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Article

In vitro antibacterial-antibiofilm effect of *Hypericum atomarium* Boiss and chemical composition

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Treatment with plant is considered an effective option against increased antibiotic resistance. In this study antibiofilm activity of methanol (CH_3OH), chloroform ($CHCl_3$), ethyl acetate (EtOAc) and water (H_2O) extracts of *Hypericum atomarium* Boiss. which is member of *Hypericum* genus was evaluated in *Pseudomonas aeruginosa* PAO1 and antibacterial performance against Gram (+) and Gram (-) strains and also bioactive compounds of extract were analysed using by HPLC and GC-MS. According to antibacterial activity test results the extracts were effective all Gram (+) bacteria and Gram (-) *Chromobacterium violaceum* (MICs ranging from 0.42 µg/ml to 4.3 mg). Inhibition effect of biofilm formation was found to be different rate in extracts (methanol-63%, chloroform-52%). The major flavonoids were detected (−)-epicatechin (2388.93 µg/ml) and (+)-catechin (788.94 µg/ml). The main phenolic acids were appeared as caffeic acid 277.34 µg/ml and chlorogenic acid 261.79 µg/ml. And according to GC results α-pinene was found main compound for three solvent extracts methanol, chloroform and ethyl acetate 67.05, 62.69, 49.28% rate respectively.

Keywords: Antibacterial activity. Hypericaceae. Phenolic acids. PAO1. Quorum sensing.

INTRODUCTION

Antibiotics have been used for a long time to treatment infection diseases. Because of this widespread and inaccurate usage, most infectious bacteria have adapted and changed, making the drugs less effective. As a result of this antibiotic resistance is becoming major problem in a worldwide (Demetrio *et al.,* 2015). Increased antibacterial resistance among bacteria made it compulsory to find different strategies to combat with bacteria (Keles *et al.,* 2001). For many years, plants have been the subject of studies for this purpose. Another promising strategy is to prevent the communication between bacteria and fight against bacteria.

The reason why plants have been used in the treatment of diseases since ancient times is because of the large number of biochemical substances they have.

The genus *Hypericum* belongs to the Clusiaceae (formerly Hypericaceae) family and consists of nearly 500 species is a medicinal plants (Akgoz, 2015). *Hypericum* is well-known in herbal medicine due to its rich bioactive constituents which have therapeutic effects against microbial infections, tumor, depression, inflammation, burn and pains (Süntar *et al*., 2015; Bertoli, Çırak, Silva, 2011). Also some species have anti-collagenase, antioxidant activities (Caprioli *et al.,* 2016*;* Mandrone *et al.,* 2015). The most active ingredients of *Hypericum* species are phenolic substances and various terpenoids, which are the most studied substance is hypericin. Besides phenolic contents, essential oils are the phytochemicals present in the *Hypericum* species, and the most prominent component in many species has been found to be alpha-pinene.

As well as the use of *Hypericum* in the treatment of some diseases, many studies also have been reported on

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antimicrobial properties (Ornano *et al.,* 2018; Chandra *et al.,* 2017; Esposito *et al.,* 2013). Plants with antibacterial effect have different mechanisms from antibiotics so they have the ability to inhibit developing bacterial resistance. However, although treatment with plant has seen as an effective and successful choice in the fight against bacteria in recent years, the inhibition of the Quorum sensing system (QS), which is responsible for the pathogenicity in the bacteria responsible for infections, has been one of the most emphasized options combat with bacteria. Biofilms, which are effective in bacterial resistance, are micro ecosystems that develop by attaching to a surface, and their control is carried out by the QS system. It is known that biofilm formation is even more important than plankton bacteria in the pathogenesis of infectious diseases (Temel, Eraç, 2018; Ceylan *et al.,* 2014). For all these reasons, in addition to antimicrobial studies with plants, antibiofilm studies have also increased. Hypericum species have been reported to be used in the treatment of many diseases as healing agents for centuries, especially *Hypericum perforatum* but no detailed study of *H. atomarium* Boiss. has been found. In this study chemical composition and antibacterial-antibiofilm effect of solvent extracts (EtOAc, $CHCl_3$, CH_3OH and H_2O) obtained from aerial parts of the *H. atomarium* Boiss. was examined. The phytochemical contents of extracts were determined by HPLC and GC-MS.

MATERIAL AND METHODS

Plant material and extraction

For the extraction, *H. atomarium* Boiss. was obtained from local commerce in Isparta/Turkey in October 2016 and identified (herbarium number: GUL 27/1/47-1) by a taxonomist Mr. Hasan Özçelik from the Department of Botany, Faculty of Science, Süleyman Demirel University. But also it still unresolved according to WCSP records.

About 5 gr flowers (for each solvent extract) of the plant was powdered and extracted with 50 mL of CH_3OH, $CHCl_3$, EtOAc, and H_2O. After 30 min in ultrasonic bath the extracts were filtered using Whatman filter no.1, samples were evaporated under vacuum using a rotary evaporator (Heıdolph Hei-Vap Rotary Evaporator) at 40 °C. The residues (in the sticky black substances) were weighed and dissolved with -96% DMSO (dimethyl sulfoxide) before testing and stored -20 °C for bacterial activity tests.

Gas chromatography mass spectrometry (GC–MS)

Different solvent extracts -$CHCl_3$, EtOAc-, of *H. atomarium* Boiss. was examined by Shimadzu GC-MS QP 5050 (Kyoto, Japan) spectrometer system. Separations were performed with the CP WAX 52 CB capillary column (50 m x 0.32 mm ID, df: 1.2 µm) purchased from Varian. Helium (99.999%), was used as carrier gas at a constant head pressure of 10 p.s.i (1 p.s.i = 6894.76 Pa). The volume of injection was 1 µl (after the extract (40 µl) was diluted with the prepared solvent (960 µl hexane). The GC oven programme steps: 1) The temperature of initial column was 60 °C. 2) The column was heated to 220 °C at a rate of 2 °C/min and held at 220 °C for 20 min. 3) The GC–MS interface and injector were kept at 250 and 240 °C, respectively. 4) The mass spectrometer was set to electron impact mode at 70 eV and mass range from 35-450 mass to charge ratio (*m*/*z*).

High performance liquid chromatography (HPLC)

Phenolic compounds were evaluated by reversed phase-high performance liquid chromatography (RP-HPLC, Shimadzu Scientific Instruments, Tokyo, Japan) with direct injection. And 23 standard compounds were investigated [gallic acid (Fluka 48630), protocatechuic acid (Fluka 37580), *p*-hydroxy benzoic acid (Sigma Cat:24,014-1), chlorogenic acid (Sigma C 3878), caffeic acid (Sigma C 0625), syringic acid (Sigma S 6881), vanillin (Sigma V 110-4), *p*-coumaric acid (Sigma C 9008), ferulic acid (Fluka 46280), sinapinic acid (Sigma D-7927), benzoic acid (Sigma 242381), *o*-coumaric acid (H 22809), rosmarinic acid (Fluka 44699), cinnamic acid (Merck S 34491 137), rutin (Fluka 78095), catechin (Sigma C 1788), hesperidin (Fluka 52040), epicatechin (Sigma E 1753), eriodictiol (Sigma 94258), quercetin (Sigma Q 0125), luteolin (Sigma L 9283), kaempferol (Sigma K 0133), apigenin (Sigma 10798)]. Briefly, 5 g of

samples were ground with blender and extracted with 50 ml of methanol, with ultrasonic water bath device about 30 min. The mixture was filtered by using a filter paper (Whatman No.1). Removal of solvent and water was carried out with a rotary evaporator 40 °C + vacuum). The extracts obtained were weighed to determine the yields volume. An HPLC equipped with a SCL-10Avp System controller, a SIL–10AD vp Autosampler, a LC-10AD vp pump, a DGU-14a degasser, a CTO-10 A vp column heater and a diode array detector (DAD) with wavelengths set at 278 nm. A gradient solvent system was used for separation of phenolic compounds, solvent A: 3.0% acetic acid in distilled water and solvent B: pure methanol. A 90 min linear gradient was programmed as follows: 0–0.10 min, 0-7% B; 0.10–20 min, 7-28%B; 20–28 min, 28-25% B; 80-90 min, 100-7% B. The flow rate was 0.8 ml min^{-1}, samples of 20 µl were injected into the reversed-phase C18 column (Agilent Eclipse XDB C-18 (250 mm × 4.6 mm length, 5 µm). The column temperature was set at 30 °C (Caponio, Gomes, Pasqualone, 2001; Karacabey, Mazza, 2008).

Bacterial strains

Total 13 strains were used to test the antibacterial activity and *Pseudomonas aeruginosa* PAO1 was used antibiofilm effect of *H. atomarium* Boiss. extracts. The antibacterial activity was screened against six Gram (+) (*Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 25923, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300 and seven Gram (-) (*Agrobacterium tumefaciens* ATCC 33970, *Chromobacterium violaceum* ATCC 12472, *Enterobacter aerogenes* ATCC 13048, *Erwinia caratovora* ATCC 15390, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* PAO1) strains.

Antibacterial activity

To determine antibacterial activity of plant extracts agar well diffusion test was used (Perez, Pauli, Bazerque, 1990) in four different concentrations for each extracts. Besides extracts, antibacterial effects of major ingredients detected by GC and HPLC were examined. Bacterial strains were cultured LB broth and incubated for 24 hours 30/37°C. Overnight cultures adjusted to equal the turbidity of 0.5 McFarland standards (1.5×10^8 CFU/mL). One hundred microliters of test microorganism with 0.5% soft agar added on surface of LB agar. 100 µl of samples were added to wells previously opened with a pasteur pipette. After incubation at 30/37°C for about 18-24 hours, the inhibition zone diameter (mm) of plant extracts was measured for all isolates. Experiment was performed triplicate and gentamicin used as positive control.

Minimum inhibitory concentrations (MIC)

Determination of MIC values of the extracts on standard strains was done according to micro dilution method with 96-well plate (Andrews, 2001). Microorganisms were prepared from 12-h broth cultures and were adjusted to 0.5 McFarland standards ($1.5x10^8$ cfu/ml). Plant extracts were added to the wells with Mueller Hinton broth medium, and two-fold serial dilutions were made, respectively. Five microliters of bacterial suspension prepared according to 0.5 McFarland (10^8/ml) turbidity was added to micro plates and left for an overnight incubation at 30/37°C. The MIC values were found the lowest concentration of the extracts that there wasn't observed visible growth of microorganisms. Dimethyl sulfoxide was used as a control and Mueller–Hinton broth as a negative control by adding well without bacteria. And gentamicin used as positive control. The experiments were repeated three times.

Biofilm formation activity

The inhibition effect of the plants on biofilm formation in PAO1 was implemented as described before (O'Toole, 2011; Dönmez, Önem, 2018) according to the crystal violet test.

Statistical analysis

In the study, random blocks trial was designed with three replications. Data were subjected to ANOVA

(analysis of variance) with JMP software and were compared by LSD test.

RESULTS AND DISCUSSION

A total 10 constituents were identified by GC–MS of plant extracts and α-pinene was found to be the major compound of *H. atomarium* Boiss. (EtOAc-49.28%; $CHCl_3$-62.69%) (Table I). There were also hydrocarbons such as nonane, decane were identified different rate. Numerous studies have been published GC analysis of *Hypericum* species especially *H. perforatum* L. but there is not much work about *H. atomarium* Boiss (Gopınath *et al*., 2013; Radulović, Blagojević, 2012).

TABLE I - GC–MS analysis of extracts % major components and their retention times

No	Name of compound	rt (min)	EtOAc (%)	CHCl3 (%)
1	*n*-Decane	5.119	10.27	*
2	*n*-Nonane	5.127	*	17.03
3	α-pinene	6.150	49.28	62.69
4	2.6-Dimethyloctane	7.336	*	8.71
5	2-Methyleicosane	7.340	15.32	*
6	3-Methylnonane	7.365	*	*
7	Cyclohexene	9.710	15.32	*
8	Hexane 3,3 dimetyl	11.409	*	5.41
9	3-Methyoctane	11.456	*	*
10	*E*-3-phenyl-2propenal	23.385	21.86	*
11	3-Nonen-1-yne	32.828	*	6.16

** Not detected*

The presence of 23 standard components were investigated by HPLC analysis. The standard phytochemicals and their quantities are given in Table II. According to data presented in the table, some phenolics acids as chlorogenic acid, *p*-hydroxy benzoic acid rosmarinic acid, caffeic acid, *o*-coumaric acid were found in very high amount. Besides phenolics acids different amount of flavonoids have been detected. Among the detected flavonoids, epicatechin has the highest level (2388.93±58.1 µg/g). The amounts of catechin, quercetin, rutin were determined 788.94±22.2 µg/g, 352.24±12.1 µg/g and 312.55±7.3 µg/g, respectively. Previous studies on *Hypericum spp.* were showed that they are rich phenolic compounds. Unal *et al*. 2008, have reported that methanolic extracts of two *Hypericum* spp. (*H. perforatum* subsp. *angustifolium* and *H. perforatum* subsp. *perforatum*) consisted of chlorogenic acid, caffeic acid, quercetin and *H. perforatum* subsp. *perforatum* also rutin. In a study using HPTLC, it was shown that even species within the same genus may have different amounts and types of phytoconstituents (Toniolo *et al*., 2014). Also in our study we found that same compounds with different amount (chlorogenic acid-261.79 µg/mg, caffeic acid-277.34 µg/mg, quercetin-352.24 µg/mg) (Demetrio *et al.,* 2015; Maltas *et al.,* 2013).

TABLE II - Phenolic composition of *H. atomarium* Boiss

Compounds	µg/g
Phenolics acids	
gallic acid	17.0±2.4
protocatechuic acid	*
p-hydroxy benzoic acid	253.61±16.0
chlorogenic acid	261.79±8.1
caffeic acid	277.34±12.7
syringic acid	*
vanillin	*
p-coumaric acid	13.41±0.5
ferulic acid	14.69±1.3
sinapinic acid	*
benzoic acid	*
o-coumaric acid	516.33±20.9
rosmarinic acid	603.36±21.3
cinnamic acid	7.33±0.2
Flavonoids	
rutin	312.55±7.3
catechin	788.94±22.2
hesperidin	*
epicatechin	2388.93±58.1
eriodictiol	3.64±0.4
quercetin	352.24±12.1
luteolin	25.06±0.03
kaempferol	*
apigenin	*

** Not detected, SD±*

Different solvents extracts showed different extraction yields and this difference is due to many reasons as time, extraction method or solvent polarities are some of them. They have also different compositions since they dissolve differently the various components in accordance with their polarity. CH_3OH had the highest yield (18.12%) and H_2O, EtOAc, $CHCl_3$ had 15.78, 8.16, 7.94 respectively. And for the antimicrobial activity test, four concentrations of these yields were done using dimethyl sulfoxide (DMSO). The screening of antimicrobial activity of CH_3OH, $CHCl_3$, EtOAc, H_2O extract of *H. atomarium* Boiss. and α-pinene (Sigma-Aldrich), epicatechin (Sigma-Aldrich- purity > 98%), catechin (Sigma-Aldrich- purity > 98%) were carried out using agar-well diffusion method against six Gram (+) and seven Gram (-) bacteria and the results were shown in Table III. The results showed that all *H. atomarium* Boiss. extracts showed antibacterial activity against six Gram (+) bacteria and one Gram (-) bacteria (*C. violaceum*) different rate (9 mm to 20.7 mm) whereas no inhibition effect on other Gram (-) bacteria.

TABLE III - Antibacterial activity of extracts and compounds (mm)

Sample/Concentration(w/v)	*B. subtilis* 6633				*B. cereus* 11778			
	1:1	1:2	1:4	1:8	1:1	1:2	1:4	1:8
H_2O	14.7^{b}	13.7^{c}	12.7^{c}	12.7^{c}	20.7^{a}	18.7^{c}	16.7^{b}	14.0^{c}
CH_3OH	20.7^{a}	20.0^{ab}	19.0^{ab}	19.0^{ab}	20.7^{a}	20.7^{a}	19.7^{a}	19.0^{b}
$CHCl_3$	21.0^{a}	21.0^{a}	20.7^{a}	20.7^{a}	21.0^{a}	20.0^{b}	20.0^{a}	19.0^{b}
EtOAc	21.0^{a}	21.0^{a}	18.7^{b}	18.0^{b}	20.7^{a}	20.7^{a}	20.0^{a}	20.0^{a}
α-pinene	11.7^{c}	*ne*	*ne*	*ne*	11.7^{c}	*ne*	*ne*	*ne*
(-)-epicatechin	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*

TABLE III - Antibacterial activity of extracts and compounds (mm)

Sample/Concentration(w/v)	*B. subtilis* 6633				*B. cereus* 11778			
	1:1	**1:2**	**1:4**	**1:8**	**1:1**	**1:2**	**1:4**	**1:8**
(±)-catechin	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*
CN (40µg/ml)	19.3^{a}	19.3^{b}	19.3^{ab}	19.3^{ab}	13.3^{b}	13.3^{d}	13.3^{c}	13.3^{d}
	***	***	***	***	***	***	***	***
	***C. violaceum* 12472**				***E. faecalis 29212***			
H_2O	10.7^{cd}	10.0^{b}	9.0^{b}	8.0^{c}	15.7^{ab}	13.0^{c}	11.7^{c}	9.0^{d}
CH_3OH	17.3^{a}	15.3^{a}	13.0^{a}	10.3^{b}	16.7^{a}	16.7^{a}	15.7^{a}	13.7^{b}
$CHCl_3$	12.0^{c}	10.0^{b}	7.7^{b}	7.7^{c}	13.7^{c}	13.7^{c}	13.0^{b}	12.0^{c}
EtOAc	9.0^{d}	8.7^{b}	*ne*	*ne*	15.0^{b}	13.7^{c}	13.7^{b}	12.7^{c}
α-pinene	*ne*	*ne*	*ne*	*ne*	9.3^{d}	*ne*	*ne*	*ne*
(-)-epicatechin	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*
(±)-catechin	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*
CN (40µg/ml)	14.3^{b}	14.3^{a}	14.3^{a}	14.3^{a}	15.7^{ab}	15.7^{b}	15.67^{a}	15.7^{a}
	***	***	***	***	***	***	***	***
	***L. monocytogenes* 7644**				***MRSA* 43300**			
H_2O	12.7^{d}	12.7^{c}	10.7^{b}	9.0^{c}	17.0^{b}	14.0^{c}	12.7^{b}	9.0^{c}
CH_3OH	18.7^{b}	19.0^{a}	18.0^{a}	18.0^{a}	18.7^{a}	18.0^{b}	18.7^{a}	18.0^{a}
$CHCl_3$	19.7^{a}	18.0^{ab}	16.7^{a}	16.7^{ab}	18.7^{a}	18.0^{b}	17.0^{a}	17.0^{ab}
EtOAc	17.7^{c}	17.0^{b}	16.7^{a}	15.7^{b}	19.3^{a}	19.3^{a}	18.7^{a}	17.7^{a}
α-pinene	*ne*	*ne*	*ne*	*ne*	10.3^{d}	*ne*	*ne*	*ne*
(-)-epicatechin	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*
(±)-catechin	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*	*ne*
CN (40µg/ml)	18.0^{bc}	18.0^{ab}	18.0^{a}	18.0^{a}	14.3^{c}	14.3^{c}	14.3^{b}	14.3^{b}
	***	***	***	***	***	***	***	***
	***S. aureus* 25923**							
H_2O	15.7c	13.7c	11.3c	9.0c				
CH_3OH	19.0a	19.0a	18.0a	18.0a				
$CHCl_3$	17.7b	17.7b	17.0a	16.7a				
EtOAc	18.7ab	18.0ab	17.0a	17.0a				
α-pinene	16.3c	*ne*	*ne*	*ne*				
(-)-epicatechin	*ne*	*ne*	*ne*	*ne*				
(±)-catechin	*ne*	*ne*	*ne*	*ne*				
CN (40µg/ml)	14.3^{d}	14.3^{c}	14.3^{b}	14.3^{b}				
	***	***	***	***				

*ne none-effective; *** The averages of zones within columns with the same letter are not importantly different by LSD's at p<0.001. CN gentamicin.*

This study showed that different solvent extracts of *H. atomarium* have antibacterial properties. According to agar well diffusion test results, the widest zone of inhibition was obtained with *Bacillus* species followed by *L. monocytogenes*, *S. aureus*, MRSA, *E. faecalis* and Gram (-) *C. violaceum*. *α*- pinene, which was the major content of the tested extracts in GC, showed antibacterial effect (9.3 mm to 16. 3 mm) only on Gram (+) bacteria at a concentration of 3 mM, and no antibacterial effect was observed on the studied concentration on Gram (-) bacteria. According to the HPLC results, antibacterial effect of the highest detected phenolic components (-)-epicatechin (2388.93 μg/g), (±)-catechin (788.94 μg/g) were also investigated in the determined amount in extract, but no antibacterial effect was observed on any tested bacteria. Phenolic components generally show their antimicrobial effects at the membrane level. For instance, catechins and epigallocatechin gallate had antibacterial activity against some resistant bacteria such as MRSA and affect the lipid layer, causing the membrane to disintegrate, resulting in death of the cell whose cell integrity is impaired (Khameneh *et al.,* 2019; Yücel, Yücel, 2015; Gibbons, Moser, Kaatz, 2004).

The chloroform extract of *H. atomarium* Boiss was the most potent with the lowest MIC of 0.42 μg/ml against *B. cereus* and *B. subtilis*. The EtOAc and $CHCl_3$ extracts was found more efficient than the other extracts against Gram (+) pathogens at MIC values of 0.42–28 μg/ml (Table IV). Gram (+) strains showed highest sensitivity to methanolic extract of *H. atomarium* Boiss than other extracts. Different inhibition zone have detected at the test concentration, which was statistically significant comparable to positive control (gentamicin 40 μg/disc) ($p<0.001$). The best antimicrobial activity was determined for ethyl acetate and methanolic extracts against *B. subtilis* and *B. cereus* (MIC = 0.42 μg/ml, MIC= 0.42 μg/ml) *B. cereus* is an important pathogen causing foodborne poisoning outbreaks, which symptoms can be either emetic or diarrheal (Majed *et al.,* 2016). In a similar study with another *Hypericum* sp., *H. humifusum*, they found that MIC value of ethanolic extract against *B. cereus* was 0.62 μg/ml (Toiu et *al.,* 2016). Although there was so many antibacterial activity study with *Hypericum sp.* especially *H. perforatum* there is lack of studies to evaluate microbial properties of *H. atomarium*. Boiss. May be this is the first report (as far as the authors area ware) of the antibacterial and antibiofilm activity of *H. atomarium*. Boiss aerial parts.

TABLE IV - MIC value of extracts (μg/ml)

Extract	Yield (%)	*S.aureus*	*MRSA*	*B. subtilis*	*B. cereus*	*E. faecalis*	*C. violaceum*	*L. monocytogenes*
$CHCl_3$	7.94	53	53	0.42	0.42	107	1730	431
CH_3OH	18.12	265	265	1.03	1.03	66	4300	2130
EtOAc	8.16	3.4	3.4	0.43	0.43	28	1775	443
H_2O	15.78	1225	1225	2.39	2.39	153	2450	1225
Gentamicin	-	1250	1250	1250	1250	1250	6250	6250

According to MIC values of the tested extracts, aqueous extract is high for all tested bacteria. So it may be considered that the traditional use of this plant will be less effective. The extracts showed lowest activity (17.3 mm-8.7 mm) against *C. violaceum* but the only Gram (-) bacteria that the extracts exhibit antibacterial activity. In contrast, in 2016, Toiu *et al.,* showed that antibacterial activity against Gram (-) strains (*P. aeruginosa*, *E. coli*) with ethanolic extract of *H. humifusum*. Although there are many reports that

Gram (+) bacteria are more susceptible to *Hypericum sp.* than Gram (-) ones, some studies have shown that they have antibacterial activity on Gram (-) bacteria also (Maltas *et al.,* 2013).

Overall, Gram (+) bacterial strains were more sensitive to the extracts than Gram (-) bacteria. The differences in the sensitivity against extracts may be due to the cell wall structure (Elisha *et al.,* 2017; Koohsari *et al.,* 2015). Unlike Gram (+) bacteria, Gram (-) bacteria have an outer membrane, and the periplasmic space between the membranes contains one to two layers of peptidoglycan. It may be due to the outer lipopolysaccharide layer in Gram (-) bacteria prevent access of phytocemical substances and act as barriers to protect bacteria from plant-derived antimicrobial (Molinaro *et al.,* 2009; Balaperiasamy *et al.,* 2014).

The bactericidal effects of plants on bacteria are realized with different effects of flavonoids. One of these is known to occur by inhibiting nucleic acid biosynthesis or by different molecular processes (Baba, Malik, 2015; Cushnie, Lamb, 2005).

Although the extracts had no antibacterial effect on PAO1, all of them had different inhibition effects on biofilm formation and the results were found statistically significant (Figure 1). According to the results, methanol and chloroform showed a high antibiofilm effect and the inhibition rates were found 63% and 52% respectively.

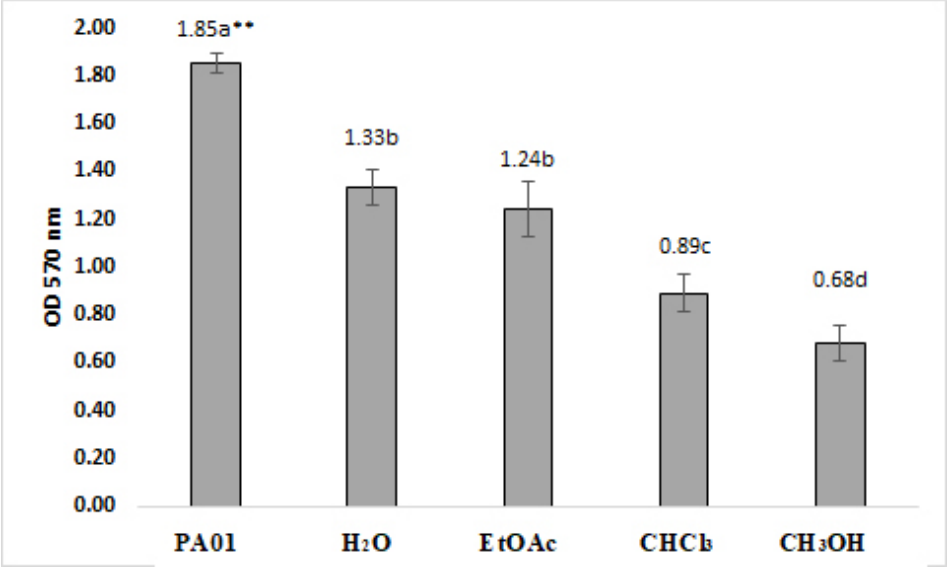

FIGURE 1 - Inhibition effect of extracts on biofilm formation in PA01. *SD±,** The averages of biofilms with the same letter are not importantly different by LSD's at p<0.01.*

Biofilm has a role in bacterial resistance by reducing the effectiveness of antibiotics in infectious diseases. Therefore, the inhibition of biofilm formation has started to be considered as an alternative in the fight against bacteria. Compounds derived from plants especially secondary metabolites are important resources for antibacterial and antibiofilm activity (Doğan *et al.,* 2019; Vattem *et al.,* 2007).

Studies on biofilm inhibition increased after it was understood that bacterial biofilms make it difficult to fight infectious diseases because the antibiotic dose acting on the planktonic form of microorganisms is ineffective on the biofilm (Demetrio *et al.,* 2015; *Lynch*, Robertson, 2008). For this purpose in addition to synthetic molecules, many herbal compounds have been tried in studies (Lu *et al.,* 2019; Ouyang *et al.,* 2015; Prabhakar *et al.,* 2013; Sánchez *et al.,* 2016; Neeraja *et al.,* 2018). Moreover some studies have shown that biological materials obtained from various organisms have an inhibition effect on biofilms in different bacteria (Pejin *et al*., 2016; Dodou *et*

al., 2020). In our study methanol and chloroform extract of *H. atomarium* Boiss. had shown significant inhibition effect on biofilm formation. Methanol extract showed 62% inhibition at 0.62 mg/ml concentration, while chloroform extract provided 52% inhibition at 0.24 mg/ml concentration. In general evaluation, it was found that the inhibition effect of plant extract on biofilm formation was statistically significant ($P < 0.01$).

CONCLUSION

In this study, it was observed that α-pinene was the main component in the extracts prepared with methanol, ethyl acetate and chloroform, and according to HPLC results, (+)-catechin and (−)-epicatechin were the major phenolic components in *H. atomarium* Boiss. The study also investigated the antibacterial and antibiofilm effects of extracts and different rates of antibacterial effects were observed on the tested Gram positive bacteria. Inhibition effect of biofilm formation was found significant for all extract compare to control PAO1. All these results show that *H. atomarium* Boiss. has potential usage in health and industrial fields and all its features can be revealed with more detailed studies.

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