



## RESEARCH ARTICLE

### Drug Resistance Modulation of Dairy MRSA through Berberine, Artesunate and Quercetin in Combination with $\beta$ -Lactams

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#### ABSTRACT

The continued use of antibiotics is bringing extensive resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) of dairy animals and demands inclusion of non-antibiotic sources in treatment protocols. The current study aims to explore antibacterial potential of berberine (BR), artesunate (AR), and quercetin (QT) and extent of their resistance modulation capacity in combination with  $\beta$ -lactam antibiotics against MRSA. For this study, a total of 674 milk samples from dairy cows were collected for isolation of MRSA and thereafter phytochemicals (drugs) alone and in combination with  $\beta$ -lactams (penicillin, oxacillin, cefoxitin, ampicillin) were evaluated against MRSA. The synergy testing showed all synergistic effects except AR with penicillin which was additive effect. The synergism of antibiotics and phytochemicals was further confirmed by time-kill assay. The killing kinetics revealed a zero bacterial count at 16h of incubation in all combinations. Additionally, the killing synergy showed a significant decline i.e. less than 60% in case of BR in combination with oxacillin and penicillin at the initial 2h of incubation followed by AR and QT in combination with ampicillin. The study thus revealed potential antibacterial effects of berberine, artesunate, and quercetin along with significant resistance modulation in terms of highly synergistic combinations with antibiotics focused on novel insights into alternatives to antimicrobials to pave the road for antimicrobial stewardship against ailments like mastitis.

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#### INTRODUCTION

Methicillin-Resistant *Staphylococcus aureus* (MRSA) refers to *Staphylococcus aureus* strain that has developed resistance to methicillin and other beta-lactam antibiotics. MRSA strains have been further established as hospital-acquired (HA-MRSA), community-associated (CA-MRSA), and livestock-associated (LA-MRSA) and hence, contributing to the complexity of the challenge (Shoaib *et al.*, 2023). The later has particular transmission potential at human and animal interface causing sever health issues particularly when people are working in close contact while

dealing with animals (Roy *et al.*, 2024). The non-judicious use of antibiotics, particularly the over-prescription, patient non-compliance, and inappropriate agricultural use has resulted in development of new resistant mechanisms in the bacterial strains leading to limited options of antibiotic treatment (Pinheiro *et al.*, 2020). The genetic basis of MRSA resistance are associated with the synthesis of penicillin-binding protein 2a (PBP2a) which reduce the affinity for  $\beta$ -lactam antibiotics and rendering this antibiotic class largely ineffective (Alghamdi *et al.*, 2023). In such scenarios, alternative to antimicrobials is a real need of the hour inclusive of which are traditional medicine.

Ayurvedic and Chinese medicine have a rich history of utilizing berberine, a quaternary ammonium salt derived from plants, possessing remarkable ability to combat a diverse array of microbes with antibacterial properties (Wang *et al.*, 2008). The antibacterial mechanism of berberine involves damaging bacterial cell membranes, inducing the generation of reactive oxygen species (ROS), reducing intracellular adenosine triphosphate (ATP) levels and limiting biofilm production (Li *et al.*, 2024). Similarly, the artemisinin, an active component of sweet wormwood has proven effectiveness against malaria and exhibits properties with anti-tumor and anti-inflammatory attributes (Dziedzic *et al.*, 2015). The antibacterial mechanisms of artesunate (derivative of artemisinin) include increasing antibiotic accumulations via the inhibition of efflux pump and inhibiting the *agr* system involved in biofilm formation (Qian *et al.*, 2021). Lastly, quercetin being a notable polyphenol express its antibacterial mechanism by disrupting cell walls and membranes of bacteria, inhibition of biofilm formation, reduction of virulence factors, inhibition of efflux pumps, interference with ATP synthesis, inhibition of enzyme activities and nucleic acid synthesis (Yang *et al.*, 2020).

Keeping in view the increasing resistance in MRSA and proven antibacterial potential of phytomedicine candidates, it was desirable to explore the interaction of plant-based drugs with antibiotics to pave a road to formulate alternative effective therapeutic regimens. Thus, this study focuses on evaluation of antibacterial potential of berberine, artesunate, and quercetin, and their resistance modulation in combination with selected  $\beta$ -lactam antibiotics.

## MATERIALS AND METHODS

**Ingredients and their preparation:** The phytochemicals; Berberine chloride (BR) (BBI Life Sciences, Shanghai, China), Artesunate (AR) (Macklin, Shanghai, China) and Quercetin (QT) (Sigma-Aldrich, USA), and antibiotics; Penicillin G sodium salt (P), Cefoxitin sodium salt (CTX), Oxacillin sodium (OXA) and Ampicillin sodium salt (AMP) were purchased from Sigma-Aldrich, USA. All antibiotics and phytochemicals were dissolved and diluted following the guidelines of Clinical and Laboratory Standards Institute.

**Isolation of MRSA and confirmation of drug resistance:** Milk samples were collected from 674 dairy cows (n= 2696 quarter samples) from multiple dairy farms located in Faisalabad, Pakistan. The samples were initially screened for subclinical mastitis by Surf Field Mastitis Test (SFMT) (Muhammad *et al.*, 2010) and shifted to Laboratory of Veterinary Preventive Medicine and Public Health, Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan. The Milk samples were subjected to microbiological and biochemical examination for the isolation of *S. aureus*. The phenotypically identified *S. aureus* isolates were further confirmed by PCR amplification of 279 bp of the *nuc* gene using the forward P1: 5'-GCGATTGATGGTGATACG GTT-3' and reverse primer P2: 5'-AGCCAAGCCTTGAC GAATAAGC-3' (Sahebnaasagh *et al.*, 2014). The clinical strains (CS) of MRSA from *S. aureus* were

confirmed by targeting the *mecA* gene using the forward P1: 5'-ACTGCTATCCACCCTCAAAC-3' and reverse primer P2: 5'-CTGGTGAAGTTGTAATCTGG-3' with a product size of 163 bp (Sahebnaasagh *et al.*, 2014). For this purpose, 25  $\mu$ L reaction mixture (1 $\mu$ L of each P1 and P2 primer, 2  $\mu$ L of template DNA, 12.5  $\mu$ L of Master Mix (Takara), and 8.5  $\mu$ L of purified water) was run in thermocycler as per condition optimized by Aqib *et al.* (2017). PCR amplicons were run on 2% agarose gel and bands were visualized under a UV light.

### Modulating the $\beta$ -lactam resistance with Berberine, Artesunate, and Quercetin

**Broth micro-dilution assay:** The minimum inhibitory concentration (MIC) of antibiotics and phytochemicals was determined by broth micro-dilution assay following the standards of The European Committee on Antimicrobial Susceptibility Testing (EUCAST). Firstly, 100  $\mu$ L of 4x Mueller Hinton broth (MHB) were added to each well of a microtiter plate. Then 100  $\mu$ L aliquot of working solutions (512  $\mu$ g/mL) of antibiotics and (1024  $\mu$ g/mL) of phytochemicals were added in first well of the microtiter plate and subsequently two-fold serially diluted with MHB from 1st well to 10<sup>th</sup> well. Afterwards, 100  $\mu$ L of overnight MRSA culture at a final concentration of  $1 \times 10^5$  CFU/mL were added from 1<sup>st</sup> to 11<sup>th</sup> well keeping 11<sup>th</sup> and 12<sup>th</sup> well as positive and negative controls, respectively. The plates were incubated for 24 hours at 37°C, and the minimum inhibitory concentration (MIC) was calculated according to the guidelines of EUCAST (Wojtyczka *et al.*, 2014).

**Well diffusion assay:** The antibiotics and phytochemicals against MRSA isolates were tested through well diffusion method. For this purpose, positive control (tigecycline 16  $\mu$ g/mL), negative control (normal saline), phytochemicals alone (512  $\mu$ g/mL for BR, 1024  $\mu$ g/mL for QT and AR), antibiotic alone (128  $\mu$ g/mL), and a combination of each phytochemical and antibiotic (1/2:1/2) were prepared. Following a 24-hour incubation at 37°C, the zones of inhibitions (ZOIs) were measured (Lodhi *et al.*, 2021).

**Checkerboard method:** A checkerboard dilution assay was performed to determine the antibiotic and phytochemical combination effects. The findings were interpreted using a fractional inhibitory concentration index. The summation of the FICs ( $\Sigma$ FICI) in each well ( $\Sigma$ FICI = FICA + FICB) was used to classify the combination of antimicrobial agents at given concentrations as synergistic ( $\Sigma$ FICI $\leq$ 0.5), additive ( $0.5 < \Sigma$ FICI $<$ 1.0), indifferent ( $1 < \Sigma$ FICI  $\leq$ 4) and antagonistic ( $\Sigma$ FICI $\geq$ 4) (Trifan *et al.*, 2022). Unit change in increase or decrease in MIC of antibiotics in combination with phytochemicals was calculated as per previous study (Haque *et al.*, 2022).

**Killing kinetics:** Growth kinetics was used to assess the bactericidal activity of the antimicrobial agents at  $\frac{1}{4}$  MIC and the tested phytochemicals at  $\frac{1}{4}$  MIC against MRSA ATCC 43300 as the results showed synergistic effect at the said concentration. Killing kinetics was found by applying 96-well microtiter plate following viable cell counts (log<sub>10</sub> CFU/mL) against time. An inactivation curve was created for each treatment at 0h, 2h, 4h, 8h, 16h

and 24h of incubation at 37°C and plotted as a graph (Alshareef, 2021).

**Statistical analysis:** The data obtained was analyzed through t-test for statistical differences between the two groups while one-way ANOVA for  $\geq 3$  treatment groups and Tukey's test were applied with post hoc test ( $p < 0.05$ ). The data were analyzed using SPSS version 22, while the graphs were made using GraphPad Prism version 8 for Windows.

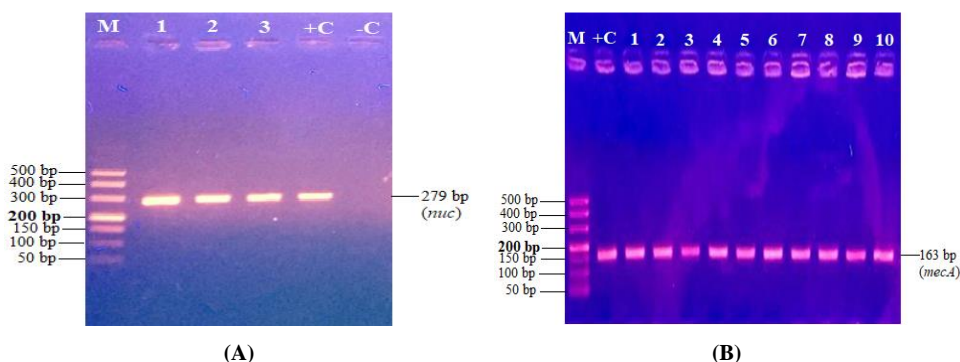
## RESULTS

**Comparative Prevalence of *Staphylococcus aureus* and MRSA:** Among the sampled isolates based on quarter level ( $n = 2696$ ), 30.1% (812/2696) were identified as subclinical mastitis samples while among them, 38.2% (310/812) were identified positive for *S. aureus* (Fig. 1A) while MRSA (Fig. 1B) prevalence was noted to be 12.3% (38/310).

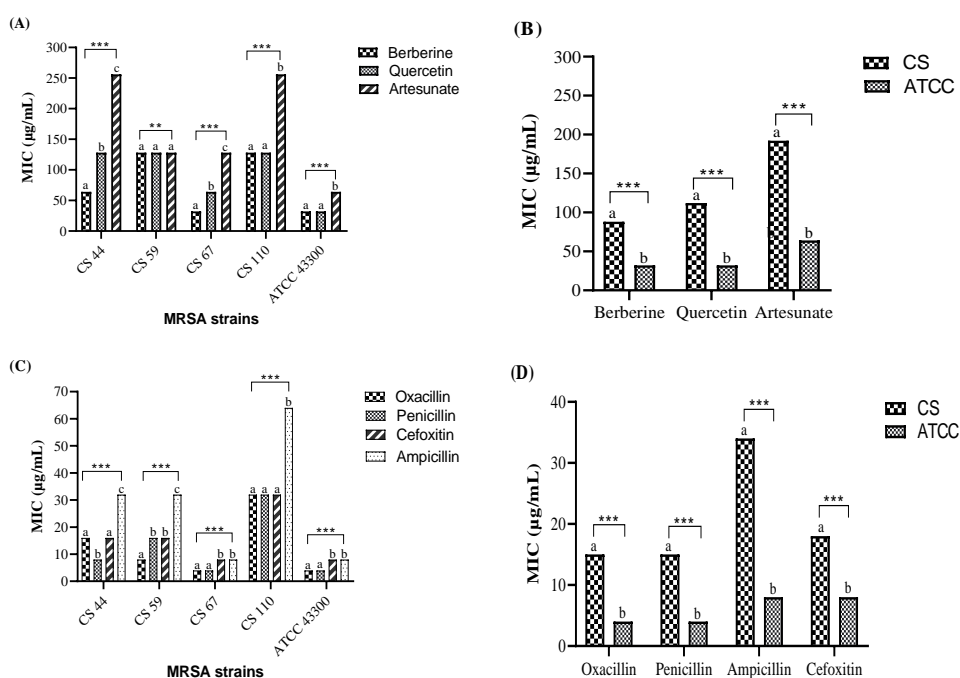
**Antibacterial potential of phytochemicals and antibiotics:** The study found a significant difference ( $p < 0.05$ ) in MIC among the tested phytochemicals within the different groups. Moreover, BR, QT and AR showed a significant difference within CS44 and CS67. Whereas in case of CS59, all the treatments showed a non-significant difference. CS110 and ATCC 43300 strain showed a non-significant difference

( $p > 0.05$ ) between berberine (BR) and quercetin (QT) having a noticeable difference with artesunate (AR) (Fig. 2A). The study also revealed greater MIC values of all the phytochemicals in clinical strains when compared to ATCC 43300 standard strain of MRSA (Fig. 2B).

The comparison of MIC values of antibiotics revealed oxacillin and cefoxitin showing no significant difference ( $p > 0.05$ ) while penicillin showed significantly lower values of MIC, and ampicillin had significantly higher values in CS44. In CS59, the MIC value of oxacillin was significantly lower than that of penicillin and cefoxitin showing non-significant difference, and that of ampicillin was higher compared to all others. Where in CS110, all the antibiotics exhibited a non-significant difference among each other except ampicillin showing higher values of MIC. CS67 and ATCC 43300 exhibited lower values for oxacillin and penicillin compared to higher values of cefoxitin and ampicillin. Overall, all the antibiotic treatments showed significant difference in their MIC values among different groups (Fig. 2C). When clinical strains compared with ATCC 43300, the results revealed a significantly higher values of MIC in clinical strains for all the antibiotics, exhibiting that oxacillin and penicillin showed the lowest MIC values followed by cefoxitin and ampicillin showing least antibacterial potential against the said bacterial isolates (Fig. 2D).



**Fig. 1:** Molecular confirmation of *S. aureus* and MRSA (A) PCR products showing *nuc* gene at 279 bp for *Staphylococcus aureus*, (B) PCR products showing *mecA* gene at 163 bp for MRSA



**Fig. 2:** Drug resistance profile of MRSA (A) Phytochemical's MIC within different isolates of MRSA (B) Comparative MIC of phytochemicals between clinical strains and ATCC 43300 (C) Antibiotics' MIC within different isolates of MRSA (D) Comparative MIC of antibiotics between clinical strains and ATCC 43300: (\*\*\*) Significant difference ( $p < 0.05$ ), (\*\*) no significant difference ( $p > 0.05$ ), a,b,c showing significant difference within the group.)

**Interaction responses of phytochemicals with antibiotics:** The results obtained from broth micro-dilution and well diffusion assays demonstrated a significant decrease in the mean MIC of antibiotics and phytochemicals when used in combination. Among the antibiotics tested, all antibiotics consistently exhibited the most favorable outcomes with BR. Apart from BR, oxacillin and cefoxitin yielded superior results when combined with AR, whereas penicillin and ampicillin showed enhanced efficacy when combined with QT. The mean MIC of phytochemicals also significantly decreased in combination with antibiotics including the best combination of phytochemicals with ampicillin followed by combination with cefoxitin (Table 1). The FIC index of all the combinations revealed that combinations showed synergistic effect except that of the penicillin with artesunate, whose effect was found to be additive (Table 1).

**Change in MIC of antibiotics in combination with BR, AR and QT:** When comparing the drug interaction assay to the antibiotics used alone, the analysis of unit changes in MIC of antibiotics in combination with phytochemicals revealed a non-significant difference ( $p>0.05$ ) between them, indicating a consistent response. Among all, cefoxitin showed the greatest reduction with BR (-0.989), AR (-0.980), and QT (-0.961) (Table 1). The same is true for other antibiotics in this experiment, whose MICs were significantly reduced in combination with the phytochemicals.

**Killing synergy of antibiotic and phytochemical combinations:** The killing synergy analysis revealed distinct patterns for each antibiotic. BR exhibited a substantial decline (<60%) in viable cell count when

combined with oxacillin and penicillin within the first 2 hours of incubation. By the 4-hour mark, significant reductions (<50%) were observed in combination with cefoxitin and ampicillin. Notably, no viable bacterial cells were detected by the 16-hour reading (Fig. 3A). In contrast, QT displayed a non-significant decline at 2 and 4 hours, followed by a significant decrease at 8 hours, with a subsequent 90% reduction by 16 hours and complete elimination by 24 hours (Fig. 3B). A smooth decline was seen in case of AR in each time interval. AR demonstrated a consistent decline in viable cell count over time intervals (Fig. 3C), with reductions to 80%, 60%, and 20% at 2, 4, and 8 hours, respectively and a further decrease to 5% by the 16-hour reading (Table 2).

## DISCUSSION

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a global health concern due to its high resistance to multiple antibiotics and ability to cause severe infections. The complexity of MRSA strains lies in their potential to adapt and evolve in response to antibiotic and host immune pressures through the factors like antibiotic resistance, biofilm formation, and virulence factors (Roy *et al.*, 2024). The emergence of such multidrug-resistant (MDR) bacteria highlights the need for alternative therapies. One promising approach to combat antimicrobial resistance (AMR) involves dual drug-delivery strategies that showed its success by targeting multiple pathways involved in bacterial resistance mechanisms. This approach in turn, enhances the efficacy of existing antibiotics while minimizing drug concentrations and target multiple resistance mechanisms, thus offer promise in the fight against antibiotic-resistant infections like MRSA (Alghamdi *et al.*, 2023).

**Table 1:** Interaction analysis of different antibiotics with Berberine, Artesunate and Quercetin

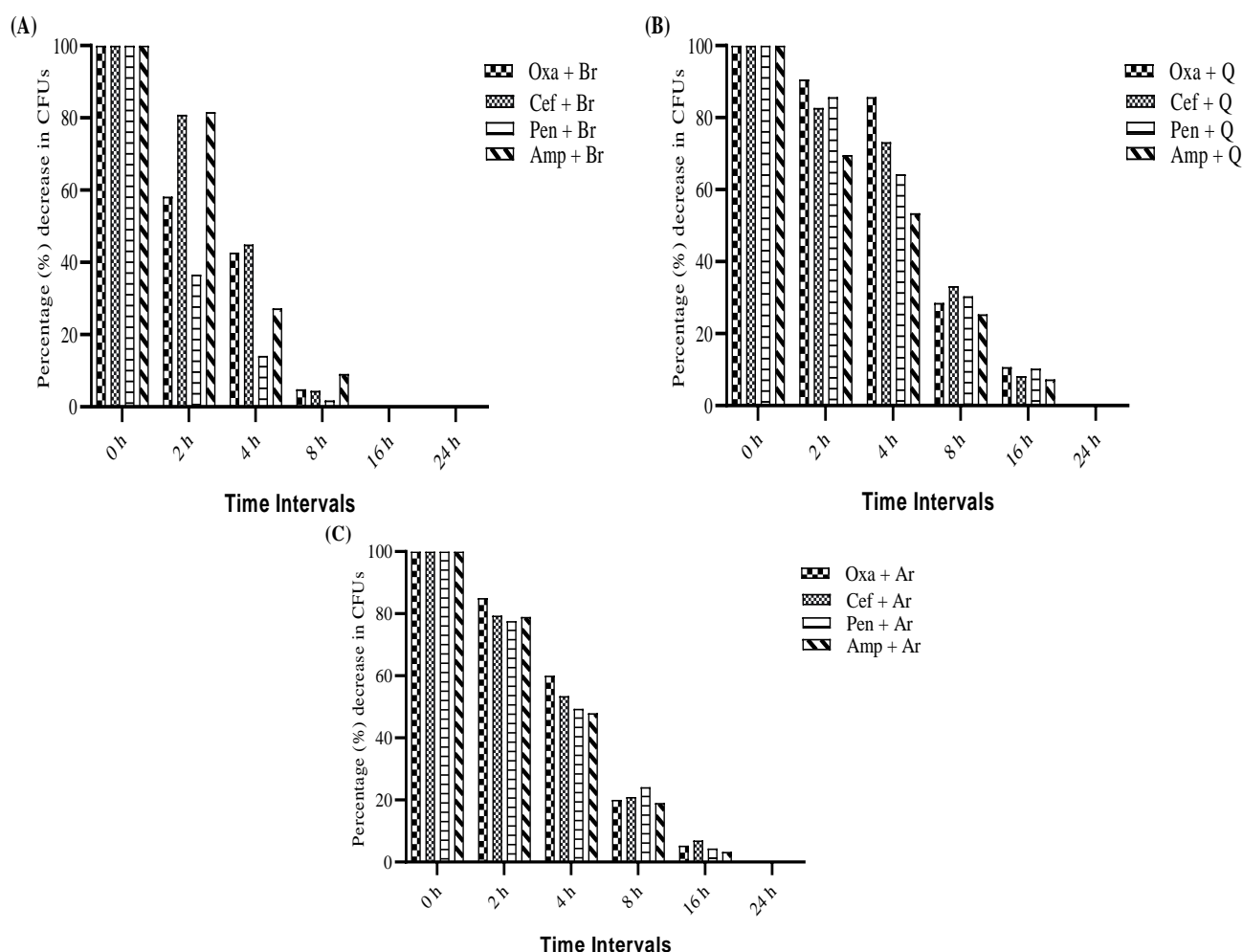
Antibiotics	Phytochemicals	Mean MIC		Mean Unit Change	Mean MIC		Mean Unit Change	FICI	Interaction Type
		Antibiotic Alone	Antibiotic Combination		Phytochemical Alone	Phytochemical Combination			
Oxacillin	BR	15	0.843	-0.943	88	28	-0.681	0.37	Synergistic
	AR		0.937	-0.937	192	72	-0.625	0.43	Synergistic
	QT		1.40	-0.906	112	32	-0.714	0.37	Synergistic
Penicillin	BR	15	0.937	-0.937	88	29	-0.670	0.39	Synergistic
	AR		1.875	-0.875	192	72	-0.625	0.5	Additive
	QT		1.062	-0.929	112	32	-0.714	0.28	Synergistic
Ampicillin	BR	18	0.937	-0.948	88	18	-0.795	0.25	Synergistic
	AR		2.375	-0.868	192	36	-0.812	0.31	Synergistic
	QT		1.781	-0.901	112	18	-0.839	0.25	Synergistic
Cefoxitin	BR	34	0.375	-0.989	88	21	-0.761	0.24	Synergistic
	AR		0.656	-0.980	192	64	-0.667	0.35	Synergistic
	QT		1.312	-0.961	112	20	-0.821	0.21	Synergistic

Minimum Inhibitory Concentration (MIC), Fractional Inhibitory Concentration Index (FICI), Berberine chloride (BR), Artesunate (AR), Quercetin (QT).

**Table 2:** Comparison of time-kill assay (bacterial count) under the effect of BR, AR and QT.

Antibiotic	Phytochemical	0h	2h	4h	8h	16h	24h
Oxacillin	BR	6696 ± 144.66	3850 ± 79.43	2842 ± 62.86	358 ± 53.71	28 ± 8.0	0 ± 0.0
	AR	6421 ± 20.13	5432 ± 52.45	3856 ± 66.9	1360 ± 80.0	326 ± 41.63	0 ± 0.0
	QT	4426 ± 61.1	4065 ± 52.2	3818 ± 89.23	1272 ± 88.22	477 ± 20.13	0 ± 0.0
Penicillin	BR	6546 ± 61.1	2406 ± 48.22	949 ± 66.61	134 ± 16.16	18 ± 15.87	0 ± 0.0
	AR	6733 ± 61.1	5334 ± 61.1	3346 ± 61.1	1644 ± 66.0	313 ± 41.63	0 ± 0.0
	QT	4453 ± 83.26	3862 ± 68.85	2860 ± 91.65	1346 ± 61.1	456 ± 26.22	0 ± 0.0
Ampicillin	BR	6933 ± 61.1	5722 ± 33.3	2024 ± 85.72	656 ± 56.86	0 ± 0.0	0 ± 0.0
	AR	4840 ± 80.0	3820 ± 40.0	2334 ± 61.1	913 ± 30.55	162 ± 16.16	0 ± 0.0
	QT	5213 ± 61.1	3634 ± 61.1	2766 ± 41.63	1330 ± 56.86	381 ± 18.03	0 ± 0.0
Cefoxitin	BR	3558 ± 62.0	2818 ± 54.45	1593 ± 30.55	160 ± 20.0	0 ± 0.0	0 ± 0.0
	AR	6866 ± 140.47	5374 ± 90.36	3646 ± 94.51	1360 ± 80.0	429 ± 50.01	0 ± 0.0
	QT	4413 ± 61.1	3670 ± 49.27	3080 ± 87.76	1426 ± 94.51	326 ± 41.63	0 ± 0.0

Berberine chloride (BR), Artesunate (AR), Quercetin (QT).



**Fig. 3:** Percent decrease in CFU/mL with respect to incubation time (A) Berberine chloride (BR) combinations, (B), Quercetin (QT) combinations, (C) Artesunate (AR) combinations,

The antibacterial response of BR in this study among different strains of MRSA is in line with the findings of Wu *et al.* (2022), who found its MIC to be 51 µg/mL indicating that BR was involved in inhibiting cell wall biosynthesis, inducing oxidative damage, reducing stress resistance, and inhibiting the synthesis of aromatic amino acids. Additionally, BR with the MIC range of 78 µg/mL, was found to damage the structure of the bacterial cell membrane and inhibit protein and DNA synthesis, as observed in studies against *Streptococcus agalactiae* by Peng *et al.* (2015). QT exhibited a gradual decline in MIC, contrasting with the findings of Adeyemi *et al.* (2020) who suggested that QT restricts bacterial growth through lipid peroxidation and kynurenine pathway activation. Researchers (Nguyen and Bhattacharya, 2022) mentioned a contradicting MIC of QT with our studies against *S. aureus* to be 20 µg/mL with proposed antimicrobial mechanisms including cell membrane damage, alteration of membrane permeability, inhibition of nucleic acid and protein synthesis, reduced expression of virulence factors, mitochondrial dysfunction, and prevention of biofilm formation. Further, our results are aligned with the studies conducted by other researchers (Pal and Tripathi, 2020) who observed disruption of cell wall and membrane integrity in various bacteria treated with quercetin showing MIC of 128 µg/mL. AR stood last in the list with the highest MIC among the phytochemicals, contradicting with

the findings of Li *et al.* (2011) and Wei *et al.* (2020) who demonstrated its MIC to be 8000 µg/mL and 512 µg/mL with a proposed the antibacterial mechanism of AR through the inhibition of multidrug efflux pump systems Acr-AB-TolC.

The synergy analysis of BR exhibited significant synergistic interactions with oxacillin and penicillin against MRSA leading to a substantial reduction in microbial growth which was corroborated by several studies, including those by Yu *et al.* (2005) highlighting its effectiveness against different bacterial strains. Similarly, QT displayed complete synergism with various antibiotics against MRSA and other strains, supported by other studies (An *et al.*, 2011; Siri Wong *et al.*, 2015; Pal and Tripathi, 2020). However, some studies, like Su *et al.* (2014), found additive interactions of QT with different antibiotics against MRSA. AR exhibited an additive effect with penicillin but showed synergistic interactions with other antibiotics, consistent with findings from Wei *et al.* (2020), Le-Tien *et al.* (2023), Jiang *et al.* (2013), and Haq *et al.* (2022). These results underscore the potential of BR, QT, and AR combinations against a range of bacterial strains, providing valuable insights for effective antibiotic therapy.

The time-kill kinetics study offered additional support for the synergistic impact of BR, QT, and AR when combined with the tested antibiotics. There was a notable reduction in CFU within 4 hours of incubation in the case

of BR, consistent with the observations made by Dash *et al.* (2020). Moreover, the studies conducted by Wojtyczka *et al.* (2014) documented a complete inhibition of bacterial growth within 24 hours aligning with our findings. The delayed and strong bactericidal effect of QT when combined with antibiotics is reinforced by the research findings of Odabaş *et al.* (2023). Conversely, AR exhibited a smooth reduction in CFU with all antibiotics over the 24h incubation period, with no growth observed after 24h, a trend also observed in the studies by Jiang *et al.* (2011).

**Conclusion:** The study found higher prevalence of MRSA from dairy animals with severe concern of emerging resistance against antibiotics. They phytochemicals, berberine (BR), quercetin (QT), and artesunate (AR), on the other demonstrated significant antibacterial activity against MRSA in that sense, the former showed highest efficacy followed by QT and AR. The interaction of these phytochemicals with selected beta-lactam antibiotics (penicillin, ampicillin, oxacillin, and cefoxitin) was all synergistic except that of the penicillin with artesunate, which was additive. The salient effective combinations of antibiotics with phytochemicals included AR and QT with ampicillin, BR with oxacillin and penicillin. The combination of phytochemicals with antibiotics showed a significant reduction in minimum inhibitory concentration at early stages of incubation indicating effective therapeutic regimen available to cover sudden infections. The study thus concludes greater room available for combination therapy consisting of phytochemicals and antibiotics to tackle emerging drug resistance and demands further studies with immediate focus on exploration of molecular mechanisms and field trials to formulate products ready to use against infections.

**Author's contribution:** Li Jianxi, Saad Ahmad and Amjad Islam Aqib conceived and designed the study. Saad Ahmad and Muzaffar Ghafoor executed the experiments. Muhammad Shoaib, Amjad Islam Aqib, Shahbaz ul Haq and Farid S Ataya analyzed the data and revised manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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**Declaration of Competing Interest:** The authors declare that they have no competing financial interests or personal

relationships that could have appeared to influence the work reported in this paper.

**Data availability:** All data supporting the conclusions of this article are included in the article.

**Ethics approval and consent to participate:** Not applicable.

**Consent for publication:** Not applicable.

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