Piperine exhibits promising antibiofilm activity against *Staphylococcus aureus* by accumulating reactive oxygen species (ROS)

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Abstract

*Staphylococcus aureus* causes numerous community-acquired and nosocomial infections in humans by exploiting biofilm. In this context, this study aims to impede the formation of *Staphylococcus aureus* biofilm by exposing the cells to a plant-based alkaloid, piperine. Our study revealed that piperine exhibited considerable antimicrobial activity against the test organism. However, we had tested the lower concentrations (up to 32 µg/mL) of piperine to observe whether they could show any antibiofilm activity against the same organism. Several experiments, like crystal violet (CV) assay, estimation of total biofilm protein, and fluorescence microscopic observations, established that lower concentrations (up to 16 µg/mL) of piperine showed efficient antibiofilm activity against *Staphylococcus aureus*. In this connection, we also noticed that the lower concentrations (8 and 16 µg/mL) of piperine showed a considerable reduction in microbial metabolic activity. Besides, it was also observed that the mentioned concentrations of piperine did not compromise the microbial growth of the target organism while exhibiting antibiofilm activity. To understand the underlying mechanism of microbial biofilm inhibition under the influence of piperine, we observed that the compound was found to accumulate reactive oxygen species in the bacterial cells that could play an important role in the inhibition of biofilm formation. Furthermore, the tested concentrations (8 and 16 µg/mL) of piperine were able to inhibit the motility of the test organism that might compromise the development of biofilm. Thus, piperine could be considered as a potential agent for the effective management of biofilm threat caused by *Staphylococcus aureus*.

Keywords *Staphylococcus aureus* · Biofilm · Piperine · Reactive oxygen species · Motility

Introduction

Microorganisms predominate in the environment as either free-floating planktonic cells or aggregates commonly termed as biofilm (Gupta et al. 2016). Bacterial biofilm is...
the aggregation of either homogeneous or heterogeneous conformations of bacteria on an abiotic or biotic surface (Hurlow et al. 2015). In this manner, the bacterial aggregates form a matured cluster by producing a self-secreted extracellular polymeric substance. This matrix has been found to obstruct the movement of antibiotics thereby limiting the action of such medicines (Abebe 2020). Moreover, the degree of resistance of this biofilm matrix against such antibiotics can increase up to 1000-folds when compared with their free-living form (Gupta et al. 2016; Paul et al. 2021a, b). As per the literature survey, almost 80% of bacterial infections are connected to biofilm, thereby posing a risk to public health (Banerjee et al. 2020). It is linked to several chronic infections, like open-wound infections, boils, cellulitis, folliculitis, pneumonia, osteomyelitis, meningitis, and so on (Moreira et al. 2017). It has also been reported that Staphylococcus aureus promotes pathogenesis when it forms biofilm (Gupta et al. 2016). In literature, several molecules have been screened in this regard to manage the threats linked to biofilm (Das et al. 2016; Chakraborty et al. 2018b, 2020). Since natural molecules are cost-effective and less toxic than synthetic ones, they have drawn more attention in biofilm inhibition studies. Moreover, among the naturally occurring alkaloids, the compound piperine (a bioactive component of pepper) has gained much interest (Quijia and Chorilli 2020). However, its antibiofilm effects (Quijia and Chorilli 2020). As per the literature survey, almost 80% of bacterial infections are connected to biofilm, thereby posing a risk to public health (Banerjee et al. 2020). It is linked to several chronic infections, like open-wound infections, boils, cellulitis, folliculitis, pneumonia, osteomyelitis, meningitis, and so on (Moreira et al. 2017). It has also been reported that Staphylococcus aureus promotes pathogenesis when it forms biofilm (Gupta et al. 2016). In literature, several molecules have been screened in this regard to manage the threats linked to biofilm (Das et al. 2016; Chakraborty et al. 2018b, 2020). Since natural molecules are cost-effective and less toxic than synthetic ones, they have drawn more attention in biofilm inhibition studies. Moreover, among the naturally occurring alkaloids, the compound piperine (a bioactive component of pepper) has gained much interest (Quijia and Chorilli 2020). However, its antibiofilm activity against Staphylococcus aureus is yet to be explored. Thus, this report has focused on exploring the antibiofilm potential of piperine against the same organism. Our experimental observations demonstrated a considerable antibiofilm activity of piperine against Staphylococcus aureus.

Materials and methods

Microbial strain, culture conditions, and growth media

The bacterial strain used for the current study was Staphylococcus aureus (MTCC 96). Luria Broth (LB) media purchased from Himedia, India, was used for securing the desired growth of the test organism. Staphylococcus aureus was grown at 37°C as per the requirement of the experiments. In this study, the test compound (piperine) was dissolved into an organic solvent, dimethyl sulfoxide (DMSO) for the preparation of a stock solution. Thereafter, the stock solution of piperine was diluted in the growth media as per the need of the experiments.

Determination of the antimicrobial activity of piperine

The antimicrobial effect of piperine was estimated against Staphylococcus aureus by determining the viable count. To test the same, Staphylococcus aureus (1 x 10^5 CFU/mL) was added to freshly autoclaved LB media in which selected concentrations (250, 500, 750, and 1000 µg/mL) of piperine were incorporated accordingly. Additionally, another set (control) was also arranged where only the test organism was added to the growth media. After an incubation of 24 h at 37°C, microbial culture was collected from each set in equal volume and serially diluted with sterile double distilled water. Thereafter, the viability of the test organism in each set was examined by the colony counting method (Chakraborty et al. 2018a).

Assessment of bacterial biofilm development

Previous literature revealed a well-reported crystal violet (CV) assay that could be used to assess the degree of biofilm formation under different circumstances (Mukherjee et al. 2013). Thus, in this study, the antibiotic potential of piperine on Staphylococcus aureus was tested by pursuing the CV assay. In this direction, microbial cells (1 x 10^5 CFU/mL) were independently transferred to the glass tubes containing autoclaved LB media (5 mL). Specified concentrations (8, 16, 24, and 32 µg/mL) of piperine were added to it. In case of the control set, the bacterium was grown without the addition of piperine. After an incubation of 24 h at 37°C, the free-living cells were removed from the glass tubes. Afterward, the tubes were further rinsed with autoclaved double distilled water. 5 mL of 0.4% CV solution was transferred to every tube followed by incubating them at room temperature for another 30 min. Then, autoclaved double distilled water was used to rinse the CV stained tubes. After that, each tube was supplemented with 5 mL of 33% acetic acid (glacial). Later, the intensity of the CV stain in each tube was measured by recording the absorbance at 630 nm.

Assessment of total biofilm protein

The microbial colonization can be indirectly determined by estimating the whole biofilm protein (Tribedi et al. 2015). The previous literature recommended that there has been a directly proportional relationship between the extent of protein recovery and the degree of microbial colonization (Paul et al. 2021a, b). A greater protein recovery could be expected from a dense microbial association and vice versa. Thus, to estimate the total biofilm protein under the selected concentrations of piperine, cells
(1 × 10^5 CFU/mL) were transferred to glass tubes containing 5 mL of autoclaved growth media (LB). Another similar set (control) was also prepared where the bacterium was not exposed to the test compound. Thereafter, the samples were kept at 37 °C for 24 h. After that, planktonic cells were removed followed by washing and drying the tubes adequately. Then, the tubes were filled with 5 mL of NaOH (0.3 N) and boiled at 100 °C for 30 min. Afterward, the respective suspensions in each tube were centrifuged at 10,000 rpm for 10 min. Thereafter, the protein content available in the recovered supernatant was estimated by following the protocol of Lowry et al. (1951).

**Analysis of microbial colonization**

The degree of microbial colonization to any given surface under different conditions could be analyzed under a fluorescence microscope as reported by Paul et al. (2021a, b). In this connection, to test the consequence of piperine exposure on the microbial colonization, the organism was inoculated into autoclaved LB (5 mL) media supplemented with the desired concentrations (8 and 16 μg/mL) of piperine. Likewise, another control set was prepared where the organism was grown devoid of piperine. After that, sterile coverslips were incorporated aseptically in each of the tubes. Then, all the growth media (treated and control) were kept in an incubator at 37 °C for 24 h. The coverslips were recovered, stained with a stain, namely, acridine orange (4 μg/mL), and viewed under a fluorescence microscope to analyze the microbial colonization under the given conditions.

**Assessment of the metabolic activity**

The metabolic activity of the test organism under the influence of piperine was estimated by adhering to the protocol of Fluorescein diacetate (FDA) hydrolysis assay as stated by Chakraborty and Tribedi (2019). To inspect the consequence of piperine exposure on the metabolic activity of *Staphylococcus aureus*, the organism (1 × 10^5 CFU/mL) was allowed to grow in autoclaved LB either in the presence (8 and 16 μg/mL) or absence of piperine. All the glass tubes were then incubated in an incubator at 37 °C for 24 h. After that, the planktonic cells were recovered from the glass tubes followed by washing the tubes with autoclaved double distilled water. Then, 5 mL (60 mM) of phosphate buffer (pH of 7.6) was added to each tube and gently vortexed. Afterward, FDA (10 mg/mL) dissolved in acetone was transferred to every tube and kept there for 1 h at 37 °C. Post incubation, the tubes were centrifuged (10,000 rpm for 10 min) and the respective supernatant was collected to record its absorbance at 490 nm.

**Assessment of biofilm disintegration**

To determine whether our test compound could disintegrate the developed biofilm of *Staphylococcus aureus*, equal numbers (1 × 10^5 CFU/mL) of cells were transferred to glass tubes containing autoclaved LB (5 mL). Then, the tubes were kept in an incubator at 37 °C for 24 h. After that, the developed biofilm was challenged with the selected concentrations (8 and 16 μg/mL) of piperine. However, one control set was also made where no piperine was exposed to the developed biofilm. Thereafter, all the glass tubes were kept in an incubator at 37 °C for another 6 h. After the completion of the incubation, the CV assay was carried out to inspect whether piperine could exhibit any disintegration of the preformed biofilm of *Staphylococcus aureus*.

**Analysis of the effect of piperine removal on the formation of microbial biofilm**

To find out whether the biofilm inhibitory effect of the test compound persists even after its removal from the growth media, cells (1 × 10^5 CFU/mL) were first inoculated in the test tubes containing autoclaved LB media (5 mL). Selected concentrations (8 and 16 μg/mL) of piperine were independently added to it. However, a control tube, in which the cells were not challenged with piperine, was also prepared. After an incubation of 24 h at 37 °C, both the piperine-treated and untreated microbial culture was centrifuged at 8000 rpm for 10 min. Then, the supernatant was removed from each tube to discard the piperine from the growth media. The cell pellet recovered from the piperine-treated and untreated culture media was separately inoculated into autoclaved LB (5 mL). Thereafter, the tubes were incubated for 6 h, 12 h, and 18 h at 37 °C. After the incubation, the well-acknowledged CV assay was pursued to estimate the degree of biofilm development by *Staphylococcus aureus*.

**Assessment of the selected concentrations of piperine on the microbial growth profile**

To inspect the antimicrobial property of the selected concentrations (8 and 16 μg/mL) of piperine against *Staphylococcus aureus*, a series of experiments were performed. At first, the pattern of microbial growth curves was analyzed under the absence and presence of piperine. To do the test, cells (1 × 10^5 CFU/mL) were added to the conical flasks carrying autoclaved LB media (100 mL). Selected concentrations (8 and 16 μg/mL) of piperine were separately transferred to it. However, another set named control was also arranged in which the test organism was grown without the exposure of piperine. All the growth media were then kept in an incubator for 24 h at 37 °C. At different time intervals, microbial cultures were recovered from every growth media and the
optical density (OD) of the same was measured at 600 nm. Furthermore, the microbial culture (1 mL) was separately collected from both piperine-treated and untreated growth media after an incubation of 24 h at 37 °C. The collected cultures were serially diluted with 9 mL of autoclaved 0.85% NaCl solution. Post dilution, the aliquots (100 μL) were collected from different dilutions of the prepared samples (treated and control) and consequently spread on sterile LB agar plates. After incubation of 24 h at 37 °C, the CFU of each sample was calculated by adhering to the protocol reported by Paul et al. (2021a, b). Besides, a spot assay was also performed in which the dilutions (10⁻¹ to 10⁻⁵) prepared from all the sets were separately loaded on the sterile LB agar plate. Again, the plate was incubated at 37 °C for 24 h. Post incubation, the developed microbial spots corresponding to each dilution were analyzed. To gain further confidence, the clear zone assay was performed to test the antimicrobial effect of the selected concentrations (8 and 16 µg/mL) of piperine against Staphylococcus aureus. To do the test, firstly, a 100 µL culture of Staphylococcus aureus was spread over the LB agar plate to prepare the microbial lawn over the plate. Thereafter, three discrete wells were prepared on the LB agar plates. Two wells were loaded with the tested concentrations (8 and 16 µg/mL) of piperine. However, in the third well, piperine was not applied. Furthermore, plates were kept for 24 h at 37 °C to observe the clear zone (if any) around the wells.

Measurement of the bacteria generated reactive oxygen species (ROS)

To determine the cellular generation of ROS under different concentrations of the test compound, a DCFDA (2‘, 7‘-dichlorofluorescein diacetate)-dependent ROS measurement assay kit (ab113851) was followed. DCFDA, a fluorescent molecule, gets converted into its non-fluorescent counterpart [2’, 7’-dichlorofluorescein (DCF)] on deacetylation by the microbial esterase. The cellular accumulation of ROS (if any) can further oxidize this DCF to a fluorogenic DCF which can be estimated with the help of a fluorescence spectrophotometer. To determine the effect of piperine on the cellular accumulation of ROS, DCFDA was added to both piperine-treated and untreated cells (1 × 10⁸ CFU/mL) followed by incubating them at 37 °C for 30 min. After that, the DCFDA-exposed cells were centrifuged at 10,000 rpm for 8 min. Thereafter, cell pellets were collected, washed with sterile LB, and subsequently exposed to the selected concentrations (8 and 16 µg/mL) of piperine. To validate our results, an antioxidant (ascorbic acid) was also incorporated in the cells which were previously exposed to piperine. A control experiment was also conducted in which the microbial cells were neither exposed to piperine nor ascorbic acid. Finally, the extent of the production of fluorogenic DCF was estimated by a fluorescence spectrophotometer (Dwivedi et al. 2014).

Assessment of the microbial motility

Literature reports stated that the motility of Staphylococcus aureus could be analyzed over soft agar media (Pollitt et al. 2015; Pollitt and Diggle 2017; Paul et al. 2021a, b). To test the same, a semi-solid agar medium was prepared (30 g/L tryptone soya broth along with 5 g/L of glucose and 8 g/L of agar), autoclaved, and poured in sterile Petri plates. After that, cells in equal numbers were separately recovered from each growth condition and spotted on the respective Petri plates at its center. Following this, the plates were kept aside under aseptic conditions for 30 min so that the microbial spots could dry. After the incubation of 24 h at 37 °C, the diameter (in mm) of bacterial motility in each case was measured from the origin of inoculation (Kumar et al. 2013).

MTT assay

To perform the MTT assay, at first Wistar Institute fetus 38 (WI 38) cells originating from human lung tissue were allowed to grow in 200 μL of Dulbecco’s Modified Eagle Medium in 96-well plates augmented with 10% fetal bovine serum (FBS) and 1% antibiotics. Furthermore, the cells were incubated with 5% CO2 at 37 °C for 24 h. Post incubation, the culture medium was treated with different concentrations (5, 10, 20, 30, 50, 75, and 100 µg/mL) of piperine followed by incubating them for another 24 h. However, the cells (WI 38) were incubated without the test compound in the control set. After the incubation, the existing media were discarded and fresh media supplemented with 0.1 mL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were transferred to it. Then, the experimental sets were incubated again for 4 h. After that, the formazan crystals formed in each well were allowed to dissolve in 200 μL DMSO. Thereafter, the intensity of the developed color in the respective wells was quantified by measuring the absorbance at 590 nm.

Statistical analysis

The statistical observations were examined by using the one-way analysis of variance (ANOVA). The mean value was presented after repeating each experiment three times. Error bars represent mean ± standard error (SE) of the mean. The P values incorporated specify the measure of significance in contrast to the control. P values less than 0.05 were demonstrated with (*), P values less than 0.01 were demonstrated with (**), and P values less than 0.001 were demonstrated with (***).
variation among the results. \( P \) values beyond 0.05 were marked as no significant difference (N.S).

**Results and discussion**

**Piperine exhibited considerable antimicrobial potential against *Staphylococcus aureus***

Since alkaloids were reported to show different biological activities, including antimicrobial properties (Othman et al. 2019), in the present report, the antimicrobial effect of piperine was estimated against *Staphylococcus aureus* by determining the colony-forming units (CFU). To estimate the same, cells in equal number were grown with or without piperine under identical conditions. Afterward, the highest number of CFU was measured in the control set among all the experimental sets. The result also revealed a notable decrease of the CFU count in the presence of the test compound (Fig. 1). The present report suggested that at the highest concentration (1000 µg/mL) of piperine, the lowest number of viable colonies was estimated, thereby indicating an inversely proportional relationship between the concentrations of the compound and microbial viability (Fig. 1). Thus, the result revealed a notable antimicrobial activity of piperine against *Staphylococcus aureus*.

Literature survey revealed that microbial infections linked to biofilm have been found to promote pathogenicity considerably (Vestby et al. 2020; Chakraborty and Tribedi 2019), thereby posing a major threat to public healthcare (Dewasthale et al. 2018). Thus, natural molecules are being explored globally for the sustainable management of the biofilm challenges (Chakraborty et al. 2021). In this connection, the antibiofilm activity of the test compound (piperine) was tested against *Staphylococcus aureus* by following the CV assay described by Mukherjee et al. (2013). The CV assay results suggested that the maximum intensity of CV stain was observed in the control set (Fig. 2A). However, the intensity of CV stain got diluted for the piperine-treated cells (Fig. 2A). It was noticed that the degree of biofilm formation got decreased by ~ 52% when the cells were incubated with piperine (Fig. 2B). Furthermore, the result revealed that 16 µg/mL could be considered as the critical concentration of piperine as beyond this concentration, no further change in the degree of biofilm inhibition was observed (Fig. 2B). Hence, these two concentrations (8 and 16 µg/mL) of piperine were selected for the subsequent experiments of the present study. To reassure the results of the CV assay, total biofilm protein was determined in both piperine-treated and untreated conditions. The previous literature revealed that a directly proportional relationship prevails between the degree of microbial association and the extent of protein recovery (Das et al. 2016). In this regard, the highest biofilm protein was determined in the control set which was not exposed to piperine (Fig. 2C). However, the biofilm protein count got decreased by ~ 30% and ~ 47% when the test organisms were treated with 8 µg/mL and 16 µg/mL concentrations of piperine, respectively (Fig. 2C). Thus, from the CV assay and protein measurement observations, it could be stated that the tested concentrations (8 and 16 µg/mL) of piperine significantly inhibited the biofilm formation of *Staphylococcus aureus*. Furthermore, a fluorescence microscopic observation was also undertaken to strongly re-establish the antibiofilm activity of piperine against the test organism. In this regard, we observed the maximum biofilm aggregates on the coverslips that were not exposed to piperine (Fig. 2D). However, such biofilm clusters got remarkably decreased in size when the cells were exposed to piperine (Fig. 2D). Thus, all the results confirmed the considerable antibiofilm activity of piperine against the test organism. Besides biofilm inhibition, efforts were put together to understand whether the compound could compromise the microbial metabolic activity of the test organism. Microorganisms in biofilm promote pathogenesis by secreting several virulence factors, including protease enzyme (Chakraborty and Tribedi 2019). Thus, the metabolic potential of the organism under

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**Fig. 1** Higher concentrations of piperine exhibited significant antimicrobial activity against *Staphylococcus aureus*. Viable microbial counts were determined in both piperine-treated and untreated growth media by performing the CFU assay as described in the Materials and Methods section. Each experiment was repeated three times. The result happened to be the average of the three experiments. Error bars represent mean ± standard error of the mean. \( P \) values were incorporated after statistical analysis using ANOVA. \( P \) value <0.001 was marked with (***).
the presence and absence of piperine was determined by following the FDA hydrolysis test. The result indicated that the microbial metabolic activity got reduced by ~47% when the cells were incubated with the highest dose (16 µg/mL) of the tested concentrations of piperine (Fig. 2E). The result also revealed that the maximum metabolic activity was shown
by the cells which were not exposed to piperine (Fig. 2E). Thus, the result indicated that the selected concentrations (8 and 16 µg/mL) of piperine were found to reduce the metabolic activity of the organism efficiently. Although piperine exerted considerable inhibition in biofilm formation, efforts were given to understand whether the compound could show any disintegration of the pre-formed biofilm of the test organism. Briefly, for the formation of bacterial biofilm, the cells were first allowed to grow for 24 h in autoclaved LB. Then, this pre-formed biofilm was either challenged with piperine or left untreated. The extent of disintegration of the pre-formed biofilm under the presence and absence of piperine was measured by following the CV assay (Paul et al. 2021a). The result revealed an inversely proportional relationship between the concentrations of piperine and the extent of residual biofilm (Fig. 2F). In other words, as the concentrations of the compound increased, the amount of residual biofilm on the glass surface decreased significantly (Fig. 2F). The result revealed that ~39% disintegration took place when the pre-formed biofilm was challenged with the highest dose (16 µg/mL) of the tested concentrations of piperine (Fig. 2F). Thus, the observations so far demonstrated that apart from the inhibition in biofilm formation, piperine could also show considerable disintegration of the pre-existing biofilm. Although piperine showed considerable antibiofilm activity, efforts were given to understand whether the cells treated with piperine could compromise biofilm even after its removal from the growth media. To understand the same, cells were independently inoculated in the growth media either supplemented with piperine or left unexposed. Post incubation, the microbial culture in each set was centrifuged to remove the piperine from the growth media. After that, the piperine exposed and unexposed cells were collected and freshly inoculated into sterile LB free from piperine. The result suggested that with the advancement of time, the pattern of biofilm formation remained similar for both piperine-treated and untreated cells in the piperine-free media (Supplementary Fig. 1). Thus, the result revealed that prolonged exposure of piperine could be an important criterion for the effective management of biofilm challenges.

**The tested concentrations of piperine had no antimicrobial characteristics against Staphylococcus aureus**

The current study aims to come up with an effective agent which does not show any antimicrobial property while exerting its antibiofilm activity. Although we observed that the lower concentrations (8 and 16 µg/mL) of piperine exhibited promising antibiofilm activities, the antimicrobial effect of the same is yet to be examined against *Staphylococcus aureus*. Thus, the bacterial growth profile was examined by treating an equal number of organisms under the influence of piperine. To compare the results effectively, another set (control) was also arranged where the cells remained unexposed to piperine. All the growth media were further incubated at 37 °C for 24 h. At regular time gap, the bacterial culture was individually collected from each set (control and treated) and its OD was recorded at 600 nm. The observations exhibited no substantial variation in the microbial growth pattern in either of the experimental sets (Fig. 3A). Thus, the result of the same indicated that the growth pattern of the test organism remained unaffected under the selected concentrations (8 and 16 µg/mL) of piperine. To validate our results further, viable microbial counts were also determined in both piperine-treated and untreated growth media by pursuing the CFU method (Chakraborty et al. 2018a). It was apparent from the results that the number of microbial colonies estimated was almost the same among the experimental sets (Fig. 3B). The result further indicated that the tested concentrations (8 and 16 µg/mL) of piperine did not show any antimicrobial action against *Staphylococcus aureus*. In support of our observations, spot assay and clear zone assay were also performed to reassure the antimicrobial effect of the selected concentrations (8 and 16 µg/mL) of piperine against the test organism. In the case of spot assay, the diluted samples collected from both piperine-exposed and unexposed cultures were spotted on sterile Luria agar (LA) plates. After the incubation, the growth pattern of the microbial spots was analyzed. The observation showed that no noticeable variation in microbial growth was spotted between the piperine-treated and untreated conditions (Fig. 3C). Thus, the result again indicated that the used concentrations (8 and 16 µg/mL) of piperine did not exhibit any significant antimicrobial property against the organism. Moreover, for the clear zone assay, the tested concentrations of piperine were placed in the wells of the microbial lawn prepared on LB agar plate. After an incubation of 24 h at 37 °C, the clear zone around the wells (if any) was examined. Post incubation, no zone of inhibition was observed around any of the wells on the LB agar plates (Fig. 3D). Thus, the results demonstrated that the used concentrations (8 and 16 µg/mL) of piperine did not offer antimicrobial activity toward *Staphylococcus aureus*.

**The tested concentrations of piperine could enhance the generation of ROS in Staphylococcus aureus**

Reactive oxygen species include highly reactive molecules formed by the inappropriate transferring of electrons to O₂. Peroxides, hydroxyl radical, singlet oxygen, superoxide, etc., belong to the components of ROS (Li et al. 2016). Previous literature reported that the generation of ROS could be considered as a potential cause of biofilm inhibition (Dwivedi et al. 2014). Hence, in this study, the cellular ROS profile
under the presence and absence of piperine was determined by following the DCFDA assay. We found a remarkable difference in the cellular ROS profile between the piperine-treated and untreated conditions (Fig. 4A). In this regard, the control set (not exposed to piperine) showed the lowest accumulation of ROS (Fig. 4A). However, the ROS generation got enhanced with the gradual increase in the concentrations of piperine (Fig. 4A). The results further showed that the maximum ROS accumulation (~4.2-fold higher than control) was observed when the cells were challenged with piperine (Fig. 4A). To further validate our results, ascorbic acid was included in the piperine-treated cells. It was noticeable from the results that the ROS which were increased by ~4.2-fold due to piperine treatment got reduced by twofold under the exposure of ascorbic acid (Fig. 4A). Thus, the result indicated that piperine could increase the accumulation of ROS in the test organism considerably. To analyze the mechanism of biofilm inhibition under the influence of ROS, a battery of experiments was conducted in which in one set, cells were treated with neither piperine nor ascorbic acid. In the next set, under similar growth conditions, the cells were exposed only to piperine. However, in the last set, the cells were challenged with both piperine and ascorbic acid. The experiments were incubated for 24 h at 37 °C. After the preferred time of incubation, the degree of

Fig. 3 The lower concentrations of piperine did not exhibit any antimicrobial activity against *Staphylococcus aureus*. A Microbial growth curve analysis. A similar number of *Staphylococcus aureus* was allowed to grow separately in glass conical flasks containing 100 mL of sterile LB media. In the control set, the organisms were grown without being exposed to piperine. All the experimental sets were incubated at 37 °C. At regular time intervals, an equal volume of microbial cultures was collected from the respective growth media and its optical density was recorded at 600 nm. B Viable microbial count analysis. An equal volume of microbial culture was individually collected from both piperine-treated and untreated growth media followed by determining the CFU count. Each of the experiments was performed thrice. Error bars represent mean ± standard error of the mean. *P* values above 0.05 were marked as N. S (no significant difference). C Microbial spot analysis. An equal volume of microbial culture was separately collected from both piperine-treated and untreated growth media followed by performing the serial dilution of the same. After that, 5 µL of sample collected from each dilution of both piperine-treated and untreated growth media was separately spotted on a sterile LB agar plate. Then, the spotted LB agar plate was incubated for 24 h at 37 °C. D Clear zone analysis. A microbial lawn of *Staphylococcus aureus* was prepared by spreading the culture of the organism over the sterile LB agar plate. Different concentrations of piperine were loaded into the wells prepared on the microbial lawn. After that, the plate was incubated for 24 h at 37 °C to observe the development of the clear zone (if any) around the wells.
bacterial biofilm formation in each of the prepared sets was determined. The result showed that the control set which was exposed to neither piperine nor ascorbic acid exhibited the maximum biofilm formation (Fig. 4B). However, the magnitude of biofilm aggregates was inhibited by ~50% under the influence of piperine (Fig. 4B). The result also revealed that ascorbic acid (25 µg/mL) was found to re-establish the biofilm-forming potential of the piperine-treated cells (Fig. 4B). Thus, the results suggested that piperine treatment could generate ROS in the test organism that could significantly inhibit microbial biofilm formation.

The selected concentrations of piperine exhibited considerable inhibition of microbial motility

Microbial motility is a crucial factor that contributes effectively to biofilm formation by enhancing microbial colonization to various surfaces (Merritt et al. 2007). The existing literature reported that microbial motility is regulated by quorum sensing (Pollitt and Diggle 2017). Quorum sensing is a phenomenon in which microorganism exhibits various functions, including microbial motility after reaching its threshold density (Gupta et al. 2016). Thus, in this report, the influence of piperine on the quorum sensing property of Staphylococcus aureus was tested by targeting microbial motility. To understand the influence of piperine on microbial motility, cells in equal numbers were independently collected from each of the sample (treated and untreated) and loaded accordingly at the middle of the plate. Post incubation, the result revealed that the piperine-untreated culture plate showed the maximum motility (Fig. 5A). On the contrary, the cells which were exposed to piperine showed reduced motility in comparison to the control (Fig. 5A). Thus, the result indicated that the increase in the concentrations of piperine could decrease microbial motility drastically. To support this observation, the zone of diameter (in mm) of microbial motility was also measured from the center of the culture plates (Fig. 5B). The result indicated that the extent of bacterial motility was found to be decreased by ~63% when the cells were treated with piperine (Fig. 5B). Thus, the results suggested that the selected concentrations (8 and 16 µg/mL) of piperine inhibited the bacterial motility by interfering with its quorum sensing property.

The tested concentrations of piperine exhibited no considerable cytotoxicity

To inspect the cytotoxicity of piperine, WI 38 cell line was challenged with different concentrations of the compound. The viability of both piperine-exposed and unexposed cells was determined by performing the MTT assay. The observations of the MTT assay indicated that piperine up to the concentration of 20 µg/mL showed no cytotoxicity against the mentioned cell line (Supplementary Fig. 2). However, the compound started showing cytotoxicity against the same cell line beyond the concentration of...
Thus, it can be affirmed that the tested concentrations (8 and 16 µg/mL) of piperine appeared to be safe for the host. Nevertheless, further studies are required in this regard before recommending it as a drug.

**Conclusion**

The alarming rise in drug resistance among the microorganisms associated with biofilm happens to be a big challenge for the sustainable management of public healthcare. In this context, the present study indicated that piperine, a natural alkaloid, could function as a potent antibiofilm agent to inhibit the *Staphylococcus aureus* biofilm by accumulating ROS. Thus, piperine could be implemented individually as well as in formulations with additional antibiofilm agents to curb the biofilm challenges in a strategic way.

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**Author contribution** SD, PP, SC, and PC performed the experiments and analyzed the results. DM designed the experiments, analyzed the results, and helped in writing the manuscript. AD and PT conceived the idea, designed the experiments, analyzed the results, and wrote the manuscript.

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**Declarations**

**Conflict of interest** The authors have no conflicts of interest to declare that are relevant to the content of this article.

**Ethical approval** Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Code availability** The software Minitab 19 was used for the current study. The software happened to be a trial version. And the authors were allowed to access the same for a period of 30 days.

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**Fig. 5** Piperine exhibited a considerable reduction in microbial motility. An equal number (~1 × 10^5 CFU/mL) of cells were independently recovered from both piperine-treated and untreated growth media and individually spotted on the plates carrying sterile TSB supplemented with glucose (5 g L⁻¹) and agar (8 g L⁻¹). Plates were then incubated at 37 °C for 24 h. Post incubation, A the microbial motility was analyzed and B the zone of colony diameter was measured in (mm). The result represented the average of three observations. Error bars represent mean ± standard error of the mean. The *P* values < 0.05 were marked with (*) and < 0.01 were marked with (**) to show the statistical difference among the observations.

20 µg/mL (Supplementary Fig. 2). Thus, it can be affirmed that the tested concentrations (8 and 16 µg/mL) of piperine appeared to be safe for the host. Nevertheless, further studies are required in this regard before recommending it as a drug.
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