RESEARCH ARTICLE

Study of Antimicrobial and Antibiofilm Effect of Essential Oils on Biofilm Producing *Staphylococcus aureus* Isolated from Mastitis Milk of Bovines

Durgesh Mishra[1], Arpita Shrivastava[1]*, Neeraj Shrivastava[2], Rajeev Ranjan[1],Swatantra K. Singh[1], Ankush Kiran Niranjan[2]

Abstract

The present work was undertaken to study antimicrobial and antibiofilm effect of essential oils on the biofilm producing *Staphylococcus aureus* isolated from the bovine mastitis milk. Out of 164 dairy cattle and buffalo milk samples screened, 24 samples were found positive for mastitis by CMT. Among these, 17 *S. aureus* isolates were identified and characterized phenotypically by standard biochemical tests. The biofilm formation ability of the isolates was studied by Congo red agar, Light microscopy method and Microtiter plate (Crystal Violet) method. The antimicrobial activity and antibiofilm effect of essential oils - Garlic and Cinnamon oil - on biofilm positive *S. aureus* were studied by agar well diffusion method and Microtiter plate method, respectively. Cefoperazone was used as standard control drug. Out of the total 17 *S. aureus* strains,70% were positive on Congo red agar, 52% by Light microscopy and 100% on Microtiter plate method. By the Microtiter plate method, 4 strains were strong biofilm producers and 13 were weak biofilm producers. Cinnamon oil showed better antibiofilm activity in all the concentration used (1%, 2%, 3%). Garlic oil showed good antimicrobial and antibiofilm activity at 3% concentration.It was concluded that biofilm producing *Staphylococcus aureus* was isolated from bovine mastitis milk samples, and sensitivity of Congo red agar, Light microscopy and Microtiter plate methods was good. Micro titre plate showed highest sensitivity for detection of biofilm production. Garlic oil and Cinnamon oil showed antimicrobial and antibiofilm producing abilityagainst *S. aureus*.

Keywords: Biofilm, Bovines, Cinnamon Oil, Garlic oil, Mastitis, Milk.

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Introduction

Bovine mastitis is one of the most significant diseases in the dairy commerce and has damaging effect on the economy and well-being of the animals. Chronic biofilm infections are disreputably known to be tough to exterminate with antibiotics and biofilm formation could be a conceivable elucidation for mastitis cases that are not resolved by standard treatment. *Staphylococcus aureus* is a common and challenging mastitis pathogen especially of transmissible bovine intra-mammary infections in dairy cattle (Zaatout *et al*., 2020). One of the highest interruptions in cure of mastitis is the antimicrobial resistance, a vital threat to global public health. Constant antibiotic treatment where antibiotics do not exterminate the microbial agents upsurges the risk of emerging antibiotic resistance, which is one of the extreme fears to human and animal health (Dugassa and Shukuri, 2017).

In this context, biofilms, an association of cell bacteria with a complex matrix of DNA, proteins, and polysaccharides, are among the most pertinent quantifiable importance, as they guard the microorganism by allowing them survive unreceptive environments, hinder antibiotic uptake, and represent more than 80% of the microbial infections worldwide (Davies, 2003; Koo *et al*., 2017). Microorganisms within a biofilm are numerous orders of size more impervious to antibiotics, related with planktonic bacteria. Antibiotic resistance in a biofilm could be due to penetrability barrier, initiation of resistance genes and expansion of resistance forms.

Essential oils ensue naturally in plants and have long been used as antimicrobials. The antimicrobic action of essential oils has been expansively verified *in vitro* against a wide range of infective bacteria (Oulkheir *et al.,* 2017). Both EO and its major component, cinnamaldehyde, significantly reduced biofilm formation (74.7 and 69.6%)(Budri *et al*., 2015). Chronic and persistent cases of bovine mastitis share

[1]Department of Pharmacology & Toxicology, College of Veterinary Science & Animal Husbandry, NDVSU, Rewa-486001, Madhya Pradesh, India

[2]Department of Microbiology, College of Veterinary Science &Animal Husbandry, NDVSU, Rewa-486001, Madhya Pradesh, India

***Corresponding Author:** Dr. Arpita Shrivastav, Associate Professor, Department of Pharmacology & Toxicology, College of Veterinary Science & Animal Husbandry, NDVSU, Rewa-486001, Madhya Pradesh, India. E-mail: arpitavet@gmail.com

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analogous characteristics with chronic biofilm infections detected in humans and other animals. Looking into its virtual destruction in both animals and humans, present study was designed to focus on the prevalence of biofilm producing microorganisms in bovine mastitis milk and likelihood of using natural composites, such as essential oils and antibiotic cefoperazone, in the fight against biofilms.

Materials and Methods

Collection of Sample

Aseptically 164 milk samples (cow 113; Buffalo 51) were collected from quarter/ composite bovine milk from participating dairies. The clinical mastitis and subclinical mastitis (SCM) were identified by standard methods (Constable *et al.,* 2017).

Phenotypic Characterization of *Staphylococcus aureus* Isolates and their Biofilm Producing Ability

300µL of milk was inoculated into 30 mL of Mueller-Hinton broth supplemented with 6.5 % NaCl incubated at 35°C for 16-20 h. Pre-enrichment culture was inoculated into Mannitol Salt agar plates at 35°C for 16-20 h then streaked onto a Baird-Parker agar plate with egg yolk-tellurite supplementat 35°C for 18-24 h. The plausible isolates were characterized by Gram's staining, standard biochemical tests, Novobiocin and Polymyxin B susceptibility. Phenotypic characterization of biofilm producing ability of *Staphylococcus aureus* from bovine mastitis milk was done using following three methods.

Congo Red Agar Method: The Congo red Agar was prepared with Brain Heart Infusion Agar (with 0.08 % Congo red and 5% sucrose), kept 35 °C for 18-24 h, black or pink colonies with a dry crystal-like surface indicated positive results (Fig. 1; Kaiser *et al*., 2013).

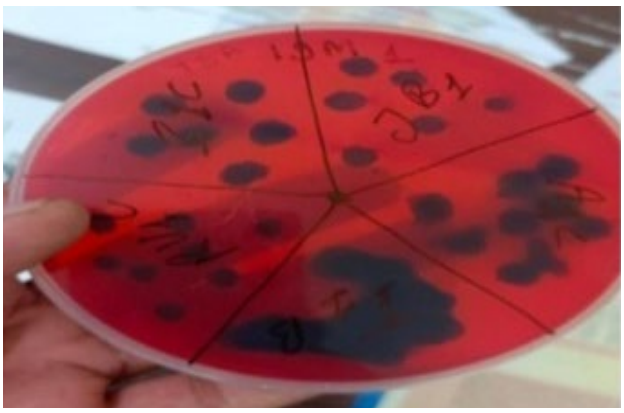


Fig. 1: Phenotypic screening of *S. aureus* for biofilm production by Congo Red Agar method

Light Microscopy Method: Overnight grown bacterial culture incubated at 37°C for 18 h **(**tryptone soy broth, 0.25% glucose; diluted 1:40 in TSB-glucose) following complete standard protocol was observed under 40 x of light microscope (Fig. 2; Silva *et al*., 2014).

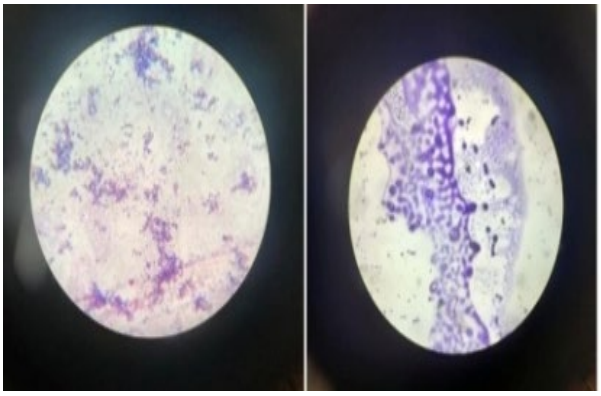


Fig. 2: Phenotypic screening of *S. aureus* for biofilm production by Light Microscopy method

Microtiter Plate Assay Method: 200 µL of overnight grown bacterial culture ;was used per well, incubated at 37°Cfor 18 h. After gentle washing three times with 200 µL of sterile phosphate-buffered saline (PBS), air dried in an inverted position, and stained with 0.1% crystal violet for 10 min. The stain released with 200 µL of the de-staining solution [50% (v/v) ethanol, 50% (v/v) glacial acetic acid] and was quantified by measuring the absorbance at 492 nm (A492) with a microplate reader **(**Cucarella *et al*., 2004; Tremblay *et al*., 2013)

Antimicrobial and Anti-Biofilm Effect of Essential Oils on Biofilm Producing *Staphylococcus aureus*

Agar Well Diffusion Method: Commercially available (100% pure) Cinnamon and Garlic essential oils were used in the concentration range of 1%, 2% and 3%. Dilution of essential oil was done in accordance with the manufacturer guideline (Drop method; 1, 2 and 3 drops of essential oil in 5 mL of DMSO yielded 1%,2%,3%, respectively.). Fresh overnight culture used, inoculum adjusted to 0.5 McFarland standards, Streakedin Mueller Hinton agar plate by standard methods. In Agar well using a sterile cork borer (5 mm in diameter) well were formed. 100 µL of each essential oil 1%, 2% 3% was added to respective wells. Following refrigeration for 30 min for oil diffusion into the agar well, incubated at 37°C for 18 h. Cefoperazone was used as the standard control. Antimicrobial activity observed after the incubation period by the zone of inhibition (including the wells diameter). DMSO at a concentration of 10% was employed as a negative control (Bazargani and Rohloff, 2016). All the experiments were conducted in the triplicates.

The biofilm producing ability of isolates was judged 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. Data on zone of inhibition was analysed using IBM SPSS software 20.

Results and Discussion

Out of 164 samples collected from different farms, only 24 samples (only cow milk) were found to be mastitis positive and 140 samples were negative. The prevalence of subclinical mastitis observed was 21%. The mastitic milk samples (24) screened for *Staphylococcus spp.,* 17 samples were found positive in selective agar media, biochemical tests, susceptibility to novobiocin (5 µg disc), and resistance to polymyxin B (300 µg disc). Out of the total 17 *S. aureus* strains, 70% samples (n=12) were positive for biofilm formation on Congo red agar, some plates gave less accuracy, whereas 52% samples (n=8) were positive on light microscopy. All the 17 samples showed biofilm production through microtiter plate method with almost 90% sensitivity (Table 1).

Table 1: Phenotypic screening of *Staphylococcus aureus* for biofilm production by micro titer plate method (Mean± SE)

Sr. No.	Sample	OD values at 492 nm	Result
1.	K3C	0.408±0.065	SBFP
2.	R1C	0.373±0.023	SBFP
3.	R2B	0.196±0.017	SBFP
4.	A1C	0.709±0.042	WBFP
5.	I1B	0.564±0.022	WBFP
6.	RV1C	1.229±0.136	WBFP
7.	S1C	0.540±0.024	WBFP
8.	JB1C	0.872±0.143	WBFP
9.	U1C	0.461±0.036	WBFP
10.	U2C	0.507±0.025	WBFP
11.	U3C	0.457±0.039	WBFP
12.	V1C	0.439±0.050	WBFP
13.	RL2C	0.408±0.037	WBFP
14.	ST1C	0.264±0.017	SBFP
15.	SK1C	0.309±0.025	WBFP
16.	KT1C	0.486±0.037	WBFP
17.	BP1C	0.432±0.035	WBFP
Sensitivity		90%	--

WBFP- Weak biofilm producer; SBFP- Strong biofilm producer

Among the commercially available cinnamon oil (100 % pure) (1%, 2% and 3%) diluted in DMSO used, the highest inhibition zone (mm) in 3% concentration (21.00±0.58 to 26.67±0.33). and lowest zone of inhibition in 1% concentration (11.33±0.88 to 13.00±0.58) was observed (Table 2; Fig. 3a). In comparison to cinnamon oil garlic oil showed high zone of inhibition only in 3% concentration (21.33±0.33 to 18.33±0.88 mm). In 2% concentration the zone of inhibition was in the range of 7.17±0.33 to 9.67±1.20 mm. The zone of inhibition was very small in 1% concentration (5.00 ± 0.00 mm) (Table 2, Fig. 3b). Cefoperazone (32 to 35 mm zone of inhibition) and DMSO were taken as positive and negative control each time. Combination of garlic oil and cinnamon oil showed zone of inhibition ranging from 20.33±1.18 to 27.33±1.33 mm in 3% concentration, while in 2% concentration it was 12.67±1.67 to 20.67±1.20 mm (Table 3, Fig. 3c).

Majority of *S. aureus* isolates from bovine mastitis cases form biofilm *in vitro* which may reduce the rate of penetration of antibiotics, thereby obscuring treatment of infections caused by these bacteria (Lister and Horsewill, 2014; Pedersen *et al.,* 2021). Microtiter plate assay with highest sensitivity in biofilm and bovine mastitis research used in our study showed 100% sensitivity. Earlier studies observed biofilm formation by microtiter plate or tissue culture plate as 64.7%, and non- or weak biofilm producers 36.3% (Darwish and Asfour, 2014; Notcovich *et al.,*2018; Bissong and Ateba, 2020).

According to the World Health Organization about 80% of the world's population uses medicinal plants to supply the primary medical care (WHO, 2002; Hassan *et al.,* 2011). Essential oils are secondary metabolites produced by plants (Martínez *et al.,* 2021). Effective antimicrobial properties, as low as 0.02% EO, were noted against *E. coli* by thyme, clove, lemon myrtle, bay laurel, lemongrass, cinnamon, tea tree, oregano, and rosewood which simulate with our findings. Preformed biofilms remained unaffected, and the inhibition rate ranged from 33% to 78% (Nazzaro *et al.,* 2019). The essential oil of garlic can be well-thought-outcapable for the development of new drugs in the deterrence of infections connected with *C. albicans* (Pourkhosravani *et al*., 2021), correlates with our findings of inhibitory effect of garlic oil on biofilm. One of the works showed significant reduction in hemolytic effect of *S. aureus* 54% and 32% by essential oils (Haney *et al.,* 2021).

Effect of garlic oil was less in comparison to cinnamon oil. Combination of garlic oil and cinnamon oil in 3% concentration was tested to see any change in the per cent inhibition of biofilm production. Only 3% concentration was taken for combination study because garlic oil did not show any significant result in 2% and 1% concentration. Combination of both oils showed higher antibiofilm property, could be due to additive effect on the biofilm inhibition. Similar findings were observed on pure EOs and their combination with Cardamom EO had the highest antibiofilm activity against biofilm formed by *E. coli* and *B. subtilis,* respectively. Garlic extract showed antibiofilm effects on six clinical bacterial isolates (Sandasi *et al*., 2010). Antimicrobial activity of garlic is imparted mainly by presence of Organo-sulfur compounds. The chief constituent, allicin is projected to wield its antimicrobial activity through manifold mechanisms. One research finding reported that garlic oil-soluble organo-sulfur compounds like allicin, ajoene, and allyl sulfides exhibit antibacterial activity (Bhatwalkar *et al*., 2021).

Conclusion

Biofilm producing *Staphylococcus aureus* was isolated from mastitis milk samples of bovines in Rewa. Presence of

biofilm producing organism is very detrimental for proper treatment of mastitis. Antibiotics therapy is found to have many drawbacks besides the development of resistance by the microorganisms. Microtitre plate showed highest sensitivity for detection of biofilm production. Garlic oil and Cinnamon oil showed antimicrobial and antibiofilm producing ability. Cinnamon oil (1%, 2%, 3%) has better effect on biofilm production. Garlic oil showed good antimicrobial and antibiofilm activity only at 3% concentration.

Table 2: Antimicrobial activity (zone of inhibition, mm) of cinnamon oil and garlic oil by agar well diffusion method (Mean± SE)

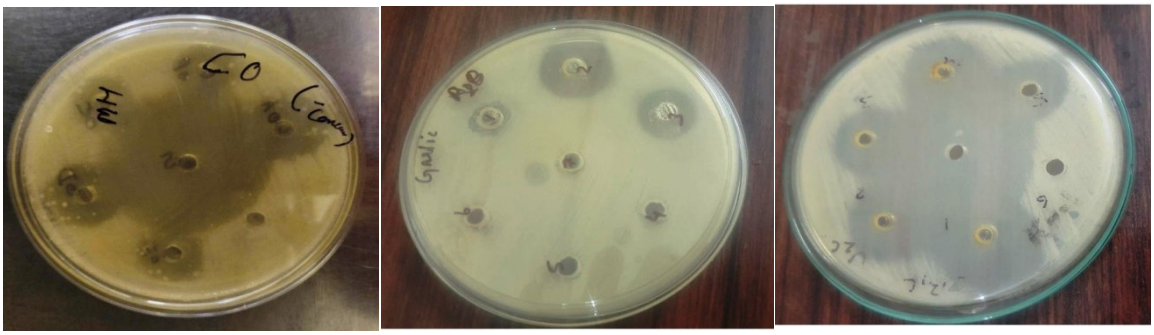
Sr. No.	Sample	Cinnamon oil (ZoI, mm)			Garlic oil ((ZoI, mm)		
		3%	2%	1%	3%	2%	1%
1.	K3C	21.00^{a}±0.58	16.3^{b}±0.88	12.00^{c}±0.58	19.67^{a}±0.33	10.00^{b}±0.58	5.67^{c}±0.33
2.	R1C	26.33^{a}±0.88	16.7^{b}±0.67	11.67^{c}±0.33	18.67^{a}±0.33	9.67^{b}±1.20	5.33^{b}±0.33
3.	R2B	22.33^{a}±1.20	15.7^{b}±0.88	12.33^{c}±0.67	20.33^{a}±0.67	9.33^{b}±0.88	6.00^{b}±0.58
4.	A1C	24.00^{a}±0.58	13.3^{b}±1.20	11.33^{b}±0.88	19.00^{a} ±1.15	8.67^{b}±0.67	5.67^{c}±0.33
5.	I1B	24.00^{a}±0.58	13.0^{b}±1.53	12.33^{c}±0.33	19.00^{a}±1.00	9.65^{b}±0.33	5.67^{c}±0.33
6.	RV1C	25.00^{a}±0.58	15.0^{b}±1.00	12.67^{c}±0.33	18.67^{a}±0.67	7.50^{b}±0.50	6.67^{b}±0.33
7.	S1C	23.33^{a}±0.67	14.7^{b}±0.33	13.00^{b}±0.58	19.33^{a}±1.67	7.33^{b}±0.33	5.33^{b}±0.33
8.	JB1C	24.33^{a}±1.20	13.3^{b}±0.3-93	12.33^{b}±0.88	20.67^{a}±0.88	8.13^{b}±0.33	7.00^{b}±0.58
9.	U1C	25.00^{a}±0.58	13.3^{b}±0.88	11.67^{b}±0.33	21.00^{a}±1.15	8.33^{b}±0.67	5.00^{c}±0.00
10.	U2C	26.67^{a}±0.33	13.7^{b}±0.33	11.33^{b}±0.33	21.33^{a}±0.33	9.33^{b}±0.67	5.67^{c}±0.33
11.	U3C	25.00^{a}±1.53	14.7^{b}±1.20	12.67^{b}±1.20	19.67^{a}± 0.88	9.00^{b}±1.00	5.33^{c}±0.33
12.	V1C	25.33^{a}±0.33	13.0^{b}±0.67	11.33^{c}±0.67	20.67^{a}±0.88	8.67^{b}±0.88	6.00^{b}±0.00
13.	RL2C	26.00^{a}±0.58	14.3^{b}±0.33	11.67^{c}±0.33	20.00^{a}±0.58	7.56^{b}±1.20	5.67^{c}±0.67
14.	ST1C	24.33^{a}±0.33	14.0^{b}±1.00	12.33^{c}±1.45	19.00^{a}±0.58	7.17^{b}±0.33	6.80^{b}±0.00
15.	SK1C	25.33^{a}±0.67	14.3^{b}±0.67	13.00^{c}±0.58	18.33^{a}±0.88	7.36^{b}±0.67	5.00^{c}±0.00
16.	KT1C	25.33^{a}±0.88	14.7^{b}±0.58	12.00^{c}±0.58	20.33^{a}±0.67	8.67^{b}±0.33	6.00^{b}±0.00
17.	BP1C	24.67^{a}±0.88	13.0^{b}±0.67	11.67^{c}±0.88	20.33^{a}±0.33	7.67^{b}±0.33	5.33^{c}±0.33

Means with different superscript within the row are significantly differentfor an oil at p<0.05.

Table 3: Antimicrobial activity of combination of cinnamon oil and garlic oil by agar well diffusion method (Mean± SE)

Sample	Zone of Inhibition (mm)		
	3%	2%	1%
K3C	25.67^{a}±1.20	17.33^{b}±1.67	11.33^{c}±1.86
R1C	27.33^{a}±1.33	14.33^{b}±1.20	11.67^{c}±0.33
R2B	25.67^{a}±1.76	12.67^{b}±1.67	11.00^{c}±0.58
A1C	26.00^{a}±2.00	15.67^{b}±0.67	10.67^{c}±1.20
I1B	25.33^{a}±1.45	15.67^{b}±0.88	11.67^{c}±0.88
RV1C	26.00^{a}±0.58	16.67^{b}±0.88	12.67^{c}±0.88
S1C	25.00^{a}±1.00	15.33±1.20	11.67±1.45
JB1C	24.00^{a}±0.08	15.00^{b}±1.00	12.00±1.53
U1C	23.67^{a}±0.67	18.00^{b}±1.00	13.33^{c}±0.88
U2C	24.33^{a}±0.33	20.67^{a}±1.20	12.67^{b}±0.67
U3C	22.33^{a}±1.40	19.00^{b}±0.58	12.67^{c}±0.67
V1C	23.00^{a}±0.58	15.67^{b}±0.33	12.00^{c}±0.58
RL2C	22.67^{a}±1.20	15.67^{b}±1.67	12.33^{c}±1.45
ST1C	22.67^{a}±0.19	13.33^{b}±0.88	11.67^{b}±0.88
SK1C	20.33^{a}±1.18	17.00^{b}±1.53	12.67^{c}±1.76
KT1C	22.33^{a}±0.88	13.67^{b}±1.20	13.33^{b}±0.67
BP1C	22.00^{a}±0.58	15.67^{b}±0.88	12.67^{c}±0.88

Means with different superscript differ significantlyat p<0.05



(a) (b) (c)

Fig. 3: Antimicrobial activity of Cinnamon oil (a), Garlic oil (b) and combination of Cinnamon oil and Garlic oil (c) by Agar well diffusion method

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