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ISOLATION AND PURIFICATION OF ANTIBIOFILM PEPTIDES FROM *TRIDAX PROCUMBENS*, A MEDICINAL PLANT

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ABSTRACT

Small peptides are being used as therapeutics in organ transplants and bacterial infections. The objective of present study was aimed to purify and evaluate antibiofilm activity of peptides from the Tridax procumbens. The aerial parts of plant material (leaf, stems and root) was collected, cleaned, shade dried and powered. The powered samples were extracted separately with acetonitrile and formic acetic acid (50:1). The extracted small peptides were partially purified by DEAE cellulose column and then concentrated by lyophilizer. The lyophilized samples were subjected to HPLC for purification. Crude and purified small peptides were analyzed by SDS-PAGE and molecular weight ascertained. Small peptides were evaluated for its biofilm inhibition of Staphylococcus aureus. The extracted small peptide yield was found to be 2%. The quantitative analysis of crude small peptides and purified was found to be 120 and 43 µg/ml respectively. The molecular weight of purified peptides was calculated and was found 4KDA. Small ppeptides exhibited biofilm inhibition of 88.49±0.65% at 100µg/mL and also showed proteolytic and exopolysaccharides at 100µg/mL against S. aureus. In conclusion, the current study showed T. procumbens is a good source for small peptides, acts as a potent biofilm inhibitor. To the best of our knowledge this is the first report on isolation, purification and evaluation of biofilm inhibition of peptides form T. procumbens.

KEY WORDS

Peptides, Tridax procumbens, DEAE cellulose, HPLC

INTRODUCTION

Small peptides (SPs) are having approximately 30 amino acids and having crucial roles in human physiology due to its short circulating plasma half-life in human body [1]. Most characteristic of SPs are known by gene sequence instead of amino acid sequence [2]. Compared to other small synthetic molecules, SPs possesses less toxicity and would not accumulate in organs [3]. The SPs are present in numerous species, ranging from plants and bacteria to mammals. These unique properties of the SPs became an attracting target for drug discovery. SPs have biological properties such as anti-microbial, anti-viral, haemolytic, immuno suppressor and anticancer [4].

Naturally occurring SP's (thionins, defensis and cyclotides) are present in numerous species, ranging from plants and bacteria to mammals [5]. Among all the species, plants are the largest producers of SP expressed in multiple tissues like flowers, leaf and seeds. In plants these peptides play a major role in host defensive system. Thus, the plants species are ideal target for the isolation of SP and evaluation of their biological activities [6]. *Tridax procubemns* is a medicinal plant having various pharmacological activities like immunomodulatory, anti-oxidant, anti-inflammatory, analgesic and antimicrobial [7]. In this scenario, the present study was aimed to isolate and purify the SPs from *T. procubemns* and evaluate its efficacy on biofilm inhibition of *Staphylococcus aureus*.

MATRERILS AND METHODS

Extraction of SPs from T. procumbens

The plant material (leaf, stems and root) was collected from Jawaharlal Nehru Technological University, Hyderabad, Telangana, India. For standardization of extraction procedure, the powdered samples were extracted with various solvents with different ratios and different concentrations (powered sample and solvents) by using percolation method. The powdered samples were extracted with acetonitrile and formic acetic acid (50:1) at 1:1 (w/v) concentrations using percolation method. The extract was filtered through Whatman No.1 filter paper and the filtrate was concentrated by lyophilizer and stored for further use [8]

Purification of SPs

Ammonium sulphate [$(NH_4)_2SO_4$)] fractionation

For concentration of SPs, various concentrations of $(NH_4)_2SO_4$ were slowly added to the extracted peptides. First it was concentrated to 0 - 15%, incubated overnight at 4ºC, the next day it was centrifuged at 6000 rpm for 10 min at 4°C, pellet was dissolved in 0.1M Tris-HCL pH-7.5 and upon assay activity was found to be very low. The pellet was discarded, and the 0-10% saturated supernatant was recovered and concentrated to 15-30% of $(NH_4)_2SO_4$, incubated overnight at 4ºC, the following day it was centrifuged at 6000 rpm for 10 min at 4°c and the pellet obtained was dissolved in 0.1M Tris-HCl pH -7.5 [9].

DEAE cellulose column chromatography

Ammonium sulphate fractionated peptides were subjected into the DEAE cellulose column and fractions were collected by passing 0.1M Tris-HCl pH-7.5. In the meantime, the absorbance spectra of each fraction was measured at 280 nm and the absorbance reached to 0.01 less than that stop passing the 0.1M Tris-HCl P^H7.5. To the column the elution buffer was passed in increasing concentrations of 25mM to 100mM, the fractions were collected, and the absorbance was measured. Once the absorbance of fraction was reached to 0.01 or less than that stop passing and started with next buffer, until eluted the proteins with all the buffers. The eluted peptide samples were lyophilized, and the peptide concentration was determined [10].

Reverse Phase –High Pressure Liquid Chromatography (RP-HPLC)

The partially purified peptides in DEAE cellulose column were subjected to RP-HPLC for further purification and identification. Briefly, a SOURCE 30RPC column was used for hydrophobic peptide purification in BioRad HPLC purification system. Once the column was settled, the column was washed with 5 column volumes of Milli-Q water containing TFA 0.05% at a flow rate of 2mL/min and equilibrated. The sample peptides were injected through 10 mL static loop in to the column at 0.5 mL/min flow rate. The unbound proteins were washed off with 2 column volumes Milli-Q water containing TFA 0.05% at a flow rate of 2 mL/min. After which the bound peptides were eluted with a gradient of 1% – 100% of Buffer-B containing (90% of ACN + TFA 0.05%) at a flow rate of 2mL/min. Two mL fractions were collected, and the eluted protein peaks were monitored at 280nm by UV-Visible detector and protein concentration was determined [11].

SDS-PAGE analysis

The crude and purified peptides were subjected to SDS-PAGE (16% resolving gel and 5% stacking gel) along with protein marker. Electrophoresis was performed with running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) at a constant voltage of 40V until the tracking dye reached to separation gel and then increased to 70V until the tracking dye reached to the end of the separating gel. The gel was stained with Coomassie brilliant blue and the image was obtained with gel dock (LAS 3000, Fujifilm). The Rf values were calculated and the molecular weight of peptides were determined [12].

Inhibition of Staphylococcus aureus biofilm formation and in situ microscopic observation

Biofilm inhibition of purified SP was assessed on *S. aureus* by using 96 well microtitre plates. Briefly, *S. aureus* was incubated in 96 well plates in the presence and absence of SP and the culture was allowed to adhere and grow without any agitation for 16 h at 37°C. After incubation, the media was discarded to remove free-floating planktonic cells and the wells were gently rinsed twice with sterile water. The wells having biofilm were stained with 0.2% crystal violet (CV) solution. After 15 min of incubation, the CV solution was discarded completely, and wells were filled with 95% ethanol to solubilize CV from the stained cells. The biofilm biomass was quantified by measuring the absorbance at 650 nm [13].

In situ microscopic observation of bacterial biofilm was carried out by incubating *S. aureus* overnight in 6 well plates having cover glass of one cm^2 along with and without SP. After

16hrs of incubation, the cover glasses were rinsed three times with distilled water to remove the free planktonic cells. The adhered biofilms were stained with 0.2% CV solution placed on glass slides and observed under light microscope [13].

Extraction and quantification of exopolysaccharides (EPS)

S. aureus was grown in presence and absence of SP in 6 well plates and incubated for 16hrs at 37°C to adhere the bacteria without any agitation. The incubated culture was centrifuged for cell pellets and was suspended in 50 mL of high-salt buffer (10 mM KPO_4, pH 7.5 mM NaCl, 2.5 mM $MgSO_4$). The cells were removed by centrifugation for 10 min at 5,000 rpm, 4°C. To the supernatant, three volumes of absolute ethanol were added to precipitate the EPS. The resulting precipitate was resuspended in 3 mL of Milli-Q water and stored at -20ºC. The extracted EPS was quantified using phenol-sulphuric acid method and absorbance was measured at 490 nm [14].

Proteolytic activity

S. aureus was grown in presence and absence of SP in 6 well plates and incubated for 16 h at 37°C to adhere the bacteria without any agitation. Proteolytic activity was determined using azocasein as the substrate. 150 µL of both treated and untreated culture supernatants were added to 1mL of 0.3% azocasein in 0.05 M Tris- HCl and 0.5 mM $CaCl_2$ (pH 7.5) and were incubated for 15 min at 37°C. The reaction was stopped by the addition of TCA (l0%, 0.5 mL) followed by centrifugation for 10 min at 5,000 rpm, 4°C and the absorbance was measured at 400 nm [15].

Statistical analysis

All experiments were performed in triplicate. Data of each experiment showed the mean ± SE.

RESULTS AND DISCUSSION

Optimization of SPs extraction

For extraction optimization various solvents with different ratios and different concentrations were tried for effective extraction. The solvent systems such as chloroform, methanol, dichloromethane, acetonitrile and formic acid were used in different ratios. Among all the solvents 50% acetonitrile and 1% formic acid in the ratio of 1:1 yielded highest amount of peptides. Acetonitrile being water-miscible serves as a solvent for extracting peptides of medium polarity where water attracts more polar peptides. The formic acid functions as a pH modifier that helps to solubilize peptides and helps to precipitation of large proteins. Along with the solvent optimization the concentration of powered sample and the volume of solvent also were optimised. The maximum yield of peptides was obtained in the ratio of 1:1 (w/v) of powered sample and solvent.

Purification of SPs

The extracted SP showed precipitation at 15-30% of $(NH_4)_2$ SO_4 and the slats were removed by dialysis. Further the SP was purified with DEAE cellulose column chromatography and the eluents were subjected to RP-HPLC. Figure 1 showed the HPLC chromatogram of SP showed single and prominent peak, which indicates purification the obtained SP was pure. The quantitative analysis of SP crude, Ammonium sulphate fractionation, DEAE cellulose and RP-HPLC purified were found to be 120, 80, 65 and 43 µg/mL respectively. The molecular weight of purified peptides was calculated and was found 4KDA (Figure 2).

Antibiofilm activity of SPs

Bacterial biofilm, the matrix of extracellular polymeric substance (EPS), serves as a protective function by reducing antibiotic efficiency and the host immune response. Bacterial behaviour within biofilms is regulated by the phenomenon called quorum sensing (QS). QS, a population density dependent mechanism present in many bacteria, is mediated through small signal molecules called autoinducers that regulate the target gene expression responsible for the phenotypes essential to pathogenicity/symbiosis [16]. Biofilms have a negative effect on wound healing as evidenced by their ability to induce keratinocyte apoptosis and inhibition of re-epithelialization. Chronic infections have been reported a global health problem, at present millions of peoples die annually [17]. In the current study decrease in biofilm formation was observed on *S. aureus* when grown in the presence of SP, the maximum was found to be 88.49±0.65% at 100µg/mL (Figure 3). *In situ* microscopic observation of biofilm formation showed a thick layer of biofilm in control wells (Figure 4). However, SP treated wells exhibited concentration dependent inhibitory activity, which showed a consistent reduction in biofilm formation of *S. aureus*. The SP also exhibited dose dependent inhibition of EPS and proteolytic activity. The maximum inhibition of EPS (76.19±0.58%) and proteolytic activity (78.72±0.49%) found at 100µg/mL (Figure 3).

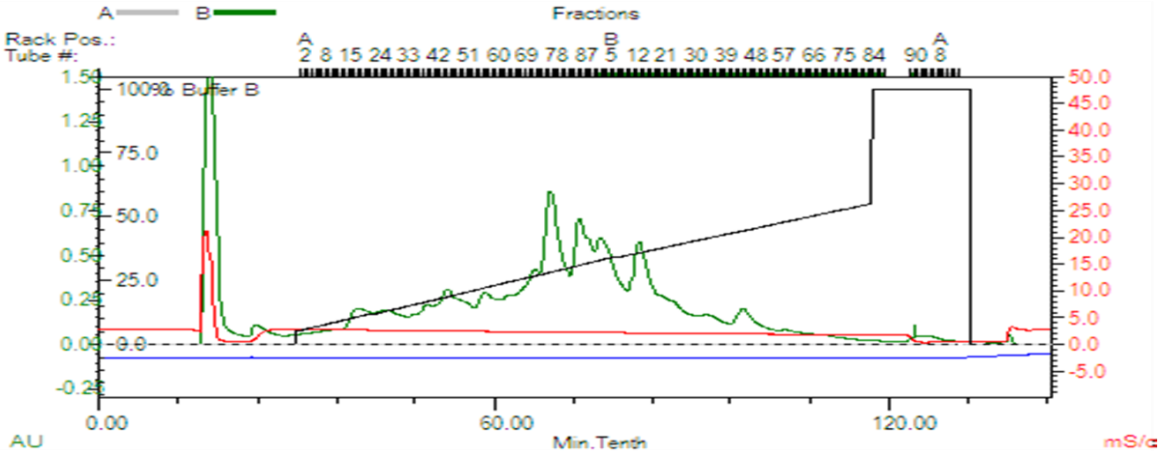

Figure 1: RP- HPLC purification of SPs isolated form *T. procumbens*

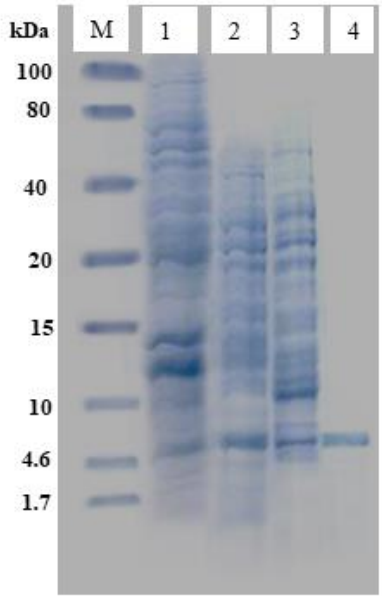

Figure 2: SDS-PAGE analysis of SP isolated from *T. procumbens*

Where – M- Marker; 1- Crude; 2- Ammonium sulphate fractionation; 3- DEAE cellulose; 4- Purified SP

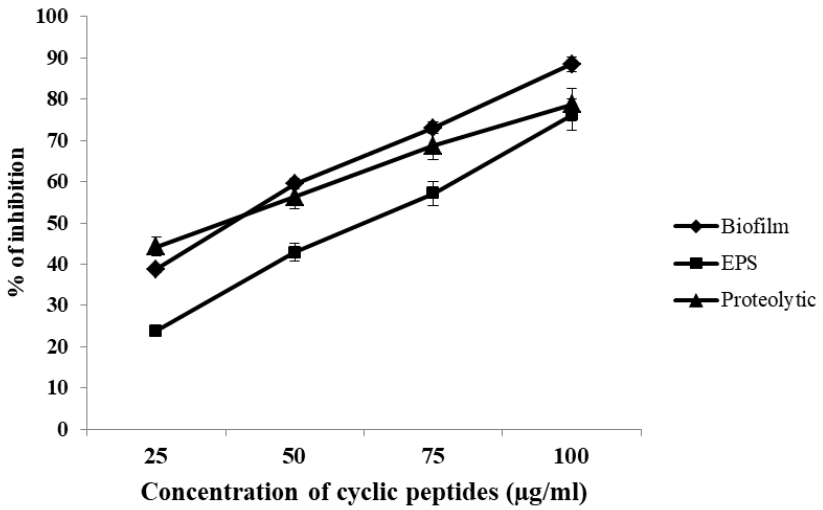

Figure 3: Biofilm, EPS and proteolytic inhibition of SP on *S. aureus*

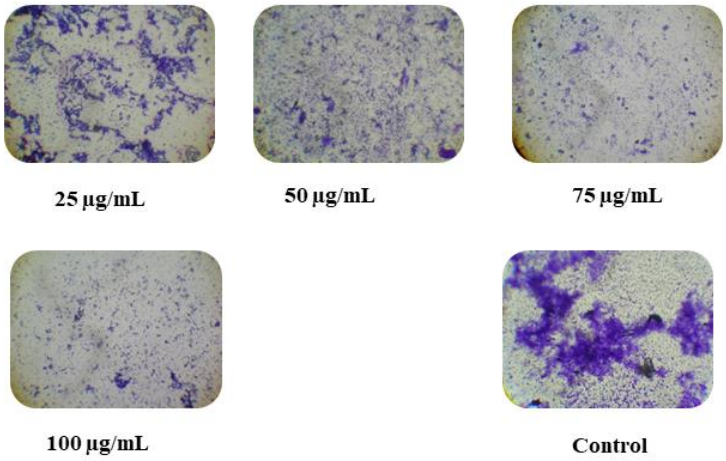

Figure 4: *In situ* microscopic observation of biofilm inhibition of SP on *S. aureus*

CONCLUSION

In conclusion, our results demonstrated the optimization of solvent system is effective for isolation of small peptides. The SP showed potent effect on biofilm inhibition and the mechanism of its activity will be evaluated in future. To the best of our knowledge this is the first report on isolation, purification and studies of its effect on biofilm inhibition of *S. aureus* of SP form *T. procumbens*.

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