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The Antibacterial Activity of *Elaeagnus angustifolia* L. Against Mastitis Pathogens and Antioxidant Capacity of the Leaf Methanolic Extracts

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Abstract: In this study, *E. angustifolia* L. was collected from different localities of Mugla in Turkey. Antibacterial and antioxidant activities of *E. angustifolia* L. have not been reported to the present day. The aim of the present study was to investigate the *in vitro* antibacterial and antioxidant activity of methanol extracts of *E. angustifolia* leaves. Methanol extracts were screened for antibacterial activity against mastitis pathogens. These bacteria include 2 *Staphylococcus aureus* and 5 Coagulase Negative Staphylococci. Antibacterial activities of the extracts were determined by the Disc Diffusion Method. Also ampicillin disc was used for comparison of inhibition zones. Methanolic leaf extract showed a maximum zone of inhibition in *Staphylococcus aureus*-17 by Disc Diffusion Method. *E. angustifolia* leaf extract showed lowest antibacterial activity against *S. aureus*-18 and Coagulase Negative Staphylococci-36 (CNS). The antioxidant activity of the leaf extract was also determined by ABTS Method using trolox as standard. The value found in ABTS Method is highly effective (84%). As a result, the *in vitro* studies clearly indicate that the methanolic leaf extract of *E. angustifolia* significant antioxidant activity and also a better source of natural antioxidant which might be helpful in preventing the process of different oxidative stress. In addition to, the *E. angustifolia* could be used in treating disease caused by the test bacteria. *E. angustifolia* methanolic leaf extracts have antioxidant and antibacterial potential.

Key words: Mastitis, Elaeagnus, antibacterial activity, antioxidant activity, ABTS Methods

INTRODUCTION

Mastitis is the most important and expensive disease of dairy industry. It results in severe economic losses from reduced milk production, treatment cost, increased labor, milk withheld following treatment and premature culling (Miller *et al*., 1993). Sharma *et al*. (2004) reported 70% incidence of sub clinical mastitis in buffaloes while Maiti *et al*. (2003) reported 70% incidence of sub clinical mastitis in cows. Worldwide, mastitis is associated with economic losses of $35 billion annually (Chockalingam *et al*., 2007).

The most common causative organisms of udder disease include: Staphylococci, Streptococci and coliforms. Other less frequent agents include: Pseudomonads, Nocardia, Mycoplasma and yeasts (McDonald, 1979). *S. aureus* and Coagulase Negative Staphylococcus (CNS) are the predominant organisms (Kapur *et al*., 1992).

The prolonged use of antibiotics in the treatment of mastitis has led to the additional problem of emergence antibiotic resistant strains hence the constant concern about the resistant strains entering the food chain (White and McDermott, 2001; Virdis *et al*., 2010; Ateba *et al*., 2010). The evolution of antibiotic resistance in *S. aureus* strains is a serious cause of concern in dairy animals (Wang *et al*., 2008). Alternative treatments to bovine mastitis with bacteriocins (Pieterse *et al*., 2010) bacteriophage therapy (Tiwari *et al*., 2013), intramammary honey infusion (Wahba *et al*., 2011) and plant derived compounds (Baskaran *et al*., 2009; Mubarack *et al*., 2011) have been described.

Medicinal plants are natural resources, yielding valuable herbal products which are often used in the treatment of various ailments (Grabley and Thiericki, 1999). The leaves of trees and shrubs are a component of most natural pastures for ruminant diets (Kumar and Vaithiyanathan, 1990). Many tree leaves have anti-microbial factors, like tannins, essential oils or other aromatic compounds (Kumar and Singh, 1984a, b). In addition to, many biological activities and antibacterial-promoting effects have been reported for plant tannins and flavonoids and their investigation is now increasingly relevant (Haslam, 1989; Scalbert, 1991; Chung *et al*., 1998).

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

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better understand their properties, safety and efficacy (Nascimento *et al*., 2000). *Elaeagnus angustifolia* is one of these herbs (Farahbakhsh *et al*., 2011). *E. angustifolia* contains flavonoids compounds, sitosterols, cardiac glycosides and terpenoids (Burgess, 2008; Taheri *et al*., 2010). Furthermore, these compounds have attracted the attention of scientists because of these flavonoid compounds have antioxidant activities (Liu and Yao, 2007).

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 *E. angustifolia* against mastitis pathogens has not been studied, the *in vitro* antibacterial activity of leaves parts of the plant growing in Mugla was evaluated using disc diffusion method. Additionally, antioxidant activities of *E. angustifolia* have not been reported. In this study, methanol extracts of plant leaves were investigated for antibacterial and antioxidant activities.

MATERIALS AND METHODS

Plant material: *E. angustifolia* samples were collected from different localities of Mugla in July 2012. The plant material was authenticated by Olcay Ceylan and a specimen was deposited in the herbarium of the Biology Department of the University of Sitki Kocman, Turkey. The identification of these specimens was carried out using the Flora of Turkey (Davis, 1965).

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

The powdered leaves of the plant samples (10 g) were extracted with methanol (100 mg mL^{-1}) using the Soxhlet apparatus. The extract was evaporated and then extracted in metanol and then kept in small sterile opac bottles under refrigerated conditions until used.

Microorganisms and cultivation: The extracts of plant leaves 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). These species including two *S. aureus* and five Coagulase Negative Staphylococci (CNS). The bacteria were grown for 24 h at 37°C in Mueller-Hinton Broth (Merck). Bacterial species identifications were studied by conventionally methods by Dr. Zafer Cantekin (Quinn *et al*., 1994).

Antibacterial activity assay: Bauer-Kirby Method applied for antibacterial activity. The methanol leaf extract of plant was tested by disc diffusion assay. The bacteria were maintained on Mueller-Hinton Agar plates (MHA, Merck) at 37°C (Bauer *et al*., 1966). Bacteria cultures adjusted 0.5 Mc Farland. The experiment was performed in triplicate. Incubations were at 37°C for 24-48 h for bacteria. After incubation, the inhibition zones formed and its were measured. Methanol used as negative control. Ampicillin (10 μg), antibiotic used as positive control.

Determination of Minimum Inhibitory Concentration (MIC): The MIC was evaluated on plant methanol extracts as antibacterial activity. The MIC was taken as the lowest concentration that inhibited growth after incubation. The microdilution assay was performed as described in the CLSI standards with some modifications (CLSI, 2003, 2006). This test was performed at final concentrations of each extract (6500, 3250, 1625, 812 and 406 μg mL^{-1}).

***In vitro* antioxidant activity:** The experiments were carried out using an improved ABTS decolorisation assay (Re *et al*., 1999). The stock solutions included 7 mM $ABTS^{\bullet+}$ [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] solution and 2.45 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL $ABTS^{\bullet+}$ solution with 10 μL methanol. Absorbance was measured 15 min after the initial mixing of 10 μL of the methanolic leaf extracts with 1 mL of $ABTS^{\bullet+}$ solution. Then the absorbance was taken at 734 nm using the spectrophotometer (Shimadzu UV-1201V, Japan). Trolox (6-hydroxy-2,5,7,8-tetramethychroman- 2-carboxylic acid; Sigma Chemical Co. St. Louis, MO, USA) was used as a reference standard. Results are expressed in mM Trolox equivalents (TE)/g dry mass. All determinations were carried out in triplicate. The scavenging capability of $ABTS^{\bullet+}$ radical was calculated using the following Equation:

$$\text{ABTS scavenging effect (\%)} = \left(\frac{1-A_1}{A_0}\right)\times 100$$

Where:

A_0 = The initial concentration of the $ABTS^{\bullet+}$ radical cation (s)

A_1 = The absorbance of the remaining concentration of $ABTS^{\bullet+}$ radical cation (s) in the presence of the extract

RESULTS

The antibacterial activity of methanol extracts of *E. angustifolia* were evaluated *in vitro* against 7 test bacteria which are known to cause bovine mastitis. Results of antibacterial activity of methanol extracts of used plant against the test bacteria are shown in Table 1. Besides, the inhibition zone diameters of the reference antibiotic to the test bacteria are shown in Table 2.

Results show that the methanol extracts of *E. angustifolia* inhibited the growth of five bacteria and the inhibition zones ranged between 9-20 mm. In addition to the methanolic extract of this plant did not determine any antibacterial effects against used 2 bacteria. The results of antibacterial activity were recorded as zone of inhibition in mm for all the materials used as follows. Methanol extract of the leaves was found to be highly effective against *S. aureus*-17. The maximum zone of inhibition was produced by methanol extract against *S. aureus*-17 (20 mm). The minimum zone of inhibition was determined by leaf methanol extract against CNS-33 (8 mm). Whereas, the inhibition zone was not produced by methanol extract against *S. aureus*-18 and CNS-36. These bacteria were found resistant to the leaf methanol extract. Ampicillin (10 μg), antibiotic used as positive control (Table 1).

MIC values of leaf methanolic extracts against the bacterial strains were studied by serial dilution method. Table 2 shows MICs of *E. angustifolia* methanol extracts obtained by the broth microdilution method. MIC values for leaf extract were applied from 6500-406 μg mL^{-1}. Four bacterial strains have shown the lowest sensitivity to 3250 μg mL^{-1} methanol extract.

The antioxidant activity of plant extract was evaluated by the ABTS radical scavenging capacity. Table 3 shows the percent of ABTS radical scavenging capacity with trolox as reference. The leaf methanol extract showed 84% inhibition at 100 mg mL^{-1} concentration (Table 3).

Table 1: Antibacteril activity of methanol extracts of *Elaeagnus angustifolia*

Bacteria	Inhibition zone (mm)	Reference antibiotic (AM; mm)
S. aureus-17	20	18
S. aureus-18	-	12
CNS-22	13	-
CNS-32	12	10
CNS-33	8	8
CNS-36	-	-
CNS-37	9	-

CNS: Coagulase Negative Staphylococci; AM: Ampicillin,10 μg; (-): zone did not occur

Table 2: Minimum inhibitory concentration of methanolic extracts of *E. angustifolia*

Bacteria	MIC (μg mL^{-1})
S. aureus-17	3250
S. aureus-18	6500
CNS-22	6500
CNS-32	3250
CNS-33	6500
CNS-36	3250
CNS-37	3250

CNS: Coagulase Negative Staphylococci

Table 3: BTS radical scavenging capacity of *E. angustifolia*

ABTS %	Trolox equivalent (mM)
84	2.29

DISCUSSION

Mastitis is a global problem as it adversely affects animal health, quality of milk and economics of milk production and every country including developed ones suffer huge financial losses (Sharma *et al*., 2007). Mastitis, the most important deadly disease of dairy animals is responsible for heavy economic losses due to reduced milk yield (up to 70%), milk discard after treatment (9%), cost of veterinary services (7%) and premature culling (14%) (Bhikane and Kawitkar, 2000). Apart from its economic importance it also carries public health significance (Vasavda, 1988).

Antimicrobial agents are commonly applied to dairy cattle either to control or to prevent bacterial infections in lactating and dry cows. The World Health Organization (WHO) states that 74% of the medicines derived from plant resources have a modern indication that correlates with their traditional, cultural uses as plant medicines by native cultures (Das *et al*., 2009). Medicinal plants have been traditionally used worldwide for the treatment of various human diseases (Chitme *et al*., 2004). They have proved to be abundant sources of biologically active compounds, many of which have been used as compounds to develop new pharmaceuticals (Palombo, 2011). *E. angustifolia* L. were selected based on their relevant ethnomedical use (Baytop, 1999; Tuzlaci *et al*., 2010). In the present study, the extracts of plant leaves obtained in methanol solvents were tested against 7 mastitis bacteria. The antibacterial activity was compared with the standard antibiotic.

In this study, the maximum zone of inhibition was produced by methanol extract against *S. aureus*-17 (Table 1). The antibacterial activity of extracts from many plant species has been extensively surveyed, this plant antimicrobial mechanism has not been reported in great detail. Since, the active antimicrobial compounds of essential oils are phenolics and terpenes in nature (Janssen *et al*., 2007; Saxena *et al*., 1994), it seems reasonable that their mode of action might be similar to that of other phenolic compounds. These compounds

have already been reported in plants (Chopra *et al*., 1981). Phytochemical studies have shown that aqueous fruit extract of *E. angustifolia* contains flavonoids compounds, sitosterols, cardiac glycosides, terpenoids (Dembinska-Migas and Gill, 1973).

The minimum zone of inhibition was determined by leaf methanol extract against CNS-33 (Table 1). Emerging antimicrobial resistance among CNS is a concern in veterinary and human medicine. Compared with *S. aureus*, CNS are more often resistant to several antimicrobials (Myllys *et al*., 1998). Resistance patterns differ among CNS species (Sampimon *et al*., 2011) and regions. The role of CNS as a reservoir of resistance genes has been discussed and some evidence for it is available (Juuti *et al*., 2005; Sampimon *et al*., 2011).

According to this study, the inhibition zone was not produced by methanol extract against *S. aureus*-18 and CNS-36 (Table 1). *S. aureus* has been the main subject of studies on antibiotic resistance because of its importance for all forms of mastitis in dairy cows (Fthenakis, 1998; Malinnowski *et al*., 2002; Kaszanyitzky *et al*., 2003). However, in recent years, the incidence of reported Coagulase Negative Staphylococci (CNS) mastitis has increased substantially (Devriese *et al*., 2002; Gentilini *et al*., 2002; Rajala-Schultz *et al*., 2004). In human medicine, antimicrobial multi-resistance is frequently encountered and methicillin-resistant S. *aureus* (MRSA) (De Neeling *et al*., 1998; McBryde *et al*., 2004) and Methicillin-Resistant CNS (MR-CNS) (De Neeling *et al*., 1998; Petinaki *et al*., 2001) strains are among the most threatening bacteria involved in nosocomial infections. In veterinary medicine, however, MRSA as well as multi-resistant *S. aureus* strains are reported occasionally (Lee, 2003; Seguin *et al*., 1999). This result may be explained by the intensive use of antibiotics in Turkey and by possible differences among the staphylococci isolated.

The research reported that four bacterial strains have shown the lowest sensitivity to 3250 μg mL^{-1} methanol extract (Table 2). Available literature results indicate a strong activity when MIC values are between 0.05-0.50 mg mL^{-1}, moderate activity in values between 0.6-1.50 mg mL^{-1} and weak activity above 1.50 mg mL^{-1} (Diaz *et al*., 2010).

In this study, the methanol extract showed 84% free radical scavenging at 100 mg mL^{-1} concentrations (Table 3). Quinn *et al*. (1994) reported that guarana seed methanol extract had high antibacterial activity when this methanol extract also possessed high phenolics content. The screening of plant extracts using the ABTS Method proved to be effective for the selection of those which could have an antioxidant activity. These extracts may be rich in radical scavengers such as flavonoids, known as antioxidants. 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 (Miller, 1998; Anderson *et al*., 1999).

CONCLUSION

The research suggest that the leaf of *E. angustifolia* may be utilized as effective and safe antioxidant and antibacterial source. However, searching for further bioactive compounds which are responsible for the biological and chemical activities of *E. angustifolia* is needed.

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