



Antibiofilm activity of berberine and capsaicin in combination with quinolones against *Staphylococcus aureus* from bovine mastitis[#]

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Abstract

Antimicrobial resistance which causes failure of antibiotic therapy has become a serious global issue nowadays. Bacteria develop resistance towards an antibacterial agent via many mechanisms. Biofilm formation by bacteria poses severe threat to the treatment of infections. The combination of plant molecules with antibiotics can combat antimicrobial resistance. In the present study, antibiofilm activity of two plant molecules berberine and capsaicin was investigated against *Staphylococcus aureus* isolates from bovine mastitis cases. Antibiotic sensitivity testing revealed that berberine and capsaicin when combined with quinolones increased the diameter of zone of inhibition in a dose dependent manner. In vitro biofilm assay using congo red agar plate method revealed the antibiofilm activity of berberine and capsaicin in combination with quinolone antibiotics. The study concludes that the combination of phytoconstituents with antibiotics can alleviate resistance mechanisms in bacteria.

Keywords: Antimicrobial resistance, biofilm, *Staphylococcus aureus*, bovine mastitis

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

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in the health care, as well as in the agricultural industry, are thought to be the primary causes of antimicrobial resistance (Dadgostar, 2019). Drug inactivation/alteration, modification of drug binding sites/targets, changes in cell permeability resulting in lower intracellular drug accumulation and biofilm formation are all different mechanisms of drug resistance in bacteria (Santajit and Indrawattana, 2016). In livestock sector, mastitis poses a serious threat as the development of resistance among mastitis causing pathogens hold back the treatment (Jisha *et al.*, 2020). In dairy cattle, *Staphylococcus aureus* is a common cause of intra-mammary infection. Its ability to endure inside the udder is based on the presence of important mechanisms such as the ability to form biofilm, polysaccharide capsules, small colony variants and the ability to invade cells, all of which will protect *S. aureus* from the cow's innate and adaptive immune response, as well as antibiotics that are no longer considered adequate against the organism (Zaatout *et al.*, 2020; Jose *et al.*, 2021).

Biofilm is an assemblage of microbial cells that has been irrevocably attached to a surface and is encased in a polysaccharide matrix. Biofilms can grow on a number of surfaces, including living tissues, medical devices, piping in industrial or potable water systems and natural aquatic systems. Biofilm-associated organisms differ from their planktonic (freely suspended) counterparts with respect to the genes that are transcribed (Donlan, 2002). When compared to their planktonic counterparts, bacterial cells in biofilms are 10 to 1,000 times less susceptible to specific antimicrobial agents. Poor antibiotic penetration into the polysaccharide matrix, the arbitrary presence of cells with a resistant phenotype and the presence of either non-growing cells or cells that induced stress responses under undesirable chemical conditions within the biofilm matrix, all contribute to the reduced susceptibility of antimicrobials against biofilm producing bacteria (Balcazar *et al.*, 2015).

Bacterial biofilms pose significant public health concern because of their ability to withstand antibiotics, immune mechanisms and other external stimuli contributing to

persisting chronic infections. As a result, the biofilm matrix provide bacteria with additional capability allowing them to not only withstand hard conditions but also become resistant to antibiotics, resulting in the development of multidrug resistant, extensively drug resistant and totally drug resistant strains (Sharma *et al.*, 2019). The majority of antimicrobial therapies have been developed and tested on bacteria that live in a planktonic (free-living) state. As a result, antimicrobial therapies are frequently ineffective against pathogenic biofilms, which could be about 1000 times more resistant to antimicrobial treatments. Biofilm recalcitrance makes it incredibly difficult to successfully treat and remove them (Verderosa *et al.*, 2019). As a result, novel solutions for bacterial biofilm prevention, dispersal and treatment are the need of the hour.

The present study was undertaken with the aim to evaluate the antibiofilm activity of two plant molecules berberine, a benzyloquinoline alkaloid from *Berberis vulgaris* L. and capsaicin, natural proto-alkaloid found in hot peppers (*Capsicum annum* L.) in combination with quinolones against *S. aureus* isolates from bovine mastitis.

Materials and methods

Collection of *S. aureus* isolates

The *S. aureus* isolates from bovine mastitis milk samples were procured from Department of Veterinary Microbiology, Teaching Veterinary Clinical Complex, College of Veterinary and Animal Sciences, Mannuthy, Thrissur.

Antibiotic sensitivity test (ABST)

Antibiotic sensitivity testing was performed by Kirby-Bauer disc diffusion assay as per the guidelines provided by Clinical and Laboratory Standards Institute (CLSI, 2012). Bacterial suspension adjusted to 0.5 McFarland standards were swabbed on sterile Mueller Hinton agar plates. The test compounds berberine and capsaicin (obtained from Sigma Aldrich in pure form) were dissolved in dimethyl sulphoxide (DMSO) and two fold serial dilutions of test compounds ranging from 8 g/L to 0.25

g/L were used in the study. Twenty microlitres of dilutions (8 g/L to 0.25 g/L) of berberine and capsaicin were impregnated into each of the antibiotic discs that already contained nalidixic acid (30 mcg), norfloxacin (10 mcg) and enrofloxacin (10mcg) separately. The antibiotic discs alone and the vehicle control (DMSO) were also added. Discs were placed on the agar plate using sterile forceps. Plates were then incubated immediately at 37°C for 16-18 h. After incubation, the zone of inhibition was measured and interpreted according to CLSI criteria. All tests were done in triplicates against six *S. aureus* isolates.

Biofilm assay

The ability of *S. aureus* isolates to form biofilm was characterised phenotypically by Congo red agar (CRA) plate method as

described by Melo *et al.* (2013). The *S. aureus* isolates were cultured in a CRA medium for the assay. The CRA medium comprised of brain heart infusion agar (52 g), saccharose (36 g) and congo red dye (0.8 g) per litre of autoclaved distilled water. The plates were then inoculated with the isolates and incubated for 24 h at 37°C aerobically. A positive result was indicated by black colonies with a dry crystalline consistency while pink colonies with occasional darkening resembling a bull's eye appearance indicated a negative result (Freeman *et al.*, 1989).

The ability of the test compounds berberine and capsaicin to inhibit biofilm formation was assessed by the CRA plate method. The antibiofilm activity was assessed in *S. aureus* isolates treated with berberine and capsaicin alone and in combination

Table 1. Zone of inhibition (mm) of quinolone resistant *S. aureus* against enrofloxacin with different combinations of berberine and capsaicin

Treatment	Zone of Inhibition (mm) Mean ± SE	
	EXB	EXC
Enrofloxacin (10 mcg)	11.67±0.21 ^f	11.67±0.21 ^g
Enrofloxacin + test 8g/L	18.83±0.7 ^a	18.67±0.56 ^a
Enrofloxacin + test 4g/L	18±0.58 ^{ab}	17.67±0.56 ^{ab}
Enrofloxacin + test 2g/L	17.17±0.48 ^{abc}	16.83±0.48 ^{abc}
Enrofloxacin + test 1g/L	16.17±0.48 ^{bc}	16.17±0.65 ^{bcd}
Enrofloxacin + test 0.5g/L	14.17±0.4 ^{cde}	14.17±0.4 ^{def}
Enrofloxacin + test 0.25g/L	13.67±0.21 ^{def}	13.83±0.31 ^{efg}
F value	31.90 ^{***}	25.54 ^{***}
P value	0.0001	0.0001

*** Significant at 0.05 level, n=6, r=3, Means bearing varying superscript differ significantly in a column. EXB → enrofloxacin + berberine, EXC → enrofloxacin + capsaicin

Table 2. Zone of inhibition (mm) of quinolone resistant *S. aureus* against norfloxacin with different combinations of berberine and capsaicin

Treatment	Zone of Inhibition (mm) Mean ± SE	
	NXB	NXC
Norfloxacin (10 mcg)	0 ^c	0 ^b
Norfloxacin + test 8g/L	12.17±0.4 ^a	12.5±0.22 ^a
Norfloxacin + test 4g/L	11±0.37 ^a	11.83±0.17 ^a
Norfloxacin + test 2g/L	10.5±0.22 ^a	11±0.26 ^a
Norfloxacin + test 1g/L	8.17±0.98 ^{ab}	10.17±0.17 ^a
Norfloxacin + test 0.5g/L	0 ^c	0 ^b
Norfloxacin + test 0.25g/L	0 ^c	0 ^b
F value	33.36 ^{***}	84.68 ^{***}
P value	0.0001	0.0001

*** Significant at 0.05 level, n=6, r=3, Means bearing varying superscript differ significantly in a column. NXB → norfloxacin + berberine, NXC → norfloxacin + capsaicin

Table 3. Zone of inhibition (mm) of quinolone resistant *S. aureus* against nalidixic acid with different combinations of berberine and capsaicin

Treatment	Zone of Inhibition (mm) Mean \pm SE	
	NAB	NAC
Nalidixic acid (30 mcg)	0 ^c	0 ^d
Nalidixic acid + test 8g/L	11.5 \pm 0.5 ^a	10.5 \pm 0.22 ^a
Nalidixic acid + test 4g/L	11 \pm 0.45 ^a	8.67 \pm 1.74 ^{ab}
Nalidixic acid + test 2g/L	7 \pm 2.24 ^{ab}	5 \pm 2.24 ^{bc}
Nalidixic acid + test 1g/L	3.67 \pm 2.33 ^b	1.67 \pm 1.67 ^{cd}
Nalidixic acid + test 0.5g/L	0 ^c	0 ^d
Nalidixic acid + test 0.25g/L	0 ^c	0 ^d
F value	18.16 ^{***}	15.81 ^{***}
P value	0.0001	0.0001

*** Significant at 0.05 level, n=6, r=3, Means bearing varying superscript differ significantly in a column. NAB \rightarrow nalidixic acid + berberine, NAC \rightarrow nalidixic acid + capsaicin

with quinolone antibiotics enrofloxacin, norfloxacin and nalidixic acid at sub-inhibitory concentrations. One milliliter dilution of test compounds berberine and capsaicin were made separately in sterile test tubes by two fold serial dilution ranging from 8 g/L to 0.25 g/L. Then one milliliter of antibiotics at sub inhibitory concentration level was added to each test tubes followed by the addition of one milliliter of bacterial suspension (adjusted to 0.5McFarland standard). Then, the tubes were incubated at 37°C for 24 h. After 24 h of incubation, the sterile CRA plates were inoculated with respective isolates. The biofilm-producing *S. aureus* isolates served as the positive control. The plates were incubated at 37°C for 48 h aerobically. The experiment was done in triplicates. Antibiofilm formation was assessed by analysing the colony morphology. Inhibition of biofilm was indicated by the formation of pink colonies in the CRA plate while biofilm producers remain as dry black crystalline colonies. Indeterminate result was indicated by the darkening of colonies with the absence of dry, crystalline colonial morphology.

Results and discussion

Antibiotic sensitivity test (ABST)

Mastitis is an important disease causing economic burden and production loss in livestock sector. The prevalent multidrug resistant (MDR) isolates in bovine mastitis impedes the treatment strategies thereby resulting in loss of producing animals.

Antibiotic sensitivity test of *S. aureus* isolates against enrofloxacin showed a zone of inhibition of 11.67 \pm 0.21mm and against norfloxacin and nalidixic acid no measurable zone of inhibition was observed. When enrofloxacin combined with berberine or capsaicin, there was an increase in zone of inhibition observed with a maximum of 18.83 \pm 0.7 mm when combined with berberine and 18.67 \pm 0.56 mm when combined with capsaicin (Table 1, Fig.1). The combinations of norfloxacin with berberine at 8g/L showed a zone of inhibition of 12.17 \pm 0.4 mm and norfloxacin with capsaicin at 8g/L showed a zone of inhibition measuring 12.5 \pm 0.22 mm. (Table 2, Fig.2). When nalidixic acid combined with different combinations of berberine or capsaicin there was an increase in the zone of inhibition at very high concentrations exhibiting 11.5 \pm 0.5mm for berberine and 10.5 \pm 0.22mm for capsaicin (Table 3, Fig. 3).

The results of the current study is in accordance with the findings of Wojtyczka *et al.* (2014) where, berberine in combination with antibiotics increased the zone of inhibition more than 5mm against coagulase negative Staphylococcal strains. The study of Nisha *et al.* (2020) on the antibacterial activity of capsaicin against tetracycline resistant *E. coli* isolates reported that capsaicin increased the zone of inhibition of tetracycline in a dose dependent manner when treated against tetracycline resistant *E. coli*. The present study showed that berberine and capsaicin when combined along with antibiotics potentiated its activity.

The disc diffusion assay on combinations of berberine and capsaicin with antibiotics is scarcely documented. However, berberine and capsaicin pose weak antibacterial activity which can be explained through the findings of Chu *et al.* (2016) and Marini *et al.* (2015).

Biofilm assay

The pathogenicity of *S. aureus* is linked to its ability to release toxins and other extracellular components, its resistance to phagocytosis as well as its ability to adhere to host surfaces and form biofilm (Vasudevan *et al.*, 2003). Biofilms are complex surface-attached congregations of bacteria bound together by self-produced polymer matrixes, which are mostly made of extracellular DNAs, polysaccharides and secreted proteins (Muhammed *et al.*, 2020). The persistent infections of bovine mastitis can be linked to formation of biofilm by microbes because

adhesive colonies are contained by a vast exopolysaccharide matrix. Apart from evasion of host defence mechanisms, one of the most striking characteristics of biofilm producing pathogenic strains is antibiotic resistance, which complicates the treatment of clinical cases (Shah *et al.*, 2019). The structural integrity, physiological and chemical characteristics of biofilm vary between microorganism species and are dependent on the development of extracellular matrix (Silva *et al.*, 2020). Thus inhibition of biofilm via conventional antimicrobials is a challenging task especially against MDR bacterial isolates.

Congo red agar (CRA) plate method is a sensitive diagnostic tool to assess the formation of biofilm by bacteria. Congo red dye can aid in visualizing the amyloid fibers and overexpressing exopolysaccharide matrix associated with the biofilm (Jones and Wozniak, 2017).

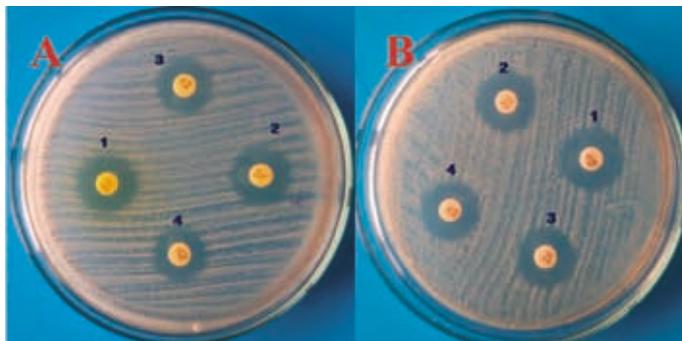


Fig. 1. Antibiogram of *S. aureus* (A)- antibiogram showing different combinations of enrofloxacin with berberine (B)- antibiogram showing different combinations of enrofloxacin with capsaicin. 1→ 8 g/L, 2→ 4 g/L, 3→ 2 g/L, and 4→ 1 g/L

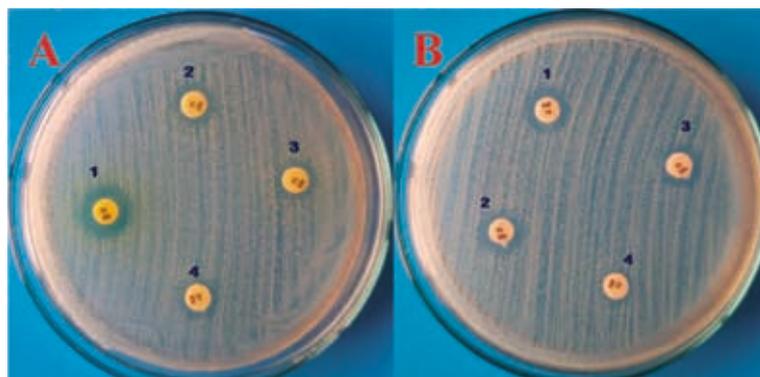


Fig. 2. Antibiogram of *S. aureus* (A)- antibiogram showing different combinations of norfloxacin with berberine (B)- antibiogram showing different combinations of norfloxacin with capsaicin. 1→ 8 g/L, 2→ 4 g/L, 3→ 2 g/L, and 4→ 1 g/L

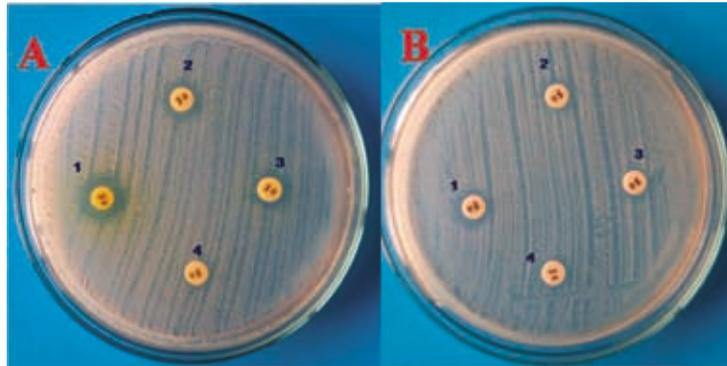


Fig. 3. Antibiogram of *S. aureus* (A)- antibiogram showing different combinations of nalidixic acid with berberine (B)- antibiogram showing different combinations of nalidixic acid with capsaicin. 1→ 8 g/L, 2→ 4 g/L, 3→ 2 g/L, and 4→ 1 g/L



Fig. 4. Biofilm producing *S. aureus*

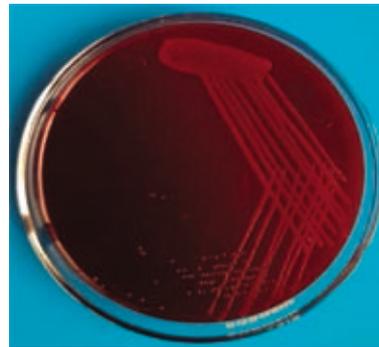


Fig. 5. Inhibition of biofilm by berberine in combination with enrofloxacin

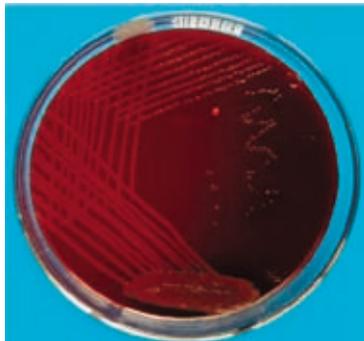


Fig. 6. Inhibition of biofilm by capsaicin in combination with norfloxacin

In the present study, all the isolates were tested positive for biofilm. The formation of biofilm is detected by formation of black dry colonies (Fig. 4). Among the isolates, six isolates were quinolone resistant and six were quinolone sensitive. The isolates that were quinolone sensitive failed to produce biofilm when treated with quinolones alone. The isolates that were quinolone resistant produced

biofilm in the presence of quinolones and test compounds when used alone. When the quinolone resistant isolates were treated with combination of quinolone and test compound, the isolates failed to produce biofilm indicating the antibiofilm activity of test compounds in combination with antibiotics. Quinolone resistant *S. aureus* isolates when treated with combination of berberine at concentration above 1g/L when combined with the antibiotics enrofloxacin, norfloxacin and nalidixic acid failed to form biofilm (Fig. 5). Similar results were reported by Liu *et al.*, 2015, Li *et al.*, 2017 and Shi *et al.*, 2018. Berberine inhibits biofilm by inhibiting the amyloid formation or by inhibiting the quorum sensing by the bacteria thus facilitating the entry of antibiotics inside the bacterial cell (Chu *et al.*, 2016; Aswathanarayan and Vittal, 2018). Jhanji *et al.* (2020) reported the efflux pump inhibiting activity of berberine to alleviate biofilm formation by bacteria. Inhibition of efflux pumps facilitates the accumulation

of antibiotics inside the bacterial cell hence destroying the bacteria. The reasons for biofilm inhibition by berberine in this study may be the above said mechanisms.

When the *S. aureus* isolates resistant to quinolones were treated with combination of capsaicin above a concentration of 0.5g/L with quinolones like enrofloxacin, norfloxacin and nalidixic acid failed to form biofilm (Fig.6). The result of the study was in accordance with Guo *et al.* (2020). The published report on the antibiofilm activity of capsaicin against *S. aureus* biofilm is scarce. The antioxidant action of capsaicin altering the permeability and oxidative stress within the biofilm can be a reason for antibiofilm activity (Silva *et al.*, 2020). Kalia *et al.* (2012) reported the antibiofilm activity of capsaicin in inhibiting the *norA* efflux pump gene conferring resistance to fluoroquinolones. It can be suggested that capsaicin inhibited the NorA efflux pump thus facilitated the accumulation of quinolones inside the bacteria cell.

In the present study, the plant molecules berberine and capsaicin inhibited the biofilm by *S. aureus* isolates from bovine mastitis sample via one or more mechanisms. The number of colonies of bacteria reduced in a dose dependent manner in each combination with the increasing dose of test compounds. Further investigation is needed in this regard to understand the mechanism of action of these compounds in inhibiting biofilm formation with suitable clinical modelling.

Conclusion

The combination of plant molecules with antibiotics can be adopted as a novel treatment strategy in alleviating antimicrobial resistance. Antibiotic sensitivity test revealed the antibacterial activity of berberine and capsaicin in combination with antibiotics. Biofilm assay showed the antibiofilm activity of berberine and capsaicin in combination with quinolones thus enhancing the activity of quinolones. Berberine and capsaicin thus can be plausible drug candidates that can be used as auxiliary molecules in treatment. Antibiofilm activity of berberine and capsaicin unveil the

scope of further exploitation of plant molecules as suitable drug entrants.

Conflict of interest

The authors declare that they have no conflict of interest.

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