BIOACTIVE PHYTOCHEMICALS TO TARGET QUORUM SENSING, VIRULENCE FACTORS AND BIOFILM FORMATION IN PATHOGENIC MICROORGANISMS

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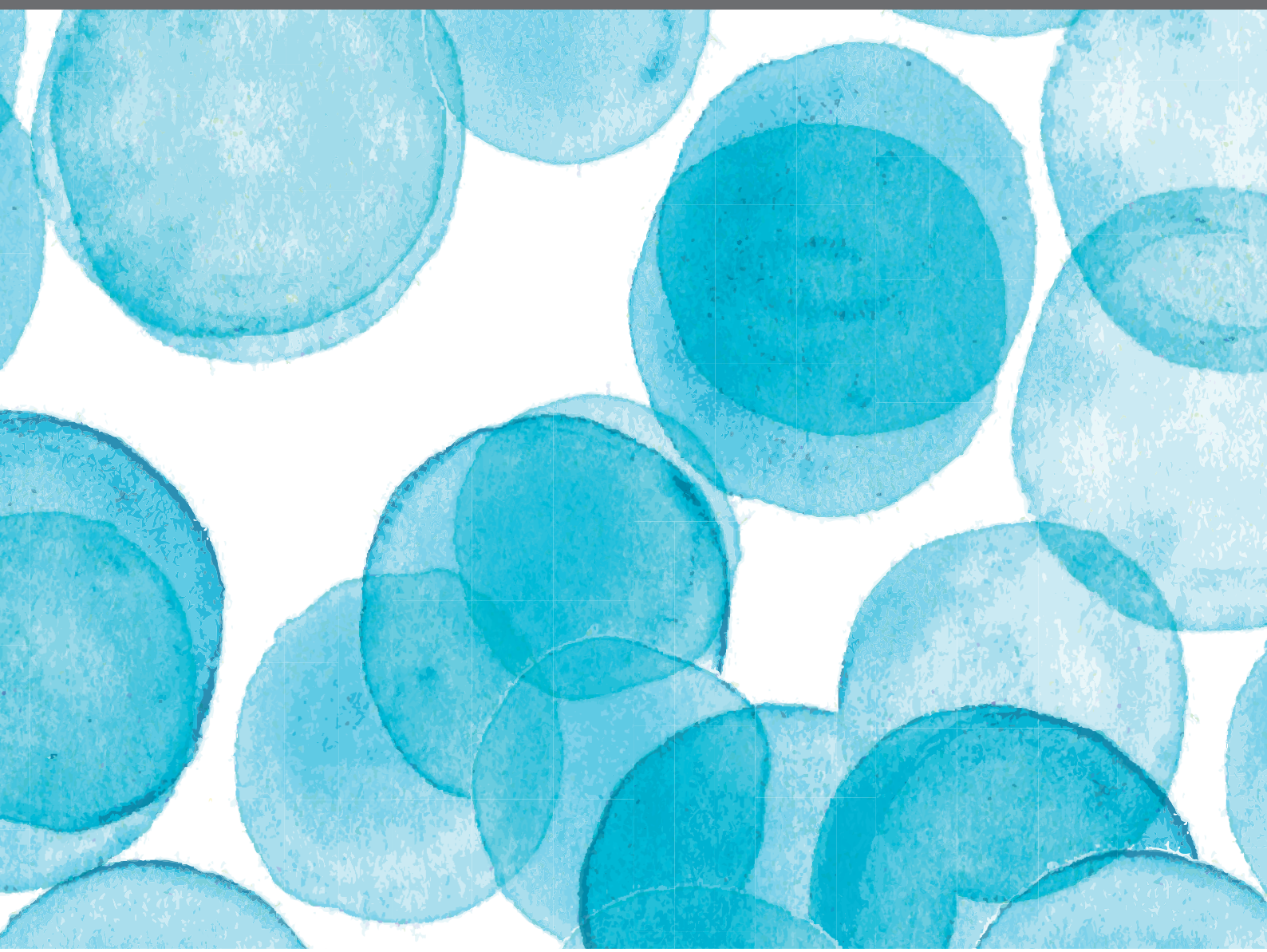

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BIOACTIVE PHYTOCHEMICALS TO TARGET QUORUM SENSING, VIRULENCE FACTORS AND BIOFILM FORMATION IN PATHOGENIC MICROORGANISMS

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ORIGINAL RESEARCH
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Quercetin Inhibits Biofilm Formation by Decreasing the Production of EPS and Altering the Composition of EPS in *Staphylococcus epidermidis*

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Staphylococcus epidermidis is an opportunistic pathogen, and its biofilm formation ability is an important virulent factor. Quercetin, a typical flavonoid ubiquitously used in dietary supplementation, is known for its antioxidant property, but its anti-biofilm activity against *S. epidermidis* remains unknown. In this study, the anti-biofilm activity of quercetin was investigated using *S. epidermidis* ATCC35984, a strong biofilm-positive strain. An attempt was made to disclose the mechanisms of the anti-biofilm activity of quercetin. *S. epidermidis* exhibited a less cell surface hydrophobicity after quercetin treatment. Also, quercetin effectively inhibited *S. epidermidis* cells from adhering to the glass slides. Quercetin downregulated the intercellular adhesion (*ica*) locus and then polysaccharide intercellular adhesin (PIA) production was reduced. Therefore, *S. epidermidis* cells became less hydrophobic, which supported quercetin's anti-biofilm effect. Our study suggests that quercetin from plants be given further attention as a potential anti-biofilm agent against the biofilm formation of *S. epidermidis*, even biofilm infections of other bacteria.

Keywords: quercetin, biofilm, hydrophobicity, *ica*, PIA, *Staphylococcus epidermidis*

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INTRODUCTION

Staphylococcus epidermidis is the most frequently encountered coagulase-negative *Staphylococci* (CoNS) species on human skin. Characteristically, the diseases caused by *S. epidermidis* and other CoNS are chronic and re-occur, which contrasts the potential of *S. aureus* to cause acute infections (Lowy and Franklin, 1998). Epidemiological studies have demonstrated the presence of the genus *Staphylococcus*, *Staphylococcus aureus,* and *S. epidermidis*, in approximately 50% of cases of bovine mastitis (Mello et al., 2020). The consequences of mastitis include economic losses due to the costs of treatment, lower milk production, changes in product quality, and culling (Halasal et al., 2007). Besides the economic losses, mastitis is a public health hazard since it can cause zoonoses and food poisoning (Fernandes et al., 2011; Gomes et al., 2016). *S. epidermidis* is notorious in particular for causing infections on indwelling medical devices, including cardiac implantable electric device (CIED) infection (Okada et al., 2021) and orthopedic device-related infection (Thompson et al., 2020), in which the pathogenesis usually involves biofilm formation. Moreover, the antibiotic therapy against pathogenic bacteria is currently decreasing, which is partly attributed to biofilm

formation (Al-Yousef et al., 2017; Singh et al., 2017). Consequently, the ability of biofilm formation by *S. epidermidis* is focused on in recent years (Vadyvaloo and Otto, 2005; Xie et al., 2019; Mu et al., 2020).

It was observed that extracts of various plants and secondary metabolites isolated from plants such as quercetin, caffeine, menthol, and chlorogenic acid have demonstrated varying levels of biofilm inhibition in gram-negative pathogens (Choo et al., 2006; Annapoorani et al., 2012; Al-Yousef et al., 2017; Satish et al., 2017; Du et al., 2018). Quercetin is a natural flavonoid antioxidant (El-Khawagah et al., 2020; Rojas et al., 2020) able to scavenge reactive species and hydroxyl radicals (Boots et al., 2008), and it bears pharmaceutical significance including anticarcinogenic (Pereira et al., 1996), anti-inflammatory (Guardia et al., 2001), and antimicrobial properties (Nitiema et al., 2012). Although the anti-biofilm/anti-virulence effects of quercetin have been explored to a wider extent in both gram-positive and gram-negative as well as a few fungi species, its anti-biofilm efficacy against *Staphylococcus epidermidis* has not yet been reported.

In this study, we performed scanning electron microscopy (SEM), matrix components analysis, and a hydrophobic assay to investigate the effect and mechanisms of quercetin on *S. epidermidis* biofilm formation.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

S. epidermidis ATCC 35984 [intercellular adhesion (*ica*)-positive], a strong biofilm-positive strain, was used in this study. Unless specified otherwise, tryptic soy agar/broth (TSA/TSB; Becton Dickinson, 211825) were used to culture cells at 37°C overnight. OD_{590} was measured using a spectrophotometer (Bio-Rad) for cell growth. Each experiment was performed using at least three independent cultures.

Assay for Biofilm Inhibition

A static biofilm formation assay was performed using 96-well polystyrene plates as previously described, with slight modifications (Pratt and Kolter, 1998; Xie et al., 2019; Mu et al., 2020). Briefly, cells were diluted 1:100 with fresh TSB and cultured with different concentrations of quercetin (0–1,000 μg ml^{-1}) for 24 h without shaking at 37°C. Biofilms were stained with crystal violet (Sigma, C3886) and dissolved in 95% ethanol (0.5%, *w*/*v*). The optical density was measured at 490 nm in an enzyme-linked immunosorbent assay reader (Bio-Rad). Cell growth in the 96-well plates was also detected at OD_{590}. Relative ability of biofilm formation was indicated as percent Relative Biofilm Formation (%) (RBF%), calculated by the following formula: RBF% = Treated OD_{490}/Untreated OD_{490} × 100%. Each data point was averaged from at least 12 replicate wells (four wells from each of at least three independent cultures).

Microscopic Visualization

Biofilms grown on glass slides were stained with crystal violet and were visualized by light microscopy (Nikon Eclipse Ti 100) at a magnification of ×400 (Nithyanand et al., 2010; Xie et al., 2019; Mu et al., 2020). SEM was used to observe biofilm cells as previously described (Lee et al., 2016). Briefly, *S. epidermidis* strain ATCC35984 cells were diluted 1:100 with fresh TSB and inoculated onto a coverslip (22 × 22 mm^2) in the presence of quercetin (125 μg ml^{-1}) at 37°C for 24 h without shaking.

Effect of Quercetin on the Production and Components of Exopolysaccharides in *S. epidermidis*

To quantify the exopolysaccharides (EPS) produced by *S. epidermidis*, the cells were diluted 1:100 with fresh TSB and cultured for 24 h with shaking at 37°C. Then, the water extraction and alcohol precipitation method was used to collect EPS as previously described, with slight modifications (Petit and Pinilla, 1995; Li et al., 2011; Jin and Zhao, 2014), and air-dried at room temperature. The crude polysaccharides were dissolved by 0.5 mol L^{-1} hydrochloric acid and 121°C heat treated for 15 min. The EPS production of *S. epidermidis* was quantified using the degrees Brix assay (Ball, 2006) with or without treatment with quercetin (125 μg ml^{-1}).

To detect the effect of quercetin on the components of EPS, the water extraction and alcohol precipitation method was used to collect EPS in the *S. epidermidis* culture, as above. Proteinase K and *n*-butyl alcohol (5:1, BOC Sciences, 71-36-3) were used to remove the proteins as described previously (Li et al., 2011). Following dialysis with distilled water overnight, the aqueous layer was collected. The liquid was lyophilized as an EPS sample for use.

Pre-column derivation high-performance liquid chromatography (HPLC) was used to detect the monosaccharide composition of EPS in *S. epidermidis* (Zhang et al., 2009; Xie et al., 2019; Mu et al., 2020). Ribose (∼1 mmol, per 50 ml) was used as the internal standard solution. A mixture of mannose, glucosamine, rhamnose, glucuronic acid, galacturonic acid, galactosamine, arabinose, glucose, galactose, xylose, and fucose (∼0.1 mmol of each monosaccharide; Sigma) was dissolved in water, followed by adding 5.0 ml of the internal standard solution. The mixture solution was then diluted to 50 ml and retained for 1-pheny-3-methyl-5-pyrazolone (PMP; Macklin, P816062) derivation.

The chromatographic conditions were generally as follows: column, Eclipse XDB-C^{18}; temperature, 25°C; solvent, 0.4% triethylamine in 20 mmol L^{-1} ammonium acetate buffer solution (pH 6.3 with acetic acid)–acetonitrile (83:17); and flow rate, 1 ml min^{-1}. The eluate was monitored at 245 nm.

The correction factor for each monosaccharide ($f_{i/s}$) and the content of every monosaccharide in the polysaccharide hydrolysis solution (W) was calculated using the equations $f_{i/s} = (W_i/W_s)/(A_i/A_s)$ and $W = f_{i/s}(A_i/A_s)W_s$, respectively. A_s and A_i are the peak areas of the internal ribose standard and the standard monosaccharide in the reference solution, respectively. W_s and W_i are the contents of the internal ribose standard and the standard monosaccharide in the reference solution, respectively.

TABLE 1 | Primers for quantitative reverse transcriptase PCR.

Genes	Primer sequences
gyrB	5′-TGACGAGGCATTAGCAGGTT-3′
	5′-GTGAAGACCGCCAGATACTTT-3′
icaR	5′-CATTGACGGACTTTACCAGTTTT-3′
	5′-ATCCAAAGCGATGTGCGTAG-3′
icaB	5′-GAAACAGGCTTATGGGACTTTG-3′
	5′-CAAGTGCGCGTTCATTTTT-3′

Cell Surface Hydrophobicity Assay

Cell surface hydrophobicity was tested as previously described (Rosenberg et al., 1980; Xie et al., 2019; Mu et al., 2020). Briefly, 1 ml of bacteria (OD_{400} = 0.6) was placed into glass tubes and 250 μl of *n*-hexadecane (Macklin, H810865) was added. The decrease in the OD_{400} of the aqueous phase was taken as a measure of *H*%, which was calculated with the formula: $H\% = [(OD_0 - OD)/OD_0] \times 100$, where OD_0 and OD are the OD_{400} before and after extraction with *n*-hexadecane, respectively. The experiments were performed using three independent cultures per condition.

Quantitative Real-Time RT-PCR Assay

To explore further the possible mechanisms of the inhibition against *S. epidermidis* biofilm by quercetin, quantitative reverse transcription PCR (qRT-PCR) was performed to investigate the transcription levels of several biofilm-associated genes in *S. epidermidis* ATCC35984 with and without quercetin treatment. Gene-specific primers were used for these genes and *gyrB* used as a housekeeping control (**Table 1**). The expression level of the housekeeping gene *gyrB* was used to normalize the expression data of the genes of interest. The qRT-PCR method was adapted from a previous study (Wang et al., 2011). qRT-PCR was performed using a SYBR green PCR master mix (TransGen Biotech) and an ABI PRISM 7500 Real-Time PCR System (Rotor-Gene Q) with two independent cultures. All experiments were performed in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to analyze the quantitative real-time PCR data.

Statistical Analysis

GraphPad Prism 5 was used to calculate the mean and the standard deviation of the mean. All experiments were performed in triplicate and the data obtained from the experiments were presented as mean values; the difference between the control and the tested groups were analyzed using Student's *t*-test. Significant differences were $P < 0.05$.

RESULTS

Quercetin Inhibited Biofilm Formation by *S. epidermidis* in a Dose-Dependent Manner

The results showed that quercetin inhibited the biofilm formation of *S. epidermidis* ATCC 35984 in a dose-dependent manner (**Figure 1A**). Specifically, it decreased the biofilm formation of *S. epidermidis* ATCC 35984 by ≥ 90% at 250 μg ml^{-1} and by ≥ 95% at 500 μg ml^{-1}.

The change in biofilm formation with or without quercetin treatment was observed using light microscopy and SEM. The results showed that the biofilms treated with 125 μg ml^{-1} of quercetin became thinner, looser, and even easier to eradicate than the untreated biofilms (**Figure 1B**). SEM analysis revealed that fewer cells attached to the coverslips when treated with quercetin (**Figure 1C**). Additionally, fewer intercellular substances were present in the treated group compared with those in the untreated group (**Figure 1D**). No morphologic abnormality was observed in the presence of quercetin. The growth curves of *S. epidermidis* cells were also measured in the presence of quercetin (125 μg ml^{-1}), and a decrease in cell growth was not observed (data not shown). The cell growth and microscopic results indicate that the inhibition by quercetin against *S. epidermidis* biofilm formation is attributed to anti-biofilm activity rather than antibacterial activity.

Quercetin Decreased Cell Surface Hydrophobicity

Surface hydrophobicity facilitates adherence to hydrophobic surfaces. Thus, it plays a crucial role in biofilm formation by *Staphylococci* (Lee et al., 2016; Xie et al., 2019). The cell surface hydrophobicity (CSH) assay was performed to explore the mechanism underlying the inhibition by quercetin against biofilm formation by *S. epidermidis*. The results showed that *S. epidermidis* cells became less hydrophobic when treated with quercetin (**Figure 2**), which at least partly demonstrates the inhibitory mechanisms of quercetin on reduced biofilm formation.

Quercetin Decreased the Production of EPS and Altered the Composition of EPS Produced by *S. epidermidis*

In our previous work, we tested the dependent type of the biofilm formation of *S. epidermidis* ATCC 35984. It was found that biofilm formation by *S. epidermidis* ATCC 35984 mainly depends on EPS consisting of reductive polysaccharides in which the dihydroxy groups are unsubstituted (Xie et al., 2019; Mu et al., 2020). Thus, we detected the effect of quercetin on EPS. The results showed that the production of EPS by *S. epidermidis* ATCC 35984 was reduced when treated with quercetin (**Figure 3**). Specifically, for strain ATCC 35984 when treated with quercetin, galactosamine (GalN) was absent and glucose (Glu) obviously appeared in the monosaccharide composition compared with the control. Additionally, the proportion of galactose (Gal) was increased while the proportions of mannose (Man) and galacturonic acid (GalA) were decreased; especially GalA was significantly reduced (**Figure 4**).

Quercetin Reduced PIA Production by Downregulated *ica* Locus

As quercetin from plant extracts exhibited potent inhibition on the biofilm formation of *S. epidermidis* ATCC 35984, we

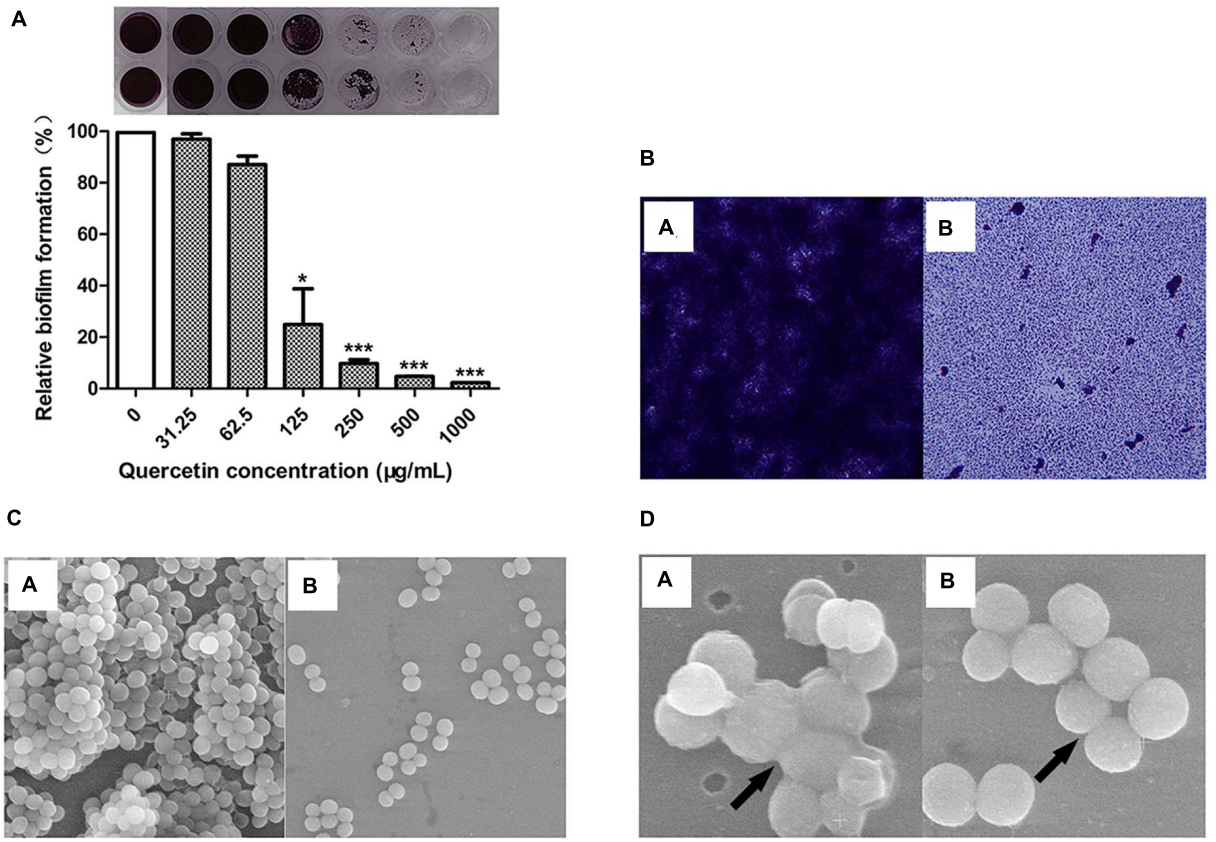

FIGURE 1 | Quercetin significantly reduced *Staphylococcus epidermidis* (ATCC 35984) biofilm formation. The biofilm formation (OD_{490}) of *S. epidermidis* **(A)** was quantified at different concentrations of quercetin (0–1,000 μg ml^{-1}) at 37°C after 24 h in 96-well plates. The relative activity of biofilm formation was indicated as percent Relative Biofilm Formation (%) (RBF%), calculated by the following formula: RBF% = Treated OD_{490}/Untreated OD_{490} × 100%. *Columns* represent the means for three independent experiments. *Error bars* indicate the standard deviations. Statistically significant differences (determined by Student's *t*-test) are indicated as $^{***}P < 0.001$ and $^{*}P < 0.05$ vs. the control group. Biofilms formed by *S. epidermidis* ATCC 35984 were visualized by light microscopy **(B)** and SEM **(C,D)**. SEM was used to examine the biofilm cells grown on coverslips in the presence of quercetin (125 μg ml^{-1}). At least three independent experiments were conducted. *a,* untreated control; *b,* quercetin treated.

were curious why it could decrease polysaccharide intercellular adhesin (PIA) production. Thus, the expression of the *ica* locus was analyzed. Quercetin treatment resulted in a downregulation of the *ica* locus which was associated with the cell adhesion in *S. epidermidis* biofilm formation (**Figure 5**). The decrease of cell-to-cell adhesion caused the reduction of biofilm formation.

DISCUSSION

S. epidermidis is the main reason for biofilm-associated infections. Unlike *S. aureus*, biofilm formation by *S. epidermidis* is an important and the sole virulence factor in the onset and development of device-related infections in clinical settings. Meanwhile, the emergence of multidrug resistance among clinical pathogens has been proven to be related to biofilm formation. Thus, novel antimicrobial and antifouling agents that cannot be surpassed by those pathogens are required. Increasing evidence of plant-derived molecules as novel antimicrobials against multidrug-resistant clinical pathogens show that local special plants may become promising candidates in which novel antibiotics could be found (Packiavathy et al., 2014; Kannappan et al., 2020).

In our previous study, we found that the extract of *Coreopsis tinctoria* Nutt, a special local plant in Xinjiang, obviously exhibited an inhibitory effect on biofilm formation in *S. epidermidis*. Also, we tested the components in *C. tinctoria* Nutt and found that quercetin is rich in this local plant. Thus, we assumed that quercetin has potential anti-biofilm activity; we then chose quercetin instead of the phytochemical as our experimental subject. In the current study, quercetin was evaluated for its potential to hamper the biofilm formation in *S. epidermidis*. In a plate incubation assay, quercetin exhibited a concentration-dependent reduction in biofilm formation. Quercetin reduced *S. epidermidis* biofilm formation up to 90.5% at the concentration of 250 μg ml^{-1} and up to 95.3% at the concentration of 500 μg ml^{-1}. The above results are different from those of Gopu et al. (2015), who reported 13–72, 8–80, and 10–61% reductions in the biofilm formation of 3 g-negative food-borne bacteria, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*,

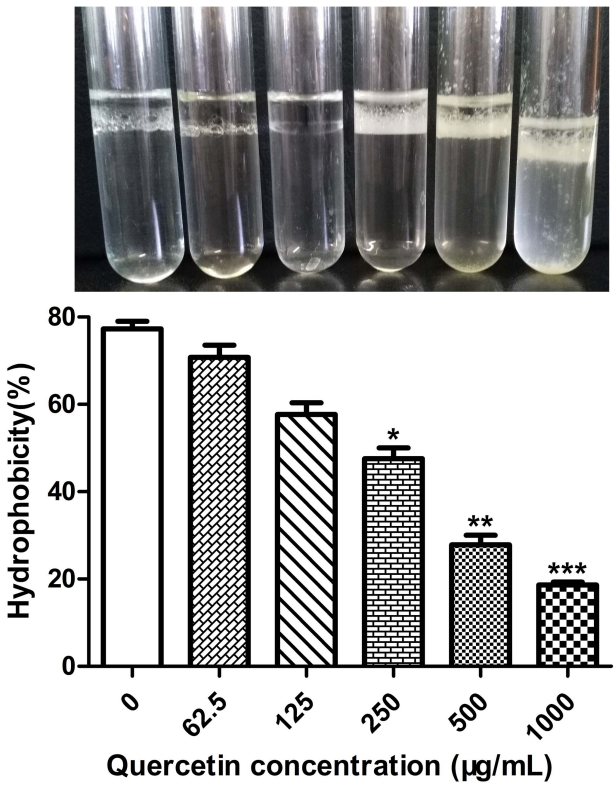

FIGURE 2 | Effects of quercetin on the hydrophobicities of cell surfaces. *Staphylococcus epidermidis* ATCC 35984 cells were grown overnight and a hydrophobicity assay was performed by hexadecane extraction. Aqueous phase absorbance was measured at OD_{400}, and high turbidity indicated less hydrophobicity. *Columns* represent the means for three independent experiments. *Error bars* indicate the standard deviations. Statistically significant differences (determined by Student's *t*-test) are indicated as $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ vs. the control group.

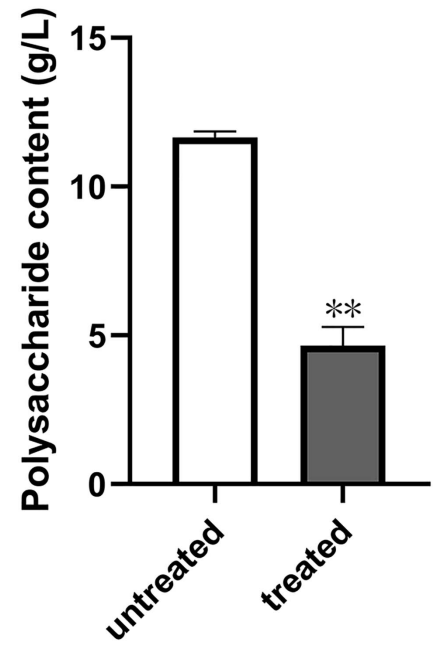

FIGURE 3 | Effect of quercetin on the exopolysaccharide (EPS) production by *Staphylococcus epidermidis*. Degrees Brix was used to detect the content of EPS in *S. epidermidis* with or without quercetin treatment (125 μg ml^{-1}). *Error bars* indicate the standard deviations. Statistically significant differences (determined by Student's *t*-test) are indicated as $^{**}P < 0.01$ vs. the control group.

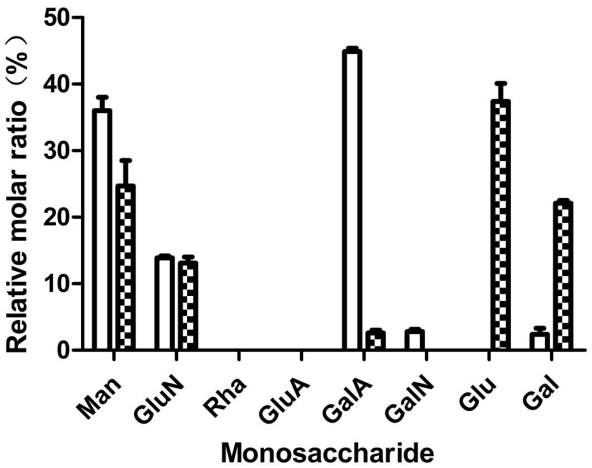

FIGURE 4 | Effects of quercetin on the exopolysaccharide (EPS) components of *Staphylococcus epidermidis* ATCC 35984. The monosaccharides include mannose (Man), glucosamine (GluN), rhamnose (Rha), glucuronic acid (GluA), galacturonic acid (GalA), galactosamine (GalN), glucose (Glu), galactose (Gal), and arabinose (Ara). *Columns* represent the means for three independent experiments. *Error bars* indicate the standard deviations. *Open rectangle*, untreated; *filled rectangle*, treated.

and *Yersinia enterocolitica*, at different concentrations of 5–40 μg mL^{-1}, respectively.

The mechanism of *S. epidermidis* biofilm formation is a complex process in which many factors are involved. Particularly, many macromolecules, such as extracellular proteins, environmental DNA (eDNA), and EPS, are the main components in biofilms (Xie et al., 2019). Among the above three substances, the production of EPS was mainly considered as a key factor which facilitates the initial attachment of *S. epidermidis*. Thus, the inhibition of EPS production may cause less biofilm formation. Our results showed that quercetin had no degradation effect on proteins and DNA (see **Supplementary Figures S2A,B**) in this study. However, a reduced production of EPS was observed in *S. epidermidis* when treated with quercetin (**Figure 3**). The above result is comparable with those of Abraham et al. (2011) and Gopu et al. (2015). Also, the composition of EPS produced by *S. epidermidis* changed with treatment of quercetin (**Figure 4**). Our previous work also showed that the composition of *S. epidermidis* EPS was altered after being treated with spent media from *Actinomycetes* (Xie et al., 2019; Mu et al., 2020). However, the changed composition of EPS is different. GalN was absent in quercetin treatment, while arabinose (Ara) and Gal were absent in the treatment of spent media from *Actinomycetes* compared with the control. GalN is a reductive glycoside that

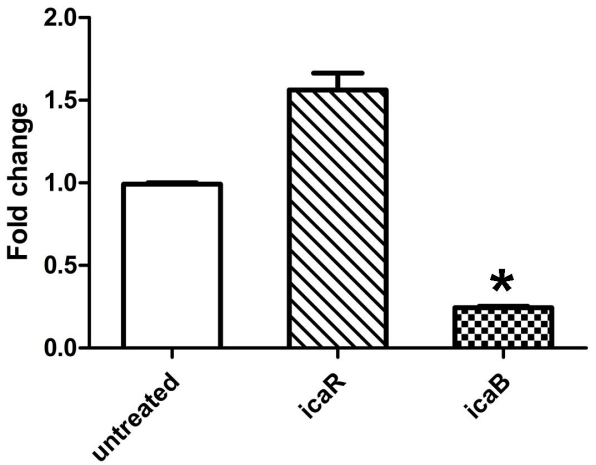

FIGURE 5 | Relative mRNA expression (mean ± SEM) of *icaB* and *icaR* in *Staphylococcus epidermidis* with or without quercetin treatment (125 μg ml^{-1}). Downregulation or upregulation of gene expression was considered significant when the relative expression was decreased or increased ≥ 2-fold. All fold changes have been normalized to *gyrB* as the reference gene. Data are the means of fold changes with standard deviations from three independent experiments amplified in triplicate. Error bars indicate the standard deviations. Statistically significant differences (determined by Student's t-test) are indicated as $^{*}P < 0.05$ vs. the control group.

plays a crucial role in *S. epidermidis* biofilm formation (Mack et al., 1992; Rohde et al., 2005; Xie et al., 2019). Thus, GalN disappeared when treated with quercetin, which may cause a weaker biofilm formation. Additionally, the proportions of monosaccharides of EPS were significantly different. When treated with quercetin, Gal was increased while Man and GalA were decreased. These results suggest that quercetin acts on EPS in a different manner. Further investigation will be required to understand the mechanisms.

The biofilm formation of bacteria is significantly influenced by cell surface hydrophobicity. It has been reported that hydrophobic surfaces are preferred (Xie et al., 2019; Mu et al., 2020). Enzyme-like biofilm inhibitors and low-concentration antibiotics reduce the hydrophobicity of *S. epidermidis* cells (Xie et al., 2019; Mu et al., 2020). In this study, it appears that a decreased cell surface hydrophobicity attenuates the attachment of *S. epidermidis* cells to the plastic wells (**Figure 1A**), glass slides (**Figure 1B**), and coverslips (**Figures 1C,D**). The above findings suggest that the combination of antimicrobials with cell surface hydrophobicity reducers in clinical cases increases drug sensitivity and improves the therapeutic effect.

EPS produced by *S. epidermidis* is responsible for intercellular adhesion. PIA is a type of EPS. PIA production is catalyzed by four glucuronyltransferases encoded by the *icaADBC* operon (Liduma et al., 2012; Mu et al., 2020), which is negatively regulated by *icaR*, encoding a transcriptional repressor of the *icaADBC* operon (Conlon et al., 2002; Mu et al., 2020). The results of the relative gene expression, the downregulation of *icaB*, and the upregulation of *icaR* when treated with quercetin in the current study are in agreement with this conclusion. To further explore the effect of quercetin on biofilm, we used SEM and observed that intercellular substances were reduced (**Figure 1D**). Meanwhile, we tested the production of *S. epidermidis* exopolysaccharides using the degrees Brix assay. In agreement with the results of SEM, a significantly decreased production of exopolysaccharides in the treated groups was found (**Figure 3**).

CONCLUSION

In conclusion, the results in this study indicate that quercetin exhibits anti-biofilm activity *via* decreasing PIA production and cell surface hydrophobicity. Quercetin warrants further attention as a potential biofilm inhibitor in biofilm-associated infections.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

WC, HZ, and YM conceived and designed the experiments. YM performed the experiments. WC and YM wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.631058/full#supplementary-material

Supplementary Figure 1 | Effect of quercetin on initial attachment stage **(A)** and aggregation stage **(B)** in *S. epidermidis* biofilm formation. Biofilm formation (OD_{490}) of *S. epidermidis* on initial attachment stage was detected when the cells were cultured at 37°C for 4 h with the treatment of quercetin (0 1,000 μg ml^{-1}) in 96-well plates. For determining biofilm production on aggregation stage the cells were cultured at 37°C for 4 h in 96-well plates, followed by the treatment with different concentrations of quercetin (0 1,000 μg ml^{-1}), and were further cultured at 37°C for 20 h. Error bars indicate the standard deviations. Statistically significant differences (determined by Student's *t*-test) are indicated as $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ vs. the control group.

Supplementary Figure 2 | Degradation activities of quercetin against proteins **(A)** and DNA **(B)**. Protein degradation assays were performed using milk agar plates **(A)**. Proteinase K and 5% DMSO were used as positive and negative controls. Protein digestion was observed by a clear zone surrounding proteinase K. DNA degradation of *Staphylococcus epidermidis* by quercetin was tested at 37°C for 2 h **(B)**. DNase I was used as a positive control.

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Fatty Acid Methyl Esters From the Coral-Associated Bacterium *Pseudomonas aeruginosa* Inhibit Virulence and Biofilm Phenotypes in Multidrug Resistant *Staphylococcus aureus*: An *in vitro* Approach

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In an attempt to study the antibacterial, antivirulence and antibiofilm potentials of bacteria residing the tissue and surface mucus layers of the pristine corals, we screened a total of 43 distinct bacterial morphotypes from the coral *Favites* sp. Among the isolates, *Pseudomonas aeruginosa* strain CBMGL12 with showed antibacterial, antivirulence and antibiofilm activity against multidrug resistant pathogenic strains of *Staphylococcus aureus* (reference strain: MTCC96; community-acquired methicillin resistant strain: CA-MRSA). Extracellular products (ECP) from the coral-associated bacterium *P. aeruginosa* were solvent extracted, fractionated by chromatographic techniques such as silica column and HPLC-UV with concomitant bioassays guiding the fractionation of metabolites. Identification of bioactive chemical moieties was performed by FT-IR analysis, GC-MS/MS equipped with NIST library, ^{1}H and ^{13}C NMR spectral studies. We report the differential production of extracellular and cell-associated virulence and biofilm phenotypes in multi-drug resistant strains of *S. aureus*, post-treatment with the ECP containing aromatic fatty acid methyl esters (FAME) such as methyl benzoate and methyl phenyl acetate produced by a coral-associated bacterium. In conclusion, this study has identified antibacterial, antibiofilm and antivirulent FAME from the coral-associated *P. aeruginosa* for its ability to attenuate virulence and biofilms phenotypes in multi-drug resistant pathogenic strains of *S. aureus.*

Keywords: coral bacteria, *Staphylococcus aureus*, antibiofilm, antivirulence, antibaceterial, *Favites* sp.

Abbreviations: BIC, biofilm inhibitory concentration; CA-MRSA, community acquired-methicillin resistant *Staphylococcus aureus*; CBMGL, coral-associated bacteria from microbial genomics laboratory; CLSI, Clinical Laboratory Standards Institute; EtOAc, ethyl acetate; FAME, fatty acid methyl esters; FT-IR, fourier transform infrared spectroscopy; FT-NMR, fourier transform-nuclear magnetic resonance; GC-MS, gas chromatography-mass spectrometry; HPLC-UV, high performance/pressure liquid chromatography-UV detection; MBC, minimum bactericidal concentration; MDR, multidrug resistant; MHA, mueller-hinton agar; MHB, mueller-hinton broth; MIC, minimum inhibitory concentration; MS/MS, tandem mass spectra; MTCC, microbial type culture collection, India; MTP, microtitre plate; NIST, The National Institute of Standards and Technology; QQ, quorum quenching; QS, quorum sensing; RBCs, red blood cells; UV-Vis, Ultraviolet-visible spectrophotometry.

INTRODUCTION

Staphylococcus aureus is remarkably a versatile microorganism that adapts tremendous interactions to its niche. It does exist on inanimate sites and animate hosts. For an instance, harmless as a commensal flora of the skin or mucous membranes; as an opportunistic pathogen for a variety of body tissues attributing several diseased conditions ranging from minor skin infections to toxicosis and as a potential drug-resistant pathogen causing systemic, life-threatening illnesses. *S. aureus* is highly regarded to be a human pathogen causing a broad range of illnesses to humanity. Infection can be either nosocomial or community-acquired in humans. Illness includes toxin-mediated diseases like Toxic Shock Syndrome (TSS), septic shock and other focal infections include infective endocarditis, bacterial arthritis, and pneumonia (Ferry et al., 2005). This kind of versatility may be due to a range of adaptive or accessory gene systems (Cramton et al., 1999; Stewart and Costerton, 2001; Baba et al., 2002; Fux et al., 2005). The accessory genetic system conferring pathogenesis in *S. aureus* is regulated by a complex network of two-component system named *agr* system, a quorum-sensing (QS) regulon, which is distributed as four variants forming four different QS groups of *S. aureus* strains. Accessory gene regulator (*agr*) locus encodes the components involved in the QS system of *S. aureus* (Novick, 2003). In *S. aureus*, *agr* system regulates the expression of virulence genes in response to an autoinduction by autoinducing peptides (AIPs) and result in cell dispersal from established Staphylococcal biofilms with the help of extracellular proteases (Mayville et al., 1999; Lyon et al., 2002; Dong et al., 2007; Boles and Horswill, 2008, 2011). It implies one has to keep track of the expression of virulence phenotypes and biofilm-forming potential in several multi-drug resistant (MDR) strains of *S. aureus* in order to explore a novel antivirulence strategy.

Coral reefs provide a suitable ecosystem for microbial interactions through signaling (Pearse and Muscatine, 1971; Rowan and Knowlton, 1995; Stanley and Swart, 1995; Gowrishankar et al., 2012; Krediet et al., 2013). Therefore, the need for finding novel bioactive molecules that can degrade/interfere with QS signals of virulent *S. aureus* may be satisfied by coral-associated bacterial populations that habitually compete with other natural biofilm formers on corals. The coral-Associated bacterium *Bacillus firmus* as the most promising source of antibiofilm agents targeting the recalcitrant biofilms of multidrug resistant-MRSA and MSSA (Gowrishankar et al., 2012), but the chemical identification of the bioactive metabolite was lacking. Gordon et al. (2013) reviewed several natural molecules and their synthetic analogs, which could attenuate the virulence gene expression of *S. aureus* in four different modes such as competitive inhibitors of AgrC–AIP interaction; inhibitors of AgrA–DNA interactions; small molecule inhibitors affecting RNAIII expression; and inhibitors of transcriptional regulators. Mansson et al. (2011) reported the production of cyclodepsipeptides such as solonamides A and B from the marine mussel surface bacterium *Photobacterium halotolerans* and their ability to interfere with *agr* QS system of *S. aureus* and the peptides were able to inhibit the expression of virulence genes in a gene reporter-agar diffusion assay. However, the drug resistance and biofilm potentials of the *S. aureus* strains used were not explored in their study. Besides, the preparations of cyclodepsipeptides for environmental and biomedical applications to control biofilms formed by MDR pathogenic *S. aureus* is quit critical unlike the fatty acid methyl esters from bacteria of pristine ecological niche like corals. Because the biofilm and adherence genes play pivotal role in enhancing the virulence of *S. aureus* (Nourbakhsh and Namvar, 2016), it is essential to study antibiofilm and antivirulence ability simultaneously in order to explore active molecules with multipotency.

Though the recent decade has collections of scientific studies pertaining to the exploration of novel bioactive molecules from marine bacterial resources, their precise targeting on multi-drug resistant and biofilm forming *S. aureus* accomplished with the determination of involved chemical groups are scanty. Herein, we studied the antibacterial, antivirulence and antibiofilm potentials of FAME secreted by the coral-associated bacterium *Pseudomonas aeruginosa* targeting MDR *S. aureus* and chemically characterized the extracellular bioactive components of interests by spectral and GC-MS/MS analysis.

MATERIALS AND METHODS

Properties of Pathogenic Bacterial Strains Used

S. aureus strain was collected from a clinical lab in Puducherry, India, and was used as a test pathogen in this study. *S. aureus* MTCC96 was used as a pathogenic reference strain. The test strain was screened for multidrug resistance potential in an antibiotic susceptibility testing by Kirby-Bauer disk diffusion assay (Bauer et al., 1966). Concentration of each antibiotics used was as per the guidelines from CLSI (Wikler, 2006). Phenotypic expression of extracellular virulence factors such as hemolysin α/β and proteases were studied using enriched plate assays for both strains (Kloos and Schleifer, 1975; McNamara et al., 2000; Miedzobrodzki et al., 2002). Potential to form biofilms *in vitro* was evaluated by modified tube assays and modified MTP assays (Stepanovic et al., 2000).

Isolation of Coral-Associated Bacteria (CB)

Sterile needleless syringes, swabs, and chisels were used to collect the surface mucus layer and tissue specimen of the coral *Favites* sp. at Palk bay, Mandapam North Sea outside Gulf of Mannar Marine National Park (9.3°S, 79.1°E), Ramanathapuram, Tamil Nadu, India, using the method adopted by Thinesh et al. (2020). Collected samples were immediately brought to the laboratory in sterile aged seawater (SAS) for further processing (Kiran et al., 2010). Tissue was homogenized with SAS using precooled sterile mortar and pestle. Both surface mucus layer and ground tissue samples in SAS were serially diluted to 10^{-7} folds and spread onto nutrient agar plates supplemented with 2% NaCl at pH adjusted to 7.8, Luria Bertani agar at pH adjusted to 7.8, Zobell Marine agar, and incubated at

30°C for 24 h to isolate coral-associated bacterial colonies (Gowrishankar et al., 2012).

Solvent Extraction of Extracellular Metabolites

For extraction of extracellular metabolites, coral-associated bacterial colonies were cultured in nutrient broth supplemented with 2% NaCl at pH 7.8 and incubated under shaking (150 rpm) conditions for 24–72 h at 30°C. Grown cultures were pellet down at 12,000 rpm for 10 min at 4°C to collect cell-free supernatants. All the supernatants were filtered through 0.2 μm filter, acidified using concentrated HCl to reduce the pH to 2.0 facilitating the protonation of water molecules releasing bioactive compounds. The acidified supernatants were twice extracted with an equal volume of EtOAc (Kiran et al., 2010). The resultant EtOAc extracts were concentrated to dryness using a rotary vacuum evaporator (R-300, BUCHI Corporation, Switzerland) and the concentrate was re-dissolved separately in different solvents such as water, dimethyl sulphoxide, methanol, ethyl acetate, and chloroform to assess the polarity. The extract was soluble in methanol, chloroform, and ethyl acetate, indicating that the active molecules were slightly non-polar to mid-polar in charge. However, for toxicity reasons, the ethyl acetate extracts were screened for further studies.

Screening for Antibacterial Potentials

For evaluation of antibacterial activity, agar well diffusion assay was employed using Muller-Hinton Agar (MHA). Overnight cultures of clinical isolates were subcultured in Tryptic Soy Broth (TSB) until turbidity of 0.5 McFarland (1×10^8 CFU / mL) was observed. Pathogens were uniformly spread across the surface of the agar plate using sterile cotton swabs. In the swabbed plates, agar wells were punched with a diameter of 6 mm; loaded with coral-associated bacterial extracts, and EtOAc (negative control) at the concentration 100 μl / well, and the plates were incubated at 37°C to observe the zone of lysis after 24 h (Gowrishankar et al., 2012). Only the extracts which exhibited a prominent zone of growth inhibition against the test and MTCC96 *S. aureus* were used for further study.

Antibiofilm Potential in 96-Well Microtitre Plate Assay

The effect of coral-associated bacterial extracts on biofilms of *S. aureus* strains was evaluated in 96-well flat bottomed polystyrene plates. Wells added with 10 μL of Staphylococcal cell suspensions prepared in 1× phosphate-buffered saline (PBS) and with each of the CB extracts (in EtOAc) in volume ranging from 10–50 μL were treated as test wells. Wells filled with 10 μL each of Staphylococcal cell suspensions and EtOAc in volume ranging from 10–50 μL was treated as the control. Wells added with 10 μL of 1× PBS, and each of the CB extracts in volume ranging from 10–50 μL served as blanks for the respective test and positive control wells. All the test, control, and blank wells were made up to a final volume of 200 μL using TSB. The experimental set up was prepared in triplicates and incubated for 24 h at 37°C. The biofilms formed were stained with 0.1% crystal violet in water (w/v) for 5 min (Kiran et al., 2010; Gowrishankar et al., 2012). The unbound stain was discarded, and the biofilms were washed twice with sterile deionized water followed by air drying. The optical density was measured at 595 nm (Kiran et al., 2010), and the level of biofilm inhibition was marked in terms of percentage using the following formula:

Percentage of

Inhibition (%) = [(Control OD595 nm − Test OD595 nm) / Control OD595 nm] × 100

The biofilm inhibitory concentration (BIC) was measured as the lowest concentration which produced a noticeable disruption of biofilms under stereo zoom microscopy and a significant reduction in the OD value at 595 nm when compared with its respective control well. Biofilm inhibitory potential was confirmed in fluorescence microscopy (blue filter for excitation wavelength with emission in green fluorescence) after staining with 0.1% acridine orange.

Effect of the CB Extract on Cell Density of *S. aureus* Strains

The effect of coral-associated bacterial extracts on bacterial cell density was assessed in 96-well polystyrene plates. Wells containing TSB were inoculated with test and reference pathogens. All the wells were loaded with coral-associated bacterial extracts at MIC and sub-MIC, and incubated at 37°C for 24 h (Andrews, 2001). Wells without extracts but inoculated with pathogens served as a control, and the medium with extract served as the blank. After incubation, the contents were gently tapped, and the cell population was quantified using spectrophotometry at 600 nm (Gowrishankar et al., 2012). A viable plate count was also performed to correlate with the reduction in the optical density values of the pathogenic strains of *S. aureus* as compared to the untreated controls.

Screening for Anti-virulence Potential

Coral-associated bacterial extracts which exhibited antibacterial and antibiofilm properties were further screened for their ability to inhibit the production of secretory virulence factors such as hemolysin α / β, proteases as well as the cell-associated virulence factors of *S. aureus* strains used in this study.

Production of Extracellular Virulence Factors

A 1% inoculum of reference and test pathogens was added to the MHB supplemented with 0.5% polysorbate 80 both in the presence and absence of CB extract at MIC and incubated at 37°C under shaking until the OD at 600 nm reached a value of 2.5. After centrifugation at 10,000 rpm twice, the supernatants containing secretory virulence factors were recovered from the pathogens (incubated with extract at MIC) and used for further assays (Gowrishankar et al., 2012).

Total Hemolysin Inhibition Assay

Hemolytic activity of the test and reference strains was initially observed on blood agar plates (Kloos and Schleifer, 1975). A total of 2% sheep erythrocytes prepared in 1× PBS was added to the buffer containing 10 mM Tris, 160 mM NaCl, and 20 mM $CaCl_2$ adjusted to pH 7.4 using HCl. A total of 300 μL of extracellular products (ECP) produced from *S. aureus* strains were added to 2700 μL of 2% sheep RBCs and incubated at 37°C for 30 min. After incubation, the mixture was kept on ice for 20 min followed by centrifugation around 12,000 rpm at 4°C. The released hemoglobin in the supernatant was estimated by absorbance at 530 nm (Gowrishankar et al., 2012). The results were represented in terms of the percentage of hemolysin inhibition for the test (pathogens treated with CB extract) compared to control (pathogens untreated with CB extracts).

Protease Inhibition Assay

Proteolytic activity due to the production of extracellular proteases from test and reference *S. aureus* strains was confirmed on skim milk agar plates incubated at 37°C for 24 h (McNamara et al., 2000; Miedzobrodzki et al., 2002). In order to evaluate the protease inhibition, wells with a diameter of 6 mm were cut onto the skim milk agar plates and loaded with aliquots of exoproteins from the test (treated with extract) and control (without extract treatment). Post loading, the plates were incubated at 37°C for 24 h.

FTIR Analysis to Study Changes in Cell-Associated Biomolecules

To study changes in cell-associated virulence factors, both test and reference *S. aureus* strains were cultured in TSB along with extracts at MIC until the OD values of respective controls (pathogens free from extract treatment) reached 2.5 at 600 nm. After incubation, cells were pelleted down by centrifugation at 10,000 rpm and dried. The potassium bromide (KBr) pellet technique was adopted for FT-IR analysis (Gowrishankar et al., 2012). FT-IR spectrum was recorded in the IR spectral region between 4,000 and 400 cm^{-1} (Thermo Nicolet-6700).

Phylogenetic Analysis of the Coral-Associated Bioactive Bacterium

For molecular taxonomic identification, the genomic DNA from CBMGL12 was extracted using standard procedures adopted from the molecular cloning laboratory manual by Green and Sambrook (2012). The quantity and quality of extracted DNA was assessed using NanoDrop spectrophotometer (ND 1000). 16S rRNA gene was amplified using the GeneAmp PCR System 9700 (Applied Biosystems). 16S gene amplicons were initially purified by ExoSAP-IT kit (USB). The purified amplicons were sequenced. The gene sequence of the coral-associated bacterium was submitted in GenBank to obtain the accession number. The phylogenetic analysis was performed using Mega software version 6.0 to establish molecular evolutionary relationships (Felsenstein, 1985; Tamura, 1992; Tamura et al., 2013).

Production and Purification of Bioactive Molecules

The strain CBMGL12 was mass produced in 1,000 mL Erlenmeyer flask containing 600 mL of nutrient broth supplemented with 2% NaCl (at pH 7.8) and incubated at 30°C for two days at 150 rpm. The culture broth was pellet down twice at 6,000 rpm for 15 min at 4°C, and the cell-free supernatant was collected. The supernatant was acidified with concentrated HCl to achieve the final pH 2.0 and extracted twice with an equal volume of EtOAc (Kiran et al., 2010). The crude solvent extract was concentrated using a rotary vacuum evaporator (R-300, BUCHI Corporation, Switzerland).

The concentrated extract was resolved initially on TLC plates using silica gel 60 Gas stationary support (Stahl, 1969) and different combinations of solvents with varying polarity as mobile phase (Landgrebe, 1977; Lade et al., 2014) to study the number of spots having active fractions (Kiran et al., 2010) and also to identify a suitable solvent system for next step purification using column chromatography. After observation of characteristic pattern of spots on TLC, the crude EtOAc extract was purified through column chromatography using chloroform: EtOAc as a solvent system and silica gel (mess size 100–200) as stationary support. The column purified active fraction was further fractionated through the C18 / ODS column of preparative HPLC-UV (UFLC-Shimadzu) equipped with a photodiode array detector. The solvent system used in liquid chromatography was chloroform : methanol (HPLC grade) in the following ratios 95: 5, 90: 10, 85:15, and 80:20 with a flow rate of 0.5 ml/min (Mansson et al., 2011). All the purified fractions were subjected to the above discussed antivirulence assays in order to confirm the presence of active molecules.

Chemical Characterization of HPLC Purified Bioactive Fractions

HPLC purified anti-virulent fraction was analyzed through FT-IR and GC-MS/MS equipped with NIST library to study the associated chemical moieties and to predict the presence of different types of fatty acid esters, respectively. The structural elucidation of the active fraction was carried out through Fourier Transform Nuclear Magnetic Resonance spectroscopy (400 MHz-FT-NMR, Bruker-Advance-II, Germany). ^{13}C (100 MHz) and proton (400 MHz) NMR spectra were recorded after overnight scanning runs (TOPSPIN NMR data system).

RESULTS

Multidrug Resistance, Virulence and Biofilm Phenotypes in Pathogenic *S. aureus* Strains

The pathogenic *S. aureus* test strain responsible for community-acquired infections was resistant to Penicillin (10 units),

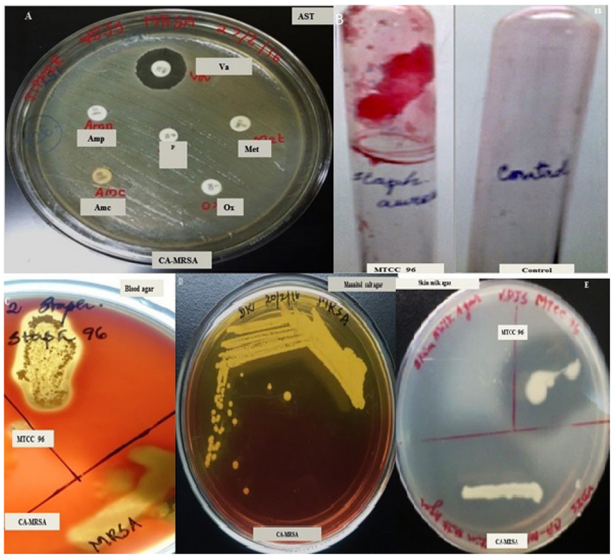


FIGURE 1 | Screening for multidrug resistance in *Staphylococcus aureus* strains [CA-MRSA **(A)**] using Kirby-Bauer Disc diffusion assay. **(B)** Recalcitrant biofilm formed by CA-MRSA in modified tube assay. **(C)** Hemolytic activity of MTCC96 and CA-MRSA on Blood agar, **(D)** CA-MRSA on Mannitol Salt Agar, **(E)** Protease activity of MTCC96 and CA-MRSA on Skim milk agar. AST, Antibiotic Sensitivity Testing; Va, Vancomycin (30 μg/disc); Met, Methicillin (10 μg/disc); P, Penicillin (10 units/disc); Amp, Ampicillin (10 μg/disc); Amc, Amoxicillin with clavulanic acid (20/10 μg per disc); Ox, Oxacillin (1 μg/disc).

Ampicillin (10 μg), Amoxicillin / clavulanic acid (20/10 μg), Oxacillin (1 μg), and Methicillin (10 μg) but found sensitive to Vancomycin (30 μg) as identified by Kirby-Bauer antibiotic disc diffusion assays (**Figure 1A**). The test strain was designated as CA-MRSA (**Figure 1D**). CA-MRSA exhibited extracellular virulence phenotypes such as hemolysin β and proteases, as confirmed by enriched agar plate assays. The potential to develop biofilm *in vitro* was absent in CA-MRSA as observed by modified tube and microtitre plate assays. On the other hand, the reference strain MTCC96 was able to develop a recalcitrant biofilm *in vitro* (**Figure 1B**) as observed in modified tube / microtitre plate assays and also produced the secretory virulence factors such as hemolysin α (**Figure 1C**) and proteases (**Figure 1E**) as identified in enriched agar plate assays. Hence, the two *S. aureus* strains were tested for further antibacterial, antivirulence and antibiofilm studies.

Screening for Antibacterial, Antibiofilm Potentials

A total of 43 morphologically distinct bacterial colonies were isolated from the surface mucus layer and ground tissue samples of the coral *Favites* sp. (**Figure 2**). The coral-associated bacterial isolates were designated from CBMGL1 to CBMGL43. Out of the 43, cell-free supernatants from the three bacterial isolates (CBMGL1, CBMGL2, CBMGL12) exhibited prominent zone of growth inhibition against *S. aureus* strains in an agar well diffusion assay when loaded at 100 μL/well (**Figure 3**). However, when extracted with an equal volume of ethyl acetate, the extract CBE 12 (from CBMGL12) exhibited antibacterial activity against CA-MRSA and MTCC96 (**Supplementary Figure 1**). The metabolite production was enhanced during nutrient limiting conditions, and a maximum was achieved after 72 h of growth at pH 7.8, 30°C, under constant shaking at 150 rpm. The MIC of CBE12 was found to be 50 and 25 μL against MTCC96 and CA-MRSA, respectively, as revealed in broth tube dilution technique. Besides, the extract exhibited significant inhibition of biofilms formed by MTCC96 (**Figures 4**, **5**) when viewed under stereo zoom and fluorescence microscopy. CBE12 extract was able to reduce the total planktonic bacterial cell density in both strains of *S. aureus* (CA-MRSA and MTCC96) at its MIC and sub-MIC when compared with respective untreated growth controls (**Figure 6**). Hence, the extract CBE12 was taken to screen for the associated antivirulence potentials.

Screening for Antivirulence Potentials

Initially, the percentage of RBC lysis was estimated from the released hemoglobin OD at 530 nm in control (ECP) and test (**Supplementary Figure 2**). From the percentage of RBC lysis, the percentage of hemolysis inhibition was deduced as an indicative of the inhibition of hemolysin production in the pathogens. Percentage of hemolysis inhibition was found to be 70.59 and 78.97% on MTCC96 and CA-MRSA, respectively (**Table 1**). Protease inhibition was confirmed on the skim milk agar plate as the reduced zone of casein hydrolysis around the wells loaded with ECP from CBE12 treated CA-MRSA and CBE12 treated MTCC96 when compared with respective control wells (**Supplementary Figure 3**).

In an attempt to observe the inhibition of cell-associated virulence factors, FT-IR spectral studies were carried out for CBE12 treated and untreated (control) MTCC96 and CA-MRSA cell pellets using KBr pelleting technique. FT-IR spectra were recorded after all the baseline corrections. CBE12 exhibited a marked reduction in the production of cell surface virulence proteins in both MTCC96 (**Figure 7A**) and CA-MRSA (**Figure 7B**). Besides, CBE12 showed a reduction in membrane fatty acid moieties of MTCC 96 when compared to the respective control cell pellet (untreated). On contrary, the membrane fatty acid moieties were increased in CA-MRSA.

Phylogenetic Analysis of Coral-Associated Bioactive Bacterium CBMGL12

Standard biochemical analysis showed the bioactive strain CBMGL12 as *Pseudomonas* sp. (**Supplementary Table 1**). Molecular taxonomic identification of CBMGL12 revealed the bioactive bacterium as *P. aeruginosa* (GenBank accession number: MF521927) through 16S rRNA gene sequencing. Phylogenetic analysis was conducted using

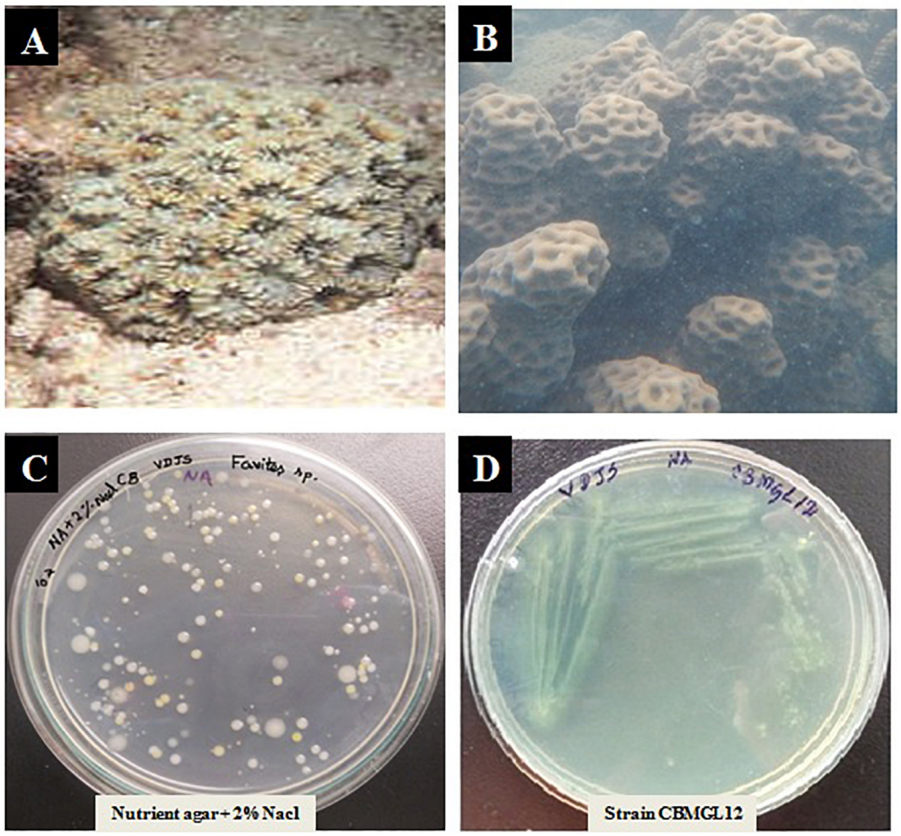


FIGURE 2 | Isolation of Bacteria from the coral *Favites sp.* **(A)** Underwater view of the coral *Favites* sp. outside the Gulf of Mannar Marine Biosphere Reserve. **(B)** *Favites* sp. after sampling the tissue and mucous using needleless syringe and swabs. **(C)** Isolated colonies of coral-associated bacteria (CB) on nutrient agar (NA) plate with 2% NaCl. **(D)** Pure culture of bioactive isolate CBMGL12 on NA plate.

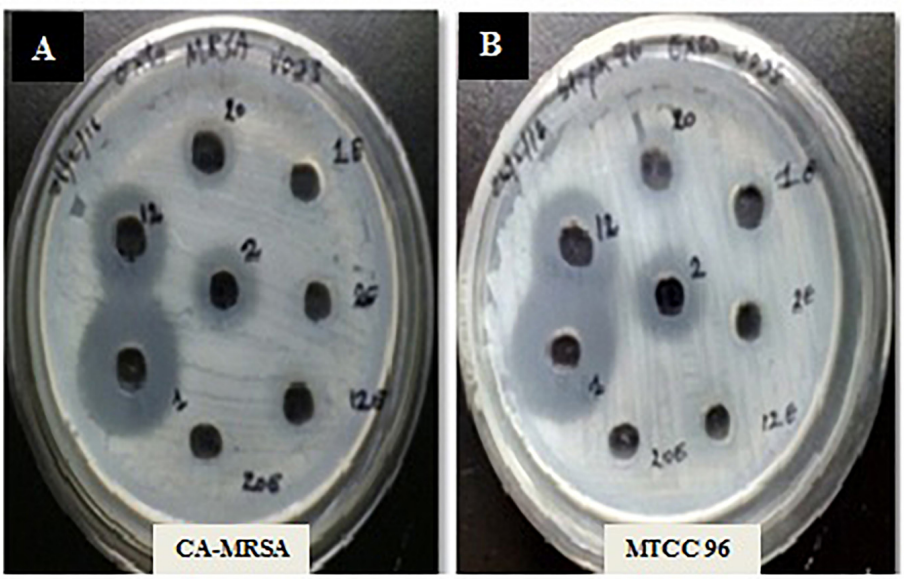


FIGURE 3 | Antibacterial activity of the CFS against *S. aureus* strains. MHA plates representing the antibacterial activity of CFS from 48 h grown cultures of CB-1, 2, and 12 against CA-MRSA **(A)** and MTCC96 **(B)**.

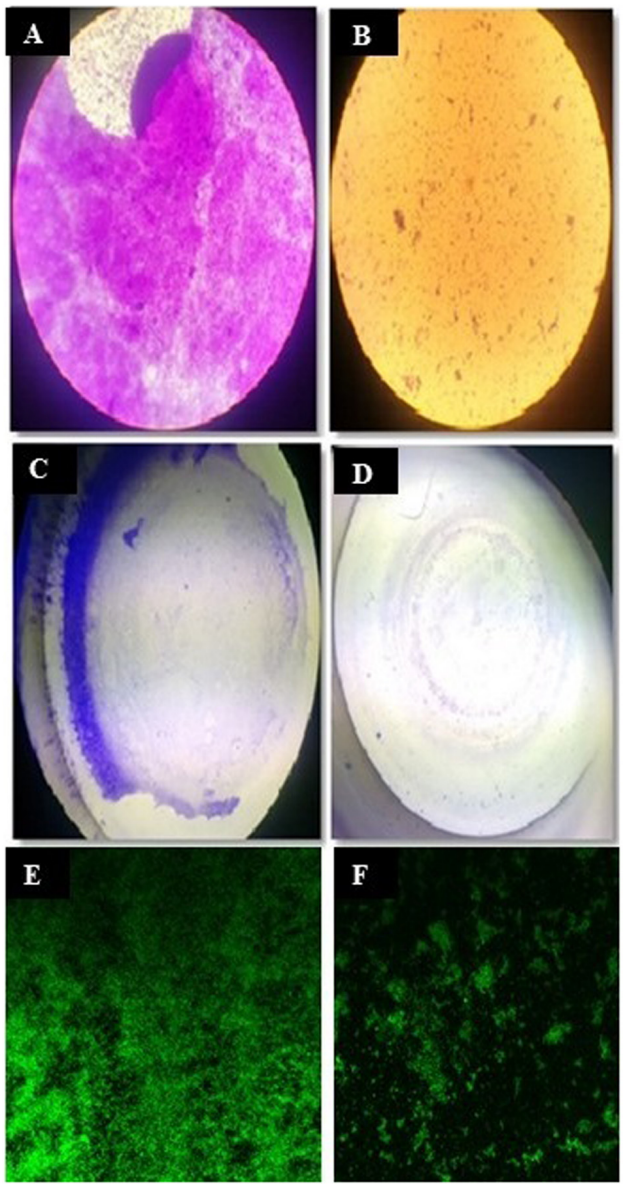


FIGURE 4 | Antibiofilm assay. Light microscopic view of *S. aureus* MTCC96 biofilm **(A)** and its disruption by CBE12 extract at MIC **(B)**. Stereozoom microscopic view of MTCC96 biofilm **(C)** and its disruption by CBE12 extract at MIC **(D)** in MT plate wells. Fluorescence microscopic view of acridine orange stained, untreated **(E)** and CBE12 extract treated **(F)** biofilm of *S. aureus* MTCC96.

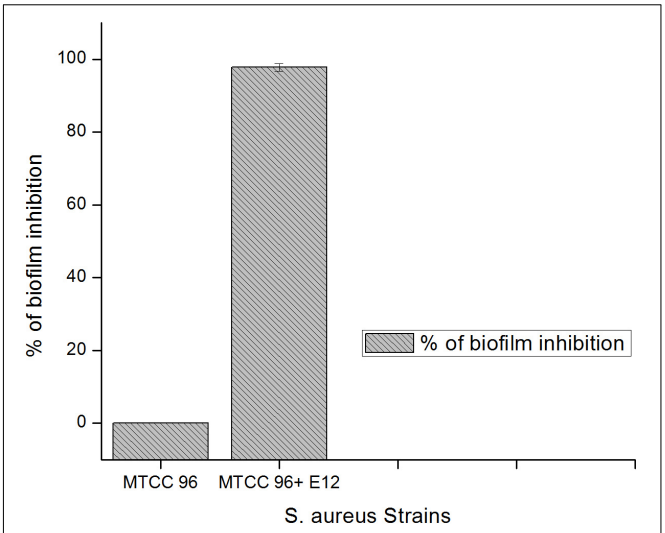


FIGURE 5 | Percentage of biofilm inhibition by CBE12 at BIC (50 μL) in 96-well MT plate as derived from OD values (triplicates) at 595 nm and compared to its untreated control. CBE12 is given as E12 in the figure.

MEGA software version 6.0. The phylogenetic tree was constructed using the UPGMA method. The tree with the highest log likelihood (−4179.1541) was shown in **Figure 8**.

Purification and Characterization of Antivirulence Fatty Acid Methyl Esters

UV-visible spectral analysis of the active crude extract records the absorbance maxima between 200 to 300 nm indicating the polygenic nature of the CBE12 extract. The crude extract CBE12 was fractionated by column chromatography. The column purified active fraction (CFA2, 10:90 (v/v) EtOAc/$CHCl_3$) was further characterized using HPLC-UV, TLC, FT-IR, and GC-MS/MS analyses. In the silica gel TLC, three discrete spots were observed with the R_f value of biologically active spot as 0.57. Bioassay directed fractionation was performed in TLC and HPLC (**Supplementary Figure 4** and **Supplementary Tables 2, 3**). The FT-IR (**Figure 9**) and GC-MS/MS (**Table 2**) analyses revealed fatty acid methyl esters such as methyl benzoate and methyl phenyl acetate may be responsible for antibiofilm and antivirulence activity. The proton NMR data marks the presence of proton types in methyl ester group at 3.686 ppm and the protons of participating carbon atom of benzene rings linked to acetic acid methyl ester at 7.8 ppm (Ortho), 7.6 ppm (Meta), and 7.7 ppm (Para). Proton peak at 8.05 ppm suggests the proton type link to the ortho carbons of benzoic acid methyl ester. ^{13}C NMR data indicates the occurrence of carbon types in the benzene ringed structures at 127.2 ppm (Meta), 128.7 ppm (Ortho), 129.5 ppm (Para). Overall, the NMR spectra suggest the presence of compounds having aromatic rings linked to the methyl ester groups in purified bioactive fraction which could possibly be involved in the antibiofilm and antivirulence activities of the coral-associated bacterial extract (**Supplementary Figures 5, 6**).

DISCUSSION

In this study, the extracellularly released bioactive compounds from coral-associated bacterium *P. aeruginosa* were extracted with ethyl acetate after acidifying the cell-free supernatant to pH 2.0 with 1N HCl in order to facilitate the protonation of water molecules which in turn releases the active molecules into

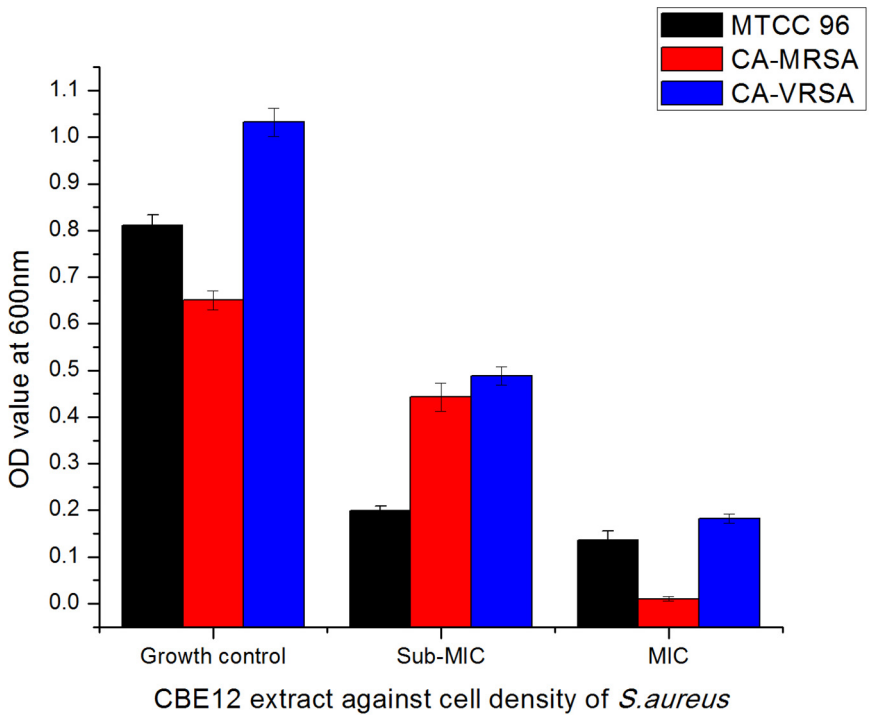


FIGURE 6 | Effect of CBE12 on cell density of *S. aureus* strains at MIC (50 μL) and sub-MIC (25 μL). CA-VRSA strain did not express virulence factors and biofilm properties *in vitro*. Hence, the strain was not taken for the later experimentations.

the solvent phase. QS is a population dependent mechanism, a decrease in cell density is usually expected at MIC and sub-MIC of the CB extracts. It is evident in our study that the extract CBE12 exerted a steady reduction in viable colony count as well as the cell density of both *S. aureus* strains as indicated by a gradual decrease in growth OD of MTCC96, and CA-MRSA strains treated at sub-MIC and MIC when compared to the corresponding untreated controls.

Biofilm inhibition was observed in MTCC96 when treated with the extract CBE12 at BIC. Though the extract CBE12 inhibited the production of secretory virulence proteins such as hemolysins and proteases and also the cell surface distributed virulence proteins in both *S. aureus* strains, the same extract slightly increased the membrane fatty acid moieties of CA-MRSA strain as observed in FT-IR analysis of extract treated *S. aureus* cell pellets.

When investigating the chemical nature of HPLC purified, active fractions of the secondary metabolites from coral-associated bacterium using FT-IR analysis and GC-MS/MS equipped with NIST library, we observed the likely involvement of aromatic fatty acid methyl esters (FAME) such as methyl benzoate and methyl phenyl acetate, in the inhibition of virulence and biofilm phenotypes in *S. aureus*. NMR data suggest the presence of participating carbon atoms of benzene ringed structures in ^{13}C NMR and the proton types of methyl ester and phenyl groups in ^{1}H NMR. As reported previously (Gordon et al., 2013), the compounds which have benzene ringed derivatives with carbonyl moieties could act as virulence factor inhibitors and interfere with the QS system in *S. aureus*. Another study in which the phenyl rings and carbonyl groups of cyclodepsipeptides such as solonamides A and B of marine mussel surface bacterium, *Photobacterium* were responsible for the inhibition of virulence gene expression in *S. aureus* through their interaction with AgrC, a receptor histidine kinase (Mansson et al., 2011). Therefore, the two fatty acid methyl esters such as methyl benzoate and methyl phenyl acetate produced by the coral-associated bacterium *P. aeruginosa* strain CBMGL12 could be responsible for the antibiofilm, antivirulence and antibacterial activities towards human pathogenic *S. aureus* as evinced in our study. In a study performed by Divya et al. (2018) in the coral *Favites abdita*, they have documented the ability of biofilm forming opportunistic pathogen *Staphylococcus sciuri* on multiple coral hosts.

As future prospects, the study can be further extended to identify the specific targets involved in the QS system of *S. aureus* which could be inhibited / interfered by the active

TABLE 1 | Percentage of hemolysis inhibition by the extract CBE12.

CBE12 treated *S. aureus* strains	Control	% of RBC lysis after CBE12 treatment	% of hemolysis inhibition by CBE12
MTCC 96	100	29.41	70.59
CA-MRSA	100	21.03	78.97

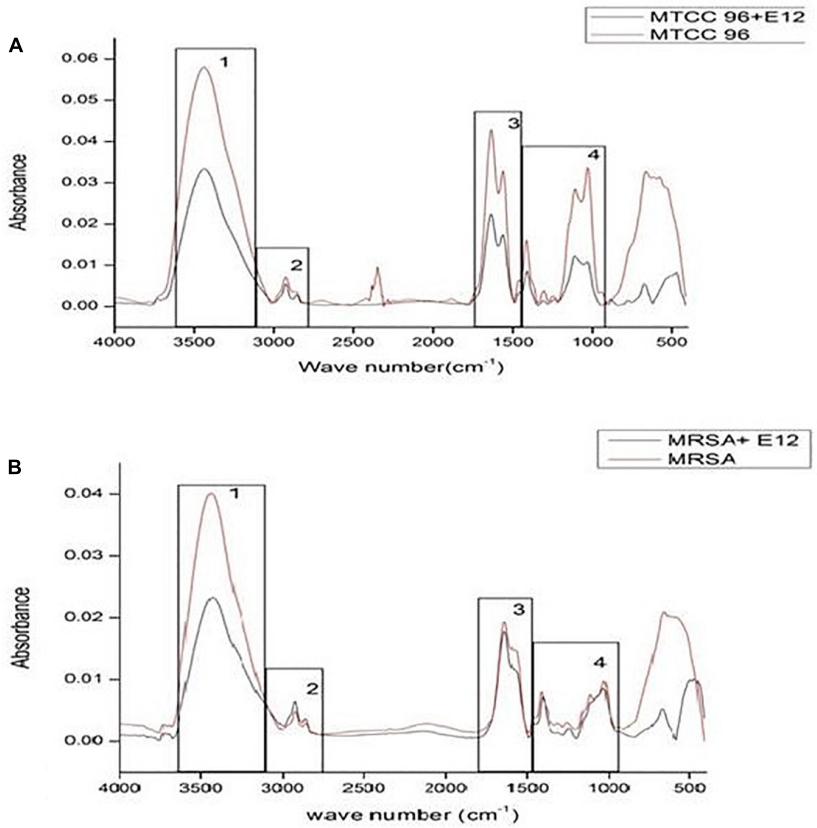


FIGURE 7 | FT-IR spectrum to study the effect of CBE12 on the cell-associated virulence factors of MTCC 96 **(A)** and CA-MRSA **(B)**.

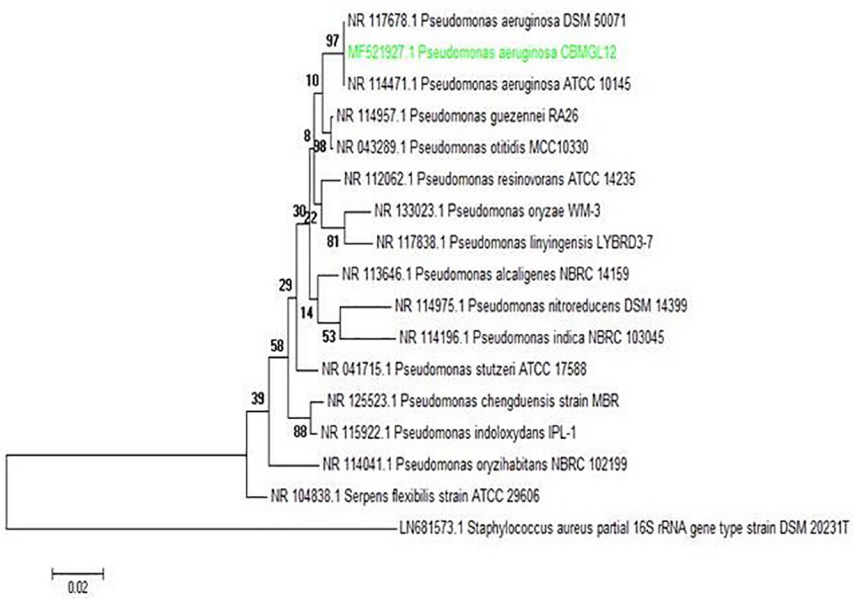


FIGURE 8 | Phylogenetic analysis of the producer bacterium *Pseudomonas aeruginosa* strain CBMGL12. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. Scale bar represents nucleotide substitutions per site.

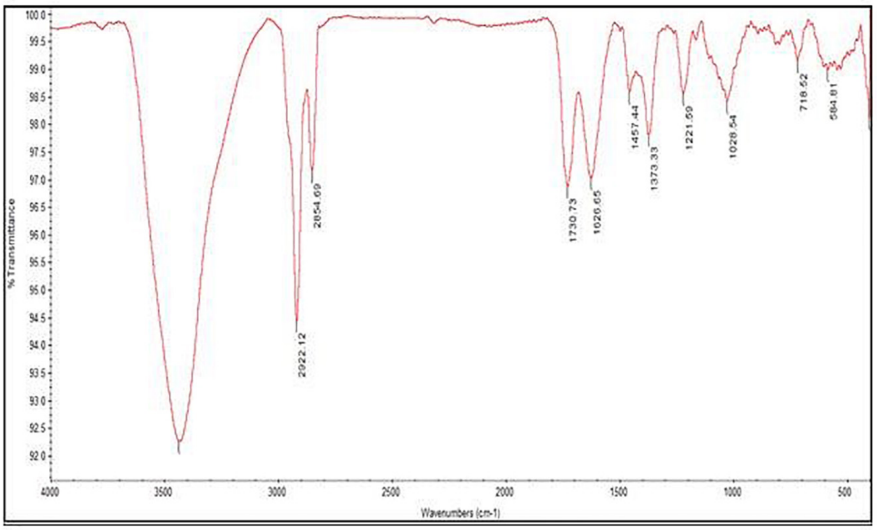
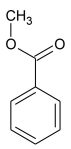
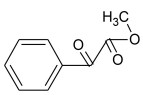


FIGURE 9 | FT-IR spectrum of antivirulence fraction separated by column chromatography.

TABLE 2 | Antibiofilm, antivirulence compounds from coral-associated bacterium *Pseudomonas aeruginosa* strain CBMGL12 as predicted by GC-MS/MS analysis assisted with NIST library.

Sl.no.	Peak name	Retention time	% Peak area	Structure
1	Name: benzoic acid, methyl ester Formula: $C_8H_8O_2$ MW: 136	17.812	1.12	
2	Name: benzene acetic acid, methyl ester Formula: $C_9H_{10}O_2$ MW: 150	21.720	13.66	

molecules produced from the coral-associated *P. aeruginosa* CBMGL12 through cloning and expression of *S. aureus* associated QS components in genetic model systems and careful observation of anti-QS activity. Also, the production of these antivirulence metabolites by *P. aeruginosa* CBMGL12 shall be over enhanced using the cheapest carbon and nitrogen sources to commercialize these bioactive molecules cost-effectively. The pathways in which *P. aeruginosa* CBMGL12 synthesized these bioactive molecules can also be a choice of study for the future young minds.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

JS and GK conceived the study, designed, supervised the research, and prompted the manuscript. KV performed *in vitro* experiments, analyzed the data, and prepared the manuscript. SD and RD assisted sample collection and *in vitro* studies. KT and ST assisted data collection and analysis. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.631853/full#supplementary-material

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Anti-biofilm Potential of *Elletaria cardamomum* Essential Oil Against *Escherichia coli* O157:H7 and *Salmonella* Typhimurium JSG 1748

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Foodborne pathogens, microbial recurrent infections, and antibiotic resistance have driven researchers to explore natural compounds as safe alternative antimicrobials. In this study, the chemical profile, antimicrobial, and mutagenic activities of the *Elletaria cardamomum* essential oil were investigated. GC-MS analysis identified the major bioactive components as α-terpinyl acetate, 1,8-cineole, linalool acetate, and sabinene, at concentrations of 34.95, 25.30, 8.13, and 5.48% respectively, of the essential oil's content. Regarding antimicrobial activity, the minimum inhibitory concentration of green cardamom essential oil was 1% against *Escherichia coli* O157:H7 and *Pseudomonas aeruginosa* ATCC 14213. Green cardamom essential oil, when used at concentrations of 0.015, 0.031, 0.062, and 0.125% (v/v) prevented biofilm formation of *Escherichia coli* O157:H7 by 64.29, 65.98, 70.41, and 85.59%, respectively. Furthermore, these concentrations inhibited 6.13, 45.50, 49.45, and 100%, respectively, of the *Salmonella* Typhimurium JSG 1748 biofilm. A mutagenicity assay confirmed that green cardamom essential oil has no demonstrable mutagenic activity against the tested strains. The study's findings suggest that green cardamom derived bioactive compounds are safe organic antimicrobials, effective in controlling biofilm formation by Gram-negative pathogens. Moreover, such compounds could possibly be used in the food industry (e.g., bakery, dairy, meat, and other food products) as a safe alternative to chemical preservatives (antimicrobials) to enhance shelf life by improving the antimicrobial status while at the same time imparting a pleasant and appealing aroma for consumers.

Keywords: green cardamom, bioactive compounds, GC-MS characterization, anti-biofilm potential, *Escherichia coli* O157:H7, *Salmonella* Typhimurium JSG 1748, mutagenic activity

INTRODUCTION

Interest in the application of bioactive phytochemicals and essential oils in food and pharmaceutical products has rapidly increased due to their health benefits, including their antioxidant, antimicrobial, and hypolipidemic properties (Deepa et al., 2013; Aghasi et al., 2019; Yousefi et al., 2019; Abdullah et al., 2020; Dehghani et al., 2020). A wide variety of herbs and spices have been used in cooking and medicine, particularly in the treatment of gastrointestinal disorders, since ancient times. Many studies have reported the health benefits of cardamom derived bioactive phytochemicals related to their antimicrobial (e.g., bacterial growth, biofilm, and quorum sensing inhibition), gastro-protective (anti-ulcer), hypocholesterolemic, and hypotensive activities (Verma et al., 2009; Sharma et al., 2011; Abdullah et al., 2017, 2020; Chakraborty et al., 2019).

Elletaria cardamomum, also called green or true cardamom, is a member of the family *Zingiberaceae*. Green cardamom is used as a functional additive in food products due to its pleasant aroma, as a flavor enhancer, and as a preservative in confectionery, beverages, bakery, dairy, and meat products. The antioxidant and antibacterial potentials of green cardamom are due to phenolic and flavonoid compounds present in the spice (Padmakumari Amma et al., 2010; Abdullah et al., 2017). For the extraction of the bioactive compounds present in the green cardamom, various techniques have been used such as conventional solvent extraction, hydro-distillation, and super-critical fluid extraction (SFE; Azmir et al., 2013; Yousefi et al., 2019). In recent years, the extraction of phytochemicals and essential oils-comprising bioactive compounds by SFE has gained a greater level of attention and is now the most widely-used method due to reduced sample degradation, higher solubilization, improved processing time and technological selectivity (e.g., temperature, pressure, and extraction time), no requirement for clean-up steps, and its eco-friendly nature (Yousefi et al., 2019). Moreover, the extraction efficiency, time, temperature, and pressure conditions can be adjusted in the SFE system according to the targeted analytes (Marongiu et al., 2004; Junior et al., 2010).

A bacterial biofilm is a three-dimensional structure, formed by mono- or multi-microbial communities embedded into an extracellular matrix, which provides resistance to microorganisms against harsh environmental conditions, antibiotics, and the defenses of the human immune system (Miquel et al., 2016; Jamal et al., 2018). Recently, Nassar et al. (2021) reported that microorganisms responsible for various human infections (~80%) and hospital-acquired infections (60–70%), have shown a biofilm origin. Therefore, to eradicate biofilm-related persistent infections and to reduce their impact on human health, it is necessary to find safe antimicrobials as anti-biofilm agents, which can inhibit biofilm formation. Furthermore, the emergence of antibiotic resistant pathogens is a key factor driving the need for novel alternative antimicrobial compounds with the ability to inhibit biofilm formation, as biofilms play an important role in the development of antimicrobial resistance (emergence and dissemination), and their persistence may eventually lead to the failure of currently available treatments for many bacterial and other species infections (Livermore, 2011; Nassar et al., 2021). Bacterial strains are becoming increasingly resistant to conventional antibiotics; therefore, safe and effective antimicrobial agents are urgently required to counteract multidrug-resistant microorganisms (Newman and Cragg, 2016). In this regard, many herbs and plants represent a promising source of biologically active compounds with antimicrobial properties (anti-biofilm) and with limited negative side effects on human health (Newman and Cragg, 2016; Abdullah et al., 2020). In recent years, some studies established the antimicrobial potential of cardamom-derived bioactive compounds against *Escherichia coli*, *Streptococcus mutans, Salmonella* Typhimurium, *Staphylococcus aureus*, and *Candida albicans* (Aneja and Joshi, 2009; Abdullah et al., 2017, 2020).

Controlling biofilm formation by pathogenic bacteria remains a challenging issue that requires the discovery and analysis of effective and safe alternative antimicrobials that may be used for the prevention of antibiotic resistance and infection recurrence. This study aimed to investigate the green cardamom essential oil (GCEO) chemical profile (bioactive components), antimicrobial, and mutagenic properties with the following objectives; (i) quantification of GCEO bioactive compounds using GC-MS, (ii) determination of the minimum inhibitory concentrations (MICs), and biofilm inhibitory concentrations of GCEO against *Escherichia coli* O157:H7 and *Salmonella* Typhimurium JSG 1748, and (iii) investigating the mutagenic activities of GCEO using *Salmonella* Typhimurium TA98 and *Salmonella* Typhimurium TA100 strains.

MATERIALS AND METHODS

Chemical Reagents and Materials

Green cardamom was procured (Faisalabad, Punjab, Pakistan), cleaned, and milled into powder. All reagents used in this study were of HPLC grade and acquired from Sigma (Sigma-Aldrich, Tokyo, Japan) and Merck (Merck KGaA, Darmstadt, Germany). Tryptic soy agar (TSA, Thermo Fisher Scientific Remel Products, Lenexa, KS, United States), and tryptic soy broth (TSB, BD Difco, Franklin Lakes, NJ, United States) were used to grow and maintain the bacterial strains. The Muta-ChromoPlate™ Bacterial Strain Kit, a 96-well microplate, was purchased and used for detection of mutagenic activity, and TA100 and TA98 were the default bacterial strains included in the Muta-ChromoPlate kit (Muta-ChromoPlate™ Bacterial Strain Kit: Product No. B5051, Environmental Bio-Detection Products Inc. (EBPI), Mississauga, Canada).

Supercritical Fluid Extraction of GCEO

GCEO was obtained using a supercritical fluid extraction (SFE) instrument (model SFT-150, Applied Separations, Inc., Allentown, PA, United States) using 99.8% pure CO_2 at 30°C, 300 bar, and 60 min according to Abdullah et al. (2020). The sample (100 g) in powder form was loaded into the extraction vessel, and the gas (CO_2) was liquefied by employing the SFE conditions (temperature, pressure, and time), which facilitated mass transfer in the form of green cardamom essential oil (GCEO). GCEO was collected in a vial and kept in the refrigerator at 4°C for further analyses.

GC-MS Analysis of GCEO

The GCEO sample (10 µl) was diluted in per ml of tertiary butyl methyl ether and examined using GC-MS according to the guidelines of Adams (2017) with minor modifications. The diluted GCEO sample (1 µl) was injected into the column (SH-RXI-5SIl MS, 30 m × 0.25 mm × 0.25 µm) by an auto sampler (AOC 6000). The GC-MS analysis of the sample was carried out under the following conditions: the initial temperature was 35°C for 4 min, which reached up to 250°C by increasing at a rate of 20°C per min, and the flow rate of helium (carrier gas) was set as constant (1 ml per min). The ionization was performed in the electron impact mode at 230°C and an ionization energy of 70 eV. Mass spectra were obtained in full scan mode (mass range *m/z* 35-450) under auto-tuning conditions. The identification of bioactive compounds was carried out by matching the spectra with the mass spectral libraries, and the identity of each compound was confirmed by comparing its Kovat's index with the libraries (Wiley275.L) and compounds from the literature (Adams, 2017). **Table 1** shows the isolated GCEO bioactive compounds listed in order of elution from the SH-RXI-5Sil MS column, and their relative percentage was computed from the chromatogram peak area by the peak integration method using a MS detector.

TABLE 1 | GC-MS quantification of *Elletaria cardamomum* bioactive compounds.

Peak No.	Retention time (min)	Bioactive compounds	Composition (%*)
1	7.619	a-Thujene	0.20
2	7.719	a-Pinene	1.81
3	8.176	Sabinene	5.48
4	8.244	b-Pinene	0.36
5	8.328	Myrcene	1.76
6	8.720	p-Cymene	0.14
7	8.777	Limonene	2.80
8	8.817	1,8-Cineole	25.30
9	9.048	g-Terpinene	0.12
10	9.169	Linalool oxide	0.15
11	9.397	Terpinolene	2.30
12	9.459	Linalool	1.87
13	10.152	Tetrahydro Linalool	0.19
14	10.259	a-Terpineol	2.79
15	10.405	cis-Sabinene hydrate acetate	1.02
16	10.557	Geraniol	0.24
17	10.609	Linalool acetate	8.13
18	10.663	Unknown	0.11
19	10.771	Geranial	0.45
20	11.143	Acetate	0.15
21	11.386	a-Terpinyl acetate	34.95
22	11.525	Geranyl acetate	1.02
23	11.972	g-Elemene	0.11
24	12.449	a-Farnesene	0.54
25	12.763	(E) Nerolidol	1.57
26	12.831	Unknown	0.15
27	14.942	n-Hexadecanoic acid	0.79
28	15.999	Fatty acids (C18)	3.27
29	16.167	Fatty acids (C18)	0.25
30	17.187	Fatty acids (C18)	0.28
		Total	98.39

**%, Relative percentage of the bioactive compounds computed from the chromatogram peak area.*

Microbial Strains and Growth Conditions

Escherichia coli O157:H7, *Pseudomonas aeruginosa* ATCC 14213, and *Salmonella* Typhimurium JSG 1748 were used as tested strains in this study. The bacterial strains were selected as representative of Gram-negative pathogens, responsible for foodborne diseases and also have medical importance. Tryptic soy broth (TSB) was used for the growth and maintenance of the bacterial strains under aerobic condition for 20–24 h with continuous shaking (200 rpm) at 37°C. Tryptic soy agar was used for bacterial plating and enumeration.

Evaluation of Minimum Inhibitory Concentrations of GCEO

A broth micro-dilution assay was performed to determine the MIC value of the GCEO according to Algburi et al. (2017), with minor modifications. Briefly, the frozen stocks of the tested strains were grown in TSB media and incubated aerobically at 37°C for 20–24 h to obtain ~10^9 CFU ml^{-1}. The spot plate method was used to confirm the bacterial cell count, then the overnight grown bacterial cultures were diluted (1:1000, v:v) in fresh TSB media to achieve bacterial cell count ~10^6 CFU ml^{-1}. GCEO was first dissolved in ethanol (95%) to prepare a 100 mg ml^{-1} stock solution and then further diluted into TSB media. The dilutions were prepared ranging from 0.015–2% (v/v) using a 96-well micro-plate. Subsequently, 100 µl of the diluted bacteria cells (10^6 CFU ml^{-1}) were added separately into each well of a 96-well micro-plate which had previously been treated with a certain concentration of GCEO. As a control, ethanol was separately diluted with TSB broth (0.1425–1.9%) and evaluated against the growth of the tested bacterial strains. The results showed that the above-mentioned concentrations of ethanol (used as control) had no antibacterial activity. Mineral oil (70 µl) was pipetted into each well of the micro-plate to avoid evaporation of the mixture during the incubation time. The micro-plate was incubated aerobically for 24 h at 37°C inside the plate reader (SmartSpec™ 3000, Bio-Rad, Hercules, CA, United States and ThermoMax, Molecular Devices, San Jose, CA, United States). MIC values of the GCEO were calculated from the kinetic growth curve readings after incubation at an optical density (OD 595 nm). The kinetic curves of bacterial growth were drawn statistically, and the experiments were repeated at least twice and in duplicate. "The MIC is defined according to CLSI (Clinical and Laboratory Standards Institute, 2010) guidance as the lowest concentration of an antimicrobial substance that inhibits bacterial growth in the wells with an OD 595 nm equal to or less than 20% of the control's mean absorbance (bacterial growth without antimicrobial addition). The MIC was determined based on the tested concentration at which a kinetic growth curve reading showed little to no increase in cell density during the 24 h incubation time indicating bacterial growth inhibition. The non-inhibitory concentrations (NICs) of GCEO were defined as the concentrations that had no measurable effect on the kinetic growth curve readings." While the minimum bactericidal concentration (MBC), which is an option to be determined, is defined as the lowest

concentration of an antimicrobial that completely kills the bacterial cells, in which the OD 595 nm of treated wells will be equal to OD of the negative control (broth only).

Evaluation of Biofilm Formation Inhibition by GCEO

The biofilm inhibitory effect of GCEO was evaluated against *E. coli* O157:H7 and *S.* Typhimurium JSG 1748. The biofilm formation inhibition analysis was performed according to Algburi et al. (2020). Briefly, *E. coli* and *S.* Typhimurium were grown aerobically using Luria Bertani (LB broth), (BD Difco, Fisher Scientific UK Ltd., Leicestershire, England) supplemented with 1% of glucose (LBG) for 24 h at 37°C. GCEO was diluted ranging from 0.007–0.125% (v/v) into a 96-well micro-plate with a final volume of 100 µl. The overnight grown bacteria were diluted (1:40) in fresh LBG culture medium and 100 µl of the diluted suspension was transferred into each well of the 96-well micro-plate which was pre-treated with GCEO and incubated for 48 h at 37°C. After incubation, the planktonic cells were carefully aspirated and enumerated using the spot plate method and the wells of the plate were washed twice with fresh culture media after incubation. The plate was then heat-fixed for 60 min at 60°C. A sample of 125 µl of crystal violet (0.1%) was added into each well and the plate was further incubated for 20 min at room temperature. The plate was then washed with sterile water three to four times. Afterwards, 200 µl ethanol (95%) was added into each well and the plate was incubated for 30 min at 4°C. Finally, 100 µl of the solubilized crystal violet was transferred into a new 96-well micro-titer plate and the absorbance was measured using a plate reader (SmartSpec™ 3000) at 595 nm. The experiment was conducted three times in duplicate.

Evaluation of GCEO Mutagenicity Using Ames Test

S. Typhimurium TA98 and TA100 strains were selected as representative mutant strains to assess the mutagenic activity of GCEO by the Ames test using a micro-titer plate method (Nighat and Shahid, 2017). Standard mutagens, i.e., potassium dichromate (30 µg 100 μl^{-1}) for *S.* Typhimurium TA98 and sodium azide (0.5 µg 100 μl^{-1}) for *S.* Typhimurium TA100 were used. Briefly, the strains were initially grown and maintained in nutrient culture media and incubated aerobically for 18–24 h at 37°C. The reaction mixture was carefully prepared using DM salt (21.62 ml), D-glucose (4.75 ml), bromocresol purple (2.38 ml), D-biotin (1.19 ml), and L-histidine (0.06 ml). Then 2.5 ml reaction mixture solution was transferred into a sterilized petri plate along with 17.5 ml deionized distilled water, 0.05 ml of the tested sample and 0.05 ml of the tested strains. The solution in the petri plate was thoroughly mixed and then 150 µl poured into each well of a 96-well plate. To check for mutagenicity, 96-well plates were wrapped with aluminum foil, tightly sealed in plastic bags, and incubated for 3 days at 30°C. The results were expressed based on the color observation (**Supplementary Figure S1**). After the incubation period, the blank 96-well plate with purple color indicated no contamination. At the time of evaluation; background, and test plates were considered positive (mutagenic) when yellow, partial yellow, and turbid colors were produced, while a purple color was reported as being negative (non-mutagenic). GCEO is considered toxic towards tested strains when all wells of the 96-well plate exhibited a purple color. However, for GCEO to be mutagenic, the number of positive wells had to be considerably higher compared to the number of positive wells in the 'background' (negative control) plate which is representative of spontaneous mutations.

Statistical Analysis

Experiments were carried out at least three times in duplicate. All calculations were made in Microsoft Excel. The experimental data was shaped using SigmaPlot 11.0 (Systat Software Inc., Chicago, IL, United States).

RESULTS

GC-MS Quantification of the Bioactive Compounds in GCEO

The GC-MS analysis of GCEO identified a total of 30 bioactive compounds including; α-terpinyl acetate, 1,8-cineole, linalool acetate, and sabinene which comprised 34.95, 25.30, 8.13, and 5.48% of the identified compounds, respectively. In addition, several other natural compounds were reported which include; g-elemene, α-farnesene, α-thujene, α-pinene, β-pinene, p-cymene, α-terpineol, limonene, linalool oxide, cis-sabinene hydrate acetate, geranyl acetate, geranial, geraniol, myrcene, nerolidol, and n-hexadecanoic acid (**Figure 1**; **Table 1**).

Minimum Inhibitory Concentrations of GCEO Against Tested Gram-Negative Bacteria

The antimicrobial activities of GCEO were assessed against selected Gram-negative pathogens. The MIC value of the green cardamom essential oil was 1% against *E. coli* O157:H7 and *P. aeruginosa* ATCC 14213 (**Figures 2, 3**). These findings indicate that GCEO was an effective antimicrobial substance against the Gram-negative bacteria and has the potential to be used in formulating novel organic anti-infective drugs to counteract multidrug resistant microorganisms. Due to its safety profile, GCEO can be considered as a potent natural formulation preventing biofilm formation by foodborne pathogens. Moreover, GCEO can also be used as an alternative to chemical preservatives (antimicrobials) in the food industry to enhance the shelf life of food products by improving the antimicrobial status while at the same time imparting a pleasant and appealing aroma to bakery, dairy, meat, and other food products.

Biofilm Inhibitory Effect of GCEO Against Tested Bacteria

Bioactive compounds and essential oils derived from aromatic herbs, spices, and plants are considered as safe and promising alternative antimicrobial therapies. *E. coli*, *S.* Typhimurium, and *P. aeruginosa* were used to evaluate the GCEO activity against

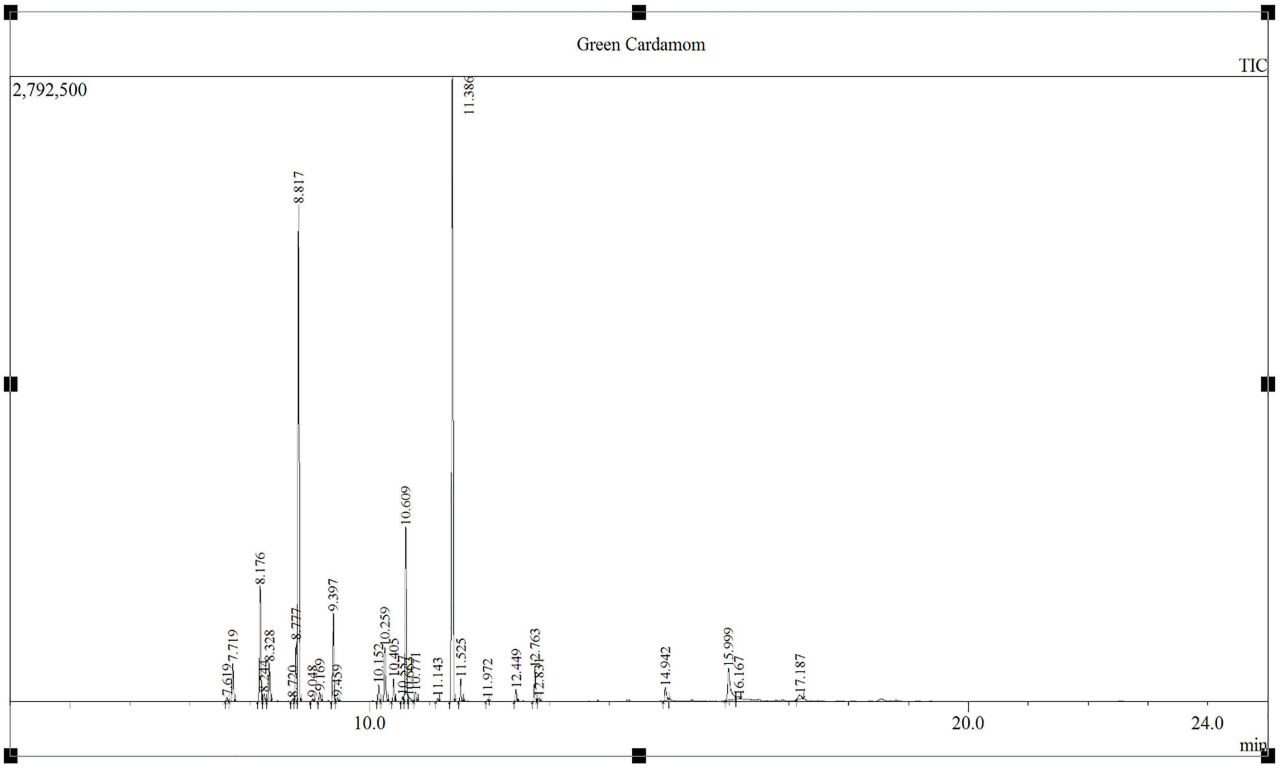

FIGURE 1 | The chromatogram elaborating GCEO bioactive compounds analyzed by the GC-MS (Shimadzu GC 2010 Plus and GCMS-TQ8040) through Shimadzu SH-Rxi-5Sil MS column (30 m long, 0.25 mm ID, 0.25 μm coated film).

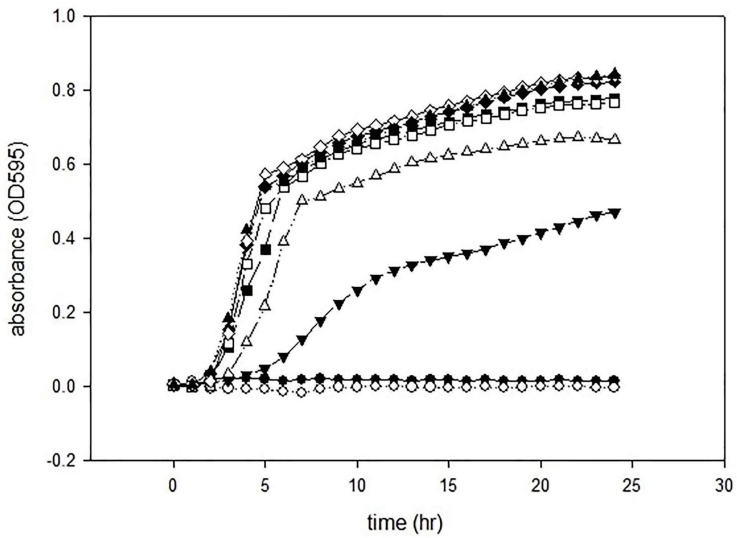

FIGURE 2 | Minimum inhibitory concentrations of GCEO against *Escherichia coli* O157:H7. GCEO used at 2% (●), 1% (○), 0.5% (▼), 0.25% (Δ), 0.0125% (■), 0.063% (□), 0.031% (♦), 0.015% (◊), and positive control (▲).

planktonic cells of the selected Gram-negative pathogens, while *E. coli* and *S.* Typhimurium were used to investigate the biofilm inhibitory activity of the cardamom derived bioactive compounds. Regarding the anti-biofilm activity of GCEO, *E. coli* and *S.* Typhimurium biofilms were found to be inhibited at sub-MIC concentrations. When GCEO at concentrations of 0.007, 0.015,

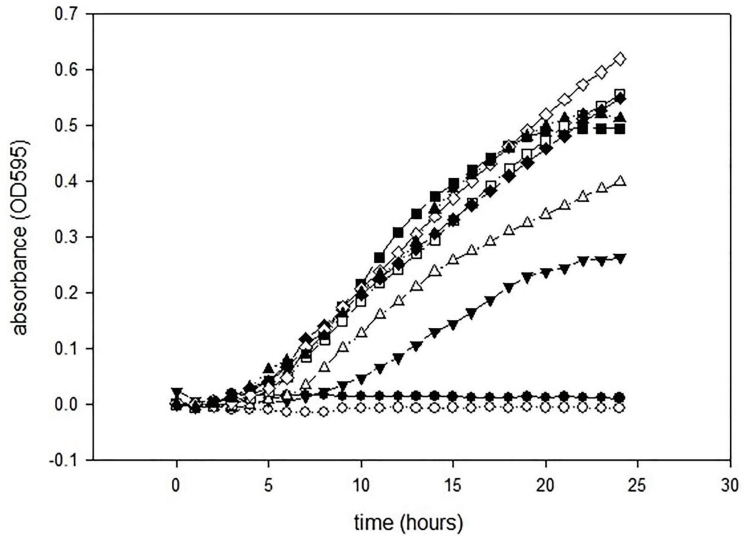

FIGURE 3 | Minimum inhibitory concentrations of GCEO against *Pseudomona aeruginosa* ATCC 14213. GCEO used at 2% (●), 1% (○), 0.5% (▼), 0.25% (Δ), 0.0125% (■), 0.063% (□), 0.031% (♦), 0.015% (◊), and positive control (▲).

TABLE 2 | Inhibition of biofilm formation by *Escherichia coli* O157:H7 and *Salmonella* Typhimurium JSG 1748 using GCEO.

Bacterial strain	Concentration (%)	Biofilm mass (%)	Inhibition (%)	Log_{10} CFU ml^{-1}
E. coli	0.000	100 ± 0.00	-	9.50 ± 0.03
	0.007	68.75 ± 0.07	31.25	9.52 ± 0.02
	0.015	35.71 ± 0.00	64.29	9.55 ± 0.01
	0.031	34.02 ± 0.00	65.98	9.55 ± 0.02
	0.062	29.59 ± 8.32	70.41	9.48 ± 0.02
	0.125	14.41 ± 4.41	85.59	9.43 ± 0.00
S. Typhimurium	0.000	100 ± 0.00	-	9.62 ± 0.01
	0.007	100 ± 0.00	-	9.75 ± 0.04
	0.015	93.87 ± 5.44	6.13	9.72 ± 0.02
	0.031	44.50 ± 5.69	45.50	9.69 ± 0.08
	0.062	40.55 ± 4.48	49.45	9.83 ± 0.03
	0.125	0.00 ± 0.00	100	9.74 ± 0.09

0.031, 0.062, and 0.125% (v/v) were used, 31.25, 64.29, 65.98, 70.41, and 85.59% of *E. coli* O157:H7 biofilm formation was inhibited, respectively (**Table 2**). Likewise, the same concentrations of GCEO (0.007, 0.015, 0.031, 0.062, and 0.125%, v/v) prevented 0.0, 6.13, 45.50, 49.45, and 100% of biofilm formation by *S.* Typhimurium. Furthermore, we noticed that the growth of the planktonic cells of *E. coli* and *S.* Typhimurium were not influenced by the tested concentrations (**Table 2**), which indicates that GCEO may be acting on the virulence factor(s) regulating biofilm formation, without interrupting the bacterial growth *via* direct antimicrobial action (**Figures 4, 5**).

GCEO Mutagenic Activity

Any substance or bioactive compound could be appraised as mutagenic if "the number of the positive-wells with the tested sample are more than twice the positive-wells in the negative control plate (background) i.e., induced mutation by standard mutagen" (Nighat and Shahid, 2017). GCEO was found to be non-mutagenic by this definition, as there were no significant results related to the mutagenicity of GCEO in the *S.* Typhimurium TA98 and TA100 bacterial strains (**Table 3**; **Supplementary Figure S1**). Thus, GCEO use is suggested in food and pharmaceutical applications as an alternative to chemical preservatives and conventional anti-infective drugs due to its safety for human health, which was confirmed in previously published reports (Al-Othman et al., 2006) and by Ames test-based evaluation of its mutagenic activity.

DISCUSSION

Many studies have reported on the health benefits of herbs and plants including different cardamom cultivars in terms of their antioxidant, antimicrobial, and hypolipidemic potentials and potential for food applications such as functional additives (Deepa et al., 2013; Abdullah et al., 2017, 2020; Aghasi et al., 2019;

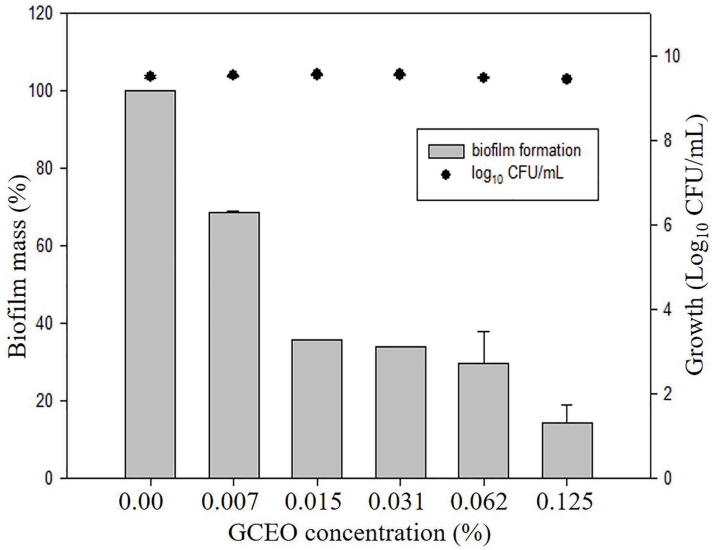

FIGURE 4 | Anti-biofilm activity of GCEO against planktonic and biofilm associated cells of *Escherichia coli* O157:H7. The columns (■) refer to the biofilm mass (%). The solid closed circles (●) refer to the growth (number) of planktonic cells (Log_{10} CFU ml^{-1}) vs. untreated cells.

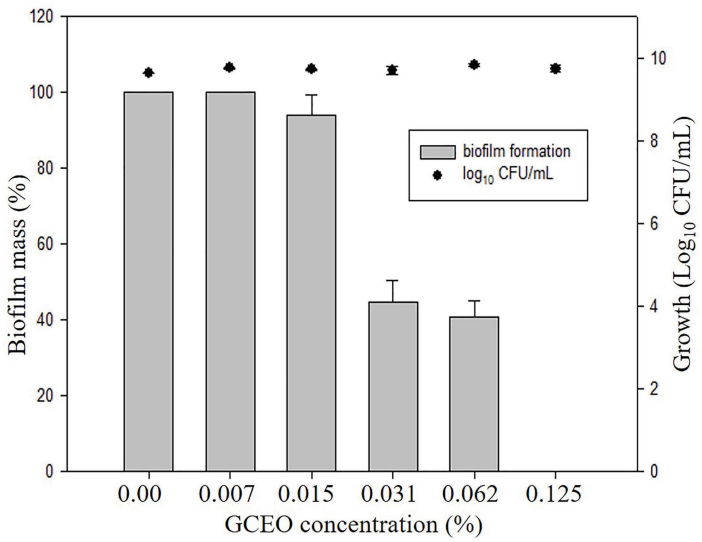

FIGURE 5 | Anti-biofilm activity of GCEO against planktonic and biofilm associated cells of *Salmonella* Typhimurium JSG 1748. The columns (■) refer to the biofilm mass (%). The solid closed circles (●) refer to the growth (number) of planktonic cells (Log_{10} CFU ml^{-1}) vs. untreated cells.

Dehghani et al., 2020). In the healthcare and pharmaceutical industries, interest in plant-derived bioactive compounds (phytochemicals) as potential medicines has attracted the attention of researchers due to their health-promoting characteristics, such as their antioxidant, antimicrobial, anti-ulcer (gastro-protective), anti-thrombotic, and anti-hypertensive effects (Verma et al., 2009; Sharma et al., 2011; Chakraborty et al., 2019). Specifically, bioactive compounds derived from different cardamom cultivars have shown promising antimicrobial activity against Gram-positive and Gram-negative pathogens (Abdullah et al., 2017, 2020). The present study investigated several key characteristics of the GCEO, including the chemical profile, antimicrobial properties (growth and biofilm inhibitory effects), and potential mutagenic activity to determine its potential use as a safe organic antimicrobial substance.

GC-MS Quantification of the Bioactive Compounds in GCEO

GC-MS analysis showed that α-terpinyl acetate and 1,8-cineole were the principle bioactive compounds in GCEO. Cardamom of Indian

TABLE 3 | The mutagenic activities of green cardamom essential oil.

	S. Typhimurium TA98		*S.* Typhimurium TA100	
Sample	**No. of +ive wells/No. of total wells**	**Results**	**No. of +ive wells/No. of total wells**	**Results**
Background	14/96	-	27/96	-
Cardamom + ethanol	24/96	Non-mutagenic	2/96	Non-mutagenic
Cardamom	18/96	Non-mutagenic	10/96	Non-mutagenic

origin contains 1,8-cineole (36.30%) and α-terpinyl acetate (31.30%) as major compounds, however, the percentages of 1,8-cineole and α-terpinyl acetate varies between 26.50–63.30% and 23.20–52.50%, respectively (Parthasarathy et al., 2008). Our results were in agreement with previous findings published by Aneja and Joshi (2009) who reported that the major constituents of green cardamom include α-terpinyl acetate, 1,8-cineole, α-terpineol, and linalool at 44.3, 10.7, 9.8, and 8.6%, respectively. Likewise, Nair (2006) reported a wide range of concentrations of 1,8-cineole (27–36.1%) and α-terpinyl acetate (38.5–47.9%) as the major volatile compounds in the GC-MS analysis of cardamom extracts. A cardamom seed oil obtained by supercritical extraction using CO_2 as a solvent was found to have 42.3% α-terpinyl acetate, 21.40% 1,8-cineole, 8.2% linalyl acetate, 5.6% limonene, and 5.4% linalool as major constituents (Marongiu et al., 2004). The GC-MS results of the present study were in line with the findings of Savan and Kucukbay (2013) who identified α-terpinyl acetate, 1,8-cineole, and linalool as major compounds at 40.7, 25.6, and 6.3%, respectively. The variations between the present findings and earlier studies regarding the chemical composition of GCEO could be due to several factors including the origin of the cardamom, extraction techniques, and variations in experimental conditions, specifically the conditions used for analytical chromatography and the stationary phase being used in the column.

GCEO Antimicrobial Perspectives

Nowadays, *E. coli* is a matter of great concern to the food industry because it can produce toxins in food commodities that ultimately result in food toxicity and food poisoning. Moreover, it is equally important to the pharmaceutical industry, as *E. coli* is also responsible for urinary tract infections (~90%) and other serious harmful effects are also associated with its presence inside the human body. Many bioactive phytochemicals possess promising antimicrobial potential and may be used as organic antimicrobial agents for food and pharmaceutical applications (Yousefi et al., 2019; Abdullah et al., 2020).

Bioactive phytochemicals play an important role in the survival of producing species as part of their defense system against pathogens. Plant-derived bioactive compounds that exhibit antimicrobial properties are most often flavonoids, phenolics, quinines, saponins, tannins, coumarins, terpenoids, and alkaloids (Choo et al., 2003; Mendez-Vilas, 2013; Nighat and Shahid, 2017). Recently, a group of researchers described the antimicrobial activities of a flavonoid extract of pummel peel; the MIC value of the extract ranged from 0.5 to 4.5 mg ml^{-1} against the *Vibrio anguillarum* and *Chromobacterium violaceum* CV026 bacterial strains (Liu et al., 2017). Likewise, Tapia-Rodriguez et al. (2017) studied the antimicrobial potential and identified the minimal inhibitory concentrations (MIC) and the minimal biofilm inhibitory concentrations (MIC-B) of carvacrol against *P. aeruginosa*. The MIC value of carvacrol was 7.9 mM against *P. aeruginosa* while biofilm formation inhibition was observed at sub-MIC values. Bahari et al. (2017) investigated the synergistic effect of curcumin in combination with antibiotics (azithromycin and gentamycin) against *P. aeruginosa*. The authors reported a reduction in the MICs of azithromycin and gentamycin when they were combined with curcumin. Similarly, McCarthy and Ogara (2015) found that garlic (*Allium sativum*) extract synergized with tobramycin to kill *P. aeruginosa*. They concluded that garlic extract not only decrease the pathogenicity of bacteria but also increased microbial susceptibility to antibiotics.

Sheng et al. (2016) explored the antimicrobial effect of grape seed extract on the generation of toxins by *E. coli*, a matter of great concern to researchers due to the harmful effects to consumers related to the expression of virulence factors in *E. coli*. This study reported that 4 mg ml^{-1} of grape seed extract inhibited the growth of *E. coli*. Recently, Amrutha et al. (2017) found that organic acids obtained from fresh fruits and vegetables have potential for use as antimicrobials. They investigated the effects of different acids such as citric acid, acetic acid, and lactic acid on biofilm development and as anti-quorum sensing compounds. They observed 39% biofilm formation inhibition with lactic acid for *E. coli* and 22% with citric acid for *Salmonella*. They also applied a lactic acid solution (2%) on cucumbers and found it to be very effective in inhibiting the growth of *E. coli* and *Salmonella*. They recommended the use of these natural acids as efficient disinfectants and natural antimicrobials to inhibit bacterial growth on fresh fruits and vegetables. *E. coli* is the most common pathogen of the urinary tract and is isolated in 90% of cases of individuals with urinary tract infections. A group of researchers explored the ability of the *Melia dubia* plant derived compound (fructose furoic acid ester) to down regulate the virulence expression of virulence factors and found it effective as an inhibitor to combat uropathogen *E. coli* biofilm formation (Vinothkannan et al., 2018).

Vasavi et al. (2016) investigated the antimicrobial activity of an extract of the traditional herb *Centella asiatca* and its impact on the biofilm formation inhibition of *P. aeruginosa* and found that the extract had antimicrobial activity against bacterial biofilms. Several studies have reported that plant-derived bioactive compounds inhibited quorum sensing by hindering synthesis of peptidoglycan, interruption in membrane structures, or inhibitory effects on signal detection and subsequently inhibit biofilm formation. A study reported that 4 mg ml^{-1} of cardamom extract obtained through conventional solvent extraction prevented biofilm formation in *S.* Typhimurium

(51.96%), and *P. aeruginosa* (45.28%) (Niu and Gilbert, 2004; Rahman et al., 2017). Likewise, *Cinnamomum verum*, *Juniperus communis*, *Origanum majorana*, *Medicago sativa*, *Syzygium aromaticum*, and *Salvia sclarea* essential oils exhibited antimicrobial activities by preventing biofilm formation (Kerekes et al., 2013; Soni et al., 2013; Newman and Cragg, 2016). Previously, ginger based phenolic derivatives showed inhibitory effects. The compounds zingerone and gingerol revealed 35 and 50% quorum sensing inhibition, respectively, at 500 ppm against *C. violaceum*, a Gram-negative bacterium (Kumar et al., 2014). The bacteriostatic properties of GCEO observed in the present study against the tested bacterial strains, provide scientific justification as a promising antimicrobial treatment. However, more studies are required regarding the safety, purification, and isolation of cardamom's bioactive compounds as anti-infective agents, to better understand their mechanisms of action in controlling microbial virulence factors and in the development of anti-biofilm therapies. Compared to the extract, the purified compounds, certainly, will possess a higher potency against pathogens, and reduce bacterial resistance to antimicrobial agents.

CONCLUSION

GC-MS analysis identified 30 bioactive compounds. Among them, α-terpinyl acetate (34.95%) and 1,8-cineole (25.30%) were present as the principal compounds in GCEO. A minimum inhibitory concentration assay revealed that a GCEO concentration of 1% inhibited the growth of *Escherichia coli* O157:H7 and *Pseudomonas aeruginosa* ATCC 14213. Anti-biofilm evaluation showed that the tested concentrations (0.015, 0.031, 0.062, and 0.125%, v/v) caused 64.29, 65.98, 70.41, and 85.59% biofilm formation inhibition in *Escherichia coli* and 6.13, 45.50, 49.45, and 100% inhibition of *Salmonella* Typhimurium, respectively. The mutagenicity assay confirmed GCEO non-mutagenic potential against *Salmonella* Typhimurium TA98 and *Salmonella* Typhimurium TA100 strains. Based on these findings, green cardamom essential oil could possibly be used as a safe antimicrobial (organic substance) to inhibit microbial biofilms and counteract multidrug resistant microorganisms. The food industry can also use green cardamom-derived bioactive compounds as safe alternatives to chemical preservatives (antimicrobials) to develop food products with an enhanced shelf life and a pleasant and appealing aroma for consumers.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Abdullah, AAl, and QH: conceptualization. Abdullah, AAl, and MC: methodology, investigation, writing, review and editing. Abdullah, TA, and HJ: writing of original draft preparation. AAs and QH: supervision. Abdullah, MC, and AE: resources and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.620227/full#supplementary-material

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AHL-Lactonase Producing *Psychrobacter* sp. From Palk Bay Sediment Mitigates Quorum Sensing-Mediated Virulence Production in Gram Negative Bacterial Pathogens

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Quorum sensing (QS) is a signaling mechanism governed by bacteria used to converse at inter- and intra-species levels through small self-produced chemicals called N-acylhomoserine lactones (AHLs). Through QS, bacteria regulate and organize the virulence factors' production, including biofilm formation. AHLs can be degraded by an action called quorum quenching (QQ) and hence QQ strategy can effectively be employed to combat biofilm-associated bacterial pathogenesis. The present study aimed to identify novel bacterial species with QQ potential. Screening of Palk Bay marine sediment bacteria for QQ activity ended up with the identification of marine bacterial isolate 28 (MSB-28), which exhibited a profound QQ activity against QS biomarker strain *Chromobacterium violaceum* ATCC 12472. The isolate MSB-28 was identified as *Psychrobacter* sp. through 16S-rRNA sequencing. *Psychrobacter* sp. also demonstrated a pronounced activity in controlling the biofilm formation in different bacteria and biofilm-associated virulence factors' production in *P. aeruginosa* PAO1. Solvent extraction, heat inactivation, and proteinase K treatment assays clearly evidence the enzymatic nature of the bioactive lead. Furthermore, AHL's lactone ring cleavage was confirmed with experiments including ring closure assay and chromatographic analysis, and thus the AHL-lactonase enzyme production in *Psychrobacter* sp. To conclude, this is the first report stating the AHL-lactonase mediated QQ activity from marine sediment bacteria *Psychrobacter* sp. Future work deals with the characterization, purification, and mass cultivation of the purified protein and should pave the way to assessing the feasibility of the identified protein in controlling QS and biofilm-mediated multidrug resistant bacterial infections in mono or multi-species conditions.

Keywords: AHL-lactonase, marine Palk Bay sediment, *Pseudomonas aeruginosa*, *Psychrobacter* sp., quorum quenching

INTRODUCTION

Biofilms are a complex aggregation of mono or mixed species of microbial populations embedded on the biotic or abiotic surfaces by a self-produced extracellular polymeric matrix (Sharma et al., 2019). Biofilm forming microorganisms are responsible for a cluster of common hospital-acquired ailments including lung infection in patients with cystic fibrosis (CF), otitis media, periodontitis, burn wound infections caused by a variety of surgical implants, endocarditis, and urinary tract infections. National Institutes of Health (NIH) recommended that approximately 60% of human infections are the consequence of biofilm formation on human mucosa. Initial attachment and subsequent maturation of biofilm are the two important steps in host tissue colonization and subsequent persistent infections (Costerton et al., 1999). Bacteria living inside biofilms are habitually able to tolerate host immune responses and are distinctly highly resistant to different antibiotics (Costerton et al., 1999). Comparatively, this level will frequently surpass the maximum dosage level, and hence limit the efficient treatment available to control bacterial infections. The underlying mechanism of resistance is multi-factorial which includes restricted penetration, heterogeneous metabolic activity, and expression of certain genes conferring enhanced resistance to antibiotics. Hence, identification of such compounds with potential antibiofilm activity is imperative to combat the pathogenesis of these detrimental pathogens.

In most of the bacterial pathogens, quorum sensing (QS) mechanism regulates the biofilm formation and other virulence factors' production, in order to establish pathogenesis in the host. This QS mechanism is also called the cell-to-cell communication system, as the bacteria communicate with each other at inter- and intra-species levels using small diffusible signal molecules called autoinducers (AIs). In Gram negative bacteria, N-Acyl Homoserine Lactone (AHL) is the prime AI responsible for QS (Papenfort and Bassler, 2016), which bind to their cognate receptor proteins that together activate the expression of QS-controlled genes (Whitehead et al., 2001). In *lux*I/R QS system, the LuxI family protein synthesizes AHL. The LuxR family protein binds with AHL and regulates the expression of many genes responsible for their coordinated behavior including motility, antibiotic biosynthesis, virulence factor production, and biofilm formation (Davies et al., 1998). Most importantly, the QS mechanism governs the biofilm formation in most of the bacterial pathogens; the QS inhibitory process termed as quorum quenching (QQ) has offered a novel target to control the biofilm-associated infections (Dong and Zhang, 2005; Costerton et al., 2007). Contrasting to antibiotics, QS inhibitors will not set bacteria under strong selective pressure to develop drug-resistance (Zhao et al., 2020). Besides QS, flagellar motility and exopolysaccharide (EPS) have also been found to be essential for bacterial aggregation and biofilm formation (Pratt and Kolter, 1998; Jaisi et al., 2007).

Secondary metabolites from marine organisms are considered an important source of biomolecules for drug discovery (Newman and Cragg, 2004; Borges and Simões, 2019). Bacteria associated with corals, sponges, and other organisms have been recognized as the factual producers of many bioactive compounds (Kelman et al., 2006; Freckelton et al., 2018). Though AHL degradation enzymes from bacterial isolates have been identified from different sources (Dong et al., 2002; Uroz et al., 2003; Ulrich, 2004; Hassan et al., 2016), the investigation of antibiofilm activity of bacteria, particularly from marine resources, are expected to act against antibiotic resistant bacterial pathogens (Huang et al., 2019; Zhou et al., 2019). Recently, a marine isolate showing a promising antibiofilm activity against *Pseudomonas aeruginosa* has been reported from red sea sediment (Rehman and Leiknes, 2018). Also, the literature evidenced these marine bacteria as one of the sources of secondary metabolites and other extracellular hydrolytic enzymes (Romano et al., 2017; Borges and Simões, 2019). Hence it is believed that bacteria from marine sediments (MSB) may also have the ability to produce several secondary metabolites that target bacterial QS mechanism. In light of this view, the present study aimed to isolate marine sediment bacteria that target the bacterial QS mechanism, and to divulge the mechanism of QS inhibition.

MATERIALS AND METHODS

Bacterial Strains, Culture Media, and Conditions

Biomarker strains *Chromobacterium violaceum* (ATCC 12472) and Tn5 mini mutant CV026 were used to determine the QS inhibitory potential of marine bacterial isolates. The bacterial pathogens such as *Serratia marcescens* (FJ584421), *Pseudomonas aeruginosa* PAO1, *Vibrio parahaemolyticus* (ATCC 17802), and *V. vulnificus* (MTCC 1145) were the target pathogens used in this study. For ring closure assay, *Bacillus subtilis* ATCC 6633 was used as positive control. All these strains were cultured aerobically in Luria-Bertani (LB) broth (Hi Media, India) and incubated at their optimum temperature (30°C for *C. violaceum* and 37°C for rest of the strains). For the experiment, the OD of the pathogens was adjusted to 0.4 at OD_{600nm} from the overnight culture (1×10^8 CFU/ml). As a standard cell suspension, 1% from the OD adjusted culture was used to inoculate the medium. As a negative control, Zobell marine broth (ZMB) was added to the wells of control sample.

Primary Screening for Quorum Sensing Inhibitors – Soft Agar Overlay Assay

Marine bacterial strains were isolated from sediment samples collected from the Palk Bay coastal region using Zobell Marine Agar 2216 (Hi Media, India). All the isolates were patched on Zobell marine agar and allowed to grow for 24 h. Following incubation, the plates were overlaid with soft agar (0.7% agar) incorporated with 1% of *C. violaceum* (ATCC 12472) at standard cell suspension. Post incubation at 30°C for 18 h, the plates were observed for the violacein pigment inhibition (McLean et al., 2004). Bacterial isolate which showed efficient violacein inhibition alone was selected and further examined against the QS-mediated virulence inhibition in other bacterial pathogens.

Preparation of MSB-28 Cell Free Supernatant

The cell free supernatant (CFS) of MSB-28 used in this experiment was prepared by inoculating 1% overnight culture of MSB-28 culture into 10 ml of ZMB. After 18 h of incubation the culture was centrifuged at 10,000 rpm for 10 min. After that, the culture supernatant was collected, filter sterilized (0.22 μm syringe filter), and stored at 4°C for further use.

Quantification of Violacein Production

The effect of MSB-28 on inhibition in violacein production was quantified by spectrophotometric analysis. Biosensor *C. violaceum* strain CV026 (OD_{600nm} = 0.1, 1% to the final volume of the growth medium) was added to the test tubes containing LB supplemented with 5 µM of N hexanoyl-l-homoserine lactone (C_6-HSL, Sigma) alone as the control, and LB supplemented with C_6-HSL and CFS of MSB-28 at various concentrations (5–20% v/v). The tubes were then incubated at 30°C for 18 h. After incubation, cell pellets of the control and treatment groups were collected by centrifugation at 8,000 rpm for 10 min. Equal volume of dimethyl sulfoxide was added to the pellet and vortexed vigorously (30 s), in order to precipitate the insoluble violacein. Post vortexing, the resultant mixture was centrifuged at 8,000 rpm for 10 min to separate the cells from the CFS, and then the CFS was measured at 585 nm spectrophotometrically (Hitachi U-2800, Japan; Choo et al., 2006).

Strain Identification

Alkaline lysis method was used to isolate genomic DNA from MSB-28. Ribosomal 16S rRNA gene was amplified using eubacterial universal primers (forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-ACGGCTACCTTGTTACGACTT-3'; Babu et al., 2009). The PCR conditions are as follows with initial denaturation at 94°C for 5 min followed by 30 cycles at 94°C for 30 s, 45°C for 30 s, and 72°C for 60 s with a Thermal Cycler (ABI). Sequencing of the 16S rRNA gene (about 1,500 bp) was done in Macrogen (Seoul, Korea). The CAP3 software was used to assemble, analyze, and to manually edit the raw sequences of 16S rRNA gene. Using BLAST analysis, the assembled 16S rRNA gene sequence of MSB-28 was then compared within the NCBI database (http://www/ncbi.nlm.nih.gov/).

Biofilm Prevention Assays

Microtiter Plate Assay

The effect of CFS from *Psychrobacter* sp. against the biofilm forming ability of bacterial pathogens was examined using 24-well microtiter plate (MTP; Santhakumari et al., 2016). MTP wells containing 1 ml of growth medium were added with 1% standard cell suspension of the target pathogens and 5–20% v/v (50–200 µl) of *Psychrobacter* sp. CFS. Plates were kept at 37°C for 24 h without agitation to allow the development of biofilm. After incubation, the wells of MTP were rinsed thrice with distilled water to remove non-adherent cells and the wells with biofilms were stained with 0.4% w/v of crystal violet solution for 5 min. The stained biofilms were than solubilized by adding 1 ml of 95% ethanol, centrifuged at 7,000 rpm for 10 min. Then, the supernatant was measured at 650 nm under UV-visible spectrometer (HITACHI U-2800, Japan).

Confocal Laser Scanning Microscope Analysis

The target pathogens were allowed to form biofilms on glass slides (1 cm^2) placed in 24-wells MTP containing growth medium supplemented with and without *Psychrobacter* sp. CFS (20% v/v). The experiment setup was incubated for 24 h at appropriate temperatures. Post incubation, biofilms in the glass slides were stained with 0.1% (w/v) of acridine orange and the stained biofilm cells were observed and imaged under a CLSM (Zeiss LSM 710, Carl Zeiss, Germany) at a magnification of 20x. The Z-stack analysis was done with the Zen 2009 software (Carl Zeiss, Germany). In addition, biofilm biovolume and average thickness of the biofilm formed in control and treated samples (z-stack images) were analyzed by COMSTAT software (kindly gifted by Dr. Claus Sternberg, DTU Systems Biology, Technical University of Denmark; Kannappan et al., 2019).

Growth Inhibition Assay

The growth inhibitory activity of CFS of *Psychrobacter* sp. was assessed by the well diffusion agar assay by following the guidelines of Clinical and Laboratory Standards Institute (2006). One hundred microliters of bacterial pathogens (0.5 Mc Farland turbidity, 1×10^8 CFU/ml) was uniformly spread over the Muller-Hinton Agar (MHA, Hi Media, India) plate. Wells were punched out (4 mm in diameter) in the bacteria-spread agar plates and were loaded with various concentrations (50–200 µl) of the CFS of MSB-28. Wells with 200 µl of ZMB served as control. Following incubation at 37°C for 24 h, the zone of inhibition was observed and recorded from the MHA plates.

P. aeruginosa PAO1 Assays

Extraction and Quantification of EPS

PAO1 was grown in test tubes in the presence and absence of *Psychrobacter* sp. CFS at 37°C and harvested at the late log phase. For EPS extraction, the cultures were centrifuged to remove the cell pellets, and the resulting CFS was syringe filtered through 20 µm nitrocellulose membrane filters. To the filtered CFS, three volumes of ice-cold absolute ethanol was added, which was then left undisturbed overnight at 2°C. The precipitated EPS was collected by centrifugation at 10,000 rpm for 20 min. The resulting precipitate was resuspended in milli-Q water and stored at −20°C until future use (Packiavathy et al., 2014).

EPS quantification was done by adding 1 ml of the EPS solution to 1 ml of cold 5% phenol and 5 ml of concentrated sulfuric acid. The reaction mixture turns to a red color. The intensity of the color is directly proportional to the EPS production. The EPS production was quantified spectrophotometrically at 490 nm (Hitachi U-2800, Japan; Dubois et al., 1956).

Swimming and Swarming Motility Assays

PAO1 was grown in test tubes in the presence (20% v/v) and absence of *Psychrobacter* sp. CFS at 37°C and harvested at the late log phase. In the center of the swarming plates (1% peptone, 0.5% NaCl, 0.5% agar and 0.5% filter-sterilized d-glucose) and swimming agar plates (1% tryptone, 0.5% NaCl, and 0.3% agar), 5 microlitres of untreated control and *Psychrobacter* sp. CFS treated PAO1 cells were placed separately and were then kept for incubation at 37°C for 24 h (Déziel et al., 2001).

Rhamnolipid Assay

Overnight culture (50 µl) of PAO1 cells grown in the absence and in the presence of varying concentration of *Psychrobacter* sp. CFS (100 and 200 µl/ml) were added separately into the wells punctured on M8 medium containing petriplates supplemented with 1 mM $MgSO_4$, 0.2% glucose, 0.5% casamino acids (CAA), 0.02% cetyl trimethyl ammonium bromide (CTAB), 0.0005% methylene blue, and 1.5% agar. The experimental setups were left undisturbed for 48 h at 37°C. Rhamonolipid production was observed by CTAB precipitation and dark blue hallow formation around the well (Caizza et al., 2005).

Drop Collapse Assay

The assay was carried out by following the protocol of Caizza et al. (2005). For this experiment, *Psychrobacter* sp. CFS treated and untreated PAO1 cells were harvested as described above. The cultures were centrifuged to remove the cell pellets. The supernatant was syringe filtered using a 0.22 µm membrane and serially diluted in distilled water. Equal volumes of the serially diluted supernatants were placed in circles on the backside of a 24 well plate lid and left undisturbed for bead formation. Samples that failed to form beads were defined to have drop collapse activity.

Biofilm Ring Assay

The assay was done by inoculating 1% of the standard cell suspension of PAO1 into glass tubes containing 1 ml of LB medium in the absence and presence (20% v/v) of *Psychrobacter* sp. CFS. After 24 h of incubation at 37°C, the cultures were removed from control and treated tubes and the biofilms were stained with 0.4% crystal violet and documented (Bordi et al., 2010).

Effect of Solvent, Heat, and Proteinase K on QQ Activity of *Psychrobacter* sp.

Polarity Extractions

Equal volumes of *Psychrobacter* sp. CFS was mixed and vortexed thoroughly with solvents from highly non-polar to highly polar including benzene (B), petroleum ether (PE), chloroform (C), and ethyl acetate (EA). The CFS and solvent mixture were left overnight for phase separation. Solvent phase was collected, evaporated, and weighted. Further, the same was resuspended in a known volume of distilled water or dimethyl sulfoxide (DMSO) and stored at −20°C. The QS inhibitory potential of every organic phase was done by following the above said protocol using QS biomarker strain *C. violaceum* (ATCC 12472).

Heat Sensitivity

To confirm the nature of the quorum quencher molecule, the CFS of *Psychrobacter* sp. was incubated at different temperatures ranging from 0 to 80°C for 10 min. After heat treatment, the CFS of *Psychrobacter* sp. was assayed against *C. violaceum* CV026 with an exogenous supply of C6-HSL as mentioned above in violacein quantification assay. Following 24 h incubation, the cultures were observed for the violacein production.

Proteolytic Activity

A treatment group composed of 1 ml CFS of *Psychrobacter* sp. was added with 1 mg/ml of Proteinase K (Sigma Aldrich, United States). Nutrient broth (pH-7.4) with Proteinase K and CFS of *Psychrobacter* sp. without Proteinase K acted as positive control and negative control, respectively. All the three groups were incubated at 55°C for 18 h. Following incubation, the samples were assayed against QS biomarker strain *C. violaceum* (ATCC 12472) for violacein pigment production.

Enzymatic Degradation of AHL by Marine Isolate *Psychrobacter* sp.

C_6-HSL (5 mM) was incubated with 500 µl of *Psychrobacter* sp. CFS and incubated for 16 h. The preparative thin layer chromatography (TLC) was performed by spotting 4 µl of the supernatant treated C_6-HSL. An equal volume of LB containing pure 5 mM C_6-HSL was used as a negative (untreated) control. TLC was developed with methanol and water in the ratio of 60: 40 v/v. The developed TLC plates were overlaid with 3 ml of top agar (0.7%, LB agar) containing *C. violaceum* CV026 (OD_{600nm} = 0.1). Further, the plates were kept at 30°C for overnight or until adequate color development was achieved.

Enzymatic Cleavage of Lactone Ring – Ring Closure Assay

As per the protocol described by Yates et al. (2002), an aliquot of the digestion media containing *Psychrobacter* sp. CFS (500 µl) treated C_6-AHL (5 mM) was added with 10 mM HCl to lower the pH to the level of 2.0, and was kept under incubation for 48 h at 4°C. On the other hand, the digestion media containing 500 µl of PAO1 CFS treated C_6-HSL (5 mM) acted as a negative control as it has acylase activity (Sio et al., 2006) and 500 µl of *B. subtilis* ATCC 6633 CFS treated C_6-HSL (5 mM) acted as a positive control as it has lactonase activity (Pan et al., 2008); both were also subjected to acidification. After 48 h of incubation, HCl was left to evaporate, and the residue was suspended in 20 µl of LB broth. The lactone recyclization was induced by the acidification of the degradation mixture. After this treatment, the acidified mixture was spotted onto TLC and overlaid with 3 ml of top agar (0.7%, LB agar) containing *C. violaceum* (OD_{600nm} = 0.1). After overnight incubation, production of violacein by CV026 confirms the degradation activity of AHL lactonase (Carlier et al., 2003).

Confirmation of AHL Degrading Activity by HPLC Analysis

AHL degrading activity was assessed by incubating 2 ml of 100 mM phosphate buffer containing 5 mM C_6-HSL (Sigma-Aldrich, United States) with 2 mg of acetone precipitated *Psychrobacter* sp. CFS at 30°C for 10 h. To exclude any alkaline lactonolysis due to pH, the pH of the mobile phase was adjusted to 6.5 and the pH of cell free lysate was 7. Post incubation, 20 µl of the digestion mixture was subjected to HPLC analysis (Shimadzu, Kyoto, Japan) with a C18 reverse phase analytical silica column (250 × 4.6 mm; 5 µm). The fractions were eluted using methanol and water (v/v) as mobile phase in the ratio of 50:50 at a flow rate of 1 ml/min (Uroz et al., 2005).

AHL Extraction and Detection

Many bacteria in the proteobacteria group have been reported to produce AHL. Consequently, the AHL produced could affect the biological activity of MSB-28. Hence, MSB-28 was examined for the synthesis of AHL molecules. MSB-28 was grown overnight in LB medium (100 ml) buffered with 50 mM 3-[N-morpholino] propanesulfonic acid (MOPS) to pH 5.5 to prevent spontaneous degradation of AHLs (Yates et al., 2002). After that, CFS was extracted twice with equal volumes of acidified ethyl acetate (0.1% v/v glacial acetic acid). The resulting extracts were concentrated to dryness under vacuum and resuspended in a minimal amount of sterile milli-Q water. The presence of AHL molecules present in the extracts was analyzed by spotting the AHL extracts on TLC plates (TLC aluminum sheets 20 cm × 20 cm, Merck, Germany). Synthetic C_6-AHL was used as a control. TLC was developed with methanol and water at a ratio of 60: 40 v/v. Then the TLC plates were overlaid with 3 ml of top agar (0.7%, LB agar) seeded with *C. violaceum* CV026 (OD_{600nm} = 0.1). Further, the plates were kept at 30°C overnight or until the development of a violet color (Chen et al., 2013).

Data Analysis

All the experiments were carried out in triplicate in three independent experiments. The results are expressed as means ± standard deviation (SD).

RESULTS

Screening Marine Bacteria for Production of QS Inhibitors

Out of 106 isolates screened, four isolates were shown to inhibit the QS mediated pigment production in *C. violaceum* ATCC 12472. Among the four isolates, marine sediment bacteria MSB-28 showed a constant and profound inhibition of violacein production (Data not shown). Moreover, the zone of inhibition was found to be opaque and not transparent, which clearly indicates QS inhibitory activity of MSB-28 and not the growth inhibition (**Supplementary Figure S1A**).

Strain Identification

The 16S rRNA gene sequence of MSB-28 was submitted to GenBank (NCBI; accession number: GU447235). Using BLAST analysis, the 16S rRNA gene sequence of MSB-28 was compared in the database and showed to have sequence similarity and identity with *Psychrobacter* sp. to a level of 98%.

Quantification of Violacein Inhibition

The CFS of MSB-28 showed a concentration dependent violacein inhibition against the QS-biomarker strain *C. violaceum* CV026; however, 20% v/v concentration of MSB-28 showed a 98% drop in violacein production (**Supplementary Figure S1B**).

Growth Inhibitory Activity of MSB-28

Antibacterial activity of MSB-28 CFS was assessed by well diffusion agar assay against the target pathogens such as PAO1, *S. marcescens*, *V. vulnificus*, and *V. parahaemolyticus*. Results revealed that none of the target pathogens displayed any growth inhibitory zones around the wells at tested concentrations (data not shown) and results are presented in **Supplementary Table S1**.

Biofilm Prevention Assay

MTP Assay

The antibiofilm potential of *Psychrobacter* sp. CFS was assessed against the bacterial pathogens such as PAO1, *S. marcescens*, *V. vulnificus*, and *V. parahaemolyticus*. *Psychrobacter* sp. CFS exhibited concentration dependent activity against all tested pathogens and maximum inhibitions of 89, 71, 58, and 60%, were recorded at 20% v/v against PAO1, *S. marcescens*, *V. vulnificus*, and *V. parahaemolyticus*, respectively, and tabulated (**Supplementary Table S1**).

Microscopy Analysis – CLSM Observation

Consistent with the results of biofilm biomass quantification assay, CLSM analysis of the biofilms developed by the tested bacteria such as PAO1, *S. marcescens*, *V. vulnificus*, and *V. parahaemolyticus* displayed disintegrated biofilm structures in the presence of *Psychrobacter* sp. (**Figure 1**). Altogether, it is confirmed that the CFS of *Psychrobacter* sp. interferes with the biofilm formation of tested bacterial pathogens. COMSTAT software analysis was a quantitative method employed to analyze the biofilm formation of the tested pathogen using the raw LSM file (Kannappan et al., 2019). The COMSTAT analysis revealed that biofilm biovolume and average thickness of the tested pathogens were considerably reduced in the presence of *Psychrobacter* sp. CFS, which authenticated the antibiofilm potential of *Psychrobacter* sp. (**Supplementary Table S2**).

Attenuation of QS Mechanisms in PAO1

Biosurfactant Inhibition-Rhamnolipid Assay

Interaction of CTAB with PAO1 surfactants leads to the precipitation of CTAB to form dark blue colonies surrounded by a white ring. In this study, the white halo surrounding the dark blue colonies around the well containing overnight culture of PAO1 clearly evidences the production of biosurfactant by PAO1, whereas wells containing cultures treated with *Psychrobacter* sp. CFS produced low levels of dark blue colonies

when compared to control, indicating the reduced production of rhamnolipids (**Figure 2A**).

Biosurfactant Inhibition-Drop Collapse Assay

The overnight culture of PAO1 showed no visible bead formation, indicating the drop collapse capability of PAO1 by the production of biosurfactant. But the *Psychrobacter* sp. CFS treated PAO1 developed beads at all tested concentrations indicated the reduced production of biosurfactant (**Figure 2B**).

Inhibition of Biofilm EPS

EPS was extracted from both *Psychrobacter* sp. CFS treated and untreated cultures of PAO1. The obtained results revealed

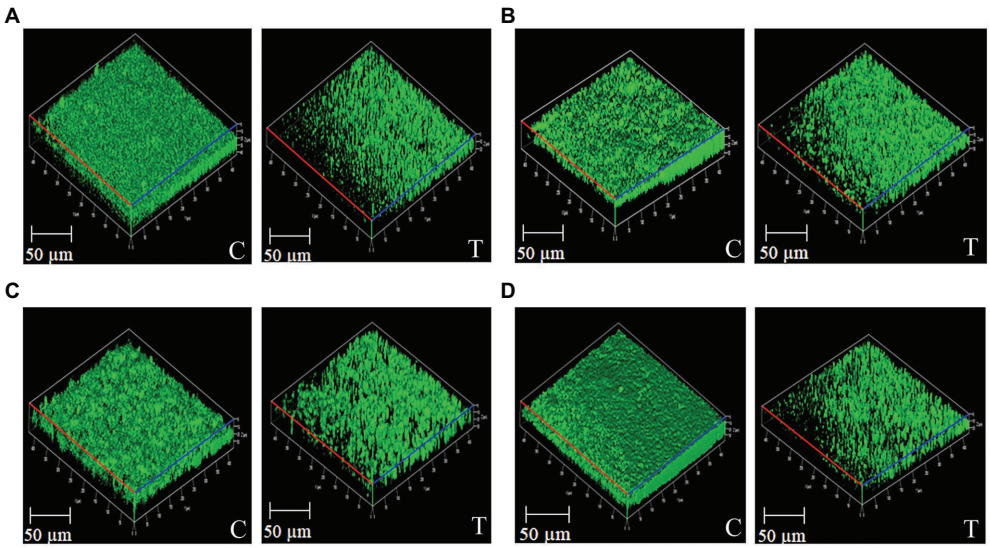

FIGURE 1 | Confocal microscopy images of bacterial biofilms harbored with and without *Psychrobacter* sp. CFS (200 µl/ml). Images represent the untreated controls (insert-C) and *Psychrobacter* sp. treated (insert-T) biofilms of PAO1 **(A)**, *S. marcescens* **(B)**, *V. vulnificus* **(C)**, and *V. parahaemolyticus* **(D)**.

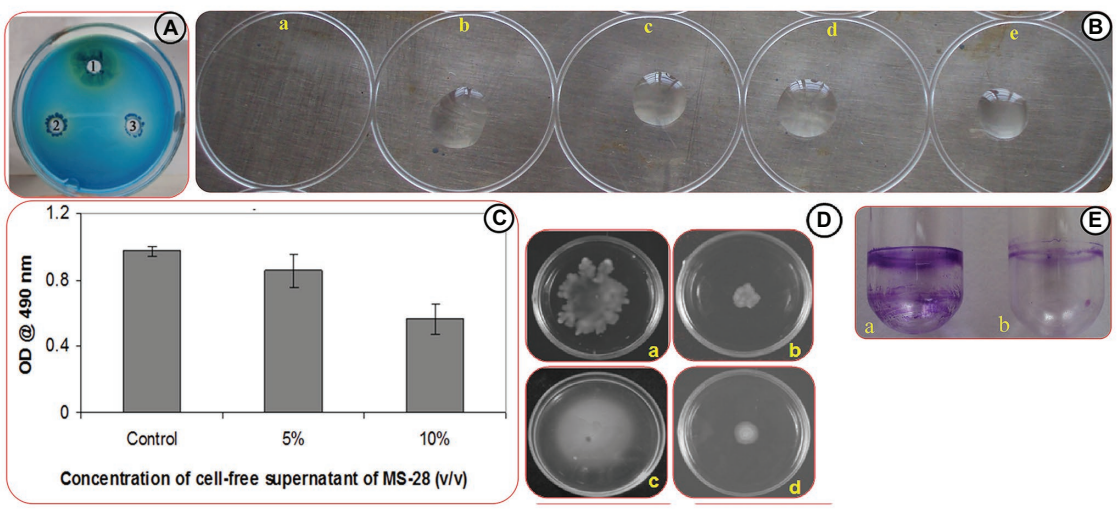

FIGURE 2 | Effect of *Psychrobacter* sp. on virulence factors production in PAO1. **(A)** Rhamnolipd production in PAO1. Image represents rhamnolipid production in untreated control (1), and PAO1 treated with CFS of *Psychrobacter* sp. at of 100 (2) and 200 µl/ml (3) Concentrations. **(B)** Assessment of biosurfactant production through drop collapse assay. Images represent the activity of untreated PAO1 **(a)**, and PAO1 treated with CFS of *Psychrobacter* sp. at concentrations of 50–200 µl/ml **(b-e)**. **(C)** Quantitative analysis of EPS inhibition in PAO1 cells treated with and without *Psychrobacter* sp. CFS. Data represent the OD values of the EPS. Mean values represent the data of three independent experiments and SD are shown. **(D)** Effect of *Psychrobacter* sp. on the motility of PAO1. Swimming and swarming motilities of untreated control of PAO1 **(a,c)** and PAO1 treated with *Psychrobacter* sp. CFS (200 µg/ml; **b,d**), respectively. **(E)** Analysis of PAO1 biofilm formation in the absence **(a)** and presence **(b)** of *Psychrobacter* sp. through ring assay.

that CFS of *Psychrobacter* sp. inhibited the EPS production in PAO1 by 90% at 20% (v/v) concentration (**Figure 2C**).

Swimming and Swarming Motility Inhibition Assays

As AHL-mediated QS regulates flagellar mediated motility of PAO1, the migration patterns of PAO1 in the presence of (20% v/v) of *Psychrobacter* sp. CFS were examined. As expected, the quorum quenching compound present in the *Psychrobacter* sp. had a great impact on the swarming and swimming migration patterns of PAO1 by displaying a significant reduction zone (**Figure 2D**).

Biofilm Ring Assay

The biofilm formation of PAO1 in borosilicate glass tubes (**Figure 2E**) was examined. For the untreated control tube, a thick layer of well-developed biofilm was easily stained by crystal violet. However, PAO1 cells treated with *Psychrobacter* sp. at a concentration of 20% (v/v) showed a remarked biofilm inhibition as evidenced by the thin biofilm ring.

Effect of Solvent, Heat, and Proteinase K Treatment on QQ Activity of *Psychrobacter* sp.

The QQ activity was lost when the CFS of *Psychrobacter* sp. was extracted with different solvents as depicted in **Figure 3A**. In the heat-inactivation assay, *Psychrobacter* sp. CFS retained its potential QQ activity upon treatment with 0–40°C temperature for 10 min by showing the inhibition percentage of around 95%. The violacein inhibition percentage was reduced to 50% upon treatment with 50°C for 10 min. Similarly, around a 22% reduction was observed when subjected to heat treatment at 60°C. At higher temperatures (70 and 80°C), *Psychrobacter* sp. CFS completely lost its violacein inhibition activity (**Figure 3B**). The obtained results confirmed that the active lead present in the CFS of *Psychrobacter* sp. was heat sensitive. To confirm the enzymatic nature of QQ molecule, the CFS of *Psychrobacter* sp. was incubated with proteinase K (1 mg/ml). Pigment deficiency was observed with proteinase K treatment, whereas CFS of *Psychrobacter* sp. without proteolytic digestion retained its QQ activity (**Figure 3C**). These results clearly revealed the enzymatic nature of *Psychrobacter* sp. and its activity is possibly attributed to the presence of an AHL-degrading enzyme.

AHL-Degrading Activity by *Psychrobacter* sp. Through TLC Analysis

The inhibition of violacein pigment was observed in C_6-HSL treated with *Psychrobacter* sp. CFS in TLC plate, which confirms the presence of AHL degrading enzymes in CFS of *Psychrobacter* sp. The inhibition was found to be dose dependent (**Figure 4**).

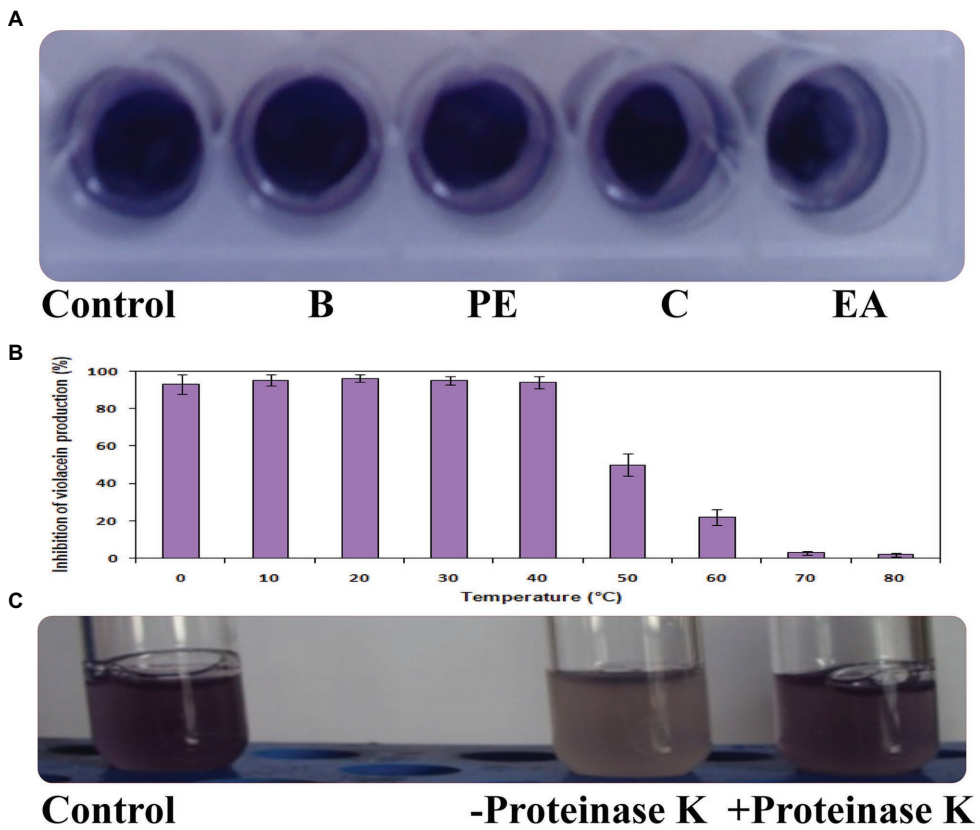

FIGURE 3 | Effect of solvents **(A)**, heat treatment **(B)**, and proteinase K **(C)** on QQ activity of *Psychrobacter* sp.

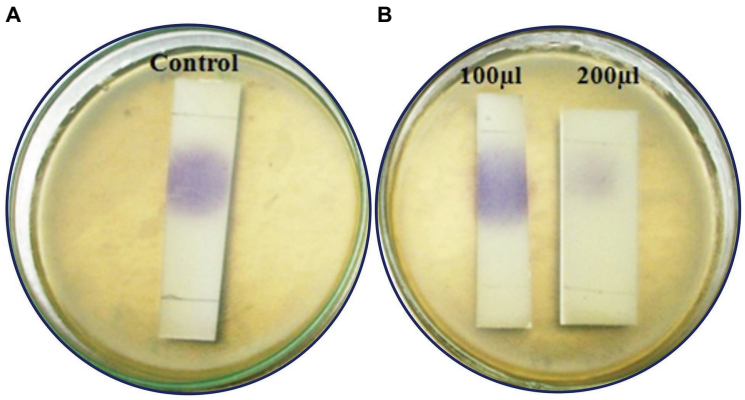

FIGURE 4 | Analysis of C_6HSL degradation by *Psychrobacter* sp. through TLC method. **(A)** C_6HSL alone. **(B)** C_6HSL treated with CFS of *Psychrobacter* sp. at different concentrations.

Confirmation of AHL-Degrading Activity of MSB-28 by HPLC Analysis

The reaction products of C6-HSL digested with partially purified *Psychrobacter* sp. CFS as well as synthetic C6-HSL (without cell free lysate) were subjected to RP-HPLC analysis. The negative control C6-HSL displayed a major peak at the retention time of 2.7 min and a solvent peak at 3 min (**Figure 5A**). After 10 h incubation, the HPLC profile of the reaction mixture containing the partially purified cell free lysate with C6-HSL revealed a peak at a retention time of 1.9 min, which might correspond to hydrolyzed products of C6-HSL and a small peak corresponding to the remaining C6-HSL at 2.8 min, in addition to the solvent peak at 3.1 min (**Figure 5B**).

Confirmation of Lactonase Activity-Lactonolysis Assay (Ring Closure Assay)

Lactonase activity is defined as the cleavage of lactone ring in AHL (active QS molecule) to become N-acyl homoserine derivative (non-active QS signal). After AHL degradation, the AHL degradation media was treated with HCl to lower the pH to 2.0 in order to induce the lactone ring closure of the AHL and to restore the activity of AHL molecules. The biosensor *C. violaceum* CV026 restored its pigmentation upon incubation with acidified AHL degradation media containing C6-HSL treated with MSB-28 as well as the positive control *B. subtilis* (**Figure 6**). In contrast, no such activity was observed with supernatant from PAO1 which has already been reported to encompass acylase activity (Sio et al., 2006). Together, the results confirm the enzymatic compound responsible for QQ activity of *Psychrobacter* sp. could be an AHL lactonase and act upon the lactone ring of AHL.

AHL Extraction and Detection

CV026 induced violacein production was not found in the TLC plate loaded with the extract from *Psychrobacter* sp. In contrast, violacein production was observed in the TLC plate loaded with synthetic C6-HSL (**Supplementary Figure S2**). The observed results lucidly revealed the nil production of AHL molecule(s) by the bacterium *Psychrobacter* sp. Hence, it is envisaged that the observed QQ activity with *Psychrobacter* sp. would not be plausibly interrupted by the AHL signaling mechanism as this bacteria lacks the ability to produce it.

DISCUSSION

The emergence of antimicrobial resistance is responsible for the failure of current antibiotics treatment on biofilm-based bacterial infections and has emphasized the urgent need for developing new alternate strategies. Bacterial QS mechanism seems to be an attractive target to develop an alternative therapeutic approach, as inhibition of QS hinders the virulence production, biofilm formation, and subsequent infection by many bacterial pathogens. Regardless, the marine bacterial organisms are being extensively investigated for their antimicrobial potentials; studies on their QQ potential are meager (Musthafa et al., 2011; Borges and Simões, 2019). Therefore, in this study, bacteria were isolated from Palk Bay sediment samples and screened against QS biomarker strain *C. violaceum*. Except the CFS of marine isolate MSB-28, none of the isolates showed pronounced QQ activity against *C. violaceum* 12472. Similar reports were made by Shepherd and Lindow (2009) in which QQ activity was observed in CFS of *P. syringae* B728a which enabled the bacteria to degrade QS signals and thus block the expression of QS-regulated traits. The isolate showing profound QQ activity was found to be *Psychrobacter* sp. and belongs to Proteobacteria. These results corroborate well with earlier studies, where the bacteria isolated from a marine environment with QQ potential were identified as Proteobacteria (Tan et al., 2015; Torres et al., 2016; Rehman and Leiknes, 2018).

QS governs the biofilm formation and maturation in several bacterial species. The marine bacterial isolate *Psychrobacter* sp. exhibited biofilm inhibition against various Gram-negative pathogens such as PAO1, *S. marcescens*, *V. vulnificus*, and *V. parahaemolyticus* at 20% v/v without growth retardation (**Supplementary Table S1**). The degree of variation in the QQ activity of *Psychrobacter* sp. could be due to the involvement

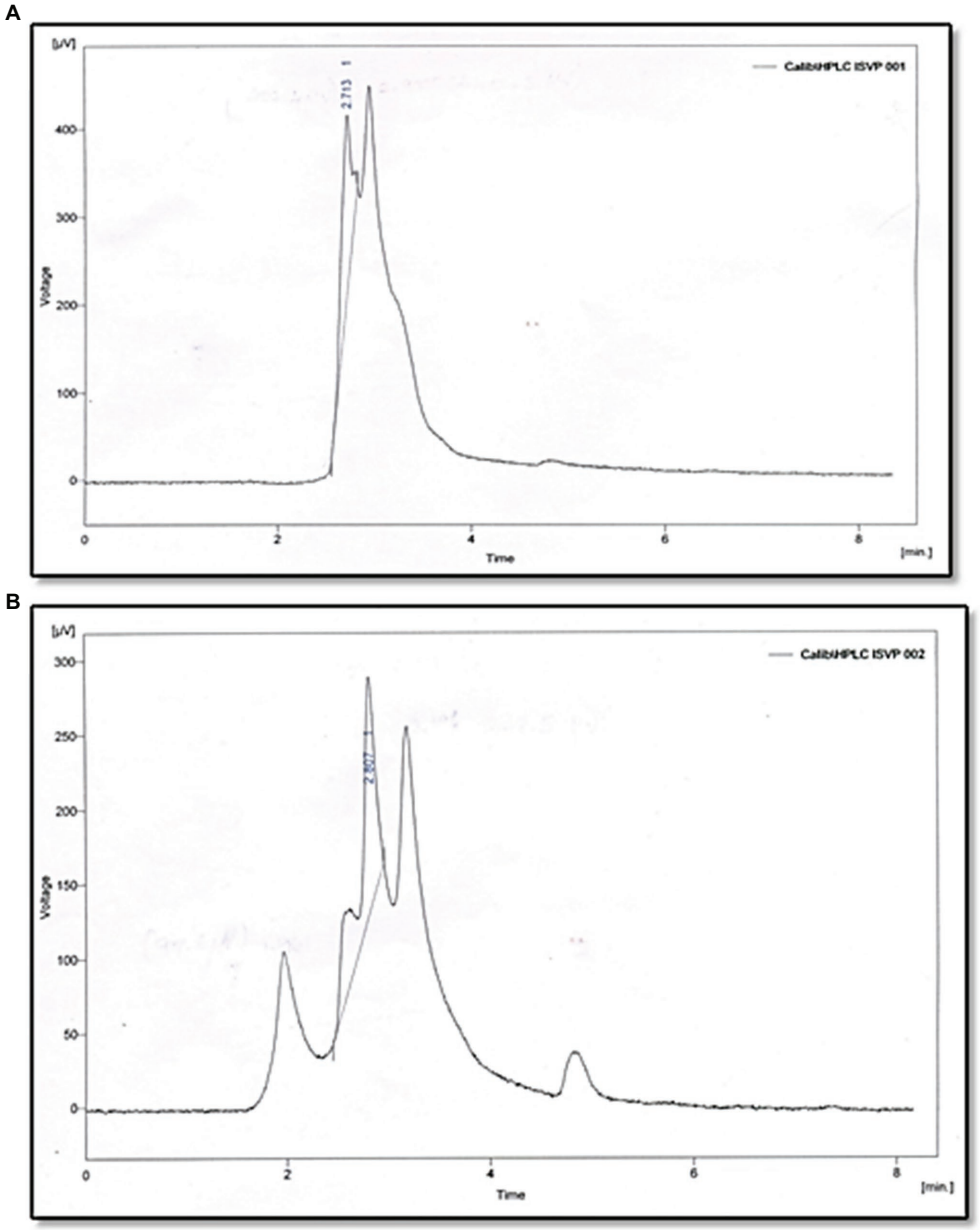

FIGURE 5 | Analysis of C_6HSL degradation by *Psychrobacter* sp. through HPLC. **(A)** C_6HSL alone. **(B)** C_6HSL treated with *Psychrobacter* sp.

of AHLs produced by the target bacteria with varied lengths of acyl side-chain. Flagella and pili aid to initiate the biofilm formation by reversible and irreversible attachment followed by microcolony formation (Shi and Sun, 2002). Hence, any interference in their expression by the metabolites of *Psychrobacter* sp. would result in the failure of biofilm formation. Development of distinctive biofilm architecture is the most important stage in biofilm formation (Kannappan et al., 2017). The attained results of CLSM analysis suggest that biofilm formation of the target pathogens was inhibited at the early stages of biofilm development. Moreover, the result of COMSTAT analysis ascertained the biofilm quantification assay. Altogether, these results suggest that the QQ agent present in *Psychrobacter* sp. might possibly interrupt the biofilm development without any negative effect on the bacterial growth. Bacteria are known to secrete antibacterial compounds. The growth analysis of pathogens clearly portrayed that the CFS of *Psychrobacter* sp. had no antibacterial activity towards the target pathogens. QS inhibition without any growth reduction is considered as the best alternative strategy to control the virulence factors' production and pathogenesis of bacterial pathogens, and leaves no scope for the development of antibiotics resistance (Rasmussen and Givskov, 2006). In this light, CFS of *Psychrobacter* sp. showed a profound QQ activity without any growth inhibition

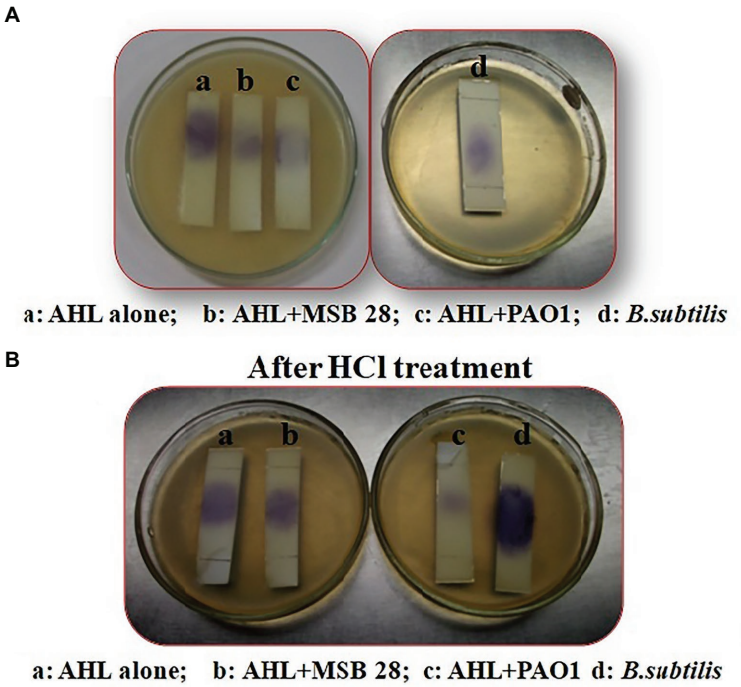

FIGURE 6 | (A) Analysis of mechanism of action of *Psychrobacter* sp. by ring closure assay. **(B)** Activity after HCL treatment. C_6HSL alone **(a)**, degradation pattern of C_6HSL by CFS of *Psychrobacter* sp., **(b)** PAO1 **(c)**, and *B. subtilis* **(d)**.

against the representative Gram-negative pathogens, which holds great clinical significance.

We also examined the QQ ability of *Psychrobacter* sp. to control other virulence factors associated with biofilms produced by PAO1. Production of EPS is known to maintain the biofilm architecture, and also correlates with an increased resistance of the biofilm-residing cells to biocides and host immune response (Kannappan et al., 2017). Hence, inhibiting EPS secretion by marine bacterium *Psychrobacter* sp. would loosen the biofilm architecture; thus, it is possible to reintroduce the use of antibiotics in treating biofilm cells along with active leads produced by *Psychrobacter* sp. In *P. aeruginosa*, several QS regulated phenotypic behaviors have been reported to be a part of the biofilm formation (Fong et al., 2019). In this study, treatment of *Psychrobacter* sp. would result in the reduced production of rhamnolipid; an important factor enhances the swarming motility by reducing the surface tension. In contrast, deficiency in surfactant production alters the swarming migration pattern and the altered bacteria would fail to colonize over the surface. The possible mode of action of the *Psychrobacter* sp. to block biofilm development is interfering either with C4-HSL signaling pathway accountable for surfactant production and swarming motility or blockage of 3-oxo-C_{12} HSL signals which have a direct control over biofilm formation. As signal-mediated QS regulates the virulence factors' production and biofilm formation, a remarkable reduction in biofilm formation and associated behaviors by *Psychrobacter* sp. might result from an effective hindrance of signal molecules by the secondary metabolites from *Psychrobacter* sp. Consistent with this result, a marine isolate VG-12 from red sea sediment inhibited the biofilm formation of PAO1 *via* QS signal degradation (Rehman and Leiknes, 2018).

The CFS of *Psychrobacter* sp. that lost its QQ activity upon being subjected to solvent extraction (**Figure 3A**), heat (**Figure 3B**), and Proteinase k (**Figure 3C**) treatments indicate that bioactive lead produced by *Psychrobacter* sp. is enzymatic in nature. Moreover, it is suggested that these QQ enzymes are heat sensitive. Hence, it is speculated that the loss of AHLs signaling was either because of AHL acylase or AHL lactonase activity. It is known that QQ bacteria able to degrade small-chain AHLs can also degrade medium and long-chain AHLs (Tan et al., 2015; Torres et al., 2016; Rehman and Leiknes, 2018). Hence, it is envisaged that investigation of QQ bacteria should have a focal point on identifying bacteria that targets small-chain AHLs, as recommended previously (Rehman and Leiknes, 2018). Interestingly, in this study the marine isolate was able to degrade the external C6-AHLs (**Figure 5**). AHL lactonases and AHL acylases are the best-known examples of AHL degrading so far reported and studied. Though the activity of AHL-acylases on short-chain AHLs remains unclear (Shepherd and Lindow, 2009; Czajkowski et al., 2011), it would be factual if the degraded C6-AHLs will be restored after acidification. In the present study, the degradation and restoration of short chain C6-AHLs suggest that the observed QQ activity of *Psychrobacter* sp. in attenuating the QS-mediated biofilm formation by bacterial pathogens such as *S. marcescens*, *P. aeruginosa*, *V. parahemolyticus*, and *V. vulnificus* is possibly due to the presence of an AHL lactonase. Altogether, the obtained results from the present study evidence that the AHL lactonase produced by the marine bacterium *Psychrobacter* sp.

is heat-liable and active against different AHLs produced by other pathogens. Moreover, this bacterium was not found to produce any AHL signal molecule and hence could be used as a potent source for AHL degrading lactonase enzyme. For the first time, the present report divulged the quorum quenching lactonase enzymes production from *Psychrobacter* sp.

CONCLUSION

Quorum quenching is of great concern in controlling infectious pathogens without interfering with growth, thus avoiding the selection pressure that often results in the emergence of resistance strains. In this study, we found marine sediment bacteria with QQ potential, identified as *Psychrobacter* sp., that is able to degrade AHLs and thereby inhibit the QS mechanism and biofilm formation of diverse bacterial pathogens. Moreover, lactonolysis and chromatograpic analysis revealed the presence of AHL-lactonase in the CFS of *Psychrobacter* sp. Thus, the attained results emphasize that the QQ activity of *Psychrobacter* sp. could potentially be used as a biocontrol agent to combat multidrug resistant bacterial infections caused by Gram-negative human as well aquatic pathogenic bacteria.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

IP: conceptualization, performed the experiments, data analysis, and writing – original draft. AK and ST: performed the experiments, data analysis, and writing and reviewing original draft. RS: data analysis, and writing and reviewing original draft. DJ and JP: data analysis. PV: data validation. AR: conceptualization, supervision, data validation, and writing – original draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.634593/full#supplementary-material

Supplementary Figure S1 | (A) Assessment of QQ activity of MSB-28 using *C. violaceum* 12472 by soft agar overlay assay. **(B)** Quantitative assessment of violacein inhibition in CV026 by CFS of MSB-28 at increasing concentrations. Mean values represent the data of three independent experiments and SD are shown.

Supplementary Figure S2 | Detection of AHL produced by *Psychrobacter* sp. *via* the biosensor CV026. **(a)** Natural C_6-HSL as positive control, and **(b)** extract from the spent culture supernatant of *Psychrobacter* sp.

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REVIEW
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Mechanistic Understanding of *Candida albicans* Biofilm Formation and Approaches for Its Inhibition

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In recent years, the demand for novel antifungal therapies has increased several- folds due to its potential to treat severe biofilm-associated infections. Biofilms are made by the sessile microorganisms attached to the abiotic or biotic surfaces, enclosed in a matrix of exopolymeric substances. This results in new phenotypic characteristics and intrinsic resistance from both host immune response and antimicrobial drugs. *Candida albicans* biofilm is a complex association of hyphal cells that are associated with both abiotic and animal tissues. It is an invasive fungal infection and acts as an important virulent factor. The challenges linked with biofilm-associated diseases have urged scientists to uncover the factors responsible for the formation and maturation of biofilm. Several strategies have been developed that could be adopted to eradicate biofilm-associated infections. This article presents an overview of the role of *C. albicans* biofilm in its pathogenicity, challenges it poses and threats associated with its formation. Further, it discusses strategies that are currently available or under development targeting prostaglandins, quorum-sensing, changing surface properties of biomedical devices, natural scaffolds, and small molecule-based chemical approaches to combat the threat of *C. albicans* biofilm. This review also highlights the recent developments in finding ways to increase the penetration of drugs into the extracellular matrix of biofilm using different nanomaterials against *C. albicans*.

Keywords: *Candida albicans*, biofilm, resistance, antifungal drugs, prostaglandins, small molecule inhibitors, naturally occurring compounds

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INTRODUCTION

Biofilms are structurally organized microbial communities attached to the surfaces of implanted devices encapsulated within a solid protective extracellular matrix (Spormann et al., 2004). Approximately, 65% of all human microbial infections embroil biofilm formation. Therefore, identifying its role, composition, and impact of microbial biofilms on human medication is an attractive proposition (Douglas, 2003). Biofilm "inhabitants," fungi and bacteria, are less sensitive or insensitive to antimicrobial agents. The property of the microorganisms to adhere to different surfaces facilitates the formation of biofilm in clinical settings such as catheters, prosthetic heart

valves, joints, and various tissues in the host leading to effective colonization and this results in persistent drug-resistant.

In recent years, the prevalence of fungal infection has increased. These mycotic diseases account for over 10 lakhs of human deaths annually, which has become a delinquent health issue across the world. In today's scenario, these opportunistic fungal infections have affected immunosuppressed or immunocompromised patients, and folks proclaimed for administration in the intensive care unit. These mycotic infections are classified from non-life-threatening mucocutaneous illnesses to intrusive infections which can affect any organ (Pfaller and Diekema, 2007; Yapar, 2014). This kind of fungal infection is often connected with the other mild to lethal fungal or bacterial infections involving species such as *Aspergillus* (Williams et al., 2016; Richardson and Rautemaa-Richardson, 2019) different species of *Candida*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Kaplan, 2014; Pleszczyńska et al., 2015; Fleming et al., 2017) and *Cryptococcus neofornams* (Aslanyan et al., 2017). Of the several known and frequently studied *Candida* species, *C. albicans* is the one most commonly recorded and encountered fungal pathogen in the human race (Su et al., 2018). Systemic candidiasis, caused by *C. albicans,* is known to be the reason for death in nosocomial and opportunistically abysmal fungal infection in patients (Su et al., 2018). followed by *Candida glabrata. Candida tropicalis* is commonly found in urinary tract infections (UTI) whereas *Candida parapsilosis* is mostly located on the epidermis of a healthy individual. The latter is also the causative agent of catheter-related infections. Likewise, all *Candida* species depict differences in terms of their biofilm formation, structure, changes in the morphology extracellular matrix (ECM), and potential to resist antifungal drugs (Cavalheiro and Teixeira, 2018).

National Nosocomial Infection Survey (NNIS) indicated that *Candida* spp. were the fourth most common cause of nosocomial bloodstream infections during the 1990s. However, in more recent studies, it has been discovered that *Candida* spp. are the third most frequent nosocomial bloodstream isolates (Perlroth et al., 2007; Lohse et al., 2018). Additionally, *Candida* is the third mainstream infection of central-line-associated bloodstream infections (CLABSIs) and the second most prominent reason for catheter-associated urinary tract infections in the United States. Lockhart (2014) Subsequently, the incidence of disseminated candidiasis which broadly refers to the mucosal, cutaneous, and deep-seated organ infection caused by *Candida* genus has increased 15 to 20-fold in the last two decades (Lockhart, 2014; Pappas et al., 2018). It has been reported that systemic candidiasis, caused by *C. albicans* is one of the major reasons for death in nosocomial as well as opportunistically abysmal fungal infection in patients because of its ability to form a biofilm which decreases the susceptibility of fungal cells toward drug treatment. Consequently, it has been reported each year that up to 50% of systemic candidiasis adults patients and approximately 30% of the young population die due to candidiasis co-related with biofilms. Moreover, there is an estimation of 100 thousand deaths because of the infection initiated by biofilm formation. Approximately, $6500 millions are spent per year in the United States toward its treatment causing a high fiscal impact on the state (Gulati and Nobile, 2016; Silva et al., 2017). The five most widespread species, *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* are the reason behind 92 to 95% of Intensive Care Unit (ICU) cases in the United States, although, distribution of all five species might vary (Kojic and Darouiche, 2004; Ten Cate et al., 2009). According to National Institute of Health (NIH) reports, pathogenic biofilms account for more than 80% of all microbial infections (Ramage et al., 2006; Perlroth et al., 2007; Tsui et al., 2016; Lohse et al., 2018), of which 75% cases are of vaginal yeast infection in women once or more than once in their lifetime (Perlroth et al., 2007; Yapar, 2014).

Candida albicans is a commensal fungi, asymptomatically associated with normal microflora of the host, but becomes invasive and virulent when converted to hyphal form covered by an extracellular polymeric substance (EPS), which is a dimorphic nature of *C. albicans* (Gulati and Nobile, 2016). It is the most stereotypical fungal pathogen in humans, causing disease ranging from apparent mucosal to lethal disseminated bloodstream infections which make a considerable contribution of more than 40% of mortality rates (Lohse et al., 2018).

The three stages of the development of *C. albicans* comprise adhesion of the yeast cells on the medical devices (early stage), differentiation of the yeast cells to hyphal cells (intermediate stage), and an increase in the matrix which is the maturation phase (Alim et al., 2018). The mature biphasic structure of *C. albicans* is promoted by the adhesive hydrophobic nature of indwelling devices with the proper growing environment. Biomedical devices inserted during transplantation catheter are the favorable ones as they provide nutrition like glucose from the excreted products (Ramage et al., 2006; Alim et al., 2018).

Candida albicans colonizes the skin, intestinal mucosa and/or genital mucosa of 30–70% of healthy individuals. Therefore, under normal circumstances, the fungus does not cause any significant disease (Ramage et al., 2014). Furthermore, about 15% of sepsis cases acquired in a hospital due to medical devices occur due to the predominant fungi *Candida* (Lohse et al., 2018). According to some recent reports, 63.5% of patients out of 224 patients suffering from septic shock showed a positive result for *Candida* and were found dead. Thus, it is the leading cause of death in hospital procured diseases (Ramage et al., 2014). In India, just 6-18% of cases of Candidemia are reported. A study exhibits 53 repeated episodes of Candidemia in 48 patients in 1.5 years. Different species made their contribution, like 45% of infections were due to *C. tropicalis*, while 23% of cases by *C. albicans* and the rest 32% by other *Candida* spp. (Kothari and Sagar, 2009). Western countries' trend on candidemia differs from India as *C. albicans* is related to more than 50% of all candidemia incidences (Kaur et al., 2014).

RECENT CHALLENGE-DRUG RESISTANCE

The extensive use of antimicrobials medicaments in a well-connected global human population has increased the cases of

antimicrobial resistance (Michael et al., 2014). According to the UN *ad hoc* Interagency Coordinating Group on Antimicrobial Resistance, drug-resistant diseases by 2050 can bring around 10 million deaths per year and cause an economic catastrophe similar to that of the 2008-2009 global financial recession. By 2030, antimicrobial resistance could affect approximately 24 million people plunging them into severe poverty (World Health Organisation [WHO], 2019). According to the Center for disease control and prevention, around 7% of all the blood samples from patients suffering from *Candida* is resistant to fluconazole. However, this resistance to azole is persistent for the last 20 years, but resistance toward echinocandins and other drugs is a huge concern. Thus, this situation will prove to be lethal because of the ever -growing human population.

Owing to its inappropriate use, antifungal drug resistance has emerged as a major challenge that needs immediate attention. This is further aggravated because of the overuse of antibiotics, which affects the normal microflora of humans, providing a favorable environment for *C. albican* to grow (Ben-Ami et al., 2012). Currently, available drugs are categorized into four major classes which include azoles, polyenes, pyrimidine analogs, and echinocandins. The primary targets of these antifungal drugs are the biosynthetic pathway of ergosterol, the cell wall of fungal cells, or the DNA/RNA of fungi (Borghi et al., 2016; Sagatova et al., 2016; Silva et al., 2017; **Figure 1**).

Azoles are widely recommended antifungal drugs to treat superficial and invasive fungal infections. The first generation azoles include clotrimazole, bifonazole, econazole, and ketoconazole containing imidazole in their ring system. Fluconazole and itraconazole are some of the renowned examples of second- generation azoles., containing triazole moiety. Voriconazole and posaconazole are the FDA- approved broad -spectrum third generation azole- based antifungal drugs. They exhibit their antifungal effects by inhibiting cytochrome P450-dependent 14α-lanosterol demethylase (Cyp51) encrypted by the ERG11 gene which converts lanosterol to ergosterol (Taff et al., 2013; Silva et al., 2017). The ancient class of fungicidal drugs, polyenes, is used to treat severe infections. It intercalates with the steroids ergosterol, found in the cell membrane, creating pores that obliterate the proton gradient of the cell, destabilizing the cell membrane causing leakage of the ions in the process. Another fungicidal drug, Echinocandins (e.g., Caspofungin), is the newest class of antifungals that acts against *Candida spp.* and is administered intravenously. It interferes with the production of β-1,3- glucans, critical polysaccharides and the major constituent of fungal cell walls (Perfect, 2017; Pristov and Ghannoum, 2019).

Analogs of nucleosides like 5-flucytosine (5-FC) are antimetabolites that imitate nucleotide bases in the course of the synthesis of nucleosides. The 5-Flucytosine acts as a pyrimidine correspondent disturbing the synthesis of fungal RNA, DNA, and protein leading to cell cycle arrest. The 5-FC in itself does not act as an antifungal agent but becomes active when changed into 5-fluorouracil. This conversion of 5-FC into 5-FU is catalyzed by the enzyme cytosine deaminase, residing in fungal cells but not present inside the host cells. Even with a vast diversity of antifungal drugs, the problem

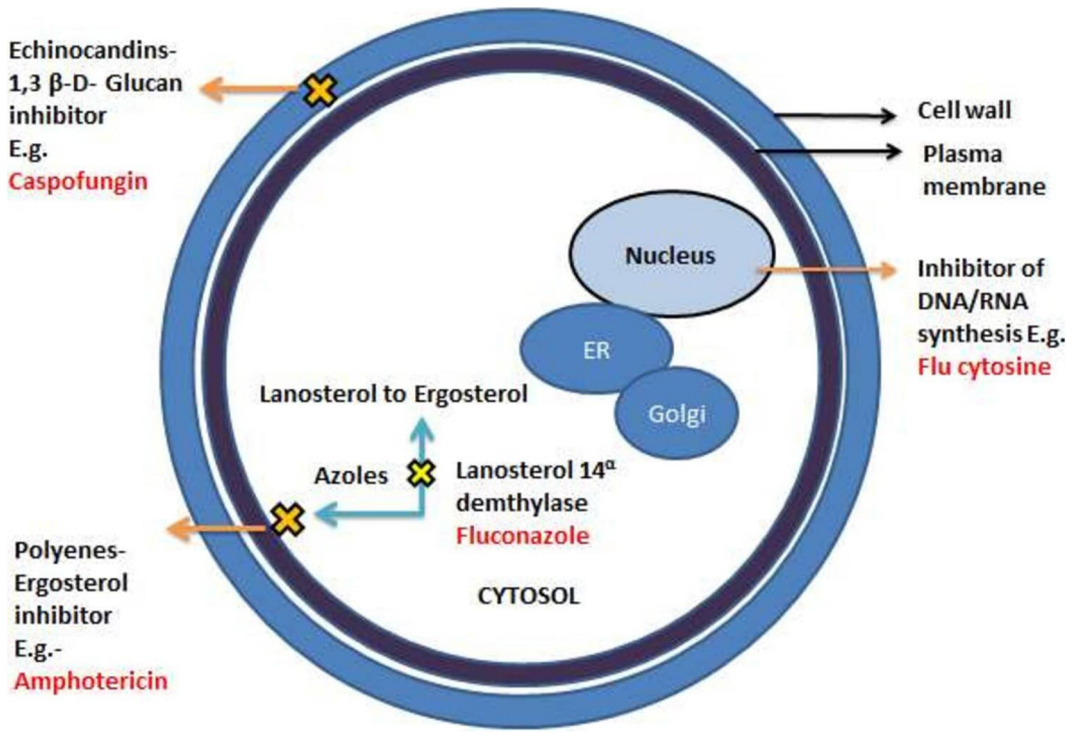


FIGURE 1 | A different mechanism of actions of commonly known antifungal drugs adapted from Spampinato and Leonardi (2013).

of resistance persists against the drug sequestration, making it challenging to fight. The classic inventions, polyenes and azoles, are also ineffective against *C. albicans* biofilms, thereby limiting the range of treatment. Thus, the indispensable development of new antifungal therapies with high efficiency against the biofilm mode of growth is required. Biofilms are the major contributors and intensifiers of antifungal resilience. As yet, none of these factors works alone. Instead, this antifungal resilience is a multifactorial phenomenon, which is still unexplored. Various factors of intrinsic resilience which alter normal vegetative cell to more virulent form have been listed briefly. These include an increase in the density of cells within the biofilm, complex association extracellular matrix, the existence of persister cells, gene expression of the antifungal resistance genes, and the proliferation of sterols on the membrane of biofilm cells (Romo et al., 2017; Silva et al., 2017; Su et al., 2018).

FUNGAL BIOFILMS

Fungal biofilms are the complex association of hyphal cells which in turn are associated with both abiotic and animal tissues. They are important virulence factors and correlated with invasive fungal infection (Borghi et al., 2015). They are the sessile microorganisms that, when attached to the abiotic or biotic surfaces, lead to new phenotypic characteristic features (Hawser and Douglas, 1995; Lohse et al., 2018). Implantable medical devices are the favorable sites where *C. albicans* form a complex association forming the biofilms, thus becoming responsible for a proportion of clinical candidiasis (Douglas, 2002).

Furthermore, adherence of the fungal cell to the available biomaterial and its relatedness to bloodstream infections might be due to hematogenous spread. Medical devices provide a perfect niche to yeast cells because of their structure to chemical properties ranging from hydrophobicity to surface roughness. These devices are surrounded by body fluids like urine, blood, saliva, and synovial fluid, which condition them with glycoproteinaceous film (Gristina et al., 1988; Subbiahdoss et al., 2010). This acclimatizing film can accord chemical properties entirely different from its origin. The mature biphasic structure of C. *albicans* is promoted by non-specific factors (cell surface hydrophobicity and electrostatic forces) and specific adhesins on the fungal surface recognizing ligands in the conditioning films, such as serum proteins (fibrinogen and fibronectin) and salivary factors (Chaffin et al., 1998; Demuyser et al., 2014). Also, *C. albicans* cells can co-aggregate to interact with bacterial cells/colonies already vested in these devices (Chaffin et al., 1998; Ramage et al., 2006). Nevertheless, the preliminary focal attachment of the fungal cell to a substratum is accompanied by multiplication and propagation of cells followed by biofilm development (Soll and Daniels, 2016; Silva et al., 2017).

Pathogenesis of Biofilm

Stages of Biofilm Development

It has been observed that biofilm development follows sequential steps over a period of 24–48 h (Mathé and Van Dijck, 2013; Taff et al., 2013; **Figure 2**). Initially, a single yeast cell adheres to the substratum making a foundation for the layer of a yeast cell (*adherence step*) (Finkel and Mitchell, 2011; Mathé and Van Dijck, 2013; Williams et al., 2013). Following this initial phase is the phase of cell proliferation where cells project out and continue to grow into the filamentous structure of hyphal cells through the surface (*initiation step*). The assembly of hyphae marks the beginning of biofilm formation accompanied by the accretion of an extracellular matrix (ECM) on maturation biofilm (*maturation step*). Lastly, non-adhering yeast cells detach themselves from the biofilm into the environment to find a favorable site of attachment (*dispersal step*).

Spreading of biofilm-associated yeast cells has tremendous clinical significance as they can start the formation of new biofilms or circulate throughout the host cell and tissues leading to disseminated invasive diseases or candidemia. Various factors promoting the pathogenesis of *C. albicans* biofilm (**Figure 3**) have been reported, which are discussed in the following section.

Extracellular Matrix Formation (ECM)

The extracellular matrix is a critically important feature of biofilms that guards the adherent cells against the host immune system and antifungal agents by forming an extensive structure of the matrix (Borghi et al., 2016). In some of the pioneer works, it was shown that *Candida* species biofilm's matrices increase when highly dynamic flow environments influence biofilm, and its quantity extensively depends upon strain and the species. Moreover, the chemical composition of *C. albicans* ECM suggests that the extracellular matrix is composed of approximately 55% of a combination of glycoproteins with carbohydrates contributing to only 25% of the total composition. Carbohydrates consist largely of α-mannan and β-1, 6-glucan polysaccharides with β-1, 3-glucans making a very little contribution. ECM also consists of 15% of lipids and only 5% of nucleic acids. β- 1, 3- glucan plays a major role in azole resilience by specific binding. Additionally, the biofilm is two times thicker than planktonic cells. Upon comparison of the chemical composition of planktonic cells with biofilm cells, it was observed that there is a difference in carbohydrate and β 1, 3 glucan composition (Mah and O'Toole, 2001; Nett et al., 2010; Silva et al., 2017; Lohse et al., 2018; Pappas et al., 2018).

Extracellular DNA and Genetic Factors

Extracellular DNA (e-DNA) present inside the extracellular matrix is a major contributor to the stability of the *C. albicans* biofilm. The e-DNA is present both in bacterial and fungal biofilms; hence, when these biofilm-forming microorganisms are treated with DNAase enzyme in combination with the respective drugs, they exhibit a decrease in the biofilm matrix (Martins et al., 2014). Various genetic factors like Bcr1 (a transcription factor required for the attachment of fungal cells to the abiotic surfaces), Rlmp, Brg1, Efg1, Ndt80, Rob1, and Tec1, Fsk1p, Smi1p (which works in unification with Fsk1p and Rlmp), Gcr1, Mnn4 are under study. All these factors work together and interact with different genes to regulate and generate biofilms, thus, has given new insight to

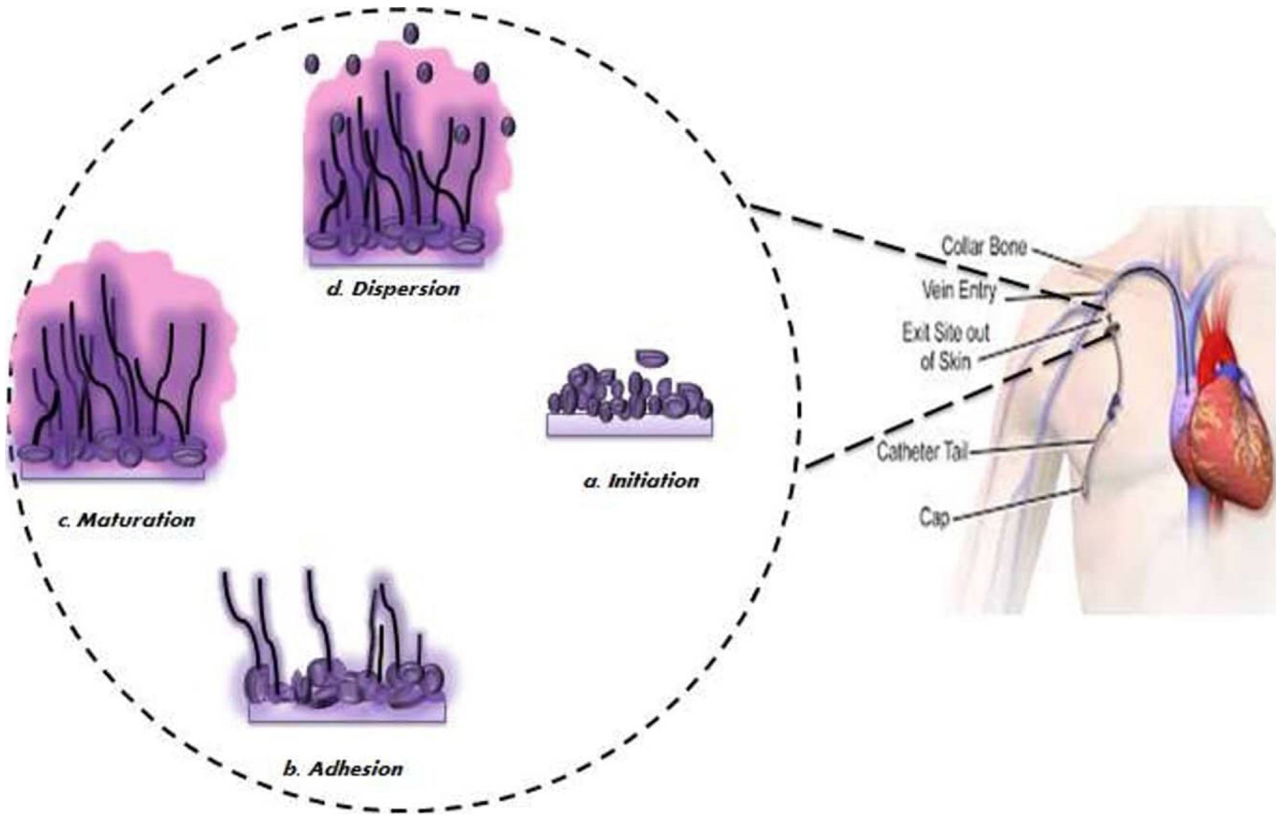


FIGURE 2 | Schematic diagram of different stages of biofilm development.

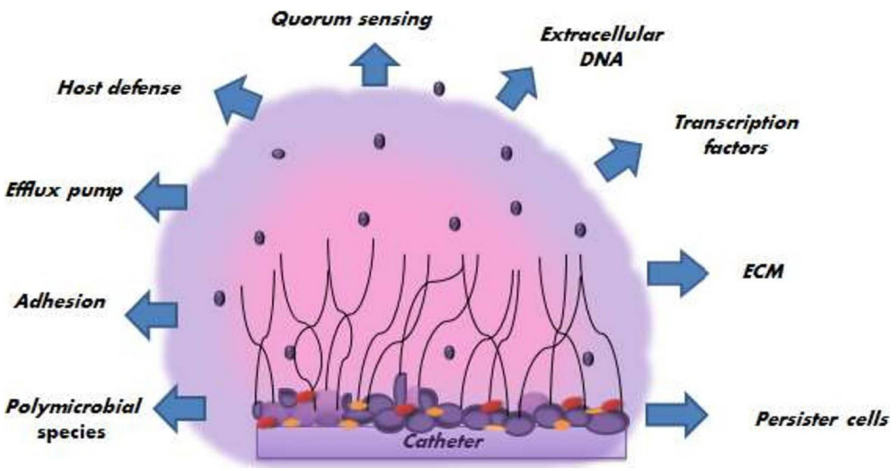


FIGURE 3 | Different factors promoting the pathogenesis of the *C. albicans* biofilm.

biofilm formation (Finkel and Mitchell, 2011; Nett et al., 2011; Nobile et al., 2011).

Quorum Sensing (QS)

Another mechanism related to extracellular properties of the matrix of the *C. albicans* is quorum sensing, which plays a significant role in the growth of biofilms. It is a density-dependent cell-cell communication mechanism in which autoinducers (signal molecules) are released in response to the increasing density, which enhances or represses the activation of certain genes or factors. This density-dependent mechanism affects different aspects of microorganisms like pathogenesis, morphology, competence, etc. Importantly, it also makes its contribution to biofilm formation. Earlier, QS was thought

to be an exclusive feature of a certain bacterial system, but the recent discovery of farnesol, a quorum-sensing molecule inhibiting *C. albicans* biofilms, has publicized QS (Ramage et al., 2002). Genetic regulation of virulent genes in pathogenic microorganisms by QS has shown an indirect connection with the emergence of multi-drug resistant pathogens. Thus, it necessitates the finding of alternative strategies to target QS and restrain the same (Finkel and Mitchell, 2011; Taff et al., 2013).

Evasion of Host Immune Responses

The immune system plays a crucial role in the recognition and elimination of the *C. albicans*. The innate immune system, which is the first line of defense, recognizes pathogen-associated molecular patterns of a pathogenic strain of *C. albicans* leading to the activation signaling channels of a host organism resulting in the extermination of the *C. albicans* cells.

According to recent studies, ten diverse surface receptors participate in this recognition pattern. They are Toll-like receptors -TLR4 and TLR2, TLR9, and NLR Family Pyrin Domain Containing 3 (NLRP3) the internal receptors, C-type lectin receptors, Dectin-1and 2, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DCSIGN), Mincle, and Mannose-binding lectin. Usually, these receptors recognize and bind to sugar moieties (β-1, 3-glucans, and mannose derivatives) present on the cell surface of *C. albicans*. Due to this binding, the cytokine complement system gets activated, phagocytizing the fungal cells. Internal uptake of these fungal cells by APCs (antigen-presenting cells) expedites the activation of internal receptors; thus, resultant activation of TLR9 or NLRP3 inflammasome occurs. This non-specific immune response (innate response) has a significant role in preventing *C. albicans* infection. Besides, the adaptive immune response also makes its contribution by producing antibodies against some specific extracellular proteins, obstructing the *C. albicans* growth.

On the other hand, there are many strategies employed by *C. albicans* to evade the immune response. Mature biofilms escape immune responses owing to the presence of a top layer of biofilm composed of hyphal cells which mask the β-glucans component of biofilm. Subsequently, these hyphal cells escape the neutrophil killing either by penetrating the epithelial cell layers in the course of invasive growth or by escaping immune response by physical penetrating inside the cell. The variance in the degree of expression of genes in planktonic cells concerning biofilms cells has a connection with a mechanism of immune evasion. There are several proteins that are highly expressed and they also restrict the stimulation of the host complement system. For example, Zinc binding cell surface protein Pra1, a cell surface glycerol-3-phosphate dehydrogenase protein (Gpd2), and all the secretory proteins of aspartyl protease (Sap) family. Another well-known protein that functions as a sensor for cell wall damage with high expression in biofilms is Msb2. This protein secretes and blocks the antimicrobial peptides preventing complement activation.

Neutrophils are the first foot soldiers of the immune system which are altered during biofilm formation. Neutrophils work with different modes of action like phagocytosis, oxidative response, and non-oxidative response against the emerging pathogen. Another known novel mechanism that was discovered in 2004, is the Neutrophil extracellular trap "NETosis." It is a complex structure that comprises neutrophil chromatin, DNA, and protein. NETosis degranulates neutrophils and releases a lytic enzyme, which is different from necrosis and apoptosis. It has killing properties against planktonic yeast cells; however, it is seemingly ineffective against *C. albicans* biofilms (Branzk and Papayannopoulos, 2013; Johnson et al., 2016, 2017; Tsui et al., 2016). Some studies also suggest that the peripheral mononuclear cells (PBMCs) -due to some unknown factors- thicken the *C. albicans* biofilms rather than phagocytizing them. Furthermore, mature biofilms do not evoke a vigorous oxidative response, a primary mechanism of neutrophil killing microbes (Lohse et al., 2018; Pappas et al., 2018).

Polymicrobial Species

Humans provide an immediate small- scale environment to a varied population of microbial species. The human microbiota embraces members from three kingdoms of life: Archaea, Bacteria, and fungi. They usually exist in a symbiotic relationship leading to a complex association of ecosystems. Imbalance in this symbiotic association due to genetic or environmental factors of the host - like changes in pH, shifts in host immunity, and transient viscosity of mucosal layers, and indiscriminate usage of broad-spectrum antimicrobial agents- lead to the disproportionate infection by the overgrowth of specific microbial species over others.

Candida albicans is the most frequent fungal pathogen present in commensalism with bacteria. Furthermore, there is incremental progress in the dual-species biofilms formation between common bacterial species and *C. albicans* that usually interact in human beings to cause infection. These kinds of interaction which lead to diseases are studied and concluded based on observational studies, arising mainly in immunocompromised patients. It is difficult to establish a clinical prognosis of polymicrobial interaction from immunocompromised individuals as they are highly vulnerable to other infections. Hence, it is tough to evaluate the degree of polymicrobial interactions and molecular mechanisms from such studies. *In vitro* studies suggest that bacterial species and *Candida spp.* isolated from different parts, like the vagina, oral cavity, etc., interact with each other in different ways. This includes exudation of signaling molecules which influences species behavior toward each other, physical interaction between microbial cells (e.g., hyphal cells provides an attachment site to bacterial cells contained within polymicrobial biofilms), biochemical alterations of the local environment like change in oxygen content and pH. For instance, in the case of the vaginal microbiota, *C. albicans* interacts with Lactobacillus species which produces lactic acid that changes local pH, thus hindering C. *albicans* growth on the mucosal surface of the vagina. Some hyphal cell wall proteins (E.g., Hwp1), transcription regulators (Tec1 and Bcr1, Efg1, Cph1, etc.) and adhesive proteins (E.g., Als1, Als2, and Als3) show a pivotal role in enhancing the expression of virulent genes and hence the resistance in bacterial species such as *Staphylococcus aureus*, *Streptococcus gordonii*

and *Staphylococcus epidermidis* (Harriott and Noverr, 2010; Pammi et al., 2013; Chinnici et al., 2019). However, deletion or mutation of these proteins results in a decrease in the number of bacterial cells and weakens their interaction with *C. albicans*. Although biofilms are formed in an oxygen-rich environment, some anaerobic bacteria can easily grow under *C. albicans* biofilms. These biofilms provide bacteria with a positive anaerobic environment to grow. In return, the bacteria augments the formation of *C. albicans* "mini biofilms" which can easily float and grow under the toxic condition (Douglas, 2003; Finkel and Mitchell, 2011; Gulati and Nobile, 2016; Alim et al., 2018).

Efflux Pumps

Overexpression of efflux pumps is one of the major contributors to antifungal drug resistance in *C. albicans* as they lead to drug sequestration by pumping out the antifungal drugs given as a treatment. Subsequently, these are primarily concerned with first-generation azoles but ineffective toward Echinocandins resistance. During usual antifungal treatment toward planktonic cells, efflux pumps prevent the intracellular accumulation of antifungal drugs by up-regulating their expression (Borghi et al., 2016; Soll and Daniels, 2016; Tsui et al., 2016). The Cdr1, 2 (ATP binding cassette transporter superfamily), and Mdr1 (Major facilitator transporter superfamily) are the two major classes of efflux pumps that control drug exportation in *C. albicans*. Moreover, initially, after a few hours of up-regulation in biofilms, they persist in being up-regulated all through the biofilm growth even in the absence of the antifungal drug. Rapid upregulation of efflux pumps happens during the primary stage of biofilm formation.

Persister Cell and Stress Response

Another major contributing element toward resistance is persister cells, which are the inconsequential subset of yeast cells with minimum metabolic activity assumed to be raised as a phenotypic variant but not a mutant type. They are highly resistant cells within biofilms. Amphotericin B treatment upon *C. albicans* led to the discovery of persister cells. These cells are acknowledged to be persister as they maintain themselves in a dormant stage. Still, in a stressful situation, they reactivate themselves to a state of active metabolism and reinstate as biofilms. Subsequently, drug sequestration by persister cells occurs due to some virulent traits like hyphal growth rather than an expression of efflux pumps and cell membrane structure (Mah and O'Toole, 2001; Tsui et al., 2016; Silva et al., 2017; Lohse et al., 2018).

ANTIBIOFILM STRATEGIES

The social burden of fungal disease is massive with approximately more than 1.5 billion people affected by the fungal disease worldwide per annum. However, these invasive fungal infections are estimated to be the cause of about 1.5 million deaths per year, making this a noteworthy health problem (Bongomin et al., 2017). While flourishing in its most usual mode of growth, a biofilm of *C. albicans* biofilm displays increased resistance to the available antifungal drugs. These persistent groups of cells are difficult to eradicate and, ever so often, accountable for treatment failures. There is a necessity to develop new strategies to exterminate and treat these emerging *C. albicans* biofilm infections in the medical setting.

Different targets have gained attention aiming to tackle this growing antifungal resistance problem. Treating these biofilms is a big hurdle in medical mycology; thus, there is a vast exigency for the development of new antifungal agents and identification of novel targets.

Considering the efforts undertaken to find solutions to the fungal biofilm infections, the reader may acknowledge the complicated mechanisms that impede the path to reach the panacea. The research was done while managing the patients so far has led to the conclusion that the study of biofilm phenotype is inevitable to restrict fungal biofilms. Besides, the counter effect of fungus on the drug should be examined not just against the planktonic cells but also against the biofilm. Since fully established mature biofilms are much more challenging to treat and eradicate, preemptive practices targeting planktonic cells further inhibiting the early development of biofilms are the need of the hour. Nonetheless, strategies to combat mature biofilms should also be kept in mind. We discuss specific strategies that are under scrutiny, globally, against the problem of drug resistance (Pierce et al., 2015b; Romo et al., 2017).

Prostaglandins

Molecular studies on biofilms have revealed the important role of lipids on antifungal resistance. The lipid profile of planktonic cells differs from that of biofilms, making lipids a censorious modulator of resistance. This change in the sphingolipids and sterol profile promotes biofilm formation affects cells morphology and physiology altering the adhesion properties in *C. albicans* (Alim et al., 2018; Su et al., 2018). Thus exploiting these prostaglandins adaptation and their properties could be targeted for treatment. The signaling cascade of Arachidonic acid (AA) is pivotal for both biology of humans and fungi morphology and growth. Very nearly each of the following enzymes of the AA cascade can be subjected to the pharmacological investigation: Cyclooxygenase (COX), Cytochrome P 450 (CYP), and Lipoxygenase (LOX) (Liu et al., 2016). Arachidonic acid could be processed into different types of eicosanoids.

Prostaglandins are synthesized by cyclooxygenase enzymes in human cells as well as pathogenic fungi. Prostaglandin E2 is the profusely synthesized prostaglandin that transfers immune responses of humans in the direction which endorses fungal establishment and chronic illness (Erb-Downward and Noverr, 2007; Alim et al., 2018). PGE_2 inhibits T-helper cells (Type-1) –Th-1 and stimulates the progression of T-helper cells (Type II)-Th-2. These cells are responsible for maintaining the inflammatory responses as Th-1 is accountable for pro-inflammatory responses, while Th-2 for producing anti-inflammatory responses (Berger, 2000). PGE_2 also down-regulates the production of chemokine and TNFα, thus augmenting the fungal colonization and germ tube formation.

Arachidonic acid, when used along with antifungal drugs, affects the level of prostaglandin E2 in a variety of *Candida* spp. The above-mentioned study suggests that lipid plays a specific role in the structural aspects, signaling cascade, fungal pathogenicity, and metabolic activity of biofilm development in *C. albicans*. However, the defined part of lipid-dependent processes in the development of *C. albicans* biofilm is still not known. Genetic modulation and pharmacological inflection of biosynthetic pathways of lipids are the two stratagems that have been browbeaten in linking lipid alterations with *C. albicans* biofilm morphology- its formation and function in this fungi. Modulating the lipid composition of the cell membrane by comprehensively targeting genes that encode enzymes for lipid biosynthesis regulates endogenic lipid levels (Noverr and Huffnagle, 2004; Erb-Downward and Noverr, 2007; Sherry et al., 2012; Fourie et al., 2016; Liu et al., 2016).

Modification of the Surface of Biomedical Devices

Biomaterial-associated-infection (BAI) is the major cause of dereliction of biomaterial implants. Microbial contamination of biomaterial devices during implant surgery (peri-operative contamination) or hospitalization (Gristina et al., 1988), causes the onset of BAI. Microorganisms present in BAI are resistant due to the biofilm mode of growth. The extensive use of indwelling catheters in recent medicine, particularly central venous and hemodialysis catheters, has contributed significantly to the increasing incidence of fungal bloodstream infections, in particular, candidiasis (Pierce et al., 2015b).

Traditionally, to decrease the cases of nosocomial infection, which occurs due to central line-associated bloodstream infections (CLABSIs), removal of the devices and providing systemic antimicrobial therapy was performed to eradicate these microorganisms.

These biomedical-assisted devices with a wide range of biomaterials are utilized by a wide range of pathogenic fungi like *C. albicans* to support adhesion, colonization, and subsequent biofilm formation (Ramage et al., 2006; Talsma, 2007). Thus, to preclude these contagious microorganisms on the surface of implanted devices with biofilms, there is an inquisitiveness in the development and improvement of unconventional biomaterials which are unfavorable to microbial (both fungal and bacterial) adhesion and colonization. Another modification strategy from some studies suggests that modifying the surface chemistry of the biomaterials could be an approach to prevent or reduce biofilm formation. This could be achieved by adding surface-modifying end groups (SMEs) or by altering the chemical composition of substrates. For example, SME Polyetherurethane when added to Elasthane 80A, a biomaterial, decreased the *C. albicans* ability to form biofilm significantly (Chandra et al., 2005).

Antibiotic Lock Therapy (ALT)

Antibiotic lock therapy is, in general, a combination of an antibacterial solution that has minimum inhibitory concentrations (MIC) 100-1000 times greater than the antibiotic used for planktonic cells in combination with the anticoagulant instilled into the lumen of a catheter. The solution is allowed to dwell or is "locked" while the Central Venous Catheter (CVC) is not used to prevent colonization or sterilize a previously infected catheter (Bookstaver et al., 2013; Justo and Bookstaver, 2014; Norris et al., 2017). The study suggests that only 10% of the patients subjected to ALT needed conventional replacement therapy compared to 33% of the patients treated with systemic antimicrobial drugs (O'Horo et al., 2011). Ethanol Lock solution at more than 40% dwelled onto catheter with *C. albicans* biofilm cleansed the catheter within 30 minutes (Öncü, 2014). Interestingly, an *in vitro* study of 40% ethanol lock solution in combination with 60IU heparin showed a significant decrease in ≥90% of *C. albicans* biofilm metabolism (Alonso et al., 2018).

Small Molecular Inhibitors and Natural Products

Microbiological resistance depends on various fungal factors that have been established due to genetic alteration in the fungi. Clinical resistance is due to host- or drug-related factors. All these factors may cause fungal resistance individually or in tandem. In addition to standardized susceptibility testing and appropriate drug dosing, one way to avoid resistance is the use of combinational antifungal therapy. Combination therapy also offers advantages in increased synergistic action with enhanced spectrum activity. Newer insights into mechanisms of drug resistance will help in the development of appropriate antifungal therapy.

For dealing with this problem of resistance, there are two ways: (i) finding of the novel anti-biofilm molecules and (ii) repurposing of the known drug to increase the activity of antifungal agents by combinational therapy. Due to the scarcity of known molecular scaffolds that inhibit/disperse fungal biofilms, high throughput screening (HTS) has been employed in an attempt to discover leads for new anti-biofilm modulators. Intending to identify novel small molecules that possessed anti-biofilm activity, screening of an extensive chemical library of compounds took a new start. It was observed that clinical isolates of *C. albicans* have varied abilities to respond to different growth media to form hyphae and also biofilms, and this variability is not associated with specific host conditions or characteristics. A high-content screen has identified many new structural series of antifungal compounds with the mode of action, namely inhibition of biofilm formation and filamentation. Many compounds among them represent a promising candidate for the development of novel anti-virulence approaches against *C. albicans* infections, aimed at minimizing the development of resistance.

Different Targets of Small Molecule Inhibitors

Small molecular inhibitors have been identified specifically for various targets along with their MIC/IC50 values which are summarized in the tables mentioned below.

Inhibitors of budded to hyphal transition

The pathogenic yeast *C. albicans* can exist in multiple morphological states, including budded, pseudohyphal, and true

hyphal forms. The ability to change between the budded and hyphal forms, termed as the budded-to-hyphal-form transition, is important for virulence and is regulated by multiple environmental and cellular signals (Toenjes et al., 2005).

Filastatin **1,** inhibits yeast to hyphal transition, impedes the adhesion of fungal cells to different biomaterials by inhibiting the hyphal specific *HWP1* promoter. Interestingly, in the *in vivo* studies, filastatin in the presence of fluconazole protected *C. elegans* against *C. albicans* infection. Moreover, it also inhibited biofilm formation in a mouse model of vulvovaginal candidal infection (Fazly et al., 2013). Similarly, Johnson and the group identified several small molecular inhibitors. This includes Buhytrin A **2**, CGP-37157 **3** (affecting the calcium metabolism of bud to hyphal transition), and ETYA **4** (inhibiting multiple signaling pathways) and Clozapine **5** (FDA approved antipsychotic drug inhibiting Efg1 pathway at Gpr1 G-protein-coupled receptor level). These molecules restrict the bud to hyphal growth in *C. albicans* and affect multiple signaling pathways that are intricate in the filamentation process which regulates the biofilm formation (Midkiff et al., 2011; Grald et al., 2012; Pierce et al., 2015b). By screening a library of 50,240 small molecules of inhibitors for yeast-to-hypha transition, Sm21 **6** was identified as a novel small antifungal molecule that showed its efficacy in both *in vivo* oral candidiasis mouse model and *in vitro* system.The study also revealed that compound **6** when given to *C. albicans* strain led to reactive oxygen species (ROS) accumulation and mitochondrial dysfunction (Wong et al., 2014; Truong et al., 2018). In another study, Chrysazin **7** (1, 8-dihydroxyanthraquinone) and Alizarin **8** (1, 2-dihydroxyanthraquinone) an anthraquinone derivative, effectively inhibits biofilm formation in *C. albicans.* Their anti-biofilm activity was mainly due to a hydroxyl group (-OH) at C1 position. Unlike other commercially available drugs, alizarin was found to be a non-toxic compound. It downregulates the expression of various hyphal-specific and biofilm related genes like *ALS3, ECE1, ECE2*, and *RBT1*. Additionally, Chrysazin and Alizarin at 2 μg/ml adequately inhibited yeast to hyphal formation and increased the survivability of *C. albicans* infected *C. elegans*, thus, proving to be a strong candidate for future investigation as an antifungal agent (Manoharan et al., 2017b).

While performing high throughput screening in a chemical library (NOVACore^{TM}), about 20,000 small molecules were identified as a novel series of a Diazaspiro-decane and its structural analogs (9a-d). These molecules inhibit progressions of the virulence-associated with *C. albicans*, especially biofilm formation and filamentation, without affecting the overall growth or prompting the resistance in *C. albicans.* These compounds displayed a potent inhibitory activity when tested against murine models of oral candidiasis (Pierce et al., 2015a). In addition to all these, when 678 small molecules were screened from the chemical library by the group scientist against the invasive hyphal growth of the opportunistic human yeast *C. albicans*, it led to the discovery of two halogenated compounds (N1-(3,5-dichlorophenyl)-5-chloro-2-hydroxybenzamide) 10 and Niclosamide 11 which is the analog of salicylanilide, an FDA-approved anthelmintic in humans, both exhibiting an anti-filamentation and anti-biofilm activities against *C. albicans* (Garcia et al., 2018).

Another compound N-[3-(allyloxy)-phenyl]-4-methoxyben zamide **12** was recognized as the lead one showing inhibition against C. *albicans* filamentation and was found effective in both *in vitro* and *in vivo* study of *C. albicans* infected murine model. The whole transcriptomic analysis revealed a total of 618 genes that were up-regulated and 702 were down-regulated. It was observed that most of the down-regulated genes such as SAP5, ECE1, and ALS3 already well-characterized were associated with filamentation and virulency. In addition, some genes affect metal chelation and utilization (Romo et al., 2019).

Homology studies using *Candida* Genome Database showed that *Candida* accounts for type III 5-α-reductase, Dfg10p, and shares a 27% sequence identity and 41% similarity to the human type III 5-α-reductase, which were identified as a target for benign prostatic hyperplasia. Finasteride **13** was recognized as one of the potent inhibitors against type III 5-α-reductase. Studying its activity showed some promising results at dose $\geq$16 mg/liter. Finasteride alone was highly efficient in preventing filamentation of *C. albicans* and demonstrated synergy with FLC against *in vitro* urinary biofilm (Chavez-Dozal et al., 2014).

LaFleur group, while filtering through 120,000 small compounds from the NIH Molecular Libraries Small Molecule Repository, identified 1,3-benzothiazole **14, 14(a-c)** and its scaffolds as an anti-filamentation drug which acts as an potentiate a known antifungal drug Clotrimazole by increasing its activity to >100 fold against *C. albican* biofilm isolates (LaFleur et al., 2011).

Enzymes

Potent inhibitors of essential microbial enzymes are significant growth inhibitors of *C. albicans,* a pathogenic fungi. The enzyme aspartate semialdehyde dehydrogenase (ASADH) is critical for the functioning of the biosynthetic pathway of aspartate in microbes and plants, an important step for the biosynthesis of other essential amino acids. Because the aspartate pathway is absent in humans, ASADH can be a promising new target for antifungal research. Deleting the ASD gene encoding for ASADH significantly decreases the survival of *C. albicans*, establishing this enzyme as essential for this organism (Dahal and Viola, 2018; Dahal et al., 2020).

The 1,4-Napthoquinone and its derivatives **15, 15 (c-e)** did possess activity against clinical isolates of *C. albicans* derived from gynecological patients by inhibiting phenotypic changes in *C. albicans*. Additionally, some other studies also establish that dichloro-derivatives of 1,4 naphthoquinone i.e., 2-chloro 1,4-Naphthoquinone **15a** and 2,3-dichloro naphthoquinone **15b** are the inhibitors of ASADH (aspartate dehydrogenase) (Janeczko et al., 2018).

Secreted Aspartic Protease-2 enzymes (SAP2) of the *C. albicans* are one of the known classes of the virulent factor for localized and systemic infection. In *C. albicans*, ten distinct SAP genes (SAP1-10) were expressed *in vitro* and *in vivo*. The SAPs are essential for the fungal nutrition process and contribute to the fungal pathogenicity due to their critical participation in several stages of the infective

process, including adhesion, invasion, and tissue damage and so on. Pepstatin and peptidomimetic are the known peptide inhibitors, but it is challenging to clinically synthesize them due to pharmacokinetic characteristics. So from the studies, compound **16,** one of the derivatives of pepstatin, came out to be a potent inhibitor against 3 of the SAP2 present in fungi. Moreover, compound **16** and fluconazole when administered to FLC resistant mouse model infected with *C. albicans* showed synergistic effect by increasing the survival rate of the mouse by 50% (Cadicamo et al., 2013).

Targeting proteinases and phospholipases, which are vital to fungal invasion of host tissues and immunosuppression, Aneja et al. (2016) developed an s triazole series–amino acid hybrids. It was found that compounds **17** and **18** significantly reduce the secretion of proteinases and phospholipases in *Candida* spp. which are vital to fungal invasion of host tissues and immunosuppression. The study showed that on treatment with compound **17**, proteinase secretion was decreased by 29, 22 and 23.5% in standard, FLC-sensitive and resistant strains of *C. albicans*, respectively, while at the same concentration, compound 18 decreased the proteinase secretion by 30, 33 and 17% against the respective strains. At similar concentrations in the same strains, compound **17** caused 40, 38, and 38% inhibition in phopholipase secretion whereas compound **18** decreased the secretions by 36, 27 and 38% respectively.

Efflux pump

One of the structural derivatives of cyclobutene-dione [3-(phenylamino)-4-{[1,3,3-trimethyl-2,3-dihydro-1H-indol-2-ylidene]methyl}-cyclobut-3-ene-1,2-dione] **19** was chemically synthesized and was identified as the puissant inhibitor of the efflux pumps residing in plasma membrane - Major Facilitator Superfamily (MFS) and ATP-binding cassette (ABC) transporters accountable for efflux pump-mediated drug resistance in the fungal pathogen *C. albicans* (Keniya et al., 2015).

Quorum Sensing Molecules

As already mentioned, quorum sensing plays an important role in mediating the formation of biofilm of *C. albicans*. One of the known quorum-sensing molecules released from *Candida* itself, "Farnesol" **20,** is known to inhibit filamentation in *C. albicans,* which is a known virulence factor enhancing biofilm formation. From the previous studies it is known that the upregulation of the sterol biosynthetic pathway gene *ERG* and the efflux pump genes *CDR* and *MDR* are one of the major contributors to the azole tolerance in *C. albicans*. Therefore, through northern blot studies, it was examined that Farnesol down-regulates the expression of partial gene expression in ergosterol biosynthesis, Ras1-cAMP-Efg1 signaling cascade, *HWP1* promoter, which encodes for hyphal –specific cell wall protein (Ramage et al., 2002; Yu et al., 2012; Dižová and Bujdáková, 2017). Further, it was observed that compound **20** is in synergism with already known antifungal drugs augmenting the accumulation of ROS, leading to early apoptosis in fungal cells (Sharma and Prasad, 2011). In a recent study, a bacterial quorum sensing quencher S8- Thiazolidinedione-8 **21** has been identified as an effective small molecular inhibitor which significantly decreases biofilm formation by *C. albicans*.Additionally, it shows, anti-filamentation, as well as anti-adhesion activity at four to eight fold decreased MIC concentrations. Nevertheless, it down-regulates *HWP1, ALS3, EAP1*, *HST7* and *CPH* transcription factors that play a key role in biofilm formation (Feldman et al., 2014). Another newly discovered Thiazolidinedione molecule- N-(oxazolylmethyl)-thiazolidinedione **22** scaffolds has been identified as a novel compound that inhibits *C. albicans* Als surface protein (Marc et al., 2018; **Table 1**).

High Throughput Screening of the Libraries

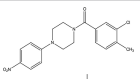
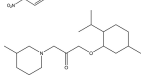
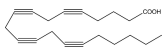
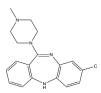
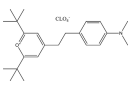
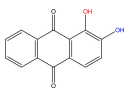
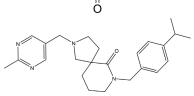
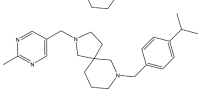
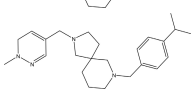
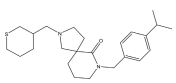
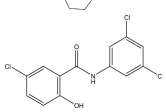
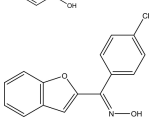
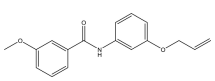
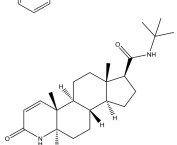
Around 400 heterogeneous drugs like molecules assembled by Medicines for Malaria Venture [MMV], Switzerland was screened with the motto to hasten the identification of medicinal molecules *C. albicans* biofilm. Three confirmed hits were obtained- MMV688768, MMV687807, and MMV687273, which exhibited activity against pre- biofilms. However, by additional exploration, it was observed that compound MMV688768- 2-methyl-3-[(4-methylpiperazin-1-yl)-thiophen-2-ylmethyl]-1H-indole **23** an anti-*Schistosoma* drugs are the best showing anti-biofilm activity against *C. albicans*, with concentrations as low as 3.12 μM (Vila and Lopez-Ribot, 2017).

Ebselen **24** an organoselenium compound, was screened out from the off-patent drugs using the Prestwick Chemical Library, a repurposing library of about 1,280 small molecules. Out of nine molecules, Ebselen illustrated 100% inhibition against multi-drug resistant species, *Candida auris* at concentration $\leq$ 2.5 μM (Wall et al., 2018). Furthermore, it elucidates its antifungal activity by accumulating reactive oxygen species (ROS) inside the fungal cells. Further, *in vivo* study of *C. elegan* model infected with *C. albicans* showed complete eradication of fungal load when treated with 8 μg/ml of ebselen (Thangamani et al., 2017). Additional screening of New Prestwick Chemical Library comprised of FDA-approved 1,200 off-patent drugs. Alexidine dihydrochloride **25** (AXD) and Thimerosal **26** showed more than 80% of inhibition mature biofilms. Subsequently, AXD showed antibiofilm potency *C. albicans* mouse model disseminated with fungal infection (Mamouei et al., 2018; **Table 2**).

Phenotypic Screening

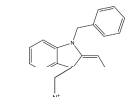
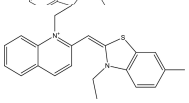
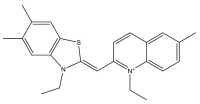
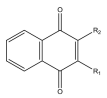
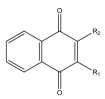
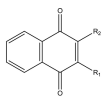
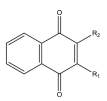
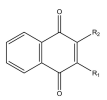
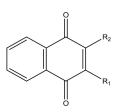
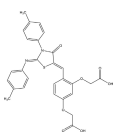
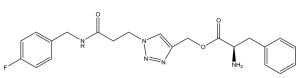
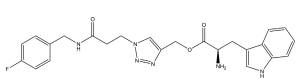
Phenotypic screening refers to the nascent methodology for the biological screening of the chemical entities to assess their therapeutic effects. It is not a target- based strategy but involves detailed cell-based studies (Aulner et al., 2019). The phenotypic screening was done to identify different small molecules which depict antibiofilm and antifilamentation activity against *C. albicans.* This study leads to the identification of about 2293 compounds from the chemical library of the National Cancer Institute which were categorized into three sets- (i) NCI Natural set, Out of all the compounds present in this set six hits were confirmed against *C. albicans* biofilm formation. These include -Trichoderonin **27**; Nanaomycin **28**; Rapamycin **29**; Anisomycin **30**; Valinomycin **31** and Bacitracin **32**. Three of these molecules (Trichoderonin, Nanaomycin, Rapamycin) showed inhibition of both filamentation and biofilm formation while the rest three showed inhibition against biofilm formation.

TABLE 1 | Chemical structures and MIC/IC_{50} values of small molecule inhibitors (1–22).

S.No.	Compound No.	Structure	IC_{50} μ M	MIC μ g/ml	References
1	1		3	-	Fazly et al., 2013
2	2			31.1	Midkiff et al., 2011
3	3		-	32.4	Pierce et al., 2015b
4	4		-	29.64	Midkiff et al., 2011; Pierce et al., 2015b
5	5		50	-	Midkiff et al., 2011; Pierce et al., 2015b
6	6		-	0.2 – 1.6	Truong et al., 2018
7	7		-	10	Manoharan et al., 2017b
8	8		-	10	Manoharan et al., 2017b
9	9a		2.70		Pierce et al., 2015a
10	9b		3.36		Pierce et al., 2015a
11	9c	 R_1=Cl, R_2=Cl	4.59		Pierce et al., 2015a
12	9d		3.15		Pierce et al., 2015a
13	10		100	-	Garcia et al., 2018
14	11		100	-	Garcia et al., 2018
15	12		1.88	-	Romo et al., 2019
16	13			≥16 × 10^3	Chavez-Dozal et al., 2014
17	14				LaFleur et al., 2011

(Continued)

TABLE 1 | Continued

S.No.	Compound No.	Structure	IC_{50} μ M	MIC μ g/ml	References
18	14a		1.7-119.4	-	LaFleur et al., 2011
19	14 b		2.8-82.1	-	LaFleur et al., 2011
20	14 c		230.9-268.2	-	LaFleur et al., 2011
21	15	 R_1=H, R_2=H	-	8-250	Janeczko et al., 2018
22	15a	 R_1=Cl, R_2=H	16.4 ± 2.0	5	Janeczko et al., 2018
23	15b	 R_1=Cl, R_2=Cl	9.8 ± 1.0	3	Janeczko et al., 2018
24	15c	 R_1=H, R_2=2',3',5-triOCH_3-Ph	-	31.2	Janeczko et al., 2018
25	15d	 R_1=H, R_2=2'4-diOCH_3-Ph	-	31.2	Janeczko et al., 2018
26	15e	 R_1=2'-OCH_3N-Ac-Ph; R_2=H	-	8	Janeczko et al., 2018
27	16		0.86	-	Cadicamo et al., 2013
28	17		70.9		Aneja et al., 2016
29	18		22.4		Aneja et al., 2016

(Continued)

TABLE 1 | Continued

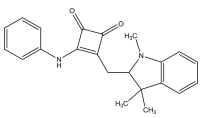
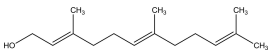
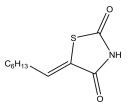
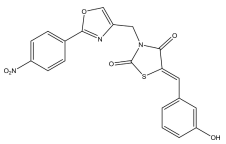
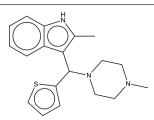
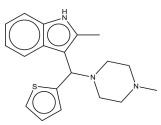
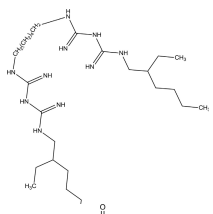
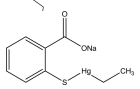
S.No.	Compound No.	Structure	IC_{50} μ M	MIC μ g/ml	References
30	19		-	0.057 with 0.157 FLC	Keniya et al., 2015
31	20		-	0.2-11.185	Ramage et al., 2002
32	21		-	64	Feldman et al., 2014
33	22		-	625	Marc et al., 2018

TABLE 2 | Chemical structures and MIC/IC_{50} values of small molecule inhibitors (23–26).

S.No.	Compound No.	Structure	IC_{50} μ M	MIC μ g/ml	References
34	23		3.12	-	Vila and Lopez-Ribot, 2017
35	24		–	≤0.725	Wall et al., 2018
36	25		5.4	-	Mamouei et al., 2018
37	26		0.2	-	Mamouei et al., 2018

Furthermore in the next (ii)-NCI-Structural diversity set, in total, there were 12 hits out of which eight were identified as biofilm inhibitor. These compounds were-Phenanthroline Hydrochloride **33**; 2-isoquinolin-2-ium-2-yl-1-phenanthren-3-ylethanone,iodide **34**;Metanilamide (3-aminobenzenesulfonamide) **35**; Mercury, (4-amino phenyl)(6-thioguano sinato-N7,S6)- **36**; 2-[7-[3-(carboxymethyl)-5,10-dihydroxy-1-methyl-6,9-dioxo-3,4-dihydro-1H-benzo[g] isochromen-7-yl]-5,10-dihydroxy-1-methyl-6,9-dioxo-3,4-dihydro-1H-benzo [g]isochromen-3-yl]acetic acid **37** are only biofilm inhibitors while Mercury,(2-aminio-1,9-dihydro-6H-purine-6-thionato-N7,S6) hexyl-,2-benzo[a]phenothiazin-12-yl-N,N-diethylethanamine **38**; 17-[1-[2(dimethylamino)ethylamino]ethyl]-13-methyl-6,7,8,9,11,12,14,15,16,17 decahydrocyclopenta[a]phenanthren-3-ol **39**. Three of these compounds inhibited both filamentation and biofilm formation. From all these compounds related to 17- aminoestradiols, a Mercury containing organometallic was the most potent compound with IC_{50} value in the range of nanomolar concentration.

Next and last was (iii)-NCI-Challenge Set. In this, there were total 11 hits, of which 10 showed inhibition against *C. albicans* biofilm formation whereas only one compound inhibited filamentation transformation. Ten hits which were identified from these compounds displayed common inhibition against both biofilm and filament formation. These include Biofilm Inhibitor- Trichopolyn-B**40**, Vengicide (Unamycin B, Toyocamycin)**41**, 4Z-4-[[4-(dimethylamino)phenyl]methylidene]-1-methyl-2-phenylpyrazolo[1,5-a]indol-1-ium-6-ol;trifluoromethanesulfonate**42**, Anisomycin**43**, Azetidinecarbo thioic acid, [1-(2-pyridinyl) ethylidene] hydrazide**4**. Additionally,

compounds with both antifilamentation and antibiofilm activity are.- 6-Hydroxy-3-((methanesulfonyloxy) Methyl)-1-((5,6,7-tri methoxyindol-2-yl) carbonyl)indoline**45**, Hydrazinecarbothioamide,N,N-dipropyl-2-(2-pyridinemethylene)-,(N,N,S) copper(II)chloridecomplex(SP-4-3)3;3-Azabicyclo[3.22]nonane-3-carboselenoicacid,[1-(2pyridinyl)ethyidene] hydrazide**46**, 2-hydroxyethyl-[(2R)-2-hydroxyheptadecyl]-dimethylazanium iodide **47**, 1H-Azepine-1-carbothioic acid, hexa hydro-, [1-(2-pyridinyl) ethylidene]hydrazide**48** (Pierce et al., 2014; **Table 3**).

Repurposing FDA Approved Drug Showing Synergistic Effect

Haloperidol **49** and its derivative (Benzocyclane derivative B10 **49a)** (Ji et al., 2020) and Bromoperidol **50** and its derivative are some of the known antipsychotic drugs that were repurposed and were utilized as anti-biofilm molecules. Haloperidol's derivative exhibited inhibition of yeast to hyphal transition in resistant strain of *C. albicans* in synergism with FLC while it also down-regulated the expression of ERG11, and MDR1 genes are leading to cell membrane damage. Moreover, compound **49a** showed significant decrease in the CFU/g from 7.63 to 7.29 in murine model of invasive candidiasis. Similarly, Bromoperidol and its derivative showed a synergistic effect in combination with different azoles. The mechanism of action is unknown but it does decrease the amount of azole administered at the time of treatment (Holbrook et al., 2017).

While looking for different antibiofilm drug groups, scientists looked for the Hsp 90 and Calcineruin inhibitors as it chemo sensitizes the fungal strains toward azole- as the first line of antifungal drugs. Piperazinyl quinolone **51** screened from MLPCN library had no antifungal activity alone, but, in combination with Azoles, it selectively inversed the fluconazole resistance in the clinical isolates of *C. albicans* (Youngsaye et al., 2011). Similarly, FK506 **52** and Cyclosporine A **53**, the other potential calcineruin inhibitors also illustrated the increase in the susceptibility of *C. albicans* toward antifungal drugs (Uppuluri et al., 2008; Cordeiro et al., 2014; Jia et al., 2016). Ribavirin **54,** already known antiviral drug, was screened through Prestwick Chemical Library against FLC-resistant strains. It illustrated a fungistatic effect against MDR species of *C. albicans* and acted synergistically with MIC < 24.4 μg/mL (Yousfi et al., 2019).

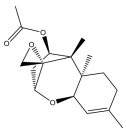
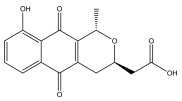
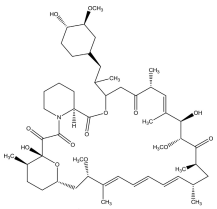
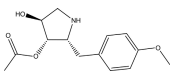
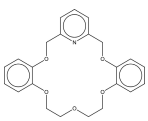
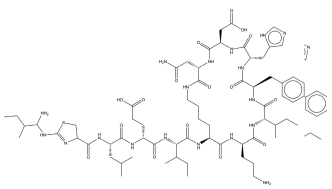
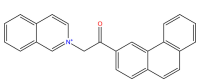
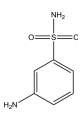
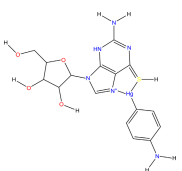
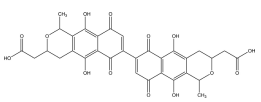
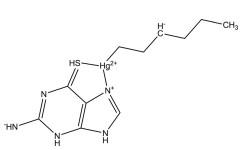
While going through 1,600 compounds present in the drug-repositioning library, 8 hits were obtained. These are Hexachlorophene **55** (anti- topical anti-infective drug) (Siles et al., 2013), Pyrvinium pamoate **56** (an anti-helminthic drug), Artesunate **57** (antimalarial drug), Broxyquinoline **58** (antiseptic drug), Dihydroartemisinin **59** (antimalarial drug), Gentian violet **60** (antibacterial and antihelminthic drug), Bithionate disodium **61** (antiseptic), and Nitroxoline (Antibacterial). Out of these 8 hits, the first seven hits showed inhibition of mature *C. albicans* biofilm in combination with miconazole at sub-inhibitory concentrations. Further, studies were performed on the best-acknowledged potentiators, Hexachlorophene, pyrvinium pamoate, and artesunate. Mechanistic synergy was most pronounced for artesunate, an artemisinin homolog, which prompted the study of different structural homologs of Artemisinia. Thus, it was observed that biofilm inhibition in combination with Micanazole was not specific only to artesunate but also applicable to dihydroartemisinin and other artemisinin derivatives, indicating artemisinin as a potential antifungal molecule that can be further investigated to establish its overall potentials in the human health care system (De Cremer et al., 2015).

In another study, the library comprehending 1600 off-patent drugs was sifted for the compound that enhances *in vitro* activity of amphotericin B when used in combination against *C. albicans* biofilms. Out of 1600 compounds, the team found 50 hits, and from these 50 hits, only seven of them illustrated $BIC_{50} < 100$ μM for biofilm inhibition. Those compounds are as follow: Prochlorperazine edisylate 5.2 μM **(62)**, Danthron 12 μM **(63)**, Chlorprothixene hydrochloride 17 μM **(64),** Toremifene citrate 19.5 μM **(65)**, Clorgiline hydrochloride 24 μM **(66)**; Perhexiline maleate 39 μM **(67)** Dicyclomine hydrochloride 60 μM**(68)**. Subsequently, all of these compounds showed a synergistic effect with caspofungin. Additionally, while studying tormifene citrate **(65)** for its synergistic effect on *C. elegans* infection model, it showed strong antifungal potency (Delattin et al., 2014). Diclofenac **69** a non -steroidal anti-inflammatory drug, increased the susceptibility of caspofungin toward the *in vitro C. albicans* biofilm. Furthermore, catheter retrieved from the animal model-rat when treated with combination therapy of caspofungin and diclofenac showed a significant reduction in the number of biofilm cells (Bink et al., 2012). Further, an off-patent antipsychotic drug Aripiprazole **70** was identified as a potent inhibitor of *C. albicans* which inhibited the early formation of pseudohyphal cells and mimicked standard azoles at higher concentrations showing different mechanisms of action (Rajasekharan et al., 2019; **Table 4**).

Natural Products

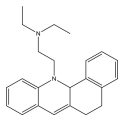
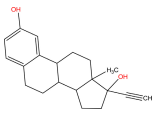
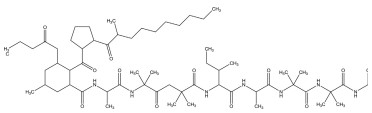
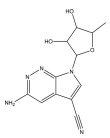
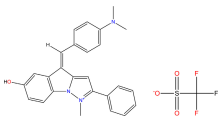
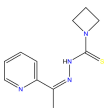
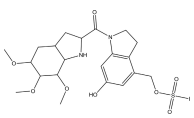
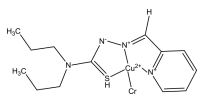
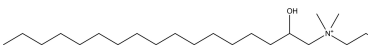
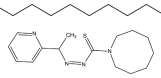
Arising fungal resistance ascribed to mutational changes has diversified the complexity fueling the thought processes along this line. To overcome this issue, we need to find some substantial solutions. For the past few decades, natural products have emerged as an essential source of antibacterial, antimalarial, and chemotherapeutic agents. Presently, around 60% of used drugs for cancer treatment are procured from natural products. Furthermore, the modification of natural products is one of the most common and productive methodologies to obtain novel therapeutic agents using medicinal chemistry (Zaki et al., 2019). Thus, targeting fungal biofilms by either natural derivatives or synthetic analogs could be a novel approach. Natural product screening has proved to be one of the promising strategies. Although anti-biofilm agents themselves might not kill the bacteria, they can make them more susceptible to conventional antibiotics and the action of the host immune system. The search for biofilm inhibitors has led to identifying a significant number of compounds of potential therapeutic use as biofilm inhibitors. A literature survey reveals that some of the most active anti-biofilm compounds discovered to date have been based upon the molecular scaffolds of natural products isolated from marine natural products and plant products (Fazly et al., 2013).

TABLE 3 | Chemical structures and MIC/IC_{50} values of small molecule inhibitors (27–48).

S.No.	Compound No.	Structures	IC_{50} μ M	MIC μ g/ml	References
38	27		0.529	-	Pierce et al., 2014
39	28		0.609	-	Pierce et al., 2014
40	29		0.514	-	Pierce et al., 2014
41	30		1.508	-	Pierce et al., 2014
42	31		0.363	-	Pierce et al., 2014
43	32		2.133	-	Pierce et al., 2014
44	33		3.354	-	Pierce et al., 2014
45	34		3.401	-	Pierce et al., 2014
46	35		3.448	-	Pierce et al., 2014
47	36		1.938	-	Pierce et al., 2014
48	37		3.309	-	Pierce et al., 2014
49	38		0.012	-	Pierce et al., 2014

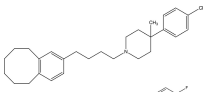
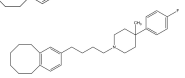
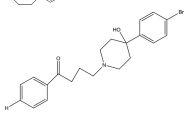
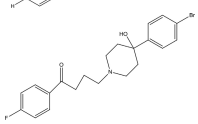
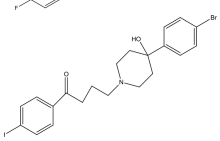
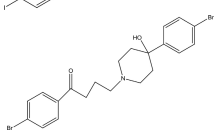
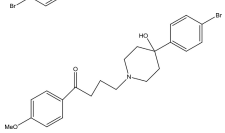
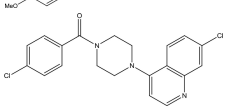
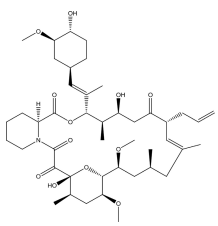
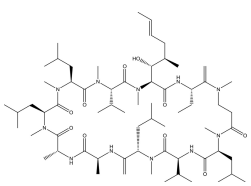
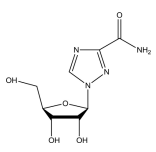
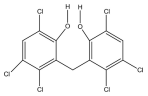
(Continued)

TABLE 3 | Continued

S.No.	Compound No.	Structures	IC_{50} μ M	MIC μ g/ml	References
50	39		3.804	-	Pierce et al., 2014
51	40		0.0097	-	Pierce et al., 2014
52	41		0.666	-	Aulner et al., 2019
53	42		1.037	-	Pierce et al., 2014
54	43		2.236	-	Pierce et al., 2014
55	44		2.298	-	Pierce et al., 2014
56	45		0.495	-	Pierce et al., 2014
57	46		0.549	-	Pierce et al., 2014
58	47		0.4273	-	Pierce et al., 2014
59	48		1.235	-	Pierce et al., 2014

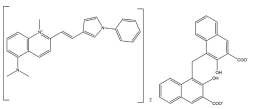
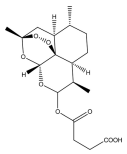
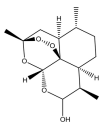
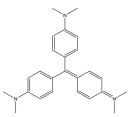
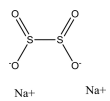
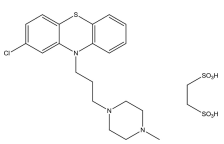
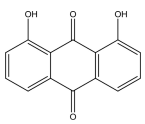
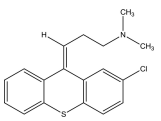
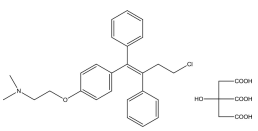
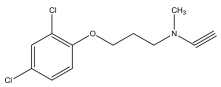
As compiled by the World Health Organization, more than 21,000 plant species containing a vast array of biologically active compounds have been used worldwide in herbal medicines (Midkiff et al., 2011). Naturally occurring alcohols magnolol **71** and its isomer honokiol **72**, carvacrol **73**, thymol **74,** and geraniol **75** have been identified as a potent inhibitor of both bacterial and *C. albicans* biofilms (de Castro et al., 2015; Magi et al., 2015; Sun et al., 2015; Sharma et al., 2016; Behbehani et al., 2017). Furthermore, when *C. elegan* was treated with 16 μg/ml of magnolol and honokiol, there was a decrease in the number of colony forming units (CFU) of *C. albicans* in the nematode which enhances its survival. Additionally, antibiofilm activity of these compounds **69** and **70** were related to Ras1-cAMP-Efg1 pathway of *C. albicans* (Sun et al., 2015). A Recent study shows that compound **70** and **71** are antifungal and induce ROS mediated programmed cell death in *C. albicans* (Sun et al., 2017; Niu et al., 2020). Saponins are one of the known natural products that bind to the ergosterol rather than cholesterol of *C. albicans*. This increases the susceptibility to photodynamic inactivation due to an increase in the permeability of photosensitizers by sapnonin. In one study, a total of 12 different saponins were identified as potential antifungal agents, but out of those 12, only two were selected. One was **76,** which belongs to the aginoside family of saponins and the secone one **77** to the barrgeniol family of the saponins. Both of these compounds were potent antifungal agents with MIC 16 and 32 μg/ml, respectively. Furthermore, to study toxicity and efficacy of the compound, *C. elegans* and *C. albicans* models were used. In an *in vivo* study, compound **76** and **77** showed no sign of toxicity and conferred high percentage

TABLE 4 | Chemical structures and MIC/IC_{50} values of small molecule inhibitors (49–70).

S.No.	Compound No.	Structure	IC_{50} μM/(μg/ml)[b]	MIC μ g/ml	References
60	49		64[b]	-	Ji et al., 2020
61	49a		2[b]	-	Ji et al., 2020
62	50a		>64[b]	-	Holbrook et al., 2017
63	50b		>128[b]	-	Holbrook et al., 2017
64	50c		>32[b]	-	Holbrook et al., 2017
65	50d		>64[b]	-	Holbrook et al., 2017
66	50e		>128[b]	-	Holbrook et al., 2017
67	51		2.4 with 8 μg/ml FLC (alone ineffective)	-	Youngsaye et al., 2011
68	52		20[b]		Uppuluri et al., 2008
69	53		-	10×10^3	Cordeiro et al., 2014; Jia et al., 2016
70	54		-	0.37-3.02	Yousfi et al., 2019
71	55		-	9.3	De Cremer et al., 2015

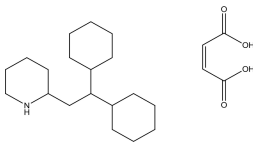
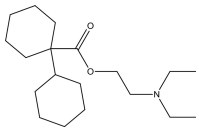
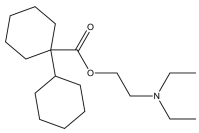
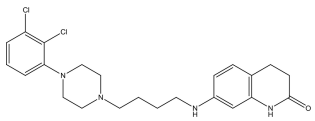
(Continued)

TABLE 4 | Continued

S.No.	Compound No.	Structure	IC_{50} μM/(μg/ml)[b]	MIC μ g/ml	References
72	56		-	3.9	De Cremer et al., 2015
73	57		-	>200	De Cremer et al., 2015
74	58		-	1.2	De Cremer et al., 2015
75	59		-	>200	De Cremer et al., 2015
76	60		-	1.5	De Cremer et al., 2015
77	61		-	6.0	De Cremer et al., 2015
78	62		5.2	-	Delattin et al., 2014
79	63		12	-	Delattin et al., 2014
80	64		17	-	Delattin et al., 2014
81	65		19.5	-	Delattin et al., 2014
82	66		24	-	

(Continued)

TABLE 4 | Continued

S.No.	Compound No.	Structure	IC_{50} μM/(μg/ml)[b]	MIC μ g/ml	References
83	67		39	-	Delattin et al., 2014
84	68		60	-	Delattin et al., 2014
85	69		6000	-	Bink et al., 2012
86	70		-	≤100	Rajasekharan et al., 2019

[b]*means that the unit of IC_{50} value is μg/ml.*

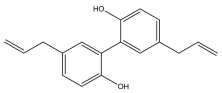
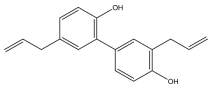
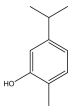
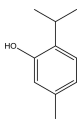
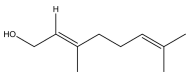
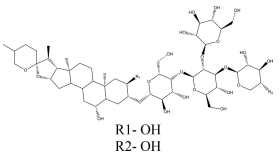
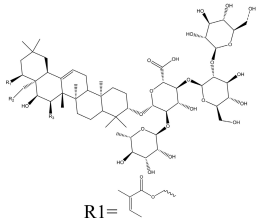
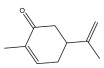
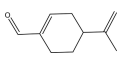
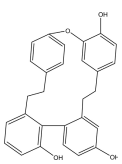
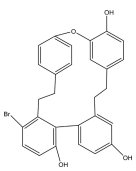
of nematode survival from upto 73% to 80%, respectively (Coleman et al., 2010).

Occasionally, other known naturally occurring compounds are found to be useful. The Carvone **78** and perillaledhyde **79** (McGeady et al., 2002; Tian et al., 2017; Moro et al., 2018) at very low concentration inhibit the filamentous formation of *C. albicans*. Furthermore, it was observed that at MIC of 4 μl/mL compound **77** increased the level of ROS, which activated the programmed cell death in *C. albicans*. Additionally, it equalizes the level of E-cadherins the epithelial barriers preventing which decreases in number due to invasion of pathogenic fungi (Tian et al., 2017; Qu et al., 2019). The Riccardin D**80, 80a** - macrocyclic bisbibenzyl isolated from *Dumortiera hirsute* chinese liverwort, exhibited an inhibitory effect on the biofilm formation of *C. albicans* by downregulating HWP1, ASL3, and EFG1 genes thus, inhibiting hyphal formation. Additionally, one of the semi-synthetic derivative of Riccardin D with a bromine atom attached to the arene ring showed better antifungal activity with MIC-2 μg/ml in comparison to Riccardin with MIC-16 μg/ml (Li et al., 2012; Sun et al., 2016). Another known compound is Emodin **81** (1, 3, 8−trihydroxy−6−methyl−anthraquinone) which is a natural secondary plant product, originally isolated from the rhizomes of *Rheum palmatum*. This compound suppressed the growth of the cells of reference and clinical *C. albicans* strains with minimal inhibitory and minimal fungicidal concentration values between 12.5 and 200 μg/mL, respectively, showing anti-virulent potential. Research showed that emodin added to *C. albicans* culture inhibited the phosphorylation of many cellular proteins, presumably, owing to the inhibition of protein kinase CK2. Notably, the enzyme isolated from the *C. albicans* cells was found to be susceptible to emodin with IC50 of 2.8 μg/mL, revealing that emodin was able to occupy the ATP−binding pocket of CK2 (Janeczko et al., 2017). In another finding, *M. sylvestris* root was also shown to inhibit *C. albicans* biofilm formation (Alizadeh et al., 2017). Studies on quorum sensing inhibition and the quest for QS inhibitors have shown plants to produce anti-QS substances. Several anti-QS methods have been used, including natural products from plant-based secondary metabolites (Berde et al., 2019). Carbohydrate derived fulvic acid (CHD-FA) **82** from pure form of fulvic acid is obtained from humic substances, also exhibits anti-biofilm activity against *C. albicans* (Sherry et al., 2012; Borghi et al., 2015). Plant alkaloid berberine **83** showed 91% biofilm inhibition against *C. albicans* in combination with Miconazole. Furthermore, it reduces the metabolic activity of early- stage biofilm formation in *C. albicans*. Individually, Berberine inactivates fungal biofilm at MIC range 0.98-31.25mg/mL (Wei et al., 2011). Resveratrol (3, 4, 5 -trihydroxystilbene) **84** is a phytoalexin, a known antimicrobial agent present in a wide range of dietary sources, including peanuts, plums, grapes and in red wines. A semisynthetic compound -EB487 **84a** was synthesized from resveratrol, which showed an antibiofilm and anti-preformed biofilm activity against *C. albicans* strain. Subsequently, the study revealed that resveratrol regulates its antifungal activity from early to late apoptosis stage. Furthermore, it increases the ROS concentration in fungal cells of *C. albicans* and modulates the activation of metacaspase release in response to mitochondrial dysfunction (Lee and Lee, 2015; Juin et al., 2019; **Table 5**).

Natural Extracts and Essential Oils

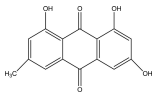
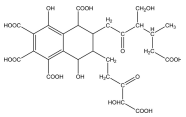
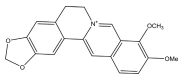
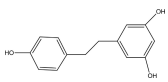
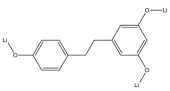
Ethanol extract of *Tovomita krukovii* led to the identification of few new xanthones like 1,3,5-trihydroxy-8-isoprenylxanthone **85a**, 1,5,7-trihydroxy-8-isoprenylxanthone **85b**, betulinic acid **86,** and 3,4-dihydroxybenzoic acid **87** targeting secreted aspartic protease SAP2 of *C. albicans* with IC_{50} values 15 μg/ml,

TABLE 5 | Chemical structures and MIC/IC_{50} values of small molecule inhibitors (71–84).

S.No.	Compound No.	Structure	IC_{50} μ M	MIC μ g/ml	References
87	71		-	16-32	Sun et al., 2015
88	72		-	16-32	Sun et al., 2015
89	73		-	16-256	Magi et al., 2015
90	74		-	4.88	de Castro et al., 2015
91	75		-	30-130	Sharma et al., 2016
92	76	 R1- OH R2- OH	-	16	Coleman et al., 2010
93	77	 R1=	-	32	Coleman et al., 2010
94	78	R2=OH R3=H 	-	312	McGeady et al., 2002; Moro et al., 2018
95	79		-	0.4	McGeady et al., 2002; Tian et al., 2017
96	80		-	16	Li et al., 2012; Sun et al., 2016
97	80a			2	Li et al., 2012; Sun et al., 2016

(Continued)

TABLE 5 | Continued

S.No.	Compound No.	Structure	IC_{50} μ M	MIC μ g/ml	References
98	81		2.8	12.5-200	Janeczko et al., 2017
99	82		-	125	Sherry et al., 2012; Borghi et al., 2015
100	83		-	98-31250	Wei et al., 2011
101	84		400	-	Juin et al., 2019
102	84a		130	-	Juin et al., 2019

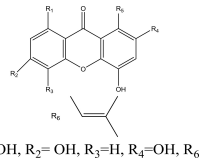
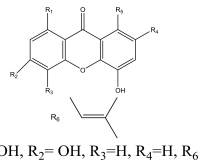
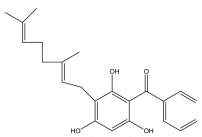
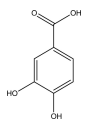
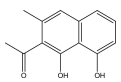
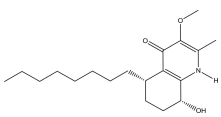
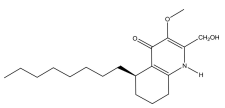
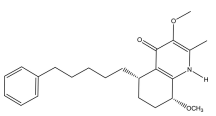
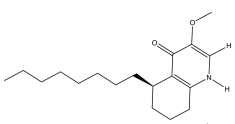
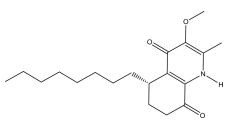
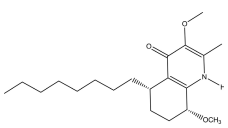
25 μg/ml, 40 μg/ml, and 6.5 μg/ml, respectively (Zhang et al., 2002). Crude hydro alcoholic extract prepared through peel extract of *Punica grantum* presented strong inhibitory activity against *C. albicans* at MIC 3.9 μg/mL and led to the two-fold decrease in the concentration of FLC when used in combination (Endo et al., 2010). In certain studies, essential oils from plants and their compounds have been scrutinized as antifungal agents. The essential oil of *Coriandrum sativum* delineates fungicidal activity in a combination of amphotericin B, mainly targeting germ tube formation in *C. albicans* (Silva et al., 2011). According to the studies, *Rumex* root extract and Nepodin **88,** a compound extracted from *Rumex* roots,were defined as anti-biofilm agents against *C. albicans.* It also increased the survivability in nematode infection model. Further, Nepodin also showed repressed expression of several genes related to bud to hyphal transition Like *ECE1*, *UME6, HWP1*, and *HGT10,* while the increase in the expression of quite a few transport genes (*CDR4*, *TPO2*, and *CDR11*) essential for phenotypic expression (Lee et al., 2019). Additionally while screening compounds from the crude extract of aerial parts of *Waltheria indica,* 10 quinoline alkaloids were identified as a potential molecule which inhibit biofilm formation in *C. albicans*: These include Waltherione N **89**, (R)-Vanessine **90**, Waltherione Q **91**, 8-deoxoantidesmone **92**, Antidesmone **93**, Waltherione E **94**, Waltherione G **95**, Waltherione I **96**, Waltherione J **97**, Waltherione F **98** (Cretton et al., 2016; **Table 6**).

The Unexplored Brazilian Organic Propolis –BOP6 crude extract was characterized as an anti-inflammatory and anti-biofilm agent. It reduces the mortality rate by 30% in *C. albicans* infected sample with MIC ranging from 50 to 100 μg/mL while in combination with Amphotericin B, it decreases to 0.25 to 1.0 μg/mL (Nani et al., 2019). Through library screening with the information regarding fungal extract, it was revealed that *Bionectria ochroleuca,* an endophytic fungus. when cultivated on cereals, lead to the production of certain secondary metabolites-Polyketide glycosides **99**. These Polyketide glycosides exhibited anti-biofilm activity against *C. albicans* and correspondingly showed a synergistic effect with Amphotericin B (Wang et al., 2014).

From HPLC, a different set of compounds were extracted from the crude dichloromethane extract from the roots of *Swartzia simplex.* In total 14 compounds were identified out of which only six compounds such as Simplexene A **100**, (5S,10S)-11,15(R)-Dihydroxy,12 methoxyswartziarboreol G **101**, Simplexene B **102**, Simplexene D **103**, 11,12-Dihydroxy-15, 16-dihydroswartziarboreol C**104**,11,12-Dihydroxy-8,11,13,15-cassatetraen-17,16-olide(11,12 Dihydroxyswartziarboreol C) **105** depicted strong biofilm inhibition against *C. albicans* (Favre-Godal et al., 2015; **Table 7**).

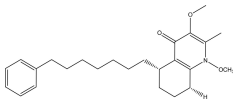
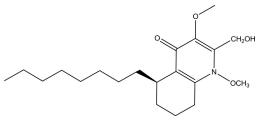
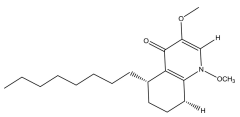
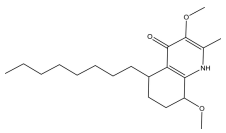
The crude extract of eucalyptus oil and its component 1, 8- cineole **106** showed antimicrobial activity against different microbes including *C. albicans* biofilm. It showed inhibitory effect alone and also in combination with chlorhexidine (Hendry et al., 2009; Simsek and Duman, 2017). While studying different components of essential oils as a treatment against invasive candidiasis, some terpenic derivatives showed promising results. These include carvacrol **73**, geraniol **75**, carvone **78**, terpinen-4-ol**107**, linalool **108**, menthol **109**, α-terpineol **110**, nerol **111**, isopulegol **112**, menthone **113,** and α-thujone **114.** On further study, tyrosol **115** –(a phenethyl alcohol), tyrosine derivative and eugenol- phenylpropanoid **116** both showed a strong inhibitory effect against *C. albicans* biofilm (Marcos-Arias et al., 2011; Raut et al., 2013). Furthermore, tyrosol **115** showed antibiofilm activity when applied exogenously at millimolar concentration. However, at micromolar concentration this compound stimulates hyphal production during the intial stage of *C. albicans* biofilm formation. This inhibition of biofilm formation by the exogenous addition of quorum sensing molecule usually occurs due to disruption in the qurom sensing mechanisms or due to the

TABLE 6 | Chemical structures and MIC/IC_{50} values of small molecule inhibitors (85–98).

S.No	Compound No.	Structure	IC_{50} μ M	MIC μ g/ml	References
103	85a	 R_1=OH, R_2= OH, R_3=H, R_4=OH, R_6	15	-	Zhang et al., 2002
104	85b	 R_1=OH, R_2= OH, R_3=H, R_4=H, R_6	25	-	Zhang et al., 2002
105	86		40	-	Zhang et al., 2002
106	87		6.5	-	Zhang et al., 2002
107	88		-	2-5	Lee et al., 2019
108	89		-	32	Cretton et al., 2016
109	90		-	32	Cretton et al., 2016
110	91		-	32	Cretton et al., 2016
111	92		-	16	Cretton et al., 2016
112	93		-	32	Cretton et al., 2016
113	94		-	4	Cretton et al., 2016

(Continued)

TABLE 6 | Continued

S.No	Compound No.	Structure	IC_{50} μ M	MIC μ g/ml	References
114	95		-	32	Cretton et al., 2016
115	96		-	32	Cretton et al., 2016
116	97		-	16	Cretton et al., 2016
117	98		-	8	Cretton et al., 2016

limitation of yeast adhesion on medical devices (Monteiro et al., 2015; Sebaa et al., 2019).

Epigallocatechin-3-gallate **117**-a Polyphenol compound from green tea extract and its analogs depicted a 75% reduction in the mature *C. albicans* biofilm (Evensen and Braun, 2009).

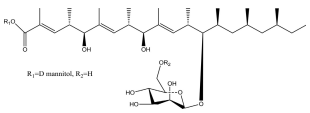
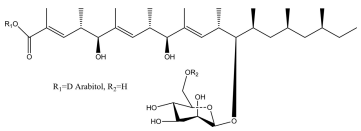
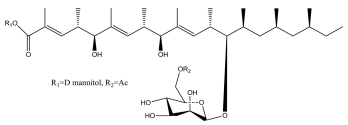
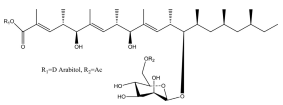
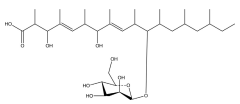
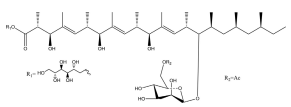
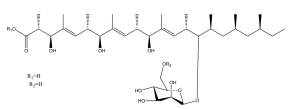
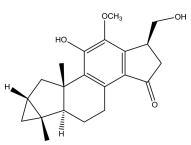
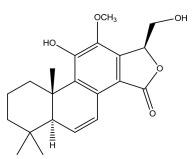
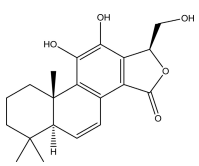
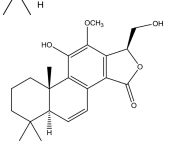
The α-Longipinene **118,** a significant component of *Helichrysum* oil and *Cascarilla* bark oil, inhibited the *C. albicans* biofilm formation without affecting planktonic cell growth and also exhibited a decrease in the virulence in *C. elegans* model (Manoharan et al., 2017a). Further, the compounds Glabridin **119**, Licochalcone A **120**, and Glycyrrhizic acid **121** derived from Licorice- a natural compound extracted from the root of the plant *Glycyrrhiza glabra* showed biofilm inhibition against *C. albicans.* It was found that Glabridin, Licochalcone A showed 35-60% of biofilm inhibition while they inhabited more than 80% hyphal growth. Furthermore, they displayed a synergistic effect with Nystatin at sub-inhibitory concentration (Messier and Grenier, 2011). Another successful study on the essential oils obtained from Thyme species – *T. camphoratus* and *T. carnosus,* earlier used in Portuguese as an antimicrobial therapy, lead to the discovery of different molecules.

In *Thymus carnosus* oil, high aggregates of Camphene and Borneol were present while *Thymus camphoratus* oil *was rich in* 1,8-cineole and α-pinene. Both the oils were highly effective against *C. albicans* biofilm and germ tube formation with very little toxicity (Alves et al., 2019). Another category of essential oils *Syzygium aromaticum* and *Cymbopogon citratus,* largely used in Asia, especially in India, for the treatment of inflammation and skin infection, displayed a potent anti-biofilm activity against *C. albicans* strain (Sajjad et al., 2012). Furthermore, Safranal **122** and its thiosemicarbazone **122a** derivative, the bioactive compounds obtained from *Crocus sativa* when added together in *C. albicans* culture, acted as a potent inhibitor of biofilm and germ tube formation at 32 times less concentration than MIC (Carradori et al., 2016; **Table 8**).

Work on small molecular inhibitors of plant origin, several molecules showed high to moderate activity toward *C. albicans* biofilm. These compounds like Cinnamaldehyde**123**, Piperidine **124**, Citral **125**, Furfuradehyde **126**, and Indole **127** showed inhibitory activity toward ergosterol biosynthesis at varying concentrations (Rajput and Karuppayil, 2013). A recent study revealed that compound **123** and **124** regulated antifungal activity against *C. albicans* by ROS mediated apoptosis (Chen et al., 2019; Thakre et al., 2020). Similarly, another set of *C. albicans* biofilm inhibitor Shearinines D and E **(128,129)** derived from *Penicillum species* showed yeast to hyphal blockage. However, it was observed that these compounds are more potent against early biofilm formation than the mature biofilm (You et al., 2013). Gymnemic acid **130,** a small molecular inhibitor obtained from the leaves of the plant *Gymnema sylvestre,* showed its activity against dual-species *S. gordonii* and *C. albicans* together, leading to biofilm formation in the mouth. It decreases the amount of e-DNA present in biofilm. It also inhibits the adhesive property of *C. albicans* by inhibiting an enzyme glyceraldehyde-3-phosphate dehydrogenase, which is thought to be responsible for the display of adhesion property in *C. albicans* (Veerapandian and Vediyappan, 2019). Similarly, Waikiloid A **131**- a prenylated indole alkaloid and polyketide metabolite Waikialide A**132** obtained from a Hawaiian *Aspergillus spp.* both acted as a potent biofilm inhibitor against *C. albicans* with a IC_{50} - 1.4 μM and 32.4 μM, respectively (Wang et al., 2012).

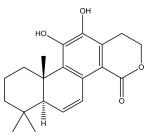
Myriocin **133** is a metabolite of the *Isaria sinclairii* - *a pathogenic insect* fungus. It disrupts the formation of sphingolipids as it is a structural analog of sphingosine precursors. It works very effectively in combination with known antifungal drugs like amphotericin and fluconazole, reducing the mortality rate from 26% to 0%, respectively. Additionally,

TABLE 7 | Chemical structures and MIC/IC_{50} values of small molecule inhibitors (99–105).

S.No.	Compound No.	Structure	IC_{50} μ M	MIC μ g/ml	References
118	99a		1.1	-	Wang et al., 2014
119	99b		1.5	-	Wang et al., 2014
120	99c		1.5	-	Wang et al., 2014
121	99d		1.2	-	Wang et al., 2014
122	99e		5.0	-	Wang et al., 2014
123	99f		1.5	-	Wang et al., 2014
124	99g		24.1	-	Wang et al., 2014
125	100		-	50	Favre-Godal et al., 2015
126	101		-	50	Favre-Godal et al., 2015
127	102		-	50	Favre-Godal et al., 2015
128	103		-	20	Favre-Godal et al., 2015

(Continued)

TABLE 7 | Continued

S.No.	Compound No.	Structure	IC_{50} μ M	MIC μ g/ml	References
129	104		-	20	Favre-Godal et al., 2015
130	105		-	50	Favre-Godal et al., 2015

G.mellonella larva infected with *C. albicans* showed a decrease in mortality rate when treated with myriocin. Furthermore, study of compound **133** indicated that it regulates *C. albicans* pathogenesis through NF-κB pathway, G protein coupled receptor and immunity (Melo et al., 2013). Acetylcholine **134,** a known soluble protein that has a neuronal function, also displays strong anti-biofilm activity against *C. albicans*. Along with this, it also reduces the damage induced due to inflammation in the host. Additionally, *in vivo* study of acetylcholine on *G. mellonella* larvae showed robust antifungal potency with a decrease in mortality rate from 80 to 100% to 25%. Further, compound **134** acts by promoting the rapid cellular immune response in host cells (Rajendran et al., 2015).

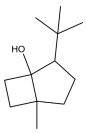
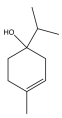
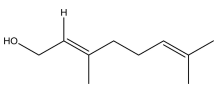
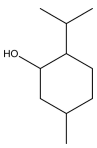
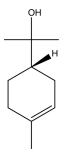
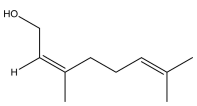
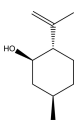
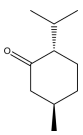
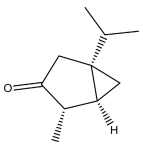
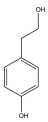
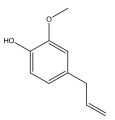
Garcinol **135** and Xanthochymol **136**, isoprenylated benzophenones obtained from *Garcinia xanthochymus* fruits, show antibiofilm activity against *C. albicans* by inducing programmed cell death in early biofilms without actually affecting the growth and viability of planktonic cells. Sampangine B **137, 137(a-b)** a naturally derived azaoxoporphine alkaloid and its derivatives showed potent activity against *C. albicans* biofilms and yeast to hyphal formation. It was found to show antifungal activity by inducing the accumulation of ROS and subsequent heme dysfunction. Moreover, it showed strong *in vivo* antifungal activity with low cytotoxicity in nematode model of *C. albicans* (Wu et al., 2017; **Table 9**).

RECENT ADVANCES TO INHIBIT *C. albicans* BIOFILM FORMATION

Throughout this article, we have seen different strategies and methods to combat antifungal drug resistance. However, after all the efforts and attempts we are still stuck at a point where biofilm formation is difficult to treat with both non-antifungal and antifungal drugs because of the poor penetration and non-specificity of the drugs. Therefore, to address this issue, researchers are working on finding ways to increase the penetration of drugs into the extracellular matrix of biofilm. Over the last few years, different metal nanoparticles have emerged as prospective candidates to treat microbial infections pertaining to their strong potential as antimicrobial agents. In this context, different research groups have initiated the exploration to identify the antibiofilm activity of different nanomaterials against *C. albicans* (Shukla, 2020).

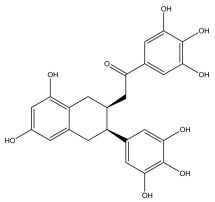
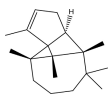
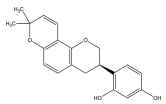
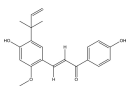
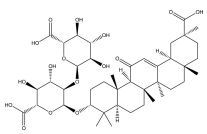
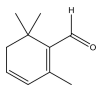
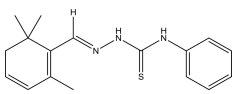
The usage of silver as an antimicrobial agent spans a century. Silver was used as a disinfectant by Greeks and Romans to decontaminate their water and food supplies. Silver was also used in ancient times to treat burns and wounds (Alexander, 2009; Barillo and Marx, 2014; Adhya et al., 2015). Studying silver nanoparticles against biofilms has gained immense recognition. Silver nanoparticles exert an inhibitory effect against fungal biofilms by damaging cell wall mostly by distortion and disruption of the outer surface of the fungal cell wall (Lara et al., 2015). In subsequent experiments, AgNPs when used to functionalize medical and environmental surfaces, demonstrated biofilm inhibition (>50%) at relatively low concentrations (2.3 to 0.28 ppm) (Lara et al., 2020). Combination therapy of Fluconazole with silver nanoparticles has also shown a substantial decrease in the MIC (Longhi et al., 2016). The resistance to fluconazole (FLC) in *C. albicans* is strongly associated with overexpression of genes encoding efflux pumps or lanosterol 14α-demethylase (Sagatova et al., 2016). Subsequently, with the disruption of the cell wall and cytoplasmic membrane of *C. albicans* by AgNPs, these silver particles cause an increase in reactive oxygen species and hydroxyl radical production, which can also contribute to cell membrane damage (Hwang et al., 2012; Monteiro et al., 2012). Recently, researchers have identified bismuth oxide (BiO_3) nanoparticles with potent antimycotic activity against *C. albicans* growth. Moreover, when these nanoparticles were compared to commercially available antifungal drugs like chlorhexidine, nystatin, they completely eradicated the biofilm formation (Hernandez-Delgadillo et al., 2013). In another study, magnesium oxide nanoparticles (MgO NP) were used to investigate their antifungal and antibiofilm property against *C. albican*s. The study revealed that MgO nanoparticles effectively inhibited *C. albicans* biofilm formation. Furthermore, adhesion experiments showed that MgO NPs repressed the preliminary adhesion of *Candida albicans* (Kong et al., 2020). In another study, when gold nanoparticles (Au-NP) were tested on the biofilms formed due to *C. albicans,* these particles showed robust inhibitory activity. Additionally, these Au-NPs increased the host immune response activity against these pathogenic organisms (Yu et al., 2016; Nani et al., 2019). From previous studies, chitosan and its nanoparticle (ChNPs) were already known for their antifungal property (Ing et al., 2012; Mohammed et al., 2017; Gondim et al., 2018). Therefore, to establish its antibiofilm activity, ChNPs were investigated, and it was found that ChNPs significantly inhibited the biofilm formation causing very less changes in the acrylic resin surface (Gondim et al., 2018). Likewise, another study conducted with selenium nanoparticles (Se-NP) showed strong inhibition on *Candida* biofilm formation as these particles out

TABLE 8 | Chemical structures and MIC/IC_{50} values of small molecule inhibitors (106–122).

S.No.	Compound No.	Structure	IC_{50} μ M	MIC μ g/ml	References
131	106		-	8	Hendry et al., 2009; Simsek and Duman, 2017
132	107		-	60-500	Marcos-Arias et al., 2011; Raut et al., 2013
133	108		-	30-250	Marcos-Arias et al., 2011; Raut et al., 2013
134	109		-	60-500	Marcos-Arias et al., 2011; Raut et al., 2013
135	110		-	60-500	Marcos-Arias et al., 2011; Raut et al., 2013
136	111		-	125	Marcos-Arias et al., 2011; Raut et al., 2013
137	112		-	125	Marcos-Arias et al., 2011; Raut et al., 2013
138	113		-	60-400	Marcos-Arias et al., 2011; Raut et al., 2013
139	114		-	500-100	Marcos-Arias et al., 2011; Raut et al., 2013
140	115		-	6.9-20	Marcos-Arias et al., 2011; Raut et al., 2013
141	116		-	30-250	Marcos-Arias et al., 2011; Raut et al., 2013

(Continued)

TABLE 8 | Continued

S.No.	Compound No.	Structure	IC_{50} μ M	MIC μ g/ml	References
142	117		-	15.6-250	Evensen and Braun, 2009
143	118		-	300	Manoharan et al., 2017a
144	119		-	6.25	Messier and Grenier, 2011
145	120		-	6.25	Messier and Grenier, 2011
146	121		-	>200	Messier and Grenier, 2011
147	122		-	1000	Carradori et al., 2016
148	122a		-	1000	Carradori et al., 2016

compete sulfur in biological process due to their similar chemical properties. Also, these nanoparticles enter into the cell to shrink and disrupt the outer membrane structure of *C. albicans* cell (Guisbiers et al., 2017).

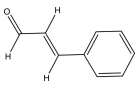
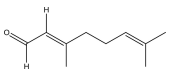
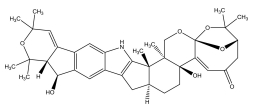
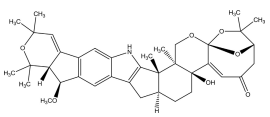
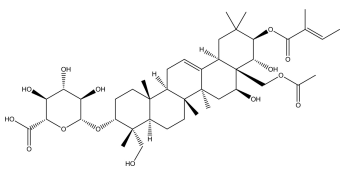
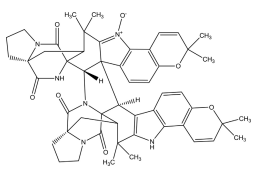
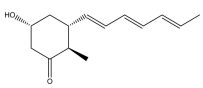
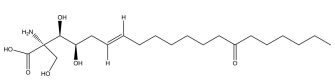
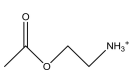
The titanium oxide nanoparticles (Ti-NP) when scrutinized for their antifungal property showed strong antifungal activity against planktonic form of *C. albicans.* They also depicted robust potential against biofilm formation (Haghighi et al., 2013). In addition, zinc oxide nanoparticles (ZnO-NPs) used on *Candida* biofilm showed a decrease in the biofilm formation by abot 62%-85% at varied concentration of 125 ppm to 250 ppm respectively. The ZnO-NPs suggested to inhibit hyphae formation in *C. albicans* by production of reactive oxygen species in a dose-dependent manner (Hosseini et al., 2018; Golipour et al., 2019; Mahamuni-Badiger et al., 2020). Additionally, another study involving copper oxide nanoparticles (Cu_2ONP and CuONP) showed potent inhibitory action against biofilm formation by *C. albicans.* The study revealed that copper oxide nanoparticles constrain the yeast for hypae transformation. Also CuO-NP elicited the reactive oxygen species while Cu_2ONP pronounced the membrane damage in *C. albicans.* In comparison to both types of copper oxide nanoparticles, CuONP was more stable and depicted better antifungal activity in comparison to Cu_2O-NP (Rasool, 2019; Padmavathi et al., 2020). In another study, iron-oxide nanoparticles (Fe_3O_4 NPs) were investigated on *C. albicans* biofilm which showed 87.2-100% inhibition based on their particle size which was 100 ppm to 200 pm respectively (Seddighi et al., 2017).

The nitric oxide nanoparticles (NO-NP) showed strong potential as an antifungal agent by hindering the extracellular matrix and biofilm formation on the surface of biomaterials. It was found that NO-NPs also decrease the metabolic activity of *Candida* cells both *in vitro* and *in vivo* (Ahmadi et al., 2016).

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

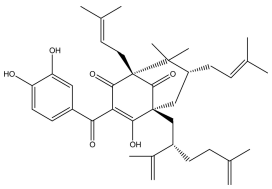
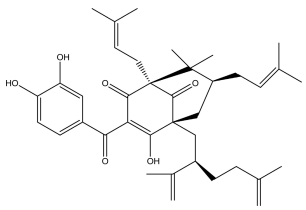
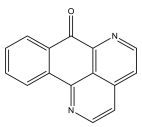
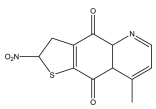
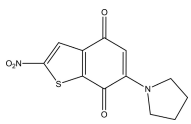
The high mortality and morbidity rate owing to *C. albicans* biofilm infection is a big challenge in medical mycology. Since the formation of biofilm and biomaterial infections is a progressively alarming problem, this warrants the development of new antifungal agents and the search for newer targets. This review elucidates the pathogenic foundation and molecular mechanism of *C. albicans* biofilms over antifungal drug resistance. A plethora of studies by several scientific groups and investigators

TABLE 9 | Chemical structures and MIC/IC_{50} values of small molecule inhibitors (123–137).

S.No.	Compounds	Structure	IC_{50} μ M	MIC μ g/ml	References
149	123		-	62.5-93.7	You et al., 2013
150	124		-	250	You et al., 2013
151	125		-	500	You et al., 2013
152	126		-	500	You et al., 2013
153	127		-	500	You et al., 2013
154	128		8.5	-	You et al., 2013
155	129		7.6	-	You et al., 2013
156	130		-	>200	Veerapandian and Vediyappan, 2019
157	131		1.4	-	Wang et al., 2012
158	132		32.4	-	Wang et al., 2012
159	133		-	0.12	Melo et al., 2013
160	134		-	800-5 × 104	Rajendran et al., 2015

(Continued)

TABLE 9 | Continued

S.No.	Compounds	Structure	IC_{50} μ M	MIC μ g/ml	References
161	135		50	-	Wu et al., 2017
162	136		30	-	Wu et al., 2017
163	137		-	0.5	Wu et al., 2017
164	137a		-	0.125-2	Wu et al., 2017
165	137b			0.5	Wu et al., 2017

have delivered essential knowledge regarding the pathogenesis associated with biofilm formation. These useful insights can represent an optimal starting point to find new therapeutic strategies related to drug resistance and mechanistic signals that govern *C. albicans* biofilm formation. Recent advances on different transcription factors, quorum sensing molecules, host response to adhesion, change in efflux pumps, enzymes, bud to hyphal transition, and change in lipid profile have broadened the knowledge of the complex mechanism underlying the biofilm resistance. Moreover, the development of new biomaterials with anti-adhesive properties, anti- infective lock therapies, high throughput phenotypic screening of small-molecule inhibitors, discovery and repurposing of naturally known compounds are under scrutiny. For medical instruments, a fine coating of nanomaterial may inhibit bacterial accumulation and biofilm formation. Recently, different metal nanoparticles have also emerged as antibiofilm agents against *C. albicans* and gaining momentum. Furthermore, different combinational therapies are harnessed for antibiofilm activity. Yet, the target-based approach is the need of the hour. For this multifaceted biofilm, the complex should be extensively studied to target different loops of phenotypic character change as they play a major role in biofilm formation. The detailed electron microscopic studies using both transmission electron microscopy (TEM) and scanning electron microscope (SEM) with much greater resolution may add additional repertoire to our knowledge. Yet another approach may be the use of some engineered enzymes that do not allow colony formation and inhibit in turn the biofilm formation. Any compound used for this purpose may be empirically optimized for its dose, sensitivity, and efficacy before a search for a target is mounted.

Given the diverse strategies to combat antifungal resistance, there is a hope that specific target-based drugs will be added to the arsenal in our fight against *C. albicans* biofilm formation in the not too distant future.

AUTHOR CONTRIBUTIONS

All authors contributed equally and approved the final version of the manuscript.

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MINI REVIEW
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The New Antibacterial Properties of the Plants: *Quo vadis* Studies of Anti-virulence Phytochemicals?

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The recent increase in bacterial resistance to antibiotics has motivated the resurgence of the study of natural antimicrobial products. For centuries, plants have been recognized for their bactericidal properties. However, in the last two decades, it has been reported that several plant derived metabolites at growth subinhibitory concentrations also tend to have anti-virulence properties, since they reduce the expression of factors that cause damage and the establishment of pathogenic bacteria. In this area of study, plants have been positioned as one of the main natural sources of anti-virulence molecules, but only a small portion of the plant species that exist have been investigated. Also, anti-virulence studies have been primarily focused on analyzing the ability of extracts and compounds to inhibit quorum sensing and biofilms formation *in vitro*. This mini-review discusses the current panorama, the trends in the study of anti-virulence phytochemicals, as well as their potential for the development of antibacterial therapies.

Keywords: antimicrobial phytochemicals, anti-virulence, quorum sensing, virulence factors, adjuvants

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INTRODUCTION

Bacteria are social cells that use quorum sensing (QS) to communicate with organisms of the same species, between species, as well as with other domains of life (Banerji et al., 2020). QS systems (QSS) involve the release of chemical signals called autoinducers, to perceive the presence and concentration of other cells (Castillo-Juárez et al., 2017). This allows them to exhibit multicellular behaviors and regulate the gene expression of various phenotypes at the population level, as among them, production of metabolites (pigments, antibiotics) and virulence factors, including the formation of biofilms (Castillo-Juárez et al., 2015). It is estimated that 80% of chronic bacterial infections form biofilms that promote adherence to host cells and allow them to withstand massive doses of antibiotics and evade the immune response (Townsley and Shank, 2017).

Anti-virulence activity (anti-pathogenic or anti-infectious) is a broad concept that refers to the ability to prevent production of the factors responsible for establishment, damage and spread, but without affecting bacterial viability (LaSarre and Federle, 2013; Totsika, 2016). It has been proposed that development of anti-virulence therapies is a viable strategy for control of bacterial infections, with the possibility of avoiding or reducing the appearance of resistance (Defoirdt, 2018; Scoffone et al., 2019). In the last two decades, many plant species and phytochemicals have been identified as having anti-QS and anti-biofilm properties

(Silva et al., 2016; Muñoz-Cazares et al., 2017). In this mini-review, the current situation of anti-virulence phytochemicals, the evidence, and the challenges faced by this field of research were analyzed.

ANTI-VIRULENCE PROPERTIES OF BACTERICIDAL PHYTOCHEMICALS

Natural products of microbial origin are the main source of bactericidal compounds, which had a "golden age" in the middle of the last century and prompted the development of commercial antibiotics (Brown and Wright, 2016). However, despite being one of humanity's greatest scientific discoveries, the alarming increase in bacterial resistance has put their efficacy and future use at risk (López-Jácome et al., 2019). Nevertheless, it should be noted that only a small proportion of the total bioactive molecules in nature have been explored, so new antibiotics continue to be sought (Li et al., 2019; Stokes et al., 2020). Different strategies are being used to avoid the "nightfall" of this class of molecules and favor the emergence of a second "golden age" (**Figure 1**).

Although the trend in development of antimicrobials has focused on their growth inhibitory properties, it has also been reported that antibiotics at sub-inhibitory concentrations can modulate QSS, virulence (Davies et al., 2006; Khan et al., 2020b), and biofilm formation (Khan et al., 2020a). For example, linezolid has been reported to reduce production of virulence factors from *Staphylococcus aureus* (Bernardo et al., 2004). Also, azithromycin interferes with QS, reducing gene expression and the production of autoinducers in *Pseudomonas aeruginosa*, while streptomycin does so in *Acinetobacter baumannii* (Nalca et al., 2006; Saroj and Rather, 2013). Interestingly, this phenomenon has also been identified in drugs of mass consumption such as aspirin (El-Mowafy et al., 2014) and ibuprofen (Dai et al., 2019), in fermented products, and in various bactericidal phytochemicals (Muñoz-Cazares et al., 2017). Thus, the effect of metabolites at low concentrations on microbial social networks and virulence regulation is a frontier issue that increases the number of molecules to be explored at sub-inhibitory concentrations (**Figure 1**).

CHALLENGES AND TRENDS IN THE STUDY OF ANTI-VIRULENCE PHYTOCHEMICALS

In recent decades, it has been reported that many natural products, especially phytochemicals, exhibit anti-virulence properties when evaluated at subinhibitory concentrations (Brown and Wright, 2016; Silva et al., 2016; Muñoz-Cazares et al., 2017; Mulat et al., 2019). Within natural products, plants are an important source of anti-virulence molecules, but most have been evaluated only *in vitro*. They are not new chemical structures, and many have been reported as bactericidal (Muñoz-Cazares et al., 2017).

The trend in studies related to identification of the anti-virulence mechanism of phytochemicals has focused on showing that they interrupt some element of the QSS. The *in silico* approach has been widely used through computational methods, such as molecular docking, to suggest the interaction of phytochemicals with LuxR-type receptor proteins and/or LuxI-type synthases (Deryabin et al., 2019). Multi-omics analysis (proteomic, transcriptomic, and metabolomic) has shown that some phytochemicals interfere with the expression of various QS genes, but also with other non-QS genes. Such is the case of coumarin, which reduces the expression of genes involved in QS, type 3 secretion system (T3SS), and metabolism of cyclic diguanylate in *P. aeruginosa* (Zhang et al., 2018). In the same way, ajoene reduces the expression of virulence factors in *P. aeruginosa* and *S. aureus* by inhibiting small regulatory RNAs (Jakobsen et al., 2017; **Table 1**). However, some reports identify natural products that can inhibit other anti-virulence targets such as other secretion systems, adhesion molecules, toxins, two-component systems, key enzymes, curli, flagellum as well as metabolic processes involved in the formation and maturation of biofilms (Muñoz-Cazares et al., 2018).

Several anti-virulence phytochemicals have been shown to reduce establishment and damage caused by bacteria *in vivo*, mainly in the nematode *Caenorhabditis elegans* in murine models (Castillo-Juárez et al., 2015) and animals of importance in aquaculture (Zhao et al., 2015). Also, they have preventive effects on phytopathogenic bacterial infections in some models with *Arabidopsis thaliana*, *Brassica oleracea* and *Solanum tuberosum*, among others (Jhosi et al., 2015; Sivaranjani et al., 2016). Although there is evidence that disruption of virulence by phytochemicals is a potential strategy to prevent disease, there are emerging issues and challenges that have been little studied and are detailed below (Mulat et al., 2019).

Anti-virulence Phytochemicals and Their Role in the Daily Diet

One of the trends is related to the role of anti-virulence phytochemicals present in edible plant species and their ability to prevent infectious processes (Givskov, 2012; McCarthy and O'Gara, 2015). Although it is thought that plants are unlikely to contain concentrations of phytochemicals high enough to counteract established bacterial infections, it has been proposed that their continuous consumption may prevent development of chronic infections (Givskov, 2012). This is still difficult to conclude, but, QS inhibitors have been identified in some edible species such as garlic (Bjarnsholt et al., 2005), oilseeds (Pérez-López et al., 2018), and hibiscus acid isolated from *Hibiscus sabdariffa* (Cortes-López et al., 2021), which have been shown to have anti-virulence properties and reduce bacterial pathogenicity in mice (**Table 1**).

Phytochemicals as Inducers de QS

Bactericidal molecules commonly have a dose-response effect, but at subinhibitory concentrations, they can exhibit multiple effects on bacterial cells (Davies et al., 2006; **Figure 1**). Hormesis is a phenomenon that commonly occurs at low concentrations and is characterized by antagonistic activities (stimulate/inhibit) exhibited by the same molecule, depending on the concentration

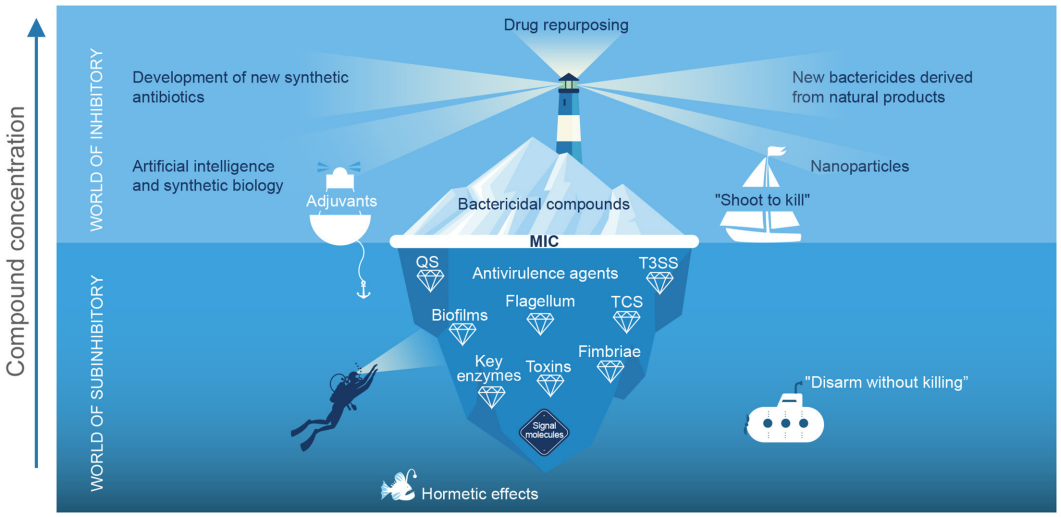


FIGURE 1 | Schematic representation of the current status of antimicrobial strategies: "The world of the inhibitory". The tip of the iceberg represents the bactericidal compounds that have been discovered, while the light of the beacon searches for current strategies to prevent the "dusk" of this class of molecules. The resurgence of research in natural products, repurposing drugs for use as antibiotics, nanoparticles, chemical synthesis of new bactericides, as well as advances in computer science, omics, artificial intelligence, and synthetic biology are playing a relevant role in the development of new bactericidal compounds (Zakeri and Lu, 2013; Pushpakom et al., 2018; Li et al., 2019; Stokes et al., 2020). However, in this analogy the strategy of "shooting to kill," allows some pathogenic microorganisms to live and generate resistance; in addition, in the "crossfire" beneficial microorganisms are eliminated. In the "world of the sub-inhibitor," the number of bioactive molecules to be explored is greater, and the strategy is based on "disarming without killing," in theory, will not induce resistance. At values below the minimum inhibitory concentration (MIC), the compounds exhibit different effects, among which are anti-virulence, and signal molecule activity, and they have hormetic and adjuvant effects (Cox et al., 2017). The term "anti-virulence agent" also includes peptides, enzymes, and antibodies. QS, quorum sensing; TS33, type 3 secretion system and TCS, two-component systems.

(Mattson, 2008; Martel et al., 2019; **Figure 1**). Although the hormetic effect of phytochemicals has been reported in other biological activities (Martel et al., 2019), their clinical use could be complicated by a change in concentration that can stimulate virulence. It has been reported that furanone and other inhibitors can inhibit or activate QS depending on the concentration (Martinelli et al., 2004; Welsh et al., 2015; Yao et al., 2019). Similarly, some natural products with no bactericidal activity can stimulate the formation of biofilms (Ranieri et al., 2018). In the case of phytochemicals, the hormetic effects have been little studied, but linalool and eugenol have been reported to have this type of effect on biofilm formation and the rhamnolipids production of *P. aeruginosa* PAO1 (Kim Y. G. et al., 2015). Also, coumarin was reported to affect swarming of *Ralstonia solanacearum* (Chen et al., 2016) and capsaicin to affect biofilm formation in *P. aeruginosa* PAO1 and *Serratia marcescens* (Rivera et al., 2019).

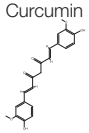
Effects of Anti-virulence Phytochemicals on the Microbiome

So far, inhibition of virulence regulation systems appears to be advantageous in combating pathogenic bacteria. However, there are still few studies on its effect on the QS systems of beneficial bacteria, the microbiome in general, or on the host (McCarthy and O'Gara, 2015; Lakes et al., 2020). Unlike *in vitro* monoculture trials, pathogenic bacteria develop in polymicrobial communities where they interact with environmental factors and different specific signaling molecules (many of them still unknown) that can determine the virulence of the pathogen (Banerji et al., 2020). We now know that the intestinal microbiome participates in many aspects of health; microbe-host interactions influence obesity, inflammatory and digestive processes, and certain psychiatric conditions, among others (Burdet et al., 2019). In this context, it has been seen that alteration of the microbiome by exposure to penicillin at sublethal doses in the early stages of development induces metabolic alterations and affects expression of genes involved in host immunity, favoring obesity induced by a high-fat diet (Cox et al., 2014). Similarly, some phytochemicals commonly ingested in the diet (phenolic compounds, terpenes, and alkaloids) affect intestinal bacterial groups and it is suggested that they may affect host microbial ecology and physiology when administered at bactericidal concentrations (Lakes et al., 2020). Morever, recent studies suggest that in complex microbial communities, interference with QS severely affects microbiome composition. However, up to the moment of this review, we did not find reports related to the effect of anti-virulence phytochemicals on the microbiome at sub-inhibitory concentrations (Nguyen et al., 2019; Waheed et al., 2020).

Development of Combination Anti-virulence Therapies

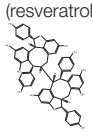
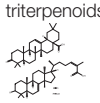
Some of the strategies to potentiate the efficacy of anti-virulence molecules are the development of combination therapies of inhibitors with different targets (Fong et al., 2018; Ranieri et al., 2018). However, although the mechanism of action of most

TABLE 1 | Main antibacterial effects of phytochemicals at sub-inhibitory concentrations.

Phytochemical	Plant species	Effect	Anti-virulence activity/target	Preclinical trials	References
Ajoene 	*Allium sativum*	Quorum quenching, anti-biofilm	Reduces biofilm formation and production of QS-regulated virulence factors/ Inhibits small regulatory RNAs, such as RsmY and RsmZ in *P. aeruginosa* and RNAIII in *S. aureus*.	Reduced the bacterial load of *P. aeruginosa* in a mouse model of lung infection.	Jakobsen et al., 2012, 2017
Baicalin 	*Scutellaria baicalensis*	Anti-biofilm, quorum quenching, and adjuvant	Reduces biofilm formation and production of QS-regulated virulence factors in *P. aeruginosa.* Improves the bactericidal effects of some conventional antibiotics.	Reduced the number of bacteria in a mouse peritoneal implant infection model.	Luo et al., 2017
Berberine 	*Coptis japonica var.major Satake, Phellodendron chinense Schne der*	Adjuvant	Inhibitor of the MexXY dependent aminoglycoside efflux	NA	Morita et al., 2016
Curcumin 	*Curcuma longa* L.	Quorum quenching, anti-biofilm	In the form of ZnO/curcumin nanocomposites, reduces expression and production of QS-regulated virulence factors in *P. aeruginosa*/CI-QS	Increased survival of specific pathogen-free albino mice injected with *P. aeruginosa*.	Prateeksha et al., 2019
b-sitosterol 	Various plant species	Anti-toxin	Prevents cell lysis caused by pneumolysin and other cholesterol-dependent toxins/ It interferes with binding sites of the toxin (Thr459 and Leu460) with cholesterol	Reduced bacterial load in lungs and mortality of mice intranasally infected with *Streptococcus pneumoniae*	Li et al., 2015
Cinnamaldehyde 	Commercially obtained	Adjuvant	NA	Increased bactericidal activity of tobramycin and baicalin hydrate, favoring elimination of *B. cenocepacia* in lungs of mice	Brackman et al., 2011

(Continued)

TABLE 1 | Continued

Phytochemical	Plant species	Effect	Anti-virulence activity/target	Preclinical trials	References
6-gingerol 	*Zingiber officinale*	Anti-biofilm and quorum quenching	In *P. aeruginosa*, reduces biofilm formation, swarming, rhamnolipid production, pyocyanin, and exoprotease activity/CI-QS	Increased survival of specific pathogen-free mice injected with *P. aeruginosa*	Kim H. S. et al., 2015
Glycyrrhizin 	Glycyrrhiza uralensis	Anti-toxin	Inhibits the interaction of the heat-labile enterotoxin of enterotoxigenic *E. coli* with G_{M1} of intestinal epithelial cells	Prevented enterotoxin-induced fluid accumulation (antidiarrheal effect) in the patented mouse gut assay	Chen et al., 2009
Hibiscus acid 	*Hibiscus sabdariffa* L.	Anti-biofilm and quorum quenching	In *P. aeruginosa*, reduces biofilm formation, swarming, alkaline protease, and elastase activity/ CI-QS	In a mouse model of dermonecrosis, it reduced establishment, damage, and systemic spread	Cortes-López et al., 2021
(-)-hopeaphenol (resveratrol tetramer) 	*Anisoptera thurifera* and *A. polyandra*	T3SS inhibitor	Block expression and secretion of the effector proteins ExoS from *P. aeruginosa* and Yop in *Yersinia pseudotuberculosis*	NA	Zetterström et al., 2013
Saturated fatty acids 	*Helianthus annuus L., Salvia hispanica L.* and *Amaranthus hypochondriacus L.*	Quorum quenching	Reduce violacein production and the activity of alkaline exoprotease/CI-QS	Increased survival of mice infected with *Chromobacterium violaceum*	Pérez-López et al., 2018
Tirucallane-type triterpenoids 	*Schinus terebinthifolia* Raddi	Quorum quenching	*S. aureus*: accessory gene regulator (*agr*) (leucocidin A (*lukA*), glycerol ester hydrolase or lipase (*gehB*), nuclease (*nuc*)) and d-toxin production	Reduce dermonecrosis in a murine model caused by *S. aureus*	Tang et al., 2020
Vitexin 		Anti-biofilm, quorum quenching, and adjuvant	The combination azithromycin and gentamicin increase antibiofilm activity and reduces the production of QS-regulated virulence factors in *P. aeruginosa*/ CI-QS	Reduced the number of bacteria in a mouse peritoneal implant infection model	Das et al., 2016

T3SS, type 3 secretion system; NA, not available; CI-QS, possible competitive inhibition with QS receptor proteins.

phytochemicals is unknown, it is highly feasible that they act at various sites to reduce virulence, as some transcriptomic and proteomic studies have revealed (Jakobsen et al., 2017; Zhang et al., 2018).

Adjuvant activity is a property that has been recently identified in several natural anti-virulence products and that helps to restore the activity of antibiotics against sensitive and resistant strains (Cox et al., 2017). Such are the cases of baicalin (Luo et al., 2017), berberine (Morita et al., 2016), cinnamaldehyde (Brackman et al., 2011), and vitexin (Das et al., 2016; **Table 1**). Although the mechanisms involved in this phenomenon are not known, in the case of berberine it has been reported that it restores the bactericidal activity of aminoglycosides because it blocks the efflux pumps that expel these antibiotics (Morita et al., 2016).

Induction of Resistance

The premise of the anti-virulence strategy is based on removing the pathogenicity of microorganisms without directly affecting their viability, so that, arguably, strong selection pressures are not generated to induce resistance (McCarthy and O'Gara, 2015). However, some reports indicate that furanone C-30 at subinhibitory concentrations generates resistance by a mechanism that involves the expression of expulsion pumps for this compound (Maeda et al., 2012; García-Contreras et al., 2016). Also, it is suggested that the presence of "cheaters" (bacteria that do not participate in collective communication but do benefit from the products that are produced) in populations may favor resistance because they would be naturally resistant to QS inhibitors (Kalia et al., 2014). Moreover, a recent finding in *Escherichia coli* suggest that QS inhibition may promote conjugation of plasmids and increase the mutation rate, hence favoring the generation of resistance (Li et al., 2021). This is one of the most debated issues in this area; however, to date no reports have shown that anti-virulence phytochemicals induce resistance.

Patents, Preclinical, and Clinical Studies

Although several patents for anti-virulence agents have been published, most focus on their ability to block QS or prevent biofilm formation, and there are few studies that corroborate the effect at the preclinical (**Table 1**) or clinical level (Kalia et al., 2019). In the specific case of phytochemicals, studies on their ability to act on biofilms abound, but clinical trials remain scarce (Reuter et al., 2016). In this regard, the study of garlic as an anti-QS agent in the treatment of cystic fibrosis stands out; the study reports a reduction in symptoms and an improvement in lung function (Smyth et al., 2010). Another is the anti-biofilm formulation based on *Hymus vulgaris*, *Eugenia caryophyllus,* and lactobacilli for the treatment of bacterial vaginosis, in which administration by slow-release capsules was able to reduce signs and symptoms in 80% of patients (Murina et al., 2018).

CONCLUSION AND PERSPECTIVES

Among natural products, plants have played a discrete role in the discovery of bactericidal compounds, but they have thus far been positioned themselves as the main source of anti-virulence molecules. However, studies of anti-virulence phytochemicals have focused mainly on analyzing their quorum quenching and antibiofilm properties *in vitro*. The few preclinical trials conducted have identified only preventive effects and they have not yet been shown to counteract established infections. In this regard, it is suggested that the anti-virulence activity registered in bacterial monocultures and ideal growth conditions (rich media) cannot always be extrapolated to the complex conditions that occur in the host (Davies et al., 2006; Turovskiy et al., 2007; Juárez-Rodríguez et al., 2021). Reports exist that indicate that host environmental factors and the presence of other microbial species may interfere with virulence expression (Sabag-Daigle et al., 2012; Ismail et al., 2016). Recently, it was reported that myristic acid, which reduces virulence *in vitro*, behaves as a signal molecule stimulating the pathogenicity of *P. aeruginosa* in a dermonecrotic mouse model (Juárez-Rodríguez et al., 2021). Furthermore, it has been discovered that in some murine models the T3SS are the main virulence determinants, while the QSS seems to have a more discrete role (Miki et al., 2010; Soto-Aceves et al., 2019; Juárez-Rodríguez et al., 2021). Thus, deciphering the ecological context in which virulence is regulated *in vivo* will be decisive for the development of effective therapies.

On the other hand, some required characteristics of an ideal anti-virulence molecule have been proposed. Most of them are the same as those expected for other bioactive compounds: high specificity, stability and absence of side effects (Kalia et al., 2019). However, other desirable properties such as not generating resistance or not negatively altering the host microbiome, have been little studied. Another important characteristic is that they should have no bactericidal activity against the pathogen or the microbiome (Davies et al., 2006). Also, hormetic effects that can stimulate virulence should be absent, and they should have the ability to inhibit several anti-virulence targets simultaneously. The latter can help reduce possible side effects derived from the administration of multi-drug therapies and decrease resistance selection.

Furthermore, it is important to expand research into other anti-virulence targets on which the phytochemicals may be acting. One of them is the T3SS, which even though various synthetic molecules have been described that inhibit it, the number of phytochemicals reported with this activity is scarce. In this regard, the preclinical results obtained with (-)-hopeaphenol are very important (Zetterström et al., 2013; **Table 1**). Also, anti-toxin properties are important, as in the case of β-sitosterol and glycyrrhizin, which protect from damage caused by bacterial toxins (Chen et al., 2009; Li et al., 2015; **Table 1**). Finally, the use of nanoparticles to potentiate the effect of phytochemicals is a strategy with which good results have been obtained at the preclinical level, as has been demonstrated with curcumin (Prateeksha et al., 2019; **Table 1**). All these trends contribute to the resurgence of the study of natural antibacterial products, with great potential to help solve the current crisis of antibiotics.

AUTHOR CONTRIBUTIONS

All the authors have contributed equally to the proposal, writing, and editing of the manuscript and also read and approved the final version of the manuscript.

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REVIEW
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Bacterial Biofilm Inhibition: A Focused Review on Recent Therapeutic Strategies for Combating the Biofilm Mediated Infections

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Biofilm formation is a major concern in various sectors and cause severe problems to public health, medicine, and industry. Bacterial biofilm formation is a major persistent threat, as it increases morbidity and mortality, thereby imposing heavy economic pressure on the healthcare sector. Bacterial biofilms also strengthen biofouling, affecting shipping functions, and the offshore industries in their natural environment. Besides, they accomplish harsh roles in the corrosion of pipelines in industries. At biofilm state, bacterial pathogens are significantly resistant to external attack like antibiotics, chemicals, disinfectants, etc. Within a cell, they are insensitive to drugs and host immune responses. The development of intact biofilms is very critical for the spreading and persistence of bacterial infections in the host. Further, bacteria form biofilms on every probable substratum, and their infections have been found in plants, livestock, and humans. The advent of novel strategies for treating and preventing biofilm formation has gained a great deal of attention. To prevent the development of resistant mutants, a feasible technique that may target adhesive properties without affecting the bacterial vitality is needed. This stimulated research is a rapidly growing field for applicable control measures to prevent biofilm formation. Therefore, this review discusses the current understanding of antibiotic resistance mechanisms in bacterial biofilm and intensely emphasized the novel therapeutic strategies for combating biofilm mediated infections. The forthcoming experimental studies will focus on these recent therapeutic strategies that may lead to the development of effective biofilm inhibitors than conventional treatments.

Keywords: antibiotics, bacterial biofilm, biofilm inhibitors, biofilm mediated infections, multidrug resistance, persistence, therapeutic strategies

INTRODUCTION

Biofilms are severe health concerns due to their multidrug resistance abilities, host defense, and other stresses (De La Fuente-Nunez et al., 2013). Therefore, it leads to chronic bacterial infections worldwide (Subhadra et al., 2018; Sharma et al., 2019). Bacterial biofilm is a multifaceted structure of communities with diverse bacterial colonies of cells in a group (Kostakioti et al., 2013). Biofilm referred to the intricate three-dimensional (3-D) aggregation of bacteria attached to a surface and buried inflexibly in an Extracellular Polymeric Substance (EPS) matrix. Bacteria form biofilms in every substratum, and their associated infections in plants, animals, and humans (Lebeaux et al., 2014; Padmavathi et al., 2017; Kannappan et al., 2020). Besides, biofilms also play destructive roles in industrial pipelines corrosion (Lenhart et al., 2014). Bacterial biofilms can attach to various materials such as metals, glass surfaces, plastic wares, tissues, and clinical devices. Bacterial communities also produce biofilm, especially on all medical implants, including vascular grafts, heart valves, intrauterine devices, pacemakers, prosthetic joints, catheters, sutures, and contact lenses to acute infections (Kannappan et al., 2017b).

Bacteria inside the biofilm can also withstand harsh conditions and hold secreted polymers such as polysaccharides, extracellular DNA (e-DNA), proteins, and amyloidogenic proteins (Sharma et al., 2019). The pathogenesis and persistence of bacterial pathogens are dependent on the formation and maintenance of intact biofilms (Xu et al., 2000; Stewart and Costerton, 2001; Pang et al., 2013; Wilkins et al., 2014). Bacterial cells swathed in the biofilm are up to 1000 fold resistant to antibiotic agents. In this biofilm form, bacteria are more resistant to various antibacterial and chemical treatments. Biofilms offer the guard to the bacteria from pH, nutrients deficiency, and mechanical forces (Bryers, 1993; Sutherland, 2001; Singh et al., 2017). Therefore, the biofilm matrix gives additional resistance to bacteria, leading to bad bug's infections like drug resistant bacteria.

Novel biofilm inhibitors have been investigated from a various sources in order to prevent biofilm formation and eliminate persistent biofilms. Although the research focused on identifying compounds able to target and inhibit this biofilm mode of bacterial growth is explicitly still inadequate (Peng et al., 2015; van Tilburg Bernardes et al., 2015; Kannappan et al., 2017a; Srinivasan et al., 2018). As a result, new therapeutic options are needed for controlling the biofilm associated infections. At present, the study of biofilm and its strategies to eliminate without any resistant development is one of the utmost significant fields of research. Several reviews on biofilm inhibitors have already been reported, but our review mainly focuses on significant novel strategies to control biofilm mediated bacterial infections.

BIOFILM FORMATION

Generally, biofilm formation by bacterial pathogens on any substratum/layer involves five major stages (Kostakioti et al., 2013; Yin et al., 2019). (1) Attachment: at an initial stage, free-swimming planktonic cells reversibly attach to the biotic or abiotic surfaces through weak interactions such as acid-base, hydrophobic, Van der Waals, and electrostatic forces. (2) Colonization: bacterial pathogens irreversibly attach to the surface through stronger interactions such as collagen-binding adhesive proteins, lipopolysaccharides, flagella, and pili. (3) Proliferation: the multilayered bacterial cells are profoundly accumulated, and the enormous amounts of EPS are produced. (4) Maturation: the attached multilayered bacterial cells grown into the matured biofilm with the typical 3D biofilm structure. (5) Dispersion: after the complete development of biofilm, it is disassembled or dispersed using mechanical and active processes (**Figure 1**).

Characteristics of Biofilm Formation

The formation of biofilm is a progressive process. Primarily, bacterial cells move onto a surface and adhere reversibly to the surface. In the second step, irreversible adherence occurs with the microcolonies expansion that produces an EPS matrix. Subsequently, the progress of the mature 3-D biofilm architecture emerges. Matured biofilms are more resistant to the host immune defenses and the action of antibacterial agents. During the dispersal of biofilm, the cells endure lysis and discharge from the biofilm community. Inside the host, bacteria produce biofilm on a biotic or an abiotic layer. The abiotic surface is typically coated with proteins or other biological molecules, forming a habituation film that changes cells adhesion. In biofilm formation, host cells can develop a fundamental part, and their components can be assimilated into the biofilm matrix (Lynch and Robertson, 2008; Romling and Balsalobre, 2012).

Components in the Biofilm Matrix

Biofilms are a group of microorganisms in which microbes produced EPS such as proteins (<1–2%), polysaccharides (1–2%), DNA (<1%), and RNA (<1%). In addition to these components, water (up to 97%) is the key portion of biofilm, distributed in a non-homogenous pattern and mainly accountable for the movement of nutrients inside the biofilm matrix (Batoni et al., 2016; Nazir et al., 2019). The capability to build and conserve an organized biofilm community mainly depends on EPS matrix components (Sutherland, 2001; Branda et al., 2005; Limoli et al., 2015). The EPS in the biofilm matrix commands a charter for the biofilms. The biofilm inhabitants are always shielded from the atmosphere (competitive microbes, temperature, host cells, antimicrobials, and desiccation) while also having access to nutrients and the capacity to react environmental changes. Bacteria generate multiple types of EPS to handle these needs in different ways. EPS can help the bacteria to adhere on many different surfaces and hosts; provide protection from the environment and reservoirs for nutrient acquisition (Ramanathan et al., 2018; Kannappan et al., 2019a).

Role of EPS in Biofilm Formation

EPS is a superglue that accounts for the biofilm communities slimy nature and is a complex blend of biopolymers, including polysaccharides, proteins, e-DNA, and phospholipids (Nazir et al., 2019). In general, EPS composition changes with the

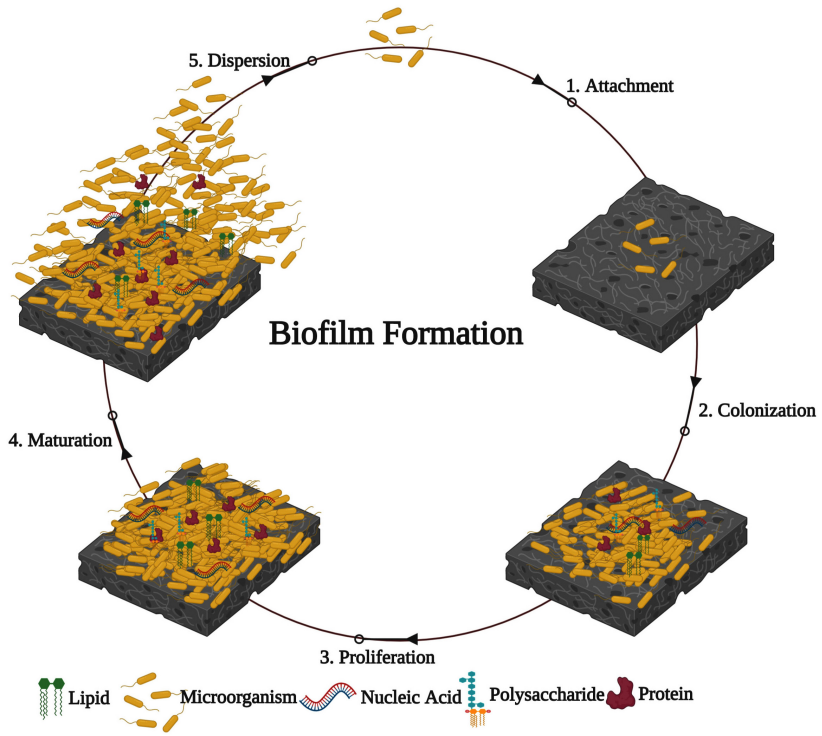

FIGURE 1 | Developmental stages involved in bacterial biofilm formation.

type of pathogens, biofilm age, and environmental conditions (desiccation, pH, oxygen, nitrogen, temperature, and nutrients availability) (Mayer et al., 1999; Kostakioti et al., 2013). The bacteria existing in biofilm suggest that they can respond to their surroundings by modifying their EPS composition and adhesion. EPS provides a physical framework for the attachment among cells and surfaces. It also acts as a blockade between biofilm cells and surroundings (Mitchell et al., 2016). It protects microbes from antimicrobial compounds, chemicals, desiccation, radiation, and unfavorable environmental conditions. They are also cherishing bacterial cells inside the biofilm with a constant supply of nutrients and keeping their capability to respond environmental variations. As related to a protein in EPS composition, polysaccharides are extremely sticky and fundamental for biofilm maintenance and its environment. Similarly, proteins from EPS matrix modify the cell wall assets, adherence, virulence, and morphogenesis; protect cells from harmful conditions and phagocytes (Chaffin et al., 1998; Bridier et al., 2011). Another vital component of EPS is e-DNA, which increases biofilm structural integrity, exchange of genetic information, nutrients provision, biofilm stability, and drug resistance (Donlan, 2002; Martins et al., 2010).

Biofilm Mediated Bacterial Infections

The level of clinical care has advanced dramatically over the few years, but bacterial biofilm infections continue to pose a significant threat to public health. Hoiby, Lam and his colleagues were the first to identify a direct correlation between the formation of biofilms and recurrent infections, especially with *Pseudomonas aeruginosa* in cystic fibrosis patients (Hoiby, 1977; Lam et al., 1980). The decades that followed embraced the idea that biofilms are an important source of tissue related infections (Lebeaux et al., 2013). There are various sites in the human body where biofilm infections may occur due to either a pre-existing condition or a hospital acquired infection. Further, tissue related bacterial biofilm infections have been noted to occur more often in immunocompromised patients, and patients with underlying chronic illness such as cardiovascular disease, diabetes, skin barrier breakage, cancer, or especially if the infection is severe or starts early in the course of the illness (Sivaranjani et al., 2018). In addition, it was understood that the usage of different forms of embedded medical devices would favor adhesion and the colonization of bacteria, resulting in infections (Marrie et al., 1982; Donlan and Costerton, 2002). Further, several types of embedded medical devices are associated with the development of bacterial biofilms **(Figure 2)**. More, ventilator pneumonia, central line bloodstream infections, urinary, pacemaker, and peripheral vascular catheter infections are the utmost common device related bacterial biofilm infections (Kamaruzzaman et al., 2018; Kannappan et al., 2019b).

Biofilm in Antibiotic Tolerance and Persister Formation

Usually, chronic tissue and device related bacterial infections are difficult to treat because the patient is exposed to the risk of recurrence (Lewis, 2007). Bacterial biofilms can spread to other

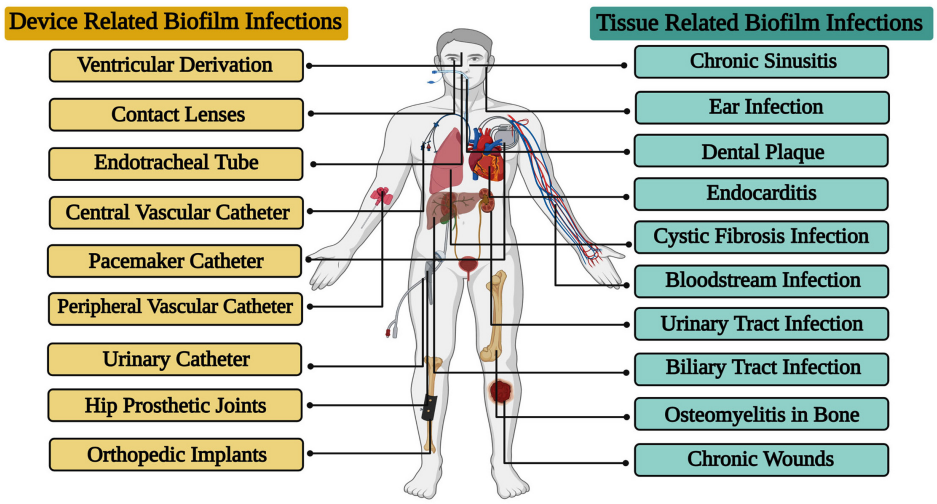

FIGURE 2 | Various types of tissue and device related biofilm infections caused by bacterial pathogens.

parts of the body or around the infection source if planktonic bacteria originate from the biofilm. Planktonic bacteria may be eradicated by combined action of host immune responses and antibiotics. However, a subset of biofilm bacteria those are not destroyed by the antibiotic treatment and can able to trigger the recurrence of infection (Lebeaux et al., 2014).

Inside the biofilm, bacterial cells reveal morphological, and physiological changes assisted by differential gene expression due to the gradient in toxic components, diffusible gasses, or nutritional pressure (Stewart and Costerton, 2001). Depleted oxygen and nutrients within biofilm stimulate asynchronous growth, which exhibits variations in the level of gene expression and may lead to drug tolerance. Phenotypic variety of bacteria within biofilm augments greater coordination, empowers genes for reprogramming, and involves the efflux of toxins, lipid biosynthesis, iron sequestration, DNA repair, and host immune modulation, etc. (Li et al., 2016). It provides persistence and selective dissemination of resilient cells enduring stress. Nowadays, resistance to antibacterial agents is the most crucial cause of non-effective therapy of biofilm-associated bacterial infections. The reason behind increased antibiotic resistance of bacteria is (1) Difficulty for the diffusion of antibiotics into the biofilm and electrostatic charge of the EPS, which attract oppositely charged antibiotics; (2) A slower growth rate; (3) Variations in phenotype acquired by bacteria forming biofilms and (4) Inactivation of antibiotics by enzymes secreted by bacteria **(Figure 3**; Lewis, 2001; Lewis, 2008, 2010; Sharma et al., 2019).

Several promising mechanisms are underlying the phenotypic resistance, which may be influenced by the type of antibiotic treatment and the host, growth rate of biofilm, transformed metabolism, and the presence of an oxygen gradient that prevents the action of some antibiotics (Li et al., 2016; Cheng et al., 2019). Besides, biofilms contain a great population of persister cells, which endure against antibiotics treatment. A limited dispersal of antibiotics into biofilms has been proposed, but in most occurrences, no direct evidence has been provided (O'Toole et al., 2000; Lewis, 2010).

Signaling in Biofilm Formation: How Bacteria "Talk to Each Other"

Bacterial cells in biofilm communicate with each other and coordinate their behavior through the signal molecules. This cell-cell communication system is called Quorum Sensing (QS), which goes beyond bacterial cell density (Miller and Bassler, 2001). Mostly, roles of QS are classified into four kinds such as (1) Cell maintenance and division (exoenzymes and siderophores production), (2) Horizontal gene transfer (conjugation), (3) Host-pathogen interactions (antibiotic and bioluminescence production), and most importantly (4) Behavior (movement and biofilm formation). The QS mediated biofilm formation has well documented in several Gram-negative and Gram-positive bacterial pathogens (Carniol and Gilmore, 2004; Labbate et al., 2004; Kong et al., 2006; Longo et al., 2014; Brindhadevi et al., 2020).

Generally, QS facilitates the physiological status of the microbial population and controls the biofilm formation through signal molecules called *N*-acyl-homoserine lactones (AHL) or auto-inducing peptides (AIP) in Gram-negative and Gram-positive bacterial pathogens, respectively (Whitehead et al., 2001; Papenfort and Bassler, 2016; Rama Devi et al., 2016; Bhatt, 2018). According to the bacterial species, varieties of AHL exist in Gram-negative bacterial pathogens, and some may vary as the strain varies. Usually, the AHL are synthesized by AHL synthase gene *luxI*. The *luxI* gene is transcriptionally expressed to the basal level at low population density. Hence, the AHL molecules are scattered in the field. At the high cell density, the LuxR family of receptor proteins senses the AHL molecules. Then, the signal molecule attaches to the receptor protein until AHL hits a particular threshold concentration. At that time, the activated LuxR-AHL complex forms multimers with other

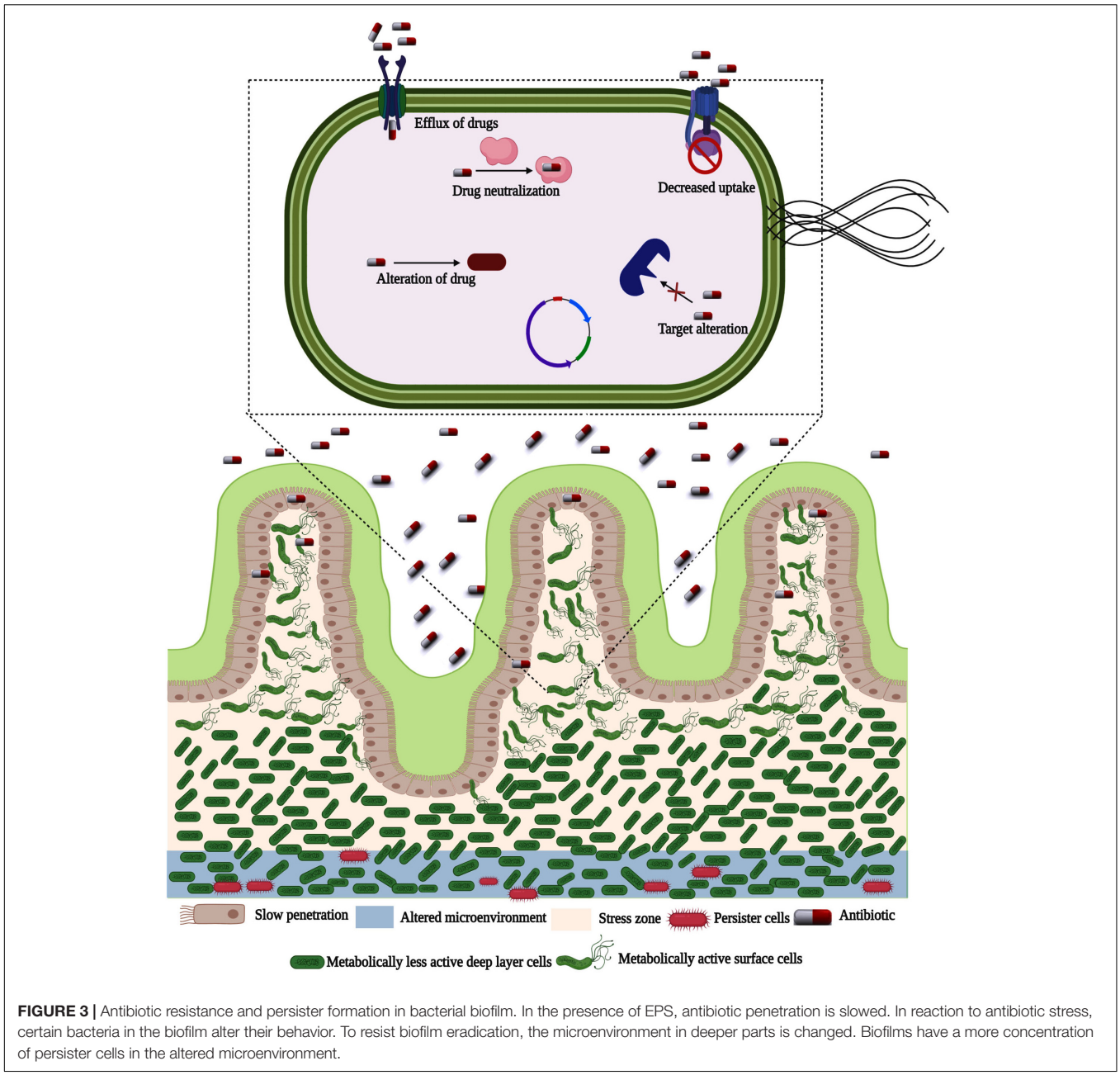

FIGURE 3 | Antibiotic resistance and persister formation in bacterial biofilm. In the presence of EPS, antibiotic penetration is slowed. In reaction to antibiotic stress, certain bacteria in the biofilm alter their behavior. To resist biofilm eradication, the microenvironment in deeper parts is changed. Biofilms have a more concentration of persister cells in the altered microenvironment.

activated LuxR-AHL complexes. Finally, these multimers control the transcription of QS regulated biofilm formation in several bacterial pathogens (Rasmussen and Givskov, 2006).

RECENT THERAPEUTIC STRATEGIES FOR BIOFILM INHIBITION

Besides the conventional antibiotics, certain promising underlying strategies extended by the prevailing biofilm inhibitors hinder the biofilm formation and reduce microorganisms' virulence. Most biofilm forming microorganisms are responsible for 80% of human infections (Shunmugaperumal, 2010) and their ill-health. Due to the EPS matrix of the biofilm, they resist the immune system of humans. Some antimicrobial peptides like defensins or existence biofilm inhibitors have an extensive part in acting upon the matrix (Lewis, 2001). However, many novel and interesting tactics or lines of attack combating against biofilms were identified, and their progression at the current scenario described in detail.

Quorum Sensing (QS) Blockage Strategy

Considering the QS system as the noteworthy comportment of biofilm synthesis by microorganisms, many researches contributed to recognizing the QS system's blockage as a vital strategy to prevent biofilm. More, bacterial pathogens in the

host can activate the QS signals for biofilm formation and virulence factors production. Therefore, inhibiting this bacterial communication through QS inhibitors makes the bacterial pathogens more susceptible to the host immune system and antibiotic responses (Ravindran et al., 2018; Jiang et al., 2019; Li et al., 2020). Consequently, it facilitates the targeting of QS as a therapeutic target for controlling biofilm mediated bacterial infections. The phenomenon of down-regulating or silencing the QS system is referred to as quorum quenching. Generally, blocking the QS system of Gram-negative bacterial pathogens can be done through three essential strategies: 1. Blocking the AHL molecule biosynthesis, 2. AHL inactivation or degradation, and 3. Interference with the signal receptor **(Figure 4)**.

Hindering the AHL Signal Molecule Biosynthesis

Previously, the *in vitro* analysis has been performed on the catalysis of AHL molecule biosynthesis in sequentially ordered reaction. The S-adenosyl methionine (SAM) is used as the amino donor to produce homoserine lactone ring moiety. An adequately charged acyl carrier protein (ACP) is used as the precursor for producing the acyl side chain of the AHL signal (Parsek et al., 1999; Hentzer and Givskov, 2003; Li et al., 2016). Other studies made by Zano et al., 2013 and Masevicius et al., 2016 have shown that several Gram-negative bacterial pathogens can synthesize the S-adenosyl-L-methionine (AdoMet) as the primary methyl donor for several methylation processes. Zano et al. (2013) have revealed that this AdoMet may also act as a precursor for the production of two different QS signal molecules; therefore, targeting the hindrance of AdoMet can lead to inhibiting the biofilm formation in various Gram-negative bacterial pathogens. So, QS inhibitors that target AHL molecule biosynthesis can be developed using knowledge of signal generation. Various analogs of SAM have been continuously revealed to be an effective inhibitor of AHL molecule biosynthesis. Some antagonists of SAM, such as S-adenosylcysteine, and S-adenosylhomocysteine have shown to ensure the capacity to effectively inhibit the AHL synthesis, which is facilitated by the *P. aeruginosa* RhlI protein (Parsek et al., 1999). Further, Christensen et al. (2013) have screened the QS inhibitors to target the AHL molecule biosynthesis against Proteobacteria such as *Burkholderia mallei* and *Yersinia pestis* through high-throughput screening. Some earlier experiments have shown that macrolide antibiotics such as azithromycin and erythromycin administered at sub inhibitory concentrations have the capacity to suppress the *P. aeruginosa* AHL molecule biosynthesis and thereby inhibited their virulence factors and biofilm formation (Sofer et al., 1999; Pechere, 2001; Tateda et al., 2001).

AHL Signal Molecule Biodegradation or Alteration

Searching for enzymes capable of breaking down the AHL signal molecules is a promising strategy to eradicate the biofilm mediated bacterial infections altogether. AHL molecules are enzymatically destroyed by various forms of enzymes, eliminating AHL accumulation in the system. Generally, the enzymatic degradation or alteration of AHL signal molecules can be catalyzed by six major classes of enzymes rendering to their catalytic sites **(Figure 5)**.

The AHL lactonases are capable of opening the homoserine lactone ring by way of breaking the bond on the leftward of the double bonded oxygen. Further, the enzyme decarboxylases are also capable of doing the same by way of breaking the bond on the rightward of the double bonded oxygen without disturbing the rest of the AHL molecule structure. AiiA 24B1, the product of the *aiiA* gene from *Bacillus* spp. 24B1, hydrolyzes the lactone ring in the homoserine moiety of AHL and which is one of the first identified and well characterized AHL lactonase (Dong et al., 2000). A recent study made by Shastry et al. (2019) has revealed the biofilm inhibitory efficacy of AHL lactonase enzyme on the *Aeromonas hydrophila* biofilm formation. The AHL acylase is a family of enzyme that corresponds to the Ntn hydrolase superfamily. It hydrolyzes the AHL signal molecules, as their names imply (Utari et al., 2017). The AHL acylase enzyme was first exposed in the *Variovorax paradoxus* strain VAI-C. It hydrolyzes the amide bond between the homoserine lactone and acyl side chain in AHL molecules, releasing homoserine lactone (HSL) and free fatty acid (Leadbetter and Greenberg, 2000). Several AHL acylase enzymes were identified from different bacterial sources for their biofilm inhibitory potential (Paul et al., 2009; Christiaen et al., 2014). The acyl side chain may also be cleaved from the HSL ring by deaminase but at a different location. Deamination encodes final products as an acyl side with NH_2 and homoserine lactone with OH (Kose-Mutlu et al., 2019). The AHL oxidase catalyzes the carbon atoms oxidation in acyl chains of AHL signal molecules (Gao et al., 2013). The AHL oxidoreductases oxidize or reduce the carboxyl group of the third carbon to attack the side chain of AHL molecules. This type of enzymes does not break down the signal, but rather it alters the AHL molecule, thereby modifying the binding efficacy of receptor proteins with signal molecules (Vogel and Quax, 2019). The AHL oxidoreductase enzyme has recently been reported for its inhibitory potential on the autoinducer-2 mediated biofilm formation in Gram-negative bacterial pathogens by modifying the AHL signal molecule (Weiland-Brauer et al., 2016).

Interference With Receptor Proteins by Analog Compounds

The membrane receptors can be interrupted by binding the antagonistic molecules so that the receptors are unavailable to bind with AHL signaling molecules. If there is no signal recognition, then there is a variation in the bacterial population's physiological behavior, especially in reducing biofilm activity, less virulence, and low antibacterial tolerance. Furanone has been first identified analog compound as the potent QS inhibitor, which effectively inhibits the biofilm formation of *Staphylococcus epidermidis* (Hume et al., 2004). Further, the study of Lonn-Stensrud et al. (2009) has highlighted the antagonistic activity of furanone with a drastic interlude in the QS signaling, isolated from the marine algae, *Delisea pulchra*. The QS system of *Vibrio harveyi* was silenced by a marine strain *Halobacillus salinus,* which synthesize an antagonistic molecule

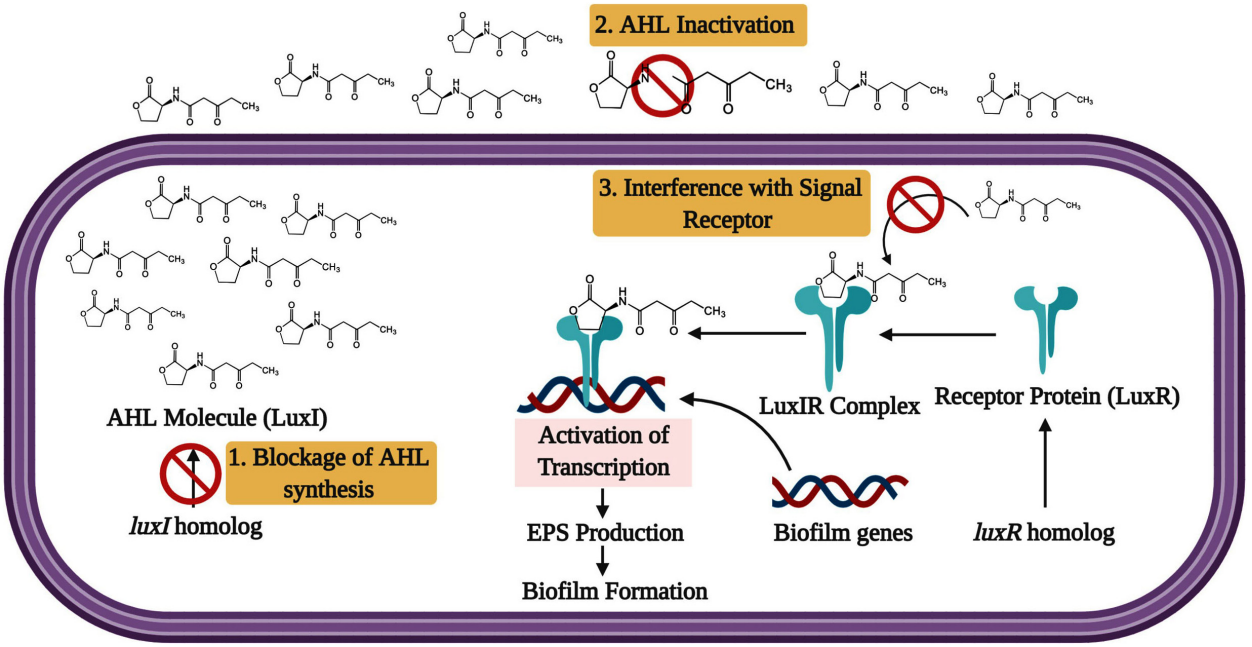

FIGURE 4 | The inhibition approaches for LuxI/LuxR QS system. The signaling molecule AHL is produced by the *luxI* synthase gene and freely diffuses from each cell. When critical concentration is reached, the synthesized signal molecules diffuse back inside the bacterial cell and binds with LuxR. Then the QS transcription is activated by the LuxR-AHL complex. The target stages for inhibition are (1) Blockage of AHL molecule synthesis, (2) Degradation of the AHL molecule, and 3. Interference with the signal receptor.

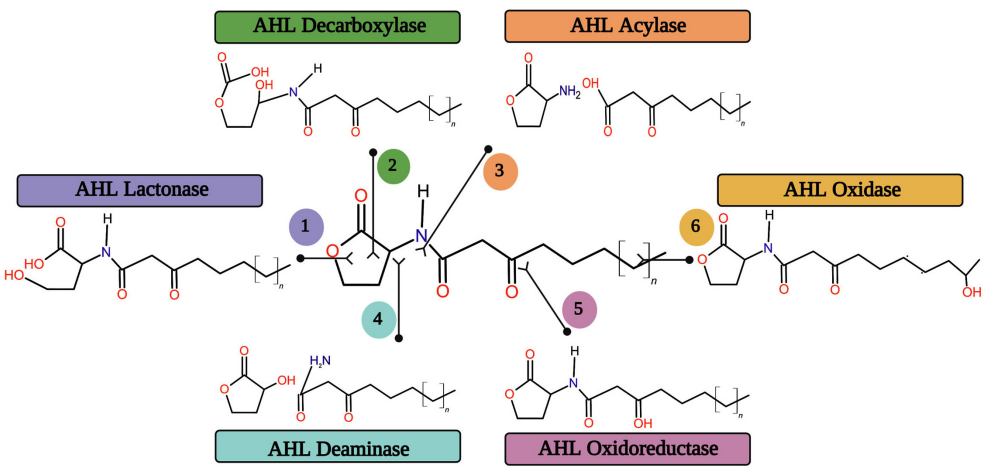

FIGURE 5 | The six major quorum quenching enzymes on the degradation or alteration of AHL signal molecule.

that suppress the activation of *lux* gene and therefore hindering the signaling molecule biosynthesis (Teasdale et al., 2009). Similarly, in yet another study by Choi et al. (2012), honaucins was synthesized by *Leptolyngbya crosbyana*, which affects cell-to-cell communication by inhibiting the QS. Furthermore, bacterial communication of QS can also be blocked by the fungal metabolite such as patulin and penicillic acid from different *Penicillium* strains, respectively. For instance, the reports of Wagner et al. (2004); Rasmussen et al. (2005); Abraham (2005) have revealed that the QS in *P. aeruginosa* could be inhibited by the penicillinic acid and patulin. The antagonistic potential of phytol on biofilm mediated infections in *Serratia marcescens* was confirmed through *in vitro* studies by Alexpandi et al. (2019) and Srinivasan et al. (2016). Further, the pre-clinical trial has been performed in a mouse model and confirmed these antagonistic molecules activity as efficient QS inhibitors. Similarly, studies made by Srinivasan et al. (2017); Arunachalam et al. (2018) have revealed the biofilm inhibitory potential of antagonistic molecules such as phytol and geraniol on *S. marcescens* associated acute pyelonephritis

infection and *S. epidermidis* associated endocarditis infection in animal models, respectively.

Apart from these three main strategies, some antimicrobial peptides could inhibit the QS system either by affecting the signal molecules transport within or outside the cell, thereby affecting the signal transduction cascade and biofilm formation. More, a newer tool in recent research is employed to block the expression of the *luxS* gene of *Escherichia coli* during QS signaling to reduce the biofilm formation by CRISPRi technology (Zuberi et al., 2017b; Sharma et al., 2019).

Biofilm Degradation by Electrochemical Method

The electrochemical method is one of the striking and promising strategies employed to put forth a great hindrance in bacterial biofilm formation. The electrochemical approach is the combinatorial effect of applying the lower dose of antibiotics in a weak electric field to disintegrate the biofilm formation or mature biofilm **(Figure 6)**, which is also denoted as the 'Bioelectric effect'. Several reports acknowledged that the electric potential lowers the antibiotics dosage to inactivate the biofilm and exerts a lethal effect on the biofilm organisms.

A contrary report has been stated by Shirtliff et al. (2005) when eliminating biofilm formation by mixed-species underwater. According to the application field, diverging hypothesis has been suggested for employing the electrochemical method to disperse the biofilm formation. The underlying principle behind electrochemical approach is that the antimicrobial binding and transport towards the biofilm matrix are enhanced due to electrostatic force under direct current and thereby it augments the efficacy of biofilm detachment (Blenkinsopp et al., 1992; Van Der Borden et al., 2004). Owing to the electric field, the media's hydrolysis occurs, resulting in the release of charged ions and hyperoxygenation with thermal stimuli (Del Pozo et al., 2008).

Usually, it is very tedious for the antibiotics to penetrate the biofilm matrix. Under the influence of the electrical field, the antimicrobial agents lead to the discharge of the biocide ions attributed to the alteration in biofilm permeability. The influx of those biocide ions into the biofilm matrix consequentially inactivates the biofilm. It destroys the bacterial cells via electrophoresis and electro-osmosis even at a low concentration (Chang et al., 1995; Stewart et al., 1999). It has been clear evidence from the study of Blenkinsopp et al. (1992) that the electric current does not have any such effect over the biofilm unless it exhibits a synergistic action with the antimicrobial agents. The hydrolysis of water and the pH change in the electrical impulses expresses the high production of oxygen molecules contributing to the improved level of minimal inhibitory concentration, thereby increases antibiotic susceptibility among biofilm and drug resistant bacteria (Borriello et al., 2004; Del Pozo et al., 2009b; Wolfmeier et al., 2018). Furthermore, the hydrated ions create an electrostatic repulsive force that aids in detachment of the biofilm from the substratum surface (Poortinga et al., 2000).

The list of other significant factors related to the bioelectric effects also depends on the voltage and electric current during the electrical stimulation as it affects the cell membrane, cellular process, behavior, and electrophysiology (Sabelnikov et al., 1991; Hancock and Rozek, 2002). Certain research suggested implementing either Alternating Current (AC) or Direct Current (DC) (Stewart et al., 1999; Del Pozo et al., 2009a) or even both (Kim et al., 2015) to produce electrical impulse for bioelectric effect with the low dosage of antibiotics. In which, the AC contributes to the direct electrostatic force, and the DC attributes to the increased permeability as a result of charged molecules vibrations. In an *in vivo* experiment of Sultana et al. (2015), a drastic reduction of the biofilm has been observed. The biofilm of *Acinetobacter baumannii* has been developed on the porcine explant, which is superimposed with the electrochemical-scaffold. When an electric field is introduced using Ag/AgCl electrodes at the constant potential of about $600mV_{Ag/AgCl}$, the biofilm formation is much reduced due to the production of H_2O_2, consequentially resulting in the synthesis of hydroxyl radicals leading to the lethal effect of the cell. In an another report using *P. aeruginosa* with a similar experimental setup employing glass-bottomed petri dishes as a biofilm model, a 10^5-fold reduction in the biofilm formation has been demonstrated (Sultana et al., 2016). The action of plasma under low current influences a decline in the EPS intensity surrounding the bacterial cells resulting in low cellular adhesion (Kovalova et al., 2016). Another innovative technique for eradicating the biofilm employing the electrochemical method is electrospray. The dispersion of liquid from the high energy potential is carried out to obtain a sterile polymer surface devoid of biofilm (Kovalova et al., 2014).

Surface Modulation of Bacterial Adhesion

There are numerous series of issues are found in marine, medical, food, and industrial fields due to biofilm formation. Especially in the marine environment, antifouling has attracted the utmost importance in recent years. Because biofouling created a severe threat in the marine environment and consequentially marine industries faced tremendous challenges. Biodeposition leads to the alteration of the nutritive source in the surrounding ecosystem, resulting in the disarray of the ecological niche in the benthic zone (Weise et al., 2009). Numerous studies have been carried out in search of innovative and novel technologies regarding the antifouling components. Antifouling compounds prevent or counteract the buildup of barnacles and other deposits, including microbial biofilms on the surface undersea. Dusane et al. (2011) demonstrated an antifouling compound from the marine strain *S. marcescens,* producing glycolipid surfactant that inhibits certain biofouling marine bacterial species such as *Bacillus pumilus*, *Candida albicans,* and *P. aeruginosa*.

Perhaps, the discovery of meticulous interaction outlay between the biofouling microbes and its substratum gives an alternative strategy against the biofouling by marine micro and macro-organisms. Modulation of the substratum or the surface to which the microbes attach is one interesting approach that has an impact over a few years in this field. Rogers et al. (1994) has sorted out various surface materials in the biofilm formation. The biocides recovered from certain microbes are

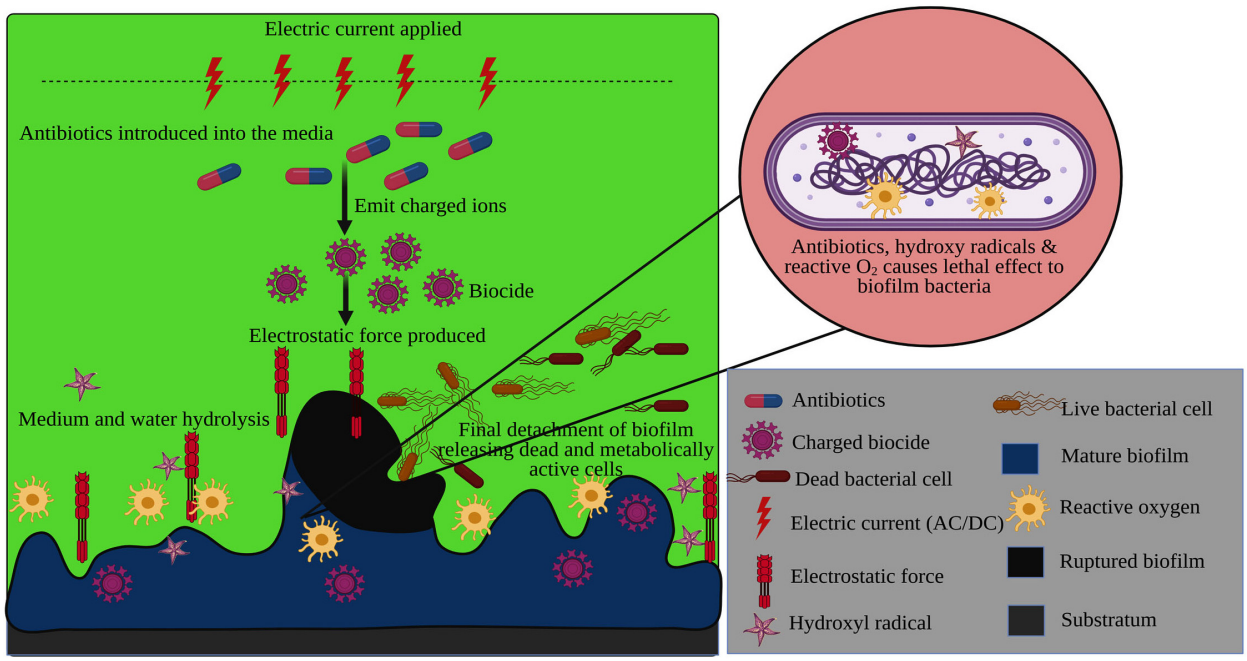

FIGURE 6 | Electrochemical degradation of bacterial mature biofilm using the electrostatic force generated by an electric current with antibiotics.

implemented to coat on the surfaces to avoid the formation of biofilms, especially in the field of medicine. Due to the toxicity and other limitations, many regulations inclusive of European Union, Biocidal Products Regulation has been laid and restricted many biocides applications (Norcy et al., 2017). Tributyltin formulations are widely used in paints in earlier days, but their usage has been prohibited owing to their toxic nature towards the marine ecosystem. Instead, natural potent biofilm inhibitors have been focused. Multispecies biofilm formed by *E. coli*, *S. aureus*, and *P. aeruginosa* have been successfully eliminated using this technology by coating silver oxynitrate effectively (Lemire et al., 2017). Further, the numerous *in vitro* studies using silver coating materials have been extensively studied by various researchers (Lansdown, 2006; Stobie et al., 2008; Lemire et al., 2015; Kalan et al., 2017).

In the case of food industries, coating of the surface using non-toxic, nonstick components like silicones and fluoropolymer derivatives are preferred, because of its ability to form non-porous surface due to the association of hydrophobicity along with low surface free energy as well as the microroughness (Sadekuzzaman et al., 2015). Other attention grabbing criteria are the absorption of nanoparticles to the surface prevents the formation of biofilm. Such implementation has a vast impact in recent years in medicine and domestic purposes like pipelines, etc. The nanoparticles attach to the microbial surface, providing them with a larger surface area and thereby react with the protein and cellular DNA, eventually resulting in the inhibition of DNA replication and gene expression (Yamanaka et al., 2005; Sadekuzzaman et al., 2015). Hetrick et al. (2009) has demonstrated the biofilm inhibitory potential of nanoparticles embedded materials towards the pathogens such as *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. epidermidis*. More, coating certain components like antibiotics, quaternary ammonium salts, polyethylene glycol, and silver ions towards the surface keeps away from bacterial colonization by weakening the cell membrane and the cellular activities (Park et al., 1998).

The application of bacteriocin is a promising approach for the control of biofilms due to their biofilm inhibitory property. Further, the biofilm formation of *Listeria monocytogenes* on the stainless steel has been destroyed using the bacteriocin derived from *Lactobacillus sakei* (Winkelstroter et al., 2011). Certain varieties of bacteriocin affect bacterial adhesion and biofilm formation even at the sub inhibitory concentration. The bovicin HC5 and nisin are the bacteriocin, which targeted the microbial cellular attachment by varying the microbial cell's hydrophobicity and the substratum at the sub-inhibitory concentration (de Jesus Pimentel-Filho et al., 2014). This study also identified that bacteriocin inhibits the expression of *icaD*, *fnbA*, *clfB*, and *rnaIII* genes related to biofilm formation in *S. aureus*. The indwelling medical device surfaces coated with bactericidal compound like aryl rhodanines has prevented the biofilm development (Chung and Toh, 2014). Hence, the surface modulation allows the prevention of bacterial adhesion towards the substratum and thereby sets an obstacle for biofilm formation.

Disruption of the Cell Membrane by Antimicrobial Peptides (AMP)

Recent research works continuously highlight that among the various biofilm inhibition strategies, the possible use of AMP, also known as host defense peptides, may denote a promising approach (Batoni et al., 2011; Jorge et al., 2012). The biofilm

is disturbed through the different AMP by the transmembrane pore mechanism, which will lead to the final condition of cell death. The study of Pulido et al. (2016) reveals that the total permeabilization effect was visualized by Confocal Laser Scanning Microscopy (CLSM) and Sytox Green permeabilization assay, where the analysis of RN3 (5-17P22-36), an AMP, at higher concentration established the permeabilizing effect in biofilm cellular population. Indeed, the AMP action over the membrane depolarization and permeabilization facilitating the antimicrobial and biofilm inhibitory activities.

Interestingly, certain AMP binds to the bacterial cells of the biofilm structure, encouraging its cellular agglutination and membrane interaction. The peptides reorganization of the membrane determinants for the lipopolysaccharides lie at the N-terminus portion on coding sequence and thus expresses a higher specificity for the affinity towards the lipopolysaccharides on the cell membrane of Gram-negative bacteria (Bhunia et al., 2009; Torrent et al., 2009). The mechanism of AMP has been described in the models named 'carpet', 'barrel-stave' or 'toroidal-pore', which highlights the factors such as cationic charge, amphipathicity, amino acid composition, and size perusing the peptide attachment, translocation, and altering membrane permeability through an alternation in cytoplasmic membrane configuration.

In yet another report, the cationic peptide interaction towards the Gram-negative bacteria occurs to the anionic surface with the lipopolysaccharides layer on the outer membrane. It disturbs the structural configuration of the cell membrane, promoting the cellular leakage causing cell death (Moore et al., 1986). Further, Zhang et al. (2000) has accepted this view, insisting that the AMP interacts to the specific divalent cationic binding site at the lipopolysaccharides of the outer cell membrane bringing up the transposition through self-promoted uptake. Peptides are rendering interaction with the cell membrane based on its charge moiety and its hydrophobic interactions. Membrane targeting peptides like RT2, KT2, and magainin II enables their hydrophobic portion to interact with the anionic moiety in the lipid head of *E. coli* cell membrane and thereby launches them in the hydrophobic core (Anunthawan et al., 2015). Therefore, the structure, shape, and design of AMP are crucial for the electrostatic interaction to interrupt cell membrane and biofilm.

Antimicrobial Lipids (AML) as Biofilm Inhibitors

Antimicrobial lipids (AML) are known as single-chain lipid amphiphiles, including fatty acids and monoglycerides (Verderosa et al., 2019). Later the 1,800s, after Koch and his colleagues first reported the growth inhibitory effects of soap, the antibacterial properties of fatty acids in soap had been identified and subsequently shown to inhibit *B. anthracis* growth, the causative agent of anthrax (Thormar, 2011). The antimicrobial potential of monoglycerides and fatty acids has been continuously exposed against various pathogens (Desbois and Smith, 2010; Desbois, 2012; Li et al., 2013; Karthikeyan et al., 2014; Wang et al., 2020). It is well understood that AML function through several pathways, such as enhanced membrane permeability, form temporary or permanent membrane pores, target the bacterial surface signal transduction system, electron transport chain destruction, cell lysis, and bacterial enzyme inhibition (Hyldgaard et al., 2012; Schlievert and Peterson, 2012; Verderosa et al., 2019).

Several studies have continuously stated the biofilm inhibitory efficacy of AML at low doses against various bacterial biofilm formations (**Figure 7**). Oh and Marshall, 1995 has first explored the biofilm inhibitory efficacy of AML (Glycerol monolaurate) against *L. monocytogenes* biofilm formation. Glycerol monolaurate (GML) has antimicrobial or immunomodulatory effects, a fatty acid composed of glycerol and lauric acid (Witcher et al., 1996; Vetter and Schlievert, 2005). GML is currently used as a dietary and cosmetic ingredient, which is generally recognized as safe (GRAS) by the U.S. Food and Drug Administration. Schlievert and Peterson (2012) also validated the biofilm inhibitory efficacy of GML against *Haemophilus influenzae* and *S. aureus* biofilm formation. They have endorsed the bactericidal effects of GML on the mature biofilm formation of both bacterial pathogens. Recently, Lopes et al. (2019) substantiates the biofilm inhibitory potential of GML nanocapsules against *P. aeruginosa* biofilm by Atomic Force Microscopy.

A study made by Harvey et al. (2019) has revealed the biofilm inhibitory efficacy of antibacterial lipids on the cariogenic organism *Streptococcus mutans* by Fluorescent Microscopy using Alexa Fluor® 647 and SYTO® 9-labeled dextran conjugate. Further, the biofilm development of Gram-positive bacterial pathogens such as *S. epidermidis, S. aureus*, and *S. mutans* has been effectively inhibited by some unsaturated fatty acids such as oleic acid, linoleic acid, and palmitoleic acid (Yuyama et al., 2020). The inhibitory efficacy of the two fatty acids includes docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) against *Fusobacterium nucleatum* and *Porphyromonas gingivalis* biofilms were recently studied by Sun et al. (2016). Obtained results of this study displayed that both DHA and EPA significantly eradicated the mature biofilm of *P. gingivalis* to the level of 61 and 47%, respectively. Further, both these two fatty acids were tested for action against *S. mutans* biofilm formation in a follow-up publication by the same research group (Sun et al., 2017). It was observed that both EPA and DHA significantly weakened the outer membrane of residing biofilm cells and thereby decreased the thickness of biofilm in *S. mutans*.

Degradation of the EPS Matrix of Biofilm

The major components of the EPS matrix of biofilm are the polysaccharides, proteins, and nucleic acids (e-DNA), and thus one of the biofilm inhibition strategies includes attack over the biofilm matrix. The biofilm degradation is possible by disrupting the EPS matrix components. The biofilm EPS matrix components vary according to the microbial strain, age, and other environmental factors like pH, oxygen tension, and nutrient abundance (Flemming and Wingender, 2010). Lipopolysaccharide, alginate, Psl (Polysaccharide synthesis locus), and Pel (Pellicle) are the major EPS of the *P. aeruginosa* biofilm (Rabin et al., 2015). Other significant mechanisms regarding the action over the dissolution of the EPS matrix

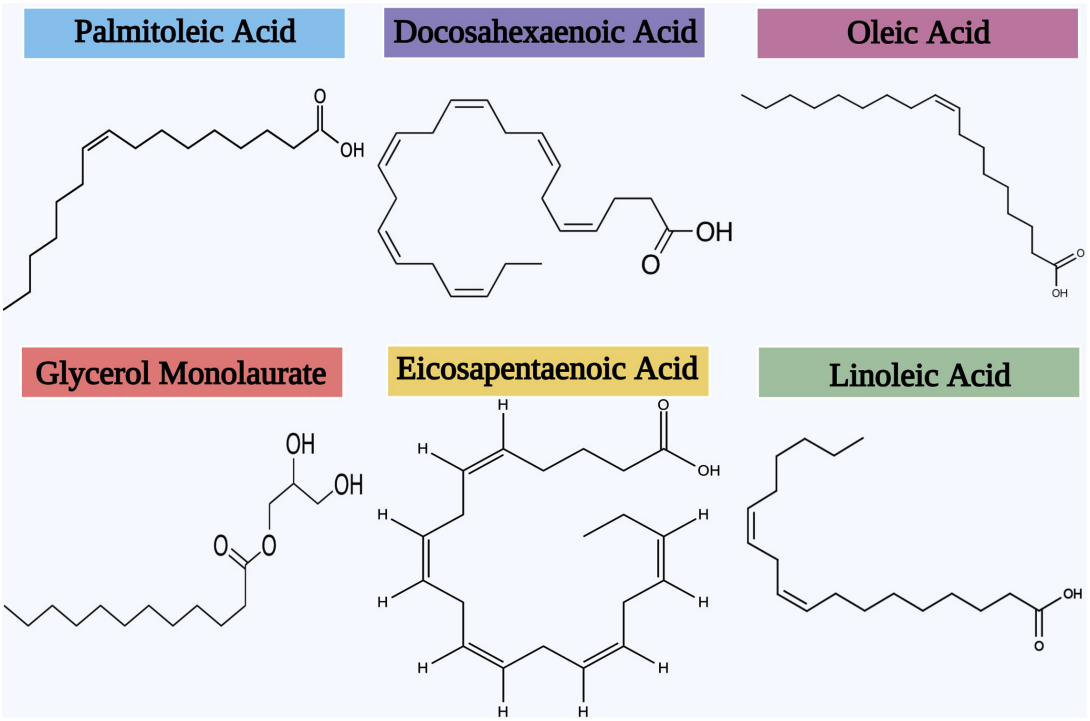

FIGURE 7 | Chemical structures of the different AML that inhibit biofilm formation.

of biofilm include alginate lyase, DNase, and hydrolase-based approach. However, Dispersin B (DspB) protein acts upon the biofilm to disperse the EPS matrix in *Actinobacillus pleuropneumoniae* (Kaplan et al., 2004b). Similarly, DspB protein derived from the *A. actinomycetemcomitans* dissolute the biofilm matrix of *S. epidermidis* (Kaplan et al., 2004a,b). Further, DspB protein also affects the linkage of the glycosidic bond in the EPS matrix's polysaccharide, and, hence the biofilm architectures are detached (Izano et al., 2007). Besides, certain phages and phage-derived enzymes like polysaccharide depolymerase can invade the EPS matrix and demolish the biofilm architecture (Sadekuzzaman et al., 2015).

The study of Powell et al. (2018) revealed the disruption of biofilm matrix through the implementation of alginate oligosaccharide (OligoG). The effect has been visualized using Lectin and ConA staining with CLSM imaging. The effects visualized by Stokniene et al. (2020) in CLSM images were similar to what was revealed in previous experiments: CLSM imaging visualized the OligoG and colistin conjugates disrupting the *P. aeruginosa* biofilm matrix. On the other hand, the interaction of divalent ions like Ca and Mg ions brings about the variation in EPS matrix through OligoG as these ions play a vital role in regulating the association of EPS and e-DNA in the mucoid matrix (Flemming and Wingender, 2010). These ions overcome the electrostatic repulsion of the negatively charged biofilm components and maintain biofilm physiology (Govan and Deretic, 1996; Whitchurch et al., 2002). It has been proved that Ca ions possess the efficacy in the stability and maintenance of the biofilm of marine species like *V. cholerae* (Kierek and Watnick, 2003). Furthermore, OligoG also influences the thickness of the biofilm (Strathmann et al., 2002).

Inhibition of Alarmone Scheme

Under stress conditions like nutrient depletion, bacteria exhibit the stringent response, which is considered to be a crucial part for the synthesis of certain molecules in the alarmone scheme such as guanidine 3′diphosphate 5′triphosphate (pppGpp) and guanidine 3′5′bis-diphosphate (ppGpp), which are jointly expressed as (p)ppGpp through the cassette of RelA and SpoT. In Eubacteria, the stringent response is regulated by the signal molecule (p)ppGpp, which is produced by RelA and hydrolyzed by SpoT (Metzger et al., 1989), especially in Gram-negative bacteria and in Gram-positive bacteria with the aid of bifunctional enzyme, Rel/Spo for both the hydrolysis and synthesis (Wendrich and Marahiel, 1997). These molecules attribute the stringent response mediation that regulates the biofilm formation in *E. coli* and *S. mutans* (Lemos et al., 2004; Aberg et al., 2006).

The AMP 1,018 degrades alarmone ((p)ppGpp) signal by acting as the biofilm inhibitor. The biofilm inhibitory efficiency of the peptides 1,018 has been reported in earlier study (De La Fuente-Nunez et al., 2014). In the same study, the genes conferring the synthesis of alarmone (p)ppGpp signal in *P. aeruginosa* have been identified as *relA* and *spoT*. The gene expression and repression suggesting their part in the biofilm formation, maintenance, and outcome of the downregulation of those genes have also been documented through the *in vitro* studies of mutant deficient with the alarmone (p) ppGpp signal.

The peptide 1,018 has more potential than the conventional antibiotics as it only suppresses the synthesis of alarmone (p) ppGpp signal rather than degradation. In addition, certain antibiotics have also been reported to affect the alarmone scheme, which eventually acts upon the alarmone (p) ppGpp signal synthesis, leading to adaptive resistance (Ikehara et al., 1985; Gilbert et al., 1990).

The exclusive investigation of He et al. (2012) provides insight into the regulation of the biofilm forming genes during stringent response in *V. cholerae*. It has been confirmed that due to the insufficiency in the synthesis of alarmone (p) ppGpp synthases, the shortage in the biofilm formation occurs. The stringent response is liable for certain regulatory factors responsible for the expression of *relA*, *spoT*, and *relV* in *V. cholerae*. It has been proved that the regulation of alarmone (p)ppGpp signal system of *vpsR* and *vpsT* in *V. cholerae* is accomplished through the transcriptional factor *rpoS*. Indeed, the sole source of alarmone (p) ppGpp synthase essential for the activation of the biofilm formation gene *vpsT* as it depends on RelA. The investigation of a novel compound named relacin has been designed to hold back the synthesis of alarmone (p)ppGpp signal molecules by inhibiting the RelA as it prompts the stringent response (Wexselblatt et al., 2012). During environmental stress like nutrient starvation, RelA induces the stringent response further affected by relacin and thereby down-regulated the biofilm formation in bacteria. Due to the reduction in alarmone (p) ppGpp synthesis, there is a low level of inosine monophosphate dehydrogenase synthesis, leading to low GTP consequences. The pyrophosphate's ribosomal transfer describes the alarmone (p) ppGpp signal synthesis from ATP to GTP/GDP. Thus the relacin plays a major role in the biofilm formation pathway and other developmental pathways in *B. subtilis*.

Other peptides like LL-37, derived from natural peptides like human cathelicidin and 1,037, a synthetic cationic peptide, have also been reported for their biofilm inhibitory activity in earlier studies (Overhage et al., 2008; De La Fuente-Nunez et al., 2012). The other peptide includes the protease-resistant D-enantiomeric peptides DJK-5 and DJK-6 are noteworthy in describing the antagonistic activity against the biofilm formation in *P. aeruginosa* through inflowing into the biofilm cells and by degrading the intracellular nucleotides (p) ppGpp even at the low concentration range of 0.5–0.8 μg/mL below the MIC (De La Fuente-Nunez et al., 2015). Further, their variation and similarity in structure, function, and association are attributed to immune-modulatory, antimicrobial, antibiofilm, and anticancer properties. The AMP, which exerts a distinctive mode of action other than the conventional antimicrobial agents, are suggested for the biofilm inhibitory activity by hindering the alarmone system.

Enzyme Mediated Biofilm Control

Certain enzymes are mediating the disruption of the biofilm of various bacterial species. Nevertheless, the bacteria themselves would synthesis certain endogenous matrix-degrading enzymes like glycosidases, proteases and DNase as it may induce the dispersion of the biofilm. DNase has been known for its biofilm inhibitory activity against Gram-positive and Gram-negative bacteria (Tetz et al., 2009). Further, DNase involve in breaking down of phosphodiester linkage in the backbone of e-DNA molecules in the formed biofilm, as the e-DNA is essential for the initial attachment and aggregation of the EPS onto the surface to make it an intact biofilm for a more extended period. Moreover, the e-DNA is one of the necessary factors for the biofilm formation, stability, and regulation (Das et al., 2010; Periasamy et al., 2012; Chagnot et al., 2013).

The synergistic action of DNase with metronidazole antibiotic established the disintegration of the biofilm formed by *Gardnerella vaginalis* at 100 μg/mL concentration. The report of Eckhart et al. (2007) also provided evidence for the suppression of biofilm through DNase in *P. aeruginosa* and *S. aureus*. For the first time, Monnappa et al. (2014) have been reported the extracellular protease and DNase from a host-independent *Bdellovibrio bacteriovorus* restrain the biofilm of Gram-positive bacteria especially, *S. aureus*. Furthermore, DNase (NucB) used as the feasible tool for dispersing e-DNA within the biofilm's EPS matrix has been depicted by Nijland et al. (2010). Similarly, the study of Bugli et al. (2016) suggested the concentration of 100 mg/L DNase can able to eliminate the biofilm formed by *Helicobacter pylori*, while Torelli et al. (2017) gives insight on the DNase and alginate lyase as effective in disintegrating the biofilm of *Enterococcus faecalis* and *E. faecium* by acting over the EPS matrix of biofilm.

A significant biofilm inhibitory effect of proteases has been extensively described by Mukherji et al. (2015). The study of Hangler et al. (2009) discloses that Esperase HPF, a protease efficacy in preventing the biofilm of the species such as *Dokdonia donghaensis, Shewanella japonica*, *Microbacterium phyllosphaerae*, and *A. lwoffi*. Further, the commercially available proteases such as proteinase K, trypsin, and chymotrypsin (serine proteases category), and serratiopeptidase, carboxypeptidase A (metalloproteases category) have been reported for their biofilm inhibitory potential against *Staphylococcus* spp. (Artini et al., 2013). Craigen et al. (2011) has revealed the inactivation and removal of biofilm formation by *S. aureus* with the aid of alpha-amylase. Alginate lyase is a potent enzyme involved in the dissolution of biofilm of certain bacteria (Germoni et al., 2016). Many studies such as Rahman et al., 2010; Singh et al., 2011; Wang et al., 2013; Daboor et al., 2019 have indicated that the marine bacterium serves as a renowned source for the production of enzyme alginate lyase, as a powerful agent in disassembling the biofilm exerted by pathogens. It may be related to the fact that marine bacteria are exposed to substantial quantities of the alginate present in their surroundings (Zhu et al., 2016). The enzyme alginate lyase is recovered from the marine bacterium *Pseudoalteromonas* active in exhibiting biofilm inhibitory activity against *P. aeruginosa*, *E. coli*, and *S. enterica* (Dheilly et al., 2010; Dufourcq et al., 2014).

Mechanical Eradication of Biofilm Formation Through a Photodynamic Approach

The photodynamic approach is the recent innovative method adapted for the disintegration of biofilms. The underlying

principle behind this approach is the implementation of photosensitizing molecules that absorb the light intensity of a specific wavelength and, by binding to the target cellular components like lipid, protein, and nucleic acid. It produces reactive oxygen radicals, which in turn will give rise to hydrogen peroxide, hydroxyl radicals and superoxide anion resulting in the lethal/toxic effect to the target. There are the latest attempts for evidencing biofilm inhibition in this regard (Darmani et al., 2018; Gholibegloo et al., 2018). The photosensitizer and the light source implemented are crucial for this kind of mechanical eradication of biofilms through photodynamic therapy. The frequently employed photosensitizer includes methylene blue, toluidine blue, or toluidine blue O. Other photosensitizer comprises 23H-porphine, tetrakis (1-methyl-pyridino)-21H, tetrakis (phenylthio)-29H, 31H-phthalocyanine, and tetra-p-tosylate salt (Collins et al., 2010; Junqueira et al., 2012; Sadekuzzaman et al., 2015).

The absorption spectrum of methylene blue is at 664 nm, while toluidine blue is at 638 nm and lies within the UV-visible range of 600–1000 nm wavelength. Though methylene blue photosensitizer is effective against various bacterial pathogens, it has more potential to eliminate Gram-positive bacteria. It is due to the reason that it constitutes additional effectiveness in transferring the negative charge towards the target cell with a negative charge cell wall owing to the presence of teichoic acid (Mirouze et al., 2018; de Oliveira et al., 2019). Although light emission is accomplished through the diode laser while employing toluidine blue as photosensitizer (Zanin et al., 2006), the study of Soukos et al. (2000) has reported that the light emission from helium/neon laser light employing toluidine blue as a photosensitizer in eradicating the 95% of oral biofilm formation. Furthermore, Andrade et al. (2013) has suggested that pre-radiating time over the target influences the elimination of the microorganism. Considering future viewpoints, more attempts have to be made to establish the complete knowledge and detailed mechanism of action for successful photodynamic therapy in eradicating bacterial biofilm formation.

Interruption/Down-Regulation of Molecular System of Biofilm Formation

Transcriptional regulation of a gene is far most important for gene expression to occur. The regulatory proteins and regulatory binding sites are also promising factors for regulatory machinery. The detailed study regarding gene expression profiling will pay for the appropriate up-regulation or down-regulation mechanism. Considering such approach, the biofilm formation can be interrupted or blocked through the downregulation of biofilm synthesis genes via certain components. Nal-P-113 is an AMP that acted against the biofilm formation of *P. gingivalis* W83 by deregulating the genes that confer biofilm formation, thereby controls the infection (Wang et al., 2017). These authors evaluated and described that when the gene *PG0282* and *PG1663* gets down-regulated through the mediation of Nal-P-113 peptide, it directly influences the ABC transporter, which plays a critical role in the biofilm formation machinery.

Upregulation of the ABC transporter is necessary for the initial biofilm formation when evaluating the *potB* gene coded for ABC transporter in *P. putida* (Sauer and Camper, 2001). This view has also been accepted by Hinsa et al. (2003). Later they have evaluated the association between the biofilm formation and the ABC transporter system. Though *lapEBC* cassette or lap gene encoded for the ABC system, the mechanism and the genes coding vary between species and strain. Furthermore, down-regulation of certain genes reduces the biofilm formation, as narrated in a few studies (Whiteley et al., 2001; Overhage et al., 2007). The study of De La Fuente-Nunez et al., 2012 highlighted the genes conferring swimming and swarming motility to be down-regulated to influence biofilm elimination using the AMP 1,037.

Several earlier studies also illustrated that the down-regulation of certain genes including *flgB*, *nirS*, *norC*, and *nosZ* (which are responsible for anaerobic biofilm formation), *fimX* (encoding for Type IV pilus assembly), *rhlB* (regulating QS and involved in the rhamnolipid synthesis), *lecB* (involved in the fucose binding of lectin) hinders the biofilm formation in different bacterial pathogens (Schuster et al., 2003; Johansson et al., 2008; De La Fuente-Nunez et al., 2012). The novel gene *rsaL* has been earlier demonstrated by De Kievit et al. (1999), which involved in the synthesis of the harmful regulatory protein that impacts the suppression of *lasI* gene expression. The gene product of *rsaL* has a negative transcription regulation of *lasI* operon in *P. aeruginosa*, thereby obstructing the QS cascade, leading to biofilm inhibition. The other approaches include altering the QS signaling cascade to prevent the biofilm formation is CRISPRi technology for deregulating the *luxS* gene expression (Seed et al., 1995; Lonn-Stensrud et al., 2009; Zuberi et al., 2017b), which is involved in QS signaling and fimbriae associated gene (*fimH*) expression, so that, it has an influencing effect to suppress the biofilm formation (Zuberi et al., 2017a).

CONCLUSION AND FUTURE PROSPECTIVE

In nature, several bacteria live in the form of biofilms. For the medical community, biofilms constitute a serious problem since not only they are linked with numerous infections in humans, but they are often particularly challenging to handle, because of their tolerance to antibiotics and immune responses. Biofilm mediated infections are difficult to control due to their complexity and increasing antibiotic resistance. It is necessary to prevent their surface colonization to restrict biofilm development, as this is the first step in the formation of biofilms. In this review, we have discussed several emerging strategies and potential perceptions for developing enhanced therapeutics to control biofilm mediated bacterial infections. The alternative approaches for preventing biofilm formation in the medical devices or marine environment are also explored in depth, with special emphasis on surface modulation of bacterial adhesion. Overall, all these

recent therapeutic biofilm inhibition strategies can open up new prospects for controlling biofilm development in diverse sectors. Future prospective of improved biofilm eradication strategies may aim to commercial intake of certain biofilm inhibitors like enzymes, AMP, AML, and QS inhibitors would make it possible as a tremendous tool to achieve the target. Nevertheless, in-depth research is necessary to clarify the effect of these biofilm inhibitors during biofilm infection in the host and prove their applicability to humans. Meanwhile, biofilm inhibitors may not cause antibiotic resistance; they have a lot of promise in the future for treating biofilm based infections in healthcare settings.

AUTHOR CONTRIBUTIONS

RS, SS, and LX conceived the concept of the review. RS, SS, PP, and MG drafted the manuscript. RS prepared the figures. RS and LX coordinated the work and acquired funding. RS, SS, MD, and LX reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Candida albicans Biofilm Inhibition by Ethnobotanicals and Ethnobotanically-Synthesized Gold Nanoparticles

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The virulence and drug resistance of globally prevalent *Candida albicans* has presented complications toward its control while advances in effective antivirulence drugs remain critical. Emerging methods are now being evaluated to facilitate development of novel therapeutic approaches against this pathogen. This study focuses on the biofilm formation inhibition of ethnobotanical crude extracts and the use of nanotechnology through the ethnobotanically-synthesized gold nanoparticles to control *C. albicans*. Control on biofilm formation was compared using crude extracts (CEs) and biologically synthesized gold nanoparticles (CEs + AuNPs). Significantly lower biofilm formation was exhibited in thirteen (13) CEs and fourteen (14) CEs + AuNPs. Biofilm-linked genes *Bcr1* and *HSP90* expression were consequently downregulated. Higher biofilm inhibition activity was noted in some CEs + AuNPs compared to its counterpart CEs. This study emphasizes the biofilm inhibition activity of ethnobotanicals and the use of nanoparticles to enhance delivery of compounds, and points to its prospects for developing anti-pathogenic drugs without evolving resistance.

Keywords: *Candida*, biofilm, quorum sensing, gold nanoparticles, ethnobotanicals, *Bcr1, HSP90*

INTRODUCTION

Candida albicans is a globally prevalent pathogen owing to its capability to survive at diverse biotic and abiotic sites (Mathe and Van Djick, 2013; Gao et al., 2016; Lee et al., 2018) with the ability to cause an array of infections that ranges from superficial to life threatening. Candida infections, commonly known as candidiasis or candidosis (Douglas, 2003; Moran et al., 2012), can occur as a consequence of its ability to develop a biofilm, a quorum sensing (QS) mechanism associated with its pathogenicity (Silva et al., 2011; Pfaller, 2012). The complexity of its biofilm offers virulence through its three-dimensional structure and innate drug resistance (Pereira et al., 2020) while withstanding host immune responses (Lee et al., 2018), thus, requiring multifaceted scheme for its control.

This high level of tolerance to multiple drugs (Duo et al., 2010; Gao et al., 2020) contribute complications to control this pathogen, and this pose serious threats to healthy and medically

compromised individuals that often lead to severe fatal infections (Hall-Stoodley et al., 2004; Lohse et al., 2018). Control of *Candida* through commercial antifungal drugs such as triazole drugs and echinocandins (Cruz et al., 2007; Peman et al., 2009) is practiced, but not without the consequences of developing fungal resistance and reduced susceptibility. Administration with these antifungal drugs inevitably produce evolving strains throughout long-term treatment (Cruz et al., 2007), hence, the increasing incidence of drug-resistant *Candida*.

To address emerging virulence and fungal resistance, Quorum Sensing (QS) mechanisms are now targeted. Quorum sensing is a cell-to-cell communication system that coordinate virulence and gene regulation through specific signal molecules that enable bacteria to adapt to changing conditions. Targeting this QS system reduces microbial virulence without disabling growth, thereby counteracting microbial resistance (Maeda et al., 2012; Zhong and He, 2021) brought about by selective pressure through overuse and mishandling of antipathogenic drugs (Borges and Simoes, 2019; Lewis, 2020; Zhong and He, 2021). Current therapies are limited and this situation has paved for the discovery of new antipathogenic agents (Duo et al., 2010). Recent studies have shown that plant metabolites offer essential agents to target drug-resistant microorganisms (Lee et al., 2018).

Among the promising sources of QSI (Quorum Sensing Inhibition) agents is a group of unexplored plants, the ethnobotanicals. These are plants that are utilized by ethnic groups for the treatment of diseases. Majority of the ethnic communities are geographically isolated and mostly depend on natural products for their medicines. One of the diverse ethnic communities in the Philippines is the *Ilongot-Eǵongot*. As one of the significant ethnic groups in the Philippines, they largely reside in the biologically diverse areas of Aurora, in the island of Luzon, Philippines. These areas comprise a deep, vast source of plants for medicinal use. Their use of ethnobotanicals, typical of the other ethnic tribes, are transferred from one generation to another, and thus, of cultural importance. Until recently, ethnobotanical evaluations are limited and this represents a recent group of plants that have gained interest for evaluating antipathogenic sources. The prospects of discovering natural QSI compounds from ethnobotanicals are evident in few existing researches that provide scientific validation of its potential use. These plants are powerful sources of natural QS inhibitors essential for the development of safe, novel therapeutic and antipathogenic agents (Fernando and Judan Cruz, 2020). Recently, the *Ilongot-Eǵongot* ethnobotanicals evaluated in this study have been demonstrated to possess QS inhibition properties against pathogenic bacteria such as *Pseudomonas aeruginosa* (Velasco et al., 2020; Santos et al., 2021), *Staphylococcus aureus* (Salamanca et al., 2019), *Aeromonas hydrophila* (Fernando and Judan Cruz, 2020), and *Streptococcus agalactiae* (Fernando et al., 2020). QSI actions of these plants against pathogenic fungi such as *Candida* have not yet been explored.

The formation of biofilm in pathogens is mediated by a network of genetic mechanisms. Among the key genes that are linked to biofilm adhesion, dispersion and regulation in *C. albicans* are the *Bcr1* and *Hsp90*. Expression of these genes impacts the formation and quality of the biofilm. *Bcr1* is among a system of transcription regulators that facilitates the formation of biofilm in *C. albicans* (Nobile et al., 2006; Mayer et al., 2013). As a transcription regulator, *Bcr1* directs functionally associated target genes that can eradicate a function that is carried out by redundant genes (Fanning et al., 2012). *Bcr1* and its downstream genes are expressed during adhesion of *C. albicans* on the substrate (Nobile et al., 2006) and this adhesion impacts the arrangement of the polysaccharide matrix (Douglas, 2003). The heat shock proteins (HSPs) unique to fungi and not present in humans have surfaced as a promising drug target for *C. albicans* management (Mayer et al., 2013). *HSP90,* a major HSP, is a key regulator in biofilm formation and virulence by suppressing dispersion (Robbins et al., 2011) and intricate cell signals (Pearl and Prodromou, 2006; Shapiro et al., 2009). *HSP90* also controls temperature- dependent morphogenesis by suppressing *cAMP-PKA* signals (Robbins et al., 2011). It also allows for the emergence of resistance to majority of existing antifungals (Robbins et al., 2011). Downregulation of these genes affects the formation, adherence and dispersion of the complex biofilm and its multi- dimensional polysaccharide matrix (Douglas, 2003). Hence, by negatively affecting *Bcr1* and *Hsp90* expression, fungal communication will be inactivated and consequently, virulence (Rasmussen and Givskov, 2006).

For a more efficient delivery of anti-pathogenic drugs from the natural metabolites, nanotechnology has gained substantial interest and relevance in drug design. Nanoparticles are used in drug delivery for an efficient transport of soluble drugs (Kamat et al., 2002; Daniel and Astruc, 2004) targeted to a specific site and bioavailability. The use of biosynthesized nanoparticles to enhance treatment of diseases increases the relay of drugs and subsequently enhances treatment of diseases due to their reduced dimensions, its efficiency due to their extremely small size and large relative surface area (Hentzer et al., 2003; Srisawat, 2007; Khatami et al., 2017).

This study evaluated the QSI properties of the ethnobotanical crude extracts as well as the biosynthesized nanoparticles using the *Ilongot-Eǵongot* ethnobotanicals to control biofilm formation and QS-related gene expression.

MATERIALS AND METHODS

Collection of Plant Samples and Ethanol Extraction Procedure

Ethnobotanicals surveyed by Balberona et al. (2018) at the *Ilongot-Eǵongot* community of Maria Aurora, Aurora, Philippines were evaluated. Necessary permits from the provincial and tribal chieftains as well as from the Department of Natural Resources (DENR), Philippines were obtained for the collection of plant samples. Voucher specimens were identified by an expert taxonomist and deposited at the Department of Biological Sciences, Science City of Muñoz, Nueva Ecija, Philippines. Plant samples were collected, sterilized, air-dried and ground. Fifty grams (50 g) of ground leaf were soaked in 500 ml of 80% ethanol in a covered flask for 72 h and was filtered. The alcohol was removed through a rotary evaporator. The crude extracts were sterilized by centrifugation of the mixture

at 10,000 × g for 30 min followed by membrane filtration using Acrodisc 25 mm Syringe Filter. The sterile extracts were kept at 2–8°C prior to use (Srisawat, 2007). Plant extracts evaluated were: *Hydrocotyle vulgaris* (leaf), *Eluesine indica* (root), *Eluesine indica* (leaf), *Mikania micrantha* (leaf), *Dillenia philippinensis* (leaf), *Dillenia philippinensis* (bark), *Ceiba pentandra* (leaf), *Cymbopogon winterianus* (leaf), Senna alata (leaf), *Urena lobata* (leaf), *Premna odorata* (bark), *Premna odorata* (leaf), *Stachytarpeta jamaicensis* (leaf), *Diplazium esculentum* (leaf), and *Phyllanthus urinaria* (leaf).

Biological Synthesis of Gold Nanoparticles

Gold chloride (Sigma Aldrich) was prepared at the 10^{-3} M concentration with sterilized Milli-Q (Merck) water. For the synthesis, 5 ml of crude extract was mixed with 45 ml 10^{-3} M gold chloride. The bottles were incubated in dark condition under room temperature with consistent stirring through a magnetic stirrer for 60 min until a pink red color was attained. This change in color indicates the synthesis of AuNPs. To assess the stability of nanoparticles, the AuNPs obtained from the solution were purified by centrifugation at 4,000 rpm for 20 min and dispersed in deionized water. The water suspended NPs were frozen at 30°C overnight and were kept under vacuum for 24 h for drying.

Preparation of Fungal Culture

Pure culture of *C. albicans* ATCC 9002 was obtained from UPLB BIOTECH, Los Baños, Laguna, Philippines. Corn meal agar was used to revive and maintain cultures of *C. albicans*. For the subsequent assays, 24 h fungal culture was suspended in sterile saline solution (0.9% NaCl) at 26–30°C and the turbidity was adjusted to 1.0 M McFarland standard.

Analysis of Antifungal Activity of Crude Extracts (CEs) and Biologically Synthesized Gold Nanoparticles (CEs + AuNPs) Against *Candida albicans*

As a pre-screening for the biofilm formation assay, antifungal activity was assessed. The absence of zone of inhibition is required for the subsequent biofilm formation assay to rule out antifungal-mediated decrease in virulence factor production (Fernando and Judan Cruz, 2020; Velasco et al., 2020). Samples with antifungal activities shall not be included in the biofilm formation assay. The protocol of Fernando and Judan Cruz (2020) was used with some modifications. Sterile paper discs (5 mm) were soaked and air dried on sterilized petri plates under a biosafety laminar flow. Prepared media of corn meal agar on petriplates were swabbed with fungal culture. Air dried discs with treatments were seeded on plates; Ketoconazole served as positive control whereas sterile distilled water served as negative control. Plates were incubated at 37°C for 24–48 h and were observed for the appearance of the zone of inhibition.

Microtiter Plate Biofilm Formation Assay

The effect of CEs and CEs + AuNPs on biofilm formation was measured using a microtiter plate assay. Overnight cultures of *C. albicans* with a volume of 180 μl were added with 20 μl of each treatment and were transferred to 96-well microtiter plates and incubated at 37°C for 40 h without shaking. After the incubation period, the microtiter plates were washed with sterile distilled water five times to discard planktonic cells. These were air dried for 45 min and stained with 150 μl of 1% crystal violet for 45 min (Fernando and Judan Cruz, 2020). Plates were rinsed with sterile distilled water five times.

To quantify the biofilm, 200 μl of 95% ethanol was added to destain the wells. From each well, 100 μl were transferred to a new microtiter plate. Optical Density (OD) values were determined at 595 nm (Djordjevic et al., 2002) using UV-visible spectrophotometer (Biotek Instruments, Inc., United States).

Gene Expression Analysis

Treatments with significant inhibition in biofilm formation were subjected to gene expression analysis. RNA extraction was done following the RNA extraction kit manufacturer's protocol (Promega Corp.) with modifications. The expression of *HSP90* and *Bcr1* in *C. albicans* was determined through qRT-PCR analysis using Bio-Rad CFX96 Real-Time System Thermal Cycler and KAPA One Step RT-PCR kit (KAPA Biosystems). The specific primers used were: *HSP90*F 5′ CGATGAATATGCCATGACT, *HSP90*R 5′ TCCATAGCAGATTCTCCAG 3′ (Mi-Kyung et al., 2004); *Bcr1*F 5′ GGCTGTCCATGTTGTTGTTG 3′, *Bcr1*R 5′ GAGCACGCATCTATGGCTTA 3′ (Alves et al., 2000); and the internal standard *16S*F 5′ATGGCCGTTCTTAGTTGGTG 3′, *16S*R 5′ GCCAAGGCTTATACTCGCTG 3′ (Zhang et al., 2000). The qRT-PCR program was as follows: incubation at 42°C for 5 min for reverse transcription; 95°C for 1 min; followed by 45 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 20 s (Nailis et al., 2006).

Statistical Analysis

Significant differences in OD values were analyzed via independent sample Tukey's Honest Significant Difference Test with 0.05 level of significance. For the gene expression analysis, Ct values were analyzed using the $2^{-\Delta\Delta CT}$ (Livak) method (Livak and Schmittgen, 2001). The statistical analysis for the gene expression was performed with the use of Kruskal-Wallis test (non-parametric ANOVA).

RESULTS

Antifungal Activity of CEs and CEs + AuNPs Against *C. albicans*

None of the CEs and CEs + AuNPs showed antifungal activity against *C. albicans*, hence, all samples were evaluated for its effects on biofilm formation.

Inhibitory Effect of CEs and AuNPs on Biofilm Formation of *C. albicans*

The optical density (OD) values of the *C. albicans* clinical isolate culture treated with 13 CEs namely *H. vulgaris* leaf (0.065 mg/ml); *M. micrantha* leaf (0.062 mg/ml); *C. pentandra*

leaf (0.066 mg/ml); *C. winterianus* Leaf (0.063 mg/ml); *S. alata* (0.063 mg/ml); *U. lobata* leaf (0.065 mg/ml); *D. philippinensis* leaf (0.080 mg/ml); *P. odorata* bark (0.060 mg/ml); *S. jamaicensis* leaf (0.067 mg/ml); *E. indica* roots (0.067 mg/ml); *D. esculentum* (0.083 mg/ml); *E. indica* L. leaf (0.067 mg/ml); and *P. urinaria* L. (0.067 mg/ml) showed significantly lower OD values in biofilm formation compared to the negative control (no extract) with a value of 0.19 mg/ml (**Table 1**). In contrast, two (2) CEs showed significantly higher OD values when compared to the control: *D. philippinensis* (0.12 mg/ml) bark and *P. odorata* leaf (0.19 mg/ml). These showed no QSI activity with increased formation of biofilm.

The OD values of 14 CEs + AuNPs showed significant decrease in *C. albicans* biofilm formation compared to the control (**Table 1**) with the following values: *H. vulgaris* (0.080 mg/ml); *M. micrantha* leaf (0.067 mg/ml); *D. philippinensis* bark (0.067 mg/ml); *C. pentandra* leaf (0.067 mg/ml); *C. winterianus* leaf (0.067 mg/ml); *S. alata* (0.063 mg/ml); *U. lobata* leaf (0.065 mg/ml); *P. odorata* bark (0.082 mg/ml); *S. jamaicensis* leaf (0.074 mg/ml); *E. indica* (0.067 mg/ml); *P. odorata* leaf (0.072 mg/ml); *D. esculentum* (0.073 mg/ml); *E. indica* leaf (0.067 mg/ml); *P. urinaria* leaf (0.071 mg/ml).

Downregulation of *Bcr1* and *HSP90* as Affected by CEs and CEs + AuNPs

The CEs and CEs + AuNPs that showed significantly lower biofilm formation were subjected to gene expression analysis. *Bcr1* expression analysis showed down regulation in all CEs and CEs + AuNPs treatments with significantly lower biofilm formation in the virulence assay (**Figure 1**). The expression of *Bcr1* in *C. albicans* was significantly downregulated in association with the CEs of *H. vulgaris* (0.071), *M. micrantha* leaf (1.036), *C. pentandra* leaf (3.033), *C. winterianus* Leaf (0.403), *S. alata* (7.459), *U. lobata* leaf (0.292), *D. philippinensis* leaf (0.485), *P. odorata* bark (3.792), *S. jamaicensis* leaf (0.87), *E. indica* roots (0.437), *D. esculentum* (0.115), *E. indica* leaf (0.213), and *P. urinaria* leaf (3.772) as compared to the control with no plant extract used (15.44). Significant downregulation of the *Bcr1* gene was also observed in CEs + AuNPs of *H. vulgaris* (0.711), *M. micrantha* (10.496), *D. philippinensis* bark (4.567), *C. pentandra* leaf (0.223), *C. winterianus* Leaf (12.898), *S. alata* (0.799), *U. lobata* leaf (0.490), *P. odorata* bark (0.161), *S. jamaicensis* leaf (0.780), *E. indica* roots (0.140), *P. odorata* leaf (0.835), *D. esculentum* (0.086), *E. indica* leaf (0.87), and *P. urinaria* (0.87) (**Figure 1**).

HSP90 also showed down regulation in *C.albicans* treated with CEs and CEs + AuNPs that showed lower biofilm formation values. The expression of *HSP90* was significantly downregulated in association with the CEs of *H. vulgaris* (0.16), *M. micrantha* leaf (0.679), *C. pentandra* leaf (1.473), *C. winterianus* leaf (0.288), *S. alata* (21.274), *U. lobata* leaf (0.683), *D. philippinensis* leaf (0.396), *P. odorata* bark (0.289), *S. jamaicensis* leaf (0.246), *E. indica* roots (0.350), *D. esculentum* (0.283), *E. indica* leaf (0.248), and *P. urinaria* (0.221) as compared to the control with no plant extract used (23.056) (**Figure 2**). Significant downregulation of the *HSP90* was also observed in CEs + AuNPs of *H. vulgaris* (0.099), *M. micrantha* (0.277), *D. philippinensis* bark (1.640), *C. pentandra* leaf (0.523), *C. winterianus* Leaf (0.674), *S. alata* (21.161), *U. lobata* leaf (0.463), *P. odorata* bark (0.024), *S. jamaicensis* leaf (0.287), *E. indica* roots (0.476), *P. odorata* leaf (0.115), *D. esculentum* (0.462), *E. indica* leaf (0.407), and *P. urinaria* (0.377) (**Figure 2**).

TABLE 1 | Biofilm inhibition in *C. albicans* as affected by CEs and CEs + AuNPs.

Scientific name	Crude extract	Biologically synthesized gold nanoparticles
H. vulgaris	0.065*[a]	0.080*[b]
M. micrantha	0.062*[a]	0.067*[b]
D. philippinensis (bark)	0.116	0.066*
C. pentandra	0.066*	0.066*
C. winterianus	0.064*	0.066*
S. alata	0.063*	0.062*
U. lobata	0.065*	0.065*
D. philippinensis (leaves)	0.080*	0.135
P. odorata (bark)	0.06*[a]	0.082*[b]
S. jamaicensis	0.067*	0.074*
E. indica (roots)	0.066*	0.068*
P. odorata (leaves)	0.186	0.072*
D. esculentum	0.083*[a]	0.072*[b]
E. indica (leaves)	0.067*	0.070*
P. urinaria	0.067*	0.071*
Control	0.190	0.190

In columns, (*) indicates significantly lower O.D. value in biofilm formation compared the control; Different letter superscripts among rows indicate significant difference.

DISCUSSION

Inhibition of biofilm formation by the ethnobotanical CEs and CEs + AuNPs in this study may be attributed to the presence of known QSI agents that are recognized to negatively affect signal receptors (Kalia, 2013) responsible for the functional communication between adjacent cells (Miller and Bassler, 2001). The *Ilongot-Egongot* ethnobotanicals evaluated in this study are known to possess active groups of metabolites against QS such as: flavonoids, saponins and tannins in *C. pentandra* (Friday et al., 2011); flavonoid, saponins, tannins, alkaloids, and geranoil in *C. winterianus* (Anosike et al., 2012); *S. alata* contains flavonoid, saponins, tannins, alkaloids, and terpenes (Sule et al., 2011), *U. lobata* with saponins, tannins, alkaloid, and terpenoid (Fagbohun et al., 2012), and *P. odorata* with flavonoid, saponins, and terpenoids (Chichioco-Hernandez and Paguigan, 2009). The isolated terpene and sterol compounds in *C. pentandra* attenuated virulence factors in *P. aeruginosa* (Muñoz-Cázares et al., 2017). Only the major metabolites have been evaluated and reported against QS while the specific bioactive components of the ethnobotanicals in this study have not yet been elucidated and presents an avenue for research in detailed phytochemistry.

The well documented mechanism of QSI action of phytochemicals is linked to their similarity in the chemical structure to QS signals and to their capacity to suppress

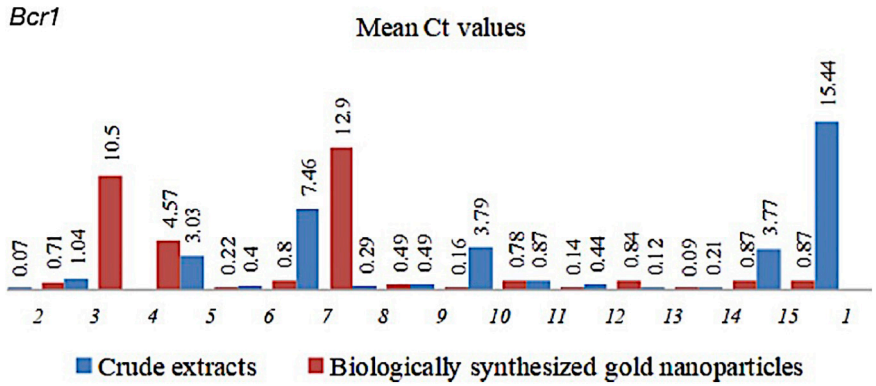

FIGURE 1 | Mean Ct values of *Bcr1* in *C. albicans* with CEs and CEs + AuNPs. (1) Control (2) *H. vulgaris* (3) *M. micrantha* (4) *D. philippinensis* (bark) (5) *C. pentandra* (6) *C. winterianus* (7) *S. alata* (8) *U. lobata* (9) *D. philippinensis* (leaf) (10) *P. odorata* (bark) (11) *S. jamaicensis* (leaf) (12) *E. Indica* (root) (13) *E. esculenta* (14) *E. indica* (leaf) (15) *P. urinaria*.

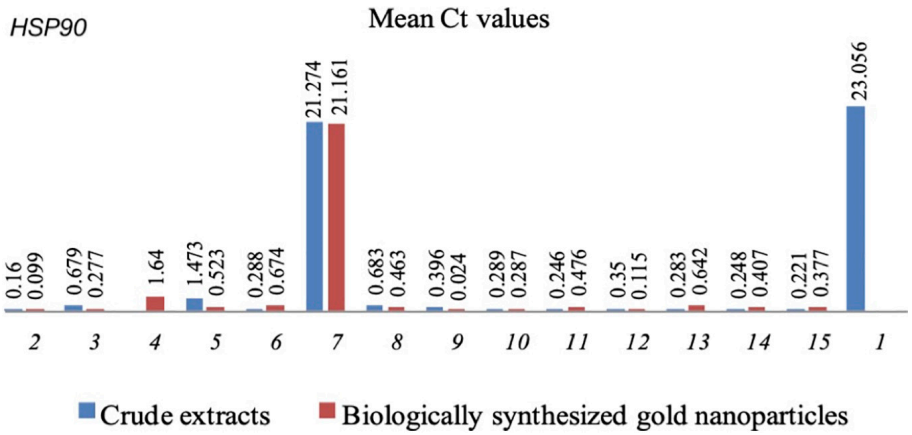

FIGURE 2 | Mean Ct values of *HSP90* in *C. albicans* with CEs and CEs + AuNPs. (1) Control (2) *H. vulgaris* (3) *M. micrantha* (4) *D. philippinensis* (bark) (5) *C. pentandra* (6) *C. winterianus* (7) *S. alata* (8) *U. lobata* (9) *D. philippinensis* (leaf) (10) *P. odorata* (bark) (11) *S. jamaicensis* (leaf) (12) *E. Indica* (root) (13) *E. esculenta* (14) *E. indica* (leaf) (15) *P. urinaria*.

signal receptors (Kalia, 2013). Plants have been known to contain phytochemicals associated with QSI activities and is considered as one of the most powerful natural sources of isolated QSI compounds. These compounds can reduce microbe pathogenicity (Rasmussen and Givskov, 2006) owing to their ability to block in intra and inter-species QS communication systems (Teplitski et al., 2000). This ability of natural compounds to suspend QS systems can serve as a defense strategy to fight against microbial penetration. As a prospective source of antivirulence agents (Rawat et al., 2016) that are safe for human health, it owes its advantage to its chemical stability and highly effective low-molecular-mass molecules (Rasmussen and Givskov, 2006) with non-toxic inhibitors of QS (Hentzer et al., 2003).

Evaluating the effects of potential QSI agents on the molecular mechanisms directing biofilm formation is a critical strategy to facilitate advances in novel antivirulence therapies. In this study, the expression of 2 biofilm-linked genes, *Bcr1* and *HSP90*, as affected by CEs and CEs + AuNPs were evaluated. Molecular expression analyses showed downregulation of both *Bcr1* and *HSP90* as affected by CEs and CEs + AuNPs. Expression of *Bcr1* and its downstream genes influences adhesion and arrangement of the polysaccharide matrix in *C. albicans*. Hence, downregulation of *Bcr1* affects the formation of the complex biofilm and its multi- dimensional polysaccharide matrix (Douglas, 2003); this means that biofilm formation will be repressed or will not yield a thick extracellular matrix. On the other hand, by targeting *HSP90* downregulation, dispersion will be suppressed and cell signals critical to biofilm formation will be blocked without developing resistance to existing antifungals (Robbins et al., 2011). The compounds in CEs and CEs + AuNPs may have acted as QSI molecules that have blocked the pathway of *Bcr1* and *HSP90,* hence, its downregulation. This showed that the production of QS molecules was reduced and have decreased in the expression of a specific receptor or transcription factor (Nobile et al., 2006). It has been recognized that expression of QS genes is important in the production of virulence factors such as the formation of biofilm, and this information gives

improved understanding of the function of the genes associated with its morphological features (Nobile et al., 2006). Thus, downregulation of *Bcr1* and *HSP90* by the CEs and CEs + AuNPs not only have the potential to inhibit the growth of biofilm but also that of antifungal resistance. Blocking or minimizing expression of these genes provides a key strategy to developing drugs for *C. albicans* management.

The efficiency on the use of nanoparticles was demonstrated in this study wherein treatments with CEs + AuNPS showed significantly lower biofilm formation in comparison with CEs alone. The CEs + AuNPs conjugation length and intensities decreased from 595 to 544 nm which signifies the decrease in size. The study of Emmanuel et al. (2017) demonstrates that the decrease in the conjugation length and intensities of AuNPs indicates the decrease in particle size. The formation of CEs + AuNPs in this study were monitored by analyzing the excitation due to the applied electromagnetic field of Surface Plasmon resonance (SPR) and absorption values. SPR peaks attained in UV–vis spectroscopy is one of the versatile techniques to confirm the formation of metal NPs and was generated due to the coherence of electrons on the surface of AuNPs. The shift to the blue or red in the λmax of the SPR peak could be related to the obtained morphology of NPs that has various shapes, sizes or extract dependencies of formed AuNPs (Vellaichamy and Periakaruppan, 2016; Emmanuel et al., 2017; Kanwal et al., 2018). The color change in reaction from yellowish to pink red and decreased conjugation length confirms the formation of CE + AuNPs (Mukherjee et al., 2016; Ovais et al., 2016). Furthermore, the pH of the solution increased from 6.0 to 6.5 after the addition of the crude ethnobotanical extracts indicating to a more stable state of the gold nanoparticles. The stability of gold nanoparticles is pH-responsive (Park et al., 2019) and its stability is pH-dependent, as shown in studies using natural compound for its synthesis (Tyagi et al., 2011).

The development of methods for integrated solution and control of pathogenic virulence and drug resistance has led many scientists to evaluate nanotechnology for efficient delivery of anti-pathogenic drugs from the natural compounds. Since its revolution, nanotechnology has been used to improve the uptake of soluble drugs (Ould-Ouali et al., 2005) due to their extremely reduced dimensions and somewhat large surface area (Kamat et al., 2002). Its safety also accompanies its advantages as it produces environmentally non-toxic molecules (Khatami et al., 2017). The results of this study may indicate expedited delivery system of the compounds through extremely reduced particle size and increased surface area that facilitates entry of compounds to the phospholipid- and glycoprotein-embedded cell membrane (Cabrera et al., 2017).

This study has shown that ethnobotanicals are a promising source of antipathogenic agents. Except for a few studies, these plants largely remain unexplored for their pharmacological potential. A number of studies have shown proof that ethnobotanicals possesses QSI actions in virulence factors in bacteria such as biofilm formation (Judan Cruz et al., 2018; Fernando and Judan Cruz, 2020; Fernando et al., 2020; Velasco et al., 2020; Santos et al., 2021), coagulase (Vias et al., 2018; Salamanca et al., 2019), pyocyanin production (Barrogo et al., 2018; Limos et al., 2018a), swarming motility (Barrogo et al., 2018; Limos et al., 2018a; Padilla et al., 2018), DNase (Limos et al., 2018b), and α- Hemolysin (Limos et al., 2018b; Vergara et al., 2018), showing the immense prospects of tapping these plants for antivirulence drug design. The QSI actions of the ethnobotanicals were confirmed through expression analyses of QS-linked genes such as *lasR, rhlR, ahyR,* and *agrA*.

Targeting virulence factors is a promising approach to design new and effective antifungal therapies (Mayer et al., 2013). Biofilm is one of the QS-regulated virulence factors that contribute to the pathogenicity as well as to the increasing development of fungal drug resistance in *C. albicans*. Despite the existing antifungal drugs, fungal resistance is evolving due to long term exposure. A novel approach for its control is to obtain plant bioactive compounds to create a wide variety of drugs (Cruz et al., 2007) that targets the formation of biofilm. Recently, a number of antifungal drugs have been designed to contain natural derivatives or compounds mimicking natural products (Newman, 2008). In *C. albicans*, numerous plant extracts and its compounds are already known to change its adhesion mechanics (Ahmad and Aqil, 2007); adhesion being the first step in its biofilm formation and significantly contributes to *C. albicans* pathogenicity (Mukherjee et al., 2003).

Diverse natural products are recognized to inhibit biofilm formation through scientific validations and studies. Therefore, the discovery of plant bioactive compounds that controls pathogenicity becomes a fundamental strategy (Ahmad and Aqil, 2007) toward *C. albicans* management. This paper highlights the great pharmacological potential of these ethnobotanical extracts for developing efficient therapeutic agents against *C. albicans* without the risk of developing drug resistance. This potential can be further improved through nanotechnology. This new understanding can be used to direct the discovery of novel approaches for preventing and controlling complex and resistant biofilms.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

KJC provided the concept and design of the study, wrote the first and final drafts of the manuscript, and performed laboratory works. EA wrote the first draft of the manuscript, performed laboratory works, and provided laboratory materials. SF performed laboratory works, wrote a section of the manuscript, and performed statistical analyses. KW wrote a section of the manuscript, provided a portion of the laboratory funding, and supervised laboratory works. All authors contributed to manuscript revision, read, and approved the submitted version.

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DEDICATION

This work is dedicated to the *Ilongot-Eǵongot* ethnic community of Maria Aurora, Aurora, Philippines.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Antibacterial and Anti-biofilm Efficacy of Chinese Dragon's Blood Against *Staphylococcus aureus* Isolated From Infected Wounds

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Chinese dragon's blood (CDB), a characteristic red resin, is an important traditional Chinese medicine (TCM), and empiric therapy of infected wounds with CDB is performed in clinical settings. For the first time, we herein report the antibacterial and anti-biofilm efficacy of CDB against *Staphylococcus aureus* (*S. aureus*). Antimicrobial susceptibility testing, growth curve assay, time-kill curve assay, crystal violet biofilm assay, scanning electron microscope (SEM) analysis, cell membrane tests, and quantitative real-time polymerase chain reaction (qRT-PCR) were used for this purpose. The results suggested that the minimum inhibitory concentration (MIC) values of CDB against *S. aureus* ranged from 32 to 128 μg/mL. Growth curves and time-kill curves confirmed that CDB could inhibit the growth of *S. aureus*. The biofilm formation ability and the expression levels of *saeR*, *saeS*, and *hla* of *S. aureus* in the presence and absence of CDB were statistically significant ($P < 0.01$). The results of SEM analysis and cell membrane tests revealed that exposure to CDB had some destructive effects on *S. aureus* cells. In conclusion, CDB exhibits positive antibacterial activity against *S. aureus*. Moreover, CDB could reduce the biofilm formation and the virulence factors of *S. aureus* by downregulating the expression levels of *saeR*, *saeS*, and *hla* genes. These findings indicated that CDB has immense potential to serve as a viable alternative for the treatment of infected wounds caused by *S. aureus* in clinical settings.

Keywords: Chinese dragon's blood, infected wounds, *Staphylococcus aureus*, antibacterial activity, anti-biofilm efficacy

INTRODUCTION

Worldwide, infected wounds are increasingly becoming a threat to human health (Ju et al., 2018). Acute wounds generally have a self-healing capacity and do not require significant external intervention; however, self-healing is often not possible in the case of chronic wounds. Therefore, external treatment is needed (Garcia-Villen et al., 2019). In fact, clinics face enormous challenges in managing chronic infected wounds. The normal recovery phases are altered significantly because of the presence of microbial contamination on the wound surface, leading to the possible impairment of the healing pathway and finally resulting in non-healing wounds (Garcia-Villen et al., 2019).

Notably, *Staphylococcus aureus* (*S. aureus*) is frequently associated with infected wounds, and the pathogen is capable of biofilm formation (Salouti et al., 2016; Davis et al., 2017). Biofilms are adherent colonies of bacteria that are covered in a self-produced extracellular polysaccharide matrix (EPS) that is of host or mixed origin. Bacteria with biofilm phenotype undergo metabolic activity alterations within the protective EPS coating (Nair et al., 2016; Davis et al., 2017; Anderson et al., 2018). The biofilm enhances the ability of the organism to adapt to the environment, which in turn leads to reduced susceptibility to most antimicrobial agents. Some studies have established that alpha-toxin contributes to biofilm formation in *S. aureus* wound isolates (Anderson et al., 2012, 2018). Therefore, *S. aureus* biofilm formation plays a significant role in non-healing wound infections. The incorporation of antibiotics in treatment regimens has effectively eliminated multiple species of pathogens (Krychowiak et al., 2014). However, with the extensive use or even abuse of broad-spectrum antibacterial drugs, resistance to antimicrobial agents has been gradually increasing. The severe drug resistance status poses a huge challenge to anti-infective treatments in clinical settings (Song et al., 2016).

In this context, traditional Chinese medicine (TCM), including Chinese dragon's blood (CDB), has been regarded as an alternative and complementary therapeutic intervention for infected wounds (Shen et al., 2019). According to the National Drug Standard [WS_3-082 (Z-016)-99(Z)], CDB is a resin with rich, deep red color, which is obtained from the fat-containing wood of the lily tree belonging to the family *Liliaceae* (Wang et al., 2011, 2017; Lin et al., 2020). Preclinical studies have shown that CDB has many phytochemicals with anti-inflammatory, antimicrobial, antifungal, and antineoplastic properties and is therefore useful in the treatment of various diseases (Pona et al., 2019). As a natural remedy for infected wounds, CDB is widely used in treating sores, diabetic foot ulcers, soft tissue injuries, etc. (Ho et al., 2016; Pona et al., 2019). Therefore, our study aimed to investigate the antibacterial and anti-biofilm efficacy of CDB against *S. aureus* isolated from infected wounds. We further attempted to provide an experimental basis for the rational use of CDB for the treatment of infected wounds in clinical settings.

MATERIALS AND METHODS

Clinical Isolates and Identification

A total of 46 *S. aureus* strains were isolated from the wound specimens of the patients from the First Affiliated Hospital of Wenzhou Medical University (Zhejiang Province, China) in 2017. All strains were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using the VITEK Mass Spectrometer (BioMerieux, Lyons, France). All strains were stored at −80°C and incubated on blood agar plates at 37°C for 18–24 h before use.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was undertaken by the agar dilution method according to the latest (Clinical and Laboratory Standards Institute, 2020). Briefly, an overnight cultured single colony was suspended in sterile NaCl (0.85%), and the suspensions were adjusted to the turbidity equal to 0.5 McFarland standard (1.5×10^8 CFU/mL). Then, the mixture was further diluted to 1:10 and evenly spotted onto the drug-containing MH agar plate; the results were observed after incubation at 37°C for 16–18 h. CDB (lot number: Z20O9B72911, Shanghai Yuanye Biotechnology Co., Ltd., China) was dissolved in dimethyl sulfoxide (DMSO) and tested over a range of 1–512 μg/mL. *S. aureus* ATCC 29213 was employed for the quality control of the strain. The minimal inhibitory concentration (MIC) values were tested in 3 independent experiments.

Bacterial Growth Curve Assay

The bacterial growth curve was determined as previously described, with minor modifications (Zhou et al., 2018). Briefly, the 6 clinical *S. aureus* strains (JP-2541, JP-2718, JP-2744, JP-2850, JP-2918, and JP-3053) were isolated from patients diagnosed with different types of infected wound, as details in **Supplementary Table 1**. *S. aureus* ATCC 29213 served as the quality control strain. The 7 isolates mentioned above were cultured in fresh Luria–Bertani (LB) broth at 37°C with shaking at 180 revolutions per minute (rpm) to obtain an OD_{600} value of 0.3, followed by further dilution to 1:100 with 20 mL of fresh LB broth in the presence of CDB (16, 32, 64, 128, and 256 μg/mL, respectively) as well as diluted 1:100 with fresh LB broth alone as the control group, followed subsequently by incubation at 37°C at 180 rpm for overnight. The OD_{600} value was measured every hour for 24 h. Therefore, a total of 24 points of data of each sample were collected. All experiments were independently repeated in triplicate.

Time-Kill Curve Assay

The time-kill curve assay for 7 *S. aureus* strains (JP-2541, JP-2718, JP-2744, JP-2850, JP-2918, JP-3053, and ATCC 29213) was performed using a previously standardized method (Foerster et al., 2015, 2016). Briefly, overnight cultures were diluted in 20 mL of fresh LB broth to a final concentration of approximately 1×10^7 CFU/mL, and CDB at 0, 1/2 × MIC, 1 × MIC, 2 × MIC concentrations were added, respectively. Finally, viable colony counts were determined at 0, 2, 4, 6, 12, and 24 h after incubation at 37°C at 180 rpm. The time-kill curves of each strain were plotted with the number of bacteria per mL (CFU/mL) with the ordinates and time (h) as the abscissas.

Crystal Violet Biofilm Assay

The biofilm formation ability assay was performed according to the methods by O'Toole with some minor modifications (Niemirowicz et al., 2016). An overnight culture of each isolate was incubated at 37°C/180 rpm up to the logarithmic phase with an OD_{600} value of 0.6, and the turbidity was adjusted to 0.5 McFarland standard, further diluted to 1:100, and CDB at 0, 32, 64, 128, 256, and 512 μg/mL concentrations were added, respectively. Then, 100 μL of the dilution was added to the 96-well polystyrene micro-test plate (Flat bottom with lid, Sterile; Corning, United States), and 3 replicate wells were set up. Following 24-h incubation at 37°C with shaking at

75 rpm, the upper planktonic bacteria was decanted, and biofilms attached to the well surfaces were stained with 100 μL of 1% (*w*/*v*) crystal violet solution (lot number: NO.20190324, Beijing Solarbio Biotechnology Co., Ltd., China) for 15 min. The bound dye was solubilized for 30 min with 100 μL of the eluent (95% absolute ethanol and 5% glacial acetic acid) and subsequently quantified by measuring the OD_{595} value by the Multiskan FC Microplate Reader.

Scanning Electron Microscope (SEM) Analysis

In addition to measuring the effects of CDB against *S. aureus*, SEM observation was performed, with some minor modifications (Jiamboonsri et al., 2011; Singh et al., 2017). Briefly, the prepared inoculum (300 μL) of JP-2541 and *S. aureus* ATCC 29213 was transferred into LB broth (2.7 mL) in the presence and absence of 1 × MIC CDB, the positively charged glass slide was placed into each well and subsequently incubated at 37℃ overnight. The bacterial cells on the coverslip were fixed in 2.5% (*w*/*v*) of glutaraldehyde at 4℃ for 4 h and rinsed with 0.1 M of phosphate buffer (pH 7.2), followed by dehydration in graded ethanol (30, 70, and 100%) and drying at the room temperature for overnight. Finally, the dried samples were covered with gold and observed under the S-3000N scanning electron microscope (SEM) (Hitachi, Japan) at various levels of magnification.

Cell Membrane Tests

Actively growing *S. aureus* culture of each isolate was treated with serial concentrations of CDB (0, 32, 64, 128, 256, and 512 μg/mL) at 37℃ for 6 h. The alkaline phosphatase release levels of bacterial cell membrane disruption of each isolate were assessed by using a commercial kit (Solarbio, Beijing, China) (Qu et al., 2019). In an alkaline environment, AKP/ALP catalyzes the formation of phenyl disodium phosphate into free phenols. Phenols react with potassium ferricyanide and 4-aminoantipyrine to form red quinone compounds with characteristic absorbance at 510 nm. The activity of CDB against *S. aureus* cell membrane was calculated by measuring the absorbance increase rate at 510 nm.

Quantitative Real-Time PCR (qRT-PCR)

All *S. aureus* strains (JP-2541, JP-2718, JP-2744, JP-2850, JP-2918, JP-3053, and ATCC 29213) were treated with or without 1/4 × MIC CDB, after which the total RNA of *S. aureus* strains were extracted from the bacterial culture using the Bacterial RNA Miniprep Kit (Biomiga, Shanghai, China). The cDNA was reversed with 1000-ng RNA templates using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, United States). These primers are listed in **Table 1** (Duan et al., 2018). The expression levels of alpha-hemolysin gene (*hla*), response regulator gene (*saeR*), and histidine kinase gene (*saeS*) were analyzed by qRT-PCR. *gyrb* was used as an internal reference. As previously described, qRT-PCR was performed using the TB Green Premix Ex Taq II (Tli RNase H Plus) (2×) (Takara, Japan) (Xu et al., 2020).

Statistical Analysis

The GraphPad Prism, version 8.02 (GraphPad Software, San Diego, CA, United States) was used for data analysis. The results were expressed as means ± SD, and comparison among the studied groups was conducted by the Student's *t*-test. Significance was considered at $P < 0.05$, and all tests were two-tailed.

RESULTS

Determination of MICs of CDB

Antimicrobial susceptibility testing revealed that the MIC values of CDB against *S. aureus* ranged from 32 to 128 μg/mL by the agar dilution method (**Table 2**).

TABLE 1 | Primers used for qRT-PCR.

Primer name	Sequence (5′→3′)
gyrb-RT-F	ACATTACAGCAGCGTATTAG
gyrb-RT-R	CTCATAGTGATAGGAGTCTTCT
hla-RT-F	TGGTAATCATCACGAACTC
hla-RT-R	GCAGCAGATAACTTCCTT
saeR-RT-F	GTCGTAACCATTAACTTCTG
saeR-RT-R	ATCGTGGATGATGAACAA
saeS-RT-F	TGTATTTAAAGTGATAATATGAGTC
saeS-RT-R	CTTAGCCCATGATTTAAAAACACC

TABLE 2 | Antibiotic susceptibility of CDB against 46 clinical *S. aureus* isolates and *S. aureus* ATCC 29213.

Strain	CDB MIC (μg/mL)	Strain	CDB MIC (μg/mL)
JP-2433	64	JP-2743	64
JP-2437	64	JP-2744	64
JP-2476	64	JP-2764	64
JP-2509	64	JP-2768	64
JP-2541	64	JP-2786	64
JP-2560	64	JP-2800	64
JP-2568	64	JP-2826	64
JP-2582	64	JP-2831	64
JP-2590	128	JP-2842	64
JP-2608	64	JP-2850	64
JP-2611	64	JP-2890	64
JP-2617	64	JP-2902	32
JP-2626	64	JP-2910	64
JP-2628	64	JP-2918	64
JP-2630	64	JP-2928	64
JP-2631	64	JP-2942	64
JP-2632	128	JP-2957	64
JP-2644	64	JP-2975	64
JP-2674	64	JP-3019	64
JP-2679	64	JP-3023	64
JP-2694	64	JP-3053	64
JP-2718	64	JP-3058	64
JP-2730	64	ATCC 29213	64
JP-2738	64		

Bacterial Growth Curve Assay

By measuring the effects of CDB with different concentrations on the growth of *S. aureus*, it was found that CDB had no effect on the growth of *S. aureus* at the concentration ≤64 μg/mL when compared with the control group; while CDB with a concentration ≥128 μg/mL could effectively inhibit the growth of *S. aureus* (**Figure 1**).

Time-Kill Curve Assay

The time−kill curve assay was performed for 6 clinical *S. aureus* isolates (JP-2541, JP-2718, JP-2744, JP-2850, JP-2918, and JP-3053) and *S. aureus* ATCC 29213. At 0–12 h, our results demonstrated that all isolates kept growing in the absence of CDB and that all these strains remained at the initial inoculation level at the 1/2 × MIC CDB. Interestingly, at 0–12 h, when compared with the initial inoculum, the colony counts of the bacteria decreased by approximately 100 times at the 1 × MIC CDB and were maintained at the level of the bacteria; while the growth of all strains was inhibited at 2 × MIC CDB. After 12 h, the results of this experiment revealed that *S. aureus* demonstrated a remarkable trend of increasing growth at all concentrations of CDB (**Figure 2**).

Efficacy of CDB on Biofilm Formation of *S. aureus*

The biofilm formation ability of *S. aureus* in the LB broth with different concentrations of CDB was compared. Our result illustrated biofilm structures of *S. aureus* in the LB broth in the presence and absence of CDB and noted that the differences in the biofilm formation ability between the drug-containing LB broth group and LB broth group be statistically significant ($P < 0.05$; **Figure 3**).

Scanning Electron Microscope (SEM) Analysis

The visualization of JP-2541 and *S. aureus* ATCC 29213 cell morphology through SEM after treatment with CDB at 1 × MIC concentration and control cells are presented in **Figure 4**. The surface of *S. aureus* cells untreated with CDB (control group) formed a thick biofilm composed of aggregates and microcolonies on the coverslip at 2500 × magnification, and the cell morphology was observed to be smooth with some typical characters of ball shape (**Figures 4A,E**). However, *S. aureus* cells treated with 1 × MIC CDB appeared sparse and dispersed, and the number of cells was significantly reduced (**Figures 4B,F**). Moreover, there was no change in the morphology of the control cells at 7000 × magnification (**Figures 4C,G**), whereas the CDB-treated cells showed partial destruction (**Figures 4D,H**).

Cell Membrane Tests

On treating these 7 isolates with different concentrations of CDB, the differences in the release levels of alkaline phosphatase between the treated and control groups were statistically significant ($P < 0.05$; **Figure 5**). Together, these data indicated that CDB could lead to increased permeability and the weakening of the cell membrane of each isolate.

qRT-PCR

The results of qRT-PCR revealed that the relative expression levels of *hla* and *saeRS* in these strains were downregulated significantly after the exposure of the 1/4 × MIC concentration of CDB ($P < 0.05$; **Figures 6A–G**). These results indicated that CDB could decrease the capacity of alpha-hemolysin production of *S. aureus* strains through the inhibition of the expression of *hla*, *saeR*, and *saeS*.

DISCUSSION

The skin serves as a protective physical barrier against invading microbes, including pathogens (Jun et al., 2015; Chaudhary et al., 2019). Wounds are inevitably infected by microorganisms in nature during the formation and healing processes. Infected wounds not only prolong the healing time but also threaten the lives of the patients at times (Meara et al., 2015). The pathogens responsible for the infection vary in different wound environments; nonetheless, *S. aureus* is the most common pathogen causing infected wounds (Alves et al., 2018; Rashid et al., 2019). In this scenario, it has been observed that CDB offers potential health benefits and could be exploited for treating infected wounds in clinical settings (Wan et al., 2019; Lin et al., 2020). Thus, we investigated the underlying antibacterial activities of CDB against *S. aureus* isolated from wound specimens and provided scientific evidence for antibiotic treatment of infected wounds with CDB.

To the best of our knowledge, the present study is the first to shed light on the antibacterial and anti-biofilm efficacy of CDB against *S. aureus*. Based on the results of bacterial growth curve assay and time-kill curve assay, we concluded that CDB could inhibit the growth of *S. aureus* in a concentration-dependent manner. Meanwhile, sub-inhibitory concentrations of CDB could reduce the biofilm formation ability of *S. aureus* and disrupt its cellular membrane. The above findings were further supported by SEM findings. We further uncovered that CDB could reduce the virulence factors of *S. aureus* by downregulating the expression levels of *saeR*, *saeS*, and *hla* and inhibiting the hemolytic activity.

CDB is mainly distributed in Hainan, Guangxi, and Southern Yunnan in China, and it is produced from *Dracaena cochinchinensis* in Indonesia, Australia, Africa, and other countries. In recent years, owing to the increasing scope of clinical application of CDB, many scholars and clinicians have conducted in-depth research on its chemical composition and pharmacological actions. Previous studies have revealed that the main components of CDB are phenolics and flavonoids (Gupta and Gupta, 2011; Stefano et al., 2014; Al-Fatimi, 2018). Moreover, saponins, terpenoids, resveratrol, and other ingredients are present in it. CDB has antibacterial, anti-inflammatory, analgesic, and anti-platelet aggregation properties and is capable of promoting blood circulation and epidermal repair, besides displaying other pharmacological effects (Luo et al., 2011; Liu et al., 2013). However, little is known about the underlying antibacterial activities of CBD against *S. aureus*. Some studies have confirmed that CBD is rich in proanthocyanidins, phenolics, and flavonoids, which constitute

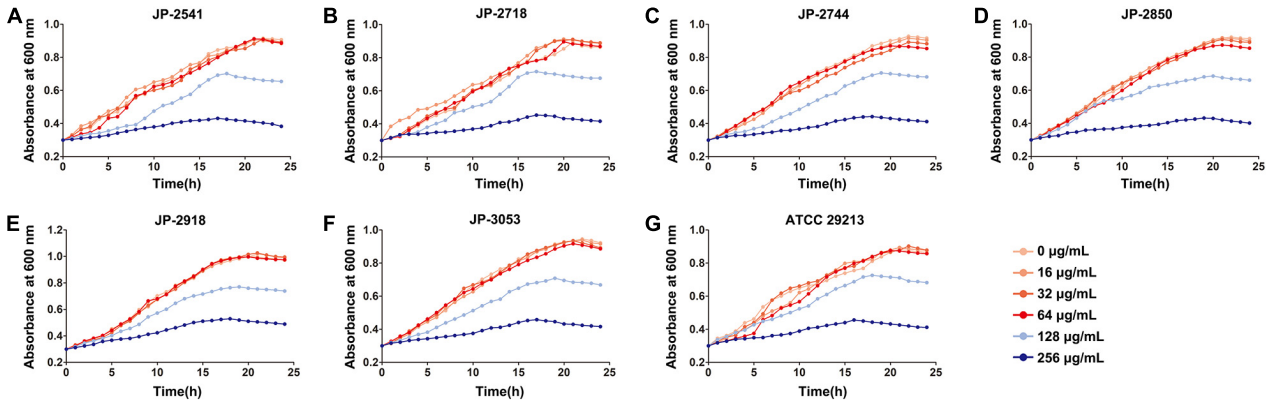

FIGURE 1 | Effects of different concentrations of CDB on the growth of *Staphylococcus aureus*. **(A)** growth curves for JP-2541; **(B)** growth curves for JP-2718; **(C)** growth curves for JP-2744; **(D)** growth curves for JP-2850; **(E)** growth curves for JP-2918; **(F)** growth curves for JP-3053; and **(G)** growth curves for *S. aureus* ATCC 29213.

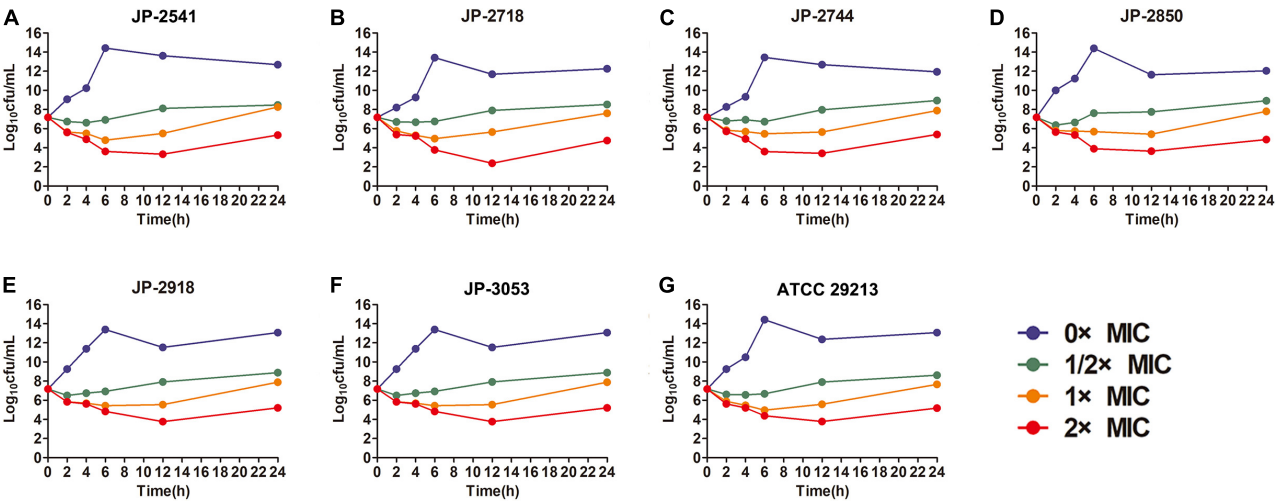

FIGURE 2 | Time-kill curves of different concentrations of CDB against *Staphylococcus aureus*. **(A)** time-kill curves for JP-2541; **(B)** time-kill curves for JP-2718; **(C)** time-kill curves for JP-2744; **(D)** time-kill curves for JP-2850; **(E)** time-kill curves for JP-2918; **(F)** time-kill curves for JP-3053; and **(G)** time-kill curves for *S. aureus* ATCC 29213.

90% of its dry weight (Jones, 2003; Al-Fatimi, 2018; Pona et al., 2019). Proanthocyanidin, as a final product of the flavonoid biosynthetic pathway, is known to effectively prevent and cure bacterial infections (Rauf et al., 2019). Also, these phenolic compounds can be easily degraded (Escobar et al., 2018). As shown in **Figure 2**, *S. aureus* exhibited remarkably increased cell growth at all concentrations of CDB after treatment for 12 h, which is consistent with previous reports. This phenomenon reminds us that we should pay attention to such time-dependent characteristics when using CDB in clinical settings.

Previous studies have confirmed that in the environment of chronic infected wounds, the formation of bacterial biofilms could make the bacteria more adaptable to the external environment by increasing the adhesion to the wound surface and evading the host's immune function (Krychowiak et al., 2014). In addition, the biofilm structure could significantly enhance the pathogen's resistance to antibiotics by preventing the drugs from entering the bacterial cell, which is the main reason for persistent inflammation of the wound and difficulty in healing (van Wamel, 2017). Therefore, it is essential to find compounds that could inhibit the formation of biofilms. In our study, crystal violet biofilm assay and SEM analysis revealed that sub-inhibitory concentrations of CDB could effectively lower the biofilm formation ability of *S. aureus*. Biofilm formation of *S. aureus* is an important factor that determines the wound healing process and patient mortality (Bhattacharya et al., 2015; Roy et al., 2020). Besides, Tsung-Jung Ho et al. confirmed that CDB could stimulate angiogenesis and promote cell proliferation and migration (Ho et al., 2016). We speculated that these might be the important reasons for CDB promoting the wound healing process.

In addition to biofilm formation, virulence factors of *S. aureus* play crucial roles in wound healing either directly or indirectly. Among the various virulence determinants, alpha-hemolysin

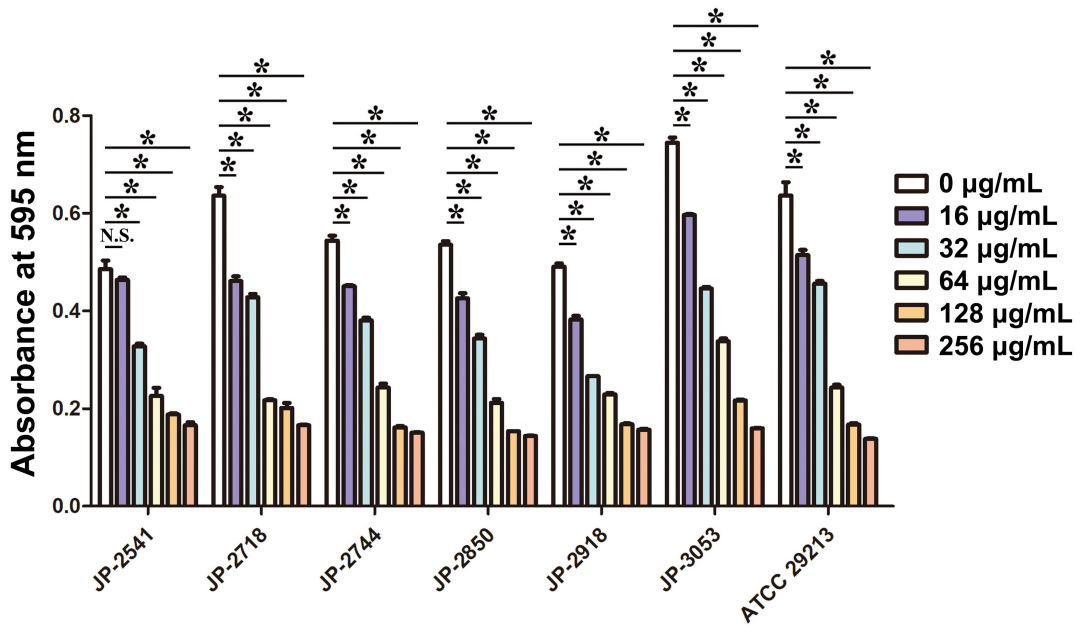

FIGURE 3 | Effects of different concentrations of CDB on the biofilm formation ability of *Staphylococcus aureus*. *$P < 0.05$; N.S., $P > 0.05$.

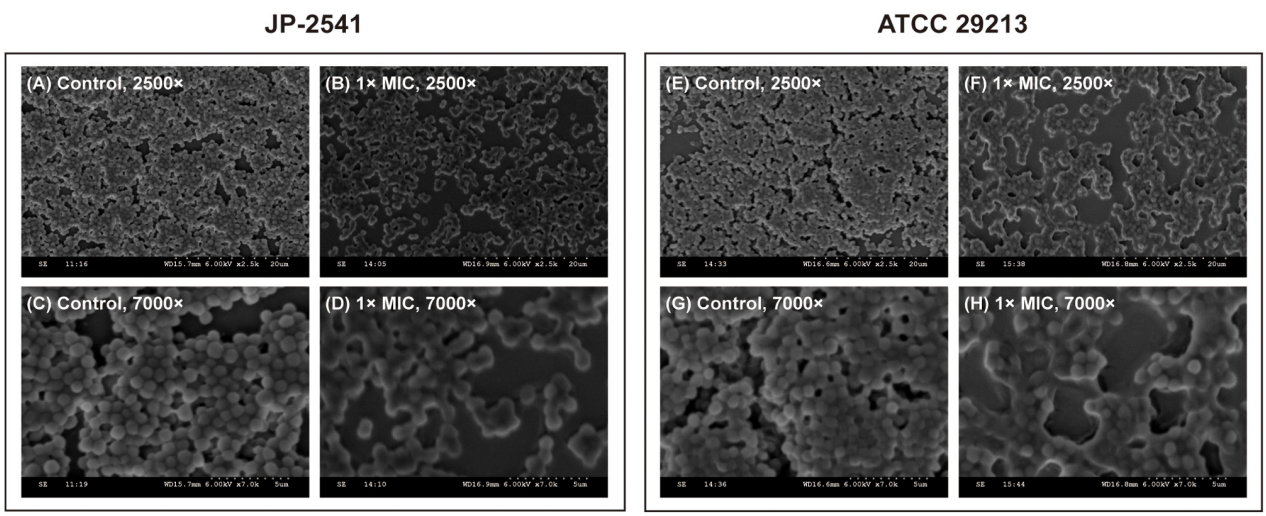

FIGURE 4 | Scanning electron microscope (SEM) images of JP-2541 and *S. aureus* ATCC 29213 after treatment with CDB. **(A)** control group of JP-2541, 2,500 × magnification; **(B)** 1 × MIC CDB against JP-2541, 2,500 × magnification; **(C)** control group of JP-2541, 7,000 × magnification; **(D)** 1 × MIC CDB against JP-2541, 7,000 × magnification; **(E)** control group of *S. aureus* ATCC 29213, 2,500 × magnification; **(F)** 1 × MIC CDB against *S. aureus* ATCC 29213, 2,500 × magnification; **(G)** control group of *S. aureus* ATCC 29213, 7,000 × magnification; and **(H)** 1 × MIC CDB against *S. aureus* ATCC 29213, 7,000 × magnification.

(Hla) is one of the most significant virulence factors in *S. aureus* wound infections, which results in attenuated production by inhibiting the expression level of the gene encoding Hla (*hla*) or global regulatory genes such as *saeS* and *saeR* (Duan et al., 2018; Gudeta et al., 2019; Putra et al., 2019). The *saePQRS* system is a global regulator of *S. aureus*, and among them, *saeS* (encoding a histidine kinase) and *saeR* (encoding a response regulator) play regulatory roles in controlling the expression of *hla* (Gudeta et al., 2019; DelMain et al., 2020). As shown in qRT-PCR results, upon comparing the gene expression data of the control group with that of the group treated with 1/4 × MIC of CDB, the transcription levels of *saeR*, *saeS,* and *hla* were downregulated. Our results revealed that CDB could decrease Hla production by *S. aureus* owing to a reduction in the expression of *saeR*, *saeS*, and *hla*, thereby potentially weakening the virulence determinants of the pathogen.

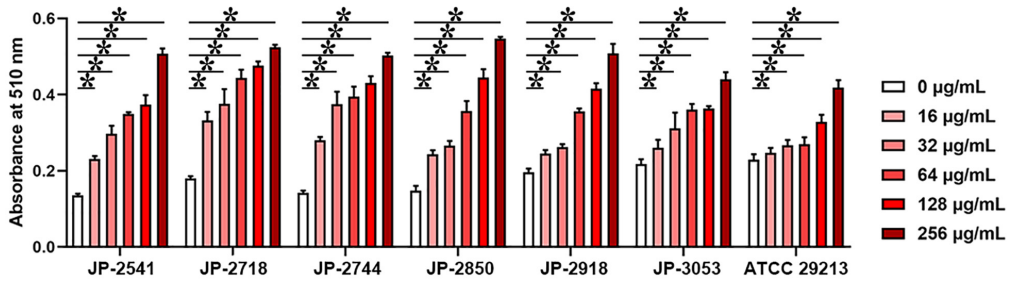

FIGURE 5 | Different concentrations of CDB used to disrupt the bacterial cell membrane integrity. *$P < 0.05$; N.S., $P > 0.05$.

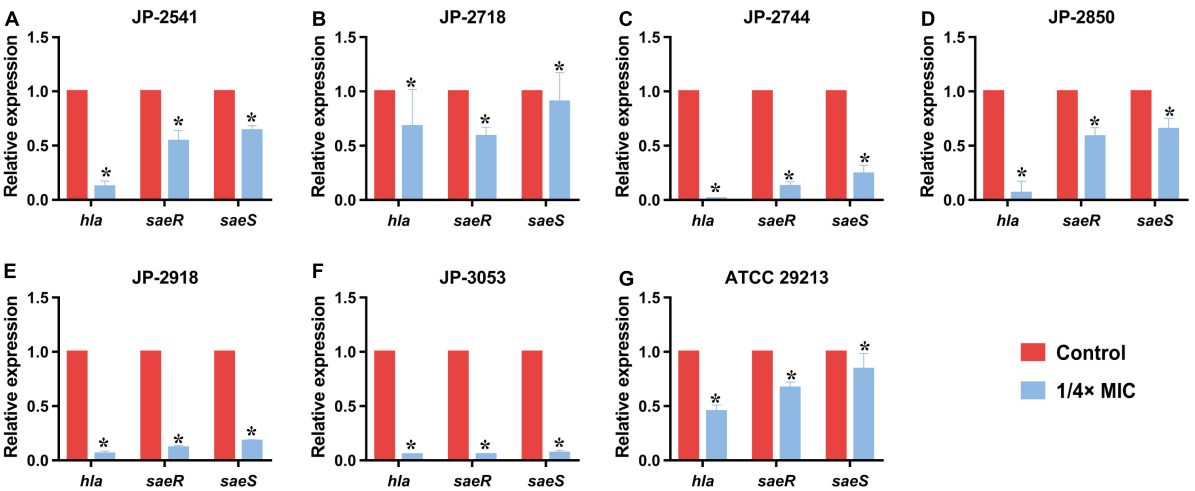

FIGURE 6 | Relative expression level of *hla*, *saeR*, and *saeS* in *S. aureus* strains after culturing with 1/4 × MIC concentration of CDB. *$P < 0.05$. **(A)** Genes expression level in JP-2541. **(B)** Genes expression level in JP-2718. **(C)** Genes expression level in JP-2744. **(D)** Genes expression level in JP-2850. **(E)** Genes expression level in JP-2918. **(F)** Genes expression level in JP-3053. **(G)** Genes expression level in *S. aureus* ATCC 29213.

Therefore, we next sought to further investigate the activities of CDB against *S. aureus in vivo* by constructing the mouse model of *S. aureus* skin infected wound, and then visual observation of surface healing, bacterial counts, histology observation, and immunohistochemical analysis can be performed in the uninfected- and infected-wound groups. Most importantly, we are aware that understanding the underlying antibacterial activities of CDB against *S. aureus* is the crucial first step in exploring the molecular mechanisms or specific pathways of CDB to curb the growth, biofilm formation, and virulence factors of the pathogen.

CONCLUSION

In conclusion, CDB, which is one of the most precious traditional Chinese medicine, exerts positive antibacterial efficacy on *S. aureus*, and can also reduce the biofilm formation and retard the virulence factors alpha-hemolysin of *S. aureus* by downregulating the expression levels of *saeR*, *saeS*, and *hla* genes. Our study provides new insights into the rational use of CDB for the treatment of infected wounds caused by *S. aureus* for the first time. These findings together indicate that CDB possesses significant potential as an alternative for the treatment of infected wounds caused by *S. aureus* in clinical settings. Furthermore, it will be worthwhile to further investigate the activities of CDB against *S. aureus in vivo*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (approval number: 2021–R003). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

XZ and LC conducted the experiments, analyzed the data, and wrote the manuscript. WZ participated in the experiments and writing. WL, ZW, XT, and RF analyzed the data. XZ and YS supervised the manuscript. TZ designed the study. All authors read and approved the final version of the manuscript for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.672943/full#supplementary-material

Supplementary Table 1 | Patient clinical data and characteristics of 6 clinical *S. aureus* isolates.

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Taxifolin, an Inhibitor of Sortase A, Interferes With the Adhesion of Methicillin-Resistant *Staphylococcal aureus*

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The evolution and spread of methicillin-resistant *Staphylococcus aureus* (MRSA) poses a significant hidden risk to human public health. The majority of antibiotics used clinically have become mostly ineffective, and so the development of novel anti-infection strategies is urgently required. Since *Staphylococcus aureus* (*S. aureus*) cysteine transpeptidase sortase A (SrtA) mediates the surface-anchoring of proteins to its surface, compounds that inhibit SrtA are considered potential antivirulence treatments. Herein, we report on the efficacy of the potent SrtA inhibitor taxifolin (Tax), a flavonoid compound isolated from Chinese herbs. It was able to reversibly block the activity of SrtA with an IC_{50} of 24.53 ± 0.42 μM. Tax did not display toxicity toward mammalian cells or *S. aureus* at a concentration of 200 μM. In addition, Tax attenuated the virulence-related phenotype of SrtA *in vitro* by decreasing the adherence of *S. aureus*, reducing the formation of a biofilm, and anchoring of *S. aureus* protein A on its cell wall. The mechanism of the SrtA-Tax interaction was determined using a localized surface plasmon resonance assay. Subsequent mechanistic studies confirmed that Asp-170 and Gln-172 were the principal sites on SrtA with which it binds to Tax. Importantly, *in vivo* experiments demonstrated that Tax protects mice against pneumonia induced by lethal doses of MRSA, significantly improving their survival rate and reducing the number of viable *S. aureus* in the lung tissue. The present study indicates that Tax is a useful pioneer compound for the development of novel agents against *S. aureus* infections.

Keywords: antivirulence, taxifolin, inhibitor, methicillin-resistant *Staphylococcus aureus*, sortase A, pneumonia

INTRODUCTION

There is currently major worldwide concern about the continuous emergence of multidrug-resistant bacterial pathogens. *Staphylococcus aureus* (*S. aureus*) is recognized as an important cause of disease worldwide. It is capable of inducing a variety of serious diseases that endanger human health, from mild skin and soft tissue infection to fatal invasive infections such as septicemia

and pneumonia, etc. (Goetghebeur et al., 2007; Tong et al., 2015; Lake et al., 2018). In clinics, antibiotics have been considered as the primary defense against *S. aureus* infections over many decades. In the years following their introduction, the therapeutic benefits of such antibiotics were remarkable. However, the considerable selective pressure and improper use of antibiotics have resulted in the emergence, prevalence, and spread of drug-resistant strains of bacteria (Liu et al., 2019; Wu et al., 2019). As strains of methicillin-resistant *Staphylococcus aureus* (MRSA) with little sensitivity to conventional antibiotics have become prevalent (Prestinaci et al., 2015), treatments for MRSA infection have become more challenging for clinicians, who require new strategies to provide effective therapeutic options against complicated *S. aureus* infections (Kali, 2015; Galar et al., 2019).

Alternative therapies such as the use of antibiotics in combination or with adjuvants, bacteriophages, antimicrobial peptides, nanoparticles (Nisar et al., 2019) and anti-virulence therapy are widely reported (Mandal et al., 2014; Kaur, 2016). Considering that *S. aureus* exploited a vast repertoire of virulence strategies that enable it to infect a host. Therefore, Therefore, exploration of the potential of these virulence factors as drug targets may represent an alternative approach to disrupt bacterial pathogenicity (Vandenesch et al., 2012). SrtA is a membrane-bound cysteine transpeptidase that plays an essential role in catalyzing the covalent anchoring of surface proteins to the bacterial cell wall (Paterson and Mitchell, 2004). A member of the sortase subfamily, it plays an active role in bacterial adhesion, biofilm formation, and immune escape (Cascioferro et al., 2014). In addition, *S. aureus* SrtA-anchored surface proteins play major roles in the infection process, with many studies demonstrating that *S. aureus* SrtA mutants do not form abscess lesions or survive when infecting mouse tissue (Mazmanian et al., 2000; Cheng et al., 2009). Specific SrtA inhibitors do not interfere with the growth of bacteria yet weaken bacterial virulence (Suree et al., 2007; Hou et al., 2018). They have the potential to prevent *S. aureus*-induced colonization and invasive diseases while exhibiting a low risk of causing bacterial resistance by blocking SrtA (Hou et al., 2018).

Novel SrtA inhibitors have been identified from libraries of natural or synthetic compounds, or may be specifically-engineered peptidomimetics (Smeltzer, 2016). Of these, there is widespread interest in natural products with diverse structures and biological properties from fungi and plants (Si et al., 2016). In the present study, characterization of the natural compound taxifolin (Tax) is reported. It is extracted from the roots of larch and is an inhibitor of SrtA. It has been widely studied due to its properties as an antioxidant and inhibitor of the synthesis of fat and cholesterol (Angelis et al., 2016; Razak et al., 2018; Ren et al., 2020). In addition, it has been observed that Tax can inhibit SrtA activity without interfering with the growth of bacteria, suggesting that the use of Tax would result in low selective pressure which would avoid the development of resistant strains. In the present study, the inhibitory mechanisms were investigated in detail and the protective effects of Tax in a murine model of MRSA-induced lethal pneumonia was evaluated. In conclusion, the results indicated that Tax represents a potential anti-MRSA drug.

MATERIALS AND METHODS

Reagents and Materials

The peptide substrate Abz-LPATG-Dap (Dnp)-NH_2 (Abz:ortho-aminobenzoic acid; Dnp:2,4-dinitrophenyl) was provided by LifeTein (Beijing, China). Dimethyl sulfoxide (DMSO) was purchased from Beyotime (Shanghai, China). A library containing 420 natural compounds derived from Chinese traditional herbs was purchased from Pufeide Biotech Company (Chengdu, China).

Bacteria and Growth Conditions

S. aureus USA300 was obtained from the American Type Culture Collection (Manassas, VA). The *S. aureus* Newman SrtA deletion mutant (Δ*srtA*) and the pET28a-SrtA strain were already used and stored within the laboratory. *E. coli* BL21 (DE3) was used as a host for protein expression and purchased from the TransGen Biotech (Beijing, China). *E. coli* and *S. aureus* were cultured in Luria-Bertani broth (LB, Hopebio, Qingdao, China) and brain heart infusion medium (BHI, Solarbio, Beijing, China), respectively, at 37°C with constant shaking.

Expression and Purification of Recombinant SrtA and Its Mutants

Site-directed mutagenesis for D170A-SrtA and Q172A-SrtA was performed based on plasmid pET28a-SrtA using a multi-site mutagenesis kit (Transgen, Beijing, China) and the desired mutation was verified via DNA sequencing by Sangon Biotech (Shanghai, China). All primers used in the study are presented in **Table 1**. Subsequently, recombinant SrtA and the SrtA mutant proteins (D170A and Q172A) were expressed and purified in accordance with a previously published procedure (Zhulenkovs et al., 2014). Briefly, bacteria were cultured until an OD_{600} value of 0.8 was achieved, after which 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce recombination of SrtA at 16°C overnight. Because the recombinant protein had 6 × His tags, it was purified using a nickel-nitrilotriacetic acid (Ni-NTA) purification system. Imidazole (10 mM) was used to wash away excess protein, while 400 mM imidazole was employed to elute the target protein.

Screening of SrtA Inhibitors

Fluorescence resonant energy transfer (FRET) was used to determine the effect of Tax on SrtA, as described previously (Kruger et al., 2002). Briefly, 4 μM purified recombinant SrtA was combined with various concentrations of Tax then added to assay buffer consisting of 50 mM Tris-HCl, 5 mM $CaCl_2$, 150 mM

TABLE 1 | Primers used in this study.

Primer name	Sequences (5′–3′)
D170A -*srtA*-F	GAGTTCTAGCTGAACAAAAAGG
D170A -*srtA*-R	CTACATCTGTAGGCTTAACATC
Q172A -*srtA*-F	CTAGATGAAGCAAAAGGTAAAG
Q172A -*srtA*-R	AACTCCTACATCTGTAGGCTTA

NaCl, at pH 7.5, to a final volume of 200 μL. Following incubation of the mixture at 37°C for 1 h, substrate peptide was added to a final concentration of 10 μM and incubated for an additional 30 min. Fluorescence intensity was measured at excitation and emission wavelengths of 309 and 420 nm, respectively.

Susceptibility Assay and Growth Curve

A broth microdilution assay was performed to determine the minimum inhibitory concentration (MIC) of Tax for *S. aureus* USA300, as described elsewhere (Sader et al., 2013; Delgado-Valverde et al., 2017). A growth curve was further evaluated by adding various concentrations of Tax (0–200 μM) to a bacterial culture, then incubating until an OD_{600} of 0.3 had been reached. *S. aureus* USA300 and Δ*srtA* were used as controls. The OD_{600} value was recorded for each sample at 1 h intervals for a total of 24 h. The growth rate between *S. aureus* treated with Tax and *S. aureus* USA300 was also calculated at each time point.

Eukaryotic Cytotoxicity

Cytotoxicity was determined using a cell counting kit-8 assay (CCK-8, US EVERBRIGHT, Suzhou, China), as described previously (Xiao et al., 2018). HEK293 or HepG2 cells were seeded in 96-well plates (Corning, United States) at a density of 5×10^4 cells/well, then incubated at 37°C in an atmosphere containing 5% CO_2 for 24 h. The culture medium was then exchanged with fresh medium containing different concentrations of Tax (0–200 μM) or vehicle and incubated for an additional 24 h. CCK-8 solution (10 μL) was carefully added then incubated for 4 h in an incubator at 37°C. The OD value at 450 nm was measured to calculate cell viability.

Fibrinogen Binding Assay

Bacterial cultures grown overnight were diluted (1:100) in fresh BHI medium containing different concentrations of Tax then cultured at 37°C until an OD_{600} of 1.0 had been achieved. The Δ*srtA* strain was used as a positive control. Aliquots of bacteria were placed in the wells of a 96-well plate pre-coated with bovine fibrinogen (20 μg/mL). After incubation at 37°C for 2 h, the cell suspension in each well was replaced with 100 μL of 25% formaldehyde then incubated for a further 30 min, after which the formaldehyde was discarded. The plates were washed twice with PBS, and then 0.1% (w/v) crystal violet was added to stain the cells. Finally, after 20 min, the wells were gently washed with PBS then dried, and the OD value at 570 nm was measured.

Effects of Tax on Biofilm Formation

Bacterial cultures grown overnight were diluted 1:100 in BHI medium supplemented with 3% NaCl and 0.5% glucose. A 200 μL aliquot of each diluted culture was added to separate wells of a 96-well plate, to which different concentrations of Tax (25–200 μM) had been added, then incubated at 37°C for 24 h. Subsequently, the culture medium was replaced with crystal violet to stain the biofilm for 15 min. The wells were then washed thoroughly with sterile deionized PBS three times. The crystal violet stain in each well was decolorized with 200 μL absolute ethanol and then the OD_{595} value was recorded using a microplate reader.

Effects of Tax on Mature Biofilms

S. aureus biofilms were formed for 24 h at 37°C on the surfaces of the wells of a 96-well plate. Following biofilm formation, different Tax concentrations (25–200 μM) were added to selected wells and the plates were incubated for 24 h at 37°C. The effects of Tax on the mature biofilms were estimated using the crystal violet stain and recorded by a microplate reader.

FITC-IgG Binding Assay for Staphylococcal Protein A (SpA)

Bacterial cultures grown overnight were diluted 1:100 and cultured in an incubator at 37°C with Tax or DMSO to a concentration of 10^8 CFU/mL. The Δ*srtA* mutant strain was assayed as a positive control. The bacteria were collected and washed three times in PBS. A 50 μL aliquot of bacterial culture was mixed with an equal volume of FITC-labeled rabbit anti-goat-IgG (1:200, Sigma, United States) then incubated in the dark for 1 h. The bacteria were then washed and suspended in PBS. A multimode microplate reader (Tecan, Spark 20M) was used to measure fluorescence intensity at 535 nm when illuminated at an excitation wavelength of 485 nm.

Invasion Assay

A549 human lung carcinoma cells were seeded at a density of 2.5×10^5 per well in 24-well plates and placed in an incubator at 37°C in an atmosphere containing 5% CO_2 for 20 h. Cultures of *S. aureus* were mixed with different concentrations of Tax then cultured at 37°C until an OD_{600} of 1.0 was achieved. The A549 cells were resuspended in medium and aliquots of bacterial suspension were added to each well to a total concentration of 2×10^7°CFU/mL. After incubation for 2 h, the invasion assay was terminated by incubation with 300 μg/mL gentamicin for 30 min. The cells were then lysed after washing and coated on a BHI agar plate. After incubation at 37°C for 12 h, the number of CFUs were counted manually.

Western Blot Analysis

To evaluate the effect of Tax on SrtA expression, Tax (25–200 μM) was added to different bacterial cultures and incubated overnight at 37°C. Equal quantities of total protein from the bacterial lysates were separated by 12% SDS-PAGE then transferred to a polyvinylidene fluoride (PVDF) membrane using a transblot semidry system. Non-specific binding to the PVDF membrane was prevented by incubating in 5% bovine serum albumin (BSA) overnight at 4°C. The membranes were washed three times with PBST, agitating each time for 5 min, then incubated with rabbit polyclonal antibody (created in the laboratory) against SrtA. Following a further washing in PBST three times, the membranes were incubated with HRP-labeled goat anti-rabbit IgG (Bioworld, China) diluted 1:10,000 in antibody diluent (PBST + 1% BSA) at 37°C for 1 h. Δ*srtA* or *S. aureus* USA300 without Tax was considered the control group. The cytoplasmic protein ClpP was analyzed as an internal control. Blots were visualized using an enhanced chemiluminescence (ECL) detection system (GE Healthcare, United Kingdom),

while bands were quantified using ImageQuant TL software (GE Healthcare).

Localized Surface Plasmon Resonance

The interaction between Tax and SrtA was determined using an OpenSPR localized surface plasmon resonance (LSPR) instrument (Nicoya, Canada) at 25°C, as described previously (Panneer Selvam et al., 2018; Song et al., 2019). Briefly, SrtA proteins were captured on a COOH chip using a standard amine coupling system. A 200 μL aliquot of blocking buffer was added to rinse the sample ring and to remove any air. After a stable baseline had been achieved, the affinity between Tax and SrtA was measured by injecting 20, 40, 80, 160, or 320 μM Tax. The kinetic parameters of the binding reaction were calculated and visualized using TraceDrawer software (Yang et al., 2011).

Molecular Docking and Dynamic Simulation

For molecular docking (MD) simulations, Tax (PubChem ID: 439533) was docked onto the SrtA structure of *S. aureus* [Protein Data Bank (PDB) ID: 1T2P] using AutoDock Vina 1.1.2 software, with default parameters (Trott and Olson, 2010). The most appropriate docked pose (conformation) for the Tax-SrtA complex obtained using molecular docking was subject to 25 ns molecular dynamic simulations using Amber14 software (Götz et al., 2012). Preparation of the complex and the molecular dynamic simulation were conducted as previously described (Chan et al., 2017; Niu et al., 2019).

Murine Model of Pneumonia

To investigate the therapeutic capability of Tax to treat acute pneumonia infection caused by *S. aureus* USA300, 6- to 8-week-old female C57BL/6J mice were infected intranasally with 2×10^8 CFU of *S. aureus* USA300, then held upright for 30 s to ensure each animal inhaled the bacteria into their lungs. Two h post-infection, the mice were subcutaneously injected with Tax (100 mg/kg). Similarly, mice were challenged with Δ*srtA* and sterile PBS containing 0.5% DMSO (control group) as controls. To assess survival, the mice were checked every 12 h for 96 h, and the percentage that had survived was recorded.

For bacterial counts in lung tissue and histopathological examination, mice were infected intranasally with 30 μL (1×10^8 CFUs) of *S. aureus* USA300. Tax (100 mg/kg) was then injected subcutaneously every 12 h after inoculation. The bacterial load in the lungs was determined after the mice in each group were sacrificed 48 h post-infection. The left lung from each animal was removed aseptically and processed to count the bacterial CFUs. The right lung of each mouse was aseptically removed and analyzed by conventional hematoxylin and eosin (H&E) staining using an optical microscope. The ratio of wet to dry lung tissue weight (W/D ratio) was recorded in each case. Wet lung weight was measured 1 min after surface moisture had been removed. Dry lung weight was determined after dehydration at 80°C. The W/D ratio is used to reveal the degree of tissue edema (Xia et al., 2016).

Acute toxicity of Tax was evaluated in mice in accordance with the guidelines for the study of acute toxicity of chemical drugs in China (H-GPT1-1) (Carmichael, 2014). Briefly, each group of 5 female BALB/c mice (6–8 weeks old, weight 18–20 g) was administered a single intraperitoneal injection of 200, 100, or 50 mg per kg bodyweight of Tax, respectively. An injection of PBS represented the control group. Symptoms of poisoning or abnormal behavior were monitored, and survival of the mice in each group was recorded after 72 h.

Ethics Approval Statement

All animal experiments and surgical procedures were carried out in accordance with guidelines approved by the Animal Welfare and Research Ethics Committee of Jilin University.

Statistical Analysis

The data were expressed as the mean ± SD for each group in the individual experiments. The experimental data in this study were analyzed using GraphPad Prism 8.0. Statistical significance was accepted as P-values < 0.05.

RESULTS

Identification of Tax as a Reversible Inhibitor of SrtA

To identify novel inhibitors of SrtA, an in-house library containing 420 natural compounds was screened, using a FRET assay. The flavonoid Tax was found to display excellent inhibitory properties against *S. aureus* SrtA, with an IC_{50} value of 24.53 ± 0.42 μM (**Figures 1A,B**). This suggests that Tax is a relatively strong inhibitor of SrtA, compared with previously reported inhibitory small molecules (Oh et al., 2004; Chenna et al., 2010). To further clarify the interacting way of SrtA and Tax, recombinant SrtA was reacted with Tax at a concentration of 10-fold IC_{50}. A total of 85.80 ± 0.67% of SrtA activity was subsequently recovered, compared with the control group (DMSO) (**Figure 1C**). These data demonstrated that Tax was a reversible SrtA inhibitor, and bound non-covalently to the active site of SrtA.

Tax Has No Significant Inhibitory Effect on the Proliferation of *S. aureus* or Animal Cells

The MIC of Tax against *S. aureus* USA 300 was 512 μg/mL. The results of the growth curve and growth rate showed that Tax had no significant effect on the growth of *S. aureus*, at a concentration of IC_{50} ($P > 0.05$, **Figures 2A,B**). Safety testing further revealed that Tax displayed no significant cytotoxicity toward HEK293 and HepG2 cells at the concentration (24.53 ± 0.42 μM) required for repression of SrtA (**Figure 2C**). Taken together, Tax was effective and safe at the concentration required to inhibit SrtA, it was a promising candidate anti-virulence inhibitor and has the potential to be developed into a small molecule drug based on inhibition of SrtA.

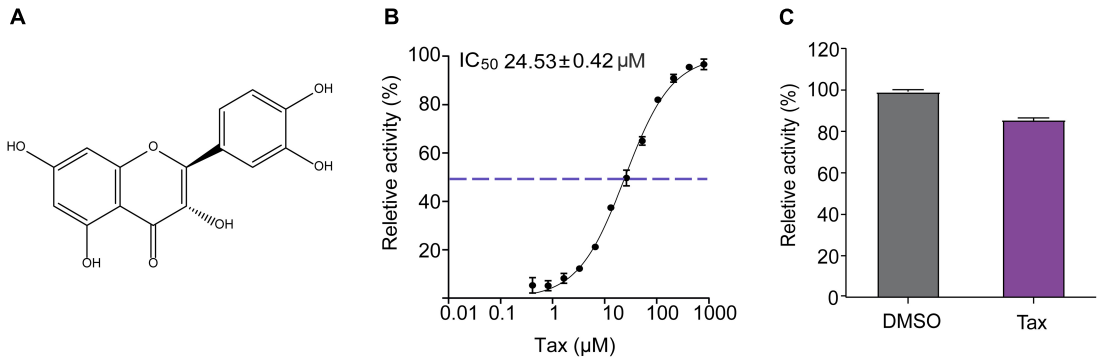


FIGURE 1 | Inhibition of SrtA transpeptidation. **(A)** Chemical structure of Tax. **(B)** Tax inhibits SrtA cleavage of a fluorogenic peptide substrate (Abz-LPATG-Dnp-NH_2) in a dose-dependent manner *in vitro*. Each reaction condition was assayed in triplicate from which the IC_{50} was determined. **(C)** Recombinant SrtA was treated with 10 × IC_{50} of Tax and then diluted, after which transpeptidation activity was measured using a FRET assay. Untreated SrtA (Control) represented 100% activity.

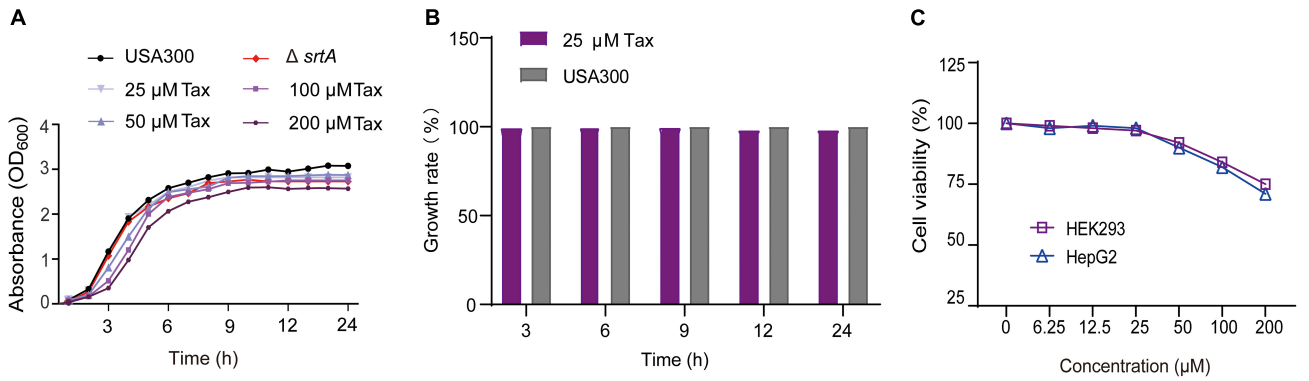


FIGURE 2 | Growth curve and cytotoxicity. **(A)** Growth curves of *S. aureus* USA300 and the Δ*srtA* group with different concentrations of Tax. **(B)** Growth rate of *S. aureus* USA300 and *S. aureus* treated with Tax (25 μM). **(C)** Percent cell viability of HEK293 (purple) and HepG2 (blue) cells were measured using a CCK-8 assay after incubation with Tax for 24 h.

Tax Represses the Adhesion of *S. aureus* to Fibrinogen

To establish whether Tax was able to reduce the adhesion of *S. aureus* to fibrinogen by inhibiting SrtA, a fibrinogen binding assay was performed. As shown in **Figure 3A**, Tax inhibited the adhesion of *S. aureus* USA300 to fibrinogen in a dose-dependent manner. Compared with the wild-type (WT) control group, 200 μM Tax significantly disrupted the adhesion of bacteria to fibrinogen, with an inhibition rate of 35.09 ± 0.49% ($P < 0.001$). This result reveals that Tax reduced the adhesion of bacteria to fibrinogen via inhibition of SrtA.

Tax Reduces Biofilm Formation and Fails to Eradicate Mature Biofilm in *S. aureus*

Initial attachment is the first stage of the development of a biofilm. Capture of the multiple surface proteins that are involved is mediated by SrtA (Otto, 2018). Consistent with this, increased expression of SrtA can cause a substantial increase in biofilm biomass in certain staphylococcal strains (Maresso et al., 2007). It can be inferred that Tax can reduce the formation of biofilms. As expected, Tax clearly inhibited biofilm biomass in a concentration-dependent manner. There was an inhibitory effect on biofilm biomass due to the coculture of *S. aureus* with 200 μM Tax, at 29.91 ± 0.51% of the mass of the untreated control. Biofilm formation by the Δ*srtA* group was 18.5 ± 1.02%, indicating that Tax reduces the formation of *S. aureus* biofilms via repression of SrtA activity (**Figure 3B**). Subsequently, we further evaluated the effects of Tax on mature biofilms, the results showed that different concentrations of Tax (25–200 μM) had no effect on mature biofilms (**Figure 3C**).

Tax Suppresses the Invasion of *S. aureus* Into A549 Cells

Since the usual initial point of infection by *S. aureus* is epithelial cells, colonization on the cell surface and their invasion through SrtA-mediated cell surface proteins may result in acute and chronic infection (Gómez et al., 2004). Therefore, the effect of Tax on the internalization of *S. aureus* into A549 cells was investigated. As demonstrated in **Figure 3D**, the number of bacteria entering cells was reduced significantly following treatment with 200 μM Tax ($P < 0.001$), suggesting that

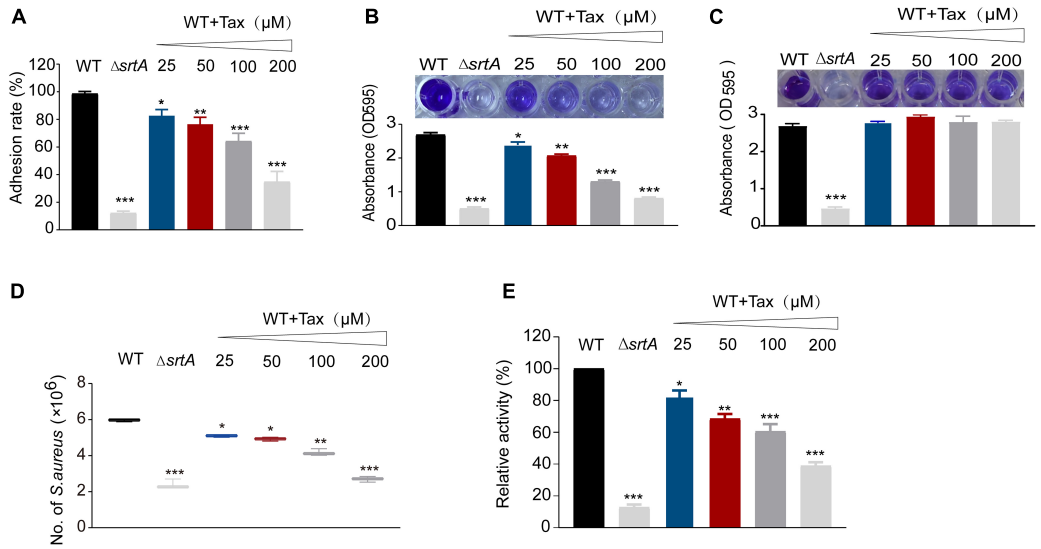


FIGURE 3 | Effect of SrtA inhibitor on virulence-related phenotypes in *S. aureus* Newman strain. **(A)** Impact of Tax on the ability of *S. aureus* to adhere to fibrinogen. **(B)** Biofilm formation of *S. aureus* in the presence of different concentrations of Tax. **(C)** Mature biofilms of *S. aureus* in the presence of different concentrations of Tax. The Δ*srtA* group represented the positive control. **(D)** Tax affects internalization of *S. aureus* into A549 cells. A549 cells were infected with *S. aureus* pretreated with different concentrations of Tax then lysed 2 h post-infection. The number of viable *S. aureus* in the cells was quantified by serial dilution on TSA agar plates. **(E)** Effects of Tax on *S. aureus* protein A (SpA) using FITC-labeled rabbit IgG. Error bars represent means ± SD of three replicates. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ calculated with a two-tailed Student's *t*-test.

Tax suppresses the internalization of *S. aureus* by inhibition of SrtA, this may be because Tax reduces the anchoring of bacterial surface proteins required for internalization by inhibiting SrtA.

Tax Influences Anchoring of SpA in *S. aureus*

In *S. aureus*, SrtA can anchor a variety of different surface proteins onto the bacterial envelope (Mazmanian et al., 2002; Falugi et al., 2013). Staphylococcal protein A (SpA) is able to specifically bind the FCγ and Fab regions of IgG. Although there was strong evidence that Tax can inhibit SrtA *in vitro*, it was of great significance to further quantify the surface SpA after treatment with Tax. In the control group, Δ*srtA* displayed weak fluorescence, indicating that it seemed to have completely lost the ability to anchor SpA to the cell wall. After treatment with 200 μM Tax, *S. aureus* displayed a clear decrease in fluorescence, with a relative activity 36.12 ± 1.59% that of the WT group ($p < 0.001$). These results suggests that Tax can reduce the amount of anchored SpA in the cell wall by inhibiting the activity of SrtA (**Figure 3E**).

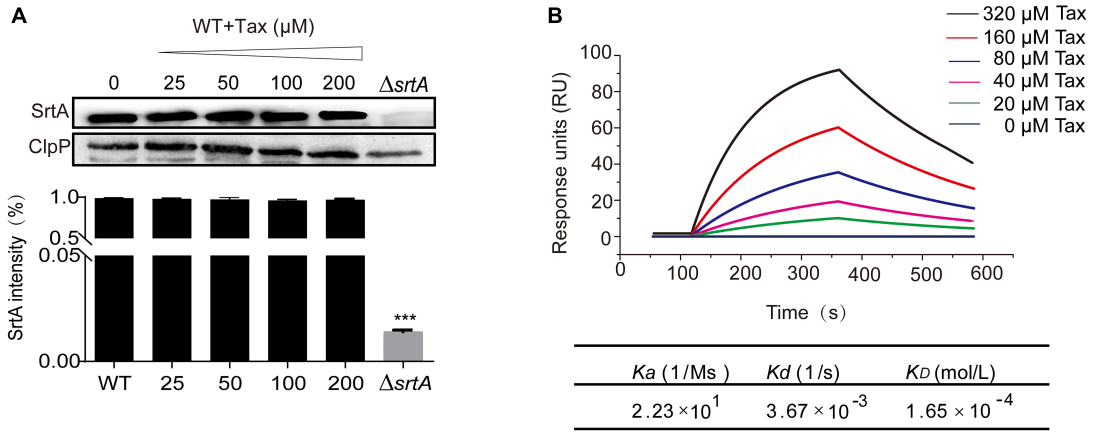
Tax Has No Apparent Influence on SrtA Expression in *S. aureus*

Western blot analysis was used to further evaluate the effect of Tax on SrtA expression Compared with the untreated group, there was no apparent effect on SrtA expression, indicating that Tax effectively blocked the function of SrtA through a mechanism other than inhibition of its expression (**Figure 4A**).

Determination of the Molecular Mechanism of the Interaction Between Tax and SrtA

To explore the interaction between Tax and SrtA, an LSPR experiment was performed. The root mean square fluctuations (RMSF) suggested that SrtA exhibited different flexibility in binding sites with and without Tax (**Figure 5A**). The results revealed that Tax interacts directly with SrtA in a dose-dependent manner, with a K_D of 1.65×10^{-4} mol/L (**Figure 4B**). These data indicate that Tax binds directly to SrtA. To investigate the mechanism of the inhibition of SrtA by Tax in more depth, molecular modeling studies were performed. Through analysis of an *in silico* model of the SrtA-Tax complex, it was found that the Asp-170 residue provides a strong electrostatic (ΔE_{ele}) contribution, with $\Delta E_{ele} < -14.0$ kcal/mol (**Figure 5B**). Asp-170 was found to have the ability to form a strong hydrogen bond with Tax, with a bond length of 2.0 Å (**Figure 5B**). In addition, Gln-172 provided a significant *van der Waals* force contribution ($\Delta E_{vdw} < -2.5$ kcal/mol) (**Figure 5B**), due to the proximity of Gln-172 to Tax (**Figure 5C**). Except for Gln-172, the majority of energetic interactions for decomposition stemmed from *van der Waals* interactions, and mostly through the hydrophobic interactions of Val-166, Val-168, Leu-169, and Val-201. Finally, the total binding free energy of the SrtA-Tax complex (ΔG_{bind}) was calculated, according to the MMGBSA method (Ylilauri and Pentikäinen, 2013), to be −19.2 kcal/mol, indicating that the complexation of Tax with SrtA is strong.

Molecular modeling studies highlight the benefits of performing site-directed mutagenesis studies. Therefore,



Ka (1/Ms)	*Kd* (1/s)	*KD* (mol/L)
2.23×10^{1}	3.67×10^{-3}	1.65×10^{-4}

FIGURE 4 | Expression of SrtA in the presence of Tax and the interaction of different concentrations of Tax with SrtA. **(A)** Western blot analysis of SrtA protein from *S. aureus* treated with various concentrations of Tax and grayscale analysis of SrtA protein bands. **(B)** LSPR analysis verified the binding affinity of Tax with SrtA. Error bars represent means ± SD of three replicates. ***$P < 0.001$ calculated with a two-tailed Student's *t*-test.

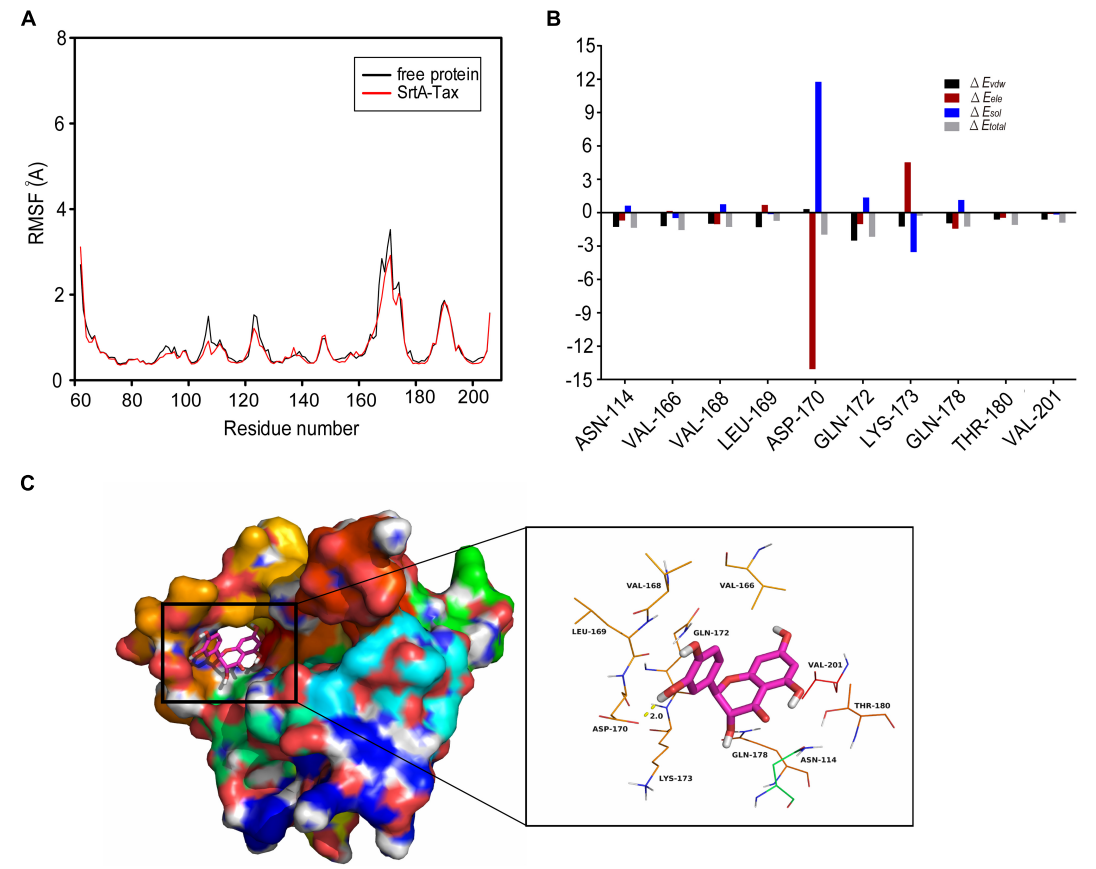


FIGURE 5 | Molecular modeling of the interaction between Tax and SrtA. **(A)** RMSF (Å) graph of free-SrtA (black) and the SrtA-Tax (red) complex during 40-ns MD. **(B)** Docking model of Tax with SrtA during a molecular dynamics simulation. **(C)** Binding free energy decomposition in each residue between Tax and modeled *S. aureus* SrtA.

fluorescence quenching experiments were utilized to determine the binding affinity of Tax with SrtA and SrtA mutants (D170A, Q172A). As displayed in **Table 2**, the binding constant K_A for Tax with each SrtA mutant (D170A, Q172A) was significantly lower than that for WT SrtA, indicating that residues Asp-170 and Gln-172 were pivotal binding sites for Tax with SrtA.

TABLE 2 | The values of the binding constants (K_A) based on fluorescence quenching assay.

Proteins	WT-SrtA	D170A	Q172A
K_A (1×10^4) l/mol	6.4	2.69	3.85
n	0.9682	0.9341	0.8912

Taken together, the results confirm that there is a direct interaction between Tax and SrtA with an inhibitory effect exerted via its direct binding with key residues of SrtA. Confirmation of this interaction allowed additional evaluation of the protective effect of Tax on *S. aureus* infection *in vivo*.

Tax Protects Mice From MRSA-Induced Pneumonia

Pneumonia caused by MRSA is clinically critical due to its complexity, the high incidence of complications, and high rates of mortality (Michalopoulos and Falagas, 2006). Therefore, the protective effects of Tax were evaluated in a murine model of MRSA-induced pneumonia.

Survival rate was examined by the intranasal administration of lethal doses of *S. aureus* USA300 to mice, with subsequent treatment with Tax every 12 h. The mortality rate was recorded at 12 h intervals for 96 h. As presented in **Figure 6A**, only 20% of mice survived when challenged with *S. aureus* USA300 96 h

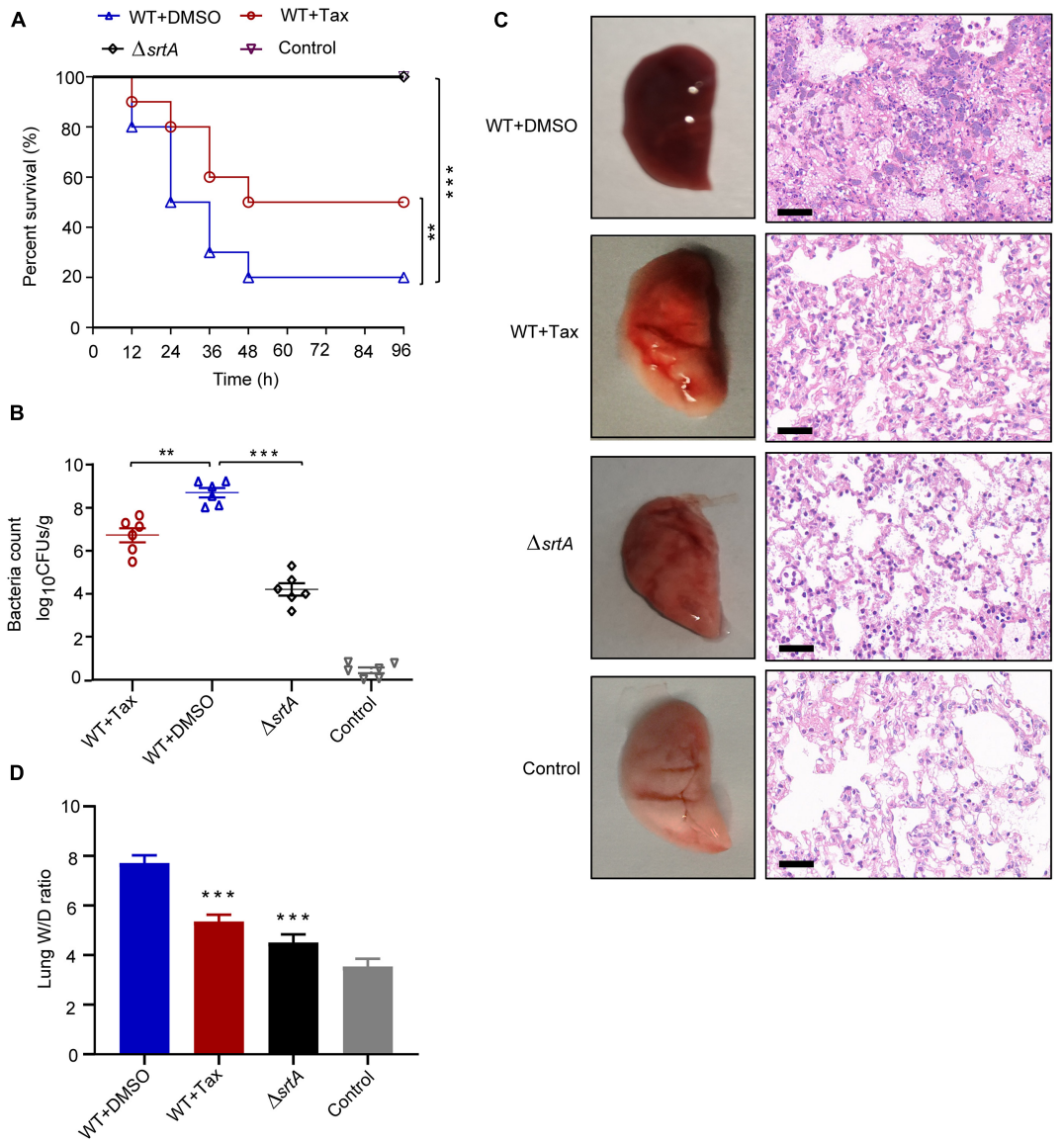


FIGURE 6 | Effect of treatment with Tax on *S. aureus*-induced pneumonia in mice. **(A)** Effect of Tax on the survival of mice (n = 10 per group) infected with a lethal dose of *S. aureus*. **(B)** Effect of Tax (100 mg/kg) on bacterial load in the lungs of mice (n = 6). **(C)** Gross pathological changes and histopathology of mouse lungs treated with Tax (100 mg/kg/d) or untreated. Scale bar: 50 μm. **(D)** Lung wet-to-dry weight ratio (W/D). Each value represents mean ± SD (n = 5 per group). $^{**}P < 0.01$, $^{***}P < 0.001$; Mann-Whitney test, two-tailed. Horizontal bars represent mean values. Animal data were obtained from two separate experiments.

post-infection, while infection in the Δ*srtA* group resulted in 100% survival. This indicates that SrtA is key to the pathogenesis of *S. aureus* pneumonia, consistent with previous reports (Cascioferro et al., 2014). Importantly, 100 mg/kg treatment of Tax significantly improved the survival rate of mice to 50% ($p < 0.01$), particularly during the early stages of infection. These findings revealed that Tax provided a strong protective effect against *S. aureus* infection *in vivo*.

The bacterial load in the lungs of the infected group was 8.56 ± 1.34 lg CFU/g (**Figure 6B**). After treatment with Tax, the bacterial load decreased to 6.73 ± 0.69 lg CFU/g, demonstrating its significant inhibition of *S. aureus* invasion in the lungs.

The pathological changes in mouse lung tissue were further evaluated. Firstly, the appearance of the lung tissue in each group was analyzed. Soft and elastic lung tissue was observed in the control (uninfected) group, whereas lungs infected with *S. aureus* USA300 displayed significant hyperemia and low levels of elasticity, consistent with previous results. Following their treatment with Tax, the lung tissue of infected mice was only slightly red and the extent of congestion was significantly reduced, while elasticity was restored (**Figure 6C**, left). In addition, histopathological examination demonstrated that the lung tissue of the WT group (*S. aureus*) was clearly hyperemic with a large number of inflammatory cells accumulated within the alveoli. However, treatment with Tax significantly reduced inflammation, as confirmed by the reduced accumulation of inflammatory cells within the alveolar spaces, and relatively complete alveolar structure (**Figure 6C**, right).

Furthermore, lung W/D ratios, recorded to evaluate the severity of lung injury (Xiao et al., 2008), increased from 3.53 ± 0.16 to 7.71 ± 0.62 after infection with *S. aureus* USA300 (**Figure 6D**). Conversely, the W/D ratio decreased significantly to 5.35 ± 0.34 in mice treated with Tax (100 mg/kg) ($P < 0.001$).

Taken together, we conclude that Tax attenuates the virulence of *S. aureus in vivo* and provides significant protection against lethal *S. aureus*-induced pneumonia. In addition, the acute toxicity test indicated that no signs of malaise, or tardy or inanimate behavior were observed following intraperitoneal injection of 200 mg/kg Tax.

DISCUSSION

The evolution, spread, and accumulation of multidrug-resistant (MDR) pathogens, such as MRSA represent a significant hidden risk to human public health (Skaar, 2010). Recent studies have shown that the proportion of MRSA isolated from patients is increasing, leading to significant incidence rate and mortality (Iwata et al., 2020). Antibiotics identified from other microorganisms have always been the primary weapon to combat bacterial infection (Rasko and Sperandio, 2010). These antibiotics rely on disrupting the key components in bacterial synthesis and assembly such as cell wall synthesis, DNA replication and protein synthesis (Mann, 2003). The effective bactericidal effect of these antibiotics also caused great survival pressure on the pathogens, making the rapid production of drug-resistant bacteria (Werner et al., 2008; Rasko and Sperandio, 2010). Therefore, it is imperative to develop alternatives to antibiotics which can not only inhibit infection but also avoid the production of drug-resistant bacteria. A promising strategy is to develop antivirulence therapies. The purpose of antivirulence strategies is not to kill pathogens directly, but to disarm them and prevent their attack on the host. This would not, therefore, exert selective pressure and so reduces the risk of drug resistance to a great extent (Hou et al., 2018). Moreover, a significant advantage of antivirulence strategies is that they reduce the damage to host

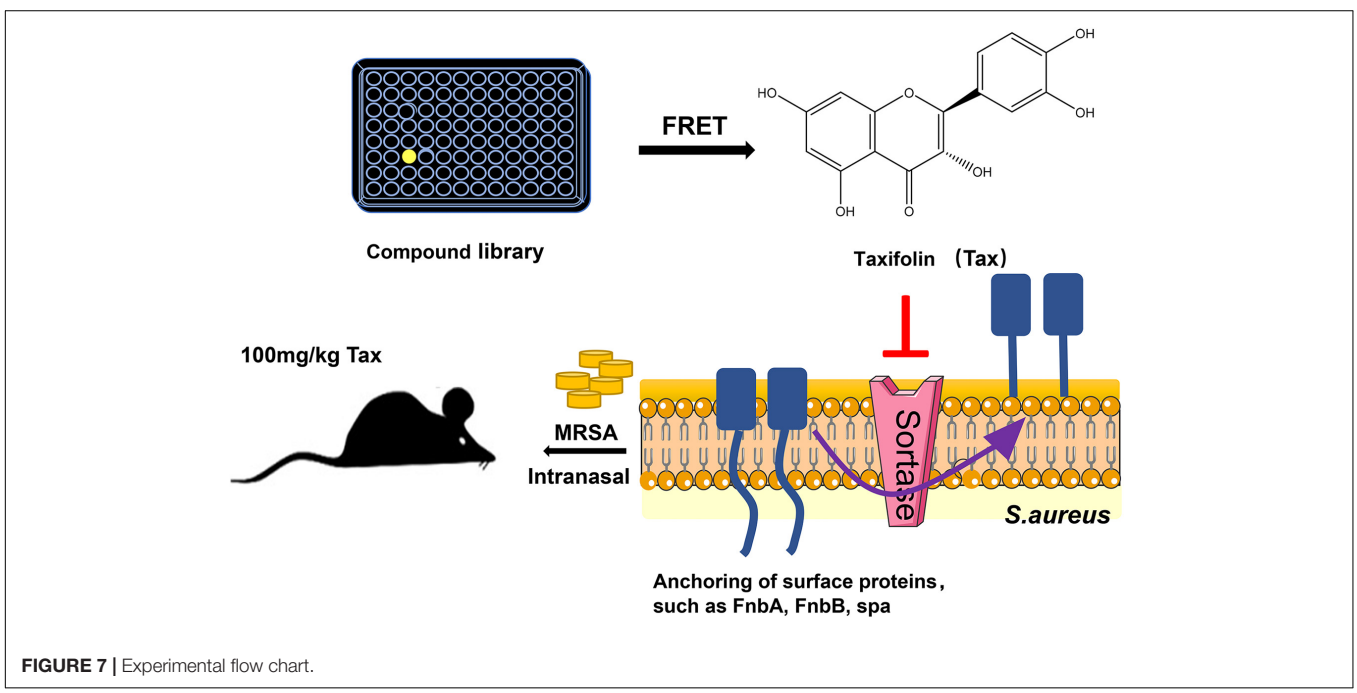


FIGURE 7 | Experimental flow chart.

microbiota, overcoming the adverse reactions and serious side effects of antibiotic therapies (Buroni and Chiarelli, 2020).

SrtA can recognize, cleave, and anchor specific LPXTG-containing proteins which contribute to the bacterial infection process (Si et al., 2016), many of those surface proteins mediate bacterial colonization to host tissues and cells, the formation of biofilms and evasion of the immune response, etc. (Geoghegan and Foster, 2017). In addition, SrtA is constitutively expressed in all clinical isolates (Marraffini et al., 2006; Foster et al., 2014). Therefore, SrtA has long been recognized to be an important virulence factor of *S. aureus* and a promising pharmacological target of antivirulence strategies.

The experimental flow chart is shown in **Figure 7**. We identified the natural compound Tax that could block SrtA activity, with an IC_{50} of 24.53 ± 0.42 μM. Inhibitors of SrtA can be categorized as either covalent or non-covalent (Jackson et al., 2017; Jaudzems et al., 2020). The formation of covalent bonds occurs via the reaction of an inhibitor with the cysteine active site (Hou et al., 2018; Barthels et al., 2020). We found that Tax is a non-covalent inhibitor that binds reversibly to SrtA. Reversible inhibitors might be a promising alternative, which overcome the drawbacks of the irreversible inhibitors, such as high toxicity, non-recoverability, non-repairability and other side effects (Beck et al., 2012). Importantly, Tax exhibited no cytotoxicity to mammalian cells (HEK293 and HepG2) at the concentration required to inhibit SrtA. This satisfies the main criteria for an ideal antibacterial agent: low selectivity pressure that avoids the generation of drug resistance (Schneewind and Missiakas, 2019). *S. aureus* is considered notorious due to its extraordinary capability to create a biofilm (Oliveira et al., 2018), a surface-attached encasement of cells and matrix. Biofilms lead to increased antibiotic resistance and evasion of the host immune system, increasing the complexity of the existing problem of antibiotic resistance (Vermote and Van Calenbergh, 2017; Graf et al., 2019). In the present study, our results showed that the co-incubation of *S. aureus* with Tax led to decreased biofilm formation but did not affect the mature biofilm, which indicates that SrtA inhibited the formation of biofilm by inhibiting the anchoring of surface protein required in the adhesion stage of biofilm (Ming et al., 2017; Vazquez-Armenta et al., 2018). Moreover, the MD simulation and mutagenesis study indicated that Tax binds to the binding pocket of SrtA, depending mostly on hydrogen bonding, and electrostatic and *van der Waals* interactions, in which Asp-170 and Gln-172 play an important role.

Bacterial strains with a mutation of SrtA have exhibited attenuated virulence in a mouse infection model (Chen et al., 2005), suggesting that SrtA performs a critical role in staphylococcal pneumonia. Therefore, inhibition of surface protein anchoring through the interference of SrtA should affect the ability of *S. aureus* to establish pulmonary infection. As expected, we observed that Tax significantly protected mice from challenge by lethal doses of *S. aureus* USA300, resulting in a reduction in bacterial burden in lung tissue. In addition, the safety of Tax was verified by acute toxicity testing which indicated that Tax has the potential for further development and clinical application.

Collectively, the present study reports on the discovery that the natural compound Tax is a specific SrtA inhibitor that is safe and efficacious, able to attenuate MRSA virulence *in vitro* and *in vivo* and the potential for development into a novel antivirulence agent. In addition, sortases possess an LPXTG sorting signal which is present in almost all strains of low G + C Gram-positive bacteria (Sillanpää et al., 2010; Spirig et al., 2011; van Harten et al., 2017; Kang et al., 2020). This suggests that SrtA inhibitors have the potential for further development and could be widely used in the treatment of multiple Gram-positive infections.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of Jilin University.

AUTHOR CONTRIBUTIONS

QL, GW, and DW conceived and designed the experiments. LW, SJ, and SG performed and analyzed the experiments. LS, HQ, and KW performed the molecular dynamics simulation. LW prepared the original manuscript and revised the manuscript.

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REVIEW
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Antibacterial Properties of Organosulfur Compounds of Garlic (*Allium sativum*)

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Garlic (*Allium sativum*), a popular food spice and flavoring agent, has also been used traditionally to treat various ailments especially bacterial infections for centuries in various cultures around the world. The principal phytochemicals that exhibit antibacterial activity are oil-soluble organosulfur compounds that include allicin, ajoenes, and allyl sulfides. The organosulfur compounds of garlic exhibit a range of antibacterial properties such as bactericidal, antibiofilm, antitoxin, and anti-quorum sensing activity against a wide range of bacteria including multi-drug resistant (MDR) strains. The reactive organosulfur compounds form disulfide bonds with free sulfhydryl groups of enzymes and compromise the integrity of the bacterial membrane. The World Health Organization (WHO) has recognized the development of antibiotic resistance as a global health concern and emphasizes antibiotic stewardship along with the urgent need to develop novel antibiotics. Multiple antibacterial effects of organosulfur compounds provide an excellent framework to develop them into novel antibiotics. The review provides a focused and comprehensive portrait of the status of garlic and its compounds as antibacterial agents. In addition, the emerging role of new technologies to harness the potential of garlic as a novel antibacterial agent is discussed.

Keywords: garlic (*A. sativum*), organosulfur compounds, antibiofilm, antibacterial, multi-drug resistance (MDR)

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INTRODUCTION

Garlic (*Allium sativum*), belonging to family *Liliaceae*, mainly the bulb of garlic, has been used as a spice in cooking worldwide especially in Italy and Southeast Asia. More importantly, garlic has been an ingredient in folk and traditional medicine since ancient times (Rivlin, 2001). Garlic is cultivated all over the world with a per-capita consumption of two pounds per year. As per the Food and

Abbreviations: MDR, multi-drug-resistant; FGE, fresh garlic extract; GP, garlic powder; GO, garlic oil; AGE, aqueous garlic extract; QS, quorum sensing; SAC, *S*-allyl cysteine; STEC, Shiga toxin-producing *Escherichia coli*; MRSA, methicillin-resistant *Staphylococcus aureus*; VRSA, vancomycin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant *enterococci*; DAS, diallyl sulfide; DADS, diallyl disulfide; DATS, diallyl trisulfide; DATTS, diallyl tetrasulfide; MIC, minimum inhibitory concentration; Bcc, *Burkholderia cepacia* complex; EPS, extrapolysaccharide; QSI, quorum sensing inhibition.

Agricultural Organization of the United Nations, China and India are first and second, respectively, in average (1961–2017) garlic production. Health benefits that are associated with the use of garlic are attributed to its anticancer, anti-inflammatory, antifungal, antiviral, and antibacterial activity. Several *in vitro*, *in vivo,* and epidemiological studies indicate that garlic exhibits anticancer activity, and the likely mechanism of action is by activating metabolizing enzymes, inhibiting reactive oxygen species, radical scavenging, preventing DNA damage, and tumor inhibition (Cao et al., 2014; Zhang Y. et al., 2019). The immunomodulatory effects of garlic are mediated through its ability to modulate cytokine production as well as activate immune response by stimulating antibody secretion and immune cells (Arreola et al., 2015). Garlic displays anti allergic properties by inhibiting antibody-mediated histamine production and modulates airway allergic response (Kyo et al., 2001; Zare et al., 2008). The anti inflammatory and anti arthritic ability of garlic comes from its ability to inhibit NF-κB signaling (Ban et al., 2009). Garlic oil (GO) exhibits antifungal activity against *Candida albicans* and *Penicillium funiculosum* by penetration into cells and organelles and causing differential expression of genes that are critical for cellular metabolism (Li et al., 2016). One of the earliest reports of garlic's antibacterial activity was by Small et al. (1947) and Stoll and Seebeck (1947). Since then, extensive research has been performed on the antibacterial effects of garlic. The antibacterial activity against various pathogenic and drug-resistant bacteria was tested using crude garlic extracts, garlic powder (GP), garlic extracts using various solvents, GO, and phytochemicals isolated from garlic. The constant and rapid emergence of antimicrobial resistance has been recognized as an alarming threat to human health, which mandates the scientific community to develop novel and effective antibacterial agents (Cheng et al., 2016). Garlic compounds exhibit multiple modes of antibacterial activity and have enormous potential to be developed into novel antibacterial agents. Most reviews about garlic discuss the antibacterial activity of garlic as one of its many health benefits diluting the importance of garlic compounds as potential antibacterial agents. This review exclusively focuses on significant antibacterial studies that were performed with garlic and its phytochemicals.

ACTIVE PHYTOCHEMICALS OF GARLIC

Most of the health benefits of garlic are attributed to a myriad of cysteine-derived sulfur-containing organic compounds present in garlic (extensively reviewed in Fenwick and Hanley, 1985a,b,c). The organosulfur compounds of intact garlic clove greatly differ from that present in garlic juice obtained after crushing garlic. The intact garlic mainly contains non-volatile γ-glutamyl-*S*-alk(en)yl-L-cysteines, namely, γ-glutamyl-*S*-allyl-L-cysteine, γ-glutamyl-*S*-*trans*-1-propenyl-L-cysteine, and *S*-alk(en)yl-L-cysteine sulfoxides such as *S*-allyl-L-cysteine sulfoxide (alliin), *S*-(*trans*-1-propenyl)- L-cysteine sulfoxide (isoalliin), and *S*-methyl-L- cysteine sulfoxide (methiin) with a small amount of *S*-allyl cysteine (SAC) (**Figure 1A**) (Block, 1992). Crushing or cutting garlic cloves releases allinase enzyme sequestered in the vacuoles, which encounters cytosolic alliin to convert it into an array of thoisulfinates of which the most prominent is allicin. The highly reactive, unstable, and volatile allicin decomposes to yield a large number of sulfides such as diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), methyl allyl disulfide (MADS), methyl allyl sulfide, ajoene, and vinyl dithiins (2-vinyl-1,3-dithiin, 3-vinyl-1,2-dithiin) shown in **Figure 1B** (Brodnitz et al., 1971). The sulfides are oil-soluble compounds that are responsible for the characteristic garlic odor and flavor. Allicin exhibits excellent *in vitro* antibacterial activity, which resulted in a huge number of studies to evaluate the potential of allicin and oil-soluble organosulfur compounds of garlic as antibacterial agents (Cavallito and Bailey, 1944a). A large body of literature supports the antibacterial potential of garlic organosulfides. The organosulfur compounds present in the aqueous and alcoholic extract of garlic include *S*-allyl cysteine (SAC), *S*-allylmercapto-L-cysteine (SAMC), and *S*-methyl cysteine (**Figure 1C**). The compounds are non-volatile, non-odiferous, and stable compounds compared to volatile organosulfides. Most health benefits of garlic are largely attributed to these organosulfur compounds present in garlic. However, garlic organosulfur compounds are very unstable with low bioavailability and the presence of these compounds depends on the processing of the garlic during the preparation of garlic supplements (Amagase et al., 2001; Amagase, 2006).

The main antibacterial organosulfur compounds of garlic are allicin, ajoene, and various aliphatic sulfides. The extraction procedure results in concentrating a particular compound rather than providing a pure compound. Extraction of garlic with water or ethanol and concentrating the extract will provide an allicin-rich product. It was noticed that yield with ethanol is better compared to water (Fujisawa et al., 2008). However, extraction of concentrated ethanolic distillate with organic solvent yields a highly concentrated and pure allicin product (Cavallito and Bailey, 1944a; Ratnakar and Murthy, 1995). Later, it was reported that extraction using acetone will yield higher allicin compared to ethanol (Canizares et al., 2004a). Recently, salting-out extraction using ethanol and ammonium sulfate result in effective extraction of allicin (Li et al., 2017). Oil-macerated garlic extract has a very high proportion of ajoene along with other thiosulfinates (Yoshida et al., 1998). Steam distillation of garlic yields GO, which mainly consists of various aliphatic sulfides (Avato et al., 2000). The components of both oil-macerated garlic extract and GO can be separated using chromatographic and distillation techniques (Casella et al., 2013).

PubMed search of "Garlic antibacterial" yields more than 350 research papers. This large body of literature comprises research papers that investigated the antibacterial activity of crude preparation of garlic, various extracts of garlic, and individual organosulfur compounds of garlic against various bacteria including MDR bacteria. **Table 1** provides a list of *in vitro* antibacterial activity of various garlic products and compounds against different bacteria. Similarly, **Table 2** provides similar information on *in vivo* studies. Some of the early research that reported the antibacterial activity of garlic against a wide variety of bacteria has been summarized

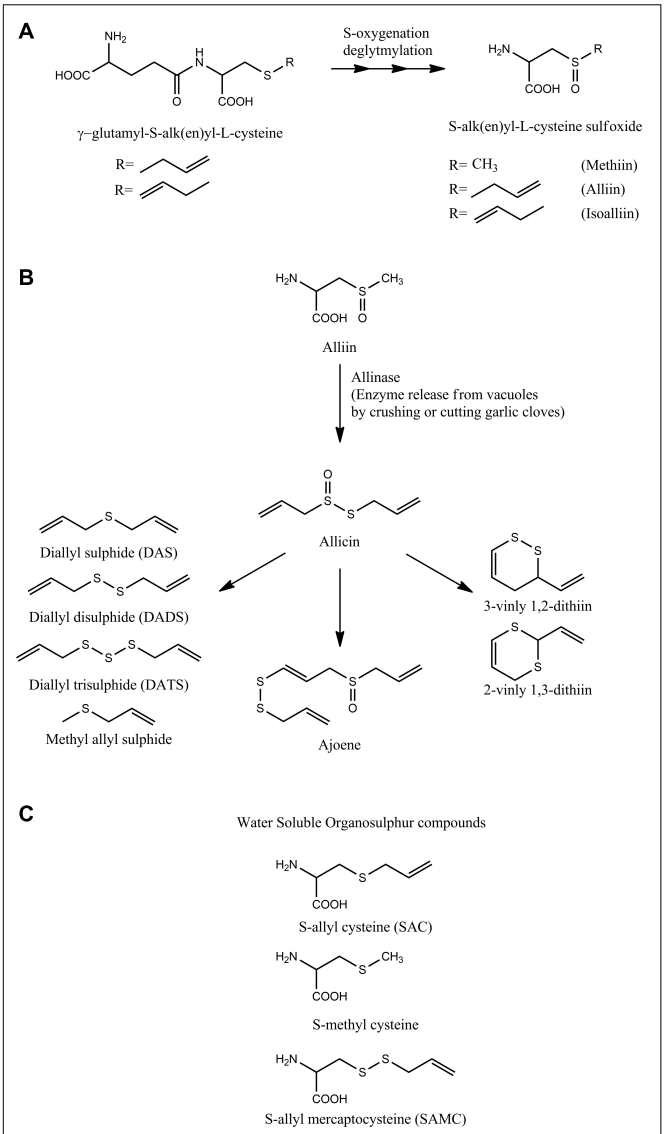

FIGURE 1 | Organosulfur compounds of garlic: The figure shows the major organosulfur compounds present in garlic. **(A)** The major compounds found in intact garlic cloves. **(B)** The crushing of garlic clove converts alliin into allicin by the action of allinase enzyme. Allicin is a highly unstable compound that degrades and rearranges itself into different organosulfide compounds shown in the figure. **(C)** Apart from oil-soluble organosulfur compounds, garlic also has water-soluble organosulfur compounds shown in the figure.

by Adetumbi and Lau (1983). The present review provides a comprehensive summary of this large body of research.

ANTIBACTERIAL ACTIVITY OF GARLIC FRESH EXTRACT AND POWDER

Garlic is one of the popular spices added to food to enhance the flavor, and it has been used in different cultures and traditions around the world to treat bacterial infections for centuries. Several studies have evaluated the antibacterial activity of various garlic preparations such as crude or fresh garlic extract (FGE), and garlic paste. The antibacterial activity of garlic paste and FGE against commensal and pathogen enteric bacteria such as *Escherichia coli, E. coli* O157:H7, *Salmonella* species, *Shigella* species, *Vibrio* species, *Campylobacter* species, *Listeria monocytogenes*, *Enterobacter,* and *Enterococcus* species*, Lactobacillus acidophilus*, *Staphylococcus aureus, Streptococcus* species, and *Clostridium difficile* has been reported by various laboratories (Johnson and Vaughn, 1969; Kumar and Berwal, 1998; Ross et al., 2001; Gupta and Ravishankar, 2005; Vuddhakul et al., 2007; Lu et al., 2011b; Jain et al., 2015a; Roshan et al., 2017). These studies suggest that garlic consumption could help in preventing food poisoning. In addition, various studies have evaluated the impact of garlic and its organosulfur compounds on the gut microbiome. Garlic was found to positively influence the gut microbiome and protect the gut microbiome damage from high-fat diet (Chen et al., 2019). Supplementing feed of farrowing sows and European bass with GO decreased pathogenic microbes from the gut microbiome (Rimoldi et al., 2020; Satora et al., 2020). Allicin treatment prevented high carnitine diet-induced dysbiosis to lower the atherosclerosis risk factor trimethylamine N-oxide that is produced by the gut microbiome (Wu W. K. et al., 2015). Oral administration of alliin, precursor of allicin, to rats resulted in decreasing the relative abundance of only *Allobaculum* genus in the cecum (Zhang C. et al., 2019). The gut microbiome was altered upon intragastric administration of DADS of rat, a low dose of DADS decreased *Bacteroidetes* phyla but increased *Firmicutes* phyla bacteria (Yang et al., 2019). Oral administration of propyl propane thiosulfonate restored the richness and evenness of gut microbiome lost due to dextran sodium sulfate-induced colitis in mice (Vezza et al., 2019). In a small-scale clinical trial, aged garlic extract supplementation for 3 months increases the richness and diversity of the gut microbiome with increase in *Lactobacillus* and *Clostridium* species (Ried et al., 2018). All the studies indicate that garlic and its compounds have a positive effect on gut microbiome composition and richness. However, the mechanistic details still need to be investigated. In a recent study from our laboratory, FGE exhibited activity against MDR Shiga-toxin producing *E. coli* (STEC) isolates from clinical and food samples (Bhatwalkar et al., 2019). In addition to antibacterial activity, garlic crude and aqueous extract exhibited anti-adherent activity against the standard strain type of *Streptococcus mutans* (Jain et al., 2015a). The data suggest that garlic could be used to preserve food and prevent foodborne infections. However, the antibacterial activity was dramatically decreased when experiments were performed with buffered peptone water and ground beef, suggesting that further research is required to utilize garlic as a food/meat-preserving agent (Gupta and Ravishankar, 2005). The causative agent of gastric ulcers, *Helicobacter pylori* (standard strains types and clinical isolates), was found to be sensitive to GP and 1,000 μg/ml of GP inactivated *H. pylori* at 6 h in a time course viability assay (O'Gara et al., 2000). Allicin-rich crude extract exhibited better antibacterial activity against *Mycobacterium phlei, Mycobacterium smegmatis,* and *Mycobacterium tuberculosis* compared to isoniazid and ethambutol. Also, disk diffusion assay with allicin-rich extract exhibited significant activity against

MRSA (Viswanathan et al., 2014). Another study also found that FGE was effective against MDR strains of *E. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Serratia marcescens,* and MRSA in both *in vitro* and *in vivo* assays (Farrag et al., 2019).

ANTIBACTERIAL ACTIVITY OF GARLIC AQUEOUS EXTRACT

There are several reports of antibacterial activity of aqueous garlic extract (AGE) against a variety of bacteria. *In vitro* assay with AGE (10%) showed complete inhibition of *Bacillus cereus* and the activity varies upon the storage conditions and heat treatment of the aqueous extract (Saleem and Al-Delaimy, 1982). AGE exhibited *in vitro* antibacterial activity against various pathogenic bacteria including *Shigella* and *Salmonella* species and enterotoxigenic *E. coli* (Arora and Kaur, 1999). In addition, AGE fully cured the rabbits that were challenged with *Sh. flexneri Y* by completely clearing them of bacteria with no significant side effects (Chowdhury et al., 1991).

Supporting the results obtained with GP, *in vitro* assays indicated that *H. pylori* is sensitive to AGE, and the sensitivity was more compared to *S. aureus* (Cellini et al., 1996; Sivam, 2001). *In vitro* antibacterial assays report that AGE is effective against various Gram-positive and Gram-negative oral bacteria, which include periodontal pathogenic bacteria *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans,* and *S. mutans* (Bakri and Douglas, 2005; Fani et al., 2007; Velliyagounder et al., 2012). Different studies reported that AGE exhibited activity against a large variety of Gram-positive and Gram-positive pathogenic bacteria including MDR strains and isolates such as MDR *M. tuberculosis* showing not only the effectiveness of garlic against drug-resistant bacteria but also its broad spectrum (Iwalokun et al., 2004; Gupta et al., 2010; Gull et al., 2012; Meriga et al., 2012). In an interesting study, it was found that counts of *S. aureus* in hamburger upon addition of AGE reduced in a dose-dependent manner during storage for different time points in the fridge and freezer, supporting the idea of using garlic for meat preservation (Mozaffari Nejad et al., 2014). To compare the antibacterial activity of various garlic health products, aqueous extracts of different products that included GP, GO, gelatinous GP suspension, aged garlic extract, and gelatinous suspension of aged garlic extract were prepared along with fresh garlic. All the extracts exhibited activity against *Neisseria gonorrhoeae, S. aureus,* and *Enterococcus faecalis*. The activity was correlated to the amount of fresh garlic constituents, namely, allicin and SAC, present in the products (Ruddock et al., 2005). The *Burkholderia cepacia* complex (Bcc) consists of 17 different species of soil bacteria that are pathogenic to allium species. These bacteria cause life-threatening lung infections in patients suffering from cystic fibrosis. AGE exhibited activity against Bcc, and this activity correlated with the allicin content of the extract (Wallock-Richards et al., 2014). In a recent study, non-aged and aged garlic cloves were pressed to remove their juices, dried, and powdered before extracted with water, ethanol, and chloroform. All three extracts of aged garlic exhibited antibacterial activity while only chloroform extract of non-aged garlic had activity

TABLE 1 | *In vitro* antibacterial activity of various garlic products and compounds.

Source	Bacteria	References
Crude or fresh garlic extract	*S. aureus* *E. coli* *S. typhi* *L. monocytogenes* MDR STEC *C. jejuni* *Vibrio parahaemolyticus* *Mycobacterium* species MRSA *Bacillus subtilis* *S. mutans* *C. difficile* *C. perfringens* *Bacteroides* species *Lactobacillus casei* MDR *P. aeruginosa* MDR *K. pneumoniae* MDR *Serratia marcescens*	Kumar and Berwal, 1998; Vuddhakul et al., 2007; Dakka, 2011; Lu et al., 2011a; Viswanathan et al., 2014; Jain et al., 2015b; Roshan et al., 2017; Farrag et al., 2019
Garlic powder	*S. typhimurium* *E. coli* *H. pylori* *B. cereus* *E. coli* (O55, O128, and O112) *Shigella* species *Vibrio* species *Yersinia enterocolitica* *L. monocytogenes* *S. enterica* *Campylobacter* species *Bacteroides fragilis* *B. subtilis* *Enterobacter aerogenes* *Enterococcus faecalis* *Klebsiella aerogenes* *Proteus vulgaris* *Lactobacillus acidophilus* *Streptococcus faecalis* *S. mutans* *Streptococcus pyogenes*	Johnson and Vaughn, 1969; O'Gara et al., 2000; Ross et al., 2001
Garlic paste	*E. coli* O157:H7	Gupta and Ravishankar, 2005
Aqueous garlic extract	*B. cereus* MDR *Shigella* species MDR *E. coli* *H. pylori* *S. aureus* *Bacillus sphaericus* *S. epidermidis* *E. aerogenes* *P. aeruginosa* *S. typhi* *S. pneumoniae* *K. pneumoniae* *Streptococcus pyogenes* *Sh*. species *E. coli* *Proteus* species *H. influenzae* *S. mutans* MDR *S. mutans* *Streptococcus* species *Actinomyces naeslundii* *E. faecalis*	Saleem and Al-Delaimy, 1982; Chowdhury et al., 1991; Cellini et al., 1996; Sivam et al., 1997; Arora and Kaur, 1999; Dikasso et al., 2002; Iwalokun et al., 2004; Bakri and Douglas, 2005; Ruddock et al., 2005; Fani et al., 2007; Gupta et al., 2010; Gull et al., 2012; Meriga et al., 2012; Velliyagounder et al., 2012; Mozaffari Nejad et al., 2014; Wallock-Richards et al., 2014; Pavlovic et al., 2017; Jang et al., 2018

(Continued)

TABLE 1 | Continued

Source	Bacteria	References
	Actinobacillus actinomycetemcomitans *Prevotella intermedia* *Prevotella nigrescens* *Porphyromonas gingivalis,* *Fusobacterium nucleatum* *Leptotrichia buccalis* *N. gonorrhoeae* MDR *M. tuberculosis* *M. tuberculosis* *B. subtilis* *Burkholderia cepacia* complex *Proteus mirabilis* *Salmonella enteritidis*	
Ethanolic garlic extract	*H. pylori* *M. tuberculosis* MDR *M. tuberculosis* *E. coli* *Enterobacter species* *P. aeruginosa* *Proteus species* *Klebsiella species* *S. aureus* *Bacillus species* VRSA *S. pneumoniae* *B. cereus* *K. pneumoniae* *S. mutans* *Proteus mirabilis* *Salmonella enteritidis* *E. aerogenes* *E. faecalis* *Lactobacillus paracasei* *Lactobacillus rhamnosus* *MRSA* *S. epidermidis* *Streptococcus oralis* *Streptococcus sanguis* *Streptococcus sobrinos* *Eikenella corrodens*	Hannan et al., 2011; Karuppiah and Rajaram, 2012; Snowden et al., 2014; Jain et al., 2015a; Liaqat et al., 2015; Mohsenipour and Hassanshahian, 2015; Pavlovic et al., 2017; Vlachojannis et al., 2018
Methanolic garlic extract	*E. coli* *P. aeruginosa* *S. aureus* *E. aerogenes* *E. faecalis* *Proteus mirabilis* *Salmonella enteritidis*	Pavlovic et al., 2017
Chloroform garlic extract	*B. cereus* *S. mutans*	Jain et al., 2015a; Jang et al., 2018
Hexane garlic waste extract	*S. aureus* MRSA	Nakamoto et al., 2020
Garlic oil	*M. tuberculosis* *H. pylori* *S. aureus* MRSA *B. cereus* *E. coli* (O55, O128, and O112) *Shigella* species *Vibrio* species *Yersinia enterocolitica* *L. monocytogenes*	Jain, 1998; Avato et al., 2000; O'Gara et al., 2000; Ross et al., 2001; Tsao S. and Yin, 2001; Tsao S.M. and Yin, 2001; Kim et al., 2004; Casella et al., 2013; Robyn et al., 2013; Mnayer et al., 2014; Viswanathan et al., 2014

(Continued)

TABLE 1 | Continued

Source	Bacteria	References
	S. enterica *Campylobacter* species *Bacteroides fragilis* *E. aerogenes* *E. faecalis* *K. aerogenes* *P. vulgaris* *L. acidophilus* *S. faecalis* *S. mutans* *S. pyogenes* *K. pneumoniae* *P. aeruginosa* MDR *K. pneumoniae* MDR *P. aeruginosa* *S. typhi* *C. jejuni*	
Ajoene	*B. cereus* *B. subtilis* *S. aureus* *Mycobacterium smegmatis* *Mycobacterium phlei* *M. tuberculosis* *M. luteus* *L. plantarum* Streptococcus species *Streptomyces griseus* *E. coli* *K. pneumoniae* *P. Aeruginosa* *X. maltophilia* *Cronobacter sakazakii*	Naganawa et al., 1996; Yoshida et al., 1998; Feng et al., 2014; Viswanathan et al., 2014
Z-10-devinylajoene and iso-E-10-devinylajoene	*B. cereus* *B. subtilis* *S. aureus* *M. phlei* *M. luteus* *E. coli* *K. pneumoniae* *P. aeruginosa* *X. maltophilia*	Yoshida et al., 1998, 1999
Diallyl sulfide (DAS)	*H. pylori* *S. aureus* MRSA *K. pneumoniae* *P. aeruginosa* *S. typhimurium* *E. coli* O157:H7 *L. monocytogenes* *Staphylococcus aureus* *C. jejuni* *A. actinomycetemcomitans* *Cronobacter sakazakii*	O'Gara et al., 2000; Tsao S. and Yin, 2001; Tsao S.M. and Yin, 2001; Yin and Cheng, 2003; Lu et al., 2011a,b; Velliyagounder et al., 2012; Feng et al., 2014
Diallyl disulfide (DADS)	*H. pylori* Clarithromycin-resistant *H. pylori* Metronidazole-resistant *H. pylori* *S. aureus* MRSA *K. pneumoniae* *P. aeruginosa*	O'Gara et al., 2000; Tsao S. and Yin, 2001; Tsao S.M. and Yin, 2001; Yin and Cheng, 2003; Liu et al., 2008; Lu et al., 2011a; Casella et al., 2013; Robyn et al., 2013

(Continued)

TABLE 1 | Continued

Source	Bacteria	References
	S. typhimurium *E. coli* *E. coli* O157:H7 *L. monocytogenes* *Staphylococcus aureus* *C. jejuni*	
Diallyl trisulfide (DATS)	*H. pylori* Clarithromycin-resistant *H. pylori* Metronidazole-resistant *H. pylori* *S. aureus* MRSA *K. pneumoniae* *P. aeruginosa* *Leuconostoc mesenteroides* *Pediococcus pentosaceus* *Lactobacillus plantarum* *C. jejuni*	O'Gara et al., 2000; Tsao S. and Yin, 2001; Tsao S.M. and Yin, 2001; Kim et al., 2004; Liu et al., 2008; Lu et al., 2011a
Diallyl tetrasulfide (DATTS)	*H. pylori* *S. aureus* MRSA *K. pneumoniae* *P. aeruginosa*	O'Gara et al., 2000; Tsao S. and Yin, 2001; Tsao S.M. and Yin, 2001
Mixture of diallyl sulfides (DASS)	*E. aerogenes* *E. coli* *S. enterica* *S. sonnei* *L. monocytogenes* *Y. enterocolitica* *M. tuberculosis*	Ross et al., 2001; Oosthuizen et al., 2017
Dimethyl trisulfide	*E. aerogenes* *E. coli* *S. enterica* *S. sonnei* *L. monocytogenes* *Y. enterocolitica* *S. aureus* *L. mesenteroides* *P. pentosaceus* *L. plantarum*	Ross et al., 2001; Kim et al., 2004
Ally methyl sulfide (AMS)	*Actinobacillus pleuropneumoniae*	Becker et al., 2012
Allicin	*S. aureus* MRSA *Streptococcus* species *Bacillus* species *V. cholerae* *M. tuberculosis* *Mycobacterium* species *Enterococci* species *H. pylori* *S. epidermidis* Methicillin-resistant *S. epidermidis* Lancefield group B *streptococci* *E. coli* *A. actinomycetemcomitans* *C. jejuni* Bcc *C. difficile* *P. aeruginosa* *S. pyogenes*	Cavallito and Bailey, 1944a; Rao et al., 1946a,b; Delaha and Garagusi, 1985; Jonkers et al., 1999a; O'Gara et al., 2000; Perez-Giraldo et al., 2003; Canizares et al., 2004b; Cutler and Wilson, 2004; Cutler et al., 2009; Fujisawa et al., 2009; Velliyagounder et al., 2012; Robyn et al., 2013; Wallock-Richards et al., 2014; Roshan et al., 2017, 2018; Fuchs et al., 2018

TABLE 2 | *In vivo* antibacterial activity of various garlic products and compounds.

Source	Bacteria	Animal model	References
Aqueous garlic extract	*Shigella flexneri Y*	Rabbit	Chowdhury et al., 1991
Aqueous extract of toluene garlic extract	*P. aeruginosa*	*Caenorhabditis Elegans*	Rasmussen et al., 2005
		Mice	Bjarnsholt et al., 2005
Ajoene	*P. aeruginosa*	Mice	Jakobsen et al., 2012
DAS	MRSA	Mice	Tsao et al., 2007
DADS	MRSA	Mice	Tsao et al., 2007
Ally methyl sulfide (AMS)	*Actinobacillus pleuropneumoniae*	Pig	Becker et al., 2012
Allicin	*H. pylori* *Aeromonas hydrophila*	Meta-analysis of clinical studies, rainbow trout (fish)	Nya et al., 2010; Si et al., 2019*

**Clinical study.*

against *B. cereus* (Jang et al., 2018). All these studies indicate that allicin is the main phytochemical responsible for the antibacterial activity of AGE. Although the ethanol extract of garlic also has allicin, AGE is more effective due to the presence of other antibacterial chemicals, which might result in a synergistic or additive effect.

ANTIBACTERIAL ACTIVITY OF GARLIC ETHANOLIC EXTRACT

HPLC analysis of ethanolic extract of garlic (EGE) revealed that it contains various thoisulfinates, the major one being allicin. The anti-*H. pylori* activity of this extract decreased with the decrease in the concentration of allicin. Furthermore, it was seen that the maturation of garlic increases the allicin yield and extract with acetone yielding a higher percentage of allicin compared to ethanol (Canizares et al., 2004a,b). *In vitro* studies have reported that EGE was found to show antibacterial activity against various pathogenic bacteria including MDR bacteria, MDR *M. tuberculosis* isolates, and vancomycin-resistant *S. aureus* (VRSA) isolates (Hannan et al., 2011; Karuppiah and Rajaram, 2012; Snowden et al., 2014; Liaqat et al., 2015). The antibacterial and antiadherence activity of organic solvent (chloroform, acetone, and ethanol) extracts of garlic was least compared to crude and aqueous extract against *S. mutans* (Jain et al., 2015a). The leaves of wild garlic (*Allium ursinum* subsp. *ucrainicum*) found in Serbia were extracted with 70 and 96% ethanol and 80% and absolute methanol, and the *S*-alk(en)ylcysteines (alliin, isoalliin, and methiin) content of the extracts was analyzed using NMR studies. The extracts exhibited some degree of antibacterial activity against test enteropathogenic bacterial strains with *Salmonella enteritidis* being the most sensitive. The tested bacteria were more sensitive to ethanolic extract compared to other extracts (Pavlovic et al., 2017). However, the study should have determined the amount of allicin in the extracts for better interpretation of the results instead of alliin, which

is a precursor of allicin. Ethanolic (30%) extract of fermented black garlic exhibited antibacterial activity against 11 bacterial strains that cause oral diseases. Short and long incubation with this extract inhibited the growth of more than 90% of salivary bacteria (Vlachojannis et al., 2018). Water extract of the Toluene extract of garlic has been reported to decrease the mortality of *Caenorhabditis elegans* from *P. aeruginosa* infections (Rasmussen et al., 2005) and clear the lungs of mice of *P. aeruginosa* by modulating inflammation (Bjarnsholt et al., 2005). Allicin along with other thoisulfinates present in EGE seems to be responsible for its antibacterial activity. Other than ethanol and methanol extract, the chloroform extract of both aged and non-aged garlic exhibited activity against *B. cereus* by disk diffusion assay (Jang et al., 2018). The hexane extract of solid waste of the GO extraction process exhibited activity against various bacteria including *S. aureus* and MRSA. DASs present in the extract were responsible for this activity (Nakamoto et al., 2020).

ANTIBACTERIAL ACTIVITY OF GARLIC OIL

Garlic oil is obtained by steam distillation of macerated or mashed garlic. Reverse-phase high-performance liquid chromatography (HPLC) studies have determined that the GO consists of a large variety of diallyl sulphides and other sulfides (O'Gara et al., 2000; Kim et al., 2004). A recent study has performed an exhaustive analysis of the content of GO and reported that the majority of GO is composed of diallyl and allyl methyl sulfides (Mnayer et al., 2014). The anti-mycobacterium effect of GO was demonstrated using *in vitro* and *in vivo* studies (Jain, 1998; Viswanathan et al., 2014). The anti-*H. pylori* effect of GO was many folds greater than that of GP. This could be because allicin is the only antibacterial thiosulfinate found in GP whereas GO has many organosulfides. The time course viability studies showed concentration-dependent inhibition of *H. pylori* by GO with 64 μg/ml resulting in complete inhibition in 4.5 h (O'Gara et al., 2000). However, two independent clinical studies indicated that administration of garlic GO was unable to ameliorate the *H. pylori* infection (Graham et al., 1999; Aydin et al., 2000). GO has been reported to exhibit antibacterial activity against 14 enteric pathogens and 11 commensal enteric bacteria with commensal bacteria being more sensitive. In time course viability studies, the inhibition of *Enterobacter aerogenes* growth increased with an increase in the concentration of GO, and complete killing was noticed at 22 mg/ml in 1 h (Ross et al., 2001). In another study, different GOs with varying percentages of DDS and DTS along with pure DDS were tested against Gram-positive (*S. aureus* and *Bacillus subtilis*) and Gram-negative (*E. coli* and *P. aeruginosa*). The antibacterial activity was not significant; however, the little activity that was exhibited was found with GO with a higher percentage of DDS. Interestingly, pure DDS showed little activity against only selected tested bacteria (Avato et al., 2000). However, disk diffusion assay found GO to be effective against *S. aureus, E. coli*, *P. aeruginosa*, *B. subtilis,* and MRSA (Casella et al., 2013; Viswanathan et al., 2014). An *in vitro* study tested the activity of GO against 40 *S. aureus* and 60 MRSA isolates and found that GO was more effective against *S. aureus* compared to MRSA, although this activity was significantly less than standard antibiotics (Tsao S.M. and Yin, 2001). Another study by the same group has reported that GO is effective against 237 clinical isolates of *P. aeruginosa* and *K. pneumoniae*, which also included drug-resistant strains. The minimum inhibitory concentration (MIC) values for *P. aeruginosa* were smaller compared to *K. pneumoniae*, and four times MIC of GO eliminated *P. aeruginosa* and *K. pneumoniae* in 16 and 24 h, respectively, in kill curve assays (Tsao S. and Yin, 2001). However, weak antibacterial activity of GO against six different bacteria has been reported using *in vitro* assays (Kim et al., 2004). Other bacteria that were reported to be sensitive to GO are *Salmonella typhi, L. monocytogenes*, and *Campylobacter jejuni* (Robyn et al., 2013; Mnayer et al., 2014). The discrepancy in the antibacterial activity of GO among various *in vitro* studies could be due to the solubility and volatile nature of GO.

ANTIBACTERIAL ACTIVITY OF AJOENE

Allicin can react with itself to yield ajoene, which is found abundantly in oil-macerated garlic. Besides, two ajoene-related compounds Z-10-devinylajoene and iso-E-10-devinylajoene were also isolated from oil-macerated garlic extract. Studies from the Fujino group reported that ajoene and its related compounds were found to display antibacterial activity against several Gram-positive and Gram-negative bacteria including *Mycobacterium* species (Naganawa et al., 1996; Yoshida et al., 1998, 1999). In all these studies, it was noticed that these compounds were more active against Gram-positive bacteria compared to Gram-negative. The same group also reported the antibacterial activity of ajoene and its related compounds against *H. pylori* (Ohta et al., 1999). Mice challenged with *P. aeruginosa* cleared the infection rapidly when treated with ajoene compared to the control group (Jakobsen et al., 2012). Pure ajoene exhibited antibacterial activity against *Cronobacter sakazakii* in a concentration-, time-, and temperature-dependent manner (Feng et al., 2014). More studies testing the activity of ajoene against more bacteria, especially clinical isolates and MDR strains, along with stability and pharmacokinetic studies are needed to better understand and utilize ajoene and its related compounds as antibacterial agents.

ANTIBACTERIAL ACTIVITY OF GARLIC ORGANOSULFIDES

The major constituents of GO are various aliphatic disulfides. DADS, which is the most abundant allyl sulfides in GO. DAS exhibits poor anti-*H. pylori* effect, but this activity increased as the number of sulfurs increased (O'Gara et al., 2000). A study from the same laboratory reported that a mixture of diallyl sulfides (DASS) and dimethyl trisulfide (DMTS) exhibits activity against six enteric pathogens with DMTS being several folds effective compared to DADS (Ross et al., 2001). The activity of DADS and DATS against antibiotic-sensitive

and -resistant isolates of *H. pylori* was confirmed by *in vitro* studies (Liu et al., 2008). *In vitro* studies indicated that DAS, DADS, DATS, and diallyl tetrasulfide (DATTS) were effective against *S. aureus*, where DAS was being least effective and the activity increases with the increase in the number of sulfurs. The activity of DATS and DATTS was comparable to standard antibiotics. It was interesting that MRSA was sensitive to all the DASs that were tested (Tsao S.M. and Yin, 2001). Similar results were reported with 237 clinical isolates of *P. aeruginosa* and *K. pneumoniae* including drug-resistant isolates (Tsao S. and Yin, 2001). The addition of DAS and DADS to meat significantly reduced the growth of aerobes and inhibited the pathogenic bacteria (Yin and Cheng, 2003). In line with the above reports, an *in vitro* study investigated the antibacterial activity of not only DASs but also dimethyl sulfides and dipropyl disulfide. The results indicate that they have moderate antibacterial activity against test bacteria, and this activity improves with an increase in the number of sulfurs (Kim et al., 2004). A later study using disk diffusion assay reported that DADS was effective, whereas dipropyl disulfide was not effective against *S. aureus, E. coli,* and *P. aeruginosa* (Casella et al., 2013). Administration of DAS and DADS to diabetic mice infected with MRSA significantly protected the mice by lowering bacteria load in the kidneys. In addition, inflammatory cytokines, namely, IL-6 and TNF-alpha, and coagulation factors C-reactive protein, fibronectin, and fibrinogen were decreased while anticoagulation factors antithrombin III (AT-III) and protein C were increased by DAS and DADS treatment. Moreover, malondialdehyde was decreased upon DAS and DADS, indicating protection from lipid peroxidation by MRSA infection (Tsao et al., 2007). Ally methyl sulfide (AMS) was shown to retard the growth of pleuropneumoniae causing pig pathogen *Actinobacillus pleuropneumonia* and protected the pigs by reducing the lung lesions by 20% (Becker et al., 2012). DAS exhibited concentration-dependent antibacterial activity against *A. actinomycetemcomitans* with and without heat treatment, indicating that DAS is heat stable (Velliyagounder et al., 2012). *In vitro* studies reported that DAS, DADS, and DATS exhibit activity against *C. jejuni* (Lu et al., 2011a; Robyn et al., 2013). The *in vitro* assay treatment of DAS also displays antibacterial activity against *C. sakazakii* and *E. coli* O157:H7 (Lu et al., 2011b; Feng et al., 2014). The enzymatic degradation of alliin, an organosulfur compound that alliinase enzyme converts into allicin and that is further degraded into a variety of organosulfides, resulted in higher percentage of DADS and diethenes showed better antibacterial activity against tested bacteria compared to alkali degradation products of alliin (Wu et al., 2017). Mixtures of DASs with various amounts of mono- to hexasulfides were prepared, and their anti-mycobacterial activity was evaluated. It was found that while all the combinations exhibited some activity, the most potent combination was the one that had higher quantity of DATS (Oosthuizen et al., 2017). Different studies, *in vitro* and *in vivo*, suggest that using GO, combinations, or individual aliphatic disulfides exhibited antibacterial activity against a wide range of microorganisms. It was noticed that the antibacterial activity increases with the increase in the number of sulfur, suggesting that antibacterial activity is mediated by formation of disulfide bonds between the compounds and bacterial protein, mainly enzymes.

ANTIBACTERIAL ACTIVITY OF ALLICIN

It is an established fact that allicin is an effective, broad-spectrum, and principal antibacterial component of garlic (Ankri and Mirelman, 1999). Allicin was identified as the principal ingredient of garlic that is responsible for the antibacterial activity of a wide variety of bacteria (Cavallito and Bailey, 1944a). Allicin was found to exhibit activity against *M. tuberculosis* including drug-resistant strains (Rao et al., 1946a; Delaha and Garagusi, 1985; Ratnakar and Murthy, 1995). Vancomycin-sensitive and -resistant clinical isolates and standard strains of *Enterococci* species were sensitive to allicin (Jonkers et al., 1999a). Allicin exhibited the best anti-*H. pylori* activity against three strains compared to DASs (O'Gara et al., 2000). A meta-analysis of clinical data indicated that adding allicin to conventional therapy improves the eradication of *H. pylori* infections (Si et al., 2019). Allicin along with related thoisulfinates, allyl methyl, and methyl allyl thiosulfinate were found and purified from acetone garlic extract. Allicin along with allyl methyl and methyl allyl mixture exhibited activity against *H. pylori* and showed synergy when used together (Canizares et al., 2004b). In a recent report, allicin was found to be active against *C. difficile* and other commensal gut bacteria, and no significant synergy was observed when allicin was tested with standard antibiotics (Roshan et al., 2017). The same group reported that allicin did not affect spore germination, but significantly inhibited spore outgrowth of *C. difficile* spores (Roshan et al., 2017). *In vitro* assay found that allicin was effective against 30 strains of *Staphylococcus epidermidis* including methicillin-resistant strains (Perez-Giraldo et al., 2003). A stable aqueous extract of allicin was found to be effective against 30 clinical MRSA isolates, some of which were mupirocin resistant. Aqueous cream of allicin also exhibited activity against tested MRSA strains (Cutler and Wilson, 2004). Similarly, aqueous allicin extract and cream demonstrated anti-Lancefield group B *streptococci* clinical isolate using *in vitro* assays (Cutler et al., 2009). A comparative *in vitro* study of antibacterial activity against *S. aureus* and *E. coli* activity of FGE, allicin, and clinically used antibiotics was performed. The results of the study indicated that fresh garlic was more potent against *S. aureus* compared to allicin and not much difference in activity was noticed against *E. coli* while both bacteria were more sensitive to antibiotics than garlic extract or allicin (Fujisawa et al., 2009). The administration of allicin to rainbow trout through its diet almost eliminated mortality when infected with *Aeromonas hydrophila,* a fish pathogen. In addition, *in vitro* studies also indicated that this bacterium was sensitive to allicin (Nya et al., 2010). It was found that *A. actinomycetemcomitans* was sensitive to allicin, and this activity disappeared upon heating, indicating that allicin is thermolabile (Velliyagounder et al., 2012). Although an *in vitro* assay found that *C. jejuni* was sensitive to allicin, *in vivo* studies indicated that allicin had no significant effect on colonization of *C. jejuni* in broilers. The possible explanation for this could be that the presence of mucus inhibited the

activity of allicin *in vitro* (Robyn et al., 2013). In addition to AGE, allicin also exhibits dose-dependent antibacterial activity against Bcc (Wallock-Richards et al., 2014). In an interesting study, allicin vapors were able to exhibit bactericidal activity against MDR lung pathogenic bacteria such as *P. aeruginosa* and *Streptococcus pyogenes* (Reiter et al., 2017). It was found that the active ingredient of Bald's eyeslave, an Anglo-Saxon medical remedy made up of garlic, onions, bovine bile, and brass effective against *S. aureus* and *P. aeruginosa*, was allicin (Fuchs et al., 2018). Allicin is the most potent antibacterial organosulfur compound found in garlic. The higher activity is thought to be due to the highly reactive sulfoxide group of allicin. However, the stability and solubility of allicin are the challenges in its clinical use. Animal studies highlight the reduced bioavailability and toxicity associated with allicin administration (Amagase et al., 2001).

ANTIBIOFILM AND ANTIVIRULENCE PROPERTIES OF GARLIC AND ITS ORGANOSULFUR COMPOUNDS

Bacterial biofilms are aggregations of bacterial cells in a matrix of extracellular polymeric substances (EPS) that include proteins, nucleic acids, polysaccharides, and lipids that are secreted by the bacteria. The formation of biofilm is a complex process that involves quorum sensing (QS) signaling. QS is also associated with the expression and release of various virulence factors that play a major role in pathogenesis. The formation of biofilm has been strongly associated with bacterial pathogenesis and antibiotic resistance. Therefore, developing strategies to inhibit biofilm formation has been a major area of research for many years. In addition to using synthetic antibiofilm agents, the use of many phytochemicals including garlic and its organosulfur compounds has gained a lot of interest. **Table 3** lists the antibiofilm and anti-QS studies that have been performed using garlic and its compounds. AGE was found to inhibit the coagulase activity of *S. aureus* using *in vitro* assays (Fletcher et al., 1974). GO was found to inhibit toxin production by *Clostridium botulinum* type A (Jc et al., 1979). Garlic ointment made by mixing GP with petroleum jelly not only prevented the formation of biofilm but also disrupted the already formed biofilm of bacteria that were isolated from burn wounds (Nidadavolu et al., 2012). DAS was found to kill both planktonic and sessile *C. jejuni* cells in the biofilm much better than ciprofloxacin and erythromycin. FTIR and Raman spectroscopy revealed that DAS treatment altered the proteins and polysaccharides of biofilm and damaged the EPS, which was visualized by electron microscopy (Lu et al., 2012). A genetic screening system was utilized to screen many herbal and pure compounds for their QS inhibition activity, and it was found that garlic exhibited significant inhibition of QS. Microarray transcriptome analysis indicated that the water extract of toluene extract of garlic affected the expression of virulence genes that were controlled by QS. In addition, garlic altered the *in vitro* biofilm to increase the penetration

TABLE 3 | Antibiofilm, antitoxin, and anti-QS activity of garlic and its compounds.

Source	Effect	References
AGE	Inhibits coagulase of *S. aureus*	Fletcher et al., 1974
Garlic oil	Inhibits production of toxin by *C. botulinum*	Jc et al., 1979
Garlic ointment	Inhibits formation of biofilm formed by bacterial cells	Nidadavolu et al., 2012
DAS	Inhibits EPS formation in biofilm of *C. jejuni* cells	Lu et al., 2012
Water and toluene extract of Garlic	Inhibits biofilm formed by *P. aeruginosa*	Rasmussen et al., 2005
Garlic extract	Inhibits biofilm and QS complex in *P. aeruginosa*	Bjarnsholt et al., 2005
Garlic extract	Inhibits QS receptors in bacterial cell	Bodini et al., 2009
Ajoene as QSI	Inhibits biofilm formed by *P. aeruginosa*	Jakobsen et al., 2012
Ajoene and 25 disulfide bond-containing compounds	Reduces QS caused infection by *P. aeruginosa*	Fong et al., 2017
Ajoene in combination with Ciprofloxacin	Reduce biofilm related diseases caused by *P. aeruginosa*	Vadekeetil et al., 2016
Allicin	Reduce EPS and virulence factor of *P. aeruginosa*	Lihua et al., 2013
Ajoene	Inhibits *Pseudomonas* quinolone signal	Vadekeetil et al., 2015
DADS	Reduce biofilm related QS and virulent gene of *P. aeruginosa*	Li et al., 2018a
DADS	Inhibits QS, virulent factors, motility, and chemotaxis of *P. aeruginosa*	Li et al., 2018b
Allicin and AGE	Inhibits group A streptococci cytolytic toxin and streptolysin O	Arzanlou and Bohlooli, 2010
DMS	Inhibits downregulation of HilA gene present in *Salmonella* invasion	Antunes et al., 2010
Allicin	Inhibits protease activity of SepB	Arzanlou, 2016
Allicin	Reduction in production of Alpha toxin of MRSA and MSSA	Leng et al., 2011
Allicin and vancomycin	Reduction biofilm formed by *S. epidermidis*	Zhai et al., 2014
Allicin	Reduce thickness of biofilm formed by *S. epidermidis* and down regulate the gene expression	Wu X. et al., 2015
Allicin	Inhibits biofilm formed by *S. aureus*	Perez-Kohler et al., 2015a
Allicin and chlorhexidine	Inhibits biofilm formation by *S. aureus*-infected rabbit hernia model	Perez-Kohler et al., 2015b
FGE	Inhibits biofilm formation of clinical isolates	Farrag et al., 2019

and killing of *P. aeruginosa* in the biofilms by tobramycin (Rasmussen et al., 2005). It was also found that pretreatment of *P. aeruginosa* biofilm to this extract made it more susceptible to tobramycin and polymorphonuclear leukocytes (Bjarnsholt et al., 2005). QS strains were used to identify that the water extract of toluene extract of garlic inhibited LuxR, AhyR, and TarR QS receptors (Bodini et al., 2009). Bioactivity-guided fractionation of garlic extract identified ajoene as quorum sensing inhibition (QSI) and microarray studies revealed that it reduced the expression of few QS-controlled virulence genes of *P. aeruginosa* such as *lasB* and *rhlA*, which increase the production of protease and rhamnolipid, respectively. Similar to previous observations, pretreatment of biofilms with ajoene increased the antibacterial activity of tobramycin on biofilm-associated *P. aeruginosa* (Jakobsen et al., 2012). The QSI activity of ajoene encouraged the screening of a library of compounds to identify a couple of sulfur-containing compounds that were similar to ajoene with QSI activity. Twenty-five disulfide bond-containing compounds were synthesized based on a quantitative structure–activity relationship (QSAR) study. These compounds could reduce the production of virulence factors, which included elastase, rhamnolipid, and pyocyanin. Besides, they were also able to inhibit the infection of *P. aeruginosa* in the murine implant infection model (Fong et al., 2017). The motility and biofilm formation of *P. aeruginosa* was significantly decreased when treated with a combination of ajoene and ciprofloxacin compared to independent treatment with each agent. Ajoene alone and in combination with ciprofloxacin significantly increased the serum sensitivity, phagocytic uptake, and killing of *P. aeruginosa* compared to no treatment. Furthermore, in the *P. aeruginosa* infection-associated murine pyelonephritis model, the combination of ajoene with ciprofloxacin significantly reduced the bacterial load of kidneys and bladder with reduced tissue damage compared to control and individual treatment of ajoene and ciprofloxacin (Vadekeetil et al., 2016). In addition to inhibiting production of long-chain acyl homoserine lactones, ajoene was also found to inhibit *Pseudomonas* quinolone signal (PQS) (Vadekeetil et al., 2015).

Allicin was also found to not only reduce biofilm formation of *P. aeruginosa* by reducing attachment and EPS production but also reduce the production of virulence factors such as exotoxin A, elastase, pyoverdine, and rhamnolipid (Lihua et al., 2013). Recently, DADS was found to decrease *in vitro* biofilm formation and swarming motility of *P. aeruginosa*. Relative gene expression studies indicated that it reduced the expression of many important QS and virulent genes (Li et al., 2018a). An interesting follow-up study performed an RNA transcriptome and proteome analysis on *P. aeruginosa* upon DADS treatment. The result indicated that all the three QS systems and virulent factors were downregulated by DADS treatment. Also, DADS treatment inhibited systems involved in motility and chemotaxis of *P. aeruginosa* (Li et al., 2018b). *In vitro* studies found that allicin and aqueous garlic (fresh and aged) extract inhibited production of streptolysin O, a cytolytic toxin by all strains of group A streptococci (GAS) (Arzanlou and Bohlooli, 2010). The transcription regulator HilA plays a crucial role in regulating the complex mechanism of *Salmonella* invasion, and it was found that dimethyl sulfide (DMS) downregulates the expression of the *hilA* gene and multiple virulent genes (Antunes et al., 2010). Another toxin produced by GAS is streptococcal pyrogenic exotoxin B (SpeB). The protease activity of SpeB was inhibited by allicin *in vitro*, and it is due to inhibition of truncation of SpeBm, the precursor protein of SpeB (Arzanlou, 2016). The reduction in the production of alpha-toxin by methicillin-susceptible and -resistant *S. aureus* upon treatment with allicin was confirmed by hemolysis and Western blot analysis. In addition, *hla* and *agrA* genes that regulate the production of alpha-toxin were downregulated by allicin (Leng et al., 2011). Administration of allicin alone or with vancomycin significantly reduced biofilm formation by *S. epidermidis* compared to vancomycin or saline treatment in a rabbit prosthetic joint infection model (Zhai et al., 2014). *In vitro* studies indicate that allicin exhibits antibiofilm property against *S. epidermidis*; however, this activity was less compared to water or ethanolic garlic extract. Allicin, water, and ethanolic extract of garlic exhibited antibacterial activity on biofilm-associated bacteria. Allicin decreased the thickness of the biofilm in a concentration-dependent manner. Gene expression studies indicated that allicin treatment of biofilm-associated bacteria resulted in downregulation of *app* and *icaA* genes that are associated with bacterial adhesion whereas only *icaA* was downregulated in planktonic cells (Wu X. et al., 2015). *In vitro* biofilm formation of *S. aureus* on reticular polypropylene mesh used in hernia was partially inhibited by allicin, but this effect diminished over time and a combination of allicin with chlorhexidine had no synergistic effect on the activity. However, allicin and chlorhexidine were cytotoxic individually, but the cytotoxicity was significantly reduced when cells were treated with the combination of both (Perez-Kohler et al., 2015a). To evaluate the effect of presoaking polypropylene mesh in allicin with chlorhexidine on biofilm formation *in vivo*, rabbit hernia model was infected with *S. aureus*, and it was found that combining allicin resulted in lower bacterial clearance and formation of biofilm compared to chlorhexidine treatment (Perez-Kohler et al., 2015b). The spectrum of antibiofilm property of FGE was tested against strong biofilm forming MDR clinical isolates of *P. aeruginosa, K. pneumoniae*, *S. marcescens*, and MRSA using *in vitro* and *in vivo* assays. *In vitro* assay indicated that FGE not only inhibited the formation of biofilm but also eradicated biofilms by these isolates on various surfaces. *In vivo* mice infection studies with *P. aeruginosa* and MRSA studies indicated that FGE significantly improved the survival of the animals and bacteria were not detected in different organs compared to control (Farrag et al., 2019).

The major organosulfur compounds of garlic, namely, allicin, ajoene, and aliphatic sulfides, pose QSI and antibiofilm activity. These activities are most explored in *P. aeruginosa* compared to other bacteria. It is evident from the data that garlic compounds downregulate QS and biofilm-associated genes. However, the precise mechanism in terms of whether the compounds bind and modify the transcription factor or interact with promoters of these genes is yet to be investigated. *In vivo* studies are encouraging to test the use of these compounds in clinical testing.

SYNERGISTIC EFFECT OF GARLIC AND ITS COMPOUNDS

The combination therapy sometimes leads to a synergistic effect, which effectively lowers the dose of individual drugs. Similarly, synergistic antibacterial effects were noticed when garlic and its compounds were used in combination with other phytochemicals and antibiotics. Both garlic crude extract and pure allicin exhibited strong synergy with vancomycin against 11 VRE clinical isolates with bacteriostatic action (Jonkers et al., 1999a). Raw garlic extract and commercial garlic tablets displayed synergistic effects against *H. pylori* when used along with omeprazole whereas no such effect was noticed when used in combination with amoxicillin, clarithromycin, and metronidazole (Jonkers et al., 1999b). In a clinical study, administration of allicin along with standard treatment (lansoprazole, clarithromycin, and amoxicillin) improved the percentage of *H. pylori* eradication by 23% in patients (Kockar et al., 2001). The combination of DATS and DATTS was either additive or synergistic when tested in combination with ceftazidime, gentamicin, imipenem, and meropenem except for DAT when used in combination with ceftazidime and gentamicin against ceftazidime and gentamicin-resistant *K. pneumoniae*, respectively (Tsao S. and Yin, 2001). Gentamicin administration induces nephrotoxicity and few reports have indicated that co-administration of aged garlic extract, garlic, S-allyl cysteine, DAS, and DADS ameliorates this nephrotoxicity. It was demonstrated that none of these agents decreased the activity of gentamicin; moreover, SAC, DAS, and DADS have enhanced the antibacterial activity of gentamicin, which makes them safe to use along with gentamicin to protect from nephrotoxicity (Maldonado et al., 2005). The MIC_{90} (concentration of the drug at which 90% of the growth is inhibited) against *S. aureus* of cefazolin and oxacillin were reduced by 128- and 64-fold, respectively, in the presence of 1/4 MIC_{90} of allicin. In the case of *Staphylococcus epidermidis,* MIC_{90} of cefazolin and oxacillin were reduced by 4- and 32-fold, respectively, in the presence of 1/4 and 1/8 MIC_{90} of allicin, respectively. The MIC_{90} of cefoperazone decreased by 16- and 8-fold in the presence of 1/2 and 1/4 MIC_{90} of allicin, respectively, against the cefoperazone-sensitive and -resistant strain of *P. aeruginosa*. The results indicate that allicin in combination with beta-lactam antibiotics results in synergy (Cai et al., 2007). The synergy of allicin with cefoperazone was confirmed against *P. aeruginosa* using the kill curve assay. In the case of tobramycin, certain synergy was observed against *P. aeruginosa* when used in combination with allicin, whereas no synergy was observed in the case of ciprofloxacin (Cai et al., 2008). It is reported that serum of patients that were administered garlic extract with standard antituberculosis had increased antitubercular activity compared to the control group, suggesting a synergistic effect when garlic is given in combination with antituberculosis drugs (Gupta et al., 1999). In the *P. aeruginosa* foreign body infection mouse model, it was noticed that in short-term infection, the bacterial clearance with the combination of ajoene and tobramycin was significantly better compared to placebo or individual treatments. The clearance of bacteria with ajoene alone was also significantly better compared to placebo. In case of long-term infection, the clearing of bacterial cells was significantly improved with ajoene and tobramycin treatment compared to control. However, there was no advantage of combining ajoene to tobramycin in clearing the bacteria (Christensen et al., 2012). Disk diffusion assay results indicated that the crude extract of garlic showed a synergistic effect when used in combination with gentamicin against *E. coli*, and it is interesting to find out that it was the only one out of the tested herbs that did not show any antagonistic effects with any test antibiotics (Ushimaru et al., 2012). In a similar study, antibiotic-resistant *P. aeruginosa* showed sensitivity to cefotaxime and ceftriaxone when FGE was added; this activity was better than FGE alone (Li et al., 2015). Another study reported that the ethyl acetate extract of garlic was antagonistic to the activity of chloramphenicol (Mahomoodally et al., 2018).

MDR *E. coli* isolated from drinking water in Bangladesh were not susceptible to AGE but were found to be sensitive to a combination of 1:1:1 combination of lime juice, garlic, and ginger extract (Rahman et al., 2011). The zone of inhibitions of Tazma honey and garlic crude extract combination were higher than when these were used individually against common pathogenic bacteria (Andualem, 2013). Garlic essential oil in combination with essential oils from several other species did not result in any synergy against tested pathogenic bacteria (Bag and Chattopadhyay, 2015). The *in vitro* interaction studies of a combination of AGE and Manuka honey against extended-spectrum beta-lactamase-producing *E. coli* showed different effects on different isolates ranging from synergy, additive, and indifferent to antagonistic effects (Idris and Afegbua, 2017). Mechanistic studies to understand the synergistic effects of garlic with antibiotics or other chemicals are lacking. Such studies are required for encouraging the use of garlic compounds to complement conventional medicine.

MECHANISMS OF ANTIBACTERIAL ACTIVITY OF GARLIC ORGANOSULFUR COMPOUNDS

The principal active ingredient responsible for the antibacterial activity of garlic was identified to be allicin (Cavallito and Bailey, 1944a). This finding was immediately followed by the observation that cysteine and other sulfhydryl-containing compounds inhibited the antibacterial activity of allicin, leading to the hypothesis that allicin might exert its antibacterial effect by reacting with sulfhydryl groups of the bacterial proteins (Cavallito and Bailey, 1944b; Cavallito et al., 1945). Allicin would react to sulfhydryl groups of cysteines irreversibly and will not be available to react with the sulfhydryl groups of the enzymes. The ability of allicin to inhibit various sulfhydryl enzymes indicates that the mechanism of antibacterial action of allicin is by reacting with the sulfhydryl groups of the many metabolically important bacterial enzymes (Wills, 1956). The action of allicin is mostly non-specific as it is found to inhibit urease, papain, amylase, and alcohol dehydrogenase.

NMR experiments identified *S*-allylmercaptocysteine, confirming the reaction between allicin and sulfhydryl group of cysteine (Rabinkov et al., 1998). The rapid permeability of allicin through lipid bilayers supports the idea of allicin able to reach and react with sulfhydryl groups of bacterial proteins (Miron et al., 2000). The treatment with reducing agents such as β-mercaptoethanol and dithiothreitol resulted in the loss of allicin's antibacterial activity, indicating that allicin forms disulfide bonds with the sulfhydryl groups of enzymes (Rabinkov et al., 1998; Jonkers et al., 1999a). Mass spectrometry and Raman spectrum analysis confirm that allicin enters the cell rapidly and reacts with cysteine and glutathione sulfhydryl groups (Fujisawa et al., 2009; Miron et al., 2010; Lu et al., 2011a). The enzymatic activity of bacterioferritin comigratory protein (BCP) from *B. cepacia*, which has two catalytically cysteines, was inhibited by allicin and mass spectrometry analysis confirmed that S-allyl thiol groups were added to these cysteines by allicin (Wallock-Richards et al., 2014). The inhibition of trypsin-like protease and general protease activity of *P. gingivalis* cell extract by AGE suggests that the antibacterial activity of garlic could be due to inhibition of proteolysis (Bakri and Douglas, 2005). Streptolysin O and mature Streptococcal pyrogenic exotoxin B of *Streptococci* that contain functionally important cysteines were inhibited by allicin and the addition of DTT reversed the inhibition (Arzanlou and Bohlooli, 2010; Arzanlou, 2016). Mass spectrometric proteomic analysis of cytoplasm of *E. coli* treated with allicin revealed 73 S-thioallylated proteins including some essential metabolic enzymes. It was shown that allicin reacts with low-molecular-weight cellular thiols such as glutathione (GSH) causing oxidative stress (Muller et al., 2016). Overall, these studies establish that the mechanism of antibacterial activity of allicin is by reacting with the sulfhydryl attaching allythio group through a disulfide bond. However, it should be noted that the mechanism of action of allicin is non-specific, which could make it cytotoxic. Alternatively, it has been reported that treatment of allicin inhibits DNA, RNA, and protein synthesis in bacteria, and the inhibition of RNA synthesis is more profound (Feldberg et al., 1988). *In vivo* study revealed that allicin modulates immunological parameters such as increased phagocytic and serum lysozyme activity to protect rainbow trout fish from *A. hydrophila* infection (Nya et al., 2010).

Allicin is highly unstable and is degraded into various organosulfide compounds (**Figure 1B**). The organosulfides also have been reported to show activity against a wide range of bacteria. Different studies have shown that organosulfides constitute a majority of GO. The mechanism of action of organosulfides, like allicin, is to react with free sulfhydryl groups of enzymes. However, organosulfides are not as reactive as allicin due to the absence of oxygen that is bound to sulfur in allicin. The activity of *H. pylori* arylamine *N*-acetyltransferase was inhibited in the presence of DAS and DADS, suggesting that these compounds exert antibacterial activity by inhibiting bacterial enzymes (Chung et al., 1998). In a more recent report, DAS was also found to inhibit the activity of the GST enzyme (Velliyagounder et al., 2012). Like allicin, addition of cysteine has reduced the antibacterial activity of GO against *E. aerogenes*, suggesting that sulfides also react with free sulfhydryl groups of enzymes (Ross et al., 2001). A recent study established that the antibacterial activity of diallyl polysulfides is due to their ability to react with sulfhydryl groups of various enzymes of *B. subtilis* such as bacillithiol and CoA and with amino acid cysteine (Kyo et al., 2001).

FTIR and Raman spectroscopy analysis revealed that treatment of *C. jejuni*, *C. sakazakii*, *E. coli* O157:H7, *L. monocytogenes,* and *Bifidobacterium* species with garlic crude extract and allyl sulfides causes many spectral changes that indicate the interaction of these agents with various cellular components; most notable changes indicate reaction with sulfhydryl groups, modification of cell membrane, and wall components to damage and destroy the integrity of the cell. These observations were consistent with the electron microscopy data that showed damage to cell wall and membrane (Lu et al., 2011a,b, 2012; Booyens et al., 2014; Booyens and Thantsha, 2014; Feng et al., 2014). Treatment of *C. jejuni* with DAS decreased the cellular ATP levels and increased the level of cellular protein in the culture, suggesting loss of cell membrane integrity (Lu et al., 2012).

A global proteomic analysis was performed to determine the mechanism of the anti-*H. pylori* effect of DATS. The results of 2D gel electrophoresis of proteins showed that upon treatment with DATS, proteins involved in metabolism, biosynthesis, bacterial virulence, and redox reactions were downregulated while stress response chaperon proteins were upregulated. The production of CagA and VacA virulent protein was decreased due to DATS treatment (Zare et al., 2008). A study performed RNA sequencing to study the changes in the global transcriptome of *C. sakazakii* upon treatment with DAS. Although there were a large number of genes that were up- and downregulated by the DAS treatment, clusters of genes that are related to cell shape and wall maintenance and lipopolysaccharide synthesis were upregulated while RNA and amino acid biosynthetic genes were downregulated. This indicates that DAS causes injury to the cell wall and membrane and decreases general metabolism (Feng et al., 2014).

The anti-*C. sakazakii* activity of ajoene was also diminished by the addition of cysteines, indicating that its antibacterial activity also involves reacting with sulfhydryl groups of bacterial enzymes. The transcriptome analysis of *C. sakazakii* treated with ajoene showed that the NADH expression factor and nitrate reductase gene were downregulated, which is related to reactive oxygen species reactions. In addition, flagellum and bacterial motility genes were downregulated, suggesting that ajoene negatively impacts biofilm formation (Feng et al., 2014).

In summary, two main mechanisms of action of garlic organosulfur compounds emerged from the reported studies: (1) the reaction of garlic compounds to the free sulfhydryl group on the proteins and/or enzymes to inactivate them, and (2) the disruption of composition and integrity of bacterial cell membrane and/or cell wall. Besides, some work also suggests that garlic compounds could also have a global effect on DNA, RNA, and protein synthesis. These mechanisms are observed in both Gram-positive and Gram-negative bacteria, suggesting that garlic and its compounds use similar antibacterial mechanisms for both groups of bacteria. However, the activity of the compounds is not specific, which could restrict their clinical application.

The mechanism of action of garlic and its compound has been summarized in **Figure 2**.

EMERGING NOVEL TECHNIQUES AND OPPORTUNITIES TO USE GARLIC AS NOVEL ANTIBACTERIAL AGENT

The emergence of various technologies such as nanotechnology, refined organic synthesis methods, and specialized drug delivery methods provide ample opportunities to use garlic organosulfur compounds as novel antibacterial agents. Green nanoparticle synthesis in recent times has emerged as a powerful tool to use phytochemicals to not only synthesize nanoparticles but also improve the antibacterial functions of these chemicals and particles (Wang and Vermerris, 2016).

Garlic extract has been used for the green synthesis and stabilization of silver nanoparticles. Eco-friendly garlic-silver nanoparticles synthesized using garlic clove extract displayed a greater antibacterial and antibiofilm activity on clinically important pathogens such as MRSA and *P. aeruginosa* compared to garlic extract or silver nitrate (Vijayakumar et al., 2019). A novel highly active molecule, (2E, 2E)-4,4-trisulfanediylbis (but-2-enoic acid) (TSDB) was synthesized through comparative molecular field analysis (COMFA) using the structure of DATS. TSDB displayed a robust inhibitory effect against *S. aureus* at low concentration. TSDB treatment increased the conductivity better than DATS, indicating better membrane penetration. The increase in the levels of protein and no change in the levels of alkaline phosphate in culture upon treatment with TSDB and DATS compared to control suggest damage to the cell membrane but not so much to the cell wall (Wu et al., 2018). Solgel prepared using tetraethyl orthosilicate was loaded with 20% ethanol extract of garlic, which displayed controlled release and stability of garlic components with increased antibacterial and antibiofilm activity against MRSA (Girish et al., 2019). GO microspheres were monodispersed in water by microemulsion technique to overcome its volatile characteristics and poor aqueous solubility. The study specified that the water-dilutable microemulsion that is formed by GO encapsulated in a nanoparticle vector is effective in preventing *S. aureus* than *E. coli* (Zheng et al., 2013). In another study, wild garlic (*Allium ursinum* L.) extract was encapsulated using spray congealing technology to shield its valued active compounds and expand its oral bioavailability. The encapsulation led to an enhancement of the extract dissolution performance as well as an improvement in the solubility of more than 18-fold compared to the pure extract. Microparticles were stable over a 3-month period, showing only a minor decrease in the content of active compounds (allicin and *S*-methyl methane thiosulfonate) and upholding a good antimicrobial activity. The study suggests that such spray congealing technology can be used to improve the solubility, bioavailability, and stability of the garlic active ingredients including allicin without affecting their antibacterial properties (Tomsik et al., 2019). The stability of phytochemicals present in GO, mainly allicin, was improved when biogenic nanoscale mesoporous silicon derived from the silicon-accumulator plant Tabasheer (*Bambuseae*) was used as a potential carrier as the antibacterial activity of this material

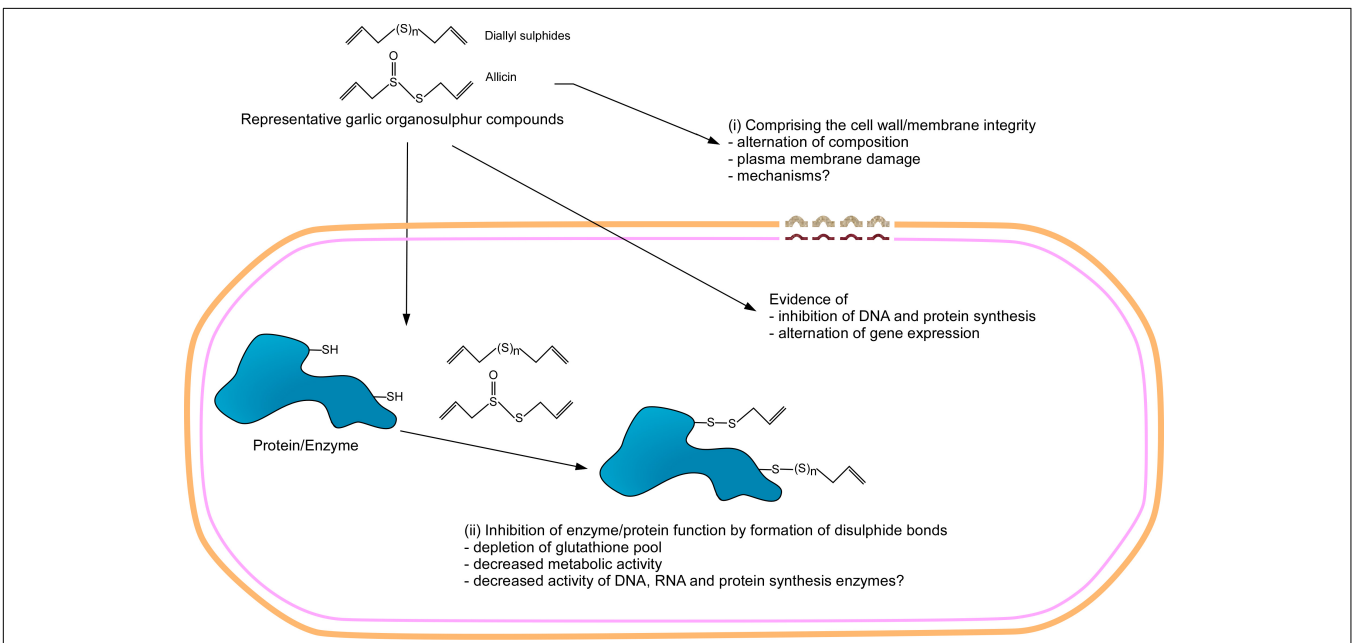

FIGURE 2 | Illustration of the mechanism of action of garlic organosulfur compounds. Garlic organosulfur compounds exert their antibacterial activity mainly through two mechanisms: (i) The organosulfur compounds are highly reactive with sulfur having the capability to form disulfide bonds with the free sulfhydryl groups of proteins including enzymes. The formation of disulfide bonds renders the enzyme inactive, resulting in the death of bacteria. (ii) The organosulfur compound interacts with the cell membrane of bacteria. This interaction compromises the integrity of the cell membranes of the bacteria leading to leakage of cell content leading to death. In addition, it is also thought that garlic organosulfur compounds interfere with protein production, DNA replication, and alter gene expression.

was better than GO only control (Le et al., 2017). More research should be focused to make similar nanoparticles, emulsions, and novel formulations using pure garlic compounds, mainly allicin, to improve their stability. *In vitro* studies with allicin aerosol and vapors using a lung model demonstrated the antibacterial efficacy of allicin with a correlation between aerosol deposition pattern and bacterial growth inhibition. Interesting synergy was observed with allicin that was administered with ethanol against *E. coli* (Reiter et al., 2020). It was interesting to note that DAS is not a strong antibacterial compound, but when given in combination with zinc oxide nanorods as an emulsion, it displayed a synergistic effect against *S. aureus* and MRSA bacteria under *in vitro* and *in vivo* conditions (Rauf et al., 2018). In a study, SAC, which is not antibacterial by itself, exhibited antibacterial activity when in complex with palladium (II) against *E. coli*, *P. aeruginosa,* and *S. aureus* (Spera et al., 2011). Organosulfides were converted into nano-iron sulfides with 500-fold superior antibacterial activity against pathogenic and drug-resistant bacteria compared to compounds themselves. The nano-iron sulfides released hydrogen polysulfanes and topical application in animal models resulted in reduced biofilm formation and accelerated wound healing (Xu et al., 2018).

Leontiev et al. (2018) developed a series of allicin analogs and evaluated their antimicrobial properties and thermal stability against bacteria and the model fungus *Saccharomyces cerevisiae*. Here, dimethyl-, diethyl-, diallyl-(allicin), dipropyl-, and dibenzyl-thiosulfinates form a series of molecules with increasing molecular mass and hydrophobicity, which would be anticipated to affect physical characteristics such as rate of diffusion, volatility, and membrane permeability, all of which are expected to affect the antibacterial properties of the molecules. In this study, the more volatile compounds showed noteworthy antimicrobial properties *via* the gas phase. Thiosulfinates differed in their effectivity against specific organisms, and some were thermally more stable than allicin. These results encourage the application of garlic-based compounds in medicine and agriculture either singly or in combination with other antimicrobials (Leontiev et al., 2018). Attaching *N*-propylthiol (similar chemistry to allicin) to ciprofloxacin increased the sensitivity of MRSA toward ciprofloxacin, suggesting that combination chemistry with garlic organosulfur could potentiate existing antibiotics (Sheppard and Long, 2016). Another study screened a chemical library composed of 19 synthesized pyridyl disulfides that emulate the chemical reactivity of allicin for antimicrobial activity against Gram-positive species including VRSA. The study identified pyridyl disulfides as stable alternatives to allicin with a similar narrow-spectrum profile and are thought to function as pro-oxidants like that of allicin (Sheppard et al., 2018).

As garlic is consumed regularly all over the world, it is considered non-toxic without any side effects. However, there are limited reports of toxic side effects of garlic and its constituents. In some individuals, contact with garlic and its constituents (especially oil-soluble sulfur compounds) leads to skin irritation and dermatitis (Jappe et al., 1999). *In vivo* studies administering garlic juice resulted in stomach damage. Garlic juice rich in allicin and allicin itself cause damage to the intestinal epithelial mucosa (Kodera, 1997). Allicin was also reported to immobilize sperms *in vitro* (Qian et al., 1986). *In vitro* cytotoxicity studies showed that DAS did not affect the cell growth or viability, whereas both DAS and allicin changed the morphology of cells. Allicin also significantly decreased the metabolic activity of cells (Velliyagounder et al., 2012; Perez-Kohler et al., 2015a). The concentration at which these effects are noticed are relatively high and more studies need to be done to evaluate the toxicity of garlic and its compounds at concentrations that exhibit antibacterial effects.

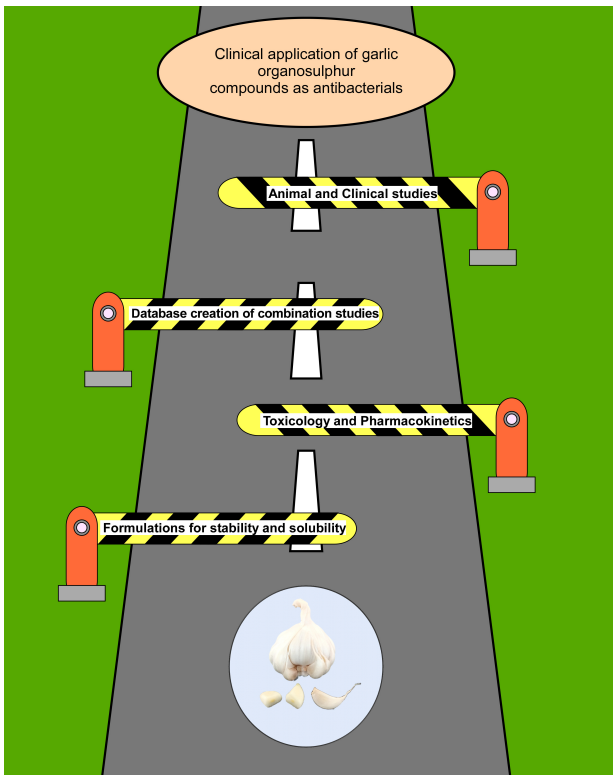

FIGURE 3 | Cartoon representation of the challenges that should be crossed for garlic/garlic compounds to be used in the clinical setting as antibacterials. The road to developing garlic/garlic compounds into novel clinically relevant antibiotics is subject to crossing the barrier shown in the cartoon, which are as follows: (1) the development of new formulations that would improve the specificity, stability, and solubility of garlic organosulfur compounds; (2) to access the safety and bioavailability of organosulfur compounds by performing toxicology and pharmacokinetic studies with various compounds administered through different routes; (3) to avoid repetition and encourage *in vivo* studies, creation of a database to record the *in vivo* and *in vitro* antibacterial studies of garlic compounds alone and along with other antibiotics or phytochemicals; and (4) more pre-clinical and clinical studies should be performed to access the safety and efficacy of these compounds. Research in these areas will ensure the use of garlic-based novel antibacterials in the clinical setting.

CONCLUSION

The extensive research strongly indicates that garlic organosulfur compounds exhibit strong antibacterial activity against a wide

range of bacteria including MDR strains. Although garlic organosulfur compounds have been known to be excellent antibacterial compounds, not much progress has been made in the direction of utilizing them clinically to tackle the problem of antibiotic resistance. The toxicity data of garlic and its compounds from animal studies are inconsistent with some studies reporting no toxic effect, whereas some report inflammation and toxic effects. The lower stability, solubility, and bioavailability of these compounds have hindered their use in the clinical setting. However, the organosulfur compounds are attractive because their bactericidal activity is exerted through multiple mechanisms, making it difficult for bacteria to develop resistance. Another concern that should be addressed for the use of garlic compounds is their toxicity and specificity to use them as antibacterial agents. Although a great deal of research has been done on the antibacterial potential of garlic and its compounds, there are recent gaps that need to be filled to utilize them as antibacterial agents in clinical settings. The first major area where more research should be focused is to develop robust and economical extraction or synthesis procedures that would yield pure garlic compounds. Besides, most of the organosulfur compounds of garlic are not water soluble and are unstable. Thus, formulations using advanced nanoparticle or emulsion techniques should be developed with improved solubility and self-life. A database should be created to curate the antibacterial data of garlic compounds by themselves and in combination with other antibiotics, which should be performed under pre-established guidelines. Such a database can be used by artificial intelligence tools to predict the most effective combinations for testing or treatment. Most of the research reports the *in vitro* antibacterial activity of garlic compounds that are important but not necessarily translate to *in vivo* conditions. Therefore, future research should be focused on validating the antibacterial activity of garlic compounds in animal infection models. Furthermore, elaborate toxicology and pharmacokinetic studies with respect to different pure organosulfur compounds, administered amount, and route should be performed. Clinical studies of garlic compounds except for anti-*H. pylori* activity has not been performed. As garlic is approved by FDA as "generally recognized as safe," garlic products should be administered in addition to standard antibiotics in the clinical setting to establish the role of garlic in complementary medicine. All these gaps in the research have been summarized in a cartoon (**Figure 3**). Recent advances in science and technology such as combinatorial and refined chemical synthesis techniques, nanotechnology, bioinformatics, computation tools, and advanced formulation can effectively overcome the challenges to develop the garlic organosulfur-based novel antibiotics. In the light of rapid emergence of antibiotic resistance, it is warranted that more research should be focused to understand the precise mechanism of action at a molecular level of organosulfur compounds. The organosulfur compounds have the potential to make a huge impact on human health by decreasing the mortality associated with bacterial infections.

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Preliminary Studies on the Antibacterial Mechanism of a New Plant-Derived Compound, 7-Methoxycoumarin, Against *Ralstonia solanacearum*

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Ralstonia solanacearum (*R. solanacearum*) is one of the most devastating plant bacterial pathogens and leads to serious economic losses in crops worldwide. In this study, the antibacterial mechanism of 7-methoxycoumarin, a new coumarin antibiotic, was preliminarily investigated by the observation of symptoms and physical and biochemical analyses. The results showed that 7-methoxycoumarin significantly suppressed bacterial growth of *R. solanacearum*, with the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values of 75 and 175 mg/L, respectively. Electron microscopy observations showed that the bacterial cell membrane was destroyed after 7-methoxycoumarin treatment. Biofilm formation of *R. solanacearum* was significantly suppressed by 7-methoxycoumarin at concentrations ranging from 25 to 100 mg/L. Furthermore, virulence-associated genes *epsE*, *hrpG*, and *popA* of *R. solanacearum* were significantly inhibited by 7-methoxycoumarin. The application of 7-methoxycoumarin effectively suppressed tobacco bacterial wilt progress in pot experiments, with relative control efficiencies of 83.61, 68.78, and 58.11% at 6, 8, and 10 days post inoculation, respectively.

Keywords: coumarins, *R. solanacearum*, antibacterial activity, tobacco bacterial wilt, membrane lysis, 7-methoxycoumarin

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INTRODUCTION

Tobacco bacterial wilt is a destructive bacterial soil-borne disease caused by *Ralstonia solanacearum* (*R. solanacearum*) that has an enormous impact on tobacco production and causes large annual economic losses (Swanson et al., 2005; Wu et al., 2015; Jiang et al., 2017). *R. solanacearum* invades host plants through root wounds, grows to high cell densities in the plant host and produces a high mass of extracellular polysaccharides, resulting in hindrance of water transportation in the plant, host wilting and death (Zeid, 2002; Li et al., 2017). Currently, the control methods of bacterial wilt include biological, chemical, cultural and integrated management (Bai et al., 2016; Cai et al., 2018). Pesticides discovered and produced by the agrochemical industry exert powerful effects for disease control management. However, long-term unreasonable use of bactericides leads to serious environmental problems inducing successive development of pesticide-resistant pathogens, disrupting the soil ecological balance, and causing the issue of environmental

safety (Fujiwara et al., 2011; Gutiérrez-Barranquero et al., 2013). Developing plant varieties with resistance to bacterial wilt is considered to be the most environmentally friendly, economical, and effective method of disease control. However, many crops with the resistance to bacterial wilt show a low yield and poor quality (Shew et al., 2019). The breeding time of resistant varieties is long, and it is difficult to meet the demand for these crops. Therefore, exploring and developing novel, eco-friendly, and efficient bactericides is important for bacterial wilt control.

Biopesticides are regarded as remarkable alternatives to classic agrochemicals, and have played a crucial role in plant disease control. Renewable plant bioresources contain a variety of plant-derived compounds (PDCs) (Hassan et al., 2009; Li et al., 2014; Cai et al., 2020). Recent studies have proven that these compounds can be used as antibacterial agents to suppress soil borne pathogens and promote plant growth (Paret et al., 2010; Abo-Elyousr et al., 2014). Certain PDCs including DIMBOA, lansiumamide B and protocatechualdehyde, suppress the growth of *R. solanacearum*, resulting in the control of plant bacterial wilt (Li et al., 2014, 2016; Guo et al., 2016). 4-Methoxy-cinnamic acid, benzoic acid and *trans*-4-hydroxycinnamohydroxamic acid are demonstrated to target the type III secretion system and biofilm formation of plant pathogens (Khokhani et al., 2013; Li et al., 2015). Currently, a variety of natural products have been developed as leader compounds to design biopesticides (Yang et al., 2018).

Coumarins are natural secondary metabolites comprised of fused benzene and α-pyrone rings produced through the phenylpropanoid pathway and coumarins accumulate in response to infection by plant pathogens (Zeid, 2002; Sun et al., 2014). Coumarins exhibited strong antibacterial activities against both clinical pathogenic bacteria (such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) and plant pathogens (such as *R. solanacearum*, *Alternaria alternata*, and *Botrytis cinerea*) (Goy et al., 1993; Souza et al., 2005). Studies have reported that coumarin exhibits strong antibacterial activity against *R. solanacearum* by inducing cell membrane lysis (Chen et al., 2016). The antibacterial activity of coumarins is related to the polarity of the oxygen-containing substituents on the benzene ring. When the C-7 position on the benzene ring has a methoxy functional group, or the C-6 and C-8 positions have hydroxyl group substituents, the compound exhibits broad-spectrum antibacterial activity (Kayser and Kolodziej, 1999). Hydroxycoumarins, including umbelliferone, esculetin and daphnetin show strong antibacterial activity against *R. solanacearum* (Yang et al., 2018). However, the inhibitory activity of methoxycoumarins against *R. solanacearum* remains largely unclear.

In this study, we demonstrated the antibacterial activity of 7-methoxycoumarin against *R. solanacearum*. The effects of 7-methoxycoumarin on the ultrastructure of bacterial cells and the leakage of intracellular constituents, as well as the activity of biofilm formation and swimming activity, were examined to understand the mechanism of action of plant-derived coumarins against *R. solanacearum*. Furthermore, the control efficiency of 7-methoxycoumarin on tobacco bacterial wilt was evaluated in pot experiments.

MATERIALS AND METHODS

Bacterial Cultures and Compounds

The *R. solanacearum* (CQPS-1) used in this study was collected by the Laboratory of Natural Products Pesticides and was isolated from an infected tobacco plant in Chongqing, China (Liu et al., 2017). *R. solanacearum* was grown at 30°C on a BG medium (Boucher et al., 1985).

The 7-methoxycoumarin (HPLC ≥ 98%) used in the study was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). This compound was dissolved in dimethyl sulfoxide (DMSO) and prepared at a final concentration of 10 mg/mL. Then, the dissolved compound was added to BG or BG agar medium to prepare compound suspensions of different concentrations, and negative control was treated with the same concentration of DMSO solvent (0.4% final concentration).

Determination of MIC and MBC

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the agar dilution method with a series of final concentrations ranging from 25 to 200 mg/L, as previously described with minor modifications (Li et al., 2014). Briefly, overnight-cultured *R. solanacearum* suspension (10^8 to 10^9 CFU/mL) was diluted with sterile water to 1×10^5 CFU/mL; then 50 μL of diluted bacterial suspension was spread directly on each antibiotic-containing agar dilution plate. The inoculated culture medium was incubated at 30°C. The MIC was defined as the lowest concentration at which no visible growth of *R. solanacearum* occurred after 48 h of inoculation. The MBC was defined as the lowest concentration at which no visible growth of *R. solanacearum* occurred after 96 h of inoculation. All assays were performed at least in triplicate.

The Growth Curve of *R. solanacearum*

The growth curve of *R. solanacearum* was investigated as in a previous study with minor modifications (Yang et al., 2017). Briefly, 125 μL of overnight-cultured *R. solanacearum* suspension (OD_{600} = 1.0) was added to 25 mL of BG medium supplemented with 7-methoxycoumarin to generate a final concentration of 10, 25, 50, 75, or 100 mg/L. The control treatment was treated with 100 μL of DMSO. Then, the triangular flask was incubated at 30°C for 24 h. Bacterial density was determined by the optical density (OD) at 600 nm every 2 h. Each treatment was repeated three times.

Biofilm Formation Analysis

The biofilm formation of *R. solanacearum* was carried out in 96-well polystyrene microtiter plates (Zhang et al., 2014). In short, BG medium and 7-methoxycoumarin were mixed in 5 mL sterilized centrifuge tubes to prepare 5, 10, 25, 50, and 100 mg/L concentration, and then 15 μL of bacterial suspension (OD_{600} = 1.0) cultured overnight was added to 96-well polystyrene microtiter plates and incubated without shanking for 24 h at 30°C. The culture was removed carefully, and plates were washed twice with 200 μL of distilled water. The

biofilm was dyed by adding 220 μL of crystal violet (0.1%) and incubation at room temperature for 30 min. After dyeing, the crystal violet was removed, and plates were washed twice with 200 μL of distilled water. After removing the floating color, plates were dried at room temperature for 30 min. Then, 200 μL of 95% ethanol was added to dissolve the crystal violet adsorbed on the biofilm for 30 min, and the absorbance value at 530 nm was determined by a microplate reader, and this represented the biofilm formation of *R. solanacearum* under 7-methoxycoumarin treatment. Each treatment was repeated at least three times.

Swimming Motility of *R. solanacearum* Under 7-Methoxycoumarin Treatment

The swimming motility of *R. solanacearum* was detected in semisolid medium as described in previous research (Tans-Kersten et al., 2004). 7-methoxycoumarin was added to the semisolid motility medium to final concentrations of 10, 25, 50, 75, and 100 mg/L. The bacterial suspension was diluted with sterile water to OD_{600} = 0.1, and 3 μL of bacterial suspension was dropped onto the plate. The inoculated plate was incubated at 30°C without shaking. The diameter of the white zone around the colony was measured 24 and 48 h after inoculation. Each treatment was repeated three times.

Transmission Electron Microscopy Analysis

The morphological changes of *R. solanacearum* after 7-methoxycoumarin treatment were further studied by electron microscopy, as in previous research with minor modifications (Chen et al., 2014). *R. solanacearum* (OD_{600} = 1.0) suspension was evenly mixed with 37.5 mg/L 7-methoxycoumarin, and incubated at 30°C for 4–6 h. After centrifuged at 8000 rpm for 5 min, the bacterial pellet was resuspended in 1 mL of sterilized deionized water, washed 3 times, and then fixed with 2.5% glutaraldehyde for 12 h. Then, the bacterial pellet was dehydrated with 30, 50, 70, 90, and 100% ethanol of different gradient series for 15 min, smoked with osmium acid for 3 h and observed directly under a scanning electron microscope. *R. solanacearum* cells were fixed with 1% aqueous OsO4 (Fluka, Los-Angeles, CA, United States) and washed with 0.1 M, pH 7.0 phosphate buffers. Thin sections containing the cells were placed on copper grids and observed under a TEM (FEI, Brno, Czechia).

RNA Extraction and Quantitative Real-Time RT-PCR

The effect of 7-methoxycoumarin on the expression of virulence-associated genes of *R. solanacearum* was evaluated as previously reported (Wu et al., 2015). Briefly, 250 μL of the overnight cultured *R. solanacearum* suspension (OD_{600} = 1.0) was added to 25 mL of BG medium, mixed with 1/2 MIC (37.5 mg/L) of 7-methoxycoumarin, and incubated at 30°C for 6–7 h. The bacterial cells were collected by centrifugation, and the total RNA of *R. solanacearum* was extracted according to the TRIzol method. After cDNA was synthesized by reverse transcription, the key genes of the type III secretion system (*hrpG* and *popA*), extracellular polysaccharide (*epsE* and *xpsR*), swimming motility (*vsrC*), and chemotaxis (*cheA* and *cheW*) were selected for evaluation of gene expression levels. The primers of the tested genes used in this study were list in **Supplementary Table 2** and the housekeeping gene *serC* was used as the control. RT-PCR was performed in a CFX96 Manager (Bio-Rad) using an Sso FastTM EvaGreen® Supermix (Bio-Rand, Hercules, CA, United States).

Control Efficiency of 7-Methoxycoumarin on Tobacco Bacterial Wilt Under Greenhouse Conditions

The naturalistic soil soak assay was used to evaluate the control efficiency of 7-methoxycoumarin on tobacco bacterial wilt as described in a previous study, with minor modifications (Wu et al., 2015). Briefly, unwounded, 6-week-old tobacco plants (*Nicotiana tabacum. L* Yunyan 87) were irrigated with 15 mL of 7-methoxycoumarin to a final concentration of 25, 50, and 100 mg/g soil. The same volume of 0.4% DMSO was used as a negative control, and as a thiadiazol copper with a final concentration of 100 mg/L was used as a positive control. After irrigation for 24 h, individual plants were inoculated by pouring 10 mL of bacterial suspension into the soil to create a final inoculation density of 1×10^7 CFU/g soil. Inoculated plants were placed in the climate room at 28°C with a 14/10 h light/dark cycle. The symptoms of each plant were scored daily using a disease index scale from 0 to 4 (0, no symptoms appeared; 1, 1–25% of leaves wilted; 2, 26–50% of leaves wilted; 3, 51–75% of leaves wilted; 4 indicated 76–100% of leaves wilted). Individual treatments contained 12 plants for each independent experiment, and the assay was repeated three times. To determine the disease index and the control efficiency, we used the following formulas:

$$\text{Disease index} = \frac{\sum(ni - vi)}{N \times 4} \times 100$$

where, *ni* = the number of plants with the respective disease index, *vi* = disease index (0, 1, 2, 3, and 4), and N = the total number of plants used in each treatment.

$$\text{Control efficiency} = \frac{(CK - T)}{CK} \times 100$$

where, T = the disease index of treatment, and CK = the disease index of the control group.

Statistical Analyses

The data were analyzed with Excel 2010 and the SPSS17.0 statistical software program (SPSS Inc, Chicago, IL, United States) using Student's *t*-test and ANOVA under the significance level of 0.05, 0.01 (*P*-value = 0.05, *P*-value = 0.01).

RESULTS

MIC and MBC of 7-Methoxycoumarin Against *R. solanacearum*

The minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the pathogenic bacterium

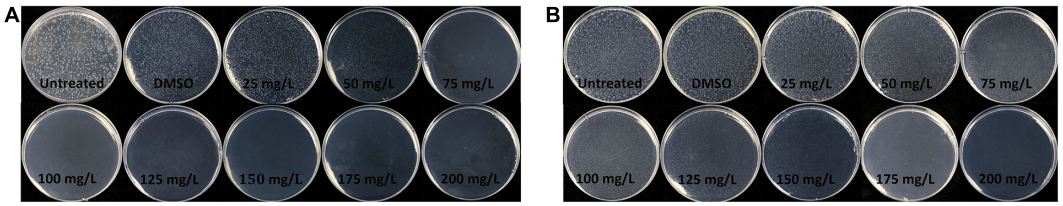

FIGURE 1 | The minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of 7-methoxycoumarin on *R. solanacearum* was determined using the agar dilution method at the concentrations ranging from 25 to 200 mg/L. **(A)** 48 h after inoculation. **(B)** 96 h after inoculation.

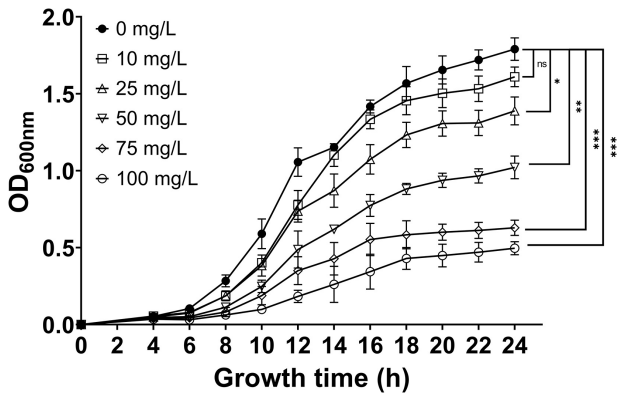

FIGURE 2 | The effect of 7-methoxycoumarin at concentrations ranging from 10 to 100 mg/L on the growth of *R. solanacearum*, (* indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$).

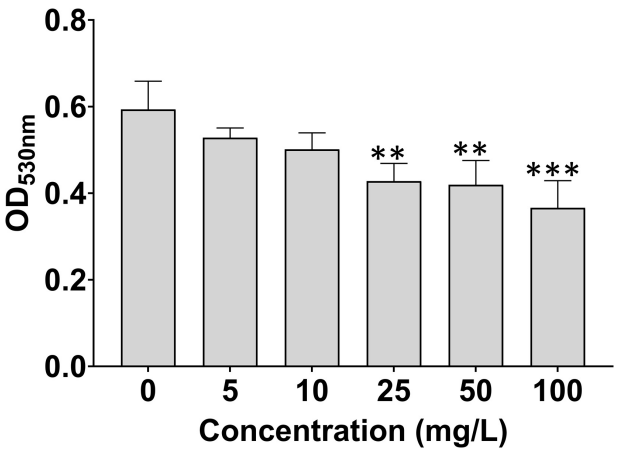

FIGURE 3 | Effects of 7-methoxycoumarin on biofilm formation of *R. solanacearum*. OD530 values were quantified after treatment with different concentrations of 7-methoxycoumarin at 30°C for 24 h in 96-well plates; ** indicates $p < 0.01$ and *** indicates $p < 0.001$).

R. solanacearum were determined by the solid dilution method. As shown in **Figure 1**, the MIC and MBC of 7-methoxycoumarin were 75 and 175 mg/L, respectively.

7-Methoxycoumarin Inhibits the Growth of *R. solanacearum*

Further investigation into the inhibitory effect of 7-methoxycoumarin against *R. solanacearum* was performed. As shown in **Figure 2**, the growth of *R. solanacearum* was significantly inhibited at concentrations of 7-methoxycoumarin ranging from 25 to 100 mg/L and the antibacterial activity of 7-methoxycoumarin against *R. solanacearum* was concentration-dependent. As shown in **Supplementary Table 1**, the IC_{50} value of 7-methoxycoumarin was 52.98 mg/L after 24 h of culture.

7-Methoxycoumarin Reduces the Biofilm Formation of *R. solanacearum*

As shown in **Figure 3**, 7-methoxycoumarin significantly reduced the biofilm formation of *R. solanacearum*. The biofilm formation treated by DMSO was 0.59, significantly high than *R. solanacearum* supplemented with 25, 50, and 100 mg/L 7-methoxycoumarin, OD_{530nm} were 0.43, 0.42, and 0.37, respectively. Specifically, the inhibitory effects of 7-methoxycoumarin were 21.25, 23.20, and 28.76% at concentrations of 25, 50, and 100 mg/L, respectively (**Supplementary Figure 1**).

As shown in **Supplementary Figure 2**, the swimming motility diameter of *R. solanacearum* 24 and 48 h after treatment with 7-methoxycoumarin was not different from that of the control treatment.

The Effect of 7-Methoxycoumarin on the Cell Morphology of *R. solanacearum*

To further study the antibacterial mechanism of 7-methoxycoumarin, the morphological changes of *R. solanacearum* after treatment with 7-methoxycoumarin were observed by SEM and TEM. As shown in **Figure 4**, negative control bacterial cells still maintain their integrity. In contrast, the surfaces of *R. solanacearum* after exposed to 7-methoxycoumarin (37.5 mg/L) were rough and obviously wrinkled. The damaged cell is also observed in TEM image as shown by the gray arrow in **Figure 5**, it is shown that 7-methoxycoumarin could damage or deconstruct cell walls to penetrate the bacterial cells, this damage may lead to the leakage of lysosomal contents resulting eventually in cell death.

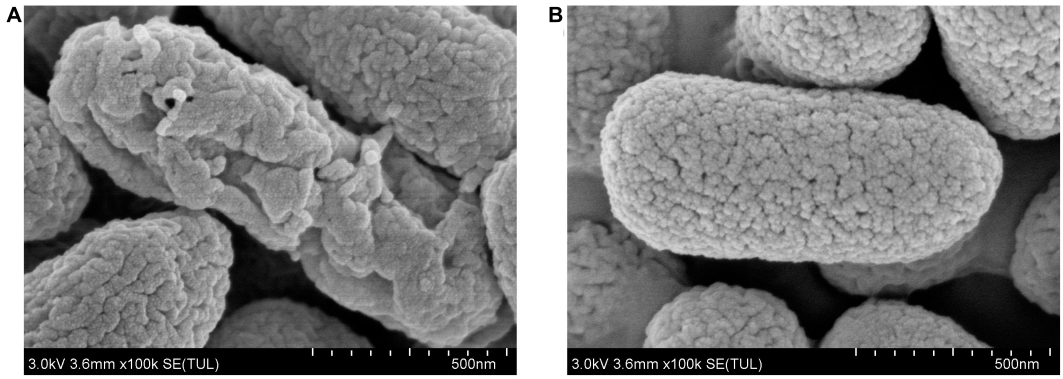

FIGURE 4 | SEM images of *R. solanacearum* cells treated with **(A)** 7-methoxycoumarin; **(B)** DMSO.

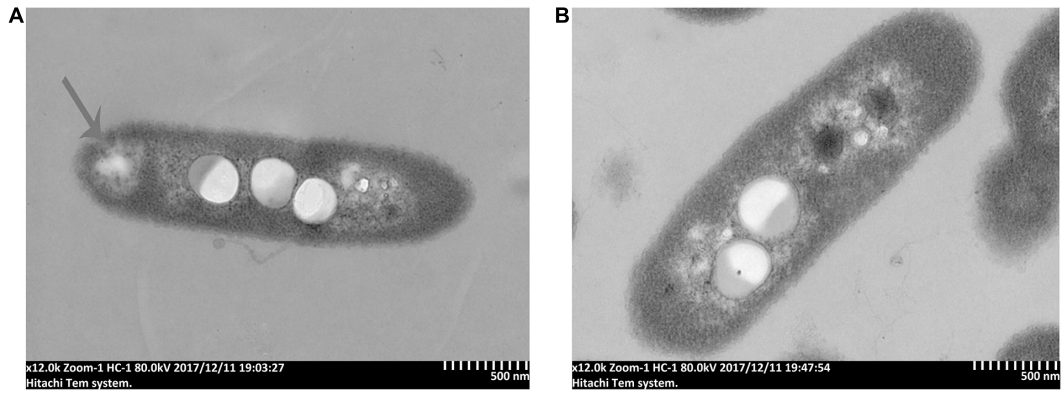

FIGURE 5 | TEM images of *R. solanacearum* cells treated with **(A)** 7-methoxycoumarin; **(B)** DMSO.

7-Methoxycoumarin Suppresses the Expression of Virulence-Associated Genes in *R. solanacearum*

In this study, qRT-PCR was used to evaluate the transcriptional expression of the major pathogenic genes of *R. solanacearum* treated with or without 7-methoxycoumarin. As shown in **Figure 6**, 7-methoxycoumarin inhibited the expression of type III secretion system associated-genes (*popA* and *hrpG*) and the extracellular polysaccharide synthesis gene *epsE*. However, 7-methoxycoumarin was found to induce the expression of *vsrC* and *cheW*. The results indicated that 7-methoxycoumarin can inhibit the genes of the type III secretory system and extracellular polysaccharides, demonstrated that this compound might has a certain preventive effect against host infection by *R. solanacearum*.

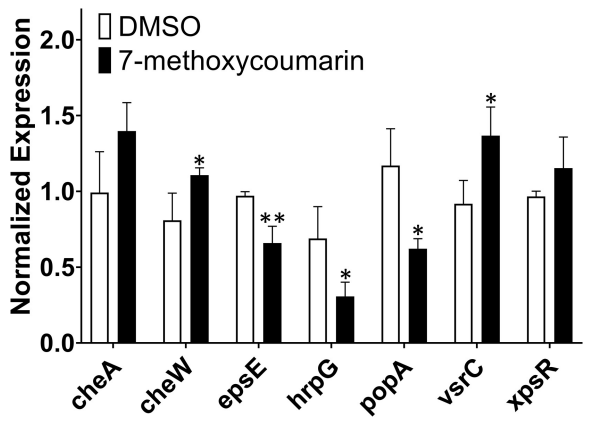

FIGURE 6 | The expression of some virulence-associated genes of *R. solanacearum* was quantified by qRT-PCR after treatment with or without 7-methoxycoumarin (* indicates $p < 0.05$ and ** indicates $p < 0.01$).

Control Effect of 7-Methoxycoumarin on Tobacco Bacterial Wilt Disease

Based on the strong antibacterial activity and biofilm formation inhibition of *R. solanacearum* by 7-methoxycoumarin, we examined the effect of irrigated roots with 7-methoxycoumarin on incidence of tobacco bacterial wilt. As shown in **Figure 7A**, the disease index of DMSO treatment was 0.53, 2.03, 3.00, and 3.58 at 6, 8, 10, and 12 days, respectively. Compared with DMSO, 7-methoxycoumarin treatments significantly altered the disease index of bacterial wilt and delayed plant

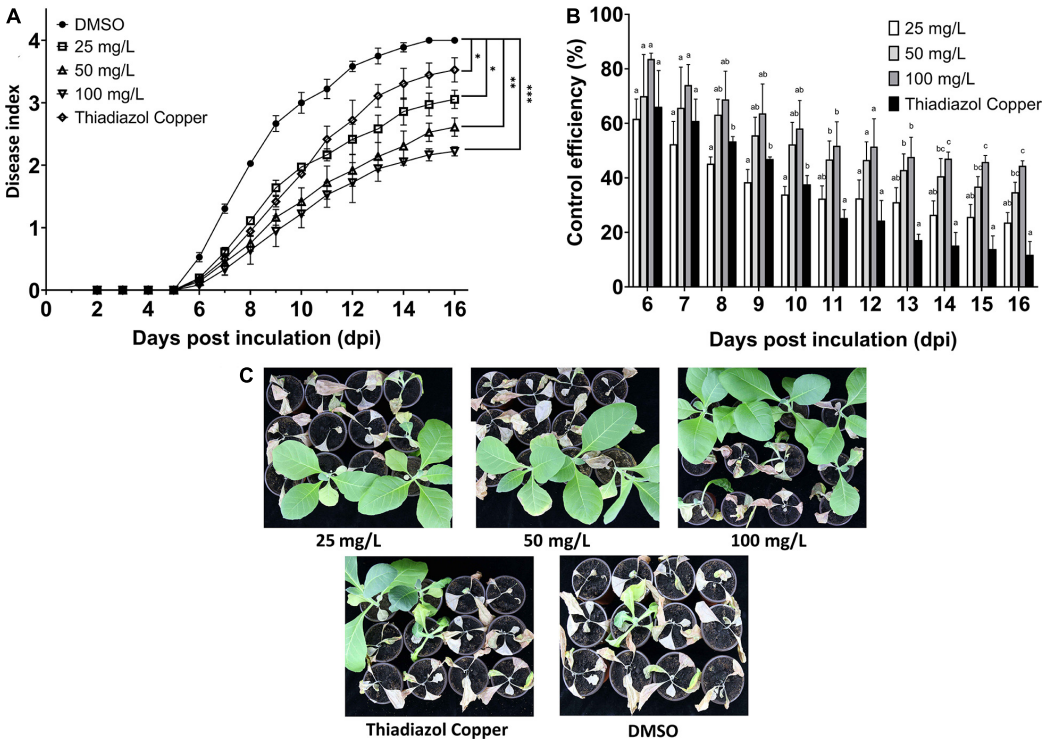

FIGURE 7 | Relative control effects of each treatment on pot experimental tobacco seedlings. **(A)**:The disease index of tobacco wilt treated with 25, 50, and 100 mg/L 7-methoxycoumarin. **(B)** The control effect of 7-methoxycoumarin. **(C)** The control effect after 16 days of inoculation with *R. solanacearum* (* indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$). Different lowercase letters on the histogram of the same group indicate significant differences between different treatments, $p < 0.05$).

wilting. As shown in **Figures 7B,C**, 7-methoxycoumarin treatment at a concentration of 100 mg/L had control efficiencies of 83.61, 68.78, 58.11, and 51.48% at 6, 8, 10, and 12 days after inoculation, respectively, which significantly higher than the positive control treatment with 100 mg/L thiadiazol copper, with control efficiencies of 66.11, 53.39, 37.61, and 24.37% at 6, 8, 10, and 12 days, respectively.

DISCUSSION

Traditional chemical pesticides produce environmental pollution and cause damage to the human body during their use (Dasgupta et al., 2007; Acero et al., 2008). Because of their ecofriendly sources and non-toxicity to humans, PCDs have gradually come to be considered a new type of pesticide that can replace traditional chemical pesticides. It has been reported that lansiumamide B, protocatechualdehyde and methyl gallate in PCDs have strong inhibitory effects on *R. solanacearum* to control the occurrence of tobacco bacterial wilt (Yuan et al., 2012; Fan et al., 2014; Wu et al., 2015). Previous studies show that hydroxycoumarins reduce the pathogenicity of *R. solanacearum* by suppressing T3SS and biofilm formation (Yang et al., 2018). However, the inhibitory activity of methoxycoumarins against *R. solanacearum*, especially the mechanism of action, has not been reported.

This article is the first study of the antibacterial effect of 7-methoxycoumarin on *R. solanacearum*, and further elucidates its antibacterial mechanism. According to our results, 7-methoxycoumarin inhibited the growth of *R. solanacearum* in both liquid medium and on solid medium (**Figures 1**, **2**). Previous studies have identified phenyl derivatives as antimicrobial agents for the control of *Pseudomonas aeruginosa*, *Bacilus subtillis* and *Xylella fastidiosa* (Alam, 2004; Maddox et al., 2010). The antibacterial mechanism of phenolic compounds is similar to that of carvacrol and thymol, resulting in a change in membrane potential (Xu et al., 2008). According to these observations, 7-methoxycoumarin caused ruffles and disruption to the cell membrane of *R. solanacearum* (**Figures 4**, **5**). Previous reports indicate that 7-methoxycoumarin has an inhibitory effect on some gram-negative bacteria, including *E. coli, V. cholerae*, and *Pneumococcus* (Céspedes et al., 2006). Therefore, consideration of strong inhibition of *R. solanacearum* and environmental ecofriendly, 7-methoxycoumarin has great potential for application for plant disease control in the future.

As a plant secondary metabolite, coumarins have inhibitory activity against diverse plant diseases, including bacteria, fungi, and viruses (Goy et al., 1993). Several coumarins obtained from different plant species revealed strong antibacterial activity against human associated *E. coli*, *S. aureus*, and *P. aeruginosa*

(Khan et al., 2010; Gnonlonfin et al., 2012). The young leaves of *N. attenuata* exhibited an increased resistance to *A. alternata*, which is associated with scopoletin accumulation (Sun et al., 2014). When Arabidopsis was infected by *Pythium sylvaticum*, scopolamine was utilized to synthesize a huge number of inhibitory substances-scopoletin, which concentrated at sites of infestation to inhibit the normal growth of *Phytophthora* (Bednarek et al., 2005). Recently, it was proven that scopoletin induces the accumulation of reactive oxygen species (ROS) such as H_2O_2 and O^{2-}, which prevents Asian soybean rust from infecting Arabidopsis (Beyer et al., 2019). Coumarins might act as plant antibacterial agents or immunity elicitors, which plays an important role in plant defense.

Like many plant pathogens, *R. solanacearum* forms biofilm-like aggregates in the roots of host plants, leading to bacterial invasion and infection (Yao and Allen, 2007). Studies have found that coumarins, such as scopoletin, umbelliferone, daphnetin, and esculetin, are secreted by plants to protect themselves from the attack of pathogens (Kai et al., 2006). In this study, 7-methoxycoumarin significantly inhibited the biofilm formation of *R. solanacearum*, but had no significant effect on the swimming motility of *R. solanacearum* (**Figure 3** and **Supplementary Figure 1**). We speculated that 7-methoxycoumarin is more likely to reduce host infection by *R. solanacearum* via affecting biofilm formation. Nevertheless, further elucidation of this mode of action may need a more systematic study of the relationship between 7-methoxycoumarin, *R. solanacearum*, and host plants.

Genes related to *R. solanacearum* pathogenicity have been comprehensively and thoroughly studied. For example, type III secretion system genes *hrpG* and *popA* are involved in the regulation of bacterial infection by *R. solanacearum* (Belbahri et al., 2001; Liu et al., 2014). The results of this study showed that 7-methoxycoumarin significantly inhibited the expression of *hrpG* and *popA*, but had little effect on the expression of *cheA* and *xpsR*. These results indicated 7-methoxycoumarin might suppress type III secretion system and biofilm formation of *R. solanacearum*. Further studies should be explained the effect of 7-methoxycoumarin on *popA*.

The control effect of the 100 mg/L 7-methoxycoumarin treatment on tobacco bacterial wilt was not significantly different from that of the control agent thiadiazol copper in the early stage of the disease, but the lasting time was better than that of thiadiazol copper, and the control effect in the later stage of the disease could still reach to 44.44%. Although 7-methoxycoumarin has shown potential as an effective plant-derived antibacterial agent against bacterial wilt, it is not clear whether this effect can be sustained in different field environments.

In conclusion, 7-methoxycoumarin is a potential antibacterial agent in plants, which is more likely to act as a crucial remedy for future field applications for bacterial wilt. Exposure to 7-methoxycoumarin could protect tobacco plants against *R. solanacearum* by strongly inhibiting biofilm formation, and killing the bacteria. Therefore, our study provides environmentally friendly and effective tactics for the research and development of tobacco bacterial wilt control agents and might be extended to the application of other plant disease control in the future.

CONCLUSION

In summary, a new plant-derived compound, 7-methoxycoumarin, exhibited strong antibacterial activity against *R. solanacearum*. 7-Methoxycoumarin significantly inhibited biofilm formation and induced bacterial cell membrane lysis. Virulence-associated genes *epsE*, *hrpG*, and *popA* were significantly suppressed by 7-methoxycoumarin. Finally, the new antibacterial agent 7-methoxycoumarin suppressed tobacco bacterial wilt progress and lead to better control effect on tobacco bacterial wilt. This study suggests that 7-methoxycoumarin has potential application in the control of plant bacterial wilt and other plant diseases in the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

WD and LY conceived and designed the experiments. SH, LY, YW, and YR performed the experiments. SH, LY, and SL analyzed the data. SH, WD, and LY wrote the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.697911/full#supplementary-material

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