Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi, 13(1), 162-169, 2023
Journal of the Institute of Science and Technology, 13(1), 162-169, 2023

ISSN: 2146-0574, eISSN: 2536-4618

Biology **DOI: 10.21597/jist.1202158**

Research Article

Received: 10.11.2022 **Accepted: 08.12.2022**

To Cite: Savaş, A. N., Hacıoğlu Doğru, N. & Demir, N. (2023). Investigation of Some Biological Activities of *Ajuga chamaepitys* subsp. *chia*. *Journal of the Institute of Science and Technology*, 13(1), 162-169.

Investigation of Some Biological Activities of *Ajuga chamaepitys* subsp. *chia*

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Highlights:
- Medical
- Plant material
- New product

Keywords:
- Antibiofilm
- time-kill assay
- antioxidant

ABSTRACT:

Ajuga L., which is an Eastern Mediterranean element, is popularly known as ground pine, dwarf grass, ground cypress, bitter gourd, wormwood, yeast grass (mayasılotu in Turkish) (Zeybek and Zeybek, 1994). *Ajuga chamaepitys* L. Schreb species has been used since the Middle Ages as a diuretic, antidote for poisonous animal stings, and as an invigorating, wound-healing and diaphoretic plant. This study evaluated antibiofilm, time-kill potential and antioxidant activity of four extracts of *A. chamaepitys* subsp. *chia* (Schreb.) Arcang. Crystal violet binding assay were used to determinate antibiofilm activities of extracts against test microorganisms (4 Gram negative, 3 Gram positive and 1yeast). Antioxidant activity was evaluated by using 2,2-diphenyl-1-picrylhydrazyl and reducing antioxidant capacity methods. The highest antibiofilm activity was obtained in acetone extract against *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 6538P bacteria. In the time-dependent killing test of plant extracts, microbicidal effect was obtained after 24 hours. The highest antioxidant activity was obtained from plants ethyl acetate extract.

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This research is a part of a Mrs. Atiye Nur SAVAŞ's master thesis.

INTRODUCTION

The Lamiaceae family, which has a high distribution area worldwide, is one of the largest families in our country. Many species in the family are used in alternative medicine to treat many diseases. The genus *Ajuga* L., which belongs to the mint family, has been used in traditional medicine since ancient times in the treatment of many diseases such as fever, toothache, dysentery, malaria, high blood pressure, diabetes, gastrointestinal disorders, diuretic and anti-fungal, anti-inflammatory (Baytop, 1984). Many chemical compounds such as essential oil, phenolic compounds, flavonoids (Ulcay, 2021) contained in the members of the *Ajuga* genus are antimicrobial (Ulukanlı, et al., 2005), antitumor (Chen, et al., 1996), anti-inflammatory (Marc, et al., 2008), antioxidant (Chenni, et al., 2007), antiviral and cytotoxic (Orhan, et al., 2009) have been shown in many different researches that it shows a wide range of biological, pharmacological and medicinal properties.

Ajuga chamaepitys L. Schreb subsp. *chia* (Schreb.) Arcang. (*Ac*) also known as ground pine naturally distributed inAlbania, Bulgaria, Central European Rus, Czechoslovakia, East Aegean Is., East European Russia, Greece, Hungary, Iran, Iraq, Italy, Kazakhstan, Krym, Lebanon-Syria, North Caucasus, Palestine, Poland, Romania, Sicilia, South European Russi, Transcaucasus, Turkey, Turkey-in-Europe, Turkmenistan, Ukraine, Yugoslavia; it can be seen in almost all regions, up to 2000 m above sea level (Davis, 1982; Coll and Tandron, 2008). Some ethnopharmacological activities of *Ac* have been also previously reviewed (Saraç and Ugur, 2007; Miti´c et al., 2011; Duran, 2015; Jakovljević et al., 2015; Salem, 2017). Till now, no scientific data exist on the time-kill evaluation of *A. chamaepitys* subsp. *chia* extracts. In this regard, this study was to evaluate comprehensive research on antibiofilm, antioxidant activities and time-kill analysis of *A. chamaepitys* subsp. *chia* extracts.

MATERIALS AND METHODS

Plant Material

The plant materials were gathered from Çanakkale in 2019 and identified by Dr. Ersin KARABACAK according to Flora of Turkey (Davis, 1982).

Preparation of Extracts

Extraction of air-dried plants materials were made with ethanol, methanol, acetone, and ethyl acetate by Khan et al., (1988) methods.

Determination of Antibiofilm Activity

Minimum Inhibitory Concentration (MIC) was investigated as recommended instruction of the Clinical and Laboratory Standards Institute (CLSI, 2006). Microplate biofilm method (Merrit et al., 2005) was used to evaluate the inhibition of biofilm formation by *Ac* extracts against test microorganisms.

Table 1. Microorganisms and culture codes used in our study

No	Microorganisms	Culture code
1	*Acinetobacter baumannii* (Gram -)	ATCC 19606
2	*Escherichia coli* (Gram -)	NRRL B 3704
3	*Proteus vulgaris* (Gram -)	ATCC 13315
4	*Pseudomonas aeruginosa* (Gram -)	ATCC 27853
5	*Bacillus subtilis* (Gram +)	ATCC 6633
6	*Staphylococcus aureus* (Gram +)	ATCC 6538P
7	*Staphylococcus haemolyticus* (Gram +)	ATCC 43252
8	*Candida albicans*	ATCC 10231

The measurement of the antibiofilm effect of the extract was made by the percentage reduction formulation.

$$\% \text{ Inhibition} = (A_{control} - A_{sample} / A_{control}) \times 100 \quad (1)$$

Some test microorganisms which were showed in Table 1 were used for MIC, antibiofilm activity and time-kill analysis of *Ac* extracts.

Analysis of the Time-Kill

The time-kill assay is used to determine the bactericidal or bacteriostatic activity of *Ac* extracts. It is performed as described by Bakari et al., 2016. Each experiment is carried out three times.

Determination of Antioxidant Activity: Free Radical Scavenging Activity (DPPH), CUPric Reducing Antioxidant Capacity (CUPRAC)

The method developed by Blois (1958) was used for the determination of DPPH and the method designed by Apak, et al., (2005) was used for CUPRAC. For DPPH and CUPRAC measurement, measurements were made in spectrophotometers with wavelengths of 517 nm and 470 nm respectively. Butylated hydroxytoluene (BHT) solution was used as a positive control. The experiment was repeated three times and the arithmetic mean of the readings was taken. The DPPH of each sample was calculated using the following equation and the results were expressed as % inhibition.

$$\text{Inhibition (\%)} = [(A_0 - A_1) / A_0] \times 100 \quad (2)$$

A_0: Extract or non-standard control absorbance, A_1: Extract or standard absorbance

RESULTS AND DISCUSSION

The extracts yields (%) of *Ac* were found that methanol (15.39%) > acetone (10.40%) > ethanol (8.75%) > ethyl acetate (1.86%), respectively.

The MIC and antibiofilm activity (at MIC and sub-MIC values) results of *Ac* plant extracts are given in Figure 1 and Table 2, respectively. The highest biofilm percentage was obtained from acetone extract against *P. aeruginosa* ATCC 27853 (93.02±0.01%), *S. aureus* ATCC 6538P (92.18.1±1.76%), and the lowest removal percentage was obtained from ethyl acetate extract against *E. coli* NRRLB 3704 bacteria (3.65%±0.25%). However, antibiofilm activity was not detected in some extracts against *E. coli* NRRLB 3704 (ethanol), *A. baumannii* ATCC 19606 (methanol), *B. subtilis* ATCC 6633 (methanol and acetone) test bacteria and *C. albicans* ATCC 10231 (methanol) yeast culture.

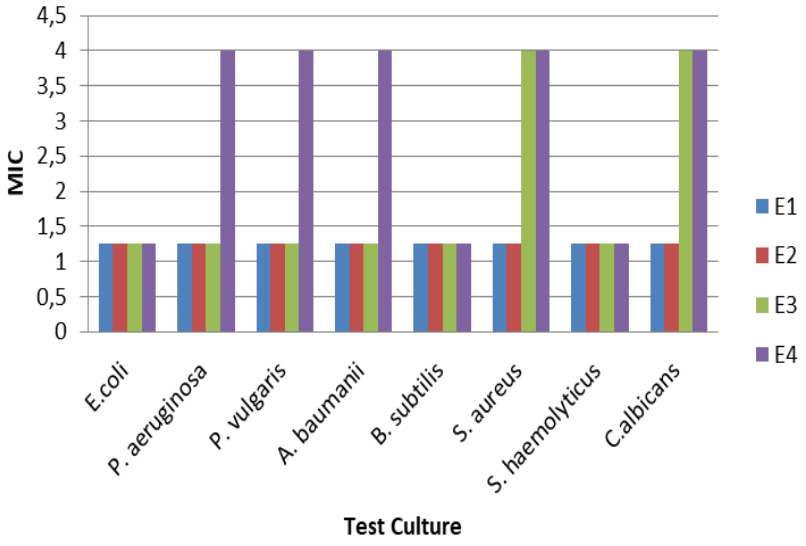

Figure 1. MIC ratios of the *Ac*. extracts against test cultures

There is only one study was conducted by Duran (2015) to determine the antibiofilm activity of the *Ac* plant. In this study, it was found that plant extract inhibited *S. mutans* biofilm formation by 80.86%. Khan, et al. (2017) investigated the antibiofilm activities of *Ajuga bracteosa* against three strain of *P. aeruginosa* and reported that biofilm removal was moderate and weak compared to the control group. In our study, high antibiotic ratios obtained from methanol, acetone, and ethyl acetate extracts against *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 6538P are the first findings that this plant extract can be used against both gram-positive and gram-negative bacteria in biofilm removal. However, literature reviews reveal that studies with this group of plants are quite limited, and our study is the first comprehensive study in this field with *Ac* species.

Table 2. Antibiofilm (% inhibition) activities of *Ac* extracts

		Test microorganisms							
Extracts	**Concentration (µg/mL)**	***E. coli* NRRLB 3704**	***P. aeruginosa* ATCC 27853**	***P. vulgaris* ATCC 13315**	***A. baumannii* ATCC 19606**	***B. subtilis* ATCC 6633**	***S. aureus* ATCC 6538P**	***S. haemolyticus* ATCC 43252**	***C. albicans* ATCC 10231**
E1	**MIC**	-	53.45±3.21	**55.54±0.20**	47.12±0.22	45.03±0.01	**50.34±1.60**	**56.12±0.11**	30.45±1.14
	MIC/2	-	-	-	33.12±0.10	-	-	-	15.45±0.99
	MIC/4	-	-	-	-	-	-	-	-
E2	**MIC**	8.93±0.62	**73.15±0.56**	**57.45±2.24**	-	-	**57.12±0.25**	**60.19±0.06**	-
	MIC/2	5.67±0.45	**66.45±0.2**	-	-	-	-	41.06±0.45	-
	MIC/4	-	-	-	-	-	-	-	-
E3	**MIC**	42.75 ±0.22	**93.02±0.01**	47.51±2.10	**55.72± 0.12**	-	**92.18±1.76**	**58.41±1.12**	**79.82±1.12**
	MIC/2	28.45±0.2	**73.12±0.24**	30.78±1.20	35.12± 0.1	-	**76.15±1.00**	28,41±0.12	45.67±0.56
	MIC/4	-	**54.12±0.12**	-		-	-	-	-
E4	**MIC**	11.01±0.2	**75.12±0.67**	**63.37±0.11**	44.22±1.21	**64.12±0.24**	**75.45±1.56**	32.57±1.10	26.28±0.14
	MIC/2	3.65±0.25	**65.44±1.12**	-	-	**58.12±0.24**	30.78±1.20	-	6.02±0.10
	MIC/4	-	-	-	-	26.12±0.30	-	-	-

E1: Ethanol, E2: Methanol, E3: Acetone, E4: Ethyl acetate

The time-dependent kinetic killing test findings of plant extracts are given in Figures 2 for each test culture. The significant microbicidal effect was achieved at the end of 24 hours for all cultures. The time-dependent microbicidal dose of plant extracts is essential to determine their ability to become potential sources of antibiotics. The Time kill method, which is one of the methods used especially in synergism tests, is used intensively for this purpose (Subaşı, 2020). The time-kill profile on *Ac* extracts is reported for the first time in this study. Therefore, their bactericidal or bacteriostatic potency on potential eight pathogen bacteria and yeast was investigated to confirm its effect and clarify its mechanism of action. Vambe, et al. (2018) reported that the time-dependent killing curve in plants used as medicinal plants in South Africa changed significantly, especially in 24 hours. Our data coincide with the study findings in this field in the literature (Yıldırım, 2011; Aumeeruddy-Elalfi, et al., 2016; Boakye, et al., 2016; Subaşı, 2020).

The antioxidant activities of 4 different extracts of the studied plant at five different concentrations were calculated by measuring the absorbance values at 517 nm by DPPH method. Although all the extracts used in the study were lower than BHT, antioxidant activity was determined at increasing rates depending on the concentration (Figure 3). However, ethyl acetate has been observed to have relatively high antioxidant activity than other solvents. Ethyl acetate extract from *Ac* has been found to have a much higher CUPRAC antioxidant effect than other extracts. At the same time, antioxidant activities were found to be elevated in all extracts due to increased concentration, especially after 400 µg/mL (Table 3).

It is known that different extracts plants reveal different active substances and functional groups (Türkoğlu, et al., 2010), two methods such as DPPH and CUPRAC were used in the antioxidant

activity study. Antioxidant activities were detected from plant extracts that were lower than positive controls but increased at certain concentrations. Haşimi (2012) found that *A. vestita* and *A. xylorrhiza* methanol extracts showed the highest antioxidant activity at a concentration of 500 µg/mL according to the DPPH method and also had a higher effect than BHT used as a positive control at the same concentration.

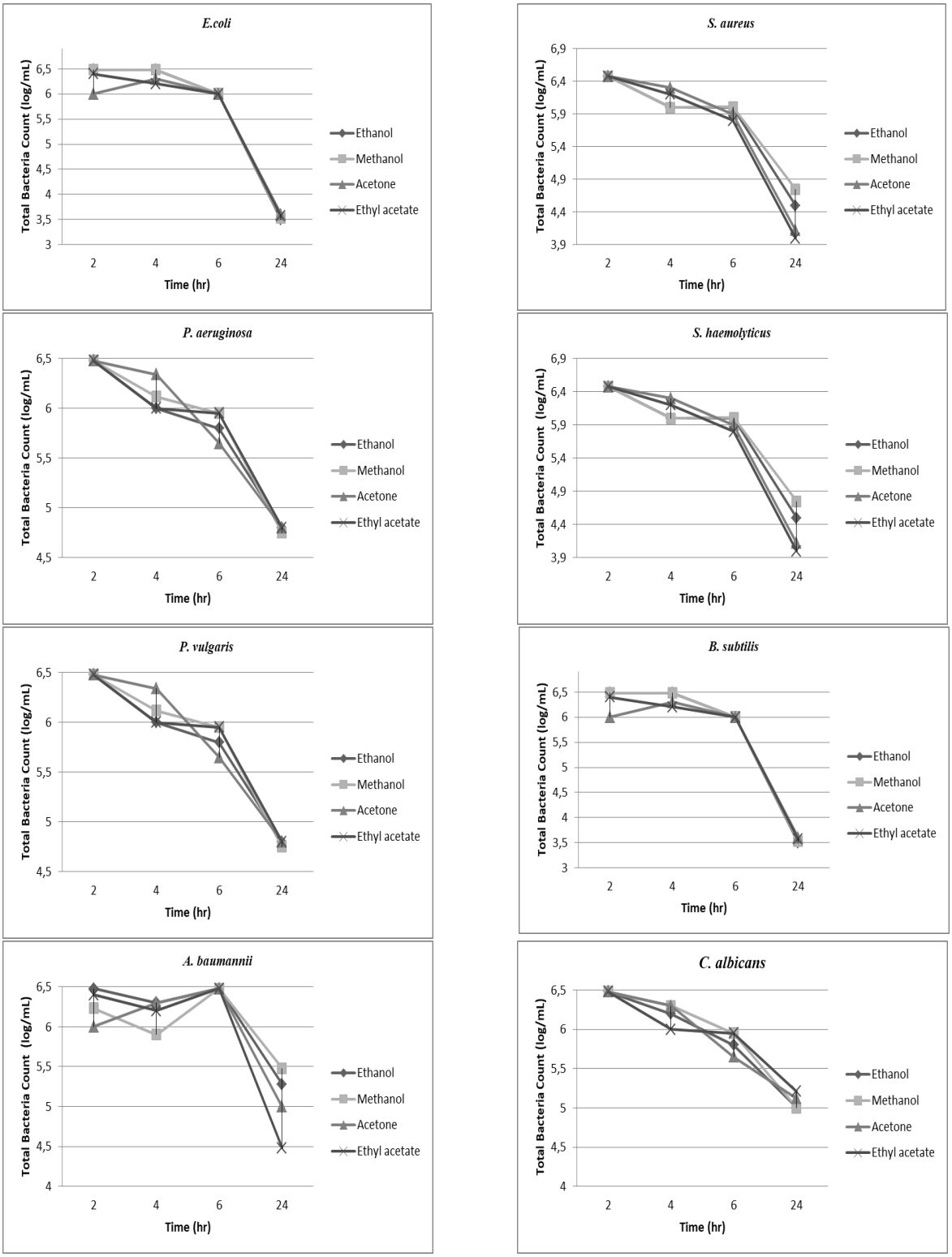

Figure 2. In vitro time-kill assessment of the *Ac* extracts

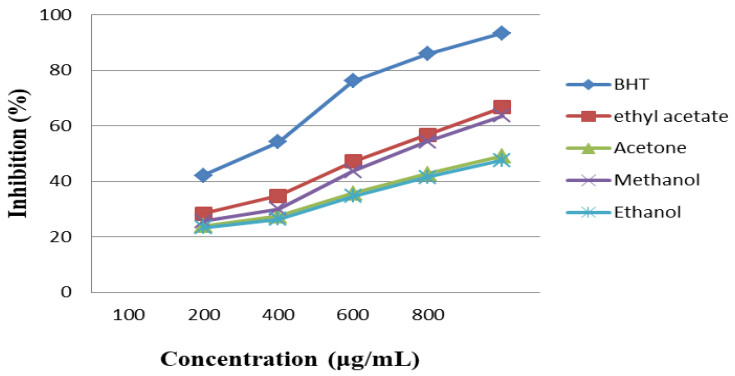

Figure 3. Free radical removal activity of four different extracts of *Ac* plant by DPPH method

In the same study, *A. vestita* methanol extract at a concentration of 1 mg/mL showed higher activity than α-tocopherol, which was used as a positive control, according to the CUPRAC method. Türkoğlu, et al. (2010) found that water extract of *Ac* was showed higher antioxidant activity than methanol and chloroform and lower than control group BHT. Mamadalieva, et al. (2013) also found high antioxidant activity in water and butanol extracts of *A. turkestanica*. The data obtained in our study coincide with the study conducted by Türkoğlu, et al. (2010). Low antioxidant activity data obtained in other literature are thought to be related to the locality characteristics, extraction conditions and methods of plant species collected.

Table 3. CUPRAC Capacitiy of *Ac* extracts

	Absorbance Values				
Concentrations of Extracts	**Ascorbic Acid**	**Ethyl Acetate**	**Acetone**	**Methanol**	**Ethanol**
100	1.39±0.07	**0.65±0.13**	0.52±0.11	0.55±0.12	0.54±0.09
200	1.51±0.18	**0.76±0.06**	0.56±0.21	0.62±0.18	0.59±0.08
400	2.±0.25	**1.02±0.12**	0.63±0.14	0.74±0.2	0.67±0.2
600	2.57±0.16	**1.17±0.21**	0.73±0.25	0.87±0.16	0.81±0.12
800	3.23±0.17	**1.53±0.09**	0.81±0.1	0.99±0.23	0.89±0.15

CONCLUSION

This study contains promising data that the comprehensive antibiofilm, time-kill assay and antioxidant capacity of different extracts of *Ac*, which are a cosmopolitan species and the subject of biological activity studies conducted by different researchers. It has been proven by tests that the biofilm-forming capacity of both bacteria with high infection capacity and virulence factors (*S. aureus* ATCC 6538P and *P. aeruginosa* ATCC 27853) is highly removed, especially by methanol, acetone and ethyl acetate extracts. This information is preliminary data for the *Ac* to be an important source of antibiofilm active ingredient in industries such as food and paper with comprehensive studies. In addition, extracts of *Ac* studied showed significant findings in terms of antioxidant activity. In the light of this information, it is recommended to conduct comprehensive studies with *A. chamaepitys* subsp. *chia* plant species. *Ac* extract trials should be performed in anti-quorum sensing studies, which are an important step in strategies in the fight against microbial biofilms and being a natural source of antioxidants that can replace synthetic antioxidants.

ACKNOWLEDGEMENTS

This research is a part of a Mrs. Atiye Nur SAVAŞ's master thesis.

Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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