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# Berberine as an Efflux Pump Inhibitor against Quinolone Resistant *Staphylococcus aureus*

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**ABSTRACT**

The emergence of antibiotic resistance has led to the search for novel strategies that can overcome this issue. In this connection, the plant secondary metabolites play a crucial role in combating the resistance. This study was undertaken with an aim to assess the resistance modulating potential of berberine as efflux pump inhibitor against quinolone resistant *S. aureus* isolates from bovine mastitis. The antibacterial activity of berberine was evaluated using broth microdilution assay. The interaction of berberine with the antibiotics under study was assessed by checkerboard assay. The resistance modulating potential of berberine was appraised by real time quantitative PCR. The broth microdilution assay revealed that berberine has a weak antibacterial activity. When used in combination with quinolone antibiotics, berberine could lower the MICs of the antibiotics. The relative gene expression study pointed out that berberine act as an efflux pump inhibitor down regulating the expression of *norA* and *norC* efflux pump genes. The study concluded that plant metabolites can be used as potential candidates in reversing the antimicrobial resistance.

**HIGHLIGHTS**

- Berberine is found to have weak antimicrobial activity.
- Berberine reduced the MIC of resistant quinolones when combined with quinolone antibiotics against *S. aureus.*
- Berberine could downregulate the mRNA expression of nor efflux pump genes, thereby overcoming antibiotic resistance.

**Keywords:** Berberine, *Staphylococcus aureus*, *nor* genes, quinolones, efflux pump inhibition

Antimicrobial resistance (AMR) has emerged as one of the most important public health risks, posing substantial challenges to the prevention and treatment of chronic diseases. Despite several steps taken in recent decades to address this issue, the worldwide AMR graph indicates no evidence of slowing down. The misuse and overuse of various antibacterial agents in health care and the agricultural industry are thought to be the primary causes of antimicrobial resistance (Dadgostar, 2019). In addition, Hofer (2019) pointed out that AMR presents many financial and social challenges.

Bacterial resistance to antimicrobial substances is an ancient characteristic that allows bacteria to survive in a changing environment. Furthermore, bacteria use their evolutionary machinery to adapt to the selective pressure exerted by antibiotic treatments, resulting in lower antibiotic efficacy against human and animal illnesses. Among various resistance mechanisms, the resistance caused by efflux pumps is increasing alarmingly. Efflux pumps, unlike most other resistance determinants, are often intrinsic. Over-expression of these efflux pumps occurs

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due to mutations in regulatory proteins or promoters, resulting in drug resistance (Webber and Piddock, 2003). Among the six identified efflux pump families, those that belong to Major Facilitator Superfamily (MFS), especially *nor* efflux pumps, result in *Staphylococcus aureus* resistant to quinolones. Nor family efflux pumps belonged to the second largest family of active transporters, the MFS, which drove the antibiotics out of the bacterial cell by proton motive force (Li and Nikaido, 2009). In *S. aureus*, *norA*, *norB*, and *norC* belonging to the MFS were the most extensively investigated efflux pumps which span around 12-14 membrane-spanning helices with cytoplasmic loop forming six or seven folds around the helices containing 380-520 amino acids, located on the chromosome (Jang, 2016).

The overexpression of *norA* gene in *S. aureus* confers resistance to hydrophilic quinolones like norfloxacin and ciprofloxacin (Hooper and Jacoby, 2015), whereas *norB* confer resistance to hydrophilic quinolones like norfloxacin, ciprofloxacin, hydrophobic quinolones like sparfloxacin, moxifloxacin, tetracycline, ethidium bromide, and cetrimide (Truong-Bolduc *et al*., 2005). In addition, Costa *et al*. (2013) revealed that *norC* encoded for the efflux pump protein NorC resulted in low-level resistance to both hydrophilic and hydrophobic quinolones when overexpressed. The pharmacological inhibition of the efflux pump by the introduction of efflux pump inhibitors along with antibiotics as adjuncts can also be done to combat efflux mediated resistance. It is predicted that the secondary plant metabolites can inhibit such overexpressed efflux pumps, thereby increasing antibiotics' efficacy.

Chen *et al*. (2005) described berberine as a quinolizinium, a nonbasic and quaternary benzylisoquinoline alkaloid. Berberine is obtained from the plant *Berberis vulgaris* L. and is used in pharmacology and medicinal chemistry. It is said to be an important natural alkaloid for the synthesis of numerous bioactive derivatives by condensation, modification, and substitution of functional groups in key locations to develop novel, selective, and potent medications. Hence, this study was undertaken to assess the efflux pump inhibiting activity of berberine against quinolone-resistant *S. aureus* isolates from the bovine mastitis sample.

## MATERIALS AND METHODS

### Bacterial strains, Berberine and Antibiotics

Biochemically identified *S. aureus* isolates from bovine mastitis milk samples were procured from Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India. The test compound berberine and the antibiotics norfloxacin and nalidixic acid were purchased from Sigma Aldrich and enrofloxacin from Himedia in powdered form and were dissolved in DMSO.

### Antimicrobial Susceptibility Testing

The procured bacterial isolates were tested for their antibiotic susceptibility testing by Kirby- Bauer disc diffusion assay. A total of eight different antibiotic discs (Himedia) were tested against twelve *S. aureus* isolates. Multiple antibiotic resistance (MAR) among the isolates were assessed and MAR Index was determined for each isolate using the formula MAR = a/b, where 'a' the number of antibiotics to which the isolate was resistant and 'b' is the total number of antibiotics to which the isolates were subjected to for the evaluation (Sandhu *et al*., 2016). Among the twelve isolates, those that were resistant to quinolones were selected for further study.

### Determination of MIC and MBC

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of berberine and MIC for the antibiotics enrofloxacin, norfloxacin and nalidixic acid were determined using broth microdilution assay as per the protocol described by CLSI, 2012.

Two-fold serial dilution of antibiotic and test compound was made in the microtitre plate. Bacterial suspension adjusted to 0.5 McFarland standards was added to the plate and plates were incubated at 37°C for 24 h. After incubation, 30 µL of 0.015 per cent resazurin was added to each of the wells and further incubated at 37°C for 2-4 h. The well with the lowest concentration of test substance and antibiotics with no colour change from blue to pink was scored as the MIC value whereas the wells that showed a colour change from blue to pink indicated the presence of viable and metabolically active bacterial cells

(Elshikh *et al*., 2016). To determine the MBC, 5 µL of each well with no bacterial growth was individually grown on Mueller-Hinton agar medium at 37°C for 18–24 hours. Berberine with the lowest concentration and no bacterial growth (with 99 percent precision) were reported as MBC.

### Checkerboard assay for synergism

The checkerboard assay was employed for assessing the antibacterial interactions between berberine and antibiotics as per the method described by Zhou *et al*. (2016) with minor modifications. The antibiotics were added into the 96 well microtitre plate at concentrations ranging from 2 MIC horizontally and berberine vertically in a similar manner. Then the bacterial suspension adjusted to 0.5 Mc Farland standards was added. The plates were incubated at 37°C for 24 h and the MIC breakpoint was observed as described by Elshikh *et al*. (2016) by adding 30 µL of 0.015 per cent resazurin.

The combination effect of antibiotics with berberine was determined by Fractional Inhibitory Concentration Index (FICI) using the formula described by Sopirala *et al*. (2010). The FICI was calculated for each antibiotic in each combination by using the following formula: $FICI = FIC_A + FIC_B$ where FIC is the MIC of drug A in combination / MIC of drug A alone and FIC is the MIC of drug B in combination / MIC of drug B alone. The FICIs were interpreted using the interpretation chart furnished by Abdolahi and Khodavandi, 2019. Likewise, FBCI for each combination were also calculated.

### Relative Gene Expression Study

The technique of real-time - quantitative polymerase chain reaction (RT-qPCR) was employed to study the relative expressions of the genes *norA*, *norB*, and *norC* in the bacterial isolates and the modulation of the gene expression by berberine. Total RNA from the bacterial isolates was isolated using TRI reagent employing the TRIzol method of RNA extraction after lysing the bacterial cell wall using enzymatic lysis buffer as per the protocol described by Villa-Rodriguez *et al*. (2018) with minor modifications. The total RNA was reverse transcribed into cDNA using a verso cDNA synthesis kit (M/s Thermo Scientific, USA) as per the manufacturer's protocol. Gene-specific primers for the efflux pump genes *norA* and *norC* used in the study were selected from the study conducted by Kwak *et al*. (2013) and for *norB* was selected from the study conducted by Ding *et al*. (2008). The cDNA of target genes *norA*, *norB*, *norC* along with reference gene was studied for mRNA expression by RTq- PCR (M/s Illumina, Eco® Q- RT PCR system). The relative change in expression of *norA*, *norB* and *norC* genes was analysed by comparative $C_T$ (Cycle threshold) method (Livak and Schmittgen, 2001) and was expressed as 'n' fold change up/ downregulation of the gene in relation to the untreated control group. A melt curve analysis was performed after the reaction for checking the specificity of amplification.

## RESULTS AND DISCUSSION

### Antimicrobial Susceptibility Testing

Efflux pumps are proteins that extrude foreign materials out of the cell. Since the drugs are foreign particles and toxic to cells, the bacterial efflux pumps efflux out the drugs and thus the action of these drugs are not available leading to emergence of resistant bacteria. In a way to combat efflux pump mediated resistance, efflux pump inhibitors can be attempted to inhibit the efflux pumps. These efflux pumps may be synthetic as well as natural and act as adjuncts fortifying the action of antibiotics. The natural efflux pump inhibitors are cost effective and are with less adverse reactions. In present study, multidrug resistant *S. aureus* isolates from bovine mastitis were used for evaluating the efflux pump inhibiting activity of berberine. Among the twelve isolates tested, eight isolates were multidrug resistant (MDR) isolates with MAR index greater than 0.25, two isolates were sensitive to all the antibiotics tested with MAR index zero. The resistance observed was high with tetracycline (75 per cent) followed by co-trimoxazole, clindamycin and ceftriaxone (58 per cent each) and ciprofloxacin (50 per cent). The antibiotic sensitivity pattern of all isolates is given in table 1.

### Determination of MIC and MBC

The present study indicated that berberine, a natural product isolated from *Berberis vulgaris* can act as an antimicrobial agent. The MIC and MBC values obtained for antibiotics- enrofloxacin, norfloxacin, and nalidixic acid and the test compound, berberine is given in table

**Table 1:** Multidrug resistance index in *S. aureus* isolates from mastitis samples

| Isolate | Zone of Inhibition (mm) | | | | | | | | MAR Index |
|---|---|---|---|---|---|---|---|---|---|
| | AMC 30 mcg | C 30 mcg | CD 2 mcg | CIP 30 mcg | COT 25 mcg | CTR 30 mcg | GEN 30 mcg | TE 30 mcg | |
| SA 1 | 28 (S) | 24 (S) | 25 (S) | 12 (R) | 23 (S) | 10 (R) | 23 (S) | 10 (R) | 0.375 |
| SA 2 | 22 (S) | 22 (S) | <10 (R) | 12 (R) | <10 (R) | 11 (R) | 19 (S) | <10 (R) | 0.625 |
| SA 3 | 31 (S) | 23 (S) | 28 (S) | 11 (R) | 22 (S) | 11 (R) | 25 (S) | <10 (R) | 0.375 |
| SA 4 | 23 (S) | 20 (S) | 12 (R) | 12 (R) | <10 (R) | 12 (R) | 18 (S) | <10 (R) | 0.625 |
| SA 5 | 26 (S) | 22 (S) | 10 (R) | 12 (R) | <10 (R) | 11 (R) | 20 (S) | <10 (R) | 0.625 |
| SA 6 | 26 (S) | 22 (S) | <10 (R) | 11 (R) | <10 (R) | <10 (R) | 19 (S) | <10 (R) | 0.625 |
| SA 7 | 32 (S) | 29 (S) | 22 (S) | 30 (S) | 25 (S) | 18 (I) | 34 (S) | 17 (I) | 0 |
| SA 8 | 34 (S) | 30 (S) | 24 (S) | 40 (S) | 28 (S) | 18 (I) | 34 (S) | 16 (I) | 0 |
| SA 9 | 40 (S) | 31 (S) | 12 (R) | 37 (S) | <10 (R) | 19 (I) | 28 (S) | 23 (S) | 0.25 |
| SA 10 | 40 (S) | 30 (S) | 12 (R) | 33 (S) | <10 (R) | 18 (I) | 27 (S) | <10 (R) | 0.375 |
| SA 11 | 39 (S) | 28 (S) | 33 (S) | 38 (S) | 20 (S) | 36 (S) | 40 (S) | 10 (R) | 0.125 |
| SA 12 | 37 (S) | 32 (S) | 12 (R) | 36 (S) | <10 (R) | 13 (R) | 27 (S) | 11 (R) | 0.5 |

n=12, r=3. SA- *Staphylococcus aureus,* AMC- amoxicillin-clavulanate, C- chloramphenicol, CD-clindamycin, CIP-ciprofloxacin, COT-co-trimoxazole, CTR-ceftriaxone, GEN-gentamicin, TE- tetracycline, MAR- Multiple Antibiotic Resistance, R- resistant, S-Susceptible, I- intermediate.

2 and 3. MIC value of berberine was in the range of 256-4096 µg/mL whereas the MBC values of berberine against quinolone resistant *S. aureus* were in the range of 1024-16384 µg/mL. MIC values of nalidixic acid, norfloxacin and enrofloxacin were in the range of 256-512 µg/mL, 512-4096 µg/mL and 64-256 µg/mL respectively.

**Table 2:** MIC values of berberine and antibiotics

| Isolate | MIC (µg/mL) | | | |
|---|---|---|---|---|
| | Berberine | Enrofloxacin | Norfloxacin | Nalidixic acid |
| RSA1 | 256 | 64 | 512 | 4096 |
| RSA2 | 256 | 64 | 512 | 4096 |
| RSA3 | 2048 | 256 | 512 | 512 |
| RSA4 | 256 | 64 | 256 | 4096 |
| RSA5 | 512 | 64 | 512 | 4096 |
| RSA6 | 4096 | 256 | 512 | 1024 |

RSA→ resistant *S. aureus*, MIC→ minimum inhibitory concentration. n = 6, r = 3.

Similar results were reported by Jin *et al.* (2010), in which *S. aureus* isolates treated with berberine showed MIC in the range of 0.1-2 mg/mL. Kim *et al.* (2004) reported a moderate antibacterial activity for berberine against gram-positive bacteria based on the MIC value (50-400 µg/mL). Li *et al.* (2020) also reported that berberine exhibited MIC value of 512 µg/mL when tested against MRSA. The antibacterial activity of berberine against gram-positive bacteria can be due to the penetration and accumulation of berberine inside the bacterial cells as confirmed by Severina *et al*. (2001).

**Table 3:** MBC values of berberine and antibiotics

| Isolate | MBC (µg/mL) | | | |
|---|---|---|---|---|
| | Berberine | Enrofloxacin | Norfloxacin | Nalidixic acid |
| RSA1 | 1024 | 64 | 512 | 4096 |
| RSA2 | 2048 | 64 | 512 | 4096 |
| RSA3 | 16384 | 512 | 1024 | 1024 |
| RSA4 | 2048 | 128 | 512 | 4096 |
| RSA5 | 2048 | 64 | 512 | 4096 |
| RSA6 | 16384 | 512 | 1024 | 2048 |

RSA → resistant *S. aureus,* MBC → minimum bactericidal concentration. n = 6, r = 3.

## Checkerboard assay for synergism

The MIC of enrofloxacin reduced to four to eight fold when combined with berberine. FICI indicated a

synergistic interaction between enrofloxacin and berberine (fig. 1, table 4). Combinations of norfloxacin with berberine resulted in two to eight fold reduction the MIC of norfloxacin. The FICI revealed that the interaction with norfloxacin and berberine was synergy, partial synergy and additivity (Fig. 2, table 5). *S. aureus* isolates treated with the combination of nalidixic acid with berberine showed only two fold reduction in the MIC. The FICI indicated an additive interaction in quinolone resistant *S. aureus* and additive interaction (Fig. 3, table 6). The results of FICI were coinciding with that of FBCI indicating synergy, partial synergy and additivity for the combination of quinolones with berberine. Berberine has reduced the MIC values of many antibiotics exhibiting synergism, additivity and even antagonism. Zhou *et al*. (2016) reported that berberine reduced the MIC of ciprofloxacin by two fold and exhibited synergy against multidrug resistant *Klebsiella pneumoniae*. The results of Li *et al.* (2017) against *P. aerugionosa* implied that berberine lowered the MIC of azithromycin concluding that berberine is competent enough to overcome antibacterial resistance. The studies of Shi *et al*. (2018) reported that berberine had a synergistic interaction with ciprofloxacin against multidrug resistant *Salmonella* strains. Li *et al*. (2021) reported that berberine exhibited synergistic effect when combined with ciprofloxacin by lowering the MIC value by 32 fold against multidrug resistant *Acinetobacter baumannii*.

**Table 4:** Checkerboard assay of enrofloxacin with berberine

| Isolate | MIC in combination (µg/mL) | | MBC in combination (µg/mL) | | FICI | FBCI | Type of Interaction |
|---|---|---|---|---|---|---|---|
| | EX | BER | EX | BER | | | |
| RSA 1 | 16 | 64 | 16 | 64 | 0.5 | 0.31 | Synergy |
| RSA 2 | 8 | 64 | 16 | 64 | 0.38 | 0.28 | Synergy |
| RSA 3 | 64 | 512 | 64 | 512 | 0.5 | 0.16 | Synergy |
| RSA 4 | 16 | 64 | 16 | 64 | 0.5 | 0.16 | Synergy |
| RSA 5 | 8 | 128 | 16 | 128 | 0.38 | 0.31 | Synergy |
| RSA 6 | 64 | 1024 | 64 | 1024 | 0.5 | 0.19 | Synergy |

n = 6, r = 3. RSA → resistant *S. aureus,* EX → enrofloxacin, BER → berberine, FICI → fractional inhibitory concentration index, FBCI → fractional bactericidal concentration index.

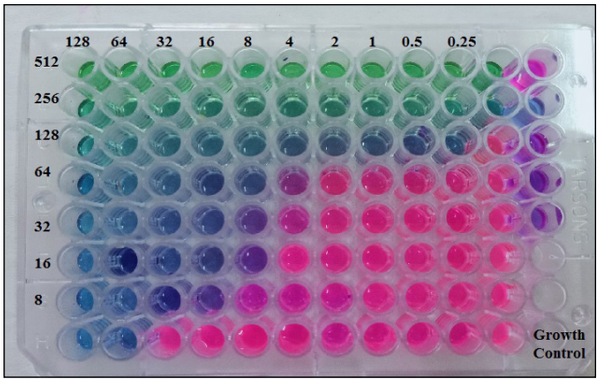


**Fig. 1:** Checkerboard assay of enrofloxacin with berberine

**Table 5:** Checkerboard assay of norfloxacin with berberine

| Isolate | MIC in combination (µg/mL) | | MBC in combination (µg/mL) | | FICI | FBCI | Type of Interaction |
|---|---|---|---|---|---|---|---|
| | NX | BER | NX | BER | | | |
| RSA 1 | 128 | 64 | 128 | 256 | 0.5 | 0.5 | Synergy |
| RSA 2 | 64 | 128 | 128 | 1024 | 0.63 | 0.75 | Partial synergy |
| RSA 3 | 128 | 1024 | 256 | 8192 | 0.75 | 0.75 | Partial synergy |
| RSA 4 | 64 | 128 | 256 | 512 | 0.75 | 0.75 | Partial synergy |
| RSA 5 | 256 | 256 | 256 | 1024 | 1 | 1 | Additivity |
| RSA 6 | 128 | 2048 | 512 | 4096 | 0.75 | 0.75 | Partial synergy |

n = 6, r = 3. RSA → resistant *S. aureus,* NX → norfloxacin, BER → berberine, FICI → fractional inhibitory concentration index, FBCI → fractional bactericidal concentration index.

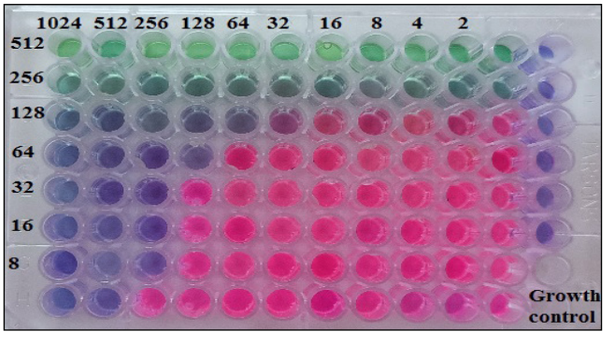


**Fig. 2:** Checkerboard assay of norfloxacin with berberine

**Table 6:** Checkerboard assay of nalidixic acid with berberine

| Isolate | MIC in combination (µg/mL) | | MBC in combination (µg/mL) | | FICI | FBCI | Type of Interaction |
|---|---|---|---|---|---|---|---|
| | NA | BER | NA | BER | | | |
| RSA 1 | 2048 | 128 | 2048 | 512 | 1 | 1 | Additivity |
| RSA 2 | 2048 | 128 | 2048 | 1024 | 1 | 1 | Additivity |
| RSA 3 | 256 | 1024 | 512 | 8192 | 1 | 1 | Additivity |
| RSA 4 | 2048 | 128 | 2048 | 1024 | 1 | 1 | Additivity |
| RSA 5 | 2048 | 256 | 2048 | 1024 | 1 | 1 | Additivity |
| RSA 6 | 512 | 2048 | 1024 | 8192 | 1 | 1 | Additivity |

n = 6, r = 3. RSA → resistant *S. aureus,* NA → nalidixic acid, BER → berberine, FICI → fractional inhibitory concentration index, FBCI → fractional bactericidal concentration index

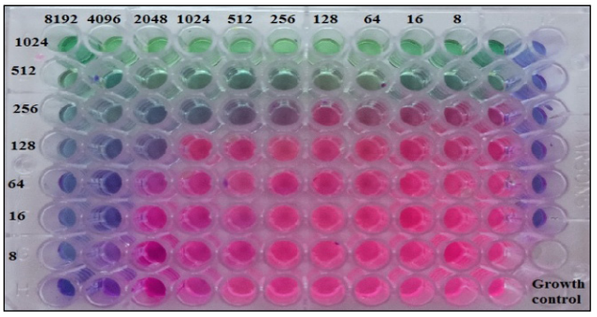


**Fig. 3:** Checkerboard assay of nalidixic acid with berberine

## Relative Gene Expression Study

The relative gene expression study revealed that berberine can inhibit nor efflux pumps by downregulating the expression of the genes. Nor efflux pumps are responsible for quinolone resistance in *S. aureus* and the overexpression of which leads to the extrusion of bacteria out of the cell. The present study pointed out the significant downregulation of the genes *norA* and *norC* in combination of berberine with quinolones. This indicates that berberine could increase the intracellular concentration of quinolones by hindering the efflux of antibiotics. Among the three genes, *norA* gene was found to be more downregulated in presence of berberine. The gene *norB* was not expressed in any of the isolates tested. Melt curve of *norA* and *norC* is given in Fig. 4.

The results of present study were in accordance with Jiang *et al*. (2013) on relative expression of *nor* genes in MRSA. The result of the study indicated that artesunate down regulated the expression of *norA, norB* and *norC* efflux genes through efflux pump inhibition by enhancing the antibacterial activity of β-lactam antibiotics treated against MRSA. The result of the present study is in accordance with Gupta *et al*. (2016) who reported the efflux pump inhibiting activity of clerodane diterpene. Clerodane diterpene extracted from *Polyalthia longifolia* down regulated the expression of *norA* gene in multidrug resistant MRSA upto two fold and potentiated the action of norfloxacin.

The efflux gene *norB* could not be detected in the *S. aureus* isolates tested. The reasons for the absence of overexpression of *norB* may be due to the fact that selective overexpression of the gene in *S. aureus* isolates as supported by the work of Truong-Bolduc *et al*. (2011) and Kosimidis *et al*. (2012) who reported that the expression of *nor* family genes varies depending on the temporal

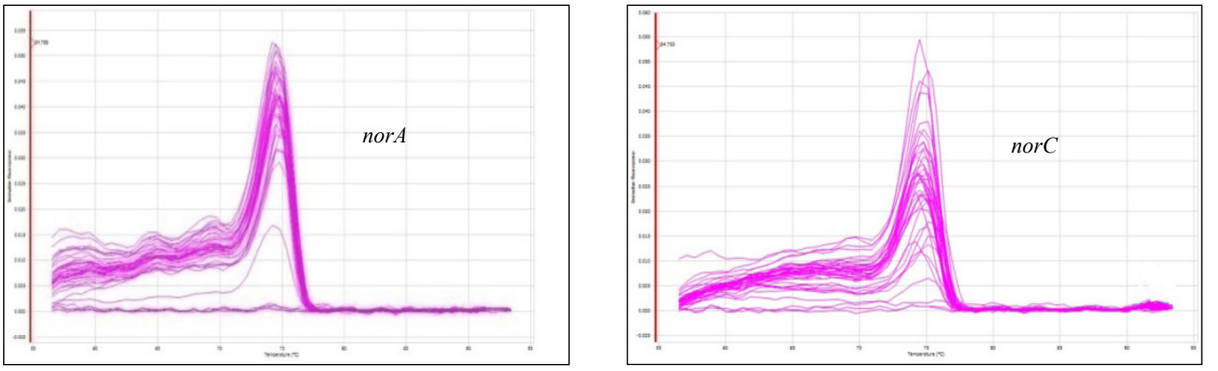


**Fig 4:** Melt curves of *norA* and *norC*

and geographical changes. The results of epidemiological studies by Hassanzadeh *et al*. (2020) on the expression and frequency of efflux genes present in *S. aureus* isolates around the world revealed that generally *norB* expression was found more in European countries (60 per cent) than in Asian and American countries. The study evidenced that a plant active metabolite can be used as an adjunct in the therapy of mastitis as the efflux pumps lays a considerable role in emergence of multi drug resistant bacteria.

## CONCLUSION

The study concluded that plant secondary metabolites can be used as resistance modulators against resistant bacteria isolates. In this study, berberine could downregulate the mRNA expression of nor efflux pump genes, thus improving the efficacy of quinolone antibiotics. In this way, there is wide scope for exploration and identification of such medicinal values of plant metabolites. The influence of efflux pumps must be considered in the design of future antibiotics, and the involvement of inhibitors must be investigated, in order to maximise the efficacy of current and future antibacterial drugs.

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