

Bioactivities of *Alchemilla mollis*, *Alchemilla persica* and Their Active Constituents

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The aim of this study was to determine antimicrobial activities of *Alchemilla mollis*, *Alchemilla persica* as well as ellagic acid and miquelianin against *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* by using microbroth dilution method and anti-inflammatory activity by using human red blood cell (HRBC) membrane stabilization method. Microbroth dilution method was used to determine the antimicrobial activities. Extracts possessed activity having MIC values of 2.5-5-10mg/mL, compounds possessed activity having MIC values of 1.25-2.5-4-5mg/mL. *A. mollis* aerial parts displayed the highest anti-inflammatory activity (IC₅₀=1.22±0.07mg/mL). Ellagic acid and miquelianin were also determined as anti-inflammatory agents with 0.57±0.01mg/mL and 1.23±0.02mg/mL IC₅₀ values, respectively. Total phenolic content and tannin content of the *A. mollis* and *A. persica* were determined as 357.00±75.80mg, 282.50±28.70mg PGE/g plant material and 18.02%, 18.63% respectively according to the method described by European Pharmacopoeia. Ellagic acid, miquelianin and catechin were analyzed by HPLC. The highest catechin content was detected in *A. persica* roots (6.69±0.05g/100g plant material). *A. mollis* aerial parts contain higher miquelianin (0.39±0.02g/plant material) and ellagic acid (1.56±0.01g/plant material) than *A. persica*.

Keywords: *Alchemilla mollis*. *Alchemilla persica*. Anti-inflammatory activity. Antimicrobial activity. Miquelianin. Ellagic acid. Biological activities of *Alchemilla* species.

INTRODUCTION

The genus *Alchemilla* L. (Rosaceae) is distributed especially in Eurasia as well as in East Africa, North America, and Australia. In Europe and Mediterranean region, 541 species of this genus are recorded and 74 of them grow naturally in North and East Anatolia (Davis, 1982; Hayırlioğlu, Beyazoğlu, 1997; Özhatay, Kültür, 2006; Özhatay, Kültür, Gurdal, 2011; Aymerich, Sae, 2015).

Alchemilla vulgaris L. known as Lady's mantle, Bear's foot, Lion's foot in Europe is the most studied

species of *Alchemilla* genus. This plant is used in folk medicine internally for menopausal complaints, gynaecological disorders such as dysmenorrhea and menorrhagia, menstrual pain, gastrointestinal disorders, inflammation, weight loss, and externally as a gargle for mouth and throat inflammation as well as for wounds and skin disorders such as eczema and rashes (Said *et al.*, 2011; Ozbek *et al.*, 2017). European Pharmacopoeia described *A. vulgaris* aerial parts and Commission E approved usage of this plant for treatment of non-specific diarrhea (Blumenthal *et al.*, 1998). On the basis of clinical studies and long-term usage, *A. vulgaris* is suggested against non-specific diarrhea, gastrointestinal disorders and dysmenorrhea by ESCOP Monographs (Mills, Hutchins, 2013).

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In Turkish folk medicine, *Alchemilla* species are used for wounds, skin disorders, gynaecological and menstrual complaints, diarrhea, liver inflammation, dyspnea, diabetes, asthma, bronchitis, kidney diseases, intestinal-gastric disorders and have sedative, tonic, diuretic, antitussive, anti-inflammatory properties (Akbulut, Bayramoğlu, 2013; Akbulut, Bayramoğlu, 2014; Kalankan, Özkan, Akbulut, 2015; Küpeli Akkol *et al.*, 2015; Ozbek *et al.*, 2017).

Previous studies indicated that *Alchemilla* species have hepatoprotective (Ozbek *et al.*, 2017), angioprotective (Mills, Hutchins, 2013), vasorelaxant (Takır *et al.*, 2015), antitumor (Mazzio, Soliman, 2009), cytotoxic (Türk *et al.*, 2011), antioxidant (Ergene *et al.*, 2010; Mills, Hutchins, 2013), wound healing (Ergene Öz *et al.*, 2016), antimicrobial (Krivokuca *et al.*, 2015) and antiviral properties (Ozbek *et al.*, 2017).

Alchemilla species are rich sources for phytochemicals such as tannins, flavonoids, proanthocyanidins, triterpenes and phenolic acids (Fraisie *et al.*, 2000; Olafsdottir, Omarsdottir, Jaroszewski, 2001; Trendafilova *et al.*, 2011; Duckstein *et al.*, 2013).

In order to reveal wound healing activities of *A.mollis* and *A.persica*, which were established previously, due to inhibition of inflammation and prevention of microbial infections, we aimed to evaluate antimicrobial and anti-inflammatory activities in current study.

Chemical composition of *Alchemilla mollis* (Buser) Rothm. and *Alchemilla persica* Rothm. aerial parts and roots were investigated in this study in order to define responsible compounds for mentioned activities. Miquelianin (**1**) and ellagic acid (**2**) were isolated from the ethylacetate part of the methanol: water extracts of *A.mollis* aerial parts by using chromatographic techniques. Catechin, ellagic acid and miquelianin were analyzed by HPLC qualitatively and quantitatively. Antimicrobial activities of the *A.mollis* and *A.persica* as well as miquelianin and ellagic acid were evaluated against *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231. Anti-inflammatory activity of the extracts and isolated compounds were also tested by measuring their HRBC membrane stabilizing effects.

MATERIAL AND METHODS

Plant Material

A.mollis was collected from Sivas, Sarıyar in 2013, while *A.persica* was collected from Erzincan, Kop Passage in 2015. *A.mollis* was identified by Mehmet Tekin and deposited in Cumhuriyet University, Faculty of Science Herbarium (CUFH 1344). *A.persica* was identified by Hayri Duman and deposited in Ankara University Faculty of Pharmacy Herbarium (AEF 26371). Aerial parts and roots of the plants were dried at room temperature separately and then ground.

Extraction and Isolation

Dried and powdered aerial parts of *A.mollis* (995.96 g) were macerated with 1500 mL; roots of *A.mollis* (54.71 g), aerial parts of *A.persica* (51.71 g) and roots of *A.persica* (51.44 g) were macerated with 150 mL methanol:water (80:20) solvent system during 24 hours at room temperature separately, stirred in ultrasonic bath for 30 minutes and then filtered. Extracts were evaporated under vacuum at 35-45 °C and then lyophilized. Crude extract of *A.mollis* aerial parts (284.4801 g) was dissolved in water and subjected to liquid-liquid extraction by petroleum ether, chloroform and ethylacetate, to yield 5.4229 g; 15.0525 g; 64.7894 g of corresponding extracts, respectively.

Ethylacetate extract (21.4730 g) was applied to silica gel column (230-400 mesh, 0.040-0.063 mm, Merck ASTM) and eluted with ethylacetate:methanol:water (100:13.5:10) solvent system. Fractions were examined on TLC plates (Merck, 1.05554), with vanillin-sulfuric acid reagent and similar fractions were combined.

Fractions 14-16 obtained from silica gel column were applied to Sephadex LH-20 (25-100 µm, Sigma-Aldrich) column and eluted with methanol. Quercetin glucuronide, namely miquelianin(**1**), (16.1 mg) was obtained from Fractions 49-54, collected from Sephadex column as yellow precipitate. Fractions 34-46 obtained from silicagel column gave us ellagic acid (**2**) as a white precipitate (31.2 mg).

Identification

MS (Waters 2695 AlliaMicromass ZQ LC/MS, C-18 column), NMR¹H (Varian Mercury 400, 400 MHz High Performance Digi NMR), ¹³C (Varian Mercury 400, 400 MHz High Performance Digi NMR) and 2D NMR (HMBC) (Varian Mercury 400, 400 MHz High Performance Digi NMR) were recorded to identify structure of the isolated compounds.

Antimicrobial Activity

Crude extracts and isolated compounds miquelianin and ellagic acid from aerial parts of *A.mollis* were dissolved in 20 % DMSO and were tested for their antimicrobial activities. *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231 were used for antibacterial and antifungal activity test respectively. Microbroth dilution method was used for determination of the minimum inhibitory concentrations (MIC). The cultures were obtained in Mueller Hinton Broth (Difco, Difco Laboratories, Detroit, MI, USA). Serial two-fold dilutions ranging from 10 to 0.078 mg/mL were prepared in medium. A set of wells containing only inoculated broth, 20 % DMSO, ampicillin, ofloxacin and fluconazole were used as control. After incubation for 18-24 h at 35±1°C for bacteria-48 h for fungi, the last well with no microbial growth was recorded to represent MIC value (mg/mL).

Anti-inflammatory Activity

The study protocol was approved by the ethics committees of the Faculty of Medicine of Ankara University, Ankara-Turkey (26.10.2015/16-695-15).

Preparation of Human Red Blood Cells (HRBC) Suspension: Fresh whole human blood was collected from healthy human volunteer who had not taken any anti-inflammatory or steroidal drug for 2 weeks prior the experiment and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min. The packed cells were washed at least three times with

equal volume of isosaline (0.85 %, pH7.2). The volume of the blood was measured and reconstituted as 10 % v/v suspension with isosaline.

Heat Induced Hemolysis: Membrane stabilizing activity of the extracts was assessed using heat-induced human erythrocyte hemolysis. A stock solution of each sample was prepared by dissolving 20 mg of extract/ acetylsalicylic acid with 1 ml of their solvents. The reaction mixture consisted of test sample (extracts) and 10 % HRBC suspension. Instead of test sample, only solvent was used as a control. Acetylsalicylic acid was used as a standard drug. All centrifuge tubes containing reaction mixture were incubated in water bath at 56 °C for 30 min. Then, the incubation tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min. The absorbance of the supernatant was measured at 560 nm (Anosike, Obidoa, Ezeanyika, 2012). Then, the efficient concentrations of the samples were determined by diluting the solution and the half maximal inhibitory concentration (IC₅₀) values were calculated.

Statistical analyses

All the experiments were performed at least in triplicates for all the test samples. SPSS 23.0 was used to examine the results. Results were expressed as mean and standard deviation (SD). One-way analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by the Fisher's least significant difference (LSD) test. P values less than 0.05 were reported as "statistically significant".

Total Phenolic Content

Total phenol content of the aerial parts and roots of *A.mollis* and *A.persica* was carried out using the method described in European Pharmacopeia 6.0. Aerial parts and roots of *A.mollis* and *A.persica* were powdered. 0.5 g of each material was heated with 150 mL water at water-bath for 30 minutes, then diluted to 250 mL with water and filtered. First 50 mL of the solution was discarded. 5 mL of each extract was diluted to 25 mL with water. Mix 2 mL of each solution with 1 mL phosphomolybdotungstic reagent (Sigma-Aldrich) and

10 mL water, then diluted to 25 mL with 29 % sodium carbonate (Atabay) solution. After 30 minutes final mixture's absorbance was measured at 760 nm as A_1 against the water as a blank solution.

Total phenolic content of the extracts were expressed as gallic acid and pyrogallol equation. For pyrogallol equation, 50 mg pyrogallol was dissolved in water and diluted to 100 mL with water. 5 mL of the solution was diluted to 100 mL with the same solvent. 2 mL of this solution was mixed with 1 mL phosphomolybdotungstic reagent (Sigma-Aldrich) and 10 mL water, then diluted to 25 mL with 29 % sodium carbonate (Atabay) solution. After 30 minutes, absorbance was read at 760 nm as A_3 , against the water. Following equation was used for calculation of total phenolic content.

$$\text{Total phenolic content} = \frac{62.5 \times A_1 \times m_2}{A_3 \times m_1}$$

m_1 = weight of test material (g),

m_2 = weight of pirogallol (g)

For gallic acid equation, standard compound, gallic acid was weighed as 50 mg and dissolved in 10 mL of water. Different concentrations of standard compound were prepared (0.5, 0.1, 0.05, 0.025 mg/mL). 2 mL of each concentration was mixed with 1 mL phosphomolybdotungstic reagent (Sigma-Aldrich) and 10 mL water, then diluted to 25 mL with 29 % sodium carbonate (Atabay) solution. After 30 minutes, final mixture's absorbance was measured at 760 nm against the water as blank solution. Calibration curve was drawn and the equation was calculated. Linear correlation between concentration and absorbance was expressed as $y = mx + n$ equation. Total phenolic content of the tested extracts was calculated by the following equation $y = 1.417x + 0.067$. y-absorbance, x-total phenolic content as gallic acid mg/g plant material equivalent.

Tannin Content

Tannin content of the aerial parts and roots of *A. mollis* and *A. persica* was carried out using the method described in European Pharmacopeia 6.0. Aerial parts

and roots of *A. mollis* and *A. persica* were powdered. 0.5 g of each material was heated with 150 mL water at water-bath for 30 minutes, then diluted to 250 mL with water and filtered. First 50 mL of the solution was discarded. 5 mL of each extract was diluted to 25 mL with water. 2 mL of each solution was mixed with 1 mL phosphomolybdotungstic reagent (Sigma-Aldrich) and 10 mL water, then diluted to 25 mL with 29 % sodium carbonate (Atabay) solution. After 30 minutes, final mixture's absorbance was measured at 760 nm as A_1 , against the water as blank solution. 10 mL of each extract was stirred with 0,10 g hide powder for 60 minutes and filtered. 5 mL of each extract was made up to 25 mL with water. 2 mL of each solution was mixed with 1 mL phosphomolybdotungstic reagent (Sigma-Aldrich) and 10 mL water, then diluted to 25 mL with 29 % sodium carbonate (Atabay) solution. After 30 minutes, final mixture's absorbance was read at 760 nm as A_2 , against the water as blank. Tannin content of the extracts was calculated from following equation and expressed as pyrogallol equation.

$$\text{Tannin content} = \frac{62.5 \times (A_1 - A_2) \times m_2}{A_3 \times m_1}$$

For determining tannin content as gallic acid, equivalent calibration curve of gallic acid was used and each extract was stirred with hide powder using the method described in European Pharmacopeia (2008) and final mixture's absorbance was read at 760 nm as phenolic compounds were not absorbed by hide powder against the water as blank. Tannin content of the extracts was calculated from the same equation by subtracting absorbance of phenolic compounds not absorbed by hide powder from absorbance of total phenolic content.

HPLC Analyses

HPLC analyses were carried out using Agilent LC 1260 chromatograph (Agilent Technologies, Darmstadt, Germany). The diode array detector (DAD) was set at wavelengths of 210 and 254 nm and peak areas were integrated automatically using Agilent Chem Station Software. ACE 5 C18 (250 mm; 4.6 mm; 5 μ m) column

was used for separation. The mobile phase was made up of acetonitrile (A) and 0.2 % phosphoric acid (B) using gradient elution: initial percentage A:B (8:92, v/v), then 0-20 min linear change from A:B (8:92, v/v) to A:B (15:85, v/v), and 20-40 min linear change from A:B (15:85, v/v) to A:B (25:75, v/v) were used. This was followed by gradient elution from A:B (25:75, v/v) to A:B (50:50 v/v) in 40-50th min and from A:B (50:50 v/v) to A:B (100:0, v/v) in 50-55th min. Isocratic flow A:B (100:0,v/v) was applied from 55th min to 60th min. The flow rate was 0.5 mL/min, column temperature was maintained at 25 °C. The sample injection volume was 10 µL. Both aerial parts and roots of *A. mollis* and *A. persica* (0.5 g) were extracted with methanol:water (80:20) mixture in ultrasonic bath for 30 min. After filtration, all extracts were adjusted to 10 mL in volumetric flask. Each extract was filtered from 0.45 µm filters before injection to HPLC system. Catechin, gallic acid, chlorogenic acid and caffeic acid purchased from Sigma; ellagic acid and miquelianin obtained from *A. mollis* were used as standard compounds. To prepare calibration curves, peak area of each solution was plotted against the concentration. Tested compounds were prepared at six different concentration levels (0.01, 0.02, 0.05, 0.1, 0.2, 0.25 mg/mL). Triplicate injections

of 10 µL of each standard solution were performed. The compounds were determined at 210 and 254 nm absorbances. LOD (Limit of detection) and LOQ (Limit of quantification) of each compound were determined as signal to noise ratio of 3 and 10 respectively and verified by six injections at 10 µL.

RESULTS AND DISCUSSION

The MIC values (mg/mL) of the methanol:water extracts of *A. mollis* and *A. persica* as well as miquelianin and ellagic acid were shown in Table I. Neither extracts nor compounds showed antibacterial activity against *E.coli*. Both of the aerial part extracts of the tested plants and also miquelianin didn't show antibacterial activity against *B. subtilis*. Root extract of *A. mollis*, ellagic acid and miquelianin didn't show antibacterial activity against *E. faecalis*. No antibacterial activity was observed against *P. aeruginosa* for ellagic acid and no antifungal activity was observed against *C. albicans* for root extract of *A. mollis*. Except these, extracts possessed activity having MIC values of 2.5-5-10 mg/mL, miquelianin and ellagic acid possessed activity having MIC values of 1.25-2.5-4-5 mg/mL against the tested microorganisms.

TABLE I - Minimum Inhibitory Concentration values (mg/mL) of *Alchemilla* species and isolated compounds against tested microorganisms

Extracts / compounds	Microorganisms					<i>C. albicans</i> ATCC 10231
	<i>S. aureus</i> ATCC 29213	<i>E. faecalis</i> ATCC 29212	<i>B. subtilis</i> ATCC 6633	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> ATCC 27853	
<i>A. persica</i> Aerial parts	5	5	-	-	5	10
<i>A. persica</i> Root	5	10	2.5	-	10	10
<i>A. mollis</i> Aerial parts	5	5	-	-	10	-
<i>A. mollis</i> Root	5	-	10	-	10	10
Ellagic acid	1.25	-	5	-	-	2.5
Miquelianin	4	-	-	-	4	4

(continues on the next page...)

Extracts / compounds	Microorganisms					<i>C. albicans</i> ATCC 10231
	<i>S. aureus</i> ATCC 29213	<i>E. faecalis</i> ATCC 29212	<i>B. subtilis</i> ATCC 6633	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> ATCC 27853	
Ampicillin	0.0003	0.001	0.006			
Ofloxacin				0.001	0.008	
Fluconazole						0.001
DMSO (10 % - 0.23 %)	-	-	-	-	-	-

‘-’=represents no activity

Anti-inflammatory activity of the samples was determined by measuring stabilization capacity against heat induced hemolysis of HRBC membrane. The extracts showed significant protection of the HRBC membrane against hemolysis induced by heat compared to acetylsalicylic acid used as a reference drug. *A. mollis* aerial part extract ($IC_{50}=1.22\pm 0.07$ mg/mL) exhibited the highest HRBC membrane stabilizing effect almost 4 times lower than acetylsalicylic acid ($IC_{50}=0.29$ mg/mL) (Table II).

TABLE II - Human red blood cell membrane stabilizing effects of methanol:water extracts of *Alchemilla* species and isolated compounds.

Plant Name	IC_{50} (mg/mL)**
<i>A. mollis</i> Aerial parts	$1.22 \pm 0.07^*$
<i>A. mollis</i> Root	$1.34 \pm 0.08^*$
<i>A. persica</i> Aerial parts	$1.52 \pm 0.09^*$
<i>A. persica</i> Root	$1.82 \pm 0.14^*$
Miquelianin	$1.23 \pm 0.02^*$
Ellagic acid	$0.57 \pm 0.01^*$
Acetylsalicylic acid	$0.29 \pm 0.002^*$

(*) Statistically significant as compared to controls, $p < 0.05$ (one-way ANOVA, SPSS 23.0).

(**) \pm Standard Deviation

Total phenol and tannin content of the *A. mollis* and *A. persica* was tested according to the method described in European Pharmacopoeia 6.0 in current study. Gallic acid and pyrogallol are both used as

standards. Results of our analysis revealed that the total polyphenol content of *A. persica* and *A. mollis* varied from 244 ± 72.7 to 331.7 ± 117.7 mg GAE/ g and tannin content varied from 8.3 ± 4.42 to 12.95 ± 3.00 g GAE/100 g plant material. As pyrogallol equivalent, total phenolic content varied from 237.8 ± 25.9 to 357 ± 75.8 mg PGE/ g and tannin content between 9.35 ± 0.29 and 18.63 ± 0.29 g PGE/100 g plant material. The highest tannin and total phenolic content as pyrogallol equivalent were determined for both aerial parts of *A. mollis* and *A. persica* extracts (Table III and Table IV). Our research reveals that aerial parts of *A. mollis* exhibited the highest red blood cell membrane stabilization activity (IC_{50} : 1.22 ± 0.07 mg/mL) with a good protection against heat-induced lysis and followed by *A. mollis* root extract (IC_{50} : 1.34 ± 0.08 mg/mL). These results can be attributed to the higher phenolic and tannin content.

TABLE III - Total phenolic content and tannin content of *Alchemilla mollis* and *Alchemilla persica* as gallic acid equivalent

Plant material	Total phenolic content (mg GAE/g plant material)	Tannin content (g GAE/100 g plant material)
<i>A. mollis</i> Aerial parts	298.52 ± 89.87	8.3 ± 4.42
<i>A. mollis</i> Root	244.00 ± 72.73	5.68 ± 2.54
<i>A. persica</i> Aerial parts	295.22 ± 80.00	12.95 ± 3.00
<i>A. persica</i> Root	331.69 ± 117.27	11.3 ± 1.56

TABLE IV - Total phenolic content and tannin content of *Alchemilla mollis* and *Alchemilla persica* as pyrogallol equivalent

Plant material	Total phenolic content (mg PGE/g plant material)	Tannin content (g PGE / 100 g plant material)
<i>A. mollis</i> Aerial parts	357.00 ± 75.80	18.02 ± 0.29
<i>A. mollis</i> Root	239.80 ± 00.00	9.35 ± 0.29
<i>A. persica</i> Aerial parts	282.50 ± 28.70	18.63 ± 0.29
<i>A. persica</i> Root	237.80 ± 25.90	9.86 ± 1.01

Total phenol contents of the *A. mollis* and *A. persica* were also determined and the highest content was observed in *A. persica* roots (331.69±117.27 mg GAE/ g plant material; 282.50±28.70 mg PGE/g plant material). Ellagic acid and miquelianin as well as catechin were also analysed by HPLC qualitatively and quantitatively (Table V) LOD and LOQ values of catechin, miquelianin and ellagic acid were also determined and are given in Table VI. Table V exhibits both roots of *A. mollis* and *A. persica*, which were found to contain catechin while aerial parts did not. The highest content of the catechin was determined in *A. persica* roots (6.69±0.05 g/100 g plant material). On the other hand, ellagic acid and miquelianin were only detected in aerial parts of both species. Ellagic acid and miquelianin were detected as major compounds in HPLC chromatogram of the *A. mollis* and *A. persica* aerial part extracts. Miquelianin percentages of the both species were higher than *A. vulgaris*. However, ellagic acid content of the *A. persica* was lower than *A. vulgaris* while *A. mollis* contained a higher amount. Both miquelianin and ellagic acid were isolated from ethylacetate part of the methanol-water extract of *A. mollis* aerial parts using different chromatographic techniques in the present study. Structures of the isolated compounds were determined according to

the NMR¹H and ¹³C NMR results and comparing with literature data (Li *et al.*, 1999; Nugroho, Lee, 2014; Vijayalakshmi, Madhira, 2014). Previous studies have revealed that *A. mollis* contains miquelianin, hyperoside, isoquercetin, cis- and trans-tiliroside, sinocrassoside D2, rhodiogin and gossypetin-3-*O*-β-D-galactopyranosyl-7-*O*-α-L-rhamnopyranoside (Trendafilova *et al.*, 2011). In acetone/water extracts of *A. mollis*, pedunculagin, agrimoniin, sanguin H-10, castalagin/vescalagin as monomeric and oligomeric ellagitannins as well as galloyl-bis-hexahydroxydiphenoyl (HHDP) hexose, gallic acid, chlorogenic acid, catechin, a procyanidin trimer, quercetin glucuronide (miquelianin), several other quercetin glycosides, kaempferol glucuronide and methylated quercetin glucuronide were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Duckstein *et al.*, 2013). Sesquiterpenes and diterpenes as well as phenolic compounds including catechin, gallic acid, chlorogenic acid, gallic acid glycoside, gallic acid methoxy glycoside, pedunculatin isomers, galloyl-HHDP-hexoside isomers, epicatechin, quercetin-3-*O*-glucuronide, sanguin isomers, casuarictin, agrimoniin, procyanidin B1, kaempferol-3-*O*-rutinoside, methyl gallate, ellagic acid, digalloyl-galloylgalloside, aromadendrin glucoside derivative were determined in *A. persica* using GC-MS and HPLC-MS techniques (Afshar *et al.*, 2015). Ellagic acid was isolated from *A. mollis* aerial parts for the first time in the current study. European Pharmacopoeia describes *A. vulgaris* dried leaves as “*Alchemillae herba*” an official pharmaceutical drug. According to the European Pharmacopoeia and ESCOP monographs, *Alchemillae herba* should contain minimum 6.0 per cent of tannins expressed as pyrogallol, approximately 2.2-2.5 % flavonoid glycosides and the miquelianin namely as main flavonoid (European Pharmacopoeia, 2008; Mills, Hutchins, 2013). Additionally, according to Fraisse *et al.* (2000), levels of the total flavonoids were detected as 2.22 %, miquelianin 1.18 %, tannins 16.0 % and ellagic acid 0.36 % for *A. vulgaris*.

TABLE V - Quantity of ellagic acid, miquelianin and catechin in plant materials

Plant material	Amounts of standard compounds in plant materials (g/100 g plant material)		
	Catechin	Ellagic acid	Miquelianin
<i>A.mollis</i> Aerial parts	-	1.56 ± 0.01	0.39 ± 0.02
<i>A.mollis</i> Root	3.11 ± 0.37	-	-
<i>A.persica</i> Aerial parts	-	1.23 ± 0.01	0.34 ± 0.00
<i>A.persica</i> Root	6.69 ± 0.05	-	-

TABLE VI - Limit of Detection and Limit of Quantitation values of ellagic acid, miquelianin and catechin

	Standard compounds		
	Catechin	Ellagic acid	Miquelianin
LOD (µg/mL)	0.152	2.6740	0.249
LOQ (µg/mL)	0.508	8.913	0.829

According to the literature survey, antibacterial activities of the *A. mollis* were tested against *S. aureus*, *S. S. epidermidis*, *S. pyogenes*, *P. aeruginosa*, *K. pneumonia* and *E. coli* previously. Water, ethanol and methanol extracts of the *A. mollis* displayed antibacterial activity against *E. coli* and *P. aeruginosa* in disc diffusion and well diffusion assays. Inhibition zones were determined as 9.33 ± 0.66 mm, 15.33 ± 0.66 mm and 9.33 ± 0.66 mm for water, ethanol and methanol extracts against *E. coli* and 22.67 ± 0.66 mm, 22.67 ± 1.33 mm for ethanol and methanol extracts against *P. aeruginosa* in disc diffusion test. Ethanolic and methanolic extracts of *A. mollis* antimicrobial activity were similar or greater than tested antibiotics; ovobiocin (23 mm), carbenicillin (22 mm), tetracycline (13 mm), bacitracin (11 mm), ampicillin (8 mm) and lincomycin (7 mm) in disc diffusion methods against *P. aeruginosa*. Same results were obtained in well diffusion method (Usta, Birinci Yıldırım, Uçar Türker, 2014). Additionally, *A. mollis* was tested against *S. aureus*, *E. coli*, *Salmonella thphimurium*, *Salmonella enteritidis* and *C.albicans aureus* in disc diffusion and

micro dilution assays. Ethyl acetate, methanol, butanol, 70% methanol and water extracts of the *A. mollis* displayed higher antibacterial activity against *S. aureus* and *Sa.enteritidis*. Inhibition zones were determined as 19.00 ± 00 mm, 18.66 ± 0.57 mm, 18.66 ± 1.52, 13.33 ± 1.15 and 19.00 ± 2.00 mm for ethyl acetate, methanol, butanol, 70% methanol and water extracts against *S. aureus* in disc diffusion test. Antimicrobial activity was similar in tested antibiotic; penicillin (19 mm) against *S. aureus*. Ethyl acetate, hexane, methanol, butanol, 70% methanol and water extracts showed moderate activity against *E.coli* and *Sa.typhimurim*. Inhibition zones were observed as 13.66 ± 1.05, 16.33 ± 3.05, 12.16 ± 1.04, 11.00 ± 00, 14.00 ± 2.64 and 9.33 ± 0.57 mm for ethyl acetate, methanol, butanol, 70% methanol and water extracts respectively and 21.00 ± 1.00 mm for penicillin against *E.coli*. Ethyl acetate, methanol and water extracts of *A. mollis* showed moderate antimicrobial activity against *C.albicans* with ranging MIC values from 125 to 500 µg/mL. (Şeker Karatoprak, İlgün, Koşar, 2017). Our study results are not similar for antibacterial activity against *E. coli*. On the other hand, antimicrobial activity was observed against *P. aeruginosa* with 5-10 mg/mL MIC values. The highest activity was observed with *A. persica* aerial part extract (MIC 5 mg/mL). Miquelianin showed antimicrobial activity against *Streptococcus mitis*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* with 0.25 mg/mL MIC values (Shahzad *et al.*, 2015). Ellagic acid was determined as one of the active constituents of *Syzygium aromaticum* for its antimicrobial activity against oral pathogens such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Streptococcus mutans*, *Actinomyces viscosus* with 1.25 mg/mL MIC value (Cai, Wu, 1996). It has been reported that ellagic acid exhibited antibacterial activity against *S.aureus* ATCC 9538, *S.epidermidis* NCTC 11047, *Micrococcus luteus* NCTC 9341, *E.coli* NCTC 8196, *B.subtilis* ATCC 6633 with 0.63 mg/mL, 0.31 mg/mL, 0.31 mg/mL, 2.5 mg/mL, 0.63 mg/mL and antifungal activity against *C.albicans* ATCC 10231 with 1.25 mg/mL MIC values (Thiem, Goslinska, 2004). Present study results revealed that similar results were obtained for ellagic acid. This compound displayed antibacterial activity against *B. subtilis* and *S. aureus* with 5 mg/mL,

1.25 mg/mL MIC values respectively and antifungal activity against *C. albicans* 2.5 mg/mL MIC value as shown in Table I. Martini *et al.* (2009) have reported that antimicrobial activity of ellagic acid on *H.pylori* G21 and *H.pylori* 10K was also observed at 0.002 mg/mL and 0.01 mg/mL concentrations, respectively. Ellagic acid inhibited growth of oral pathogens such as *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus salivarius*, *Actinomyces naeslundii*, *Actinomyces viscosus*, *Lactobacillus rhamnosus*, *Porphyromonas gingivalis* and *Bacteroides forsythus* (Loo *et al.*, 2010). Antibacterial activity of ellagic acid against *Staphylococcus epidermidis*, *Bacillus cereus*, *Klebsiella pneumonia* and *Salmonella typhi* was also tested using plate-agar method. Activity of the compound was determined as more effective than gentamycin and streptomycin. Minimum effective dose of ellagic acid was determined as 0.15 mg/mL while gentamycin and streptomycin were 0.3 mg/mL and 2 mg/mL respectively (Ghudhaib, Hanna, Jawad, 2010).

According to current study results, *A.mollis* aerial parts contain tannin, miquelianin and ellagic acid in a proper percentage when compared to *A.vulgaris* as an official pharmaceutical drug. *A. persica* also contains a higher amount of tannin and miquelianin than *A. vulgaris* and a lower amount of ellagic acid. These results may explain the traditional usage of *A. mollis* in European traditional medicine for same purposes with *A. vulgaris* as a commercial drug “Herba Alchemillae” for its astringent, diuretic, antispasmodic, wound healing activities and against excessive menstruation (Trendafilova *et al.*, 2011; Özbek *et al.*, 2017).

In conclusion, antimicrobial activity was observed with aerial parts and roots of *A. mollis* and *A. persica* as well as miquelianin and ellagic acid as isolated compounds against tested microorganisms. All the plant extracts tested as well as ellagic acid and miquelianin stabilized the HRBC membrane against heat significantly. Miquelianin, ellagic acid and tannin content of the *Alchemilla* species could be responsible for their antimicrobial and anti-inflammatory activities. Additionally, tannin and miquelianin content of *A. mollis* was determined as high as *A. vulgaris*. These results may suggest the usage of *A. mollis* instead of *A. vulgaris* in countries where *A. vulgaris* does not grow naturally.

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