International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 8, Issue 11, 2016

Original Article

COMBINATORIAL EFFECT OF D-AMINOACIDS AND TETRACYCLINE AGAINST *PSEUDOMONAS AERUGINOSA* BIOFILM

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Received: 16 Oct 2015 Revised and Accepted: 21 Sep 2016

ABSTRACT

Objective: The present study attempted to evaluate the anti-biofilm activity of D-amino acids (D-AAs) on *Pseudomonas aeruginosa* and determine if the combination of D-AAs with tetracycline enhances the anti-biofilm activity *in vitro* and *ex vivo*.

Methods: Different D-AAs were tested for antibiofilm activity against wild type *P. aeruginosa* PAO1 and two multidrug resistant *P. aeruginosa* clinical strains in the presence of sub inhibitory concentrations of tetracycline using crystal violet microtitre plate assay. Results were further validated using *in vitro* wound dressing and *ex vivo* porcine skin models followed by cytotoxicity and hemocompatibility studies.

Results: D-tryptophan (5 mmol) showed 61 % reduction in biofilm formation of *P. aeruginosa*. Interestingly combinatorial effect of 5 mmol D-tryptophan and 0.5 minimum inhibitory concentration (MIC) (7.5µg/ml) tetracycline showed 90% reduction in biofilm formation. 5 mmol D-methionine shows 28 % reduction and combination with tetracycline shows 41% reduction in biofilm formation of *P. aeruginosa*. D-leucine and D-tyrosine alone or in combination with tetracycline did not show significant anti-biofilm activity. D tryptophan-tetracycline combination could reduce 80 % and 77 % reduction in biofilm formation in two multi drug resistant *P. aeruginosa* clinical strains. D-tryptophan-tetracycline-combination could also reduce 76% and 66% reduction in biofilm formation in wound dressing model and porcine skin explant respectively. The cytotoxicity and hemocompatibility studies did not show significant toxicity when this combination was used.

Conclusion: The results established the potential therapeutic application of D-tryptophan alone or in combination with tetracycline for treating biofilm associated clinical problems caused by *P. aeruginosa.*

Keywords: D-amino acid, Tetracycline, Biofilm, *P. aeruginosa*

DOI: http://dx.doi.org/10.22159/ijpps.2016v8i11.9531

INTRODUCTION

Many bacteria form biofilm, which is an assemblage of surface-associated microbial cells enclosed in an extracellular polymeric substance matrix. Biofilm, which consists of polysaccharide, protein and DNA [1] has high significance in agricultural, industrial, environmental and clinical settings. The soil bacterium, *Bacillus subtilis* forms biofilm on the roots to protect plants from variety of pathogens [2]. The cause of most of the persistent infections is biofilm formation, as these groups can tolerate very high doses of antibiotics that would kill planktonic cells. *P. aeruginosa,* the most predominant pathogen in immuno-compromised patients [3, 4] forms biofilm virtually on any surface at any nutritional or environmental conditions. The ability to form biofilm is thought to be one of its main survival strategies when infecting a host and it is considered to be important for pathogenesis [1].

Classically biofilm development can be divided into three events, attachment, maturation and dispersion [5, 6]. The final event contributes to biological dispersal, bacterial survival, and disease transmission. It provides a mechanism whereby biofilm bacteria can spread throughout an infected organ [7] or to the whole body, in which a chronic infection can cause an acute blood stream infection [8-10]. Bacterial detachment and dispersal are triggered by various chemical signals [7]. Some of them are D-AAs, lysostaphin, farnesol, and cis-2-decenoic acid [11]. Given that many bacteria produce D-AAs, these molecules may provide a general strategy for biofilm disassembly [12]. Combining biofilm dispersal and inhibition agents with antibiotics can greatly enhance the susceptibility of bacterial biofilm to antibiotic therapy [13]. D-AAs such as D-tryptophan reported to have inhibitory effect on biofilm development by food borne organism *Cronobacter sakazakii*.[14]. Similarly combination of D-tyrosine with tetrakishydroxymethyl phosphonium sulphate has the ability to mitigate sulfur reducing bacterial biofilm[15]. In addition to biofilm inhibitory properties D-AAs also have other properties. Administration of D-methionine after noise exposure leads to rescue from noise induced auditory brain stem response threshold shift [16]. D-leucine has potential anti-seizure properties. [17]. In the present study we evaluated the combinatorial effect of D-AAs with antibiotics. The antibiofilm activities of the tetracycline and D-AAs combination against *P. aeruginosa* strains were tested.

MATERIALS AND METHODS

Bacterial strains and treatments

P. aeruginosa wild strain, PAO1 (*P. aeruginosa* ATCC 15692) obtained from ATCC and two multidrug resistant clinical strains PACL-W (*P. aeruginosa* clinical strain-surgical wound swab(leg) and PACL-S (*P. aeruginosa* clinical strain-sputum) were treated with amino acids, D-leucine, D-tryptophan, D-methionine and D-tyrosine (Sigma Aldrich, USA). All the other aminoacids except D-tyrosine was dissolved in distilled water and D-tyrosine was dissolved in 1N HCl.

Antibiotic sensitivity of bacterial strains was assessed by disc diffusion method. Antibiotic discs (Himedia India) were placed on the surface of Müeller Hinton agar plates (Himedia, India). Subsequent to overnight incubation at 37 °C, growth inhibition zones formed around the disks were measured. The minimum inhibitory concentration (MIC) was evaluated by broth dilution method. Briefly, bacterial suspensions were prepared in 0.9% saline from overnight cultures and adjusted to attain the 0.5 McFarland turbidity standards. Suspensions were inoculated onto Müeller-Hinton broth containing antibiotics at concentrations 5-50 µg/ml and incubated at 37 °C for 20 to 24 h [18].

Determination of biofilm formation

Biofilm formation was assessed by microtiter plate method [19]. Briefly, overnight bacterial cultures were diluted to 0.1 OD at 600

nm.100 µl of diluted sample was incubated with D-AAs and 0.5 MIC tetracycline combination in culture medium (LB broth) at 37 °C for 24 h in 96-well polystyrene micro titer flat bottom plate (Nest Biotech Co. Ltd). After incubation, the medium was discarded; wells were washed with water and incubated with crystal violet (0.1%) for 10 min. Subsequently the plates were washed with water to remove the unbound dye. The crystal violet bound to the biofilm was eluted using 33% acetic acid and optical density (OD) was recorded at 600 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT). Percentage inhibition of biofilm formation was calculated using the following formula

$$\% \text{ inhibition} = \frac{\text{OD of Control} - \text{OD of test}}{\text{OD of Control}} * 100$$

Cell viability assay

Cell viability assay was performed according to the protocol described earlier [20]. 3T3-L1 mouse fibroblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % foetal bovine serum (FBS) (v/v), 1% penicillin, 1% streptomycin and 0.1% amphotericin B. Cell viability assays were performed for cells treated with/without D-tryptophan in DMEM by using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT). 3T3-L1 cells were seeded at a density of 7500 cells/well in a 96-well microtiter plate and incubated overnight. Cells were treated with and without D-Tryptophan at a concentration of 5 mmol and 10 mmol in DMEM for 24 h. 20 µl of 5 mg/ml MTT was added to each well and incubated for 3 h at 37 °C. The media was removed after incubation and 100 µl of MTT solvent (4 mmol HCl and 0.1% Nonidet P-40 in isopropanol) was added for solubilisation. After shaking briefly for 5 min, the absorbance was read at 590 nm with a reference filter of 620 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT).

Determination of hemolysis

Extent of hemolysis was determined according to previously described method [21]. Briefly 1 ml blood was diluted 10 times with Ringer buffer. 200 µl of diluted blood was incubated with D-tryptophan and tetracycline at 37 °C for 24 h and centrifuged at 3,500 rpm for 15 min. OD of the supernatant was recorded at 540 nm using microplate reader. Triton-X 100 (0.01%) and 0.9 % saline treated blood samples served as positive and negative controls respectively.

***In vitro* wound dressing model**

The *in vitro* wound dressing model was done as previously described [22]. Overnight bacterial cultures were diluted in fresh LB broth to contain 1x108colony forming units (CFU)/ml and placed on sterile polyethylene absorbent wound dressing (1.5 cm x 1.5 cm) in freshly prepared LB agar plates. The plates were kept undisturbed for 30 min while the inoculum was allowed to dry. Fresh gauze squares (2 cm x 2 cm) with D-tryptophan and tetracycline were placed over the inoculated polyethylene pads and the plates were incubated at 37 °C. After 24 h of growth period, the samples were removed from the plates and the polyethylene pads or gauze pieces were separated and processed to determine the biofilm formation. Polyethylene pads were washed with normal saline and vortexed in saline for 1 min. The bacterial suspension was then serially diluted and 1 ml aliquots of each dilution were placed on LB agar plates. After 24 h of incubation at 37 °C, the colony forming units were calculated.

***Ex-vivo* porcine skin model**

Ex-vivo porcine skin model was prepared according to the protocol described earlier [21, 23]. Hides from carcasses of freshly killed adult pigs obtained from a local slaughter house were thoroughly washed and de-haired. The excess fat deposits were removed and cut into sections of 2 cm × 2 cm. Subsequently the skin explants were washed in PBS, disinfected with 70 % ethanol for 5 min and allowed to dry in sterile petri plates. The explants were incubated at 37 °C for 24 h with 100 µl bacterial suspension with/without test compounds. They were submerged in Tryptic soy broth. After incubation; sections were rinsed with sterile PBS to remove the unattached bacterial cells. Subsequently the sections were aseptically transferred to sterile test tubes containing sterile PBS with 5 µl/l Tween 80 and sonicated to liberate bacteria from the biofilm. This suspension was then serially diluted and 100 µl aliquots of each dilution were spread on Tryptic Soy agar (TSA). After overnight incubation at 37 °C, the colonies were counted and the colony forming unit (CFU) was calculated.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Values are expressed as mean±SD. Dunnett-ANOVA test was employed to compare between the test and control. Significance at **p*<0.05 Vs control, ***p*<0.01 Vs control, ****p*<0.001. Vs control

RESULTS

Antibiotic susceptibility and minimum inhibitory concentration (MIC)

Antibiotic susceptibility was tested using ciprofloxacin, ceftazidime, amikacin, co-trimoxazole, gentamycin, streptomycin, chloramphenicol and tetracycline. All the three *P. aeruginosa* strains were susceptible to tetracycline. The lowest concentration of tetracycline which inhibits the visible growth of bacterium i.e. the MIC value was found to be 15µg/ml. Hence sub inhibitory concentration (0.5MIC) i.e. 7.5 µg/ml of tetracycline was selected for further experiments. The results of MIC of tetracycline is shown in fig. 1.

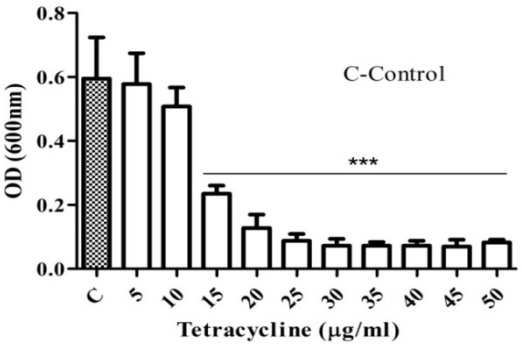
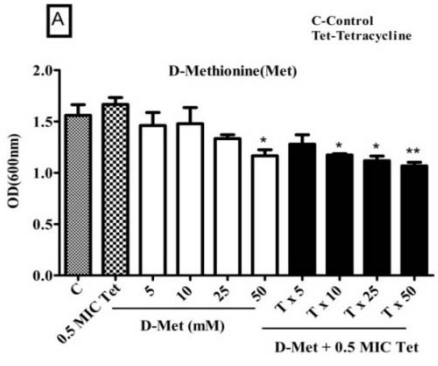


Fig. 1: Minimum inhibitory concentration (MIC) of tetracycline. MIC of tetracycline against *P. aeruginosa* was determined at a concentration range from 5-50 µg/ml. Mean values of triplicate independent experiments and SD is shown. Dunnett's test demonstrates significant difference between the tests and the control. Significance at ***p<0.001

Dose dependent effect of D-AAs and D-AAs-tetracycline combination on biofilm formation of *P. aeruginosa*

To evaluate the potential of D-AAs and a combination of D-AAs with tetracycline on antibiofilm activity against *P. aeruginosa* PAO1, different doses of D-AAs ranging from 5-50 mmol in combination with 0.5 MIC tetracycline (7.5µg/ml) were selected for testing.(fig. 2 (A). As shown in fig. 2 (B) among the four D-AAs tested D-tryptophan showed maximum antibiofilm activity at 5 mmol (61%), while D-tryptophan-tetracycline treatment showed 90% inhibition. 5 mmol D-methionine showed 28 % inhibition and combination with tetracycline augmented the effect to 41%. In contrast, D-leucine and D-tyrosine had minimal antibiofilm activity even at a concentration of 50 mmol.



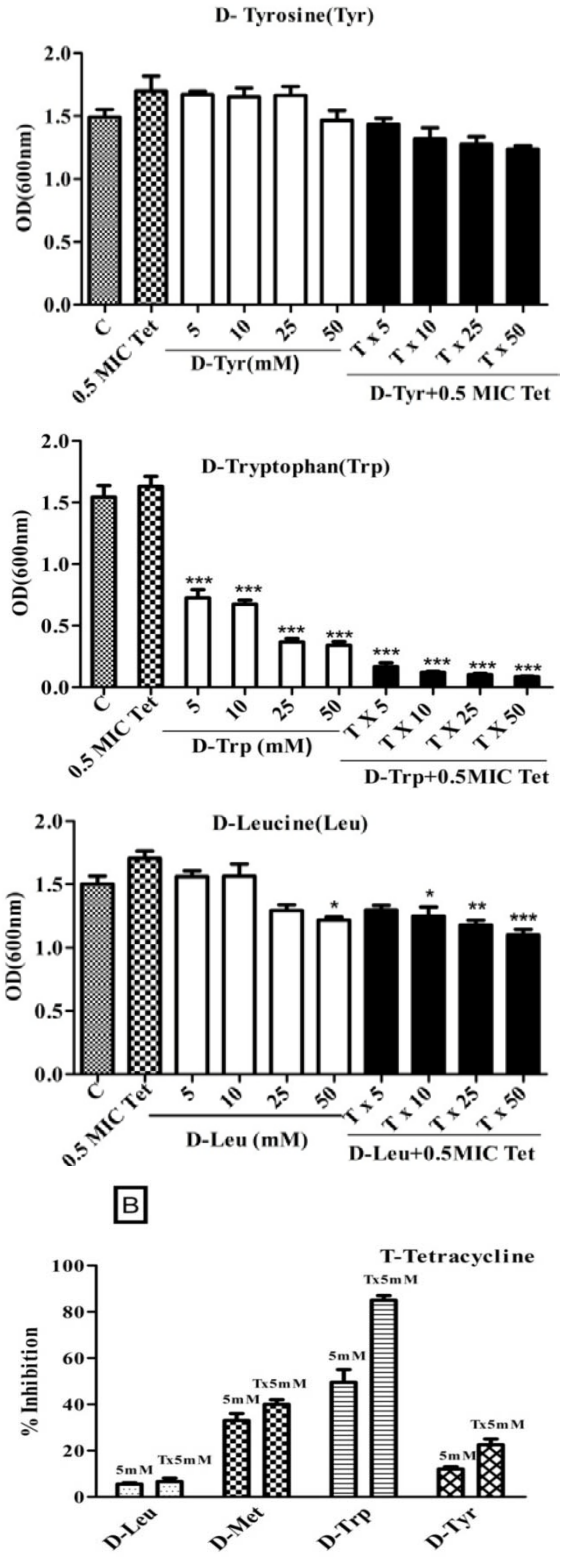


Fig. 2: (A). Dose-dependent effect of D-AAs against *P. aeruginosa* biofilm formation. Screening of antibiofilm activity of individual D-AAs, D-methionine, D-tyrosine, D-tryptophan and D-leucine at concentrations ranging from 5 mmol to 50 mmol and its combination with 0.5 MIC tetracycline (7.5 µg/ml) (Tet). Mean values of triplicate independent experiments and SD are shown. Dunnett's test demonstrates significant difference between the tests and the control. Significance at *p<0.05, **p<0.01, ***p<0.001, (B). Percentage inhibition of biofilm formation by *P. aeruginosa* PAO1 with different D-aminoacids (5 mmol) and its combination with 0.5 MIC (7.5 µg/ml) tetracycline (T)

D-tryptophan-tetracycline combination disperses the biofilm formed by clinical isolates of *P. aeruginosa*

Treatment with 5 mmol D-tryptophan-0.5 MIC tetracycline combination on multi-drug resistant *P. aeruginosa* strains PACL-S (*P. aeruginosa* clinical strain-sputum) and PACL-W **(***P. aeruginosa* clinical strain-leg wound swab) demonstrated significant anti biofilm activity. This activity of D-AAs against biofilm was partly strain dependent. Treatment with 5 mmol tryptophan inhibited biofilm formation in PACLW and PACLS by 51% and 50.5 % respectively, while treatment with D-tryptophan and tetracycline in combination augmented this effect by 80 % and 77 % respectively in PACLW and PACLS (fig. 3).

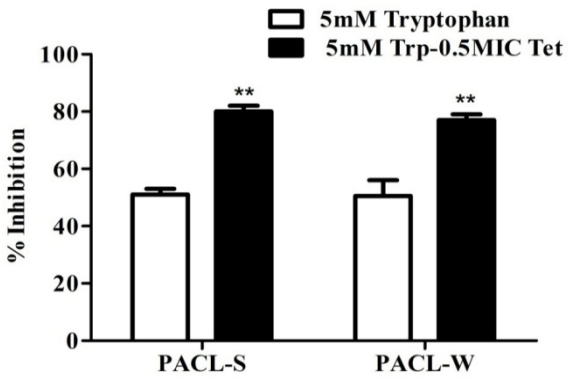


Fig. 3: Percentage inhibition of the biofilm formed by clinical strains of *P. aeruginosa*. *P. aeruginosa* strains isolated from sputum (PACL-S) and wound (PACL-W) treated with 5 mmol tryptophan (Trp) alone or in combination with 0.5 MIC tetracycline (Tet). Mean values of triplicate independent experiments and SD are shown

***In vitro* wound dressing model and *ex-vivo* porcine skin model**

To better understand anti-biofilm activity of D-tryptophan-tetracycline combination in other biofilm models, we used *in vitro* wound dressing and *ex-vivo* porcine skin models. In wound dressing model, 5 mmol D-tryptophan in combination with 0.5 MIC tetracycline reduced biofilm formation (76%) when compared to controls (fig. 4).

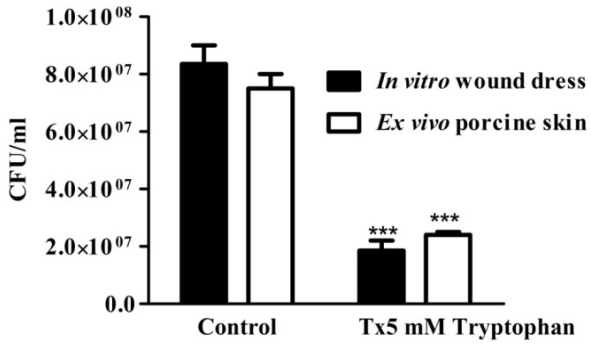


Fig. 4: Effect of D-tryptophan and 0.5 MIC tetracycline combination on the biofilm formed by *P. aeruginosa* using *in vitro* wound dress model and *ex vivo* porcine skin model. Wound dress/porcine skin inoculated with bacterial cultures were treated with 5 mmol tryptophan and 0.5 MIC tetracycline. Planktonic bacteria were removed and the bacteria present in the biofilm were detected by plated into LB agar plates. Mean values of triplicate independent experiments and SD are shown

Further, the anti-biofilm activity of 5 mmol D-tryptophan in combination with 0.5 MIC tetracycline was tested using *ex-vivo* porcine skin model. Consistent with the previous result, D-

tryptophan in combination with tetracycline inhibited biofilm formation in porcine skin explants by 66% compared to the untreated explants (fig. 4). These results confirm the efficacy of D-tryptophan-tetracycline combination in inhibiting biofilm formation.

Cell viability assay

To investigate the effect of D-tryptophan on viability of cells, MTT assay was performed.3T3-L1 fibroblast cells were incubated with 5 mmol and 10 mmol D-tryptophan with/without 0.5 MIC tetracycline for 24 h. Results from MTT analysis showed no significant cytotoxicity on treatment when compared to the untreated control (fig. 5).

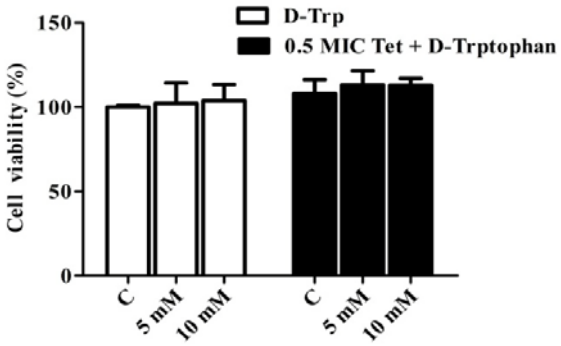


Fig. 5: Effect of D-tryptophan in combination with 0.5 MIC tetracycline on cell viability of 3T3-L1 fibroblast cells. Cell viability using MTT cytotoxicity assay was performed for the 3T3-L1 cells treated with D-tryptophan in combination with 0.5 MIC tetracycline in DMEM supplemented with 10% FBS. Mean values of triplicate independent experiments and SD are shown. Dunnett's test demonstrates no significant difference between the test and the control

Hemocompatibility testing of D-tryptophan in combination with tetracycline

The hemocompatibility of 5 mmol D-tryptophan-0.5 MIC tetracycline mixture was analyzed. No significant hemolysis was observed when this combination was used (fig. 6).

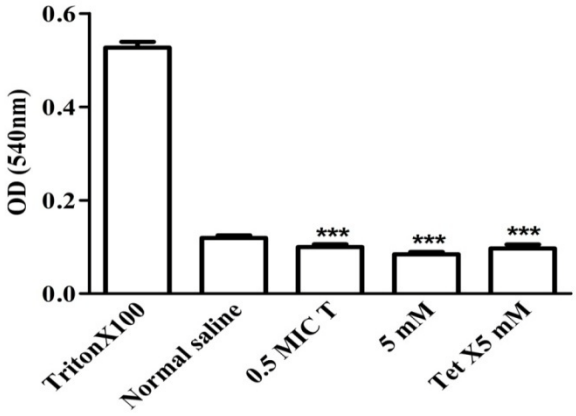


Fig. 6: Hemocompatibility of D-Tryptophan in combination with 0.5 MIC tetracycline (Tet). A representative bar diagram showing hemolysis by Triton X 100, normal saline, 0.5 MIC tetracycline, 5 mmol tryptophan and 5 mmol tryptophan-0.5 MIC tetracycline combination. Mean values of triplicate independent experiments and SD are shown. Dunnett's test establishes significant difference between the test and the control. Significance at ***p<0.001 Vs control

DISCUSSION

Most of the commercially available antibiotics are developed against planktonic and metabolically active bacteria. However, bacteria in the biofilm are different and can be 10-1,000 times more resistant to antibiotics than planktonic bacteria [24]. Designing an anti-biofilm strategy is complicated due to the characteristic properties possessed by biofilm [25] that include protective shielding by the matrix, specific expression of microbial proteins for nutrient scavenging, ramping down of metabolic activity, induction of a secluded state in which bacterial cells are in a phenotypically variable state and resistant to antimicrobial treatment [26, 27].

Recent studies have pointed out the possible use of antibiofilm/biofilm dispersing agents like D-AAs as potential therapeutic molecules [12]. Combining these agents with antibiotics can have significant promising effects. In *Bacillus subtilis,* a mixture of D-AAs (D-leucine, D-methionine, D-tyrosine and D-tryptophan) is reported to prevent biofilm formation and induce existing biofilm disassembly [11]. Previous studies have reported anti-biofilm activities of D-AAs in *Staphylococcus aureus* [12] and *P. aeruginosa* [13]. These results indicate that D-AAs might prove widely useful in medical and industrial applications for the prevention or eradication of biofilm.

Consistent with previous studies [13], D-tryptophan showed highest antibiofilm activity followed by D-methionine, while marginal activity was observed in the case of D-leucine and D-tyrosine. A dose dependent antibiofilm activity was seen in the presence of D-tryptophan. Although tetracycline alone did not show any significant activity against biofilm at 0.5 MIC, combination of tetracycline and D-AAs showed enhanced antibiofilm activity. We also observed that co-treatment of D-tryptophan-tetracycline had promising antibiofilm effect compared to other combinations. Our results are in line with previous studies that have demonstrated that the activity of various antimicrobial agents can be significantly enhanced by the addition of dispersal agents [28].

As a model designed to assess the efficacy of topical application of tetracycline and D-tryptophan combination, gauze squares saturated with this combination was checked for their antibiofilm activity. The decreased bacterial load recovered from the dressing containing 5 mmol D-tryptophan-0.5 MIC tetracycline mixture when compared to the untreated control supported the observations from the biofilm tests done in microtitre plate. Further, the antibiofilm activity of D-tryptophan-tetracycline combination was validated using porcine skin infection model. Porcine skin model for bacterial biofilm attachment was selected because it fulfills the requirements of an *ex vivo* model due to its similarity with human skin physiology. The other advantage of using such a model is that a large number of trials can be conducted economically [23]. Our study clearly elucidated that a single application of 5 mmol D-tryptophan-0.5 MIC tetracycline combination was capable of reducing *P. aeruginosa* biofilm attachment to porcine skin explants. Cell viability assay performed in 3T3-L1 fibroblast cells showed that 5 mmol and 10 mmol D tryptophan in combination with 0.5 MIC tetracycline did not have any cytotoxic effect. The results of hemocompatibility studies were also in strong agreement with the cytotoxicity studies suggesting the potential of D-tryptophan-tetracycline combination as an effective therapy against *P. aeruginosa* infections.

The mechanism through which the D-AAs and its combination with antimicrobials act against biofilm have not been fully addressed yet. It is possible that D-AAs cause altered protein expression, disrupt cell wall integrity and metabolic status. A recent study reported that epigallocatechin gallate,the major catechin present in green tea extract enhances tetracycline activity against resistant staphylococcal isolates by impairment of tetracycline efflux pump activity thereby increasing intracellular retention of the drug leading to effective synergistic drug combination [29, 30]. The results obtained demonstrate that antibiotic treatment with D-AAs could be more effective to reduce infections with minimal systemic toxicity. Moreover antibiotic concentrations required could be reduced by co-delivery with D-AAs, by which antibiotic toxicity can potentially reduced.

CONCLUSION

In conclusion, we have demonstrated *in vitro* anti-biofilm activity by D-AAs in *P. aeruginosa*. Anti-biofilm activity by individual amino

acids was dose dependent, and amino acids in combination with tetracycline potentiated the anti-biofilm activity against diverse isolates of *P. aeruginosa*. The *in vitro* studies were validated using *ex vivo* porcine skin model. This combination may find commercial advantages in topical applications and treatment of infections caused by *P. aeruginosa.*

ACKNOWLEGMENT

The authors graciously acknowledge the chancellor Sri Mata Amritanandamayi for the constant motivation. The authors would like to thank the BRITE initiative at Amrita School of Biotechnology for the financial support.

CONFLICT OF INTERESTS

Declared none

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How to cite this article

- H Jayalekshmi, C Harikrishnan, Sajin Sali, N Kaushik, Norin Mary G Victus, R Anoop, TM Sarath, O Athira, Geetha B Kumar, Bipin Nair. Combinatorial effect of d-aminoacids and tetracycline against *Pseudomonas aeruginosa* biofilm. Int J Pharm Pharm Sci 2016;8(11):216-220.