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The enzymatic and non-enzymatic antioxidant activities of *Arbutus andrachne L.* leaf and flower and its antibacterial activities against mastitis pathogens

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ABSTRACT

For solving the antibiotic resistance problem it needs to explore new antimicrobial substances. Plants can be good sources for these purposes. This study was planned to evaluate the antibacterial and antioxidant activities of Arbutus andrachne leaf and flower. The antibacterial and antioxidant activities were conducted for methanolic extracts of the plant using conventional methods. Antioxidant activity was analyzed using the DPPH free radical scavenging method and high antioxidant activity was found (81%), reached up to 2.10 and 2.11 mmol Trolox equivalent (mM (TE) /g dry biomass), respectively. It was determined that the activities of catalase (CAT) and ascorbate peroxidase (APX) enzymes were higher at the flowers of Arbutus andrachne L. compared to the leaves. The extracts were screened for its antibacterial activity against seven mastitis pathogens. The methanol extract of Arbutus andrachne showed minimal inhibitory activity (MIC) at 3250 µg/mL. It can thus be concluded that A. andrachne is a good source of antibiotics and antioxidants.

Key words: *Arbutus andrachne*, Antibacterial activity, Antioxidant activity

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INTRODUCTION

Mastitis is a complex disease, which is defined as inflammation of parenchyma of mammary glands and is characterized by physical, chemical and usually bacteriological changes in milk and pathological changes in glandular tissues [40]. Sharma *et al*. reported 70.32% incidence of subclinical mastitis in buffaloes [44], while Maiti *et al.* determined 70.37% incidence of subclinical mastitis in cows [33]. More than 130 different microorganisms can cause mastitis [48]. Mastitis is caused by many bacteria, which include the *Staphylococci, Streptococci, Corynebacteria, Pasteurella, Mycoplasma, Leptospira, Yersinia, Mycobacteria, Pseudomonas, Serratia* etc. Coagulase-negative *staphylococci* (CNS) have been considered to be minor mastitis pathogens, especially in comparison with major pathogens such as *Staphylococcus aureus*. The main reason for this is that mastitis caused by CNS is very mild, and usually remains subclinical [45]. The significance of CNS, however, needs to be reconsidered as in many countries they have become the most common mastitis-causing agents [38, 47].

Mastitis has been and continues to be recognized as one of the serious problems concerning the dairy industry. It causes heavy economic losses to the dairy industry worldwide. Traditionally, the mastitis control programmes are focused at use of chemical disinfectants, antiseptic or herbal teat applications [34] and antibiotic therapy. The

antibiotic treatment may be help in minimizing the losses but simultaneously may lead drug resistance.

Medicinal plants are represent a rich source of antimicrobial agents. Many plants have been used due to their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. Rajaperumal *et al.* reported that the methanolic root extract of *Indigofera aspalathoides aspalathoides* (Vahl ex DC) have found antimicrobial, antioxidant and cytotoxic effect [52]. Amin *et al.* reported that the ethanolic extract of *Ocimum sanctum* leaves showed 90% radical inhibitions at 100 µg/mL [54]. *Arbutus andrachne* L. and *A. unedo* L. are two members of the *Ericaceae* family. The species are distributed in coastal parts of Anatolia and have edible fruits. *Arbutus andrachne* L. is the strawberry tree and it is native to the Mediterranean region and southwestern Asia. *Arbutus andrachne* can reach a height of about 12 meters. The smooth bark is exfoliating during the summer, leaving a layer with a pistachio green color, which changes gradually to a beautiful orange brown. The flowers bloom in Spring and are white or yellowish green. Its fruits ripen in Autumn. Its small trees are usually less than 4 m high; the wood is used for several purposes including making carved spindles, stools and small furniture [3, 15, 23, 24, 26, 37].

Arbutus unedo is well-known in folk medicine for beneficial properties [10]. *A. andrachne* is used traditionally as astringent and urinary antiseptic and for the treatment of urinary system, and also used as a blood tonic, against joints ache and for treating wounds. These uses caused increase demand for this species and consequently it became threatened [41, 42]. *A. andrachne* was found to be the highest among 51 other medicinal plant species in Jordan that have antioxidant content [46]. According to the available literature, a lot of compounds were isolated from bark, and fruits. Tawaha *et al.* [46] reported that triterpenoids and steroids were also isolated from fruits. The leaf extracts contain a number of different compounds such as flavones, steroids, terpenoids, aromatic hydroxyacids. However, tannins are not the only phenolics present in leaf extracts. In fact, catechin gallate [31], myricetin [29], ά-tocopherol [27], phenolic glycosides and in particular quercitrin, isoquercitrin, hyperoside and rutin [35], have been identified. These substances are important for treatment of diseases. For example, ethanol leaf extracts have shown antimicrobial activity against Gram positive and Gram negative bacteria [27].

However, to the best of our knowledge there is no report on the biological activities of *A. andrachne* from Turkey. The aims of the present work were to evaluate antibacterial and antioxidant activities of this wild growing species in Turkey.

MATERIALS AND METHODS

Plant material and extraction

Arbutus andrachne samples were harvested from Hatay in May 2013. The plant material was deposited in the herbarium of the Biology Department of the Mustafa Kemal University, Turkey. The identification of these specimens was carried out using the Flora of Turkey [15].

The leaves and flowers were washed thoroughly 2-3 times with running water and once with sterile distilled water. The samples were dried in the shade at room temperature for 10 days. Plant parts were ground to a fine powder using a laboratory mill, passed through a 24 mesh sieve, to provide homogeneous powder for the analysis. All samples were stored at ambient temperature until initial sample preparation and protected from light until the analyses. Finally, it was packed in a plastic bag for further use.

The air dried and powdered leaves and flowers of the plant samples (10 g) were extracted with methanol (100 mg/mL) using the Soxhlet apparatus until colorless extract obtained on the top of the apparatus. The extracts were evaporated and then deposited in methanol. These extracts were filtered through a Whatman filter paper No.1 (Sargent- Welch, USA), and kept in amber-coloured glass bottles at refrigerated conditions until the analyses.

Microorganisms and cultivation

The leaf and flower extracts were individually tested against mastitis pathogens. Mastitis pathogens obtained from previous studies by Dr. Zafer Cantekin, Mustafa Kemal University, TURKEY (Project number: 1101 M 0103; Ethics council number: 2010 / 02- 30: 12). Seven bacteria were used in these studies: two *S. aureus* and five coagulase-negative *staphylococci* (CNS). The bacteria were grown for 24h at 37°C in Mueller- Hinton Broth (Merck). The bacteria were identified by traditional biochemical tests [39].

Determination of antibacterial activity

Bauer and Kirby method applied for antibacterial activity [4]. The bacteria were maintained on Mueller-Hinton agar plates (MHA, Merck) at 37 °C [4]. The inocula were prepared daily and stored at +4°C until use. The extracts of *A. andrachne* leaf and flower were then dissolved in methanol to a final concentration of 100 mg/mL and sterilized

by filtration with 0.45-µm Millipore filters. For assay of the antibacterial activity of the extracts, the disc diffusion technique was used.

Bacterial inoculums that were 18 h old. Bacterial cultures adjusted to 0.5 McFarland were deployed on the surface of the Mueller-Hinton agar media with the help of a sterile cotton swab. Mastitis pathogens were incubated at 37°C for 24h. The assessment of antibacterial activity was based on measurement of the diameter of the inhibition zones around the discs after 24 h. Methanol used as negative control. Oxacillin (5µg) used as positive control. All tests were performed in triplicate and the mean values were given.

Determination of minimum inhibitory concentration (MIC)

The MIC was measured on plant extracts as antimicrobial activity. The serial dilution assay was performed as described in the CLSI standards [12, 13]. This test was studied at final concentrations of each extract (6.5; 3.25; 1.625; 0.812; 0.406 mg/mL). Before gelling, 20 ml of agar medium were added to each of the Petri dishes containing the plant extract, 20 µl of each bacterial strain (0.5 McFarland) were inoculated on the Mueller Hinton agar surface. MIC was defined as the lowest extract concentration, showing no visible bacterial growth after incubation time (37°C for 24h). The experiment was studied three times and the mean values were presented.

Determination of enzymatic and non-enzymatic antioxidant activities

The antioxidant activities were determined using DPPH as a free radical. The stable 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts. Extract (0.1 ml) was added to 3.9 mL of a 0.1 mM methanol DPPH solution. After incubation for 30 minutes, absorbance of extract was measured at 515 nm using spectrophotometer. Methanol was used as a blank, while methanol with DPPH solution was used as a control [6]. Trolox was used for reference antioxidant. The DPPH scavenging capacity expressed in percentage (%) was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [\text{Abs}_{(control)} - \text{Abs}_{(extract)}] \times 100.$$

Where, $\text{Abs}_{(control)}$ is the absorbance value of the DPPH- blank sample and $\text{Abs}_{(extract)}$ is the absorbance value of the test solution.

Within the scope of the enzymatic antioxidant studies, antioxidant enzyme activities were determined in the flowers and leaves of the fresh plants. In this study ascorbate peroxidase and catalase enzyme activities were tested. Ascorbate peroxidase (APX, E.C. 1.11.1.11) activity was determined by measuring ascorbate oxidation rate in 290 nm according to [8, 9]. Catalase enzyme activity (CAT, E.C. 1.11.6.1) on the other hand was measured with a spectrophotometer as per the decomposition rate of H_2O_2 at 240 nm. APX and CAT enzyme activities were calculated and recorded in terms of (µM/min/g FW) and fresh weight. All tests were conducted simultaneously with three replications and the results were expressed as averages.

RESULTS

The results of antibacterial activity were measured as zone of inhibition in mm for all the materials used as follows. The antibacterial activities of *A. andrachne* methanol extracts were evaluated *in vitro* against test pathogens, which are known to cause mastitis. Results of antibacterial activity of methanol extracts of used plant parts against the test bacteria are shown in (Table 1).

Table 1: Antibacterial activities of *Arbutus andrachne* methanol extracts

Bacteria	İnhibition zone diameters (mm)		
	Plant extracts (100 mg/mL)		Antibiotic
	Leaf	Flower	O
S. aureus- 17	9	17	10
S. aureus- 18	8	17	8
CNS-22	-	-	-
CNS-32	-	-	7
CNS-33	9	16	7
CNS-36	9	16	-
CNS-37	9	16	-

CNS: coagulase negative Staphylococci; O: oxacillin, 5µg; (-): zone did not occur

Results show that, the methanol extracts of *A. andrachne* inhibited the growth of five bacteria and the inhibition zones ranged between 8- 17 mm. In addition to the extract of this plant did not determine any antibacterial effects against used 2 bacteria. The highest antibacterial activity shows on *S. aureus*-17 and 18 (17mm). The lowest activity

was found on *S. aureus*- 18 as 8mm. However the inhibition zone was not produced by both of extracts against CNS-22 and 32. These bacteria were found resistant to the both of extracts. Oxacillin (5µg), antibiotic used as positive control. Data of antibacterial activities of the extracts are demonstrated in (Table 1).

Antibacterial activity studies have been tested against mastitis pathogens by using serial dilution method. In Table 2, MIC values of methanol extracts belong to leaves and flowers of *A. andrachne* were summarized. MIC values for plant extracts were applied from 6500 to 406µg/mL. Four bacterial strains have shown the lowest sensitivity to leaf methanol extract. The leaf extracts from *A. andrachne* possessed antibacterial activity, and showed minimal inhibitory concentration (MIC) effect at 3250 µg/mL.

Table 2: Minimum inhibitory concentrations of *A. andrachne* methanolic extracts

Bacteria	Leaf (µg/mL)	Flower (µg/mL)
S. aureus- 17	3250	6500
S. aureus- 18	3250	3250
CNS-33	3250	3250
CNS-36	3250	6500
CNS-37	6500	3250

CNS: coagulase negative Staphylococci

Antioxidant activity was analyzed using the DPPH free radical scavenging method. The results of DPPH scavenging assay of *A. andrachne* extracts are shown in (Table 3). Table 3 shows the per cent of DPPH radical scavenging capacity with trolox as reference. The both of parts methanol extracts showed 81% inhibition at 100 mg/mL concentration (Table 3).

Table 3: Enzymatic and non-enzymatic antioxidant activities of *A. andrachne*

Extracts	DPPH radical scavenge (%)	Trolox equivalent (mM (TE) /g dry biomass)	CAT (µM/min/g FW)	APX (µM/min/g FW)
Leaf	81	2,10	0,35	7,61
Flower	81	2,11	0,25	8,8

CAT: Catalase activity APX: Ascorbate peroxidase TE: Trolox equivalent FW: fresh weight

In this work, antioxidant enzyme activities of the fresh leaves and flowers of *A. andrachne* were established. In enzymatic antioxidant activity studies, ascorbate peroxidase and catalase enzyme activities were studied. According to the results of the study, it was calculated that when considering the aboveground organs of *A. andrachne*, antioxidant enzyme activities are high in the flower component. While the catalase activity of *A. andrachne* flower was determined to be 0.25 µM /min /g FW, ascorbate peroxidase activity was determined as 8.8 µM /min /g FW. In addition, the CAT activity of the leaf of *A. andrachne* was found to be 0.35 µM /min /g FW, APX activity was determined as 7.61 µM /min /g FW (Table 3).

DISCUSSION AND CONCLUSION

This study confirms that the leaf and flower of *A. andrachne* possess antimicrobial and antioxidant activities. The properties commonly found in the plants, and they have been reported to have multiple biological effects including antimicrobial and antioxidant activities. In this study, the highest antibacterial activity was showed as 17 mm against *S. aureus*- 17 and 18 for flower extract (Table 1). In Gram-positive bacteria, cell wall allows the essential oil and hydrophobic constituents to be in direct contact with the phospholipid bilayer of the cell membrane. Researchers reported that where they bring about their effect, causing either an increase in ion permeability and leakage of vital intracellular constituents, or impairment of the bacterial enzyme systems [14, 49]. Dua *et al.* reported that antibacterial activities of *Foeniculum vulgare* Miller seeds were found as 11 mm inhibition zone against *S. aureus* [51].

In this study, the antibacterial activity for methanolic extract was also high against the two tested mastitis pathogens, results indicated the polarity of the solvent plays an important role in the extraction of the active ingredient and consequently on its antimicrobial activity. The antibacterial activity of the phenolic fractions could be due to the investigated strain sensitivity to tannins, flavonoids and the other phenolic components [18, 19]. The tannin components demonstrated strong antimicrobial activity against bacteria and fungi [25]. Flavonoids are synthesized by plants in response to microbial infection [17] and are effective against a broad range of microorganisms.

In this work, the inhibition zone was not produced by both of extracts against CNS-22 and 32. These bacteria were found resistant to the extracts (Table 1). Sawsan *et al.* reported that the extract of *A. andrachne* was not inhibited three test bacteria. This report also supports the results we obtained from our study [43].

According to our results, the leaf extract from *A. andrachne* possessed antibacterial activity, and showed minimal inhibitory concentration (MIC) effect at 3250 µg/mL (Table 2). Previous antibacterial studies of *Arbutus pavarii* indicate that methanolic extract exhibited antibacterial effect against *Staphylococus aureus*, with zone of inhibition of 20 mm, and the minimum inhibitory concentrations (MICs) were 4.86 mg/mL [1]. In this study, MIC value was generally measured as 3250 µg/mL, and our results are better than those of Alsabri *et al*. and Sharma *et al***.** [1, 53]. The effects of the methanol extract against test bacteria are of weak activity.

The results of DPPH scavenging assay of *A. andrachne* extracts are shown in (Table 3). The both of methanol extracts showed 81% inhibition at 100 mg/mL concentration (Table 3). Lee *et al.* studied the fruiting body and mycelia of *Hypsizygus marmoreus* with different extraction [30]. According to their results, the scavenging effects of the fruiting body and mycelia ethanol extracts at 5 mg/mL concentration were both 75.5%, while the scavenging effects of the fruiting body and mycelia hot water extracts were 36.8% and 55.5%, respectively. Compared with the inhibition values reported in this study, *A. andrachne* extract was more effective at 100 mg/mL concentration. Phytochemical studies have shown that the leaf extract contains phenolic antioxidant compounds, such as flavonoids [35, 36], tannins, phenolic glycosides, anthocyanins, gallic acid derivatives etc. [3, 20, 24, 27, 28, 37, 50]. Also several compounds have been isolated from the roots of *A. unedo* such as (+)-catechin, (+) catechin gallate and a number of phenolic compounds were also identified by GC–MS such benzene acetic acid 4-hydroxy, caffeic acid, gallic acid etc. [10, 11, 16, 20]. Plants contain flavonoids compounds, sitosterols, cardiac glycosides, terpenoids [5, 7]. Furthermore, these compounds have attracted the attention of scientists because of these flavonoid compounds have antioxidant activities [32].

Catalase is antioxidant enzyme which plays an important role in the body defense mechanism against the harmful effects of the reactive oxygen species (ROS) and free radicals in biological systems [22]. In plants, antioxidant enzymes namely catalase and peroxidase have been shown to increase when exposed to stress condition. Peroxidase reduces H_2O_2 to water while oxidizing a variety of substrates. In this work, the highest catalase activity was determined in leaf, whereas the highest ascorbate peroxidase activity was calculated in *A. andrachne* flower (Table 3). Ao *et al.* reported high antioxidant activity from leaf compared to bark and fruit extracts of *Ficus microcarpa* [2]. In other crops, similar differences in enzymatic antioxidant responses between callus and intact plants have been reported [21]. These studies also support the results we obtained from our study.

Our findings suggest that *A. andrachne* has significant antibacterial activity and it could be very useful in the discovery of novel antibacterial agents of plant origin. Further phytochemical studies are required to determine and isolate compounds responsible for the antibacterial effects of these species. In conclusion, *A. andrachne* might be considered as a potential source of metabolites which could be developed as precursors for antimicrobial and antioxidants drugs.

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