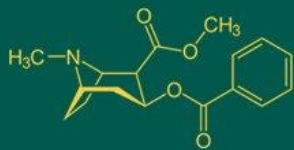


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B Aravinda
Veterinary Officer, Department of
Animal Husbandry and Veterinary
Services, Govt. of Karnataka,
Karnataka, India

JP Singh
Professor, Department of
Veterinary Medicine, College of
Veterinary Science and Animal
Husbandry, Acharya Narendra
Deva University of Agriculture &
Technology, Kumarganj, Ayodhya,
Uttar Pradesh, India

SV Singh
Professor, Department of
Veterinary Medicine, College of
Veterinary Science and Animal
Husbandry, Acharya Narendra
Deva University of Agriculture &
Technology, Kumarganj, Ayodhya,
Uttar Pradesh, India

Vibha Yadav
Associate Professor, Department of
Veterinary Microbiology, College of
Veterinary Science and Animal
Husbandry, Acharya Narendra
Deva University of Agriculture &
Technology, Kumarganj, Ayodhya,
Uttar Pradesh, India

Rajkamal Yadav
Veterinary Officer, Department of
Animal Husbandry, Govt. of Uttar
Pradesh, Uttar Pradesh, India

Ankur Singh
Ph.D. Scholar, Department of
Veterinary Medicine, Sardar
Vallabhbhai Patel University of
Agriculture and Technology,
Meerut, Uttar Pradesh, India

Dinesh Kumar Yadav
Veterinary Officer, Department of
Agriculture, Animal Husbandry &
Co-Operative, Govt. of Jharkhand,
Jharkhand, India

Vijaya Mahantesh SK
Veterinary Officer, Department of
Animal Husbandry and Veterinary
Services, Govt. of Karnataka,
Karnataka, India

Corresponding Author:
B Aravinda
Veterinary Officer, Department of
Animal Husbandry and Veterinary
Services, Govt. of Karnataka,
Karnataka, India

In-vitro efficacy of *Cinnamomum zeylanicum* against *Staphylococcus aureus* isolated from buffaloes with mastitis

B Aravinda, JP Singh, SV Singh, Vibha Yadav, Rajkamal Yadav, Ankur Singh, Dinesh Kumar Yadav and Vijaya Mahantesh SK

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Abstract

Mastitis causes a significant proportion of economic losses to the dairy farmers in India. Buffalo farming contributes significantly to the economy of the country. The emergence and spread of antibiotic resistance, as well as the evolution of new strains of disease-causing agents, are of great concern to the global health community. Microbial resistance to antibiotics especially among *Staphylococcal* strains is a major threat to public health. Therefore, plant extracts are suitable alternatives now to treat resistant organisms. Present study was designed to study the *in-vitro* antimicrobial efficacy of *Cinnamomum zeylanicum* bark extracts against *Staphylococcus aureus* that causes mastitis. The *in-vitro* efficacy of *Cinnamomum zeylanicum* extracts-aqueous, methanolic and hydro-methanolic extracts were studied by disc diffusion sensitivity test and it was observed that at 50 mg/ml both aqueous and methanolic extracts showed no zone of inhibition. At 100 mg/ml, 150 mg/ml, 200 mg/ml and 250 mg/ml both aqueous and methanolic extracts showed zones of inhibition and comparatively aqueous extract had higher zone of inhibition than methanolic extract at similar concentration. No zone of inhibition was observed for hydro-methanolic extract at any concentration against *S. aureus*. The MIC value of both aqueous and methanolic extract of cinnamon against *S. aureus* was 12.5 µg/ml, while MIC value of hydro-methanolic extract was 25 µg/ml. MBC value of the all three extracts of cinnamon against *S. aureus* was 25 µg/ml.

Keywords: Mastitis, antibiotic resistance, *Cinnamomum zeylanicum*, *Staphylococcus aureus*

Introduction

Mastitis is an economically important disease of intensive buffalo dairy farming worldwide. Mastitis is common problem in buffaloes with significant impact on the economy of rural India. Of the total milk production, buffaloes contribute about 53% while cows have a 43% share only (Kala *et al.*, 2021) ^[11]. Mastitis is one of the most common reasons for which management dairy cows are given antibiotics (Lago *et al.*, 2011; Birhanu *et al.*, 2017) ^[13, 5]. In conventional dairy farming, more than 80% of antibiotics were solely used to cure or prevent diseases (Pol and Ruegg, 2007) ^[18]. Antibiotic overuse has a number of negative consequences, including antibiotic-tainted milk, the development of antibiotic resistance in microorganisms, and human health risks (Ji *et al.*, 2020; Khan *et al.*, 2020; Shoaib *et al.*, 2020) ^[10, 12, 22].

S. aureus has been the most common pathogen identified in mastitis (McParland *et al.*, 2019) ^[15]. The ability of *S. aureus* which causes persistent infection is major concern for antibiotic therapy failure, which are the first choice to fight against bacterial infections, the emergence of multidrug-resistant pathogens and the presence of antibiotic residues in the milk and milk products are major concerns. Current situation requires the need for the exploration of alternative treatments since antibiotics are no longer considered as a useful tool against bovine mastitis caused by *S. aureus* (Pietrocola *et al.*, 2017) ^[17].

Herbal therapy is a promising area in treatment of mastitis as no adverse effect is associated with it. Ethno-veterinary medicine is a branch of veterinary medicine that deals with the treatment of diseases with herbal preparations (Tiware *et al.*, 2018) ^[25]. Medicinal plants can be used as an alternative therapeutic option or as an adjunct agent in managing bovine mastitis.

They can be used as an anti-bacterial, anti-inflammatory, and immunomodulatory agent for the treatment of mastitis (Mushtaq *et al.*, 2018) ^[16]. Several studies reported that natural medicinal plants with varying degree of antimicrobial properties can be used as antibacterial agents (Akter *et al.*, 2014) ^[12]. The alternative approaches to overcome multidrug-resistance by using natural bioactive components including essential oils, extracts of various parts of plant and plant bioactive compounds (Gadisa *et al.*, 2019) ^[8].

Cinnamomum zeylanicum, a member of the family Lauraceae, is a tropical evergreen tree, native to Sri Lanka and the Malabar Coast of India, called differently in different languages such as dalcini in Hindi, cannelle in French, kaneel in German, canela in Spanish, yook gway in Chinese and kurunda in Sinhalese. *Cinnamomum zeylanicum* is mainly used as a culinary herb like other cinnamons in the traditional Eastern and Western medicine (Sudan *et al.*, 2013) ^[23]. Plants extracts of cinnamon have been traditionally used by people for various purposes in different parts of the world (Al-dhaher, 2008) ^[3]. Cinnamon primarily contains vital oils and other derivatives, such as cinnamaldehyde, cinnamic acid and cinnamate (Rao *et al.*, 2014) ^[20].

The present study was undertaken in order to evaluate the *in-vitro* antibacterial activity of aqueous, methanolic and hydro-methanolic extracts of cinnamon against *Staphylococcus aureus*.

Materials and Methods

Staphylococcus aureus Isolates

About 10 ml of midstream milk samples were collected in sterile vial from affected quarters. Tests namely White side test and California mastitis test were used to detect sub clinical mastitis in buffaloes and those samples which showed strong positive reaction were selected for isolation of *S. aureus*. Milk samples collected from buffaloes with subclinical mastitis were incubated in sterile nutrient broth at 37 °C for 24 hr to check growth of bacteria; and then bacteria were streaked on mannitol salt agar (MSA) plate and incubated in incubator, the bacterial colonies were stained with Gram's staining. Gram positive bacteria were transferred to nutrient agar slant and biochemical test namely Catalase, Nitrate reduction test, Coagulase, IMVIC test etc., were performed followed by Beta-Haemolysis in Blood agar. Each sample of milk was directly inoculated onto mannitol salt agar (MSA) and incubated at 37 °C for 24 hours to identify *S. aureus*, as described by Talan *et al.* (1989). Mannitol fermented colonies from primary cultures were purified by subculture onto nutrient agar slants and incubated at 37 °C for 24-48 hour and stored at 4 °C until further use. For biochemical characterization of bacteria, Methyl Red test, Voges-Proskauer test, catalase test, Slide and Tube coagulase test, Nitrate reduction test and haemolysis on blood agar were performed.

Processing and testing of *Cinnamomum zeylanicum*

Cinnamomum zeylanicum bark was collected from local market, Kumarganj, Ayodhya and bark was dried and then ground into powder form. Powder was soaked in hydro, alcoholic and hydro-alcoholic solvent and it was sieved by several layers of muslin cloth. Then it was finally filtered by Whatman Filter Paper No.1 for a clear solution of *Cinnamomum zeylanicum* powder and filtrate was

concentrated in incubator at 45 °C to obtain the final solid extract. Lastly, sterilized blank discs were soaked in different concentration of hydro, alcoholic and hydro-alcoholic extract of *Cinnamomum zeylanicum* and ABST was carried out with comparing antibiotic discs.

From the powder of *Cinnamomum zeylanicum* bark, extract was prepared by using aqueous, methanolic and hydro-methanolic solution. aqueous, methanolic and hydro-methanolic extraction was done separately by using distilled water, methanol and distilled water + methanol in 1:1 ratio respectively. Total 50 gm of *Cinnamomum zeylanicum* bark powder was soaked in 250 ml of water, methanolic and hydro-methanolic solution (125 ml distilled water + 125 ml of methanol) in glass container separately for 48 hrs at room temperature. The beakers with lid were stirred at frequent intervals to properly dissolve the powder in the aqueous (A), methanolic (M) and hydro-methanolic (HM) solvent. After 48 hrs the contents were sieved separately through several layers of muslin cloth. Then these solutions were collected in bottle and filtered by the Whatman filter paper no.1 using funnel separately. The final filtrate was collected in a glass bowl and kept in incubator with circulating fan at 45 °C for drying it completely for 5-6 days. Afterwards the dried material was removed and kept properly with labeled and final weight in air tight in colored bottles at refrigerated temperature at 4 °C.

Disc preparation

Stock solutions of each types extract were prepared with respective solvents (sterile distilled water for aqueous extract and 99.9% dimethyl sulfoxide for methanolic extract) and the final concentrations of 50 mg/ml, 100 mg/ml, 150 mg/ml, 200 mg/ml, 250 mg/ml were then used to impregnate in sterilized blank discs (Bauer *et al.*, 1966) ^[4]. Distilled water and dimethyl sulfoxide-loaded discs were used as negative controls for aqueous and methanolic extracts respectively. All impregnated discs were ensured to be fully dried in 45 °C in incubator for 18 to 24 hours prior to the application on bacterial lawn (Zaidan *et al.*, 2005) ^[27]. Enrofloxacin discs were used as positive controls.

Determination of minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC)

For this test, Nutrient broth media was used, the media was prepared as per the standard composition and autoclaved for sterilization. In the test, a series of 10 test tubes was used starting from the highest concentration of antibacterial agent followed by the lower ones, one blank test tube (media without test organism and test material), media control (with test material and without test organism) and growth control (with test organism and without test material).

The antibacterial agent was serially diluted in the tubes containing sterile culture media (Nutrient broth media). The respective concentrations of the antibacterial agents were prepared in each tube, that is CA, CM, CHM 50 µg/ml. After this step, each tube containing the media and antibacterial agent was loaded with 100 µl of 24 hr old bacteria culture that is *S. aureus*, separately.

After addition of Bacteria culture, the tubes were plugged and incubated at 27 °C for 24-48 hrs. After incubation, the tubes were observed for the turbidity in the medium, which is an indication of the bacterial growth. The strength of turbidity in each tube was recorded as high turbidity (HT),

weak turbidity (WT) and no turbidity (NT) that is flourished growth, partial growth and no growth of the bacterial isolate respectively.

The lowest concentration of the antibacterial agent at which no turbidity was seen is taken as the MIC value for that set of analysis. The minimum bactericidal concentration (MBC) is determined by subculture from each tube on nutrient agar plate without any antimicrobial agent and the concentration which show no growth on nutrient agar plate is MBC value.

Results and Discussion

Antimicrobial sensitivity pattern of extracts of *Cinnamomum zeylanicum* bark

Each type of Cinnamon extract was tested against the isolates of *Staphylococcus aureus*. The mean diameter of zone of growth inhibition was measured in millimeters and the values were recorded.

Aqueous extract of cinnamon at 50 mg/ml concentration exhibited no antibacterial activity. Highest antibacterial activity was observed at 250 mg/ml with 14.2±0.78 mm zone of inhibition, followed by 200 mg/ml (12.6±0.51 mm), then 150 mg/ml (11.1±0.73 mm), the disc with concentration 100 mg/ml exhibited 8.1±0.56 mm diameter zone of inhibition. Al-dhaher (2008) [3] found that the mean of the diameter of inhibition zone were 7 mm, 8 mm, 11 mm, 13 mm, 15 mm, 15.5 mm, 17 mm for the concentrations of 15%, 20%, 30%, 40%, 50%, 60% and 70% respectively.

Highest antibacterial activity of methanolic extract was noticed at 250 mg/ml with 13.8±0.73 mm zone of inhibition, followed by 200 mg/ml (11.7±0.67 mm), 150 mg/ml (10.6±0.84 mm) and at 100 mg/ml (7.3±0.48 mm). No zone of growth inhibition was found at 50 mg/ml. Mazimba *et al.* (2015) [14] showed 9 mm zone of inhibition. Elhag *et al.* (2015) [7] showed that the average zone of inhibition of methanolic extract against *Staphylococcus aureus* was 20.5 mm at 100 mg/ml concentration, which is more in compare to the present study.

No zone of growth inhibition was found at any concentration of hydro methanolic extract of cinnamon, which indicates that there is no antibacterial activity of hydro methanolic extract of cinnamon against *S. aureus* by disc diffusion method.

Among aqueous and methanolic extracts of cinnamon at similar concentrations aqueous extract is having better antibacterial activity. At 100 mg/ml aqueous extract showed 8.1 mm±0.56 ZOI which is > methanolic extract which showed 7.3 mm±0.48 ZOI, at 150 mg/ml aqueous extract showed 11.1 mm±0.73 ZOI which is > methanolic extract which showed 10.6 mm±0.84 ZOI, at 200 mg/ml aqueous extract showed 12.6 mm±0.51 ZOI which is > methanolic extract which showed 11.7 mm±0.67 ZOI, at 250 mg/ml aqueous extract showed 14.2 mm±0.78 ZOI which is > methanolic extract which showed 13.8 mm±0.73 ZOI. Table 1 summarizes these findings.

Earlier reports also mention the antibacterial activity of Methanolic cinnamon extract (Elhag *et al.*, 2015 and Mazimba *et al.*, 2015) [7, 14] and Aqueous cinnamon extract (Al-dhaher, 2008) [3] against *S. aureus*.

Similar findings were reported in animals affected with mastitis in cow by Hatem and Sabeeh (2013) [9] who reported antibacterial activity of ethanolic extract of cinnamon against *S. aureus*. Seenivasan *et al.* 2006 [21]

reported that cinnamon extracts possess antibacterial activity against *S. aureus*.

Table 1: In-vitro antimicrobial sensitivity pattern of extracts of *Cinnamomum zeylanicum* bark

Disc Code	Concentration (mg/ml)	Inhibition zone (mm) against <i>S. aureus</i>
CA	50	NIL
CA	100	8.1±0.56
CA	150	11.1±0.73
CA	200	12.6±0.51
CA	250	14.2±0.78
CM	50	NIL
CM	100	7.3±0.48
CM	150	10.6±0.84
CM	200	11.7±0.67
CM	250	13.8±0.73
CHM	50	NIL
CHM	100	NIL
CHM	150	NIL
CHM	200	NIL
CHM	250	NIL
Positive control (Enrofloxacin)	10 mcg	22.7±0.82

* CA-Aqueous extract of *Cinnamomum zeylanicum*

* CM-Methanolic extract of *Cinnamomum zeylanicum*

* CHM-Hydro-methanolic extract of *Cinnamomum zeylanicum*

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Both MIC and MBC values are summarised in table 2. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) values of Aqueous extract of cinnamon were found at 12.5 µg/ml and 25 µg/ml respectively. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) values of Methanolic extract of cinnamon were 12.5 µg/ml and 25 µg/ml respectively. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) values of Hydro-methanolic extract of cinnamon were 25 µg/ml and 25 µg/ml respectively.

According to Al-dhaher (2008) [3] minimum bactericidal concentration (MBC) of cinnamon aqueous extract for *Staphylococcus aureus* was 300 mg/ml which is much higher than our findings. Mazimba *et al.* (2015) [14] found that Methanolic extract of cinnamon stem bark against *Staphylococcus aureus* 2.5 µg/mL MIC value. MIC of Aqueous extract of cinnamon *S. aureus* was at 246.6 µg/mL according to Rakshit and Ramalingam (2011) [19].

In vitro efficacy of cinnamon can be attributed to Cinnamaldehyde, that possess antioxidant and antibacterial activity as reported by Adarsh *et al.* (2020) [1], Al-dhaher (2008) [3], Hatem and Sabeeh (2013) [9], Vangalapati *et al.* (2012) [26] and Bowles *et al.* (1995) [6].

Table 2: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Test Sample	Minimum Inhibitory concentration	Minimum Bactericidal Concentration
CA	12.5 µg/ml	25 µg/ml
CM	12.5 µg/ml	25 µg/ml
CHM	25 µg/ml	25 µg/ml

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