Original Article

The Antibacterial Activities of *Piper nigrum* L. Against Mastitis Pathogens and its Antioxidant Activities

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ABSTRACT

Objective / Purpose: Bacteria causes one of the most common types of chronic mastitis. The most common causative organisms of mastitis include: *Staphylococci*, *Streptococci* and coliforms. The scope of this work was to research the antibacterial effects of *Piper nigrum* extracts against mastitis pathogens, and its antioxidant capacity. **Materials and Methods**: In our study, 2 *Staphylococcus aureus* and 5 Coagulase Negative *Staphylococcus* were used for experiments. Additionally, *Piper nigrum* were collected from Mugla herbalists in Turkey. The plant extracts were tested by disc diffusion assay for antibacterial activity. The antioxidant activities of plant extracts were also determined by ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] method. **Results:** The methanol extract of plant showed maximum inhibition zone against 2 bacteria. MIC values of extracts are 6500 μg/mL. The value found in ABTS method is highly effective (75.7%). **Discussion/Conclusion:** As a result, the *in vitro* studies indicate that the extracts of *Piper nigrum* have significant antibacterial and antioxidant activities. In addition to, the plant extracts could be used in treating mastitis caused by the test bacteria.

Keywords: Piper, Mastitis, *Staphylococcus*, Antibacterial Activity, Antioxidant Activity.

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.[1] In this disease, the most common causative organisms are *Staphylococci*, *Streptococci* and coliforms.[2] 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.[3]

The continuous evolutions of bacterial resistance to currently available antibiotics are increasing problems. Drug-resistant bacteria create additional cases of illness, longer recuperation times and unnecessary deaths that necessitated the search for novel and effective antimicrobial compounds.[4,5] This situation has forced scientists to search for new antimicrobial substances from various plants which are the good sources of novel antimicrobial chemotherapeutic agents.[6,53,57] Because of the concern about the side effects of conventional medicine, the use of natural products as an alternative to conventional treatment in healing and treatment of various diseases has been on the rise in the last few decades.[7,54]

Black pepper or *Piper nigrum* is one of the most popular spice products in oriental countries (mostly in Southeast Asia). *P. nigrum* is a plant of the Piperaceae family, largely used as a flavouring agent in foods. Its characteristic aromatic odour is due to the volatile oils in the cells of the pericap.[8] *Piper nigrum* L. is used to treat asthma, chronic indigestion, colon toxins, obesity, sinus,

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congestion, fever,[9] intermittent fever, cold extremities, colic, gastric ailments and diarrhea.[10] Black pepper has been shown to have antimicrobial activity.[11,12] Both aqueous and ethanolic extracts of black pepper have been screened for antibacterial activity against a penicillin-G resistant strain of *Staphylococcus aureus*,[13]*Bacillus cereus* and *B. subtilis*.[14] Piperine ([1-[5-[1,3 benzodioxol-5-yl]-1-oxo-2, 4, pentadienyl piperidine), a pungent alkaloid present in black pepper, enhances the bioavailability of various structurally and therapeutically diverse drugs. Larvicidal[15] and anti-cancer[16] activities of *Piper nigrum* Linn. have been reported.

Reactive oxygen species (ROS) have been implicated in degenerative diseases such as cancer, inflammation, atherosclerosis and aging as also in food deterioration.[17-19] Restriction on the use of synthetic antioxidants due to their carcinogenic nature[20] has lead to a growing interest in recent years in natural antioxidants of plant origin for application in food industry to combat food deterioration. Spices and herbs are recognized as sources of natural antioxidants and thus play an important role in the chemoprevention of diseases and aging. Many studies have reported that phenolic compounds in spices and herbs significantly contributed to their antioxidant and pharmaceutical properties.[21-24,56] Kapoor *et al.*[25] recently demonstrated that the protective effect of piperine is most likely due to its antioxidant activity. Although a few reports on the antioxidant activity of cultivated *P. nigrum* are available in the literature.[26-30]

The aim of the investigation presented in this paper is to evaluate the antibacterial and antioxidant activities of various extracts of *Piper nigrum* on several mastitis pathogens, as there is a significant lack of information on such activities in literature.

MATERIAL AND METHODS

Plant material

Piper nigrum were obtained from the local herbal market from Mugla in 2016. Taxonomical identification of plant was performed by Dr. Olcay Ceylan from the University of Mugla Sıtkı Kocman, Turkey. In the identification of this spice was used the Flora of Turkey (Herb no: MUH 1045).[31]

The *Piper* fruits were washed 3 times with running water and sterile distilled water. Then the spice material was air-dried. The fruits were powdered in a laboratory blender. All samples were stocked at room temperature, and they were stored at 4°C until required for analysis.

Preparation of extracts

The air dried and powdered fruits of the spice (50 g) were extracted with solvents using the Soxhlet. In this study, methanol, ethanol and ethyl acetate were used as solvent. After solvents have been evaporate, extracts (250 mg/mL) were kept in small sterile opac bottles under refrigerated conditions until used.

Microorganisms and cultivation

The bacteria obtained from previous studies by Dr. Zafer Cantekin, University of Mustafa Kemal (Project number: 1101 M 0103; Ethics council number: 2010 / 02- 30: 12). Seven bacteria were used in these studies. Two of them were *S. aureus*, and five of them were Coagulase- Negative Staphylococci (CNS). The bacteria were grown in Mueller- Hinton Broth for 24 h at 37°C (MHB; Merck). The bacteria were identified by conventional biochemical tests.[32]

Determination of antibacterial activity

In this study, the extracts were individually tested against mastitis bacteria. The antibacterial activities of extracts were determined by disc diffusion assay. The bacteria were grown on Mueller-Hinton agar plates (MHA, Merck) at 37˚C.[33] The cultures of bacteria set to 0.5 Mc Farland. Incubations of bacteria were at 37˚C for 24 h. The assessment of antibacterial activity was based on measurement of the diameter of the inhibition zones around the discs after 24 h. Methanol, ethanol and ethyl acetate used as negative control. Ampicillin (10µg) antibiotic used as positive control. The extracts were impregnated to discs as 25 mg/mL. All tests were performed in triplicate and the mean values were given.

Determination of minimum inhibitory concentration (MIC)

The other antibacterial activity test is MIC. The MIC value was taken as the lowest concentration that inhibits growth of bacteria after incubation. The broth dilution assay was made according to CLSI standards.[34,35] The final concentrations of the extracts are 13000, 6500, 3250, 1625, 812.5 µg/mL.

Non- enzymatic antioxidant activity assay

ABTS decolorization assay was used for non- enzymatic antioxidant activity experiments.[36] Main stock liquid include 7 mM ABTS•+ [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] solution and 2.45 mM potassium persulfate solution. The absorbances were measured at 734 nm by spectrophotometry (Shimadzu UV–1201V, Japan). Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid; Sigma) was used as standard. Results of ABTS scavenging activities were given as mM Trolox equivalents (TE)/g dry mass.

RESULTS

Results of antibacterial activities of different extracts are given in Table 1. The inhibition zones were recorded as mm for all the materials. The all of extracts of *Piper nigrum* were inhibited *S. aureus*-18, CNS-32, and CNS-36. The none of extracts of *Piper nigrum* were not inhibited *S. aureus*-17, CNS-32 and CNS-33. The highest inhibition zones were shown in *S. aureus*-18 and CNS-36, and its zone was 10 mm. Ampicillin (10 µg) used as positive control.

MIC values of *Piper nigrum* are shown Table 2. In this study, 2 bacteria showed lowest sensitivity to various extract of plant (6500 µg/mL). These bacteria are *S. aureus*-18 and CNS-36 (Table 2).

Table 1: Antibacterial activities of *Piper nigrum* various extracts against mastitis bacteria

Bacteria	Inhibition zone (mm)			
	Methanol	Ethanol	Ethyl acetate	A
S.aureus- 17	(-)	(-)	(-)	18
S.aureus- 18	10	8	8	12
CNS - 22	(-)	(-)	(-)	(-)
CNS - 32	8	8	8	10
CNS - 33	(-)	(-)	(-)	8
CNS - 36	10	8	8	(-)
CNS - 37	(-)	(-)	8	(-)

CNS: Coagulase negative Staphylococci; A: ampicillin,10µg; (-): zone did not occur

Table 2: Minimum inhibitory concentrations of *Piper nigrum* various extracts (µg/mL)

Bacteria	Minimum inhibitory concentration (µg/mL)		
	Methanol	Ethanol	Ethyl acetate
S.aureus- 17	(nt)	(nt)	(nt)
S. aureus- 18	6500	6500	(-)
CNS - 22	(nt)	(nt)	(nt)
CNS - 32	(-)	(-)	(-)
CNS - 33	(nt)	(nt)	(nt)
CNS - 36	6500	6500	6500
CNS - 37	(nt)	(nt)	(-)

CNS: Coagulase negative Staphylococci (nt): Not tested; (-): inhibition did not occur

Table 3: Antioxidant activities of *Piper nigrum* extracts (250 mg/mL)

Extracts	ABTS (%)	TE
Methanol	8.6	1.5
Ethanol	13.2	1.54
Ethyl acetate	75.7	2.3

TE: Trolox equivalent (mM/gDW); DW: dry weight

In our study, antioxidant capacities of extracts are very different. Two extracts of *Piper nigrum* have low radical scavenging ability. Table 3 shows these results. The ethyl acetate extract showed about 76 % inhibition at 250 mg/mL concentration. Trolox equivalent value was 2.3 mM/g DW. Whereas, other extracts showed lower inhibition at 250 mg/mL concentration (Table 3).

DISCUSSION

The antimicrobial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may have clinical value in treatment of resistant microbial strains. Because of the side effects and bacteria resistance against the antibiotics, the scientist developed new drugs from natural sources such as plants, which have been extensively used as alternative treatment for disease.[37,38] This study confirms that the fruits of *Piper nigrum* possess biological activities.

In the present study, the highest inhibition zone was determined on two bacteria (Table 1). Then one of extracts of *Piper nigrum* were not inhibited *S. aureus*-17, CNS-32 and CNS-33 (Table 1). Obasola, *et al.*[39] and Kloos and Bannerman[40] reported that CNS pathogens are resistive to most antibiotics. They have multi-resistance genes in plasmids which can be changes and they can spread among the different species covering also *S. aureus*. Parekh and Chanda[41] reported that *Piper nigrum* was not inhibited *S. epidermidis*. The mode of action of antimicrobial agents also depends on the type of microorganisms and is mainly related to their cell wall structure and the outer membrane arrangement. The reports supported our results.

All of extracts of *Piper nigrum* were inhibited S. aureus-18, CNS-32, and CNS-36 (Table 1). Dorman and Deans[11] reported that *Piper nigrum* inhibited *S. aureus* as 14.5 mm. Reddy *et al.*[42] reported that *Piper nigrum* suppressed *S. aureus* as 8-11 mm. Nazia and Perween[43] published that *P. nigrum* inhibited *S. aureus* as 23 mm inhibition zone. Shan *et al.*[44] determined that *P. nigrum* repressed *S. aureus* as 4.6 mm. Karsha and Lakshmi[45] shown that *Piper nigrum* inhibited *S. aureus* as 14-20 mm. Weerakkody *et al.*[46] found that *P. nigrum* repressed *S. aureus* as 5-6 mm inhibition zone. Zarai *et al.*[47] reported that inhibition zone of *Piper nigrum* shown 8-12 mm for *S. aureus* and 7-14 mm for *S. epidermidis*. The studies supported our results.

Sethi, *et al.*[48] reported that minimum inhibitory concentration value (MIC) of *Crocus sativus* against *S. aureus* was found as 65 mg/mL. Weerakkody *et al.*[46] found that MIC value of *P. nigrum* as 5 mg/mL. Karsha and Lakshmi[45] shown that MIC value of *Piper nigrum* found as 125 ppm.

In this study, MIC value is 6500 µg/mL, and these data are better than other studies (Table 2). Whereas, Zarai *et al.*[47] reported that MIC values of *Piper nigrum* as 312.5 and 1250 µg/mL. The differences in the antimicrobial activities with the reported one may be due to different geographical environment, age of the plant, different method followed for isolation of oil, cultivar type, seasonality etc. According to Aligiannis, *et al.*[52] the extracts of *Piper nigrum* can be considered a weak inhibitor against mastitis pathogens.

Plants play a major role in providing the required antioxidants for the body. Although, traditionally, spices have been used in food preparations to improve flavour and taste.[26] In our results, the ethyl acetate extract showed 76 % inhibition at 250 mg/mL concentration. Whereas other extracts showed different inhibition at same concentration (Table 3). Zarai *et al.*[47] reported that the ethanolic extract of *Piper nigrum* showed a high antioxidant activity 65.6 % at final concentration of 50 µg/mL. Shan *et al.*[44] found that the antioxidant activity of fruit of black pepper as 4.6 mmol TE/ 100 g DW. Ahmad *et al.*[49] determined that callus of *Piper nigrum* have 40 % antioxidant activity. Khalaf *et al.*[50] reported that *Piper nigrum* inhibited DPPH. The inhibition rate is about 60 %. Singh *et al.*[27] told that the highest DPPH inhibition found 61 % for ether extract. Gülçin[21] reported that the highest DPPH inhibition of spice found 55 % for ethanol extract. Our results are better than these studies. Furthermore, Fogden and Neuberger[51-55] cited that the active constituents themselves are influenced by a wide range of factors: harvest season, preparation methods, plant species, and location including altitude and climate, and quality control.

CONCLUSION

Piper nigrum 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 shown that *Piper nigrum* has biological activity. It would be very useful in the finding of novel antibacterial agents. Furthermore, plant extracts have great importance as antioxidant activities. Also, more researches involving more detailed studies to determine which components of spice extracts offer the best biological activity are advised.

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CONFLICT OF INTEREST

None

ABBREVIATION USED

CLSI: Clinical and Laboratory Standards Institute MIC: Minimum inhibitory concentration; ABTS: 2,2'-azinobis(3-ethyl benzothiazoline-6-sulfonic acid); TE: Trolox equivalent; MHA: Mueller-Hinton Agar; MHB: Mueller-Hinton Agar; DPPH: 2,2-diphenyl-1-picrylhydrazyl; h: hour; mg/mL: milligram/milliliters; ug/mL: microgram/milliliters; nm: nanometer; °C: Celsius degree; mm: millimeter.

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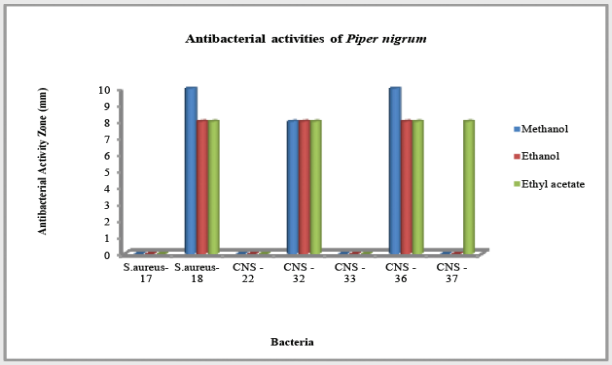
PICTORIAL ABSTRACT



SUMMARY

- The highest antibacterial activity was determined from methanol extract for *Staphylococcus aureus*-17 and CNS-36. In this study, all of the bacteria were shown sensivity for 6500 μg/mL concentration. The highest antioxidant activity was shown at ethyl acetate extract. Our results suggest that *Ribes nigrum* has significant antibacterial and antioxidant activity and it could be very useful in the discovery of novel antibacterial and antioxidant agents of plant origin.

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