RESEARCH ARTICLE Asian Journal of Dairy and Food Research, Volume 45 Issue 1: 36-45 (February 2026)

Antimicrobial and Antibiofilm Activity of Cinnamon Oil on *Staphylococcus aureus* Isolated from Cattle Milk with Mastitis

Durgesh Mishra[1], Arpita Shrivastav[1], Neeraj Shrivastava[2], Rajeev Ranjan[1], Nitesh Kumar[1], Swatantra K. Singh[1], Yogesh Chatur[3], Ankush Kiran Niranjan[2], Namrata Upadhyay[1]

10.18805/ajdfr.DR-2266

ABSTRACT

Background: Dairy sector is a leading industry in our country. Mastitis being most concerned and frightful disease, not only affecting health of animals but also the economy of the livestock owner. Prevailing status of the antimicrobial resistance makes the treatment of mastitis much more difficult. Biofilm formation in mastitis milk could place difficulty in the treatment of animals as it further increases the possibility of antimicrobial resistance. Essential oils generally being less toxic, could be optimal alternatives to conventional antibiotics, most of these essential oils higher MIC values than antibiotics, needed to be given in increased concentration in feed/water.

Methods: Different dairy farms were screened for mastitis (164 dairy cattle and buffalo) *Staphylococcus aureus* isolates were identified and characterized phenotypically by standard biochemical tests. The biofilm formation ability was studied by different methods along with the quantitative analysis and MIC of standard drug cefoperazone.

Result: Out of total milk samples 17 samples were identified *Staphylococcus aureus* positive with Congo red agar (70% positive), Light Microscopy method (52% positive) and Microtiter plate (Crystal Violet) method. (100% sensitivity). The antimicrobial and antibiofilm effect of Cinnamon oil were studied by Microtiter plate method, SEM analysis and confocal techniques. Cinnamon oil gave antibiofilm activity in all the concentration used (1%, 2%, 3%). The MIC of Cefoperazone was in the range of 64 µg/to 16 µg/ml. No significant changes were observed on biofilm production at sub-MIC of cefoperazone.

Key words: Antibiofilm, Cefoperazone, Cinnamon oil, CLSM, Mastitis, Microtiter plate assay, Milk.

INTRODUCTION

Bovine mastitis is one of the leading disease of animals, negatively affecting economy and welfare of dairy industry. Treatment failures are increasing antibiotic use, as some cases do not respond to standard therapies (Pedersen *et al*., 2021). *Staphylococcus aureus* due to its pathogenicity and ability to form biofilm is the most imperative and widespread environmental pathogen which spread through other contaminants, apart from contaminated milk of mastitis cows (Vargová *et al*., 2023). Biofilm are the communities of bacteria attached to a surface with a slime layer. It protects bacteria against treatments and help in persistence of bacterial infections (Pizauro *et al.,* 2021). Plant essential oils (EOs) with their antibacterial, antiviral, antifungal and insecticidal agents could be a better alternative to antibiotic therapy against multidrug-resistant bacteria (Razni *et al*., 2019; Caneschi *et al.,* 2023; Shrivastava *et al*., 2020). Cinnamaldehyde present in Cinnamon oil increase membrane permeability and compromise membrane integrity, leading to the removal of preformed biofilms and significantly reducing biofilm formation (74.7 and 69.6%, respectively) (Budri *et al.,* 2015; Ganic *et al*., 2022; Didehdar *et al*., 2022; Mohini and Alexander, 2024). Present study focuses on the use of essential oils with test drug cefoperazone, against biofilm formation.

MATERIALS AND METHODS

A total of 164 bovines (cow = 113 and Buffalo = 51) from dairy farms were screened in and around Rewa (M.P., India) for mastitis by California Mastitis test (CMT) and clinical mastitis (CM) as abnormal milk, abnormal udder and/or abnormal cow (Constable *et al*., 2017; Rust *et al*., 2023). Collected milk samples (300 µl) was inoculated in 30 ml of Mueller-Hinton broth supplemented with 6.5% NaCl, incubated at 35°C for 16-20 hours. Work was conducted after prior permission of IAEC (No71/IAEC/Vety/Rewa/6.4. 2022).

[1]Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, Rewa, Kuthuliya-486 550, Madhya Pradesh, India.
[2]Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Rewa, Kuthuliya-486 550, Madhya Pradesh, India.
[3]Department of Public Health and Epidemiology, College of Veterinary Science and Animal Husbandry, Rewa, Kuthuliya-486 550, Madhya Pradesh, India.

Corresponding Author: Arpita Shrivastav, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, Rewa, Kuthuliya-486 550, Madhya Pradesh, India. Email: Vetrewa12@gmail.com
ORCHID: 0000-0002-2711-2243

How to cite this article: Mishra, D., Shrivastav, A., Shrivastava, N., Ranjan, R., Kumar, N., Singh, S.K., Chatur, Y., Niranjan, A.K. and Upadhyay, N. (2026). Antimicrobial and Antibiofilm Activity of Cinnamon Oil on *Staphylococcus aureus* Isolated from Cattle Milk with Mastitis. *Asian Journal of Dairy and Food Research*. **45(1):** 36-45. doi: 10.18805/ajdfr.DR-2266.

Submitted: 10-08-2024 **Accepted:** 21-11-2024 **Online:** 30-12-2024

One loopful of pre-enriched culture was streaked into Mannitol Salt agar plates (35°C for 16-20 h) and thereafter in Baird-Parker agar plate supplemented with egg yolk-tellurite (at 35°C for 18-24 h). Colony was streaked onto a Tryptone soy agar plate (HI Media) incubated at 35°C for 18-24 hour to confirm *Staphylococcus aureus*. The colonies were checked for purity by Gram's staining for gram's reaction, cellular morphology. Later *Staphylococcus aureus* was phenotypically characterized by standard biochemical tests, Novobiocin and Polymyxin B susceptibility (EFSA 2012). Biofilm producing ability of *Staphylococcus aureus* was phenotypically characterized by following methods.

Congo red agar method

The Congo red Agar was prepared as per the earlier method with slight modifications (Kaiser *et al*., 2013; Raksha *et al*., 2020).

Light microscopy method

2 ml of overnight bacterial culture in tryptone soy broth (TSB) supplemented with 0.25% glucose (TSB-glucose). diluted 1:40 in TSB-glucose, was used per petri plate (30 mm) containing a cover slip. Incubated at 37°C for 18 h, gentle washing thrice with 1-2 ml of sterile phosphate-buffered saline (PBS), air dried and stained with 0.1% crystal violet for 10 minutes. Cover slips were put on a clean oil free glass slide and was observed under 40× of light microscope (Prabu and Umamaheswari, 2020).

Microtiter plate assay method

Overnight bacterial culture was diluted 1:40 in tryptone soy broth (TSB) supplemented with 0.25% glucose (TSB-glucose), 200 µl of this cell suspension was inoculated in sterile, 96-well microtiter plates. After incubation at 37°C for 18 h, wells were gently washed thrice with 200 µl of sterile phosphate-buffered saline (PBS), air dried and stained with 0.1% crystal violet for 10 minutes. The biofilms were washed with distilled water, dried at 37°C for 15 min and destained with 200 µl of the destaining solution [50% (v/v) ethanol, 50% (v/v) glacial acetic acid], The amount of released stain was quantified by measuring the absorbance at 490 nm (A490) with a micro plate reader (Campo-Pérez *et al*., 2023). For scanning electron microscope (SEM) analysis, Glass slides were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2-12 hours at 4°C followed by gradual dehydration (by 70, 85, 90 and 100% ethanol). The samples were air-dried, sputter-coated with colloidal gold and observed under an EVO18 scanning electron microscope at an operating voltage of 15 kV. For confocal microscope analysis, Glass slides having biofilm were fixed by absolute methanol for 2 minutes, air-dried and further subjected to staining with 0.1% acridine orange stain (dissolved in PBS) for 5 minutes. Finally, stained slides were examined with a confocal laser scanning microscope (CLSM) (Fig 1, 3).

The antimicrobial and anti-biofilm effect of cinnamon oil on biofilm producing *Staphylococcus aureus*

Commercially available (100% pure) Cinnamon oil were used after dilution in the DMSO by drop method for the study in the concentration range of 1%, 2% and 3%. Phytochemical analysis of cinnamon was done for saponins, flavonoid, steroids/terpenes, Tannins, alkaloids, Resins, Phenols and glycosides (Effiong *et al.,* 2020).

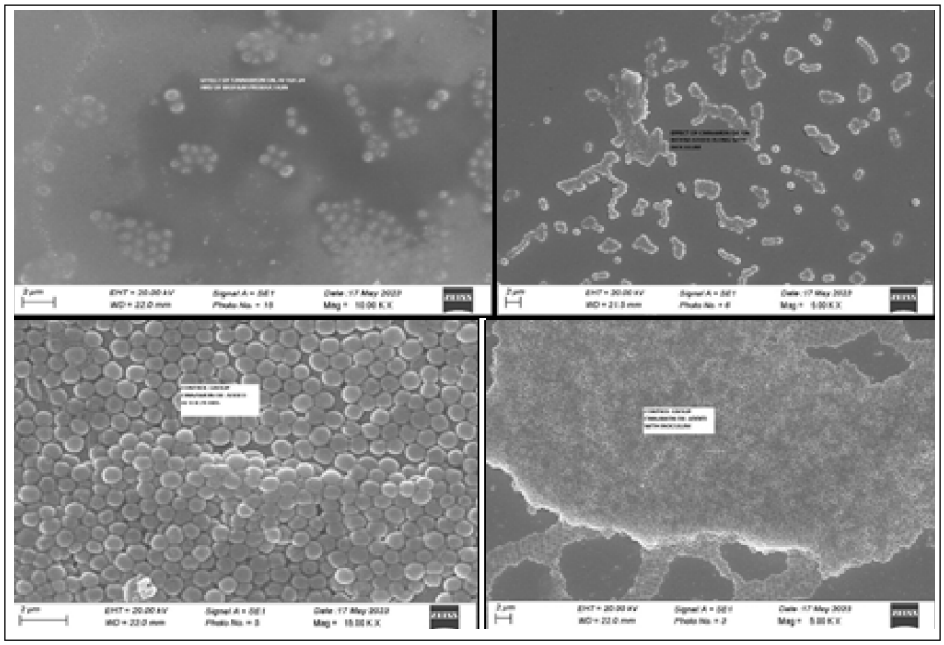


Fig 1: SEM analysis of control group and effect of cinnamon oil added with inoculum and after 24 hrs. Images recorded at 10kX, 5kX, 15kx magnification.

Quantitative analysis of essential oil by gas chromatography

Phytochemical analysis was performed through GCMS-QP2010 Plus equipment (outsourced from JNU New Delhi). Experimental Conditions were Ion Source Temp: 220.00°C; Interface Temp: 270.00°C; Solvent Cut Time: 2.50 min;Detector Gain Mode: Relative; Detector Gain: 0.00 kV; Threshold: 100; Column Oven Temp 50.0°C; Injection Temp -260.00°C; Injection Mode-Split Flow; Control Mode-Linear; Velocity Pressure-69.0 kPa Total Flow-137.3 mL/min; Column Flow-1.21 mL/min; Linear Velocity 39.9 cm/sec; Purge Flow-3.0 mL/min; Split Ratio-110.0Sample Inlet Unit: GC (Effiong *et al*., 2020).

Antibiofilm effect of cinnamon oil *by* Microtiter plate assay method

Fresh overnight bacterial culture (adjusted to 0.5 McFarland standards) (20 µl) was inoculated in each well with, cinnamon oil (1%, 2% and 3%) Cefoperazone (5 µgm/ml) 200 µl per well and MHB, DMSO used as positive and negative controls, respectively plate incubated at 37°C for 18 h without any shaking. Stained with 1% crystal violet, washed, destained and absorbance recorded at an optical density of 490 nm. The percentage inhibition was calculated as (Sarah *et al.,* 2023).

$$\text{OD of negative control-} \frac{\text{OD of experimental data}}{\text{OD of negative control}} \times 100$$

Determination of minimum inhibitory concentration of Cefoperazone by Resazurin-based 96-well plate micro dilution method

The inoculums were prepared in accordance with the CLSI recommendation (as per 0.5 McFarland standard). diluted by 1:100 in MHB broth. Dilutions of the antibiotic prepared with the CLSI recommendation in the range of 256 µg/ml to 0.25 µg/ml (Peng *et al*.,2022). 100 µl, added in each column leaving column 11 and 12 for positive and negative control. 100 µl of the adjusted (0.5 McFarland standard) bacterial suspension (diluted by 1:100 in MHB) was added to each well. In each column 17 different biofilm isolates were added in triplicates In column 11 only MH broth and in column 12 MH broth with bacterial inoculums was added as negative and positive control respectively. After incubation for 24 h at 37°C, resazurin (0.015%, 30 µl per well) was added), colour was observed after incubation of 30 minutes. Columns with no colour change (blue colour) were scored as above the MIC value (Elsheikh *et al*., 2016).

MIC of cinnamon oil by Resazurin-based 96-well plate micro dilution method

After dilution of cinnamon oil in the concentration range of 6%,5%,4%,3%,2%,1%,0.5%,0.4%. in the MH broth with DMSO as emulsifier MIC was performed like antibiotic. Color change was observed with resazurin (0.015%,), columns with no colour change were scored as above the MIC value.

Determination of sub-minimum inhibitory concentrations (sub-MICs) of antibiotics and its effect on biofilm formation

The MIC and Sub-MIC for the tested cefoperazone were determined, as per the standards of CLSI. Culture diluted to 1:100. Cefoperazone diluted in the range from 1/2,1/4 and 1/8 µg/mL of the MIC values of cefoperazone determined previously. *Staphylococcus aureus* was sub-cultured on Mueller Hinton, all wells in the first antibiotic-broth plate were dispensed with 50 µL of culture, while the wells of the second antibiotic-broth plate were inoculated with 50 µL of free culture. The same procedure was repeated to the antibiotic-free broth plates as control. After incubation for 72 hrs 30 µL of 0.1% crystal violet was added to each well and incubated at room temperature for 30 min. The bound bacteria were quantified by addition of ethanol 70% and measurement of the dissolved crystal violet at optical density (OD) of 630 nm using 96-flat wells

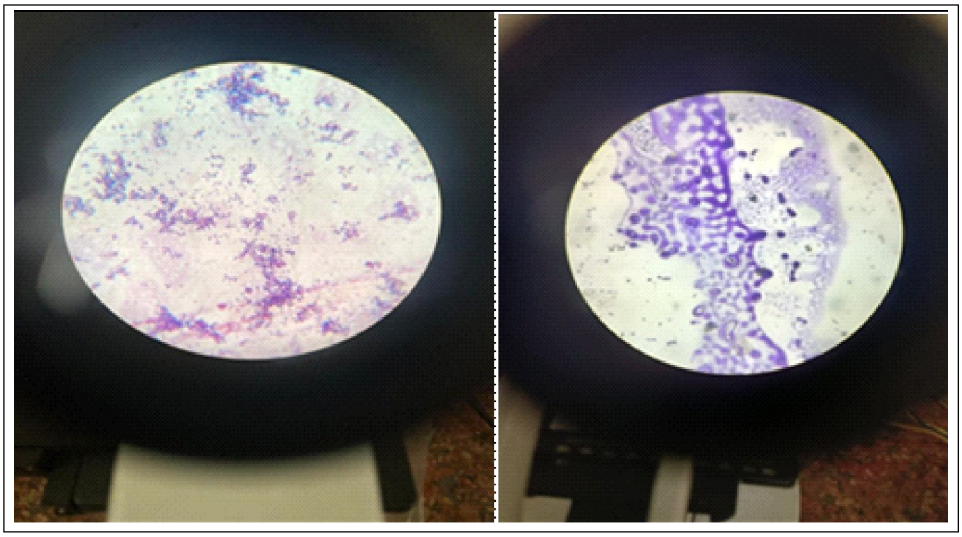


Fig 2: Biofilm under light microscopy 40x magnification.

microtiter plate The effect of sub -MIC level of antibiotic on the biofilm was estimated.

RESULTS AND DISCUSSION

Phenotypic screening of *staphylococcus aureus* for biofilm production

Out of 164 bovines (cow = 113 and Buffalo = 51) screened for mastitis by California Mastitis test (CMT), 24 samples were found to be mastitis positive and 17 characterized as *Staphylococcus aureus.* Among the 17 *Staphylococcus aureus* samples 70% (n=12) were positive for biofilm formation on Congo red agar, where as 52% samples (n=8) were positive for biofilm on light microscopy (Fig 2). All the 17 samples showed biofilm production through micro titer plate method with almost 90% sensitivity. Subclinical mastitis is of concern in veterinary hospitals because contagious mastitis pathogens might be unknowingly transmitted to susceptible cows and then back to their farm of origin. Crystal violet staining binds to negatively charged molecules of bacteria and exopolysaccharides and is therefore used to quantify the total biomass of these systems (Fig 4). Multiple studies observed that majorities of *Staphylococcus aureus* isolate from bovine mastitis cases can be studied *in vitro* by this assay (Bonsaglia *et al*., 2020). Congo red agar method giving very little correlation with the other methods with specificity (92%) and very low accuracy (41%), some reports do not recommend the Congo red agar method for biofilm detection in their study (Othmany *et al.,* 2021). In a study undertaken previously (Sandasi *et al*., 2010) isolates showing biofilm formation by micro titer plate or tissue culture plate was 70 (64.7%), non or weak biofilm producers were 40 (36.3%) (Table 1). Regional data from India also showed that out of 152 isolates tested, the number of biofilm producers identified by Micro titer plate method was 53.9 % and non-biofilm producers were 46%.

Table 1: Phenotypic screening of *Staphylococcus aureus* for biofilm by microtiter plate assay.

Sample	O.D. values at 492 nm wavelength	
	Mean± SE	Result
K3C	0.408±0.065	SBFP
R1C	0.373±0.023	SBFP
R2B	0.196±0.017	SBFP
A1C	0.709±0.042	WBFP
I1B	0.564±0.022	WBFP
RV1C	1.229±0.136	WBFP
S1C	0.540±0.024	WBFP
JB1C	0.872±0.143	WBFP
U1C	0.461±0.036	WBFP
U2C	0.507±0.025	WBFP
U3C	0.457±0.039	WBFP
V1C	0.439±0.050	WBFP
RL2C	0.408±0.037	WBFP
ST1C	0.264±0.017	SBFP
SK1C	0.309±0.025	WBFP
KT1C	0.486±0.037	WBFP
BP1C	0.432±0.035	WBFP
Per cent Sensitivity	90%	

WBFP- Weak biofilm producer; SBFP- Strong biofilm producer Based on the OD values: OD≤OD control = Non-biofilm producers; OD control<OD≤2×OD control = Weak biofilm producer 2×OD control<OD≤4×ODcontrol = Moderate biofilm producers; 4×OD control<OD = Strong biofilm producers.

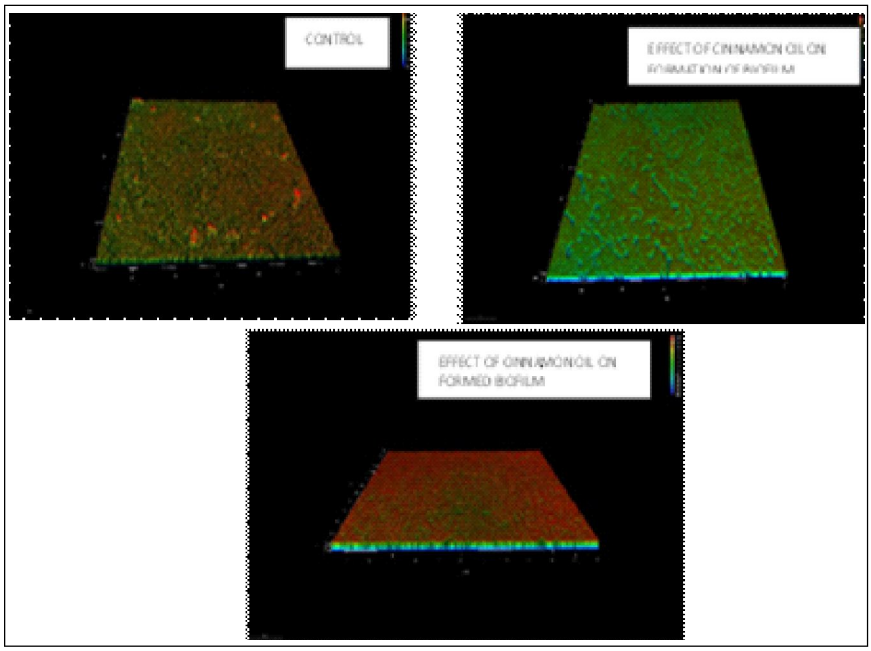


Fig 3: CLSM analysis of biofilm and cinnamon oil. Images recorded as 3 D view.

GC-MS analysis of cinnamon oil

Phytochemical screening of the Cinnamon oil was undertaken through GC-MS analysis led to the identification of a number of compounds. The various components present in the cinnamon oil Pinene alpha, BICYCLO [2.2.1] HEPTANE, 2, 2-DIMETHYL-3-METHYLENE- BENZALDEHYDE, Pinene beta- Phellandrene alpha-Terpinene alpha-Cymene para-, Phellandrene beta, CYCLOHEXENE, 1-METHYL-4-(1-ETHYLETHYLIDENE)- Linalool, ACETIC ACID, PHENYLMETHYL ESTER , Terpinen-4-ol, OCTADIEN-3-OL, DIMETHYL-, PROPANOATE, 3-Phenylacrylaldehyde ,Cinnamaldehyde, 2-PROPENAL, 2-METHYL-3-PHENYL- Hexa-2,4-dienylbenzene, Eugenol, Copaene alpha-Benzene, 1,3-hexadienyl- Caryophyllene, 2-Propen-1-ol, 3-phenyl-, acetate, (E)- Humulene alpha-, PHENOL, 2-METHOXY-4-(2-PROPENYL)-, ACETATE, Caryophyllene oxide Tetramethyl-12-oxabicyclo [9.1.0]dodeca-3,7-diene BENZOIC ACID, PHENYLMETHYL ESTER, .alpha.-Phellandrene, dimer Myrac aldehyde (Fig 5). Phytochemical screening of the cinnamon oil showed presence of Saponins Alkaloids Steroids/ Terpenes Tannins Alkaloids Resins in cinnamon oil phenols and Flavonoids were absent in cinnamon oil. Twenty-nine compounds were identified (Table 2, Fig 5). Cinnamaldehyde was identified as the main compound with the highest peak area percentage of 70.61%, Eugenol was the second major compound detected with area % of 17.54%. (1.21%). Ainane *et al*. (2019) showed that the main component of the essential oil of cinchona bark was cinnamaldehyde (89.31%) and (69.15%) respectively which were similar to our results. Tsukatani *et al*. (2020) also suggested inhibitory activities in 498 different plant foodstuffs on biofilm formation of *Streptococcus mutans* by micro titer plate assay and concluded five of the extracts with potent inhibitory activities which further correlates with our finding. The antibacterial activity of Cinnamomum cassia (cinnamon) essential oil (EO) alone and in combination with some classical antibiotics against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa as* reported earlier further correlates with our finding of antibiofilm and antimicrobial activity of essential oil (Atki *et al*., 2021). The bioactive components of essential oils supposed to be affecting the integrity of cell membranes by altering membrane permeability, resulting in leakage of electrolytes and loss of important intracellular contents (Kegang *et al*., 2023).

Table 2: Compounds identified in cinnamon oil with its peak report.

Peak#	R.Time	Area	Area%	Name
1	6.928	10270528	0.45	Pinene <alpha->
2	7.486	3402657	0.15	BICYCLO[2.2.1]HEPTANE, 2,2-DIMETHYL-3-METHYLENE-
3	7.999	2267636	0.10	BENZALDEHYDE
4	8.511	3638473	0.16	Pinene <beta->
5	9.669	9937373	0.44	Phellandrene <alpha->
6	10.107	1079526	0.05	Terpinene <alpha->
7	10.442	8492705	0.37	Cymene <para->
8	10.665	9958799	0.44	Phellandrene <beta->
9	13.058	729675	0.03	CYCLOHEXENE, 1-METHYL-4-(1-METHYLETHYLIDENE)-
10	13.877	27619433	1.21	Linalool
11	16.605	966943	0.04	ACETIC ACID, PHENYLMETHYL ESTER
12	17.372	938906	0.04	Terpinen-4-ol
13	18.097	3919070	0.17	1,6-OCTADIEN-3-OL, 3,7-DIMETHYL-, PROPANOATE
14	19.169	23704754	1.04	(Z)-3-Phenylacrylaldehyde
15	23.377	1607549154	70.61	Cinnamaldehyde <(E)->
16	24.330	755450	0.03	2-PROPENAL, 2-METHYL-3-PHENYL-
17	24.812	3292661	0.14	Hexa-2,4-dienylbenzene
18	25.946	399291804	17.54	Eugenol
19	26.073	6656824	0.29	Copaene <alpha->
20	26.740	3183859	0.14	Benzene, 1,3-hexadienyl-
21	27.864	39296970	1.73	Caryophyllene <(E)->
22	29.164	14867437	0.65	2-Propen-1-ol, 3-phenyl-, acetate, (E)-
23	29.274	5754475	0.25	Humulene <alpha->
24	31.964	32092278	1.41	PHENOL, 2-METHOXY-4-(2-PROPENYL)-, ACETATE
25	34.288	9955350	0.44	Caryophyllene oxide
26	35.332	1343187	0.06	(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene
27	41.157	42948076	1.89	BENZOIC ACID, PHENYLMETHYL ESTER
28	48.279	1071401	0.05	.alpha.-Phellandrene, dimer
29	52.300	1606955	0.07	Myrac aldehyde
		2276592359	100.00	

Antibiofilm activity by micro titer plate assay method

Antibiofilm activity of essential oils by micro titer plate assay method was measured using the dye crystal violet (CV) at 490 nm wavelength. (In triplicates) (Mean±S.E.). Maximum inhibition of cinnamon oil was observed at 3% concentration with O.D values ranging from 0.021±0.005 to 0.084±0.007 with per cent inhibition of 96% to 86% respectively. In 2% and 1% concentration per cent inhibition was 16 to 20%. (Table 3,4, Fig 6).

***In situ* visualization of *S. aureus* biofilm**

At the 5,10,15 KX magnification, SEM and confocal images of the *S. aureus* control group and the cinnamon oil treatment group at MIC were shown (Fig 1, 3). The treated group had reduced biofilm, slime production and dismantling of cell layers on one another. As the microorganisms grew in the media supplemented with glucose, the biofilm production and growth rate were higher at MBC compared to the growth when MIC was determined. This reduction in the biofilm thickness and dismantling of the cell indicates the cinnamon oil inhibitory effect.

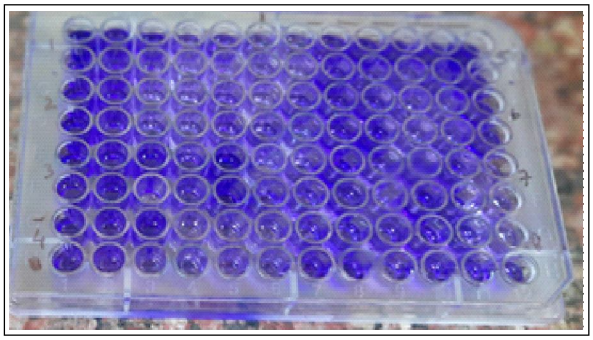


Fig 4: Phenotypic screening for biofilm production by microtiter plat sassy.

Determination of MIC of cefoperazone and essential oil against biofilm positive isolate

MIC of cefoperazone was in the range of 32 µg/ml to 8 µg/ml Minimal inhibitory concentration (MIC) states *in vitro* planes of susceptibility or resistance of explicit bacterial strains to applied antibiotic (Table 5) Cinnamon oil showed MIC of 0.5% in almost all the samples. Dependable assessment of MIC has a significant effect on the optimal of a therapeutic strategy, which marks efficiency of an infection therapy. In view of our observations, Sader *et al.* (2020) reported MIC breakpoints of Cefoperazone sulbactam combination in the commercial product to be 16 µg/ml for susceptible organisms and 64 µg/ml for resistant organisms. Earlier studies showed MIC in the range of 32 µg/ml to 2 µg/ml which correlate with our findings. Comparison of MICs in different antibiotics is based on how far the MIC is as of the breakpoint, the site of the infection and other contemplations, such as the age, species and health of the animal (Millezi *et al.,* 2019).

A positive correlation between cinnamon oil and cefoperazone MICs, was observed may be associated withantimicrobial resistance in some cases. In particular, increased resistance to multiple antibiotics was reflected in increased susceptibility to cinnamon oil. Although highly hypothetical but this suggests that acquired resistancemay decrease the probability of the bacterium to counteract

Table 3: Antibiofilm activity of cinnamon oil by microtiter plate assay.

S.no.	Sample	O.D. Values (Mean of triplicates) Mean±SE		
Concentration of essential oil		1%	2%	3%
1.	K3C	0.312^{a}±0.002	0.220^{b}±0.007	0.064^{c}±0.012
2.	I1B	0.273^{a}±0.013	0.156^{b}±0.022	0.084^{b}±0.007
3.	R1C	0.308^{a}±0.002	0.208^{b}±0.006	0.047^{c}±0.009
4.	RV1C	0.306^{a}±0.005	0.280^{a}±0.164	0.049^{b}±0.005
5.	R2B	0.214^{a}±0.010	0.116^{b}±0.006	0.061^{b}±0.013
6.	SlC	0.311^{a}±0.051	0.112^{b}±0.008	0.063^{b}±0.010
7.	A1C	0.195^{a}±0.050	0.089^{b}±0.021	0.064^{c}±0.017
8.	JB1C	0.203^{a}±0.017	0.164^{a}±0.024	0.055^{b}±0.019
9.	U1C	0.210^{a} ±0.053	0.150^{b} ±0.033	0.055^{b} ±0.003
10.	U2C	0.239^{a}±0.038	0.183^{b}±0.005	0.049^{c}±0.003
11.	ST1C	0.207^{a}±0.061	0.115^{a} ±0.005	0.029^{b} ± 0.008
12.	U3C	0.216^{a}±0.014	0.164^{a} ±0.025	0.037^{b} ± 0.005
13.	SK1C	0.160^{a}±0.010	0.106^{b} ±0.004	0.023^{c} ± 0.007
14.	V1C	0.174^{a}±0.031	0.092^{b}±0.018	0.027^{c}±0.007
15.	KT1C	0.265^{a}±0.021	0.154^{b} ±0.022	0.021^{c} ± 0.005
16.	RL2C	0.223^{a}±0.024	0.124^{b} ±0.006	0.025^{c} ±0.011
17.	BP1C	0.218^{a}±0.015	0.141^{b}±0.032	0.035^{c} ±0.013
CEFOPERAZONE	0.012± 0.002	Control	0.642± 0.004	0.683 ± 0.003

Mean with different superscript differ significantly ($p<0.5$).

the activity of this essential oil (Jadhav *et al*., 2018; Van *et al.,* 2022). The sub- MIC effect bacterial virulence factor expression and rational use of antimicrobials in clinical practice. No effect of sub-MIC of cefoperazone was observed by us on the biofilm production differing with the earlier findings where sub-MICs of most antibiotics inhibit the development of *S. aureus* biofilm, oxacillin, cefazoline, mupirocin and rifampicin even promoted staphylococcal biofilm formation. Bernardi *et al.* (2021); Chen *et al.* (2021) reported the increase in the ability to form biofilms over a

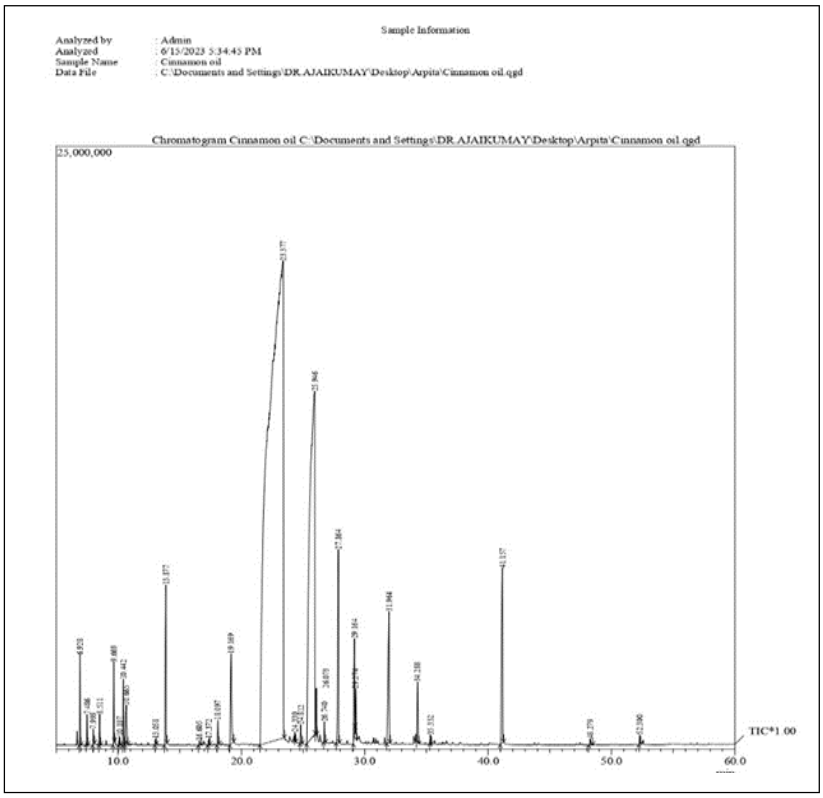


Fig 5: Chromatogram of cinnamon oil.

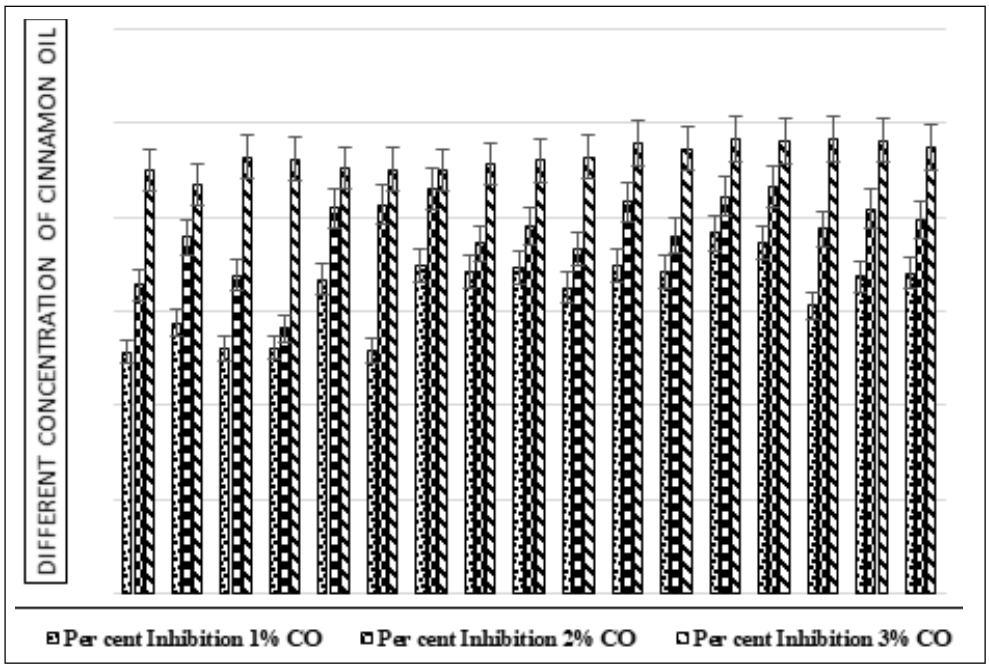


Fig 6: Per cent inhibition of cinnamon oil.

Table 4: Per cent inhibition of cinnamon oil on positive isolates.

Sample no.	Concentration of cinnamon oil (CO)		
	1% CO	2% CO	3% CO
K3C	51	66	90
I1B	57	76	87
R1C	52	68	93
RV1C	52	56	92
R2B1	67	82	90
SlC	52	83	90
A1C	70	86	90
JB1C	68	75	91
U1C	69	78	92
U2C	65	73	93
ST1C	70	83	96
U3C	68	76	95
SK1C	77	84	97
V1C	75	86	96
KT1C	61	77	97
RL2C	67	82	96
BP1C	68	79	95

Table 5: Minimum inhibitory concentration of Cefoperazone (µgm/ml).

Sample	MIC (µgm/ml)
K3C	64
R1C	32
R2B	64
A1C	32
I1B	64
RV1C	32
S1C	16
JB1C	16
U1C	32
U2C	32
U3C	32
V1C	64
RL2C	64
ST1C	32
SK1C	32
KT1C	64
BP1C	64
7 samples	64 µgm/ml
8 samples	32 µgm/ml
2 samples	16 µgm/ml

concentration range from 1/2 to 1/64 of the MIC concentration. Efficacy of bacteriophage was also evaluated against Staphylococcus in mastitis (Srujana *et al*., 2022).

CONCLUSION

On the basis of our findings, we can conclude that presence of biofilm producing organism is very detrimental for proper treatment of Mastitis. Sensitivity of Congo red agar method, Light microscopy method is lower than Microtiter plate method for detection of biofilm production. Cinnamon oil showed antimicrobial and antibiofilm producing ability in all the concentration used (1%, 2%, 3%) Cefoperazone could be used in the biofilm producing *Staphylococcus aureus* causing mastitis in bovines but reduced concentration of cefoperazone has no effect on the biofilm formation as reported earlier since, cefoperazone showed no antibiofilm effect at sub-MIC concentration.

ACKNOWLEDGEMENT

Authors are highly thankful to Dean, College of veterinary science and A.H. Rewa for providing necessary facilities. We are also thankful to Director, Advance Instrumentation Research facility, Jawaharlal Nehru University and All India Institute of Medical Sciences New Delhi for necessary help in our research work.

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal Care and Handling Techniques were approved by the University of Animal Care Committee (certificate attached).

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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