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CONTENTS

Antibacterial activity of Clove bud extract on MDR bacteria

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ABSTRACT: Mastitis, a prevalent disease in high-yielding dairy animals, leads to substantial economic losses, further complicated by the rise of multidrug-resistant (MDR) bacteria, limiting conventional treatment options. This study aimed to evaluate the antibacterial efficacy of ethanolic clove (*Syzygium aromaticum*) extract against MDR pathogens isolated from mastitis-infected milk. Total 20 milk samples were collected, yielding MDR *Staphylococcus aureus* and *Escherichia coli*. The extract, prepared by maceration and confirmed to contain bioactive phytochemicals (flavonoids, tannins, phenols, etc.), was tested at varying concentrations using agar well diffusion assay. Results showed dose-dependent antibacterial activity, with the highest inhibition at 200 mg/ml. *E. coli* exhibited greater sensitivity (22.33±0.88 mm) than *S. aureus* (19.17±0.17 mm), and MIC values were 6.25 mg/ml for *E. coli* and 1.5625 mg/ml for *S. aureus.* The findings highlight clove extract's promising potential as a natural alternative for treating MDR mastitis pathogens.

Keywords:Antibacterial activity, mastitis, MDR, *Syzygium aromaticum*

Mastitis is the major production disease that affects the high-yielding animals causing significant financial losses to the farmers. In lactating animals, mastitis is very common. It causes tremendous losses to Indian dairy farmers. Dairy farming makes a substantial contribution to the nation as well as the state's economy (Audarya *et al.,*2021). The prevalent bacteria isolated from mastitis milk are *Staphylococci spp*., which are followed by *Klebsiella spp., Escherichia coli spp.* and *Streptococci spp*. (Ali *et al.,* 2021). Singh *et al.* (2018) reported that there was highest prevalence of *Streptococcus agalactiae* and *Staphylococcus aureus* followed by *E. coli* and *Klebsiella pneumoniae* in mastitis milk. Traditional remedies have been employed for centuries across various cultures to address a wide range of health conditions. The development of new antimicrobials is vital, but treating infectious diseases has become more challenging due to the alarming increase in antibiotic resistance in microbes brought on by their indiscriminate use. As a result, there is currently a great deal of interest in investigating the antibacterial properties of medicinal herbs.80% of the world's population is dependent on medicinal plants, and India's ancient medical system still heavily emphasizes the use of herbs as therapeutic agents. The biologicals and antibacterial properties of numerous plants have been studied (Arora and Kaur,2007). According to the World Health Organization (WHO), "Traditional medicine is the sum of the knowledge, skills, and practices based on the theories, beliefs, and experiences from indigenous to different cultures whether explicable or not. " Traditional medicines include formalized forms with well-documented remedies, such as European Cloister medicine, Ayurveda, Ancient Iranian medicine, Islamic medicine, traditional Chinese medicine, etc., in addition to more informal practices that are verbally transmitted from generation to generation (Efferth and Greten, 2014).

Numerous botanicals have antimicrobial qualities. Because they contain a spectrum of bioactive substances, also known as phytochemicals, which operate as defence mechanisms against microbial infections, plants are thought to be effective treatment agents for a variety of infectious illnesses. An important alternative approach to controlling antibiotic resistance seems to be the extraction of several bioactive components from higher plants. Research in the field of ethnopharmacology spe-

cifically on medicinal plants possessing antimicrobial property is also encouraged by the notion that green medicine is safer and more effective against expensive commercial drugs (Javale and Sabnis,2010).

Syzygium aromaticum or Clove is a dried flower bud native to the Indonesian Maluku islands. It belongs to Myrtaceae family. *Syzygium aromaticum* has been traditionally used for millennia to cure a variety of ailments, including nausea, vomiting, flatulence, liver, intestine and stomach issues. They are also used for nerve stimulation. Cloves have shown to alleviate variety of pathogenic infections, including scabies, cholera, malaria and tuberculosis in tropical Asia. In America, they have traditionally been employed to combat food-related pathogens and to manage infections caused by viruses, worms, candida as well as various bacterial and protozoan ailments. Besides the uses of clove, clove essential oil is considered as monster antioxidant due to its exceptionally high antioxidant activity (Bhowmik *et al*., 2012).

MATERIALS AND METHODS

The dried flower buds (clove) of *Syzygium aromaticum*were purchased from the local market in Udgir. The dried *Syzygium aromaticum* clove was identified and authenticated by a botanist. The cloves were purchased and shed dried. Using a dry mechanical grinder, the dried cloves were powdered. The dried powder was sieved to get fine powder and kept in an airtight container until further usage.

Preparation Syzygium aromaticum clove extract

The ethanolic extract of *Syzygium aromaticum* clove was prepared by using a maceration process. Two conical flasks containing 50 gm of *Syzygium aromaticum* clove powder collected and were filled with 250 ml of 95% ethanol. The flasks were closed tightly with a stopper and kept for 48 hrs. The contents of the flasks were shaken intermittently during the maceration process. After 48 hrs the contents of the flask were filtered through muslin cloth. The filtrate obtained was once again filtered through Whatman no.1 filter paper. The filtrate so obtained was transferred to large petri plates and kept under fan at room temperature for evaporation. After complete evaporation, the obtained extract was transferred to labelled airtight glass containers and stored in the refrigerator for further use.

Test Organisms

20 Mastitis milk samples were collected from mastitis infected animals (bovine),which were reported to TVCC, College of Veterinary and Animal Sciences, Udgir.

Isolation of bacteria from clinical samples

The collected samples i. e. mastitis milk were subjected to isolation of bacteria.
The samples were subjected to bacteriological study in the Department of Microbiology, College of Veterinary and Animal Sciences, Udgir by inoculating approximately 0.01 ml of milk sample onto the Nutrient agar, MacConkey's agar and Eosin methylene blue agar plates and the plates were incubated under aerobic conditions at 37! for 24 hours. Identification of bacteria was carried out based on their morphology and colony characteristics suggested by Cruickshank *et al.* (1975)and biochemical screening test suggested by Agrawal *et al.* (2003).

Screening of MDR bacteria

Multi drug resistant (MDR) is defined as an isolate that is not susceptible to at least three antimicrobials, (Magiorakos *et al.,* 2012). The bacteria isolated in pure culture were subjected to drug sensitivity test as suggested by Bauer *et al.* (1966). Antibiotic discs used for the study were Amoxicillin/sulbactam (AMS), Chloramphenicol (C), Ceftriaxone/ tazobactam (CIT), Enrofloxacin (EX), Oxytetracycline (O), Gentamicin (GEN), Ciprofloxacin (CIP) and Ampicillin/cloxacillin (AX). The bacterial isolates from mastitis milk samples showing resistance to more than or equal to any 3 antibiotics/ drugs were selected for the present study.

One standard MDR strain of bacteria *Escherichia*

coli ATCC 25922 was purchased from HI-media.

Agar well diffusion assay

Ethanolic extract of *Syzygium aromaticum* clove was screened against test organisms isolated from mastitis milk samples by agar well diffusion method as mentioned by Perez (1990). *Syzygium aromaticum* stock solution of 200 mg/ml were prepared by dissolving 200 mg of the extract into 1 ml of DMSO (1%) and consequently diluted further to obtain serial dilutions of 100 mg/ml, 50 mg/ml, 25mg/ml, 12.5 mg/ml, 6.25 mg/ml and 3.125mg/ml. The 4-hr. old broth culture of test organisms (0.2 ml according to 0.5 McFarland standards) was prepared. Oxytetracycline (30 µg) was used as the standard reference antibiotic against MDR isolates of *Staphylococcus aureus* and *E. coli* and ciprofloxacin was used as the standard reference antibiotic for standard *E. coli* (ATCC 25922) bacteria. Antibiotic showing highest zone of inhibition are taken as reference antibiotics. The plateswere incubated at 37°C for 16-18 hrs. and after incubation the diameter of the zone of inhibition was measured in mm with scale. For each test organism, three replicates were used. Furthermoreethanol (95%) was taken as a negative control.

Minimum Inhibitory Concentration

A broth microdilution assay was performed using 96 well microtitre plates and resazurin. It was carried out to assess the microbial growth and determine the minimum inhibitory concentration as suggested by Sarker *et al.* (2007) with slight modification as described below.

Statistical analysis

After completion of conduction of antibacterial activity by Well Diffusion method and MIC by broth microdilution method, observations were noted and data was recorded; accordingly results obtained were processed for further analysis. Then the data was statistically analyzed by IBM SPSS software using the method of ANOVA single factor and Dunkan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Qualitative phytochemical analysis

The ethanolic extract of *Syzygium aromaticum* showed the presence of flavonoids by shinoda test, ferric chloride test and lead acetate test; tannins by ferric chloride test lead acetate test and gelatin test; sterols by Salkowski test; glycoside by benedict's test; terpenes by salkowski test; phenolic compounds by ferric chloride test gelatine test and phenolic proteins by million's test. These results align with those of Gupta *et al.* (2014) and Jimoh *et al.* (2017).

Isolation and confirmation of the test organisms

Table 1: Zone of inhibitions (mm) along with standard error of bacteria against ethanolic extract of *Syzygium aromaticum*

Organism	Concentrations (mg/ml)							Oxytetracycline/
	200	100	50	25	12.5	6.25	3.125	Ciprofloxacin
S. aureus	19.17 ± 0.17	17.17 ± 0.17	15.27 ± 0.27	14.20 ± 0.20	12.13 ± 0.13	10.57 ± 0.29	0.00 ± 0.00	32.67 ± 0.33[b]
E. coli	22.33 ± 0.88	18 ± 0.58	16 ± 0.58	15 ± 0.58	13.67 ± 0.88	11.33 ± 0.88	0.00 ± 0.00	30.33 ± 0.33
E. coli ATCC 25922	20 ± 0.58	18± 0.58	17 ± 0.58	15 ± 0.58	13 ± 0.58	11 ± 0.58	0.00 ± 0.00	29.67 ± 0.33

Table 2: Minimum Inhibitory Concentration of ethanolic extract of *Syzygium aromaticum* againstMDR bacteria

MDR Bacteria	Colour change occurred well no.	Concentration of extract in the well	MIC
Staphylococcus aureus (A2 to C9)	A7, B7 and C7	1.5625 mg/ml	1.5625 mg/ml
Escherichia coli (D2 to F9)	D5, E5 and F5	6.25mg/ml	6.25mg/ml
Escherichia coli ATCC 25922 (G2 to H9)	G5 and H5	6.25mg/ml	6.25mg/ml

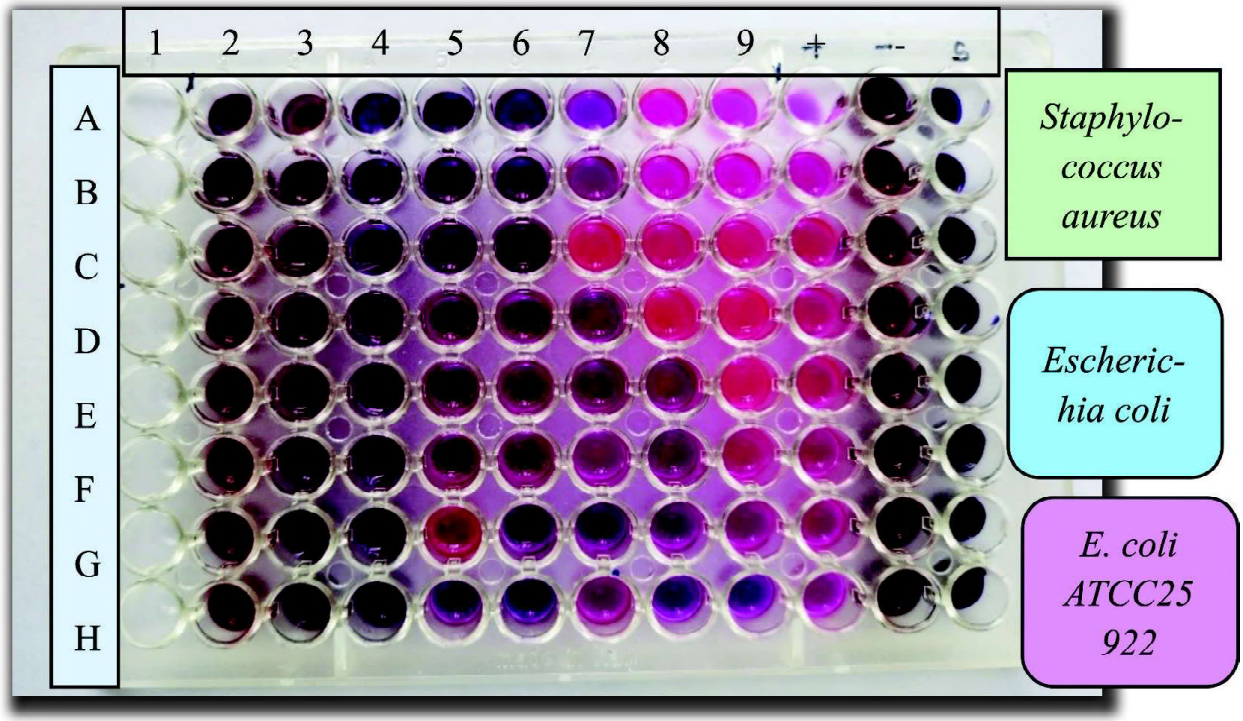
Two MDR (multi drug resistant) organisms were isolated and identified, *Staphylococcus aureus* and *Escherichia coli.* Confirmation of *Staphylococcus aureus* and *Escherichia coli* was done by using the following tests: Nitrate reduction test, Methyl Red (MR) test, Voges-Proskauer (VP) test, Indole test and Catalase test.

Staphylococcus aureus isolated from the mastitis milk found to be resistant to ciprofloxacin (CIP), Amoxicillin/sulbactam (AMS), Ampicillin/cloxacillin (AX), and Enrofloxacin (EX) with the zone of inhibition of 12mm, 13mm, 9mm, and 16mm respectively. Whereas, *Escherichia coli* isolated from the mastitis milkwas found to be resistant to ciprofloxacin (CIP), Amoxicillin/sulbactam (AMS), Ampicillin (AX), and Enrofloxacin (EX) with the zones of inhibition of 12mm, 13mm, 9mm, and 7mm respectively. While Standard *Escherichia coli* ATCC 25922 was found resistant to Enrofloxacin (EX), Ceftriaxone/tazobactam (CIT) and Ampicillin (AX) with zones of inhibition of 11mm, 11mm and 14mm respectively.

Munita and Arias (2016) discussed main ways that bacteria may adapt to antibiotics: 1) Gene alterations that are directly related to the compounds' modes of action 2) the horizontal gene transfer (HGT) acquisition of foreign DNA encoding resistance determinants 3) enzymatic inactivation, which modifies the antibiotic molecule; and 4) reduced antibiotic efflux and penetration. Additionally, overuse and misuse of antibiotics, which are selected in many bacterial species, is the most common cause of antibiotic resistance, making antimicrobial treatment useless.

Evaluation of the antibacterial activity of the extractof Syzygium aromaticum

The Table 1also indicates that the zones of inhibi-



1. From A2 to C9- *Staphylococcus aureus;* 2. From D2 to F9- *Escherichia coli*; 3. From G2 to H9- *Escherichia coli* ATCC 25922; 4. From A2 to H9- Serial dilution of extract+ bacteria+ broth+ resazurin; 5. +: -Positive control (bacteria + broth + resazurin dye except extract); 6. -: - Negative control (broth + resazurin dye except bacteria); 7. S – Sterility control (bacteria + broth + resazurin dye)

tion observed at concentrations of 200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25 mg/ml were highly significant ($P \leq 0.001$). The *Syzygium aromaticum* extract demonstrated a slightly higher antibacterial activity against both the *Escherichia coli* strains than against *Staphylococcus aureus*. In all cases, the antibacterial activity of the ethanolic extract of *Syzygium aromaticum* was dose-dependent with higher concentrations producing larger inhibition zones. No zone of inhibition was observed at 3.125 mg/ml concentration for all bacterial strains and by negative control. The maximum inhibition was observed at 200 mg/ml concentration for both *Staphylococcus aureus* and *Escherichia coli* strains; confirming the extract's dose-dependent nature.

These observations are in accordance with the study performed by Salisu *et al.* (2022) who observed that *Syzygium aromaticum* exhibited antibacterial activity against *Staphylococcus aureus* with the highest inhibition zone19±0.8mm at 200mg/ml and the lowest inhibition zone 6± 0.8 mm at 6.25mg/ml. Similar results were found by Pandey and Singh (2011) who evaluated the antibacterial activity of the extract of *Syzygium aromaticum* against *Staphylococcus aureus* and found 16mm of the zone of inhibition. Zhang *et al.* (2016) determined the antibacterial activity of clove against *Escherichia coli* isolated from chicken meat and found the mean zone of inhibition of 20.79mm which is similar to the present study's result at 200 mg/ml. Ginting *et al.* (2021) screened *E. coli* ATCC 25922 against clove essential oil and found the zones of inhibition ranging from 17mm to 24.3mm which closely aligns with the present study's range of zones of inhibition 11 mm to 20 mm.

Minimum inhibitory Concentration

Plates were prepared under aseptic conditions. A sterile 96-well plate was labelled. A volume of 50 µl ethanolic extract of *Syzygium aromaticum* of 100mg/ml concentration in 1% (v/v) DMSO was pipetted into the first column of the plate from A1 to H1. To all other wells, 50 µl of nutrient broth was added from A2 to H12.50 µl of the extract was pipetted into column 2 (A2) from one (A1) and serial dilutions were performed. Tips were discarded after use such that each well had 50 µL of the test material in serially descending concentrations. Finally, 50 µl of bacterial suspension (1.5×10^6 cfu/ml) was added from A2 to H12. The wells were prepared in triplicate and placed in an incubator set at 37 °C for 18–24 h. Finally, to each well, 20 µL of resazurin indicator solution was added except the first column from A2 to H12. The colour change was then assessed visually. Any colour changes from purple to pink were recorded as positive. The lowest concentration at which colour change occurred was taken as the MIC value. The average of three values was calculated and that was the MIC for the ethanolic extract of *Syzygium aromaticum* against particular bacteria. Table 2 and photograph indicates the values of MIC. Hoque *et al.* (2008) reported that, MIC of ethanolic extract of clove ranged from 1.5mg/ml to 2.5 mg/ml against four strains of *Staphylococcus aureus;* these findings are consistent with the present study's result of 1.5625 mg/ml. Similarly, Behbahani *et al.* (2019) found the MIC of *E. coli* ATCC 25922 to be 6.25 mg/ml. Egwuatu *et al.* (2023) screened the clove essential oil for its antibacterial activity against ESBL (Extended Spectrum Beta-Lactamase) producing *Escherichia coli* in urinary tract infections and found the MIC at concentration 6.25mg/ml. These results were consistent with the present study's results for *E. coli* isolates. Similarly, Marouf *et al.* (2023) found the MIC of *E. coli* ATCC 25922 at a concentration of 6.25 mg/ml.

CONCLUSION

The ethanolic extract of clove (*Syzygium aromaticum*) exhibited significant antibacterial activity against MDR bacteria isolated from mastitis milk samples. The findings suggest that clove extract could be developed as an alternative or complementary treatment for mastitis caused by MDR bacteria. Further studies on *in vivo* efficacy, toxicity, and mechanism of action are recommended to establish its therapeutic applications in veterinary medicine.

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