Anti-quorum sensing activity of clove (*Syzygium aromaticum*) bud extract and its combined efficacy with ceftazidime against multidrug-resistant *Pseudomonas aeruginosa*

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Abstract

In this study the quorum sensing inhibitory activity of methanol extract of clove (Syzygium aromaticum) bud, ceftazidime (cefta) and their combination was determined against multidrug-resistant Pseudomonas aeruginosa. The pigment inhibitory activity as anti-quorum sensing (AQS) activity of methanol extract of clove was preliminary confirmed against Chromobacterium violaceum ATCC 12472 (CV12472). The inhibition of quorum sensing dependent virulence factor formation such as swarming motility, pyocyanin formation and biofilm formation by clove, ceftazidime (cefta) and their combination was investigated at their sub-minimum inhibitory concentration (sub-MICs) in clinical isolates (CI 2,3,4) and reference PA01 of Pseudomonas aeruginosa.

The concentration dependent reduction (%) was found in swarming motility, pyocyanin formation and biofilm formation with clove, cefta and their combination against different CIs and PA01. The combination of clove and cefta reduced the tested QS regulated virulence factor formation more efficiently as compared to either of two alone. Our results suggest that the plant-antibiotic combination may be used as an alternate for safe and effective treatment of bacterial infection as well as for reduction in emergence of drug resistance at the reduced dose (sub-MIC).

Keywords: Anti-quorum sensing (AQS) activity, Clove (*Syzygium aromaticum*) bud, Ceftazidime (cefta), Minimum inhibitory concentration (MIC), *Pseudomonas aeruginosa*.

Introduction

Quorum sensing (QS) is a cell to cell communication and population density dependent mechanism which regulates the gene expression involving in formation of virulence factors in *Pseudomonas aeruginosa*. The QS system relies on the self-generated signalling molecules controlling regulation of gene expression known as autoinducers[28]. The survival and pathogenesis of *P. aeruginosa* depend on the expression of various virulence factors (exopolysaccharide synthesis, swarming motility, pyoverdin, pyocyanin pigment production, LasA protease, LasB elastase, alginate) and biofilm formations are under the control of QS mechanism[23,34].

P. aeruginosa is a gram negative opportunistic bacteria and can cause nosocomial infection, burn wound infection, urinary tract infection and also infects the patients of AIDS, cystic fibrosis and pulmonary diseases[14,27]. Antibiotic therapy commonly used to treat pseudomonas infection but increasing resistance of bacteria against antibiotics is a serious side effect of this therapy[12,22].

In *P. aeruginosa*, lasI-lasR and rhlI-rhlR are two interrelated QS systems which function in a hierarchical manner[9,29]. These quorum sensing systems involve inducer and regulatory proteins and a cognate autoinducer signal molecule, N-(3-oxododecanoyl) homoserine lactone (OdDHL) and N-butanoyl homoserine lactone (BHL) respectively[11,32]. Furthermore a third intercellular signal termed as Pseudomonas quinolone signal (PQS), (2-heptyl-hydroxy-4-quinolone) has been investigated in *P. aeruginosa*[26].

The antibiotic combination therapy including β–lactam antibiotics (ceftazidime, cefepime, ceftolozane/ tazobactam) and aminoglycoside / fluoroquinolones (ciprofloxacin) is generally used for the treatment of *P. aeruginosa* infections[4]. During the infection process *P. aeruginosa* forms biofilms which may be eradicated using higher dose of antibiotics because bacterial cells living as biofilms are much more tolerant and resistant to antibiotics in comparison of planktonic cells[2,6].

The use of higher dose of antibiotic to remove biofilm imposes the bacteria for emergence of resistance and leads to serious side effects in patients. These problems related to antibiotics necessitated requirement of alternative of this approach.

Phytoconstituents of medicinal plants having antibacterial and anti-quorum sensing activity may beneficial in reducing dose of antibiotics, thereby minimizing the risk of toxicity as well as resistance development. Inhibition of quorum sensing of bacteria to regulate the virulence factor formation may be a target because anti-quorum sensing compound do not kill or inhibit the bacterial growth rather than only reduce the formation of virulence factor by interfering quorum sensing mechanism.

Additionally, these compounds do not impose selective pressure on bacteria, so problem of drug resistance may also be overcome with anti-quorum sensing strategy[15]. Previous research has demonstrated that natural plant extracts have the abilities to interfere with quorum sensing mechanism and inhibition of virulence factor production against pathogens[5,18]. Medicinal plants which exhibit anti-quorum sensing activity may be used for treatment of pseudomonas infection as well as to reduce the emergence of drug resistance.

Previously we determined the multidrug-resistance (MDR) pattern of seven clinical isolates of *P. aeruginosa* (CIs) in which CI 2,3,4 were selected on the basis of their MDR pattern and subjected for further experiments (own unpublished data). As an extension of our research, present study was aimed to evaluate the anti-quorum sensing and antibiofilm activity of clove and cefta alone and also to investigate their combined efficacy to inhibit virulence factor production such as swarming motility, `pyocyanin pigment and biofilm formation against multidrug-resistant *P. aeruginosa*.

Material and Methods

Collection of plant material and preparation of methanolic extract: The fresh buds of clove (*Syzygium aromaticum*) were collected and authenticated by experts in Central Council for Research in Ayurvedic Sciences-Regional Ayurveda Research Institute (CCRAS-RARI), Jhansi (Uttar Pradesh), India. Dried clove buds were powdered and subjected to extraction in methanol (100%). Briefly dried powder (10 gm) was soaked in methanol and subjected to mixing at room temperature for 3 successive days. The crude extracts were pooled, filtered through Whatmann filter paper no. 1 and centrifuged at 3000 rpm for 10 min. The supernatant was concentrated with rotary evaporator. Prepared crude methanol extract of clove was preserved at 4℃ in air tight bottle for further experiments.

Collection of bacterial strains and culture condition: Clinical isolates of *P. aeruginosa* were collected from Department of Microbiology, Sarojini Naidu Medical College, Agra (Uttar Pradesh), India. *Chromobacterium violaceum* ATCC 12472 (CV12472) and reference strain (PA01) of *P. aeruginosa* were collected from Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh (Uttar Pradesh), India. *C. violaceum* (CV12472) produces quorum sensing regulated purple coloured pigment (violacein) and maintained on nutrient broth at 28℃. Clinical isolates (CIs) and reference strain *P.aeruginosa* (PA01) were maintained on Luria- Bertani (LB) broth at 37℃ for proposed research study.

Determination of minimum inhibitory concentration (MIC) of clove extract, cefta and their combination: Previously antibacterial activity of cefta and clove extract was determined with agar well diffusion method and their MICs were determined by the broth microdillution method according to the guidelines of Clinical and Laboratory Standard Institute[8]. Previously combined efficacy of cefta and clove extract was determined using standard checkerboard method calculating fractional inhibitory concentration (FIC) and FIC index (FICI) against CI 2,3,4 and reference strain (PA01) of *P. aeruginosa*[7,33].

Determination of anti-quorum sensing (AQS) activity: Agar well diffusion method was used to determine the preliminary AQS activity (as pigment inhibition activity) of clove extract against CV12472 using the standard method with slight modifications[36]. Briefly Mueller Hinton agar plates with 100 µl of diluted freshly grown bacterial suspension (2.5 x 10^6 CFU/ml) were prepared and wells of 6-8 mm diameter were made with sterile cork borer and sealed at the bottom using soft agar. 50µL of crude clove extract (500 mg/ml) were aseptically poured in the wells and plates were incubated at 28℃ for 18-20 h. DMSO (dimethyl sulfoxide) was used as negative control. The diameter of inhibition zone of pigment inhibition (mm) around the well was measured to determine the AQS activity.

Effect on Quorum sensing regulated virulence factors: The sub-MICs (1/2,1/4,1/8 MIC) of clove extract and cefta alone and their combination were prepared and subjected to AQS activity (Table 1 and 2) (own unpublished data).

Swarming assay: The swarm plates for determination of motility were prepared with 50 µL of sub-MICs of clove extract and cefta and their combination using nutrient broth (8 gm/l) supplemented with glucose (5 gm/l) and solidified by addition of bacto agar (0.5%)[3]. The plates were subjected to absorb and point-inoculated with freshly grown over night culture of CIs and reference strain (PA01) of *P. aeruginosa*. The plates were incubated at 37℃ for 18 -24 h. Swarming motility was determined by measuring the diameter (mm) of circular turbid zone. Diameter of swarms (mm) of treated and untreated strain (as control) was taken to calculate inhibition in swarming motility (%).

Pyocyanin assay: The effects of sub-MICs of cefta and clove extract alone and their combination against pyocyanin formation in CIs and reference strain (PA01) of *P. aeruginosa* were analysed using the chloroform extraction method[10]. Briefly 5 ml of freshly prepared bacterial broth culture were mixed with 150 µL of sub-MICs of clove, cefta and their combination and incubated at 37℃ for 18 -24 h. Chloroform (3 ml) was added to the incubated culture supernatant and mixed vigorously. Chloroform layer was separated and mixed with 1 ml HCl (hydrochloric acid) (0.2 M). The solution was centrifuged (8,000 rpm for 10 min at room temperature) and absorbance of pink to deep red coloured HCL layer was measured at 520 nm. Absorbance (A) of treated and untreated strain (as control) was taken to calculate inhibition in pyocyanin formation (%).

Assay for biofilm inhibition: The effects of sub-MICs of cefta and clove extract alone and their combination on

biofilm formation were determined by microtitre plate assay with slight modifications[25]. Briefly biofilms were developed in the presence of sub-MICs of clove extract, cefta and their combination at 37℃ for 18 -24 h. After incubation, the wells were drained properly and washed three times with sterile PBS (phosphate- buffered saline) to remove planktonic cells. 150 µl of 0.1% crystal violet solution was added to wells for staining and plate was incubated at room temperature for 5-10 min.

After incubation wells were washed with sterile PBS to remove excess dye. The plate was air dried and dye adopted by biofilm cells in the wells was solubilised by adding 150 µL of glacial acetic acid (33% v/v). Absorbance (A) of treated and untreated strain (as control) was taken at 570 nm to calculate inhibition (%) in biofilm formation.

Statistical analysis: The experiments were performed in triplicate and the mean and standard deviation were calculated. The data of pyocyanin, swarming motility and biofilm inhibition (%) was further analyzed for statistical significance among the sub-MICs level (1/2, 1/4, 1/8 MIC) of clove, cefta and their combination as compared to untreated control with One-way ANOVA and level of significance was expressed as P value ($P < 0.001$, $P < 0.005$, $P < 0.01$ and $P < 0.05$ denoted with ****, ***, ** and * respectively). Comparison between mean values of treated (with clove, cefta and their combination) among sub-MICs level was based on Tukey's HSD post hoc test and the mean difference was expressed as significant at the 0.05 level. Statistical analyses were performed in IBM SPSS statistics 20 (Version 20.0. Armonk, NY: IBM Corporation).

Results

Anti-quorum sensing (pigment inhibition) activity of clove methanolic extract against CV12472: CV12472 produced a purple pigment violacein under the control of quorum sensing signals. In preliminary determination of anti-quorum sensing activity, the significant inhibition in violacein pigment formation against biosensor strain CV12472 was shown by clove extract (Figure 1).

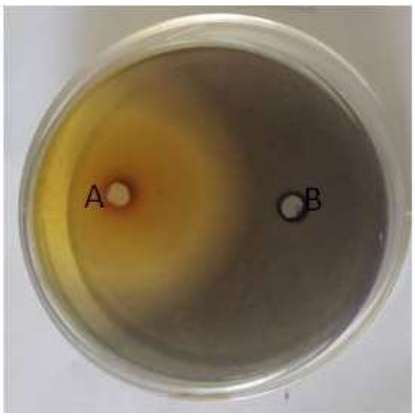

Figure 1: Antiquorum sensing (violacein pigment inhibition) activity of methanol extract of clove against *C. violaceum* CV12472. (A: methanol extract of clove; B: negative control)

Effect on Quorum sensing regulated virulence factors of *P. aerugnosa*: The individual and combined MIC of clove extract and cefta against CI 2, 3, 4 and reference strain (PA01) of *P. aeruginosa* were previously determined and mentioned in table 1 and table 2 (own unpublished data).

Table 1
MIC and sub-MICs of clove and cefta against clinical isolates (CI) 2, 3, 4 and PA01 of *P. aeruginosa* (own unpublished data)

Isolates	MIC and sub MICs (mg/ml) (Clove)				MICs and Sub MICs (µg/ml) (cefta)			
	MIC	1/2 MIC	1/4 MIC	1/8 MIC	MIC	1/2 MIC	1/4 MIC	1/8 MIC
CI 2	3.90	1.95	0.97	0.48	31.25	15.62	7.81	3.90
CI 3	15.62	7.81	3.90	1.95	31.25	15.62	7.81	3.90
CI 4	7.81	3.90	1.95	0.97	31.25	15.62	7.81	3.90
PA01	3.90	1.95	0.97	0.48	15.62	7.81	3.90	1.95

Table 2
Combined MIC and sub-MICs of clove and cefta against clinical isolates (CI) 2, 3, 4 and PA01 of *P. aeruginosa* (own unpublished data)

Isolates	Combined MIC of clove and cefta		Combined sub MICs of clove (mg/ml) + cefta (µg/ml)		
	Clove MIC (mg/ml)	Cefta MIC (µg/ml)	1/2 MIC	1/4 MIC	1/8 MIC
CI 2	0.97	3.90	(0.48) + (1.95)	(0.24) + (0.97)	(0.12) + (0.48)
CI 3	7.81	15.62	(3.90) + (7.81)	(1.95) + (3.90)	(0.97) + (1.95)
CI 4	1.95	3.90	(0.97) + (1.95)	(0.48) + (0.97)	(0.24) + (0.48)
PA01	0.97	1.95	(0.48) + (0.97)	(0.24) + (0.48)	(0.12) + (0.24)

As an extension of our previous findings, the sub-MICs (1/2,1/4,1/8 MIC) of clove extract and cefta alone and their combination were prepared and subjected to evaluate their inhibitory effect on swarming motility, pyocyanin pigment formation and biofilm formation.

The effects of clove extract, cefta and their combination on growth of PA01 and CI 2,3,4 at their sub-MICs were evaluated by growth curve analysis. This study revealed that the growth was not affected with sub-MICs of clove extract, cefta and their combination (data not shown).

Effect on swarming motility: The cefta and clove extract showed concentration dependent reduction in swarming motility against PA01 and different CIs within the range of 61.03%-69.06% (at 1/2 MIC), 45.5%-54.69% (at1/4 MIC), 25.96%-39.77% (at 1/8 MIC) and 50.48%-58.01% (at 1/2 MIC), 34.80%-40.37% (at 1/4 MIC) and 20.19%-26.51% (at 1/8 MIC) respectively. Moreover combination of cefta and clove extract showed highly significant reduction in swarming motility with range 74.73%-82.15% (at 1/2 MIC), 55.28%-71.82% (at 1/4 MIC), 37.01%-47.51% (at 1/8 MIC) of PA01 and clinical isolates (CI) as compared to their individual effect (Figure 2a, 2b, 2c, 2d).

Effect on pyocyanin formation: The pyocyanin formation in PA01 and CI 2, 3, 4 was significantly reduced at the level of sub MICs of cefta, clove extract and their combination. The maximum reduction was found up to 85.80% at 1/2 MIC of combination against clinical isolate 4. The individual pyocyanin inhibitory activity (%) of clove and cefta was found to be increased by approximate 24% and 13% respectively when combined with each other at 1/2 MIC (Figure 3a, 3b, 3c, 3d).

Effect on biofilm formation: The significant reduction in biofilm formation was observed in different CIs and PA01 ranged (68.12%-74.10%), (54.43%-59.55%), (26.11%-38.08%) with cefta and (55.30%-67.69%), (38.08%-52.94 %), (18.83%-29.16%) with clove extract at 1/2 MIC, 1/4 MIC and 1/8 MIC respectively. The combination of clove and cefta reduced biofilm formation more efficiently as compared to either of two and ranged 31.62%-87.97% at different sub- MICs. The biofilm inhibitory activity (%) of clove and cefta was found to be increased approximate by 20% and 14% when used as combination at 1/2 MIC (Figure 4a, 4b, 4c, 4d).

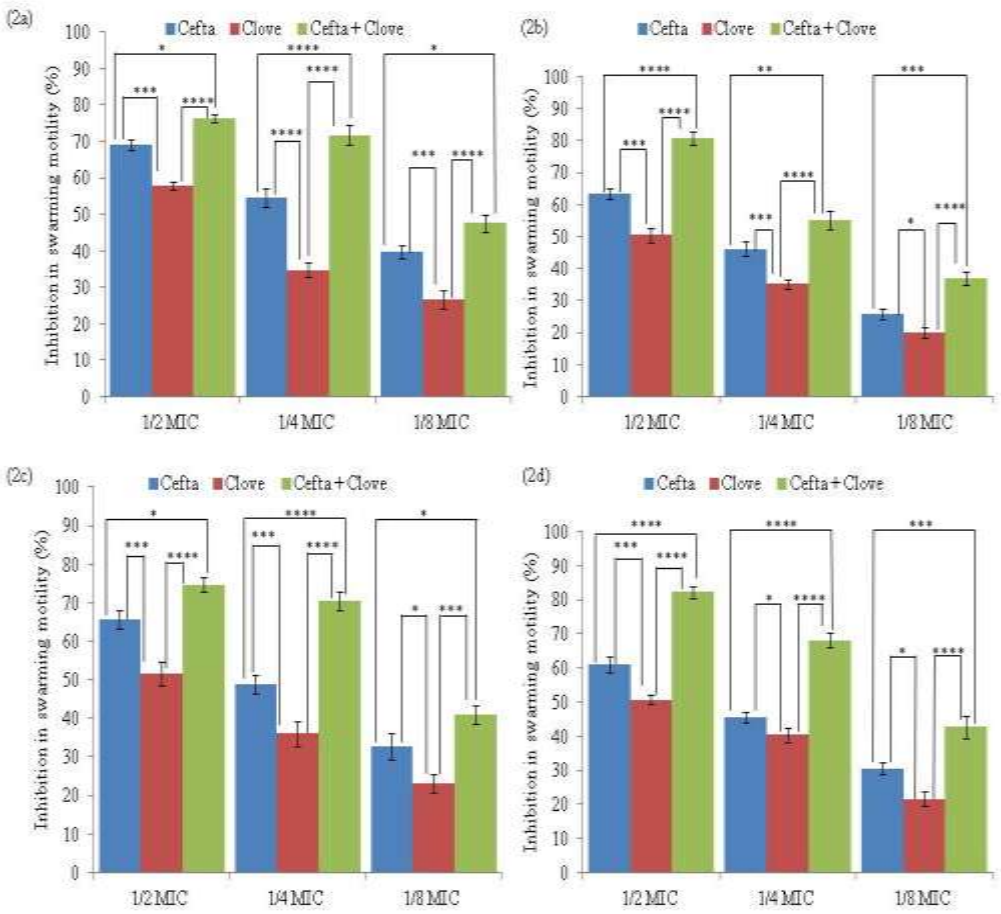

Figure 2 (a,b,c,d): Inhibition of swarming motility by cefta, clove extract and combination of cefta and clove extract at their sub-MICs against (a) PA01 (b) CI 2 (c) CI 3 (d) CI 4 of *P. aeruginosa*. Each bar represents mean value of three independent replicates and error bar showed standard deviation. **** P < 0.001, *** P < 0.005, ** P < 0.01, * P < 0.05 (significance level as compared to untreated control)

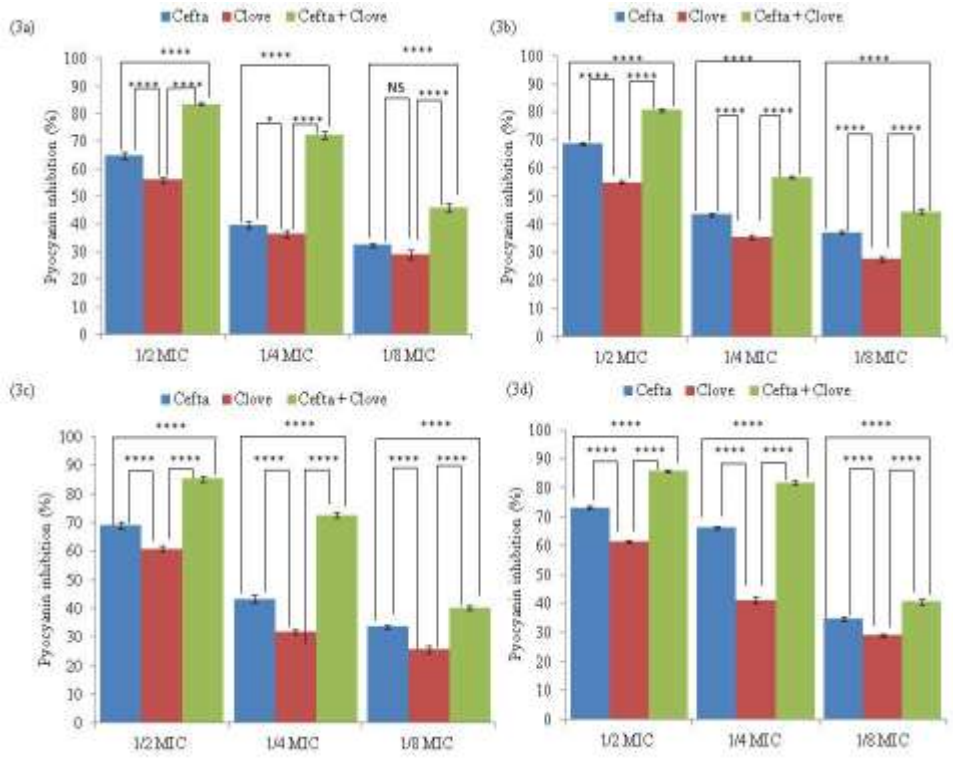

Figure 3 (a,b,c,d): Inhibition of pyocyanin formation by cefta, clove extract and combination of cefta and clove extract at their sub-MICs against (a) PA01 (b) CI 2 (c) CI 3 (d) CI 4 of *P. aeruginosa*. Each bar represents mean value of three independent replicates and error bar showed standard deviation.
****** P < 0.001, * P < 0.05, NS: Not significant (significance level as compared to untreated control).**

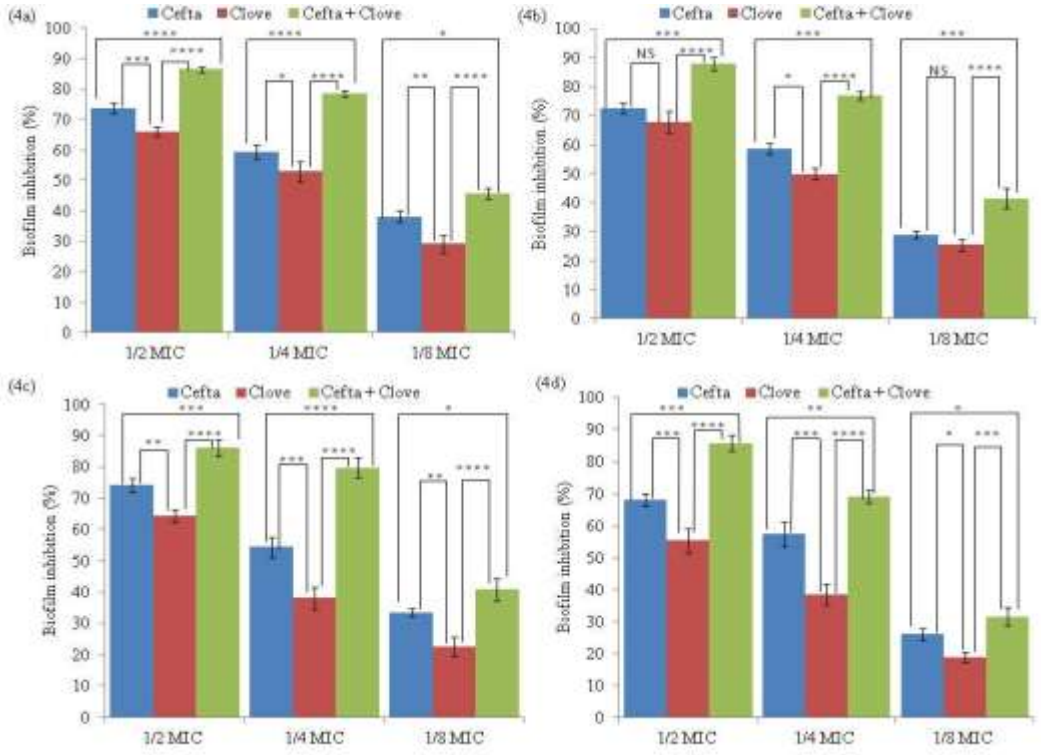

Figure 4 (a,b,c,d): Inhibition of biofilm formation by cefta, clove extract and combination of cefta and clove extract at their sub-MICs against (a) PA01 (b) CI 2 (c) CI 3 (d) CI 4 of *P. aeruginosa*. Each bar represents mean value of three independent replicates and error bar showed standard deviation.
****** P < 0.001, *** P < 0.005, ** P < 0.01, * P < 0.05, NS: Not significant**
(significance level as compared to untreated control)

Discussion

In many bacteria including *P. aeruginosa,* the production of virulence factor is regulated by a quorum sensing. Disruption in quorum sensing regulatory mechanism has been established as a novel target to attenuate the pathogenecity of bacteria[36]. Antibiotic combination therapy is generally used for treatment of several bacterial infections. Ceftazidime, a third generation cephalosporin is commonly used in the treatment of *P. aeruginosa* infection and is a QS inhibitor, decreasing the formation of various virulence factors regulated by QS system[21,30,31]. Wang et al reported that the combination of cefta and azithromycin inhibits the biofilm formation effectively and reduces the bacterial load of *P. aeruginosa* in comparison of treatment with either of these two antibiotics alone[33].

The down regulation of QS regulatory gene cascade to block QS and attenuate the activity of *P. aeruginosa* with antibiotics at their sub-MICs level was previously reported by Skindersoe et al.[30] In present study, we evaluated the combined AQS activity of clove and cefta as an alternative of antibiotics combination therapy and compared with their individual effect against *P. aeruginosa*. The dual activities of clove extract and cefta i.e. bacteriostatic or bactericidal and QS inhibitor (at sub-MICs) may explain their role in the treatment of pseudomonas infection.

CV12472, a biosensor strain produces violacein under the regulation of CviIR dependent QS system[24]. In present study, preliminary anti-quorum sensing (AQS) activity of clove extract was confirmed by inhibition of violacein formation in CV12472. Further AQS activity of clove extract, cefta and their combination was evaluated against different CIs and reference strain (PA01) of *P. aeruginosa*. The inhibitory effect on virulence factors such as swarming motility, pyocyanin pigment formation, biofilm formation regulated by quarum sensing mechanism was targeted to determine interference in QS cascade.

We found that clove extract and cefta reduced the formation of tested virulence factors but their combination was more efficient as compared to clove alone or cefta alone. Pyocyanin is a well characterized QS regulated virulence factor associated in cystic fibrosis *P. aeruginosa* infections[35]. A significant dose dependent decrease in pyocyanin formation was found with clove extract, cefta and their combination against all CIs and reference PA01 ($P<0.001$). The maximum reduction was found with combination of clove and cefta range 80.63%-85.80% at1/2 MIC while it was 64.91%-73.27% with cefta alone and 54.97%- 61.52% with clove alone.

Similar dose dependent decrease in pyocyanin production (37%-75%) at different sub-MICs (0.2–1.6% v/v) of clove oil concentration against PA01 was reported by Husain et al.[16] Swarming motility mediated by type IV pilli and flagella contributes to keeping cell compact and in close proximity within biofilm of *P. aeruginosa*[19]. In our study, swarming motility of all CIs and PA01 was significantly reduced at sub-MICs with clove extract and cefta but maximum reduction was found with combination of cefta and clove. Slightly similar concentration-dependent reduction in swarming motility of PA01 ranged from 40%-78% of clove oil at different sub-MICs 0.2 - 1.6 (% v /v) reported by Khan et al.[18]

Abd El-Aziz et al[1] also reported the reduction in swarming motility by sub inhibitory concentration of clove buds extract. Krishnan et al[20] reported the inhibitory effect of clove extract against QR regulated phenotype including pyocyanin and swarming motility. Biofilm, a physical barrier against defence system of host, contributes to increasing resistant against antibiotics and making antibiotic monotherapy insufficient to treat bacterial infection[13,21]. We found significant reduction ($P<0.001$) in biofilm formation with clove and cefta combination ranging 31.62%-87.97% at different sub-MICs which was more efficient as compared to their individual effect against different CIs and PA01. Similar dose dependent reduction in biofilm formation with clove oil (0.2%-0.8% v/v) against PA01 was observed by Husain et al[16].

Concentration dependent (at sub-MICs) reduction in virulence factors including swarming motility, pyocyanin formation and biofilm formation with ceftazidime against *P. aeruginosa* was demonstrated by Husain et al.[17] In our study, the inhibition of swarming motility (%) in reference PA01 was less than clinical isolate CI 2 and CI 4 but more than CI 3 (at 1/2 MIC) while pyocyanin inhibition (%) was slightly less than CI 3 and CI 4 but more than CI 2 (at1/2 MIC). Biofilm inhibition (%) in PA01 was slightly less than CI 2 and more than CI 3 and CI4 (at1/2 MIC).

We observed that the inhibition (%) in swarming motility, pyocyanin and biofilm was independent from strain type either PA01 or clinical isolates (at various sub-MICs level) and the requirement of dose for their inhibition varied independently. These inhibitory activities suggest that tested combination may interfere more efficiently in QS cascade which regulates the gene expression of related virulence factor. Although molecular investigations are required for confirmation of the target sites of this combination, yet the effectiveness of plant-antibiotic combination over their individual effect has been revealed through this study. In future we should focus on such type of combination as a possible and effective alternative of antibiotic mono and combination therapy against bacterial infections.

Conclusion

Our results suggest that clove extract and cefta combination has effective QS inhibitory activity at a very low concentration (sub-MIC) and may be a dominating and better approach over mono or combination therapy of antibiotics. This combination may be used for the safe and effective treatment of bacterial infections as well as reduction in emergence of drug resistance.

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