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ASSESSMENT OF *IN VITRO* ANTIBACTERIAL EFFICACY OF GERANIOL AGAINST MASTITIS PATHOGENS

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ABSTRACT

Antimicrobial resistance (AMR) is a serious global dispute that is threatening both human and animal health in the current millennium. As an outcome, it violates the monetary stability of millions of rural farmers as well as the nation's food security. The foremost predecessor for the formation of drug-resistant superbugs is the acquisition of newer resistant strategies by microorganisms against the conventional antibiotics. In light of the projected rise in drug resistance, essential oils seem to be a feasible candidate for existing antibiotics. Bovine mastitis milk sample was found to contain both gram-negative and gram-positive bacteria. Isolated bacteria were identified by its growth in selective and differential medium as well as with various biochemical tests specific to the pathogen. By using the Kirby-Bauer disc method, its multiple antibacterial resistance was tested. Minimum inhibitory concentration (MIC) and disc diffusion assays were done using geraniol as a test substance and results were compared with nalidixic acid. Inhibition was observed at different concentrations. This provides preliminary evidence that geraniol might be a superior alternative for treating bacteria that are resistant to medications.

Keywords: Essential oil, antimicrobial resistance, Mastitis, Geraniol

INTRODUCTION

Bovine mastitis ranks as the most ubiquitous disease inflicting monetary damage to the dairy business because of drop in milk production and inferior quality of milk. It is characterized by a complicated disease pattern which is governed by the host, the infection, and the environment. Physical damage and microbial infections in the udder tissue leads to the onset of inflammatory signs in mammary gland commonly known as mastitis. It mostly occurs due to the entry of different Gram positive and negative bacterial species. The

main predominant contagious pathogen responsible for the occurrence of mastitis is multiple strains of *Staphylococcus aureus*. Extent of infection may be acute, subacute, chronic or gangrenous form which depends upon the strain (Arya *et al.,* 2022).

Subclinical form of mastitis is more challenging due to constraints in diagnosis, but it is of utmost significance for the herd to be free of the disease state (Atyabi *et al.,* 2006). The most common form of mastitis in dairy herds across the world is subclinical mastitis which characterised by an increase in somatic cells in the milk as well as variations in its physical and chemical composition (Malek dos Reis *et al.,* 2013). Along with huge economic implications, mastitis is correlated with faster development of drug resistant variants worldwide due to its profound zoonotic risk (Pascu *et al.,* 2022). Globally, to combat the intramammary infections, the most routine class of antibiotics prescribed are Beta-lactam groups such as penicillin, cephalosporins, lincosamides, macrolides and aminoglycosides (Molineri *et al.,* 2021). At present, veterinary healthcare and public hygiene lay the significant emphasis on the secure usage of antibiotics by physicians and also the quest for additional curative approaches to mitigate the use of antibiotics and the corresponding antibiotic resistance in dairy products (Molineri *et al.,* 2021).

Essential oils

Essential oils from scented and therapeutic plants have drawn a special concern because of its diverse pharmacological actions including its ability to neutralise and forage the free radicals (De sousa Barros *et al.,* 2015). The key attribute for essential oils and their ingredients is hydrophobicity, which allows them to make a bond with the lipids of bacterial membrane and mitochondria, making them to more porous by disrupting the cell structures (Chouhan *et al.,* 2017). Geraniol, an essential oil chemically it is an aliphatic acyclic monoterpene alcohol which is transparent and exists as liquid state in its natural form. It is abundant in a variety of flowers and has a distinctive flavour and aroma. It has been granted authorization by the US Food and Drug Administration to be used as a flavouring compound in food industry (Bhattamisra *et al.,* 2018).

MATERIALS AND METHODS

Geraniol was purchased from M/s Sigma- Aldrich India Ltd., India. *Staphylococcus aureus* and *Escherichia coli* samples were collected from mastitis milk. Samples were plated on mannitol salt agar and EMB gar to confirm the presence of *E. coli* and *S. aureus* (CLSI, 2008). Gram staining was done to differentiate Gram

positive *S. aureus* and Gram-negative *E. coli*.

Antibiotic sensitivity test

According to the guidelines described by Clinical Laboratory Standards Institute antibiotic sensitivity testing was performed for the bacterial isolates by Kirby-Bauer disc diffusion assay (CLSI, 2020). Multidrug resistance in *S. aureus* and *E. coli* was detected using disc diffusion assay. The antibiotic discs employed in the assay included penicillin (10 units), clindamycin (2mcg), ciprofloxacin (5mcg), ceftriaxone (30 mcg), enrofloxacin (10 mcg), ampicillin (10mcg), co-trimoxazole (25mcg), chloramphenicol (30 mcg), gentamicin (10 mcg), tetracycline (30 mcg), cloxacillin (5mcg), cefotaxime (30mcg) and novobiocin (5 mcg).

A sterile non-toxic cotton swab on a wooden applicator was dipped into the standardized bacterial inoculum. Then streaked over the agar with two to three times in a clockwise direction. Later, antimicrobial discs were placed over the agar by aseptic manner and the streaked plates were incubated at 37°C for 16-18 h. After incubation, zone of inhibition was measured using scale and interpreted as either susceptible or resistant to the exposed antimicrobial disc according to CLSI criteria. Multiple antibiotics resistant to the bacterial isolates were assessed and Multiple antibiotic resistance (MAR) index was calculated (Sandhu *et al.,* 2016). The MAR was calculated using the formula as a/b a= Number of antibiotics resistant to isolates; b= number of antibiotics susceptible to isolates.

Minimum inhibitory concentration

MIC of geraniol and nalidixic acid were carried out by resazurin based microtiter plate assay as per CLSI (2020) and Elshik *et al*. (2016). Ninety-six well plate with CAMHB medium, test compound and inoculum were incubated at 37°C for 24 h. Three controls were performed like growth control with bacterial suspension, sterility control with CAMHB alone and vehicle control with CAMHB and vehicle used for dilution. After incubation, 30 µl of 0.015 percent resazurin were added to each of the wells and further incubated at 37°C for 2-4 h. The MIC breakpoint was assessed visually after incubation. The well with the lowest concentration of test substance and antibiotics with no colour change from blue to pink was taken as the MIC value and the well with colour change from blue to pink indicated the presence of viable bacterial cells (Elshik *et al.,* 2016).

Disc diffusion assay

The antibacterial activity of geraniol was evaluated by employing the Kirby-Bauer disc diffusion assay.

Various concentrations of geraniol were impregnated into sterile discs. The plates were incubated at 37°C for 24h and the zone of inhibition was measured.

Checkerboard assay for synergism

Checkerboard assay was done for determining the antibacterial interactions between test compound and antibiotics. Combination used in the study was nalidixic acid and geraniol. It was performed using resazurin based microtiter plate assay (Bhattamisra *et al.,* 2018). The two substances to be assayed were added to a 96-well microtiter plate to get two-fold dilutions in the vertical and horizontal direction respectively. The range of substances was chosen based on their MIC values. Column 1-10 was dispensed with antibiotic and row from A-G was received with geraniol concentration. Each well in 96-well plate was added with 50µl of CAMHB except 12th column. 50 µl of antibiotic was added to the first column of the plate. The two-fold serial dilution of geraniol was done in sterile tubes with 2 MIC as starting value and 50 µl was added to respective wells in 96-well plate vertically. 100 µl of bacterial suspension was added to each well. The final concentration of antibiotic in the wells were from 2 MIC to 1/128 MIC and geraniol from 2 MIC TO 1/16 MIC.

$$\text{FICA/B} = \frac{\text{MIC of drug A/B in combination}}{\text{MIC of drug alone}}$$

RESULTS

S. aureus fermented mannitol in mannitol salt agar with yellow colonies. *E. coli* was cultured and streaked on EMB agar which formed characteristic greenish metallic colonies. Both *S. aureus* and *E. coli* were stained with Gram staining. *S. aureus* was identified as Gram-positive cocci with bunch of grapes appearance and *E. coli* with appearance of Gram-negative bacilli.

Detection of multidrug resistance in *S. aureus* and *E. coli*

S. aureus and *E. coli* were tested with eight varying groups of antibiotics (penicillin, cephalosporins,

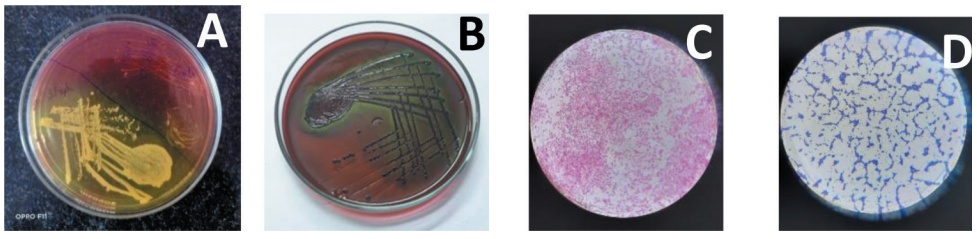

Figure 1. (A) Yellow colonies on mannitol salt agar (B) Greenish metallic sheen colonies on EMB agar (C) Gram-negative bacilli (E. c*oli*) and (D) Gram- positive cocci (*S. aureus*)

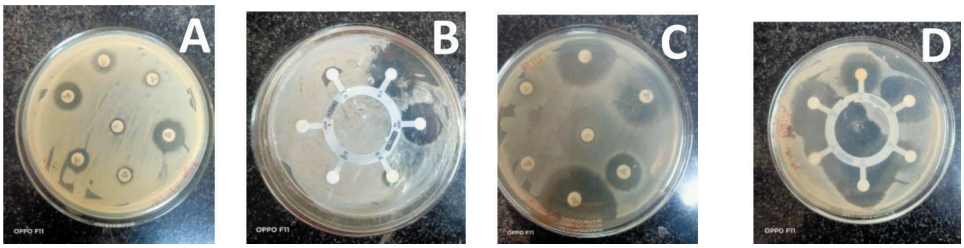

Figure 2. (A) and (B) Drug resistant pattern of *S. aureus* (C) and (D) Drug resistant pattern of *E. coli*.

aminoglycosides, tetracyclines, lincosamides, sulphonmaides, amphenicols and quinolones) for assessing the multidrug resistance pattern. Both of the isolates were multidrug resistant with *S. aureus* having MAR index of 0.53 and *E. coli* with MAR index of 0.23. *S. aureus* showed resistance towards penicillin, ciprofloxacin, ceftriaxone, cloxacillin, cefotaxime and co-trimoxazole. *E. coli* showed resistance against antibiotics such as novobiocin, clindamycin and penicillin.

Sensitivity of *S. aureus* and *E. coli* against geraniol

Different concentrations of geraniol viz. 1mg/ml, 2mg/ml, 4mg/ml, 8mg/ml, 16 mg/ml and 32mg/ml were used against *S. aureus* and *E. coli*. *S. aureus* produced no zone of inhibition at 1,2, and 4mg/ml. *E. coli* produced zone of inhibition with a range of 15mm and 20mm at 8mg/ml, 16mg/ml and 32mg/ml.

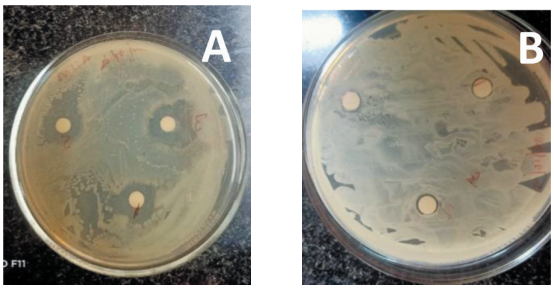

Figure 3. (A) Antibiogram of *E. coli* against different concentrations of geraniol (B) Antibiogram of *S. aureus* against different concentrations of geraniol

Determination of MIC

The MIC value of geraniol against *S. aureus* was in the range of 2-32 mg/ml whereas that *E. coli* it ranged from 16-32 mg/ml. Minimum inhibitory concentration value of nalidixic acid against *S. aureus* were found to be from 4-8mg/ml and for *E. coli* it was in the range of 0.5-1mg/ml.

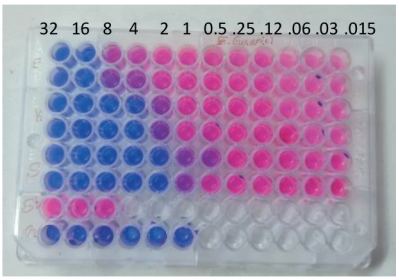

Figure 4. MIC value of geraniol against *E. coli* and *S. aureus*

Checkerboard assay for synergism

Resazurin based

checkerboard broth microdilution assay was done to assess the activity of geraniol with antibiotic, nalidixic acid

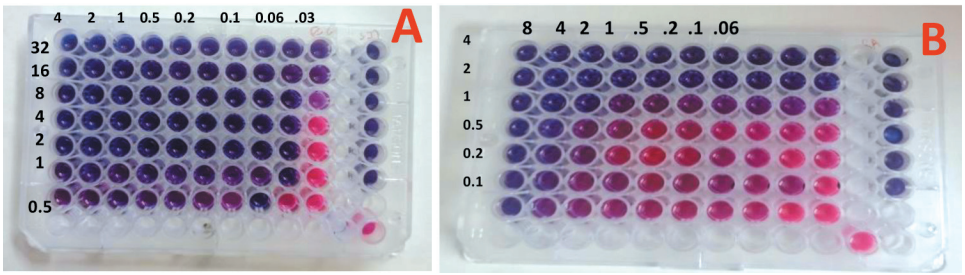

Figure 5. Checkerboard assay (A) Combination of nalidixic acid with geraniol against *E. coli* (B) Combination of nalidixic acid with geraniol against *S. aureus*

in different combinations. Fractional inhibitory concentration index (FICI) values were calculated. MIC of nalidixic acid with geraniol against *E. coli* ranged from 0.03 mg/ml to 2mg/ml and 4mg/ml to 8 mg/ml against *S. aureus*. Fractional inhibitory concentration index value was determined as 0.25 indicating that the synergistic combination existed between geraniol and nalidixic acid for *E. coli* whereas for *S. aureus* FICI showed a value of 1 which is suggestive of additive action between geraniol and nalidixic acid.

DISCUSSION

Miladinovic *et al.* (2014) reported that savory oil with its main constituent as geraniol showed synergistic combination with antibiotics like chloramphenicol and tetracycline and also exhibited significant reduction in MIC values against Gram-negative bacteria.There is a notion that geraniol interrupts the bacterial function by its contact with bacterial cell membrane and thereby it exerts its antibacterial effect(Rasoul *et al.,* 2012). Bibiana *et al.* (2012) found that geraniol exhibited maximum zone of inhibition in the disc diffusion assay against both Gram-positive and Gram negative bacteria such as *E. coli*, *S. aureus, Streptococcus pyogenes, Klebsiella pneumoniae, Bacillus subtilis, Proteus vulgaris, Vibrio cholerae, Streptococcus fecalis, Citrobacter* and *Vibrio parahemolyticus*. Coutinho *et al.* (2015) found that geraniol increased the action of aminoglycoside antiotic, kanamycin against MRSA strain *Staphylococcus aureus* 358. Based on the study conducted by Kannappan *et al.* (2019) revealed that combination of geraniol and cefotaxime averts the potential of *S. aureus* to form biofilm. Hence under *in vivo* conditions, it boosts the immune system to unload the pathogen from an individual.

CONCLUSION

Surge in bacterial resistance to antibiotics and the shortage of new drug in the market led to seeking of novel strategies to treat ailments driven by drug resistant bacteria. Geraniol exhibited

substantial efficacy against drug-resistant Gram-negative bacteria and Gram-positive bacteria when combined with nalidixic acid respectively. Synergistic and additive action of geraniol against *E. coli* and *S. aureus* might be presumably due to its robust activity against efflux pump. Synergistic property is evidenced by upsurge in the activity when two substances are coupled together compared to that of individual agent alone. Minimization of standard antibiotic dose will be worthwhile in the therapeutic setup to stamp out the adverse effects and in the mitigation of antimicrobial resistance. The bursting instances of acquiring drug resistance by the pathogens are the formidable menace to the global health sector.

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