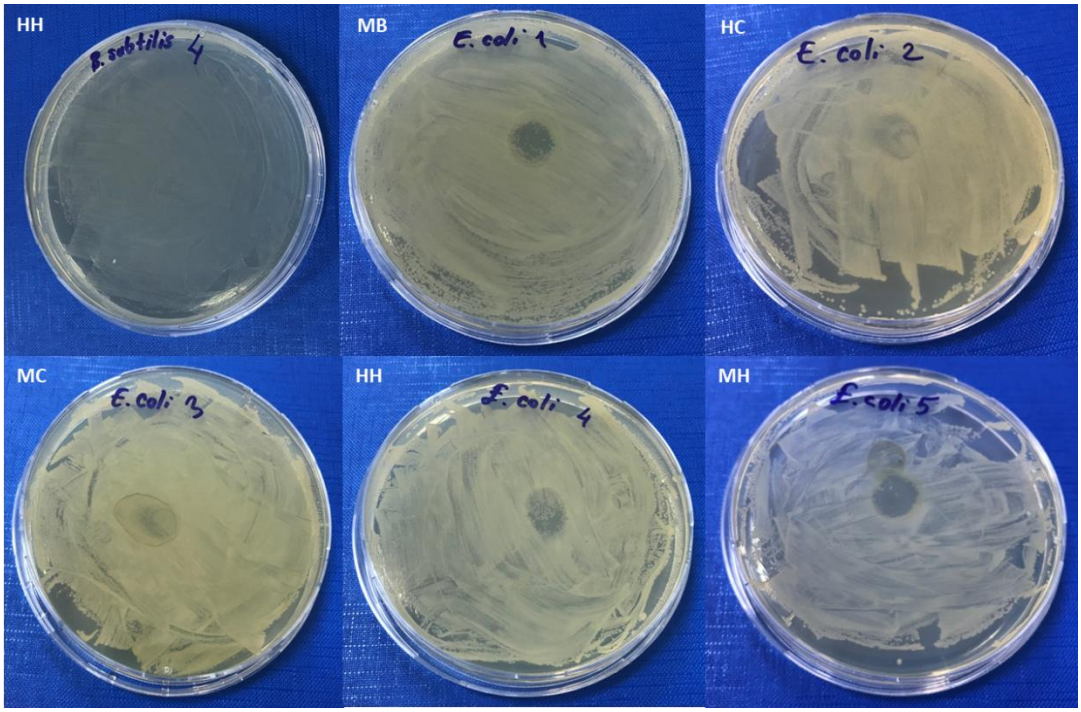


Figure 4. Antimicrobial activity of the five extracts from *A. rupicola*

4. Conclusion

A. rupicola is a plant that is not consumed because of its toxicity when fresh. However, it can be consumed after it is dried or boiled. There are many publications about this herb and similar species. With this study, five extracts of *A. rupicola* were obtained using the Scheme 1 protocol and a variety of solvents that were polar and apolar. From these extracts, methanol and hexane were the main extracts, while the others were sub-extracts. Different extracts, including different polarity compounds, were obtained. These compounds in the five extracts were analyzed by GC-MS, HPLC-TOF/MS, and UV spectrophotometry, and their antioxidant and antibacterial activities were determined. The contribution of this study is that it provides more comprehensive information about *A. rupicola*.

Statement of Research and Publication Ethics

The study is complied with research and publication ethics.

Acknowledgments

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Abbreviations: ABTS, 2,2-azinobis (3-ethylbenzothiazoline 6-sulfonate); GC-MS, Gas chromatography-mass spectrometry; DMSO, dimethyl sulfoxide; HPLC-TOF/MS, High-performance liquid chromatography-time-of-flight mass spectrometry; HPLC, High-performance liquid chromatography; BHA, Butylatedhydroxyanisole; BHT, Butylhydroxytoluene.

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