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Efficacy of Chrysin as an Adjuvant to Vancomycin Against MRSA and VRSA

Harika Mudumbai[1]*, Lakshmi Velaga[2], Sri Ramya Balijepalli[3]

[1] * Research Scholar,Department of Biotechnology, Andhra University, Visakhapatnam, Andhra Pradesh, India
harikakumar2301@gmail.com

[2]Associate Professor, Department of Human Genetics. Andhra University, Visakhapatnam, Andhra Pradesh, India
lakshmivelaga8@gmail.com

[3]Quality Analyst, Excelra Knowledge & Solutions, Hyderabad, Telangana, India
sriramyabalijepalli1@gmail.com

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ABSTRACT

The emergence of antibiotic resistance in Staphylococcus aureus, particularly Vancomycin-resistant Staphylococcus aureus (VRSA), has posed significant challenges in clinical treatment. This study investigates the potential synergistic effect of combining vancomycin with chrysin, a natural flavonoid, to combat VRSA. Employing a multi-faceted approach including disc diffusion assays, minimum inhibitory concentration (MIC) determination, and Scanning Electron Microscopy (SEM), we assessed the antimicrobial and antibiofilm efficacy of the vancomycin-chrysin combination. Our findings demonstrate enhanced antibacterial activity and significant disruption of biofilm structure, suggesting that chrysin augments the effectiveness of vancomycin against VRSA. These results offer promising insights into alternative therapeutic strategies for treating antibiotic-resistant infections.

Keywords: Antibiotic resistance,*S. aureus*, Chrysin, vancomycin resistance, Docking

Introduction

Staphylococcus aureus is a pervasive pathogen responsible for a range of serious infections in both humans and animals. In the livestock industry, it is particularly problematic, causing conditions such as mastitis in dairy animals and gangrenous dermatitis in poultry, which result in substantial economic losses and pose significant treatment challenges (Seegers et al., 2003; Ravikumar et al., 2011).

Methicillin-Resistant Staphylococcus aureus (MRSA) and Vancomycin-Resistant Staphylococcus aureus (VRSA) have emerged as major hurdles in the effective management of these infections. MRSA, resistant to methicillin and other beta-lactam antibiotics, complicates treatment due to its altered penicillin-binding protein, encoded by the mecA gene, which prevents the antibiotics from inhibiting cell wall synthesis (Grema et al., 2015). VRSA, which resists vancomycin, one of the last lines of defense against MRSA, carries genes like vanA or vanC that alter the antibiotic's target site, reducing its effectiveness (Arthur et al., 1996).

The prevalence of MRSA and VRSA in livestock not only restricts treatment options but also raises public health concerns due to the risk of zoonotic transmission to humans, particularly those in close contact with animals, such as farmers and veterinarians (Weese et al., 2006).

Challenges in Managing *S. aureus* Infections

The management of *S. aureus* infections is further complicated by the bacterium's ability to form biofilms. These biofilms are structured communities that protect the bacteria from antibiotics and the host immune system, leading to persistent and hard-to-treat infections (Melchior et al., 2006). The biofilm mode of growth significantly contributes to the chronicity of infections, necessitating prolonged and intensive treatment regimens.

Potential of Natural Compounds as Adjuvants

With the limitations of traditional antibiotics in mind, natural compounds such as **chrysin** have been investigated for their potential to enhance antibiotic efficacy. Chrysin, a flavonoid found in honey, propolis, and various plants, has demonstrated antimicrobial properties in several studies (Bhattacharya et al., 2015). Recent research suggests that chrysin can act synergistically with antibiotics, improving their ability to combat resistant bacterial strains (Amin et al., 2015; Liu et al., 2014).

This study aims to explore the combined effects of chrysin and vancomycin on MRSA and VRSA isolates from buffalo mastitis and broiler chicken gangrenous dermatitis. Specifically, it examines their impact on antibacterial and antibiofilm activities, proposing chrysin as a promising adjuvant in treating resistant *S. aureus* infections.

Materials &Methods

Sample Collection and Isolation

Sample Sources:130 Clinical samples were collected from buffaloes diagnosed with mastitis and 86 samples from broiler chickens with gangrenous dermatitis across twelve districts in Andhra Pradesh. The selection of these sites was based on the regions' high prevalence of livestock diseases and the need for diverse sampling to understand the distribution of *S. aureus* infections.

Sample Collection:

- **Buffalo Mastitis Samples**: Milk samples were collected aseptically from the mammary glands of buffaloes showing clinical signs of mastitis. Using sterile techniques, approximately 10 mL of milk was drawn directly into sterile collection tubes to avoid contamination.
- **Broiler Chicken Samples**: Swabs were taken from lesions of broiler chickens exhibiting symptoms of gangrenous dermatitis. Sterile cotton swabs were used to collect samples by gently rotating them over the infected areas.

Transportation and Storage:

- Samples were immediately placed in sterile containers and transported to the laboratory at 4°C to preserve their integrity and viability.All samples were processed within 24 hours of collection to ensure the accuracy of subsequent microbiological analyses.

Isolation of *S. aureus*:

Samples were collected and cultured on Mannitol Salt Agar (MSA) plates, which is a selective medium used for isolating ***S. aureus*** due to its high salt concentration. The plates were incubated at 37°C for 24-48 hours (Quinn et al., 2000). Colonies that exhibited yellow coloration on the MSA plates were selected for further analysis. The yellow coloration indicates mannitol fermentation, which is characteristic of ***S. aureus***.

Isolates were then confirmed as ***S. aureus*** through Gram staining and a series of biochemical tests:

- **Gram Staining**: Isolates were stained to observe Gram-positive cocci in clusters.

- **Catalase Test**: A drop of 3% hydrogen peroxide was added to the bacterial culture. The presence of bubbling indicated a positive reaction for catalase production (Raus & Love, 1983).
- **Coagulase Test**: The ability of the isolates to clot rabbit plasma was tested. Clot formation confirmed coagulase production, a distinguishing feature of ***S. aureus*** (Devriese et al., 1985).

Qualitative Assessment: Disc Diffusion Assay

The disc diffusion assay was performed to evaluate the antimicrobial activity of vancomycin, amoxicillin-clavulanic acid, penicillin, linezolid, and chrysin, both individually and in combination.

- **Antibiotic and Disc Preparation**:
    - Vancomycin (30 µg/mL), amoxicillin-clavulanic acid (30 µg/mL), penicillin (10 µg/mL), and linezolid (30 µg/mL) were procured from Sigma-Aldrich.
    - Chrysin was prepared as a stock solution of 10 mg/mL in DMSO and diluted to 10 µg/mL for use.
    - Antibiotic discs were prepared using Whatman filter paper and loaded with either the antibiotics alone or in combination with chrysin.

- **Inoculation and Assay**:
    - ***S. aureus*** cultures were standardized to the 0.5 McFarland standard (approximately 1.5 x 10^8 CFU/mL).
    - Cultures were then spread onto Mueller-Hinton Agar (MHA) and Congo Red Agar (CRA) plates.
    - The prepared antibiotic discs were placed on the inoculated plates, which were then incubated at 37°C for 18 hours.
    - Zones of inhibition around the discs were measured to determine antimicrobial activity (CLSI, 2018).

Quantitative Assessment: Minimum Inhibitory Concentration (MIC) Determination

The MIC of vancomycin and chrysin, alone and in combination, was determined using a microtiter plate method.

- **Microtiter Plate Setup**:
    - 96-well microtiter plates were prepared with varying concentrations of vancomycin and chrysin presented in table 2
    - The bacterial inoculum was standardized to the 0.5 McFarland standard and added to each well.
    - Plates were incubated at 37°C for 24 hours.
    - Turbidity was observed to determine the MIC, defined as the lowest concentration inhibiting visible bacterial growth (Christensen et al., 1985).

Scanning Electron Microscopy (SEM)

SEM was employed to assess the impact of the antibiotic combination on the morphology and structure of bacterial biofilms.

- **Sample Preparation**:
    - After MIC determination, ***S. aureus*** cells were fixed in 2% formaldehyde.
    - The cells were then washed with PBS and dehydrated using increasing concentrations of ethanol.
    - Samples were coated with gold particles and mounted on SEM stubs.
    - SEM imaging was performed to observe changes in bacterial biofilm morphology and structure.

Results & Discussion

1. Qualitative Assessment: Disc Diffusion Assay

Antimicrobial Activity: The combination of vancomycin and chrysin showed significantly larger zones of inhibition compared to vancomycin alone across various *S. aureus* isolates on MHA and CRA media plates (Figures I and II respectively). This suggests enhanced antimicrobial activity on MHA media and antibiofilm activity on CRAmedia when the two agents are used together. The increase in zone of inhibition values were represented in Table 3.
Notably, the combination of vancomycin and chrysin consistently produced larger inhibition zones compared to vancomycin alone, indicating a synergistic effect.

2. Quantitative Assessment: MIC Determination
MIC Values: Table 4show the MIC values for vancomycin and chrysin, alone and in combination, against *S. aureus* isolates. The combination of vancomycin with chrysin significantly reduced the MIC for most isolates, further supporting the enhanced efficacy of the combined treatment.

3. SEM Analysis
Biofilm Disruption: SEM images revealed significant disruptions in biofilm structure when *S. aureus* was treated with the vancomycin-chrysin combination compared to vancomycin alone. Figures III shows the intact biofilm structures of untreated controls and those treated only with vancomycin. In contrast, Figure IV illustrates the disrupted biofilms with the combined treatment, indicating a potent antibiofilm effect.

Figures

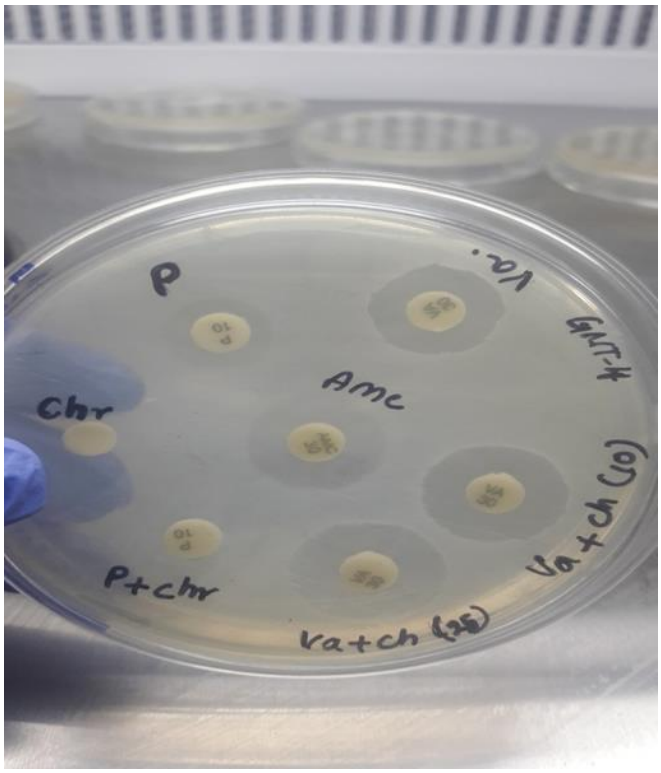

Figure 1 Qualitative assessment of antimicrobial activity of chrysin with vancomycin in VRSA isolates on MHA plate

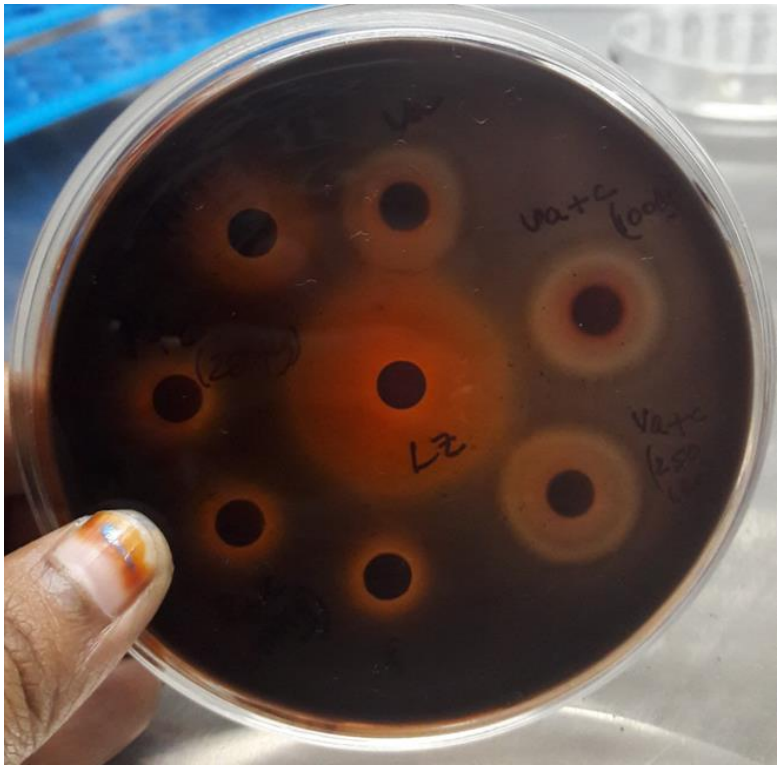

Figure 2 Qualitative assessment of antibiofilm activity of chrysin with vancomycin in VRSA isolates on CRA plate

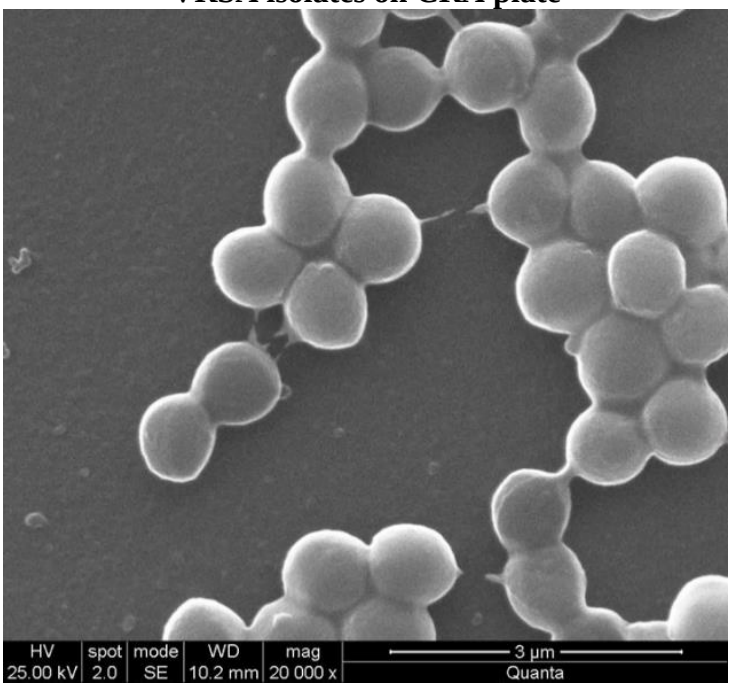

Figure 3 SEM analysis of *S. aureus* isolates exposed to only vancomycin (Control)

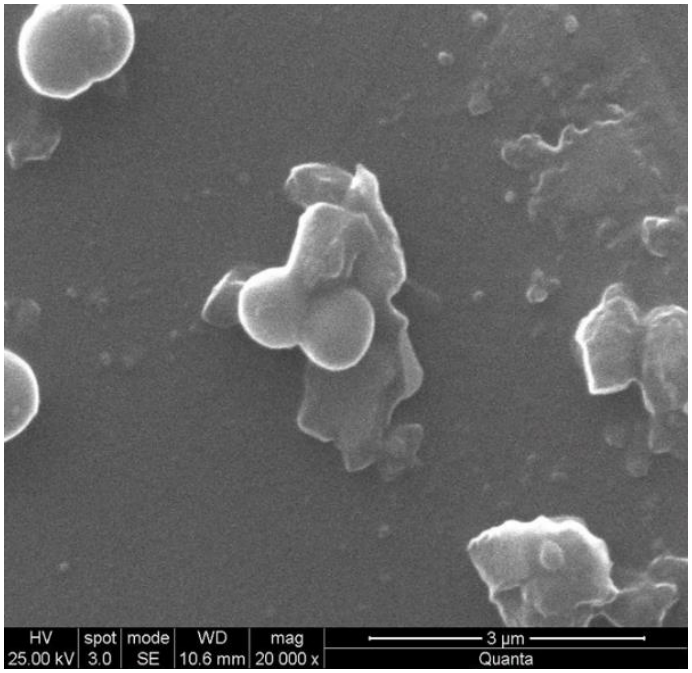

Figure 4 SEM analysis of*S. aureus* isolates exposed to the combination of vancomycin along with chrysin

Tables

Table 1 Different concentrations of Antibiotics used

Antibiotic	**Concentration (µg/mL)**
Vancomycin	30
Vancomycin + Chrysin (100)	30 + 100
Vancomycin + Chrysin (250)	30 + 250
Penicillin	10
Penicillin + Chrysin (100)	10 + 100
Penicillin + Chrysin (250)	10 + 250

Table 2 Different Concentrations of Vancomycin and Chrysin Used

	Drug Combinations	**1 µg/ml**	**2 µg/ml**	**3 µg/ml**	**4 µg/ml**
A	Vancomycin	25	12.5	6.25	3.13
	Chrysin	6.25	6.25	6.25	6.25
B	Vancomycin	25	25	25	25
	Chrysin	6.25	3.13	1.57	0.79
C	Vancomycin	25	12.5	6.25	3.13
	Chrysin	25	12.5	6.25	3.13
D	Only Vancomycin	25	12.5	6.25	3.13
E	Only Chrysin	25	12.5	6.25	3.13

Table 3 Increase in Zone of inhibition on MHA and CRA media

Isolate	Vancomycin on MHA	Vancomycin + chrysin on MHA	Vancomycin on CRA	Vancomycin + chrysin on CRA
GNT 2	**9**	**13**	**11**	**13**
GNT 4	**10**	**12**	**7**	**11**
GNT 24	**13**	**14**	**14**	**17**
GNT 25	**10**	**11**	**16**	**17**
AG 26	**14**	**16**	**14**	**16**
AG 31	**8**	**11**	**15**	**17**
AG 32	**7**	**8**	**15**	**19**
PDR 2	**17**	**18**	**6**	**7**
PDR 6	**9**	**13**	**17**	**18**

Table 4 Decrease in MIC values of Vancomycin + Chrysin compared to Vancomycin alone

Isolate	Vancomycin MIC (µg/mL)	Vancomycin + Chrysin MIC (µg/mL)
GNT 2	25	6.25 + 6.25
GNT 4	25	6.25 + 6.25
GNT 7	25	6.25 + 6.25
GNT 9	25	12.5 + 12.5
GNT 14	25	6.25 + 6.25

Conclusions

The results of this study underscore the potential of combining vancomycin with chrysin to combat VRSA. The enhanced antimicrobial and antibiofilm activities observed suggest that chrysin can potentiate the efficacy of vancomycin. This combination could be a promising therapeutic strategy, reducing the reliance on high doses of vancomycin and potentially overcoming resistance mechanisms. Further research, including in vivo studies and clinical trials, is warranted to validate these findings and explore the clinical applications of this combination therapy.

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