Journal of Animal Research: v.12 n.04, p. 483-495. August 2022

DOI: 10.30954/2277-940X.04.2022.4

Antibacterial and Antibiofilm Activities of Cinnamon Bark Oil and Thyme Oil Against Clinical Isolates of Multidrug Resistant *Staphylococcus aureus*

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Received: 06 July, 2022 **Revised:** 28 July, 2022 **Accepted:** 30 July, 2022

ABSTRACT

The rising tides of multidrug resistant *Staphylococcus aureus* (MDRSA) infection necessitate the development of potential antibacterial and antibiofilm agents. Essential oils derived from medicinal plants are gaining popularity as prospective antibacterial agents in recent years. Hence, the current investigation was carried out to evaluate the antibacterial and antibiofilm properties of cinnamon bark oil (CNBO) and thyme oil (THMO) against MDRSA isolated from bovine mastitic milk. Eighteen isolates of *Staphylococcus* spp. were recovered from 30 bovine mastitic sample collected, among which six were identified as MDRSA organisms. Further, chemical composition of CNBO and THMO were determined by GC-MS analysis, which revealed the existence of several terpenoids. The antimicrobial susceptibility of these MDRSA isolates against CNBO and THMO was tested by disc diffusion and microbroth dilution tests which specified significant antimicrobial activity of CNBO and THMO as evident from their zone of bacterial growth inhibition, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Subsequently, antibiofilm assays were performed using Congo red agar method and tissue culture plate-based crystal violet assays. The CNBO and THMO produced remarkable inhibition of biofilm formation and preformed biofilm, manifested as significantly reduced minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC). Moreover, CNBO was found to be more effective as compared to THMO against the MDRSA isolates. Thus, the present findings are indicative of the vital role of cinnamon bark oil and thyme oil in inhibiting the multidrug resistant *S. aureus* and associated biofilm formation.

HIGHLIGHTS

- Chemical composition of CNBO and THMO was assessed by GC-MS analysis.
- Disc diffusion, Minimum inhibitory concentration and Minimum bactericidal concentration were used for assessing antibacterial activity.
- Antibiofilm activity was measured using Congo red method and tissue culture plate-based crystal violet assay.

Keywords: Multidrug resistant *Staphylococcus aureus*, antibacterial, antibiofilm, cinnamon bark oil, thyme oil

Antimicrobial resistance (AMR) is upsurging as a major public health concern, hastening the quest for novel antimicrobial compounds in nature. Human pathogens have inexorably evolved resistance to a variety of currently accessible medications, resulting in significant morbidity and mortality around the world (Subramani *et al*., 2017). *Staphylococcus aureus*, a Gram-positive

How to cite this article: Sreeshma, T.S., Suja Rani, S., Uma, R. and Ambily, R. (2022). Antibacterial and Antibiofilm Activities of Cinnamon Bark Oil and Thyme Oil Against Clinical Isolates of Multidrug Resistant *Staphylococcus aureus*. *J. Anim. Res.,* **12**(04): 483-495.

Source of Support: None; **Conflict of Interest:** None

bacterium that produces multiple virulence factors engenders the development of various diseases as well as resistance against a multitude of antimicrobial agents. A wide array of illnesses ranging from simple skin and soft-tissue infections to more severe disorders like necrotizing pneumonia, toxic shock syndrome, and endocarditis in humans and also the most prevalent mastitis in dairy cattle can be precipitated by *S. aureus* infection (Tacconelli *et al*., 2014). In addition, biofilm formation is a significant factor in the development of multidrug resistance (MDR) in *S. aureus*. Biofilms are referred to as the multicellular communities bound together by an extracellular matrix created by the cells themselves. It enables the bacteria to be resistant to different antibiotics paving the way for various life-threatening infections and serious allied complications (Lopez *et al*., 2010).

Plants have long been employed in the battle against diseases as a source of pharmaceuticals and alternative medicine. The phytochemicals generated from plants, especially the secondary metabolites such as quinones, tannins, terpenoids, alkaloids, flavonoids, and polyphenols are gaining huge demand in medical therapy due to their potential effectiveness and low adverse effects. Consequently, essential oils (EOs) derived from many plant species have been explored intensively and reported to have a diverse spectrum of pharmacological effects, including anti-inflammatory, antibacterial, antioxidant, anticarcinogenic and other properties. Amongst these, its microbicidal potential against bacteria, fungi and viruses is highly conspicuous particularly in the current era of AMR (Cowan, 1999). Moreover, the Food and Drug Administration (FDA) typically deems numerous individual components of EO to be safe, allowing its usage in a variety of applications in the medical, pharmaceutical, food, cosmetic and health industries (Boskovic *et al*., 2015).

Cinnamomum zeylanicum, belonging to Family Lauraceae, is considered as one of the oldest spices in the world and has been used as a potent therapeutic agent in ethnomedicine and as a flavouring agent in foods. Cinnamon is reported to have antioxidant, antimutagenic and antimicrobial activities apart from reducing cardiovascular diseases, risk of colon cancer and boosting cognitive function (Gotmare and Tambe, 2019). *Thymus satureioides* is an annual plant in the Lamiaceae family, with grassy appearance that grows in many parts of the world. It is commonly used in folk medicine for its expectorant, antitussive, antibroncholytic, antispasmodic, antihelminthic, carminative and diuretic properties. It is also used as culinary herbs and is a well-known source of flavoring agent. Essential oil extracted from both the plant species are reported to have antibacterial activity against a wide range of pathogens (Al-Asmari *et al*., 2017).

Keeping these facts under consideration, the present study was undertaken to evaluate the antibacterial and antibiofilm potentials Cinnamon bark oil (CNBO) and Thyme oil (THMO) against the multidrug resistant *S. aureus* isolates.

MATERIALS AND METHODS

Bacterial Isolates

The *Staphylococcus aureus* organisms were isolated from bovine mastitic milk samples, from Teaching Veterinary Clinical Complex (TVCC), Mannuthy and University Livestock Farm (ULF), Mannuthy, Thrissur, Kerala.

Test Substances

Cinnamon (*Cinnamomum zeylanicum*) bark oil and Thyme (*Thymus satureioids*) oil were obtained from Synthite Industries Pvt. Ltd., Kerala as gift samples. They were kept tightly closed and were stored in a dry ventilated area, protected from light.

Isolation of *S. aureus*

A loopful of mastitic milk sample was inoculated into nutrient broth and incubated at 37ºC for 24 h to enrich the culture and further streaked onto mannitol salt agar (MSA) which is a selective media for *S. aureus.* The inoculated plates were incubated at 37ºC for 24-48 h for the preliminary detection of *S. aureus*. Subculturing and maintenance of the isolates were further done by streaking the culture on to Mueller Hinton agar (MHA) plates containing 2 per cent sodium chloride and further incubation at 35ºC for 24 h (CLSI, 2017). After incubation, the plates were again wrapped with parafilm and kept in refrigerator at 4ºC. The isolates were kept viable by sub culturing on weekly basis.

Identification of *S. aureus* Isolates

The isolates were identified based on morphological characteristics and biochemical tests as per Barrow and Feltham (1993) and Quinn *et al*. (2002).

Morphological Characterisation of *S. aureus* Isolates

The colony characteristics of the *S. aureus* culture grown in MSA were observed after 24-48 h of incubation at 37ºC and the yellow colonies with yellow zones were apparently identified as *S. aureus*. Consequently, Gram's staining of these presumptive isolates from MSA was done as per the manufacturer's instructions and the colony characteristics *viz*. Gram-positive cocci with 'bunches of grapes' appearance was verified for the authentication of *S. aureus* isolates.

Biochemical Characterization of *S. aureus* Isolates

These putative isolates of *S. aureus* were further subjected to conventional biochemical tests like catalase, oxidase, sugar fermentation and coagulase tests for confirmation of *S. aureus* isolates.

Identification of Multidrug Resistant *S. aureus* Isolates

Antimicrobial susceptibility testing of the confirmed *S. aureus* isolates was done using six different antibiotic discs such as methicillin (M, 30 µg), tetracycline (TE, 30 µg), enrofloxacin (EX, 5 µg) chloramphenicol (C, 30 µg), cephalexin (CE, 30 µg) and co-trimoxazole (COT, 25 µg) of 6 mm diameter on MHA media by Kirby-Bauer disc diffusion method (Bauer *et al.,* 1966) as per the Clinical and Laboratory Standards Institute guidelines (CLSI, 2017) in order to identify the MDRSA isolates phenotypically.

Direct colony suspension was prepared by transferring 4-5 isolated colonies with wire loop into a vial containing 4-5 mL of nutrient broth and incubated for 4-6 h at 37ºC until slight to moderate turbidity was developed. The turbidity of the suspension was adjusted to 0.5 McFarland standards. A sterile cotton swab on a wooden applicator was dipped into the standardized inoculum and the entire agar surface of the MHA plates was streaked with the swab three times, turning the plate at 60° angle between each streaking to ensure even distribution. The antibiotic discs were placed equidistant from each other on the agar surface and the plates were incubated at 35°C for 18-24 h. After incubation, the zone of inhibition was measured at three different directions and the mean of the measurements of inhibition was used for interpretation of the results as either susceptible or resistant to the exposed agent according to CLSI criteria.

Analysis of the Chemical Composition of Cinnamon bark oil and Thyme oil

The GC –MS analysis was conducted using Shimadzu GC-MS Model Number: QP2010S (Software: GCMS Solutions) equipped with ELITE- 5MS Capillary column (30 m x 0.25 mm, 0.25 µm thickness) for analysis of the chemical composition of CNBO and THMO. The column temperature was held at 60°C for 2 min and then increased to 280°C at the rate of 5°C /min and held at 280°C for 5 min. The injector and interface temperature were 220°C and 250°C respectively. The ion source temperature was 200°C. For GC-MS detection, an electron ionization system with ionization energy 70 e V was used over a scan range of 50- 500 m/z. Carrier gas was Helium at flow rate of 1.00 ml/min in split 1:50 with injection volume of 1 µl. Libraries used were NIST 11 & WILEY 8.

Antimicrobial Susceptibility of MDRSA Isolates against Cinnamon bark oil and Thyme oil

Disc diffusion assay and microdilution methods were employed to assess the antimicrobial susceptibility of the MDRSA isolates against CNBO and THMO as per the procedure described by Balouri *et al*. (2016) with minor modifications.

Disc Diffusion Assay

Kirby Bauer disc diffusion assay of CNBO and THMO was done on MHA media by the standard disc diffusion method as detailed earlier. Under aseptic conditions, 6 mm diameter sterile discs were impregnated with 10 µl of different dilutions of CNBO and THMO in 0.5 per cent Tween 20 and were placed symmetrically by means of sterile disc holding forceps on the surface of agar plates. All Petri dishes were sealed with parafilm to avoid eventual evaporation of the test samples. The plates were

incubated for 24 h at 35ºC to obtain zone of bacterial growth inhibition and the diameter of the zones of complete inhibition measured as >10 mm was considered as significant inhibition as denoted by Fu *et al*. (2007).

Microdilution Method for Determination of Minimum Inhibitory Concentration (MIC)

A modified resazurin microtitre plate assay was employed as reported by Balouri *et al.* (2016) with minor modifications. The stock microbial suspension was prepared in cation adjusted Mueller Hinton broth (CAMHB) supplemented with 2 per cent NaCl from a 24 h old MDRSA culture and incubated for 4-6 h at 37ºC until slight to moderate turbidity develops. Turbidity of bacterial inoculums suspension was made to 0.5 McFarland standards and then diluted to 1:100 in sterile broth medium to get a final concentration of 5×10^5 CFU/ml in the microtitre well. An aliquot (50 µl) of CAMHB supplemented with 2 per cent NaCl, containing 0.5 per cent (v/v) tween 20 was added to wells of a sterile 96-well microtitre plate. Two-fold serial dilutions of CNBO and THMO were made by using a multichannel pipette, for which 50 µl of test substance was added initially in the first well followed by sequential transferring of 50 µl to the subsequent wells after proper mixing, and finally, 50 µl from the last well was discarded. Then 50 µl of bacterial suspension was added to each well. The final concentrations of CNBO/THMO obtained in the wells were 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.0312, 0.0156, 0.0078, 0.0039 and 0.0019 per cent (v/v). The growth control (inoculum/positive control) wells contained 50 µl each of CAMHB medium and bacterial cells without test substances (CNBO/THMO) while the sterility control (media/vehicle/negative control) wells contained 100 µl CAMHB only. The plates were incubated at 35°C for 24 h and subsequently 30 µl of 0.01 per cent resazurin was added in each well, mixed by gentle shaking and the plates were again incubated at 37°C for 3 h. Bacterial growth was monitored visually as colour change from blue to pink, which indicated the presence of viable cells in cultures (Elshikh *et al*., 2016). The MIC was defined as the lowest concentration at which visible growth was inhibited i.e., the lowest concentration that remained blue in colour as the colour change from blue to pink was inhibited (Sanchez *et al.,* 2016). Each test reactions were done in triplicates, against six MDRSA isolates (n=6).

Determination of Minimum Bactericidal Concentration (MBC)

The MBC was determined by employing the method described by Misra and Sahoo (2012). The bacterial suspension in the 96 well microtitre plate with more than or equal to the MIC, were used for MBC determination. The suspension was streaked onto sterile nutrient agar (NA) plates and observed for the bacterial growth after incubation at 35ºC for 24 h. The lowest concentration of test substance which inhibited the visible growth of bacteria on NA was defined as MBC. Each test was done in triplicates against six MDRSA isolates.

Detection of Biofilm forming ability of the MDRSA Isolates

For the detection of biofilm forming ability of the MDRSA isolates, Congo red method was employed as described by Hassan *et al.* (2011) with slight modifications. Congo red agar plates inoculated with test organism were incubated at 37 °C for 24-48 h aerobically. Black colonies with a dry crystalline consistency indicated biofilm production, whereas weak producers usually remained as pink colonies.

Analysis of Antibiofilm activity of Cinnamon bark oil and Thyme oil against the MDRSA Isolates

Congo Red Method

For the analysis of antibiofilm activity of CNBO and THMO against the MDRSA isolates, Congo red plate method was employed as reported by Seo *et al.* (2021) with minor modifications. The biofilm forming MDRSA suspension in BHIB was incubated with test substances at concentration of 1/2 MIC, MIC, 2MIC and 4MIC for 24 hours at 37 °C. Further, the sterile CRA plates were streaked with the respective treated MDRSA isolates and the strains were then incubated aerobically for 24 h at 37 °C. The colour of the colonies was observed after 24 h while, sterile CRA plates served as positive control. The lowest concentration of test substance which inhibited the formation of black crystalline colony was defined as biofilm inhibitory concentration.

Tissue Culture Plate Method

Tissue culture plate assay/microtitre plate-based crystal violet assay was performed for quantitative analysis of antibiofilm activity of CNBO and THMO against MDRSA isolates. Minimum biofilm inhibitory concentration (MBIC) and Minimum biofilm eradication concentration (MBEC) were assessed to determine the inhibitory potential of these agents against biofilm formation and preformed biofilm respectively in MDRSA isolates as per the method described by Adukwu *et al.* (2012). The MBIC/MBEC was determined as the concentration of test substances at which the OD ≤ negative control (Sandoe *et al*., 2006). Each experiment was performed in triplicates and performed on six different isolates. The percentage of inhibition was calculated by the equation given below:

Percentage of inhibition =

$$\frac{\text{OD positive} - \text{OD sample}}{\text{OD positive}} \times 100$$

STATISTICAL ANALYSIS

All the results were expressed as Mean ± SEM with 'n' as number of replicates. Statistical analysis was performed using SPSS software version 21. The data were analyzed by one way analysis of variance (ANOVA) followed by Duncan's multiple comparison test. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Culture and Isolation of *Staphylococcus aureus* Isolates

A loopful of milk inoculated into nutrient broth from all the 30 mastitic milk samples showed turbidity after 24 h of incubation. The inoculum was subsequently streaked on to mannitol salt agar (MSA) for the preliminary identification of *S. aureus*.

Identification of *S. aureus* isolates

Morphological Characterisation of *S. aureus* Isolates

The colony characteristics of the culture grown in MSA revealed a colour change in the media from red to yellow in 18 isolates, while seven samples showed red colonies, indicating 60 per cent mannitol fermenters and 23 per cent mannitol non-fermenters among the 30 isolates tested. As a result, the yellow colonies on MSA were positively identified as *S. aureus* colonies, whereas the red colonies were identified as other Staphylococci species. However, five samples did not show any growth in the medium. Furthermore, these prospective *S. aureus* isolates (18 isolates) when subjected to Gram staining, all of them appeared as bunches of grapes, suggestive of *S. aureus* isolates. Besides, streaking of the *S. aureus* culture to MHA plates containing 2 per cent sodium chloride followed by incubation at 35 ºC for 24 h for the subculturing and maintenance of the *S. aureus* isolates revealed yellow colonies. The morphological characteristics of the culture are depicted in Fig. 1.

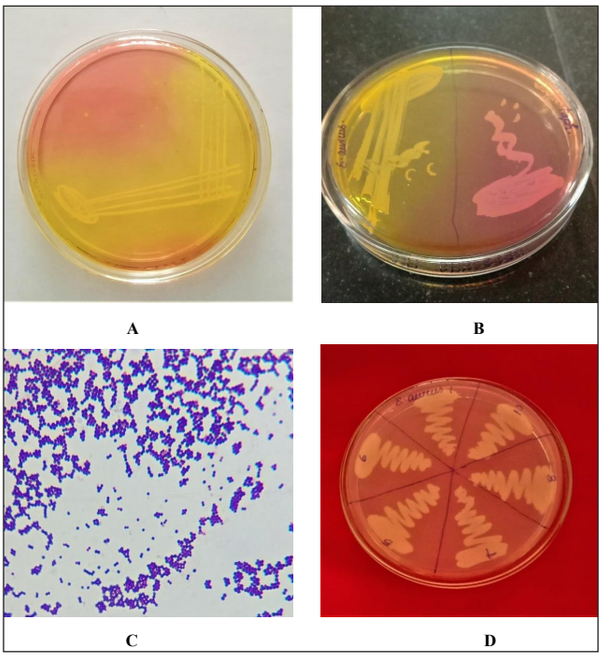


Fig. 1: Morphological characterisation of *S. aureus* isolates **(A)** and **(B)** Yellow colonies on mannitol salt agar **(C)** Colonies resembling bunches of grapes in Gram staining **(D)** Yellow colonies on Mueller Hinton agar

Biochemical Characterisation of *S. aureus* Isolates

Various conventional biochemical tests performed on the 18 presumptive isolates of *S. aureus* revealed 12 isolates to

be catalase positive, oxidase negative, coagulase positive and sugar fermentation positive for sucrose, lactose, glucose, fructose, trehalose, maltose, mannitol, mannose, while negative for xylose and cellobiose, confirming the presence of *S. aureus* isolates in the samples. The summary of the morphological and biochemical characterisation of *S. aureus* isolates is tabulated in Fig. 2 and table 1, based upon which 12 out of the 30 isolates collected were found to be *Staphylococcus aureus* and was selected for further study.

Identification of Multidrug Resistant *S. aureus*

The result of antibiotic susceptibility test against *S. aureus* isolates are depicted in Fig. 3 and table 2. The per cent of isolates resistant to methicillin, tetracycline, co-

Table 1: Morphological and biochemical characterisation of *S. aureus* isolates

Particulars of identification tests	Observation	Number of positive samples
Growth on mannitol salt agar	Yellow colony	18 (60%)
	Red colony	7 (23%)
	No growth	5 (16.67%)
Gram staining	Gram positive cocci in clusters	18
Catalase test	Appearance of effervescence	18
Oxidase test	Absence of blue colour on disc	18
Sugar fermentation test	Change of colour of the medium to dark pink colour with specific sugar discs	18
Coagulase test	Clot formation	12

* Values in parenthesis indicates the per cent of positive samples out of total of 30 isolates.

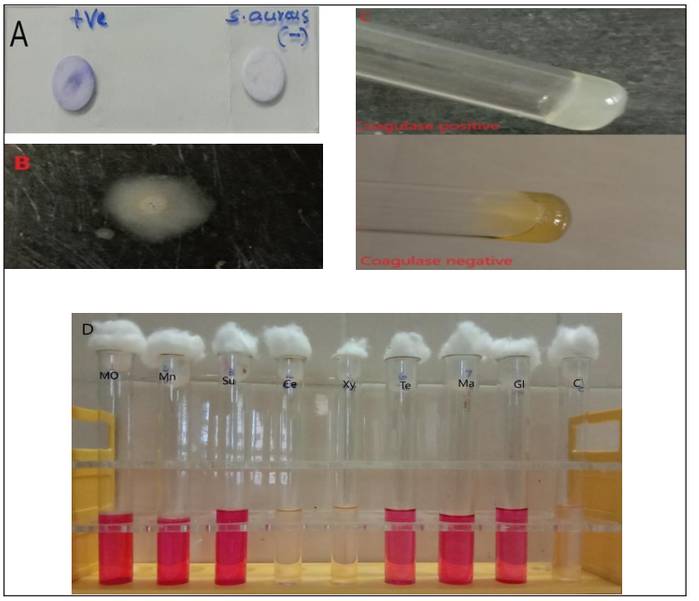


Fig. 2: Biochemical characterization of *S. aureus* isolates **(A)** Oxidase test **(B)** Catalase test **(C)** Coagulase test **(D)** Sugar fermentation test (Mo- mannose, Mn- mannitol, Su-sucrose, Ce- cellobiose, Xy-xylose, Te-Trehalose, Ma - maltose, Gl- glucose, C- control)

Fig. 3: Antimicrobial susceptibility test of *S. aureus* isolates using individual antibiotic discs (MET-Methicillin, TE-Tetracycline, C-Chloramphenicol, COT- Cotrimoxazole, CN- Cephalexin, EX-Enrofloxacin)

trimoxazole, cephalexin and enrofloxacin was 66.67, 50, 41.67, 33.33, 41.67 % respectively and all the isolates were susceptible to chloramphenicol. Two out of the 12 isolates were found to be susceptible to all the antibiotics used and methicillin resistance was found in eight *S. aureus* isolates. Finally, six out of the twelve *S. aureus* isolates were found to be multidrug resistant (resistant to atleast three antibiotic classes) with MAR index ranging from 0.5 to 0.83.

Analysis of the Chemical Composition of Cinnamon bark oil (CNBO) and Thyme oil (THMO)

Chromatogram and list of active compounds obtained on phytochemical analysis of CNBO and THMO using GC-MS analysis are given in Fig. 4 and 5, Table 3 and 4 respectively. The major constituent of CNBO detected was cinnamaldehyde which represented 76.16 per cent of the total constituents, while some of the other pertinent compounds included β- caryophyllene (9.91 %), β-

Table 2: Antibiotic susceptibility profile of S. aureus isolates

Isolate ID	Zone of inhibition (mm)						MAR index
	MET	TE	C	COT	CN	EX	
SA1	8 (R)	- (R)	24 (S)	- (R)	18 (S)	13 (R)	0.67
SA2	13(S)	8 (R)	26 (S)	- (R)	20 (S)	14 (R)	0.5
SA3	- (R)	- (R)	25 (S)	- (R)	14 (R)	11 (R)	0.67
SA4	- (R)	- (R)	27 (S)	23 (S)	7 (R)	17 (I)	0.5
SA5	- (R)	- (R)	23 (S)	- (R)	19 (S)	13 (R)	0.67
SA6	- (R)	6 (R)	27 (S)	- (R)	- (R)	13 (R)	0.83
SA7	8 (R)	20 (S)	26 (S)	20 (S)	14 (R)	21 (S)	0.3
SA8	10 (R)	19 (S)	24 (S)	22 (S)	19 (S)	18 (I)	0.16
SA9	13 (S)	19(S)	23 (S)	19 (S)	17 (I)	21 (S)	0
SA10	17(S)	21 (S)	26 (S)	18 (S)	21 (S)	22 (S)	0
SA11	14 (S)	24 (S)	19 (S)	16(S)	23 (S)	20 (I)	0
SA12	9 (R)	19 (S)	25(S)	23 (S)	18 (S)	24 (S)	0.16
Resistant %	66.67	50		41.67	33.33	41.67	
Intermediate %					8.33	25	
Sensitive %	33.33	50	100	58.33	58.33	33.33	

*Values of zone of inhibition are mean of triplicates, n=12; S-Sensitive, I-Intermediate, R-Resistant, MAR index- Multiple antibiotic resistance index, MET- Methicillin, TE-Tetracycline, C-Chloramphenicol, COT- Co-trimoxazole, CN- Cephalexin, EX- Enrofloxacin

Table 3: List of compounds from cinnamon bark oil on GC-MS analysis

Sl. No.	Name of the compound	Class of compound	Molecular formula	Molecular weight (g/mole)	Retent ion time (min)	Height %	Peak area %
1	Cinnamadehyde	Phenylpropanoid	C_9H_8O	132.16	20.18	63.14	76.16
2	β- Caryophyllene	Bicyclic sesquiterpenes	C15H24	204.35	26.29	15.57	9.91
3	β- Phellandren	Cyclic monoterpenes	C10H16	136.24	9.61	5.39	3.93
4	4-Cymene	Monoterpenoid	C10H14	134.22	9.44	5.24	3.42
5	β- Linalool	Monoterpenoid	C10H18O	154.25	12.50	4.46	2.66
6	α- Humulen	Monocyclic sesquiterpenes	C15H24	204.36	27.69	2.45	1.56
7	α-Copaene	Tricyclic sesquiterpenes	C15H24	204.35	24.44	2.34	1.44
8	Trans-3-caren-2- ol	Bicyclic monoterpenoid	C10H15O	152.23	8.69	1.41	0.91

Table 4: List of compounds from thyme oil on GC-MS analysis

Sl. No.	Name of the compound	Class of compound	Molecular formula	Molecular weight (g/mole)	Retent ion time (min)	Height %	Peak area %
1	Borneol	Bicyclic sesquiterpenes	$C_{10}H_{18}O$	154.25	15.43	34.93	41.01
2	α-Terpenol	Monoterpenoid	$C_{10}H_{18}O$	154.25	16.50	19.02	20.41
3	Bicycloheptane- 2, 2-dimethyl-3methylene	Monoterpenoid	$C_{10}H_{16}$	136.23	6.83	11.20	7.58
4	β- Caryophyllene	Bicyclic sesquiterpenes	$C_{15}H_{24}$	204.35	26.28	7.31	7.73
5	Bicycloheptane- 2, 2-dimethyl-3methylene	Monoterpenoid	$C_{10}H_{16}$	136.23	6.79	7.09	5.29
6	α-Pinen	Monoterpenoid	$C_{10}H_{16}$	136.23	6.37	6.79	4.58
7	4-Cymene	Monoterpenoid	$C_{10}H_{14}$	134.22	9.43	5.22	5.40
8	β- Linalool	Monoterpenoid	$C_{10}H_{18}O$	154.25	12.50	3.01	2.78
9	2-Camphanyl acetate	Oxygenated monoterpenes	$C_{12}H_{18}O_2$	194.27	20.61	2.32	2.07
10	Camphor	Monoterpenoid	$C_{10}H_{16}O$	152.23	14.40	1.92	2.06
11	Methyl carvacrol	Monoterpenoid	$C_{11}H_{16}O$	164.24	18.7	1.20	1.11

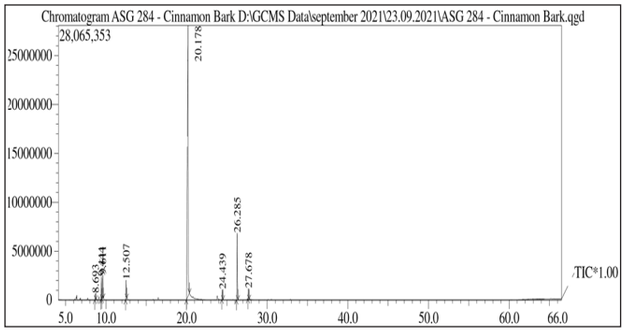


Fig. 4: GC-MS chromatogram of cinnamon bark oil

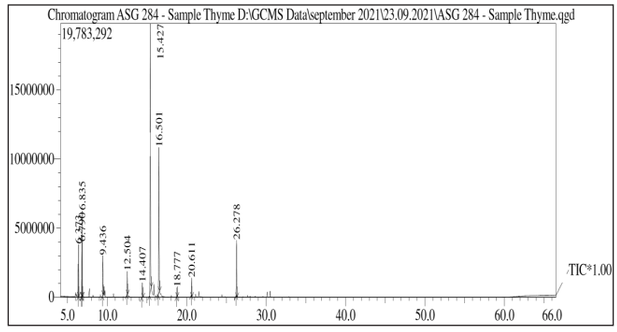


Fig. 5: GC-MS chromatogram of thyme oil

phellandren (3.93 %) and 4-cymene (3.42 %). While, borneol (41.01%), α-terpenol (20.41%), bicycloheptane-2, 2-dimethyl-3-methylene (7.58 %) and β- caryophyllene (7.73 %) which together represented 65.15 per cent of the total constituents.

Antimicrobial susceptibility of MDRSA isolates against cinnamon bark oil and thyme oil

Disc Diffusion Assay

For CNBO, significant inhibition zone (>10 mm) observed at 1% v/v with an inhibition zone diameter of 12.5 mm, whereas, THMO exhibited significant inhibition zone at 32% v/v concentration with an inhibition zone diameter of 11.33 mm (Fig. 6 and Table 5).

Moreover, CNBO was found to be more effective as compared to THMO and a dose dependent decrease in the diameter of zone of inhibition was noticed for both CNBO and THMO. Similar results were observed in the study conducted by Oulkheir *et al*. (2017), in which CNBO displayed higher zone of inhibition as compared to THMO against antibiotic resistant *S. aureus*.

Determination of MIC

The MIC values of CNBO and THMO were 0.0625 and 2% v/v respectively (Fig. 7 and table 6), indicating that CNBO possessed higher potency in comparison to THMO. This was in line with the findings by Xiao *et al.* (2020), in which CNBO showed higher bacterial growth inhibition as compared to THMO as evident from the lower MBC and MIC values.

Table 5: Diameter of zone of inhibition of cinnamon bark oil and thyme oil against MDRSA isolates, mm

Cinnamon bark oil		Thyme oil	
Concentration (%v/v)	**Diameter of inhibition zone (mm)**	**Concentration (%v/v)**	**Diameter of inhibition zone (mm)**
2%	18.33 ± 0.51[c]	64%	14.00 ± 0.63[c]
1%	12.50 ± 0.76[b]	32%	11.33 ± 0.49[b]
0.5%	7.00 ± 0.63[a]	16%	7.00 ± 0.63[a]

*Values are expressed as Mean± SE, n=6: Values bearing different superscript vary significantly at P< 0.05. No inhibition zone was observed with vehicle control; Diameter values of zone inhibition are inclusive of disc diameter of 6 mm.

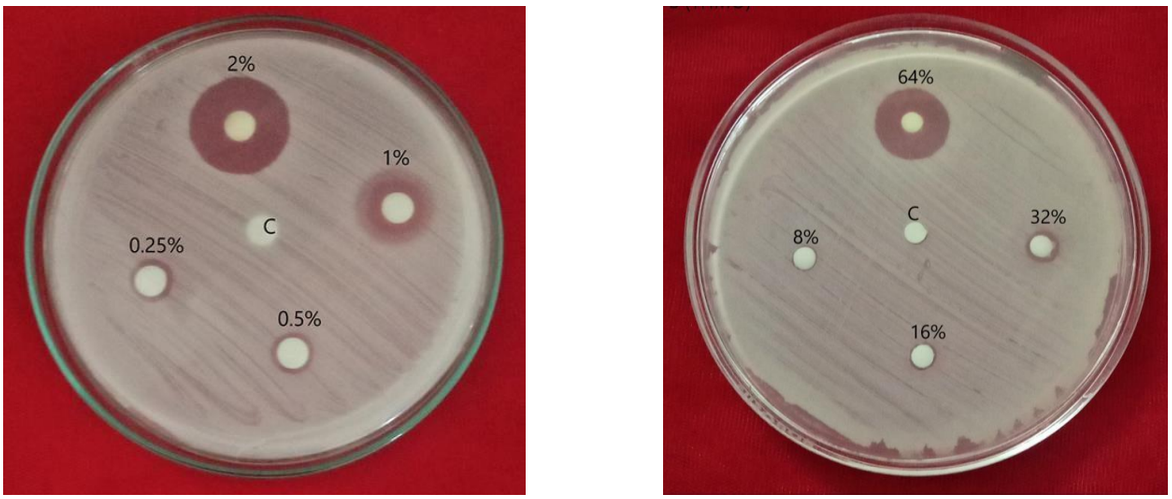


Fig. 6: Disc diffusion assay of MDRSA isolates **(A)** Cinnamon bark oil **(B)** Thyme oil

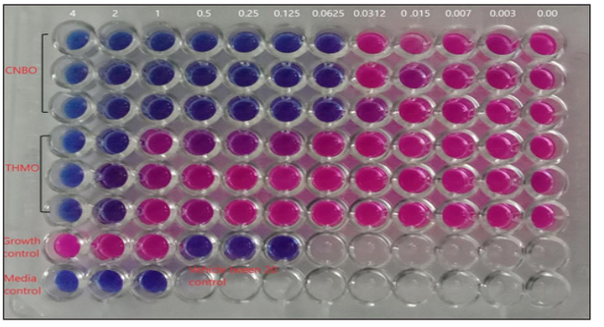
Table 6: Minimum Inhibitory concentration of cinnamon bark oil and thyme oil against MDRSA isolates, % v/v

Isolate ID	Minimum inhibitory concentration	
	Cinnamon bark oil	**Thyme oil**
SA1	0.0625	2
SA2	0.0625	2
SA3	0.125	4
SA4	0.0625	2
SA5	0.0625	2
SA6	0.0625	2

*Value of MIC of each isolate is mode of triplicates, n=6.

Determination of MBC

The MBC values for CNBO against MDRSA isolates varied from 0.125 to 0.25 % v/v, whereas MBC values for THMO ranged from 16 to 32 %v/v. Cinnamon bark oil at 2 fold MIC and THMO at 8 fold MIC inhibited bacterial growth, indicating that CNBO had a greater bactericidal effect than THMO.



CNBO: Cinnamon bark oil; THMO: Thyme oil

Fig. 7: Determination of MIC of Cinnamon bark oil and Thyme oil

The MBC values of CNBO and THMO were determined by subculturing the bacterial suspension with more than or equal to the MIC, and incubating at 35°C for 24 h and the findings are depicted in Fig. 8 and table 7.

Table 7: Minimum bactericidal concentration of cinnamon bark oil and thyme oil against MDRSA isolates

Isolate ID	Cinnamon bark oil		Thyme oil	
	Concentration (%v/v)	Folds of MIC	Concentration (%v/v)	Folds of MIC
SA1	0.125	2	16	8
SA2	0.125	2	16	8
SA3	0.50	4	32	8
SA4	0.125	2	16	8
SA5	0.125	2	16	8
SA6	0.125	2	16	8

* Values of MBC of each isolate is mode of triplicates, n=6.

Detection of Biofilm forming ability of the MDRSA Isolates

Congo Red Method

The biofilm production of the MDRSA isolates was evaluated by culturing the organism on Congo red agar (CRA) medium. The results revealed that all the six isolates produced black colonies with darkening at the centre of the colony, indicative of biofilm production (Fig. 9).

Antibiofilm activity of Cinnamon bark oil and Thyme oil against MDRSA isolates

Congo Red Method

Antibiofilm activity of CNBO and THMO were tested against MDRSA isolates by culturing them on Congo

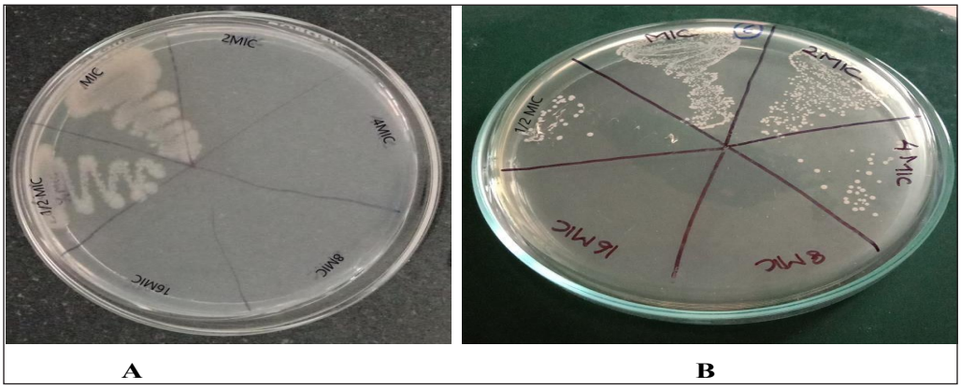


Fig. 8: Determination of MBC against MDRSA isolates **(A)** Cinnamon bark oil **(B)** Thyme oil

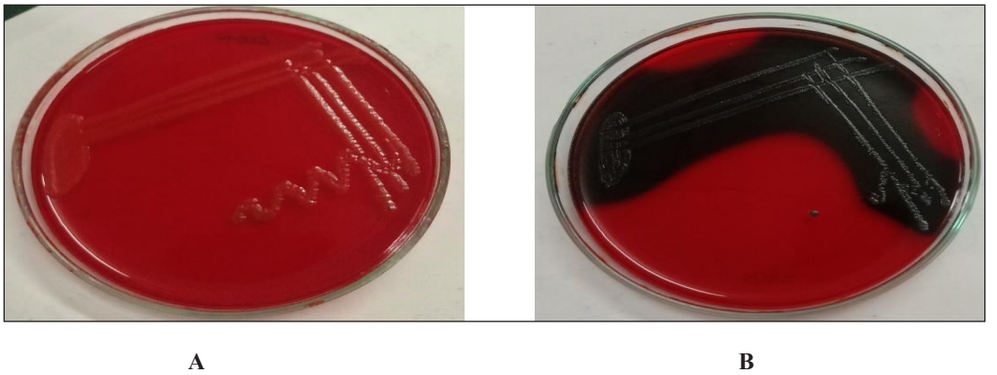


Fig. 9: Appearance of MDRSA strains on Congo red agar **(A)** Red/pink colonies indicating lack of biofilm production **(B)** Black colonies indicating biofilm formation

red agar after 24 hours of incubation with different concentrations of test compound, as shown in Fig. 10 and table 8. The results revealed that CNBO inhibited biofilm development at 2 fold the MIC while THMO inhibited biofilm formation at 4 fold the MIC. For CNBO and THMO, the values ranged from 0.125-0.25 %v/v and 8-16 %v/v respectively.

Table 8: Antibiofilm activity of cinnamon bark oil and thyme oil

Isolate ID	Cinnamon bark oil		Thyme oil	
	Concentration (% v/v)	Fold of MIC	Concentration (%v/v)	Fold of MIC
SA1	0.125	2MIC	8	4MIC
SA2	0.125	2MIC	8	4MIC
SA3	0.25	2MIC	16	4MIC
SA4	0.125	2MIC	8	4MIC
SA5	0.125	2MIC	8	4MIC
SA6	0.125	2MIC	8	4MIC

*MIC: Minimum inhibitory concentration; CNBO: Cinnamon bark oil; THMO: Thyme oil.

Tissue Culture Plate Method

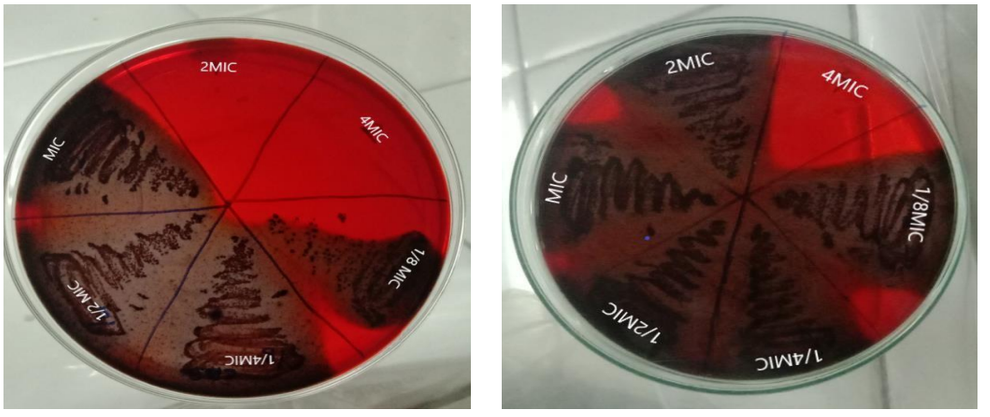
Determination of Minimum Biofilm Inhibitory Concentration (MBIC)

The MBIC of CNBO and THMO against biofilm formation of MDRSA isolates was determined by tissue culture plate-based crystal violet method and the results are illustrated in table 9. For CNBO and THMO, MBIC values ranged from 0.03-0.06 %v/v and 2-4 %v/v respectively, indicating that CNBO had more potential to attenuate biofilm formation. The MBIC determined from the triplicates of six MDRSA isolates obtained for CNBO were half of the MIC, whereas for THMO, MBIC was equivalent to the MIC. Firmino *et al*. (2018) have reported MBIC Values of CNBO against *S. aureus* as half of the MIC value, and MBEC values as four fold of MIC, which was in corroboration to our findings.

Table 9: Minimum biofilm inhibitory concentration and respective fold of MIC for cinnamon bark oil and thyme oil against MDRSA isolates

Isolate ID	Minimum Biofilm Inhibitory Concentration			
	Cinnamon bark oil		Thyme oil	
	MBIC (%v/v)	Folds of MIC	MBIC (%v/v)	Folds of MIC
SA1	0.03125	0.5	2	1
SA2	0.03125	0.5	2	1
SA3	0.0625	0.5	4	1
SA4	0.03125	0.5	2	1
SA5	0.03125	0.5	2	1
SA6	0.03125	0.5	2	1

*Values of MBIC of each isolate is mode of triplicates, n=6; MIC: Minimum inhibitory concentration; MBEC: Minimum biofilm inhibitory concentration; MDRSA: Multidrug resistant *Staphylococcus aureus.*



A B

Fig. 10: Antibiofilm activity evaluation by Congo red method **(A)** Cinnamon bark oil **(B)** Thyme oil

The per cent inhibition of biofilm formation by CNBO and THMO against MDRSA isolates calculated at various concentrations (2MIC, MIC, 1/2MIC, 1/4MIC) is presented in table 10. The results revealed that CNBO and THMO reduced biofilm formation to >85% level at all concentrations except for THMO at 1/4MIC.

Table 10: Per cent inhibition on biofilm formation of MDRSA isolates on different concentration of cinnamon bark oil and thyme oil

Concentration	% Inhibition of MDRSA biofilm formation	
	Cinnamon bark oil	Thyme oil
2MIC	91.1±0.41^{b}	90.65±0.42^{c}
MIC	90.36±0.39^{b}	89.75±0.44^{c}
1/2MIC	89.48±0.44^{b}	85.45±1.32^{b}
1/4MIC	85.39±0.77^{a}	78.58±1.64^{a}

*Values are expressed as Mean ± SE, n=6: Values bearing different superscripts vary significantly at P>0.05 MIC: Minimum inhibitory concentration; MDRSA: Multidrug resistant *Staphylococcus aureus*

Determination of Minimum Biofilm Eradication Concentration

The MBEC values of CNBO and THMO against preformed biofilms produced by MDRSA isolates are shown in table 11.

Table 11: Minimum biofilm eradication concentration and respective fold of MIC for cinnamon bark oil and thyme oil against MDRSA isolates

Isolate ID	Minimum biofilm eradication concentration			
	Cinnamon bark oil		Thyme oil	
	MBEC (%v/v)	Folds of MIC	MBEC (%v/v)	Folds of MIC
SA1	0.25	4	8	4
SA2	0.25	4	8	4
SA3	0.5	4	16	4
SA4	0.25	4	8	4
SA5	0.25	4	8	4
SA6	0.25	4	8	4

*Values of MBEC of each isolate are mode of triplicates, n=6; MIC: Minimum inhibitory concentration; MBEC-Minimum biofilm eradication concentration.

The MBEC values of CNBO and THMO ranged from 0.25-5% and 8-16% v/v respectively which were four times greater than their MIC values. Furthermore, the efficacy of CNBO to eradicate preformed biofilm was greater than that of THMO. However, the study conducted by Carvalhoa *et al.* (2020) reported that THMO could able to disrupt the preformed biofilms by antibiotic sensitive *S. aureus* isolates even at a concentration 0.25 folds of their MIC, which was not in alignment to our findings and might be due to the MDR feature of *S. aureus* isolates in this study.

The per cent inhibition of preformed MDRSA isolates by different concentrations (8MIC, 4MIC, 2MIC, and MIC) of CNBO and THMO calculated is presented in table 12. The CNBO at concentrations 4MIC and 8MIC as well as THMO at 8MIC, showed >90% inhibition of preformed biofilm. The results revealed that the biofilm eradicating effect of CNBO and THMO were higher than that of the MIC value. Moreover, a dose dependant increases in the per cent of inhibition of preformed biofilm of MDRSA isolates was observed for both CNBO and THMO.

Table 12: Per cent inhibition of preformed biofilm of MDRSA isolates by different concentration of cinnamon bark oil and thyme oil

Concentration	% Inhibition of MDRSA biofilm formation	
	Cinnamon bark oil	Thyme oil
8MIC	92.43 ± 0.58^{c}	90.43 ± 0.77^{c}
4MIC	90.41 ± 0.79^{bc}	89.08 ± 0.89^{c}
2MIC	88.63 ± 0.95^{b}	86.08 ± 0.62^{b}
MIC	84.68 ± 0.82^{a}	82.13 ± 0.92^{a}

*Values are expressed as Mean ± SE, n=6; Values bearing different superscripts vary significantly at p>0.05, MIC: Minimum inhibitory concentration; MDRSA: Multidrug resistant *Staphylococcus aureus.*

CONCLUSION

The present study findings are suggestive of the potential role of cinnamon bark oil and thyme oil in inhibiting the multidrug resistant *Staphylococcus aureus* and associated biofilms.

ACKNOWLEDGEMENTS

Authors are sincerely thankful to Kerala Veterinary and Animal Science University for their valuable support for conducting the research.

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