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Susceptibility of biofilm forming *Pseudomonas aeruginosa* and *Staphylococcus aureus* to antibiotic-adjuvants

Mala Rajendran*, Ruby Celsia Arul Selvaraj, Hari Prasath Nagaiah, Keerthana Ravi Chandran and Gopika Shri Janaki Raman

Department of Biotechnology, Mepco Schlenk Engineering College, Sivakasi-626005, Tamil Nadu, India.
Email: maalsindia@mepcoeng.ac.in

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ABSTRACT

Aims: The objective of the present study is to evaluate the possibility of reversing the resistance of pathogens to antibiotics using phytochemicals from plant extracts as antibiotic-adjuvant.
Methodology and results: Twenty-one plants were collected from Podhigai Hills, Tamil Nadu, India and tested in this study. The susceptibility of burn wound isolates (*Pseudomonas aeruginosa* and *Staphylococcus aureus*) to antibiotics and the adjuvant activity of the aqueous plant extracts were tested using well diffusion assay. The impact of the plant extracts on quorum sensing was assessed using *Chromobacterium violaceum* as the model organism. The antibiofilm activity of the adjuvant and antibiotics was determined by crystal violet assay. The isolates which were resistant to more than one class of antibiotics (aminoglycoside, cephalosporin, fluoroquinolone and penicillin) were designated as multi-drug resistant bacteria. Combination of cefdinir-*Citrullus colocynthis* showed 17 mm inhibition zone which is greater than cefdinir (0 mm) against *P. aeruginosa.* The combination reduced quorum sensing with an inhibition zone of 30 mm. The same combination reduced 96% and 95% of the biofilm formed by *P. aeruginosa and S. aureus*, respectively at 16 h. Besides, cefdinir with *Leucas aspera* reduced quorum sensing with an inhibition zone of 28 mm. The combination reduced 94% and 95% of biofilm formed by *P. aeruginosa and S. aureus*, respectively at 16 h. The aqueous extract of *C. colocynthis and L. aspera* revealed the presence of flavonoids that possess adjuvant activity.
Conclusion, significance and impact of study: Cefdinir-*C. colocynthis* and cefdinir-*L. aspera* reversed the resistance of multi drug resistant bacteria to cefdinir. The flavonoids of *C. colocynthis* and *L. aspera* served as an adjuvant that potentiates the activity of cefdinir.

Keywords: Flavonoids, adjuvants, *Citrullus colocynthis*, *Leucas aspera*, quorum sensing

INTRODUCTION

Biofilm is a pattern of life adopted by bacteria on living and non-living solid surfaces. It is an association of bacteria encased inside self-assembled extracellular polymeric substances (EPS). EPS is composed of polysaccharides, proteins and DNA (Wolcott *et al*., 2010). Biofilm formation by bacteria in lungs causes cystic fibrosis, tuberculosis and pneumonia. Dental plague is caused by *Streptococcus mutans*. Approximately 60% of infectious diseases are caused by biofilm (Chen and Wen 2011). Biofilm is not only associated with tissue infections. It is the major hurdle to the success of medical devices like contact lens, prosthesis, heart valves, central venous catheter and urinary catheter (Percival and Kite 2007; Jamal *et al*., 2018). Biofilm formation occurs in two sequential stages namely, adhesion and maturation. Adhesion involves attachment to the substratum and maturation involves the multiplication and differentiation of the adhered cells. Maturation stage is determined by quorum sensing, the method of cell to cell communication in bacteria. WHO (2018) reported that pathogens causing common infection are resistant to antibiotics. β-lactam class of antibiotics are widely used to control a broad range of infectious agents. Development of resistance to β-lactam antibiotics is due to the modification of target, presence of efflux pumps and production of β-lactamases. Exner *et al*. (2017) reported that five classes of β-lactamases are responsible for the alarming resistant to β-lactam class of antibiotics observed in health care unit. The key organisms resistant to antibiotics are *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp. (ESKAPE). Quorum sensing facilitates bacteria to behave as multi-cellular organisms. Pathogens within the biofilm develop multiple strategies to resist antibiotics and evade immune system of the host. They prevent the entry of antibiotics into the

*Corresponding author

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biofilm through EPS (Francolini and Donelli 2010). EPS neutralizes and dilutes the antimicrobial agents (Hall-Stoodley *et al*., 2004). Quorum sensing inhibitors are the major targets for controlling and eliminating biofilm, as they are the key regulators of biofilm formation, virulence and pathogenicity (Zhu and Mekalanos, 2003).

One possible way to counteract the effect of drug resistance is to discover new antibiotics. However, it takes approximately 20 years to commercialize a lead molecule. Many plant-derived compounds and metals like silver and zinc are used for potentiating the power of antibiotics. Many phytochemicals serve as an adjuvant that has no intrinsic antimicrobial action. Abreu *et al.* (2016) described the alarming increase in multi-drug resistance (MDR) among the pathogens that drives scientist to search for phytochemicals that serves as an adjuvant to antibiotics. Their study evaluated the adjuvant activity of phytochemicals from twenty-eight plants. Among the twenty-eight plants, *Acacia dealbata, Pyrus communis, Prunus avium, Prunus domestica, Prunus persica, Centaurea nigra, Eupatorium cannabinum, Ficus carica* and *Buxus sempervirens* potentiated the activity of ciprofloxacin, tetracycline and erythromycin (Abreu *et al*., 2016). The study identified betulinic acid and terpenoids as adjuvants. The investigation by Abreu *et al.* (2017) explored the role of isoflavonoids from *Cytisus straitus* as an adjuvant.

Exploiting adjuvants to sensitize the pathogens to already ineffective antibiotics is a cost effective and promising technology to combat antibiotic resistance threat. The effect of four adjuvants on the activity of novobiocin against *Escherichia coli* as model organism was reported earlier (Kalan and Wright, 2011). The study concluded with a hypothesis that, changes in the morphology of the cell leads to a change in the entry or exit of chemotherapeutic agents (Tegos *et al*., 2011). Adjuvants are broadly classified into class I and II (Zaheer *et al*., 2017). Class I works in association with antibiotics and class II improve the antimicrobial activity of the host. Class I is subdivided into I.A and I.B by their different mechanism of action. Class I.A acts as an adjuvant by inactivating the enzymes that confer resistance to antibiotics, block the efflux pump or by altering their targets. Class I.B functions by evading intrinsic antimicrobial resistance mechanisms. Class I.B and II are still in pre-clinical trials (Worthington and Melander, 2013).

Plants are the richest source of therapeutic agents since ancient times, but no antibiotic has been produced from plants till now. It is due to the poor activity or toxicity of purified components. Minimum inhibitory concentration of phytochemicals against pathogens is high. Plants are constantly exposed to a variety of biotic and abiotic stress. They co-evolve strategies to combat the stress by producing a wide array of bioactive compounds. Even though no single potent antimicrobial agent was produced by plants, it produces structurally diverse compounds that act synergistically with antibiotics, that reverse the resistance of pathogens to antibiotics (Gonzalez-Lamothe *et al*., 2009). Plants also produce compounds that alter the physico-chemical properties of antibiotics by increasing their solubility, stability, availability and absorption. Efflux pump inhibitors produced by the plants prevent the efflux of antibiotics (Abreu *et al*., 2016). Clavulanic acid is a β-lactamases inhibitor with weak antibacterial activity, combination of clavulanic acid with amoxicillin remarkably improved the therapeutic efficiency of amoxicillin (Abreu *et al.*, 2016). The present study evaluates the twenty-one plants extract for the production of antibiotic–adjuvants with the aim to reverse the resistance of pathogens to antibiotics by the combination of antibiotic-adjuvant and to evaluate their role as biofilm breakers.

MATERIALS AND METHODS

Collection of clinical isolates

The burn wound pathogens (*S. aureus* and *P. aeruginosa*) used in the study were isolated from third degree burn wound. The swab of burn wound was collected as per ethical approval (Ref No: 75/E1/2016:dt. 22.08.2016) from the Government Hospital, Sivakasi, Tamil Nadu, India.

Collection of plant samples for screening of antibiotic-adjuvant activity

Twenty-one medicinal plants were collected from Sivakasi

Table 1: Plants collected for screening of antibiotic-adjuvant activity in this study.

1) *Citrullus colocynthis* (F)	8) *Allium cepa* (R)	15) *Solanum procumbens* (L)
2) *Leucas aspera* (L)	9) *Zingiber officinale* (R)	16) *Lawsonia inermis* (L)
3) *Anisomeles malabarica* (L)	10) *Ocimum tenuiflorum* (L)	17) *Asystasia gangetica* (L)
4) *Microstachys chamaelea* (L)	11) *Ficus racemosa* (F)	18) *Ocimum basilicum* (L)
5) *Tribulus terrestris* (L)	12) *Aegle marmelos* (L)	19) *Catharanthus roseus* (L)
6) *Kalanchoe pinnata* (L)	13) *Cynodon dactylon* (L)	20) *Morinda citrifolia* (L)
7) *Piper betle* (L)	14) *Ricinus communis* (L)	21) *Indigofera tinctoria* (L)

L: Leaves; R: Roots; F: Fruits

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and Podhigai Hills, Coutrallam for screening of adjuvant (Table 1). The plants were shade dried. A total of 1 g of respective parts of the plants as mentioned in Table 1 were weighed and surface sterilized with ethanol. Aqueous extract of the plants were prepared with 20 mL of double distilled water. The extract was centrifuged at 10,000 rpm for 10 min and the supernatant was concentrated and dried in rotary evaporator at 50 °C for 10 h.

Susceptibility of isolates to antibiotics and adjuvants by diffusion method

Susceptibility of the clinical isolates was tested against amikacin-30µg (aminoglycoside), ciprofloxacin-5µg (fluoroquinolone) and cefdinir-5µg (cephalosporin), amoxycillin-30µg (penicillin) and amoxyclave-30µg (penicillin). Optical density (OD) of the overnight grown culture was adjusted to 0.05 and swabbed on to Mueller Hinton agar. Wells of 8 mm diameter was punctured on the plates. Then, 100 µL of plant extracts were added and the plates were incubated at 37 °C for 16 h (Smânia 2007). After incubation, the diameter of inhibition zone was measured in millimeters (mm). The organisms were designated as resistant (R), susceptible (S) and intermittent (I) based on the inhibition zone as per the CLSI guidelines (CLSI, 2017). The plant extract was considered to possess adjuvant activity based on the diameter of inhibition zone of antibiotic and plant minus inhibition zone of antibiotic (Abreu *et al*., 2015). Based on result, the activity of plant extracts can be classified into three categories namely, indifferent (<4 mm), additive (≥4 mm), potentiation (>6 mm).

Effect of antibiotic-adjuvants on quorum sensing

Quorum sensing is an intercellular communication between bacteria through autoinducers. It is demonstrated using *Chromobacterium violaceum* strain (MTCC 2656). These bacteria specifically produce violet color pigmentation during quorum sensing. Lack of purple pigmentation from *C. violaceum* is an indicator of inhibition of quorum sensing. The OD of *C. violaceum* was adjusted to 0.1 and 100 µL of culture was swabbed on agar plate. Well of 8 mm diameter was punctured and 100 µL of respective plant extracts were added. The plates were then incubated at 37 °C for 16 h. Violet pigmentation in the plates indicate the quorum sensing. The zone of clearance of violet colour indicates the quenching of quorum sensing. The diameter of zone of clearance is proportion to the ability of the test compound to inhibit quorum sensing (Fuqua *et al*., 1994). Cefdinir was used as the antibiotic control for assessing quorum sensing.

Effect of antibiotic-adjuvants on biofilm and planktonic cells

Biofilm formation was evaluated by crystal violet assay in a sterile 24 well plate. 2 mL of nutrient broth and 200 µL of culture (0.05 OD) were added to all wells. Each treatment was replicated thrice. The plate was incubated for 16 h and 50 h as an adherence period. After incubation, the medium was drained off and 2 mL of fresh broth along with 200 µL of antibiotic-adjuvants (1:5) were added. A control well was maintained with 200 µL of phosphate buffered saline. After incubation for 24 h, the planktonic cells were collected in a separate tube. The OD of the planktonic cells was read at 600 nm. The adhered biofilm was stained with crystal violet. The dye was solubilized with 1% glacial acetic acid. The OD of the samples was read at 660 nm for the quantification of biofilm (Walker and Horswill, 2012). Reduction in biofilm and planktonic cells were assessed by comparing with the control at 16 h and 50 h, respectively using the formula:

Reduction (%) =
(Control OD − Test OD)/Control OD × 100

Phytochemical analysis of plants

Aqueous extract of plants (1 g/20 mL of double distilled water) were qualitatively analyzed for the presence of flavonoids, alkaloids and tannins (Reynolds *et al*., 1998).

Test for flavonoids

The presence of flavonoids was confirmed by adding 5 drops of 1 N sodium hydroxide to 3 mL of the plant extract. Formation of yellow color that disappears on the addition of 0.1 N hydrochloric acid confirms the presence of flavonoids.

Test for alkaloids

The presence of alkaloids was tested by Mayer's test. To 5 mL of the plant extract, 3 drops of concentrated sulphuric acid was added followed by the addition of 5 drops of Mayer's reagent. Formation of orange colored precipitate is an indication of positive test.

Test for phenols

5 drops of neutralized ferric chloride was added to 5 mL of the plant extract. Formation of green color is an indication of positive test.

Statistical analysis

All experiments were repeated thrice. The data represented in the graph are the mean of three values with standard error of the mean. All the calculations and graphs were drawn using in GraphPad Prism 5.

RESULTS AND DISCUSSION

Sensitivity of isolates to antimicrobial agents

Antibiotics

The sensitivity of the isolates to amikacin

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Table 2: Sensitivity of the bacteria isolates from burn wound to antibiotics.

Bacteria	Inhibition zone (mm)				
	Amikacin	Cefdinir	Ciprofloxacin	Amoxycillin	Amoxiclav
S. aureus	10.0 ± 0.5	5.0 ± 0.1	9.0 ± 0.2	8.0 ± 0.1	10.0 ± 0.1
P. aeruginosa	7.0 ± 0.4	0.0 ± 0.0	2.0 ± 0.0	5.0 ± 0.2	8.0 ± 0.1

(aminoglycoside), cefdinir (cephalosporin), ciprofloxacin (fluroquinolone), amoxycillin (penicillin) and amoxiclav (penicillin and β-lactam inhibitor) are represented in Table 2. The inhibition zone exhibited by all antibiotics against *S. aureus* and *P. aeruginosa* were ≤10 mm. The isolates were resistant to more than one class of antibiotic (aminoglycoside, cephalosporin, fluoroquinolone and penicillin) and hence they were interpretted as MDR bacteria. From Table 2, the inhibition zone exhibited by cefdinir was 5 mm and 0 mm against *S. aureus and P.* aeruginosa, repectively, which show *S. aureus* and *P. aeruginosa* are highly resistant to cefdinir. Cefdinir is a β-lactam antibiotic from cephalosporin class. β-lactam antibiotics are widely used to control infection. Bacteria develop resistance to β-lactam antibiotics by the synthesis of β-lactamase which is a threat to human being (Wright, 2016).

Adjuvants

Antimicrobial activity of the twenty-one plants collected were tested against two MDR organisms and the results are depicted in Figure 1a and Figure 1b representing *S. aureus* and *P. aeruginosa*, respectively. As the inhibition zone obtained for all plant extracts were less than 3 mm, it was considered to be insignificant for antibacterial activity (Abreu *et al*., 2017). To be used as an adjuvant, phytochemicals need not to possess antibacterial activity. But phytochemicals need to interact together with different compounds to enhance their activity mutually. They can improve the efficiency of antibiotics or reverse the resistance developed by pathogens due to efflux pump or β-lactamases (Chevalier *et al*., 2004).

Antibiotic-adjuvants

Among the antibiotics used for evaluating the sensitivity of bacteria, the inhibition zone exhibited by cefdinir was very low with 5 mm and 0 mm against *P. aeruginosa* and *S. aureus*, respectively (Table 2). So, the adjuvant activity of plant extracts was tested with cefdinir only. The effect of adjuvant from plant extracts and their combination with cefdinir against *S. aureus* and *P. aeruginosa* are

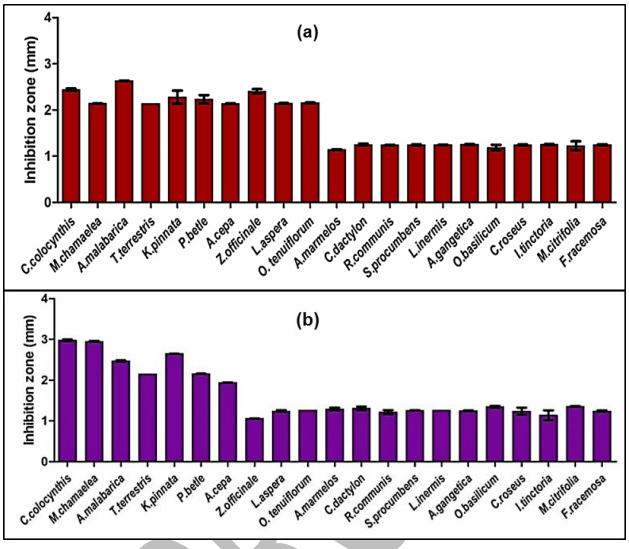

Figure 1: Sensitivity of bacteria (a) *S. aureus* (b) *P. aeruginosa* to various plant extracts.

represented in Figure 2a and Figure 2b, respectively. Aqueous extract from six plants namely *C. colocynthis, L. aspera, K. pinnata, A. malabarica, M. chamaelea* and *T. terrestris* exhibited high adjuvant property when compared to others. Inhibition zone exhibited by cefdinir, *C. colocynthis* and cefdinir-*C. colocynthis* against *P. aeruginosa* were 0 mm, 3 mm and 17 mm, respectively. On the other hand, the inhibition zone exhibited by cefdinir, *C. colocynthis* and cefdinir-*C. colocynthis* against *S. aureus* were 5 mm, 2.5 mm and 17 mm, respectively. The adjuvant activity of *L. aspera* was high next to *C. colocynthis* with an inhibition zone of 16 mm against *P. aeruginosa* and *S. aureus.* The other plants such as *M. chamaela, A. malabarica* and *K. pinnata* exhibited an inhibition zone of 16.8 mm, 15.6 mm and 15.5 mm respectively against *P. aeruginosa and S. aureus*.

Similar observation of phytochemicals potentiating the activity of antibiotics is supported by Abreu *et al.* (2017) and Gill *et al*. (2015). Abreu *et al*. (2017) reported that the compounds from *Cytisus striatus* was not active in *in vitro* tests when assessed alone. Different compounds can interact together and enhance their activity mutually. In the present study, plant extracts alone exhibited no antibacterial activity. But when combined with cefdinir, the activity was significantly enhanced. The results clearly demonstrated the potency of plant extracts in improving the effectiveness and reversing the resistance of antibiotics. Therefore, to identify the chemical nature of phytochemicals present in the extract, qualitative screening was done, and the results are shown in Table 3.

Effect of antibiotic-adjuvants on quorum sensing

Quorum sensing is one of the important virulence factors for biofilm formation. Antimicrobial agents (antibiotics,

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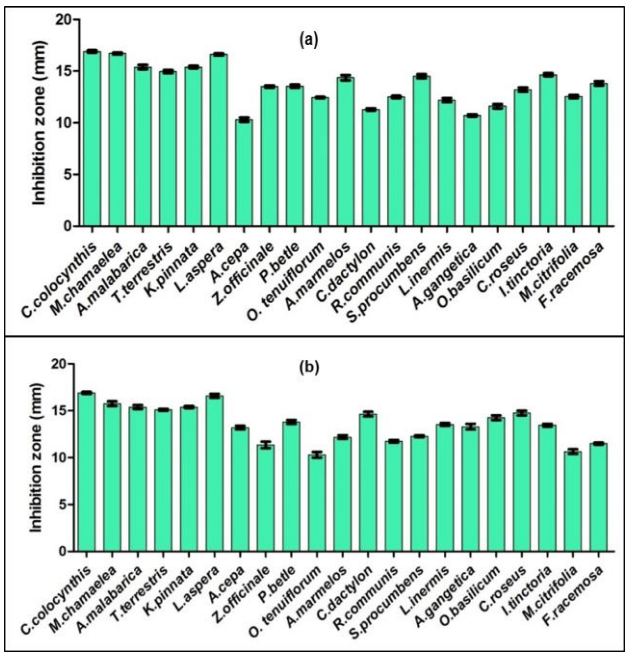

Figure 2: Sensitivity of bacteria (a) *S. aureus* (b) *P. aeruginosa* against cefdinir-plant extracts.

adjuvants and antibiotic-adjuvants) were assessed for their quorum quenching activity using *C. violaceum* (Zhu *et al*., 2011). Among the 21 different plant extracts, only four plants (*C. colocynthis, L. aspera*, *A. malabarica* and *M. chamaelea*) exhibited quorum quenching activity and the results are represented in Figure 3a and Figure 3b. The plants which lack quorum quenching activity had no zone of clearance and the results are represented in Figure 3c. Among the four quorum quenching plants, *C. colocynthis* and *L. aspera* were found to have a clearance zone of 14 mm and 13 mm, respectively when used alone. The same adjuvants in combination with cefdinir showed an increased inhibition zone to 30 mm and 28 mm, respectively. The results were supported by the observations of Bacha *et al.* (2016). Their study demonstrated that tannins and flavonoids are responsible for quorum quenching activity. Thus, the antibiotic-adjuvants have significant quorum quenching activity than existing antibiotics. Among the 4 plants which were tested for quorum quenching activity, *C. colocynthis* and *L. aspera* are widely distributed than other plants in Podhigai hills. So, they were used for biofilm and planktonic cells reduction in present study.

Effect of antibiotic-adjuvant on biofilm and planktonic cell reduction

Biofilm formation in tissues and medical devices starts from 8 h to 12 h. Later it fully matures in 48 h. Hence in the present study, the effect of antibiotic-adjuvant on the biofilm forming ability of *S. aureus* and *P. aeruginosa* were evaluated at 16 h and 50 h by crystal violet assay and the results are depicted in Figure 4 and Figure 5.

Table 3: Phytochemical analysis of the aqueous extract of plants.

Plants	Flavonoids	Alkaloids	Phenols
C. colocynthis	+	-	+
L. aspera	+	-	+
K. pinnata	+	+	-
A. malabarica	+	+	+
M. chamaelea	+	-	-
T. terrestris	+	-	+

(+): Presence; (-): Absence

C. colocynthis reduced 55% of biofilm of *P. aeruginosa* at 16 h and 43% at 50 h. Cefdinir inhibited 57% of biofilm at 16 h and 50% at 50 h. But the combination of cefdinir-*C. colocynthis* inhibited 96% and 90% at 16 h and 50 h, respectively. *L. aspera* reduced 44% and 36% of biofilm formed by *P. aeruginosa* at 16 h and 50 h, respectively. When cefdinir was used with *L. aspera* extract, 94% and 88% of biofilm formed by *P. aeruginosa* at 16 h and 50 h, respectively were reduced. The study demonstrated that the biofilm reduction was higher at 16 h than 50 h. *C. colocynthis* reduced 51% and 50% of biofilm formed by *S. aureus* at 16 h and 50 h, respectively. Cefdinir inhibited 55% of *S. aureus* biofilm at 16 h and

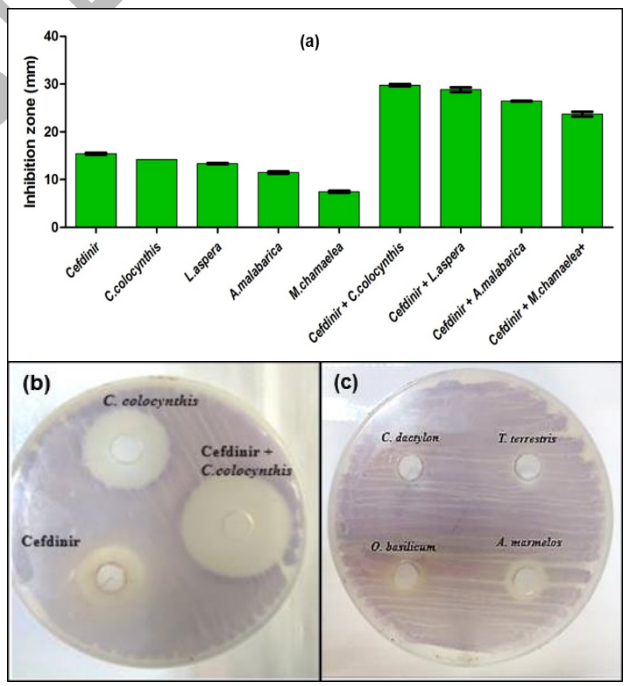

Figure 3: Influence of cefdinir-plant extracts on quorum quenching of *C. violaceum*. (a) Inhibition zone exhibited by cefdinir-plant extracts (b) Plate showing quorum quenching by cefdinir and *C. colocynthis* plant extract (c) Plant extracts with no quorum quenching activity.

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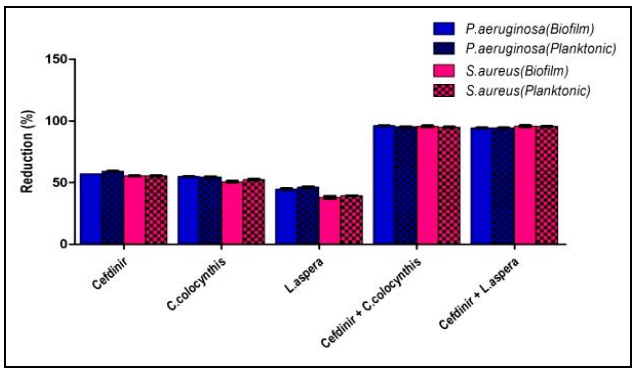

Figure 4: Influence of antibiotic-adjuvant on biofilm and planktonic cells reduction in 16 h.

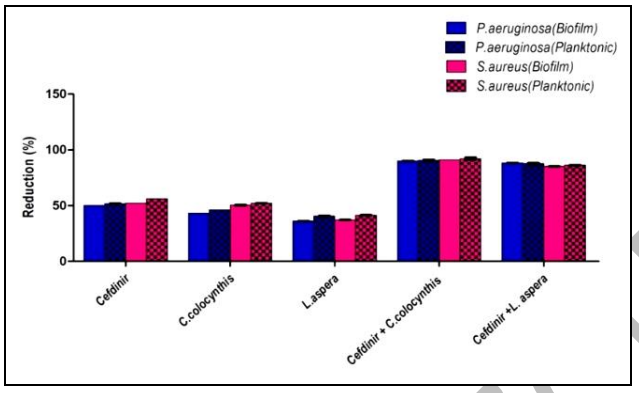

Figure 5: Influence of antibiotic-adjuvant on biofilm and planktonic cells reduction in 50 h.

52% at 50 h. But the combination of cefdinir-*C. colocynthis* inhibited *S. aureus* biofilm 95% and 91% at 16 h and 50 h, respectively. *L. aspera* reduced 37% and 37% of biofilm formed by *S. aureus* at 16 h and 50 h, respectively. When cefdinir was used with *L. aspera* extract, 95% and 85% of biofilm formed by *S. aureus* was reduced at 16 h and 50 h, respectively.

The results confirmed that the bioactive compounds in the plant functions as an adjuvant by improving the efficiency of cefdinir (Grassi *et al*., 2017). The plant might have compounds that inhibited β-lactamase or efflux pump inhibitors that are responsible for the resistance of pathogens to cefdinir. The antibiotic-adjuvant potentiated the antibiotic and reversed the resistance (Wright, 2016). Bacterial biofilm are resistant to antibiotics and are comparatively susceptible to antibiotic-adjuvant combination. There is a possibility of phytochemicals increasing the permeability of bacterial cell to antibiotics that result in reduction in biofilm and planktonic cells. The data represented in Figure 2a and 2b also supported the reduction in the bacterial load. The plant extracts that exhibited quorum quenching activity which reduced the multiplication of bacteria. Biofilm formation is favored by the extensive communication among bacteria through quorum sensing. The antibiotic-adjuvant reduced the planktonic cells and biofilm by targeting multiple points. Quorum sensing is one of the virulent factors that facilitate the formation of biofilm. Blocking quorum sensing and increased permeability of antibiotic has resulted in the reduction of biofilm and planktonic cells.

Phytochemical screening of plants

The phytochemicals present in the selected plants are represented in Table 3. The table clearly reveals that flavonoids are present in all the four plants which play a promising role as an antibiotic adjuvant. Alkaloids are present in the aqueous extract of *K. pinnata* and *A. malabarica.* The role of flavonoids as an adjuvant was reported by Abreu *et al*. (2017). In the present study, it is also confirmed that flavonoids are present in aqueous fraction that served as an adjuvant. Further studies are needed to purify and characterize the specific flavonoids from the plant extracts that possess adjuvant activity to antibiotics.

CONCLUSION

The study demonstrates the aqueous plant extracts of *C. colocynthis* and *L. aspera* in combination with cefdinir possessed ability to reverse the resistance of *S. aureus* and *P. aeruginosa* to cefdinir and potent antibiofilm property towards clinical isolates of *S. aureus* and *P. aeruginosa*. Therefore, the present study reveals the phytochemicals from *C. colocynthis* and *L. aspera* plant extracts can be used as an antibiotic adjuvant to improve the antibacterial and antibiofilm activities of cefdinir against *S. aureus* and *P. aeruginosa*.

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