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Research Article

IN-VITRO SYNERGISTIC INTERACTIONS OF BAICALEIN IN COMBINATION WITH β-LACTAM ANTIBIOTICS AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

Srishti Soni[1*], Reena Mukherjee[1], Ujjwal Kumar De[1], Deeksha Bharti[1], Babul Rudra Paul[1]
Jitendra Singh Gandhar[1], Varun Kumar Sarkar[1], Amit Kumar[2]

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ABSTRACT: The pressing issue of the moment affecting public health is antimicrobial resistance. Methicillin-resistant *Staphylococcus aureus* (MRSA) is an emerging pathogen that is proven to be resistant to a range of antibiotics like penicillins, cephalosporins, other β-lactam antibiotics, and carbapenems. Baicalein, a phytochemical of the traditional Chinese herb, *Scutellaria baicalensis* has an inhibitory effect on different bacteria. The current study aimed to investigate the synergistic effect of baicalein and β-lactam antibiotics (amoxicillin and ampicillin) against methicillin-resistant *S. aureus* (MRSA). The minimum inhibitory concentrations (MICs) of baicalein and β-lactam antibiotics (amoxicillin and ampicillin) against the MRSA were determined through the broth dilution method. The determination of synergism was tested by checkerboard tube dilution and time-kill assay procedure. The combination of baicalein with amoxicillin showed more potent synergy against MRSA strain compared to the combination of baicalein and ampicillin. The fractional inhibitory concentration indices (FICI) of amoxicillin + baicalein varied from 0.19 to 0.50. The fractional inhibitory concentration (FIC) indices of ampicillin + baicalein varied from 0.19 to 0.25. These findings offer significant evidence that the amoxicillin combined with baicalein showed potent synergistic activity against MRSA *in vitro* and might offer promising therapeutic implications for MRSA infections.

Keywords: Antibiotic, Baicalein, β-lactam, MRSA, Synergetic activity.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is the most prevalent bacterial organism which causes the most severe form of cow mastitis and poses a significant challenge to clean milk production [1]. *S. aureus* mastitis causes significant economic losses in terms of a sharp drop in milk revenue, elimination of superior animals, higher veterinary care expenses, and the need to replace tainted milk [2, 3]. Even though antibiotics are the cornerstone of treatment for *S. aureus* mastitis, the excessive use of proven antimicrobial agents in the management of *S. aureus* mastitis has culminated in a dramatic rise in the number of antibiotic-resistant pathogenic microorganisms and the poor production and introduction of new antimicrobial medications poses a serious threat to the health of the dairy animal [4]. It is found that control of microbial infections becomes more difficult every day due to biofilm formation and gaining resistance even before facing the antibacterial substances [5].

MRSA is generated when the *S. aureus* susceptible to methicillin acquires the mecA gene (the penicillin-binding protein structural gene, PBP2a), which helps the bacteria sustain the synthesis of the cell wall. Acquisition of the mecA gene induces tolerance to methicillin and reduces its sensitivity to β-lactams [6].

[1]Division of Medicine, [2]Division of Animal Genetics, ICAR-Indian Veterinary Research Institute, Izatnagar-243122, Bareilly, Uttar Pradesh, India.
**Corresponding author. e-mail: srishusoni987@gmail.com*

There are already significant indications that MRSA strains are immune to the final antibiotic defense line, contributing to the search for other effective methods to combat MRSA [7, 8]. Drug formulations provide a successful method for eliminating processes of bacterial resistance and restoring antibiotic efficacy. Baicalein, a plant-based flavonoid is well known for its potent ability to suppress *S. aureus* quorum sensing and biofilm development. [9]. Liu *et al.* (2020) demonstrated that the combined therapy of linezolid and baicalein showed significantly greater antimicrobial properties against MRSA biofilms compared to either drug alone. This combination was able to effectively reduce the bacterial load in the biofilms and inhibit biofilm formation [10]. It was also observed that baicalein could also decrease the minimum inhibitory concentration (MIC) of several conventional antimicrobials. Given the significant bacteriostatic properties of plant ingredients and the remarkable outcomes of current combination therapies, we assessed baicalein synergistic *in vitro* activities with two β-lactam antibiotics, amoxicillin and ampicillin, to determine whether such combination therapy could improve the antibacterial activity of β-lactam antibiotics that are usually MRSA resistant.

The minimum inhibitory concentrations (MICs) of baicalein and β-lactam antibiotics, amoxicillin, and ampicillin against the Methicillin-resistant *Staphylococcus aureus* were determined through the broth dilution method. The determination of synergistic activity was assessed by checkerboard tube dilution method via measurement of fractional inhibitory concentration indices of combination (FICIs) of baicalein with amoxicillin and ampicillin and time-kill technique.

MATERIALS AND METHODS

Procurement of drug and plant metabolite

Powders of amoxicillin and ampicillin and baicalein were purchased from Sigma-Aldrich, MO, USA. The Mueller-Hinton agar (MHA) and Mueller-Hinton broth (MHB) were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India.

Source of bacteria and its revival

The Methicillin-resistant *S. aureus* (MRSA; *S. aureus* R1/BOV-ACCESSION NO-KX181857) was isolated earlier from mastitis-affected dairy animals of a dairy farm of IVRI by our laboratory and kept as 30% glycerol stock and stored at -80°C. The bacteria were sub-cultured on Mannitol salt agar (Sigma Aldrich) plates followed by on tryptic soya agar overnight at 37°C. The colonies were transferred into 500 µL sterile phosphate buffer saline (PBS) and rotated at 3000 rpm. The cell pellet was washed two times and finally, the suspension was matched with the McFarland 0.5 and the density of 1x105 colony forming unit/mL was adjusted.

Antibiotic sensitivity test and determination of MIC

The ABST of antibiotics against MRSA was performed as per the guidelines of the Clinical Laboratory Standards Institute [11]. According to CLSI, 2018 guidelines, the antibiotics used in this study were chosen based on the most commonly prescribed and approved treatments for intramammary infection in bovine mastitis in the study region. Antibiotics used were ampicillin (10 µg/disk), cefoxitin (30 µg/disk), cefalexin (30 µg/disk), amoxicillin (30 µg/disk), penicillin G (10 IU/disk), cef-tazo (10 µg/disk), methicillin (10 IU/disk), clindamycin (2 mcg/disk) (Fig. 1). The broth dilution method was used to calculate the Minimum Inhibitory Concentration (MIC) in MHB [12]. Overnight broth culture was diluted 1:100 (10 µl in 990 µl of fresh broth). For broth dilution, a series of tubes numbered 1 to 10 were aseptically dispensed with 950 µl of fresh broth, and then 50 µl of respective amoxicillin dilutions (64 µg/mL to 0.25 µg/mL) were added from tubes 1 to 9 and tube number 10 was kept as control. 10 µl of 1:100 diluted broth cultures was added to all the tubes. Tubes were kept for incubation overnight at 37ºC. Similarly, the MIC was performed for ampicillin (64 µg/mL-0.25 µg/mL). While plate dilution method was performed for the determination of MIC of Baicalein (8000 µg/mL - 500 µg/mL) (Fig. 2).

Evaluation of synergy

The synergistic activities of baicalein combined with antibiotics (amoxicillin and ampicillin) were examined separately by using the checkerboard technique (broth microdilution). The cumulative effect is calculated by using measurements of the MIC to calculate the FIC Index as follows:

The FICI was calculated as $FICI = FIC_A + FIC_B$

Where $FIC_A = C_A/MIC_A$, $FIC_B = C_B/MIC_B$

C_A = MIC of drug A in combination,

C_B = MIC of drug B in combination,

MIC_A = MIC of drug A alone,

MIC_B = MIC of drug B alone.

Mean FICI = Sum of FICI calculated / Number of FICI calculated.

The following formula was used to calculate the fractional inhibitory concentration indices (FICIs) for each combination:

FICI = MIC of drug A in the combination/MIC of drug A alone + MIC of drug B in the combination/ MIC of drug B alone.

The results of antibacterial effects of these combinations were categorized as synergistic (mean FICI < 0.5), additive (mean FICI > 0.5 and < 1.0), indifferent (mean FICI 1.0 to 2.0), or antagonistic (mean FICI > 2.0) according to the Schwarz *et al.* (2020) [13].

Time kill assay

Time kill assay is one of the most appropriate methods to quantify the bactericidal effect as well as to detect the synergism or antagonism between different drugs. Time-kill curves were conducted in triplicate by inoculation of MRSA at the concentration of 5 × 105 cfu/mL of into 3.0 mL of fresh cation-adjusted Mueller-Hinton broth. For every strain, amoxicillin was added at concentrations of 1×, 2×, and 4× MIC. 10µl aliquots of broth were sampled at 0, 2, 4, 6, 8, 24, and 48h of incubation, for determination of bacterial counts. The number of cfu/mL was measured after each aliquot was diluted in series onto MH agar plates in duplicates and incubated for 18-24 h overnight at 37ºC [14].

RESULTS AND DISCUSSION

Antimicrobial sensitivity testing and MIC

In ABST, clindamycin has shown a significant zone of inhibition, and ampicillin and amoxicillin exhibited weak *in vitro* activities against the clinical isolates of MRSA (Fig. 1). The MICs of ampicillin, amoxicillin, and baicalein against MRSA bacteria were summarized in Table 1. The baicalein could inhibit the growth of MRSA, and the MIC of it was 1000 µg/mL whereas, the MICs of ampicillin and amoxicillin were 64 µg/mL and 32 µg/mL respectively.

FICIs of amoxicillin-baicalein and ampicillin-baicalein combination

The combination of BC with 500 µg/ml has shown a partial synergistic or additive effect with a 50% reduction in MIC of amoxicillin (16 µg/ml) (FICI=1). However, a 25% reduction in MIC of amoxicillin (8 µg/mL) was seen for the combination of baicalein (250 µg/mL) revealing a synergistic effect with FICI equal to 0.5. Further reduction in MIC was remarkable with 125 µg/mL concentration of baicalein showing observable synergistic activity (FICI <0.5). There was a 25% and 50% reduction in MIC of ampicillin seen with the concentration of 750 and 500 µg/mL of baicalein with partial synergistic or additive activity respectively. The baicalein with concentrations of 250 and 125 µg/mL showed a reduction in MIC of ampicillin with evidence of synergistic effect (FICI <0.5) (Table 1).

Herbal phytochemicals have the unique characteristic of presenting customers with synergistic benefits of metabolites stored inside extracts and also generate results in different systems. This heterogeneity is also recognized in the biological mechanisms that various phytochemicals have on multidrug-resistant bacteria [15]. Studies found that flavonoid compounds have a definite pharmacological activity and found synergistic potential when combined with several antibiotics [16, 17, 18]. In the present investigation, the checkerboard and time-kill testing protocols were used to examine the *in vitro* ability of baicalein in combination with ampicillin and amoxicillin to kill MRSA. The antibacterial activity of β-lactams on trans peptization is attributed to the attachment of penicillin-binding proteins (PBPs) leading to the inactivation of its transpeptidase activity, therefore lysis and cell killing is assumed to decline in peptidoglycan formation and rise in autolysin [19]. Two established mechanisms of *Staphylococci* for resistance to beta-lactam antibiotics are the biosynthesis of beta-lactamases, enzymes that breakdown beta-lactams via hydrolytic reaction, and the regulation of penicillin-binding protein 2a (PBP 2a), which is resistant to inhibition by beta-lactam group of antibiotics [20]. The main goal was therefore to identify successful anti-MRSA medications that could curb the resistance of antibiotics to avoid or eliminate MRSA infections. Here the effectiveness of β-lactam antibiotics (amoxicillin and ampicillin) in conjunction with variable concentrations of baicalein was examined against MRSA to assess if the antibacterial function of the combination was more effective than each antibiotic independently.

Time kill assay

When 5×10^5 colony-forming units (CFU)/mL of MRSA was incubated with baicalein at 1x, 2x, and 4x concentrations of MIC, the bacterial growth was inhibited to 4.6, 4.2, and 3.8 $\log_{10}$ CFU/mL at 48 h of incubation from 0 h. When the same inoculum of MRSA was incubated with Amoxicillin at 1x, 2x, and 4x concentrations of MIC, the bacterial growth was inhibited to 6, 5.4, and 5 $\log_{10}$ CFU/mL at 48 h of incubation from 0 h. When MRSA of this inoculum

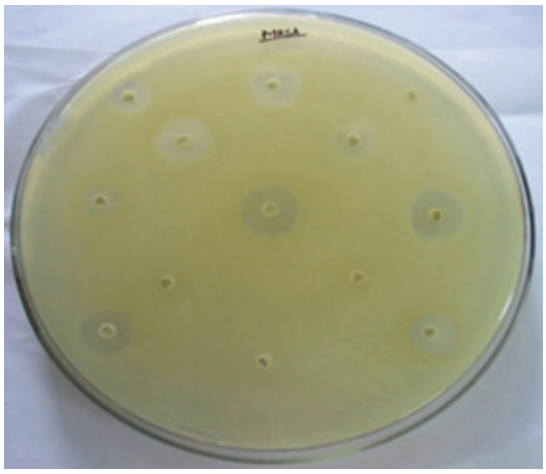

Fig. 1. Antibiotic susceptibility pattern of MRSA isolates.

[The antibiotics were used: clindamycin (2 mcg/disk), amoxicillin (30 µg/disk), cefoxitin (30 µg/disk), cefalexin (30 µg/disk), ampicillin (10 µg/disk), penicillin G (10 IU/disk), cef-tazo (10 µg/disk), gentamicin (10 µg), sulphamethoxazole/trimethoprim (25 µg), vancomycin (30 µg), sulphamethoxazole/trimethoprim (25 µg), and vancomycin (30 µg)].

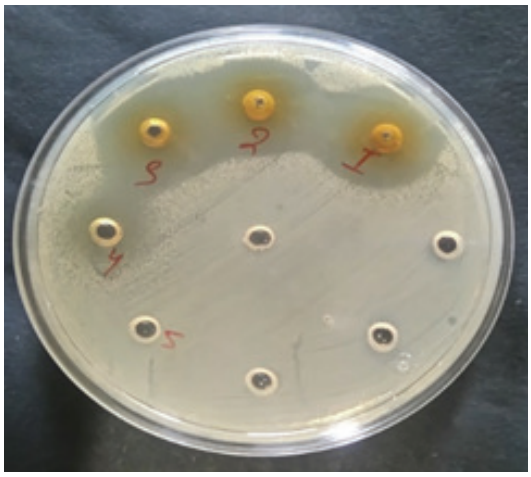

Fig. 2. Minimum Inhibitory Concentration (MIC) of Baicalein using plate dilution method.

[1st well: 8000µg/ml, 2nd: 4000 µg/ml, 3rd: 2000 µg/ml, 4th: 1000 µg/ml, 5th (Control): 500 µg/ml]

Table 1. Minimum inhibitory concentrations (MICs; µg /mL) of antibiotics and baicalein and FICI of their combination against Methicillin-resistant *Staphylococcus aureus*.

Drugs/ Plant metabolite		MIC of drug Combination		FICI	Effect	MIC of drug Combination		FICI	Effect
Name	Individual MIC	AMX	Baicalein			AMP	Baicalein		
AMX	32	16	500	1	A	32	750	1.25	A
AMP	64	8	250	0.50	S	16	500	0.75	A
Baicalein	1000	4	250	0.25	S	8	250	0.25	S
		2	125	0.19	S	4	125	0.19	S

[AMX = Amoxicillin; AMP = Ampicillin; FICI = fractional inhibitory concentration index; S = Synergy; A = Additive].

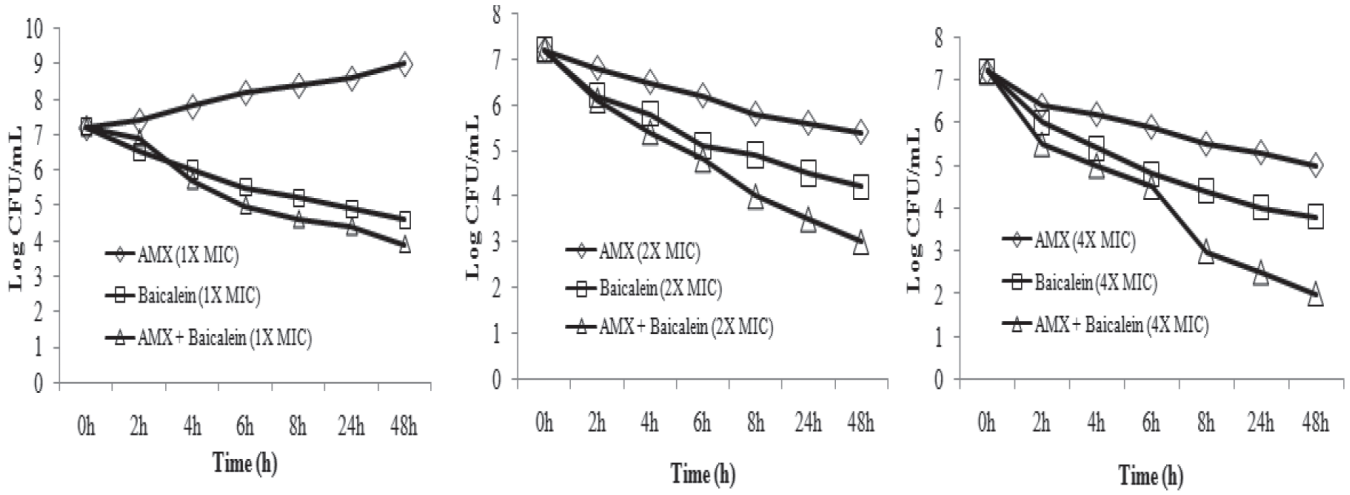

Fig. 3. Mean log reduction of CFU of MRSA by time kill kinetics assay of 1X, 2X and 4X MIC of amoxicillin (AMX), baicalein and both AMX + baicalein. [Results are expressed as log10 CFU/mL at different time intervals (h=hour)].

size was incubated with the combination of baicalein (1x MIC) and amoxicillin (1x MIC) and baicalein (2x MIC) and amoxicillin (2x MIC), the inhibitory effect against MRSA persisted for at least 48 h, and at this time point, the colony count was approximately decreased to 3.9 and 3 $\log_{10}$ than the starting inocula. Interestingly, while 5×105 CFU/mL of MRSA was incubated with the combination of baicalein (4x MIC) and amoxicillin (4x MIC), the inhibitory effect against bacteria persisted for at least 48 h, and at this time point, the colony count reached approximately 2 $\log_{10}$ lower than the starting inocula and approximately 2 $\log_{10}$ lower than when baicalein, the more active single agent, was used alone (Fig. 3).

The findings of the time-kill test protocol show that the baicalein and amoxicillin combination at different doses relative to ampicillin significantly inhibited the development of MRSA. The gradual decrease in the cell viability method has demonstrated that the formulation of amoxicillin and baicalein will overcome β-lactam antibiotic resistance in MRSA. Additionally, in the checkerboard titration technique, Baicalein demonstrated a synergistic phenomenon with amoxicillin as compared to ampicillin by a significant reduction in MIC. Therefore, the present study indicates that baicalein improves the antibiotic activity of β-lactam against MRSA whereas potentiation with amoxicillin is noted. From earlier studies, it has been reported that the synergistic activity of β-lactam-baicalein combinations against *S. aureus* takes place from three different types of action. The first of these is due to a weak to moderate direct antibacterial activity of baicalein on cell growth. Secondly, the mechanism derives from the capacity of baicalin to prevent the β-lactamase hydrolysis of susceptible penicillins and hence restore cell sensitivity to antibiotics. Thirdly, the mechanism involves an effect against MRSA, which is non-dependent β-lactamase inhibition but may well be associated with the inhibitory interactions between β-lactams and penicillin-binding proteins [21, 22].

Thirdly, the mechanism involves an effect against MRSA that may be related to the inhibitory interactions between β-lactams and penicillin-binding proteins but is not reliant on β-lactamase inhibition [21, 22].

CONCLUSION

From this study, it can be concluded that plant flavone baicalein has a significant antibacterial effect on MRSA strain, and such activity potentiated β-lactam antibiotic activity against MRSA. However, an *in vivo* toxicity assessment of the current compound is necessary before developing it as a potential alternative to β-lactam therapies against MRSA.

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