

## **The antibacterial activities against mastitis pathogens of *Cyclamen mirabile* Hildebr. tubers and its non-enzymatic antioxidant activities**

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### **ABSTRACT**

Milk is an important food for human nutrition. Mastitis reduces milk yield and alters milk composition. Economically, bovine mastitis is the most important and costly disease of dairy herds. Antibiotics are widely used in the treatment of the disease. However, this widespread use of antibiotics causes both antibiotic residues in milks and antibiotic resistance developed in bacteria. Mastitis cases caused by antibiotic-resistant *S. aureus* is both suffered more severely and contaminated milk and bacteria are spread to the other cattle and infected to human by either direct contact or by food chain. Today's researches are focused on discovering and using new antibiotics against bacteria. The aim of this work was to investigate the antibacterial effects of *Cyclamen mirabile* extracts against mastitis pathogens, and its antioxidant potentials. The extracts were screened for antibacterial activity against mastitis pathogens. The both of extract showed maximum inhibition zone against Coagulase-negative staphylococci-36 (CNS-36), and the zone was 12 mm. CNS-36 and CNS-37 showed the lowest sensitivity to *C. mirabile* ethanol extract (1625 µg /mL). In addition, the extracts were tested against the stable DPPH (2,2-diphenyl-1-picrylhydrazyl-hydrate) free-radical for antioxidant activity. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was chosen as a standard antioxidant. As a result, the ethanol extract displayed a strong antioxidant activity (Trolox equivalent = 2.26 mM). The extracts of *Cyclamen mirabile* have antibacterial, and antioxidant potential.

**Key words:** mastitis, *Cyclamen*, antibacterial, antioxidant

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### **INTRODUCTION**

Worldwide, mastitis is associated with economic losses of \$35 billion annually [1]. 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 [2]. The most common causative organisms of udder disease include: *Staphylococci*, *Streptococci* and coliforms [3]. 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 [4]. The significance of CNS, however, needs to be reconsidered as in many countries they have become the most common mastitis-causing agents [5, 6].

The use of antibiotics in the treatment of this disease has led to the additional problem of emergence antibiotic resistant strains, hence the constant concern about the resistant strains entering the food chain [7, 8, 9]. The evolution of antibiotic resistance in *S. aureus* strains is a serious cause of concern in dairy animals [10]. Alternative treatments to bovine mastitis with bacteriocins [11], bacteriophage therapy [12], intramammary honey infusion [13] and plant derived compounds [14, 15] have been described.

Medicinal plants are natural resources, yielding valuable herbal products which are often used in the treatment of various ailments [16]. According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy [17].

*Cyclamen mirabile*, is an endemic species grown in Anatolia (C2 Mugla, C3 Isparta), Turkey, where it grows in *Pinus brutia* forests and hill slopes with maquis, on limestone, metamorphic and granitic rocks, at altitudes of 400 to 1600 m and flowers from September to November [18]. The tubers of *C. mirabile* contain glycosides such as starch, glue, organic acids and saponins. Calis et al. [19] had previously reported the study of the triterpenoid saponins of *C. mirabile*. This resulted in the isolation of six saponins and their biological activities.

Many plants have been used due to their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. The antibacterial activity of *C. mirabile* against mastitis pathogens has not been studied. Additionally, antioxidant activities of *C. mirabile* Hildebr. tubers have not been reported. In this study, various extracts of the plant tubers were investigated for antibacterial and antioxidant activities.

## MATERIALS AND METHODS

### Plant material

*Cyclamen mirabile* tubers were collected from Mugla, Kavaklıdere in Turkey. Samples were collected from N37° 27' 10' E28° 21' 78' in November 2012. Taxonomical identification of plant was performed by Olcay Ceylan from the Mugla Sıtkı Kocman University, Turkey and a specimen was deposited in the herbarium of the Biology Department of Mugla Sıtkı Kocman University. The identification of these specimens was carried out using the Flora of Turkey [20].

The tubers of plant were washed thoroughly 2-3 times with running water and once with sterile distilled water. Fresh plant material was air-dried. The dried tubers were powdered in a laboratory mill. All samples were stored at ambient temperature until initial sample preparation, after which they were stored at 4°C until required for analysis.

### Plant extraction

The air dried and powdered tubers of the plant (15 g) were extracted with methanol and ethanol (60mg/mL) using the Soxhlet apparatus. The extracts were evaporated and then extracted in methanol or ethanol and then kept in small sterile opac bottles under refrigerated conditions until used.

### Microorganisms and cultivation

The tuber 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 [21].

### Antibacterial activity assay

The tuber extracts were tested by disc diffusion assay. Bauer- Kirby method applied for antibacterial activity. The bacteria were maintained on Mueller-Hinton agar plates (MHA, Merck) at 37°C [22]. Bacterial cultures adjusted to 0,5 Mc Farland. Incubations were at 37°C for 24 h for bacteria. The assessment of antibacterial activity was based on measurement of the diameter of the inhibition zones around the discs after 24 h. Methanol and ethanol used as negative control. Ampicillin (10µg) antibiotic 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 evaluated on plant extracts as antibacterial activity. The MIC was taken as the lowest concentration that inhibited growth after incubation. The broth dilution assay was performed as described in the CLSI standards with some modifications [23, 24]. This test was performed at final concentrations of the extract 6500; 3250; 1625, 812.5, 406.25 µg/mL.

### Determination of non-enzymatic antioxidant activity

The non-enzymatic antioxidant activity was determined using DPPH as a free radical. The stable 2,2-diphenyl-1-picrylhydrazyl- hydrate radical (DPPH) was used for determination of free radical-scavenging activity of the tuber 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 [25]. Trolox was used for reference antioxidant. The DPPH scavenging capacity expressed in percentage (%) was calculated using the following equation:

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

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

## RESULTS

The antibacterial activities of the tuber extracts were evaluated *in vitro* against different microorganisms, which are known to cause mastitis. Results of antibacterial activity of the extracts of used plant against the test bacteria are shown in Table 1. The results of antibacterial activity were recorded as zone of inhibition in mm for all the materials used as follows.

Results show that both of the extracts inhibited the growth of seven bacteria and the inhibition zones ranged between 8-12 mm. In addition antibacterial effects against all of bacteria determined by both of extracts. Results also show that the methanol extracts of *Cyclamen mirabile* tubers inhibited the growth of 4 bacteria with 10 mm inhibition zones. The highest inhibition zone was shown in CNS-36, and its zone was 12mm. Ampicillin (10 $\mu$ g), antibiotic used as positive control. Ampicillin very strongly inhibited the growth of *S. aureus*- 17 (Table 1).

**Table 1. Antibacterial activity of *Cyclamen mirabile* tuber extracts**

Bacteria	Inhibition zone (mm)		
	Methanol	Ethanol	Reference antibiotic (AM; mm)
<i>S. aureus</i> - 17	11	10	18
<i>S. aureus</i> - 18	10	9	12
CNS - 22	10	9	-
CNS - 32	9	8	10
CNS - 33	10	8	8
CNS - 36	12	12	-
CNS - 37	10	10	-

CNS: coagulase negative Staphylococci; AM: ampicillin, 10 $\mu$ g;  
(-): zone did not occur

Table 2 shows MICs of *C. mirabile* tuber extracts obtained by the broth dilution method. CNS-36 and CNS-37 showed the lowest sensitivity to ethanol extract of *C. mirabile* (1625  $\mu$ g/mL) (Table 2).

**Table 2. Minimum inhibitory concentration of *C. mirabile* tuber extracts**

Bacteria	Minimum Inhibitory Concentration ( $\mu$ g/mL)	
	Methanol	Ethanol
<i>S. aureus</i> - 17	3250	3250
<i>S. aureus</i> - 18	6500	3250
CNS - 22	6500	6500
CNS - 32	3250	3250
CNS - 33	3250	3250
CNS - 36	3250	1625
CNS - 37	3250	1625

CNS: coagulase negative Staphylococci

The non-enzymatic antioxidant activities of the plant extracts were evaluated by the DPPH radical scavenging capacity. Table 3 shows the percent of DPPH radical scavenging capacity with trolox as reference. The methanol extract showed 77 % inhibition at 60 mg/mL concentration. Trolox equivalent value was 1.99 mM/g DW. Whereas ethanol extract showed 87% inhibition at 60 mg/mL concentration, trolox equivalent value was 2.26 mM/g DW (Table 3).

**Table 3. DPPH radical scavenging capacity of *C. mirabile* tuber extracts (60 mg/mL)**

Tuber extracts	DPPH (%)	Trolox equivalent (mM/gDW)
Methanol	77	1.99
Ethanol	87	2.26

DW: dry weight

## DISCUSSION

Medicinal plants have traditionally been used worldwide for the treatment of various diseases [26]. They have proved to be abundant sources of biologically active compounds, many of which have been used as compounds to develop new pharmaceuticals [27]. This study confirms that the tubers of *C. mirabile* possess antibacterial and antioxidant activities.

In the present study, extracts of the plant tubers obtained in various solvents were tested against the test bacteria. The antibacterial activity was compared with the standard antibiotic. The ethanol extract was found to be lowly effective in inhibiting the growth of CNS-32 and CNS-33 (Table 1). Similar results with four *Ficus* sp. extract were obtained by Nair and Chanda [28]. Obasola *et al.* reported that CNS pathogens are resistant to most antibiotics [29]. They have multi-resistance genes in plasmids which can be changed and they can spread among the different species covering also *S. aureus*. Kloos and Bannerman reported that CNS resistance is more for different antimicrobial agents and this is clinically important and the resistance development [30]. The reports also support the results you obtained from our study.

In this study, antibacterial activity of the methanolic extract was found against *S. aureus*- 17 as 11 mm. Dua *et al.* reported that antibacterial activities of *Foeniculum vulgare* Miller seeds were found as 11 mm inhibition zone against *S. aureus* [35]. In addition to, Kumari *et al.* reported that antibacterial activities of *Indigofera aspalathoides* roots were found as 12 mm inhibition zone against *S. aureus*. The reports also support the results you obtained from our study [36].

According to this study, CNS-36 and CNS-37 showed the lowest sensitivity to ethanol extract of *C. mirabile* (Table 2). Alsabri *et al.* reported that minimum inhibitory concentration value (MIC) of *Arbutus pavarii* against *S. aureus* was found as 4.86 mg/mL [31]. In this study, MIC value was measured as 1625 µg/mL, and our results are better than those of Alsabri *et al.*, Sharma *et al.* and Ergun *et al.* [31, 37, 38]. According to Aligiannis *et al.* who reported a classification of plant materials based on MIC results, the both of extracts from *C. mirabile* can be considered a weak inhibitor of mastitis pathogens [32].

It has been reported that free radical scavenging and antioxidant activity of many medicinal plants are responsible for their therapeutic effect against cancer, tissue inflammatory, cardiovascular disease [33]. The results of DPPH scavenging assay of *C. mirabile* extracts are shown in Table 3. The methanol extract showed 77 % inhibition at 60 mg/mL concentration. Trolox equivalent value was 1.99 mM/g DW. Whereas ethanol extract showed 87% inhibition at 60 mg/mL concentration. Trolox equivalent value was 2.26 mM/g DW (Table 3). In a study about *Liquidambar formosana* essential oils performed by Liu *et al.* essential oil in high concentration was reported to have powerful antioxidant effect [34].

## CONCLUSION

In conclusion, *C. mirabile* tuber extracts tested in the study were determined to have potential antibacterial activities against *S. aureus* and CNS pathogens isolated from subclinical cow mastitis. Our findings suggest that *C. mirabile* has significant antibacterial activity and it could be very useful in the discovery of novel antibacterial agents of plant origin. Results of our study show that the plant can be supplemented in the treatment of subclinical cow mastitis and tuber extracts of the plant may have the potential to be used as antibacterial agents for searching new medicines. Furthermore, tuber extracts of the plant have great importance as antioxidant activities. However, further investigations involving more detailed *in vitro* and *in vivo* studies to establish which components of the extracts offer the best antioxidant and antibacterial activity are recommended.

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