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Antibacterial synergy between rosmarinic acid and antibiotics against methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

Aim/Background: Medicinal plants have ability to resist microorganisms by synthesizing secondary metabolites such as phenols. Rosmarinic acid (RA) is a phenylpropanoid widely distributed in plants and well known as therapeutic and cosmetic agent. Methicillin-resistant *Staphylococcus aureus* (MRSA) which is resistant to all kinds of β-lactams, threatens even most potent antibiotics. To improve the efficiency of antibiotics against multi-drug resistant bacteria and to reduce the antibiotic dose, the antibacterial activity and the synergistic effect of RA with standard antibiotics against *S. aureus* and MRSA was investigated. **Materials and Methods:** Antibacterial activity of RA against *S. aureus* and a clinical isolate of MRSA was evaluated by agar well diffusion method. Minimum inhibitory concentration (MIC) of RA was determined by broth dilution method. Synergism of RA with various antibiotics against *S. aureus* and MRSA was studied by broth checkerboard method and time-kill kinetic assay. Effect of RA on microbial surface components recognizing adhesive matrix molecules (MSCRAMM's) of *S. aureus* and MRSA was studied using sodium dodecyl sulfate - polyacrylamide gel electrophoresis. **Results:** MIC of RA was found to be 0.8 and 10 mg/ml against *S. aureus* and MRSA, respectively. RA was synergistic with vancomycin, ofloxacin, and amoxicillin against *S. aureus* and only with vancomycin against MRSA. The time-kill analysis revealed that synergistic combinations were a more effective than individual antibiotics. MSCRAMM's protein expression of *S. aureus* and MRSA was markedly suppressed by RA + vancomycin combination rather than RA alone. **Conclusion**: The synergistic effects of RA with antibiotics were observed against *S. aureus* and MRSA. RA showed inhibitory effect on the surface proteins MSCRAMM's. Even though RA was shown to exhibit a synergistic effect with antibiotics, the MIC was found to be higher. Thus, further studies on increasing the efficacy of RA can develop it as an adjuvant for antibiotics.

KEY WORDS: Antimicrobial resistance, methicillin-resistant *Staphylococcus aureus*, microbial surface components recognizing adhesive matrix molecules, rosmarinic acid, synergy

INTRODUCTION

Antibiotic resistance of micro-organisms is a major challenge confronted by all scientists who are involved in antibiotic drug discovery. Bacteria widen its resistance to antibiotics by mutating existing genes (vertical evolution) or by acquiring new genes from other strains or species (horizontal gene transfer). The sharing of genes between bacteria by horizontal gene transfer occurs by many different mechanisms [1].

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a nosocomial and community-acquired pathogen that has developed resistance to various antibiotics such as β-lactams, quinolones, aminoglycosides, vancomycin, oxazolidinone, and streptogramin type antibiotics [2-4]. Newly emerging community-associated MRSA (CA-MRSA) is transmissible from healthcare acquired MRSA (HA-MRSA). Some CA-MRSA strains display enhanced virulence, spreading more rapidly and causing illness much more severe than HA-MRSA infections, affecting vital organs which lead to sepsis, toxic shock syndrome, and necrotizing pneumonia [5]. One of the strategies employed to triumph over the bacterial resistance is the use of a combination of drugs. The secondary metabolites of the plants are the good sources for combination of drugs to act as multidrug resistant modifiers with varied mechanisms of action [6]. Polyphenols is a prominent class of plant metabolites possesses

efficient antimicrobial action. A number of reports are available on the synergistic interactions of polyphenols with antibiotics to overcome microbial resistance like epigallocatechin gallate from green tea [7], tellimagrandin I and rugosin B from rose red (*Rosa macdub*) [8], baicalein from *Scutellaria amoena* [9], and corilagin from *Arctostaphylos uva-ursi* [10].

Rosmarinic acid (RA) is a well-known phenylpropanoid and chemically it is a dimer of caffeic acid and 3, 4-dihydroxyphenyl lactic acid, bound by an ester linkage [Figure 1]. RA belongs to the group of polyphenols. It is known for its therapeutic and cosmetic properties. It is well accounted as an antioxidant [11,12], anti-inflammatory [13,14], and antimicrobial agent [11,15,16]. RA was described to possess antimicrobial activity against wild strains of *Bacillus subtilis*, *Micrococcus luteus* and *Escherichia coli* [17], *Pseudomonas aeruginosa*, *S. aureus*, *Shigella* sp., and *Enterobacter* [15], *Candida albicans*, and *Aspergillus niger* [16]. Further, it was reported that RA possesses bactericidal activity against acne causing pathogens such as *S. aureus*, *Staphylococcus epidermidis*, and *Propionibacterium acne* through its membrane-damaging effect [18]. Previously, the synergistic effect of *Rosmarinus officinalis* extract with cefuroxime against MRSA was reported [19]. Although RA is stated to be an outstanding antimicrobial agent, its effect on MRSA is not yet studied in detail. Furthermore, there is nil report on the synergistic effect of RA with antibiotics against *S. aureus*.

Thus, the current study was designed to evaluate the antibacterial activity and to determine the minimum inhibitory concentration (MIC) of RA against *S. aureus* and MRSA. To develop RA as an adjuvant to antibiotics, the synergistic effect of RA with antibiotics against *S. aureus* and MRSA was explored. Further to understand the mechanism of action, the role of the effective synergistic combination of RA on microbial surface components recognising adhesive matrix molecules (MSCRAMM's) – surface proteins of *S. aureus* and MRSA was also studied.

MATERIALS AND METHODS

RA

RA (90% pure) was obtained from Sami Labs, Bengaluru, India.

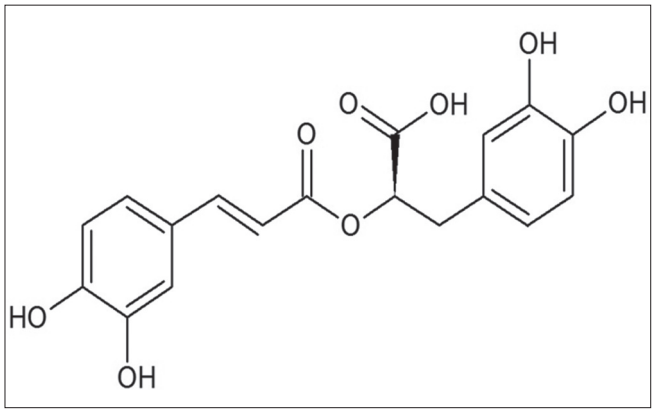

Figure 1: Structure of rosmarinic acid

Bacterial Strains and Culture Medium

S. aureus ATCC 25923 was used in the study. Clinical isolate of MRSA was obtained from Doctors Diagnostic Centre, Trichy (India), and resistance against methicillin was observed by Kirby-Bauer method. Mueller-Hinton agar (MV173) and Mueller-Hinton broth (MV391) were purchased from Hi Media Laboratories Pvt. Ltd., Mumbai, India. The bacteria were rejuvenated in Mueller-Hinton broth at 37°C for 18 h and then stocked at 4°C in Mueller-Hinton agar. Subcultures were prepared as required. The inoculum size of the bacterial culture was standardized according to the National Committee for Clinical Laboratory Standards Guideline [20]. Further, they were analyzed by Mannitol salt agar test (+), catalase test (+), fermentation and oxidation of glucose (+), DNAse test (+), and tube coagulase test (−) for the confirmation of the strain. The antibiotics amoxicillin, ofloxacin, and vancomycin were obtained from Cipla Ltd., Mumbai, India.

Evaluation of Antibacterial Potency of RA

Agar well diffusion method

Antibacterial activity was evaluated by agar well diffusion method according to the National Committee for Clinical Laboratory Standards [20]. Petri plates containing 20 ml Mueller-Hinton agar were seeded with 24 h culture of bacterial strains with 0.5 McFarland standard equivalent using spread plate method. The diameter wells of 6 mm were cut and 20 μl of RA at various concentrations were added. The plates were incubated at 37°C for 24 h and measured for antibacterial activity by observing the diameter of the zone of inhibition. The above experiment was done in triplicate to establish the statistical value.

MIC

MIC of RA was determined by microbroth dilution method as described by NCCLS, 2000 [20]. Increasing concentration of 1-10 mg/ml of RA was prepared using Mueller-Hinton broth as diluent and inoculated with 20 μl of bacterial inoculums with turbidity equivalent to 0.5 of McFarland scale. The mixture was incubated for 24 h at 37°C for the growth of bacteria. The lowest concentration at which there was no bacterial growth determined using ultraviolet spectrophotometer was taken as the MIC of RA [21].

Evaluation of Synergy with Antibiotics

Broth checkerboard method

Broth checker board method was used to find the synergism of RA with the standard antibiotics amoxicillin, ofloxacin and vancomycin against MRSA [22]. RA was taken in the microfuge tube in ascending concentration from lowest inhibitory concentration to double the concentration of MIC and was arranged in a row. All the antibiotics were prepared in a similar manner and were arranged in a column. Using the checker board, various combinations of RA with antibiotics were obtained. The minimal concentration at which there

was no growth of organism was fixed as MIC. The fractional inhibitory concentration index (FIC index) was calculated for each combination by the formula:

$FIC_A + FIC_B = FIC_I$,

Where, FIC_A = MIC of RA in combination/MIC of RA alone; FIC_B = MIC of any antibiotic in combination/MIC of antibiotic alone. If FIC index ≤0.5 – synergy; FIC index >0.5 to <1 - additive; FIC index >1 to <4 - no interaction; FIC index >4 – antagonism.

Time Kill Analysis

Time-kill kinetics was analyzed only for synergistic combinations of antibiotics and RA confirmed by checker board method [23]. Mueller-Hinton broth with combinations was inoculated with organism which was adjusted to 0.5 McFarlands standards and kept in a shaking incubator at 35°C. Samples were taken at 0, 6, 12, 24 and 30 h after inoculation. The extent of growth inhibition was analyzed at standardized $OD_{620\ nm}$.

Effect of RA on Surface Protein Fraction

Mueller-Hinton broth was inoculated with the overnight bacterial culture of *S. aureus*, MRSA, and RA. The mixture was then incubated and centrifuged to form pellet. The pellet was resuspended in 1 M LiCl in about one-tenth of the media volume. The suspension was then incubated in a shaker at 42°C for 2 h. The bacteria were pelleted and the supernatant containing noncovalently attached surface proteins to the plasma membrane was removed. The proteins were quantified by recording the absorbance at 280 nm. 1 M LiCl was used as blank. The protein fraction was then run on sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and compared with the standard proteins [24].

RESULTS

Antibacterial Activity

The antibacterial activity of RA against *S. aureus* and MRSA was evaluated by agar well diffusion method by measuring the inhibition zones. RA showed inhibition zone with 12.4 mm diameter against *S. aureus* and 11.6 mm against MRSA. Inhibition zone for RA and antibiotics were compared and shown in Table 1. MIC of RA was evaluated by broth dilution method and shown in Table 2. MIC of RA was found to be 0.8 mg/ml against *S. aureus* and 10 mg/ml against MRSA.

Synergistic Evaluation of RA and Antibiotics

To improve the antibiotic efficiency as well as to reduce the antibiotic dose, the antibacterial activity of the combinations of RA and standard antibiotics (amoxicillin, ofloxacin, and vancomycin) on susceptibility of *S. aureus* and MRSA by broth checker board method was investigated. The calculated FIC index is shown in Table 2. RA at ¼ × MIC value reduced the MIC of vancomycin, amoxicillin, and ofloxacin by ¼ times against *S. aureus*. However against MRSA, only vancomycin was found to be synergistic. All the synergistic combinations have shown FIC index value of 0.5. As MIC value for amoxicillin and ofloxacin was not observed against MRSA until the dose level of 80 μg/ml, further determinations for its MIC was not carried out and thus these two antibiotics were not studied for synergism by broth checker board method.

Time-Kill Kinetic Assay

The effect of RA, antibiotics, and synergistic combinations were studied on the bacterial growth rate by time-kill kinetic

Table 1: Antibacterial activity of RA against *S. aureus* and MRSA strains (as inhibition zones in mm)

Compounds	Diameter of inhibition zones (mm) against *S. aureus*	Concentration (volume – 20 μl)	Diameter of inhibition zones (mm) against MRSA	Concentration (volume – 20 μl)
RA	12.4±0.4	0.2 mg/ml	11.6±0.4	2 mg/ml
	15.4±0.5	0.4 mg/ml	14.2±0.2	4 mg/ml
	16.4±0.4	0.6 mg/ml	17.6±0.2	6 mg/ml
	18.4±0.6	0.8 mg/ml	20.0±0.4	8 mg/ml
Streptomycin	27±0.1	10 μg/disc	9.0±0.2	10 μg/disc
Ofloxacin	14±0.4	5 μg/disc	-	5 μg/disc
Ciprofloxacin	22±0.2	5 μg/disc	7.0±0.3	5 μg/disc
Chloramphenicol	28±0.2	30 μg/disc	20±0.5	30 μg/disc

S. aureus: Staphylococcus aureus, MRSA: Methicillin-resistant *Staphylococcus aureus,* RA: Rosmarinic acid

Table 2: MIC values and synergism of RA with antibiotics

Strains	Antibiotics	MIC of antibiotics	MIC of RA	FIC index	Interpretation
S. aureus (ATCC 25923)	Vancomycin	20 μg/ml	0.8 mg/ml	0.5	Synergy
	Ofloxacin	20 μg/ml		0.5	Synergy
	Amoxicillin	30 μg/ml		0.5	Synergy
MRSA (Clinical isolate)	Vancomycin	40 μg/ml	10 mg/ml	0.5	Synergy
	Ofloxacin	>80 μg/ml		-	-
	Amoxicillin	>80 μg/ml		-	-

S. aureus: Staphylococcus aureus, MRSA: Methicillin-resistant *Staphylococcus aureus,* RA: Rosmarinic acid, MIC: Minimum inhibitory concentration

assay. The results are shown in Figures 2-5. It was observed that the synergistic combinations of RA and antibiotics have shown better time kill kinetics as compared to RA and antibiotics alone against *S. aureus*. The synergistic combinations retained a strong effect on decreasing bacterial growth in lag phase, log phase, and stationary phase when compared with individual amoxicillin and RA as shown in Figure 2. Amoxicillin and RA alone showed stronger effect only on the stationary phase and a lower effect in lag phase of *S. aureus*. The synergistic combination of RA + ofloxacin showed a much better inhibition in the log and stationary phase compared to RA and ofloxacin individually as shown in Figure 3. RA + vancomycin combination showed a better effect than individual compounds on log phase, while in lag and stationary phase showed effects similar to singular drugs as shown in Figure 4. Similarly, on synergistic combinations against MRSA, there was marked inhibition in lag and stationary phase on comparing with vancomycin and RA individually as shown in Figure 5. The antibacterial effects of all synergistic combinations were much potent in log phase compared to the individual effects.

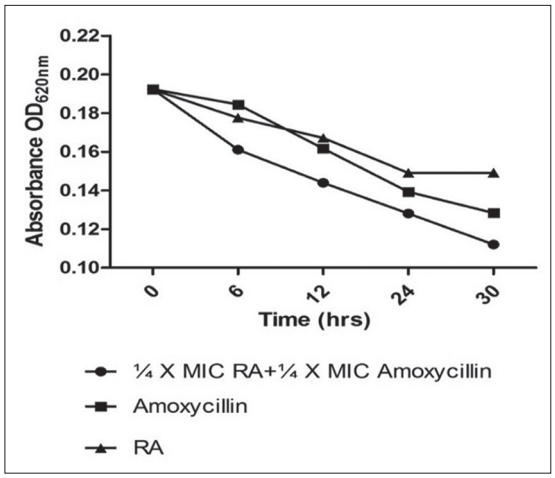

Figure 2: Time kill curve for rosmarinic acid, amoxicillin and its synergistic combination against *Staphylococcus aureus*

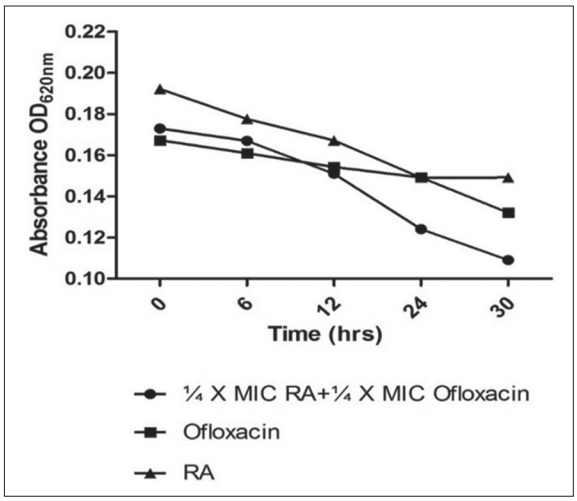

Figure 3: Time kill curve for rosmarinic acid, ofloxacin and its synergistic combination against *Staphylococcus aureus*

Effect of RA on Surface Protein Fraction of MRSA

MSCRAMMs were isolated by protein precipitation method from *S. aureus* and MRSA and were then studied by running the protein fractions on SDS-PAGE. The gel picture [Figure 6] showed expression of proteins from 40 to 90 kDa in *S. aureus* control. A single band was expressed in the RA as well as vancomycin-treated sample, whereas the RA + vancomycin combination treated sample showed no protein expression. No bands were observed. Similarly, the protein expression in MRSA control showed intense bands, which were appeared to be suppressed in vancomycin and RA alone treated samples [Lane 2 and Lane 3 in Figure 7]. The RA + vancomycin combination have shown marked reduction in number and intensity of bands when compared to control lane.

DISCUSSION

RA, a polyphenol found in higher concentration in *R. officinalis* is a well-known therapeutic and cosmetic secondary metabolite

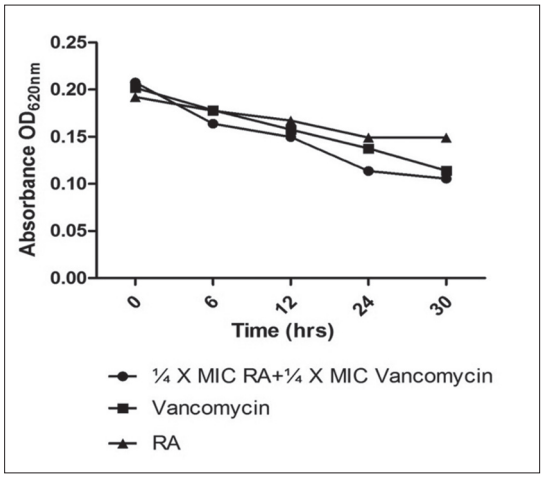

Figure 4: Time kill curve for rosmarinic acid, vancomycin and its synergistic combination against *Staphylococcus aureus*

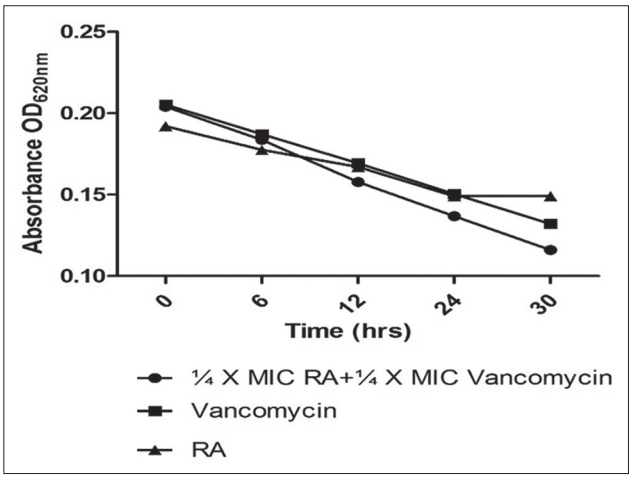

Figure 5: Time kill curve for rosmarinic acid, vancomycin and its synergistic combination against methicillin-resistant *Staphylococcus aureus*

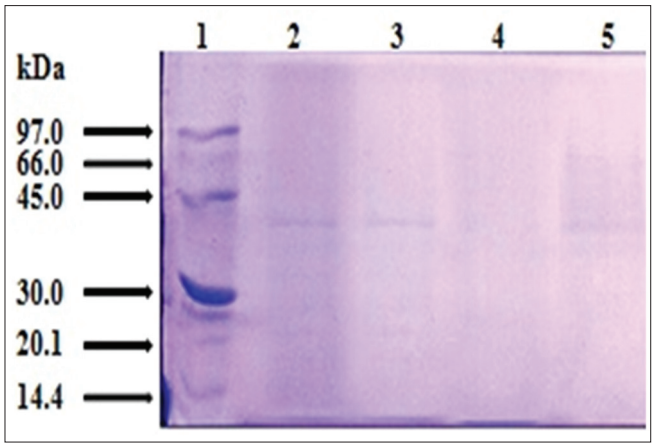

Figure 6: Sodium dodecyl sulfate - polyacrylamide gel electrophoresis analysis of microbial surface components recognizing adhesive matrix molecules in *Staphylococcus aureus*. Lane 1 - Protein marker; Lane 2 - Vancomycin; Lane 3 - RA; Lane 4 - Rosmarinic acid + Vancomycin; Lane 5 – *S. aureus* control

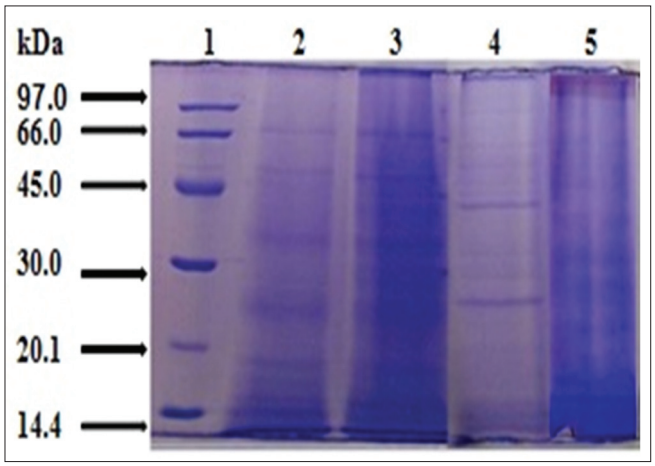

Figure 7: Sodium dodecyl sulfate - polyacrylamide gel electrophoresis analysis of microbial surface components recognizing adhesive matrix molecules in Methicillin-resistant *Staphylococcus aureus* (MRSA). Lane 1: Protein marker; Lane 2: Vancomycin; Lane 3: Rosmarinic acid (RA); Lane 4: RA + vancomycin; Lane 5: MRSA control

with antimicrobial [11,25-27] and antioxidant [11,28,29] properties. RA was reported to have antibacterial activity against *S. aureus* [11,18,30-33], whereas it was reported to be inactive up to 480 μg/ml against MRSA [26]. The efficacy of RA on MRSA was not addressed systematically. In an aim to know the actual MIC of RA against MRSA, this study evaluated the antibacterial potency of RA by measuring inhibition zones and also evaluated the MIC by broth dilution method. The MIC of RA was found to be 0.8 mg/ml against *S. aureus* and 10 mg/ml against MRSA. The MIC values were found to be on the higher side stating the lesser efficacy of RA against *S. aureus* and MRSA. There was an earlier report that the antimicrobial activity of phenolic compounds was mainly due to the inactivation of cellular enzymes which was highly dependent on the rate of penetration of the compounds into the cell or its ability to cause membrane permeability changes [34]. In the current study, the higher MIC value of RA might be due to its poor penetration capability into the bacterial cell wall. Further, as the MIC of RA against MRSA (10 mg/ml) was found to be higher in concentration, an effort was taken to study the synergistic possibilities of RA with antibiotics to develop RA as an adjuvant to the antibiotics. The results proved that RA was synergistic with the commercial antibiotics amoxicillin, ofloxacin and vancomycin against *S. aureus* and only with vancomycin against MRSA. The synergistic effect of RA was further confirmed by time-kill study, where the combinations have shown marked reduction of bacterial growth over time especially in the log phase of bacterial growth when compared with the individual antibiotics and RA against both *S. aureus* as well as MRSA. These results clearly show that RA combined with antibiotics could produce maximum bactericidal effect may be because of its higher efficacy in the log phase of bacterial growth. RA at the specified MIC was found to be synergistic with the antibiotics.

There are several virulence factors in the surface membrane proteins of bacteria. MSCRAMMs are a major virulence factor, which is present on the cell surface. They are covalently anchored transmembrane molecules in bacteria and have been shown to be a prominent antimicrobial target for antibiotics. MSCRAMMs are the major factors to cause infections as they mediate the initial host-bacterial interactions. It includes clumping factor A, protein A and fibronectin binding protein A, which are the major factor for initiating host-pathogen interactions [35,36]. Apart from MSCRAMMs cell surface proteins also contains penicillin-binding protein 2a and fmt B gene encoded protein, which is the major cause for resistance in MRSA. To study the possible mechanism of action of RA, the effect of RA on surface proteins was explored. The expression of MSCRAMM's in *S. aureus* was comparatively lesser in respect of MRSA, as resistant strains (MRSA) express a large number of surface proteins as its virulence factors. The combination of RA and vancomycin could able to suppress the expression of surface proteins (MSCRAMM's) completely in *S. aureus*, whereas its effect on MRSA was only moderate as the combination showed minimum number of intense bands when compared with its control. The results clearly indicate that the antibacterial activity of RA might be due to its action on the surface proteins MSCRAMM's of *S. aureus* and MRSA.

CONCLUSION

To conclude, RA is an antibacterial agent against *S. aureus* and MRSA, but the MIC values are on the higher side. Even then, RA may act as an adjuvant or resistant modulating agent confirmed by its synergistic effect with antibiotics. The suppression of surface proteins MSCRAMM's by RA in *S. aureus* and MRSA might be one of the mechanism responsible for its antibacterial action. However, future studies on increasing the efficacy of RA and identification of the detailed mechanism for its synergism with antibiotics has to be studied.

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