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ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF *VITEX AGNUS-CASTUS* L. AGAINST MASTITIS PATHOGENS

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ABSTRACT

It is now widely reported that mastitis agents have developed resistance to antibiotics, due to the widespread use of antibiotics. In total, seven mastitis-causing bacteria were used in the study two are *Staphylococcus aureus* strains and five are Coagulase-Negative Staphylococci (CNS) strains. The plant material of *Vitex agnus-castus* was collected from Mugla-Turkey. Kirby-Bauer assay was used to screen for the antibacterial activities of the extracts. Antioxidant activities were assayed using the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) technique. Results indicated that the extracts of *V. agnus-castus* had antibacterial activity against mastitis pathogens, and the range of inhibition zone was 8-10 mm. The largest inhibition zone was obtained from the methanol extract of *V. agnus-castus*. In MIC assays, no inhibition was observed in any of the tested concentrations. Among the extracts, the water extract exhibited the highest antioxidant activity (78.95%). In summary, it was found that the extracts of *V. agnus-castus* exhibit antibacterial activity against mastitis-causing *Staphylococcus aureus* and CNS pathogens, and thus, the plant extracts can be used to treat mastitis caused by these test bacteria.

KEYWORDS:
ABTS assay, Antibacterial activity, Antioxidant activity, Mastitis, *Staphylococcus aureus*, *Vitex agnus-castus*

INTRODUCTION

The control of microorganisms that are widely known as environmental pathogens (e.g. coliform bacteria and species of Streptococci) is of great importance in preventing economic loss [1]. Mastitis is an inflammatory disease of the mammary glands, which affects the milk yield in the dairy industry, thereby contributing to economic loss. This disease is caused by various bacterial species. While *Staphylococcus aureus* and *Streptococcus agalactiae* are the main pathogens causing mastitis, strains of coagulase-negative *Staphylococci* (CNS) have also been linked to the disease [2-5]. In recent years, Coagulase-negative *Staphylococci* have been identified to cause majority of intra-mammary infections in many herds. Although many countries have developed many traditional and non-traditional control programs in the treatment of mastitis, this disease is still common and is very expensive to treat. The most preferred method of treatment is using antibiotics due to their short time response. However, when looking at this disease from the perspective of environmental/public health, it seems likely that the risk of antibiotic resistance posed by the preferred treatment method can extend beyond cattle. General public consuming dairy products, such as milk, yogurt, or cheese can indirectly be exposed to antibiotic-resistance [6, 7, 8]. Additionally, it is suggested that chemical contaminants in milk and other dairy products can contribute to etiology of Alzheimer's disease, cancer, and Parkinson's disease [8]. Therefore, the issue of antibiotic resistance is no longer just an environmental health problem, but a global threat to all countries. There is no doubt that *S. aureus* has superior ability to develop resistance when compared to other bacteria. This calls for an urgent need for newer antibiotics and strategies that can mitigate the disease [9, 10]. For this reason, today's scientists are focusing on discovery and development of new antibiotics against drug-resistant bacteria.

In the field of public health, there is an increasing interest in the use of traditional or plant-derived medicine for treatment of various diseases, particularly in developing countries [11]. In the treatment of diseases, there is an increasing interest in alternative natural resources such as plants, rather than chemical drugs that adversely affect the environment and human health [12]. Medicinal plants are the backbone of traditional medicine and they serve as a natural and potential source for production of new compounds. Various studies have been conducted to extract novel therapeutic agents from different parts of a plant. As discussed above, the main purpose of such studies is to identify phytochemicals that can overcome antimicrobial resistance of pathogens to existing antibiotics and antifungal drugs. Moreover, such studies contribute

towards identifying natural alternative sources to generate antimicrobials that are cheap, reliable, and nontoxic to the environment and humans [13]. For instance, plant-derived antioxidants, especially polyphenols, have great importance in terms of preventing oxidative damage [14]. It is well known that antioxidants are highly effective in removing free radicals and thus, an antioxidant-rich diet could help prevent chronic illnesses such as cancer, Alzheimer's disease, and Parkinson's disease [15-17]. For this reason, phytochemicals are the most studied among alternative sources.

Lamiaceae is one of the most studied plant families due to its rich medicinal properties. It comprises more than 3000 species of plants that exist worldwide [18]. *Vitex* L. is one of the largest genera of the *Lamiaceae* family with more than 250 species and is a little deciduous, aromatic, and decorative tree or large shrub. *Vitex agnus-castus* is also known as monk-pepper or chaste tree. According to historical records from ancient Iran, Egypt, Rome and Greece, the plant (specifically, its fruit) has been used medically for over 2500 years [18-20]. The plant is originally native to southern Europe and western Asia, and it is also cultivated in different subtropical areas. Flavanoids are one of the main active components of the plant. Because of their hormone-like effect, these plants have been preferred as a medical treatment for many women's diseases, specifically menstrual problems [11, 21]. There are many studies conducted in Turkey that provide evidence for therapeutic use of different parts of this plant and their significance in traditional medicine [22, 23]. However, to the best of our knowledge, the antimicrobial effect of *V. agnus-castus* against mastitis pathogens has not yet been investigated.

Hence, this study aimed to investigate the antibacterial activities of the various extracts of *V. agnus-castus* against mastitis pathogens, and to reveal the antioxidant potential of this plant. Although several studies on the biological activities of this species already exist in the literature [24-26], the antioxidant and antibacterial activities of *V. agnus-castus*, against mastitis-causing pathogens, for which it is widely grown and used in Mugla (Turkey), have not been extensively explored.

MATERIALS AND METHODS

Plant material. The flowers of *Vitex agnus-castus* were collected from Mugla (Turkey). The material was identified by Dr. Neslihan Balpinar and stored in the Department of Biology at Burdur Mehmet Akif Ersoy University. The identification of plant material was made according to *Flora of Turkey and East Aegean Islands* [27-29].

Microorganisms. The mastitis pathogens used in this study include two strains of *S. aureus* and five types of Coagulase-Negative Staphylococci (CNS) strains. All mastitis pathogens were supplied from Dr. Zafer Cantekin's project (project no: 1101 M 0103, ethics committee no: 2010/02-30: 12). The diagnosis of test pathogens was performed using biochemical assays and classical culture methods [30].

Preparation of the plant material for extraction. The flowers of *V. agnus-castus* used in the analysis of the antibacterial activity were washed several times with sterilized distilled water. The flowers were then dried at room temperature in the laboratory before being powdered in a blender. During preparation of the samples, all the materials were stored at room temperature.

Extraction process. The powdered plant flowers (50 g) were processed using a Soxhlet extractor (Isotex). Ethanol, methanol, and water were the solvents used in this process. The extracts were evaporated under a fume hood and transferred into sterilized opaque falcon tubes containing their own solvents. The concentrations of all extracts were set at 300 mg/mL, and they were preserved in a refrigerator until analysis. The temperature of freezer was -20°C.

Culture of microorganisms. Mastitis pathogens mentioned above were used as a source of test microorganisms. The extracts were analyzed one at a time to examine their antimicrobial activity against test mastitis pathogens. The bacteria used in the study were cultivated in Mueller-Hinton Broth (MHB, Merck) medium. Incubation was carried out at 37 °C for 24 h.

***In vitro* antibacterial assay.** The Bauer-Kirby method was used to determine antimicrobial activity [31]. All the ethanol, methanol, and water extracts of the flower were analyzed by disc diffusion method. Test bacteria were cultured on Mueller-Hinton Agar (MHA, Merck) medium at a temperature of 37°C for 24 h. The turbidities of bacterial cultures were set to 0.5 McFarland. Zones of inhibition occurring after the incubation were recorded in mm scale. Standard antibiotic discs (ampicillin 10 μg; oxacillin 5 μg) were used as positive controls, while solvents were used as negative controls against the test pathogens. All measurements were performed in triplicate.

Minimum inhibitory concentration (MIC). The other antibacterial activity test applied to bacteria was minimum inhibitory concentration (MIC). The experiments were performed by broth dilution assay as defined by CLSI standards [32, 33]. The MIC values of all leaf extracts were recorded after

the incubation. The final concentrations of each extract were set to 13000, 6500, 3250, 1625, and 812.5 µg/mL.

Detection of non-enzymatic antioxidant activities. In this study, ABTS decolorization technique was used for determining the antioxidant capacity of each of the plant extracts [34]. This assay is called the ABTS or trolox equivalent antioxidant capacity (TEAC) assay. Main stock liquid consisted of 7 mM ABTS• [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] solution and 2.45 mM potassium persulfate solution. The absorbance values were measured at 734 nm by spectrophotometry (Shimadzu UV-1201V, Japan). Trolox was used as a positive control for antioxidant in this assay. The test values represented as mM Trolox equivalents (TE)/g dry mass.

RESULTS

The antibacterial activities of the extracts obtained from flowers of *Vitex agnus-castus* were tested against seven mastitis-causing pathogens. The results were summarized and compared to standard antibiotics in Table 1. As seen in Table 1, these flower extracts exhibited antibacterial activity against some mastitis pathogens. After a closer look at the data obtained from this study, it was seen that the largest zone of inhibition of 10 mm was obtained from the methanol extract.

In addition, it was determined that the methanol extract of the plant showed antibacterial activity against CNS-22 (ZOI = 9 mm). As for the ethanol extracts of *V. agnus-castus*, the zone of inhibition was observed against CNS-33 (ZOI = 8 mm) and CNS-36 (ZOI = 9 mm). The value obtained against CNS-33 was recorded as the lowest zone of inhibition of the study. In contrast, it was found that all of the bacteria were resistant to water extracts of the plant. Moreover, none of the ethanol, methanol, and water extracts of *V. agnus-castus* developed inhibition zones against *S. aureus*-17 and *S. aureus*-18 and CNS-32 and CNS-37. The solvents used as negative controls had no antibacterial activity against test microorganisms (Table 1).

The broth dilution method was also used to examine antibacterial activity of the plant extracts that had previously shown antimicrobial activity by Bauer-Kirby method. The MIC results of the flower extracts from this assay are summarized in Table 2. No inhibition was observed in any of the tested concentrations (Table 2)

The non-enzymatic antioxidant activity of the flower extracts was also tested via the ABTS• decolorization method with trolox used as a reference antioxidant. The data obtained from this assay was recorded as trolox equivalents (Table 3). Ethanol, methanol, and water extracts of *V. agnus-castus* flower have different radical scavenging capacity from one another. While the water extract of *V. agnus-castus* had highest antioxidant activity (78.95%) and its trolox equivalent was 2.08, antioxidant activities of the ethanol and methanol extracts were 5% and 7.38%, respectively (Table 3).

TABLE 1
The antibacterial activities of the flower extracts of *Vitex agnus-castus* (300 mg/mL) against mastitis pathogens

Bacteria	Inhibition zone (mm)			Antibiotics (mm)		Solvents		
	E	M	A	AM	O	E	M	A
Staphylococcus aureus-17	(-)	(-)	(-)	18	10	(-)	(-)	(-)
Staphylococcus aureus-18	(-)	(-)	(-)	12	8	(-)	(-)	(-)
CNS-22	(-)	9	(-)	-	-	(-)	(-)	(-)
CNS-32	(-)	(-)	(-)	10	7	(-)	(-)	(-)
CNS-33	8	10	(-)	8	7	(-)	(-)	(-)
CNS-36	9	(-)	(-)	-	-	(-)	(-)	(-)
CNS-37	(-)	(-)	(-)	-	-	(-)	(-)	(-)

CNS: Coagulase-Negative *Staphylococci*; (-): No inhibition; AM: Ampicillin (10µg); O: Oxacillin (5µg); E: Ethanol; M: Methanol; A: Aqueous

TABLE 2
The MIC results of the flower extracts of *Vitex agnus-castus* (µg/mL)

Bacteria	Ethanol	Methanol	Water
CNS-22	(nt)	-	(nt)
CNS-33	-	-	(nt)
CNS-36	-	(nt)	(nt)

(nt): not tested; (-): No MIC was observed at concentrations tested up to 13000 µg/mL.

TABLE 3
The antioxidant activities of the flower extracts of *Vitex agnus-castus* (%) and their trolox equivalent

Plant material (300 mg/mL)	EE		ME		WE	
	ABTS (%)	TE	ABTS (%)	TE	ABTS (%)	TE
Vitex agnus-castus	5	0.53	7.38	0.79	78.95	2.08

EE: Ethanol extract; ME: Methanol extract; WE: Water extract; TE: Trolox equivalent (mM TE/g DW); DW: Dry weight

DISCUSSION AND CONCLUSIONS

As more and more active compounds of medicinal plants are discovered and their clinical importance is identified, the demand for alternative natural resources to develop various therapeutic agents will gradually increase. In line with this purpose, ethnobotanical and *in vitro* research is being conducted at an increasing pace in many countries. Similarly, in this study we proposed to determine the antimicrobial effects of different extracts obtained from the flowers of *Vitex agnus-castus* against some mastitis-causing pathogens. We found ethanol and methanol extracts of *Vitex* flowers to have antibacterial activity against test strains of mastitis-causing bacteria. The inhibition zone was in the range of 8-10 mm (Table 1). In their study, Bayraktar et al. [26] obtained the zones of inhibition of 7.5, 8, and 7.5 mm against *Staphylococcus aureus* in their study, which was performed using the disc diffusion method for and ethanol extract of *V. agnus-castus* leaves collected from Izmir during October, November and December, respectively. Arokiyaraj et al. [35] reported that the ethyl acetate extracts of *V. agnus-castus* leaves were highly active against almost all potent clinical pathogens used in their study. In their study on the essential oil of *V. agnus-castus* fruit against six mastitis-causing bacteria, Eryiğit et al. [24] reported the zone of inhibition of 10 mm against *S. aureus*, and suggested that the composition of the essential oil and its related compounds might be responsible for antimicrobial activity. In addition, they observed that the essential oil of *V. agnus-castus* fruit exhibited lower antimicrobial activity compared to ampicillin used as a control antibiotic.

Nonetheless, in a study by Maltas et al. [36], no antimicrobial activity was observed using disc diffusion method for methanol extract from Vitex leaves against seven bacterial strains including *S. aureus*. Antimicrobial activity was only observed against *Candida albicans* (ZOI = 11 mm). Likewise, no antibacterial activity was detected against *S. aureus-17 and S. aureus-18* in our study. Compared to the standard antibiotics, ampicillin and oxacillin, which were utilized as positive controls, the methanol extracts showed relatively higher activity against CNS-22 and CNS-33, and the ethanol extracts against CNS-36. However, none of the plant extracts showed any antibacterial activity against CNS-32 and CNS-37. There are some studies in the literature suggesting that CNS pathogens are resistant to many antibiotics [37]. Furthermore, previous studies have shown that differences in antimicrobial activity of these plant extracts may be genetic, environmental, and climate-based, or due to harvest season or varied extraction methods [26, 38, 39]. Although antibacterial activity against some mastitis pathogens was observed in this study, the minimum inhibitory concentration value of the extracts was not reached at any of the concentrations tested.

Plants possess broad-spectrum natural antioxidant properties due to their secondary metabolites. In this study, the scavenging effect of the water extract of *V. agnus-castus* flowers on ABTS radical was measured as the highest value compared to other extracts, and the Trolox equivalent was 2.08 mM TE/g dry mass. Across various studies on *V. agnus-castus,* though the plant was collected from different countries or from different regions of the same country, its leaves specifically were found to be rich in iridoid glycosides (agnoside), alkaloids (viticin), volatile oils (eucalyptol, linalool), fatty acids (palmitic acid, linoleic acid), and flavonoids (casticin, vitexin) [36, 40-42]. In addition to these compounds, vitexilactone is widely known to have medical and economic importance as an antioxidant [43]. Bayraktar et al. [26] reported that one of the many factors affecting polyphenolic content is the harvest time of the plant and they found December to be the optimum time to collect leaves of *V. agnus-castus*. Additionally, Rashed et al. [44] found that the ethyl acetate extracts of *V. agnus-castus* collected from Egypt exhibit significant antioxidant capacity (88.46%). Gökbulut et al. [45] found that the DPPH values of the methanol leaf and fruit extracts of *V. agnus-castus* were 0.45% and 0.61% for IC_{50} values, respectively. Other studies have also observed that the total phenolic content of leaf extracts of Vitex plant is high.

It was determined that the highest antioxidant activity of *V. agnus-castus* was 78.95% for the water extract. The findings of our study support results from the literature where antioxidant mechanisms of volatile oils of herbal extracts are explained by presence of phenol and flavonoid compounds. In recent years, in the hope of replacing synthetic antioxidants, various extracts of plants have been studied with respect to their antioxidant properties.

To summarize, in this study we found that the extracts obtained from flowers of *Vitex agnus-castus* grown in Mugla (Turkey) were effective against some mastitis pathogens and exhibited a strong antioxidant activity. These results show that this plant could be a great source for potent natural antibacterial agents against mastitis-causing pathogens and also act as a natural antioxidant. However, further investigations concerning bioactive and phytochemical compounds of this plant are needed. In addition, wider selection of bacterial populations need to be tested in our future studies.

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