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In vitro antibacterial activity of venom protein isolated from sea snake *Enhydrina schistosa* against drug-resistant human pathogenic bacterial strains

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ABSTRACT

Objective: To evaluate the antibacterial activity of sea snake (*Enhydrina schistosa*) venom protein against drug-resistant human pathogenic bacterial strains.
Methods: The venom was collected by milking process from the live specimens of sea snake are using capillary tubes or glass plates. Venom was purified by ion exchange chromatography and it was tested for *in-vitro* antibacterial activity against 10 drug-resistant human pathogenic bacterial strains using the standard disc diffusion method.
Results: The notable antibacterial activity was observed at 150 μg/mL concentration of purified venom and gave its minimum inhibitory concentrations values exhibited between 200-100 μg/mL against all the tested bacterial strains. The maximum zone of inhibition was observed at 16.4 mm against *Salmonella boydii* and the minimum activity was observed at 7.5 mm against *Pseudomonas aeruginosa*. After the sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis there were a clear single band was detected in the gel that corresponding to purified venom protein molecular weight of 44 kDa.
Conclusions: These results suggested that the sea snake venom might be a feasible source for searching potential antibiotics agents against human pathogenic diseases.

1. Introduction

The discovery of penicillin by Alexander Fleming in 1928 changed the world of medicine. Since that time, the challenge between bacteria and antibiotics started and continues[1]. Bacterial infections are among the 10 leading causes of death worldwide according to the World Health Organization[2,3]. The presence and current emergence of drug resistant strains makes the risk of these infections become more threatening as the treatment becomes unreachable. In fact, bacterial resistance to clinical antibiotics has been the major factor responsible for increasing morbidity, mortality and health care costs of bacterial infections[2,3]. Therefore, new antimicrobials or antibacterial archetypes are continuously essential for drug design and development for treatment of infections involving multi drug-resistant microorganisms[2-4].

Snake venoms are the secretion of venomous snakes, which are synthesized and stored in specific areas of their body *i.e.* venom glands. Most of the venoms are complex mixture of a number of protein, polypeptides, enzymes, toxins and non-protein inclusions. They display potential biological activities which may lead to the production of new drugs of potential therapeutic value[5-7]. Snake venoms are a rich source of biologically active substances affecting blood circulation and neurotransmission, including metalloproteinases, peptidases, phospholipase A_2, nucleotidases, nucleases, phosphatases, parkinson's, alzheimer's, and various pain disorders, *etc*[5,8,9].

The capability of some snake venom toxins to cause toxicity is associated with their high specificity and affinity for cell and tissues. In spite of their toxicological effects, several isolated snake venom proteins and peptides have practical applications as pharmaceutical agents[10]. For example, thrombolytic agents have been used in several cases of vascular disorder[11], antimicrobial activity against Gram-positive and Gram-negative bacteria[12,13], antiviral activity against several types of viruses including the herpes simplex virus[14], yellow fever and dengue[15], antiparasitic activity against *Leishmania*[16] and *Plasmodium falciparum*[17], and antifungal activity[18], among other examples.

Sea snakes are the most venomous reptilian group in the world. They are encountered around coast which including reefs, in estuaries, rivers and sea. Sea snake venoms are more toxic than

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those of terrestrial snakes and sea snakes are rarely aggressive or menacing. The sea snakes envenomation are unusual with the advent of modern snaking methods[19], but serious hazard of the marine environment as a sea snake venom contains potent neurotoxin[20]. However, the venom contains purely active compounds such as, anticoagulant and anticancer agent[21].

On the basis of their biological tricks, some of venom proteins are useful for basic studies of hemostasis, thrombosis, pharmacological and clinical applications[22]. All these studies demonstrated the significance of snake venom proteins as valuable tools for basic research, disease diagnosis and drug development. However, most snake venoms remain poorly characterized despite being a rich source of biologically active proteins with therapeutic potential. Thus, this present work was aimed to evaluate the antibacterial activities of sea snake venom against 10 different human pathogens bacterial strains such as *Salmonella typhi* (*S. typhi*), *Shigella flexneri* (*S. flexneri*), *Klebsiella pneumonia* (*K. pneumonia*), *Vibrio cholera* (*V. cholera*), *Vibrio parahaemolyticus* (*V. parahaemolyticus*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Salmonella boydii* (*S. boydii*) (Gram-negative bacteria), as well as *Staphylococcus aureus* (*S. aureus*), *Streptococcus pyogenes* (*S. pyogenes*) (Gram-positive bacteria) strains. We hope that this study might useful to find some alternative source for developing the new lead compounds.

2. Materials and methods

2.1. Collection of venom

Sea snakes [*Enhydrina schistosa* (*E. schistosa*)] were collected from various fishing ports (Cuddalore to Nagapattinam) along the southeast coast of India and were brought to the laboratory in living condition. Live sea snakes were maintained for several weeks and the venom was collected by milking process using capillary tubes or glass plates in weekly intervals. The collected venom was suspended in deionized water (MilliQ) and centrifuged for 15 min at 3 000 r/min, and supernatant was lyophilized and stored at 4 ℃ for further experiments.

2.2. Ion exchange chromatography

Purification of *E. schistosa* venom sample was carried out by column ion exchange chromatography using diethyl-aminoethanol (DEAE)-cellulose (Sigma, India). One hundred milligram of fresh lyophilized crude venom was dissolved in 5 mL of 0.01 mol/L phosphate buffer at pH 6.4, applied to a DEAE-cellulose (1.5 cm × 16 cm) column eluted with the same buffer with a linear gradient of 0-0.5 mol/L NaCl. The column was eluted at the flow rate of 25 mL/h and collected in 4 mL per tube using sterile screw cap test tubes. Totally 70 fractions were assayed for protein content and were pooled according to protein content and used antibacterial activity. The protein concentration of each fraction was simultaneously determined by UV-spectrophotometer (UV-160A, Shimatzu, Japan) at optical density 280 nm. Antibacterial activities were assayed according to the method as indicated below under antimicrobial assay. The active fractions were pooled and concentrated by lyophilisation and the purity was checked by sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Protein quantification

The protein concentration of crude venom and its purified fractions was determined by the method of[23] using bovine serum albumin as a standard.

2.4. Molecular weight determination

The molecular mass of venom protein was determined through SDS-PAGE was performed by following Laemmli[24] method using 10% resolving gel and 4% stacking gel. Venom sample (200 μg) were diluted (1: 1) with sample buffer (50 mmol/L Tris pH 6.8, 2% sodium dodecyl sulfonate, 20% glycerol, 2% 2-mercaptoethanol and 0.04% bromophenol blue) and were then boiled at 100 ℃ for 2 min, shaken for 30 seconds in vortex and loaded onto the gel. After electrophoresis, gel was stained in 0.3% Coomassie brilliant blue R-250 solution followed destaining with 30% methanol, 10% acetic acid and water to reveal protein bands. The molecular size marker, 29-205 kDa (Protein standards, Genei pvt Limited, Bangalore, India), was run parallel with venom samples for molecular weight determination.

2.5. Collection of bacterial strains

The pathogenic strains used in the present study are clinical isolates collected from the Department of Medical Microbiology (Raja Muthiah Medical College and Hospital) Annamalai University, Annamalainagar, Tamilnadu, India. These clinical isolates were identified based on the standard microbiological techniques[25] and drug susceptibility test for each clinical isolate was done following the standard agar disc diffusion method[26]. Ten different bacterial pathogenic strains including Gram positive: (*S. pyogenes*, *S. aureus*) and Gram negative (*S. typhi*, *S. flexneri, K. pneumonia, V. cholera, V. parahaemolyticus, E. coli, P. aeruginosa,* and *S. boydii*) were used to determine the antibacterial activity of sea snake venom.

2.6. Pure culture techniques of bacterial strains

Collected human pathogenic bacterial strains were maintained in the laboratory on nutrient agar (Hi-Media, India) by slant streak technique for further pure cultures[27]. The nutrient agar Hi-Medium composed of 5 g peptone, 2 g beef extract, 5 g sodium chloride and 20 g agar. Agar was dissolved in 1 L of double distilled water and pH was maintained at 7.0 ± 0.2. The mixture of contents was later transferred into a sterile conical flask and plugged with cotton for air tightening. The conical flask with contents was autoclaved and the flasks were cooled and stored at 5 to 10 ℃. Under sterile conditions, the contents when needed were dissolved on heating mantle and 10 mL of medium was poured into sterile test tubes and cooled in laminar air flow by placing in slanting position. The solidified medium was streaked with specific bacterial strains using sterile inoculation loop. The slants with strains were incubated for a period of 24 to 48 h in bacterial incubator at 35 to 37 ℃ and the slants of strains were stored at 4 ℃.

2.7. Maintenance of pure bacterial culture suspension in nutrient broth

Under aseptic conditions, pure colonies of bacterial isolates from slants were picked with an inoculating loop and suspended in 3 to 4 mL of nutrient broth in sterile test tubes and incubated for 24 h at 37 ℃. The contents were transferred into sterile conical flask and plugged with cotton.

2.8. Determination of inimum inhibitory concentration (MIC)

MIC was performed as previously described by Kelman *et al.*[28] with some modification. MIC was measured by determining the smallest amount of test sample or standard antibiotic needed to inhibit the growth of a test microorganism. MIC for bacterial strains was performed using 96-well plate and the assay plates each wells were filled with Mueller-Hinton broth medium containing different concentrations (250, 200, 150, 100 and 50 μg/mL) of

venom samples, positive control (ampicillin), negative controls were maintained simultaneously without venom used for the experiment and the test microorganism 10^8 CFU/mL. After 24 h incubation periods at 37 ˚C, the bacterial growth was measured at 600 nm using Versa MaxTM tunable microplate reader. All tests were carried out in triplicate and the results were averaged.

2.9. Antibacterial activity tests

The agar disc diffusion method[26,29] was employed to test the antibacterial activity of venom of sea snake and standard antibiotics against bacterial pathogens. Inoculum was prepared using fresh cultures of bacterial strains on nutrient agar and a loop full culture was inoculated into a sterile nutrient broth medium and incubated at 37 ˚C for 24 h. The size was adjusted to 0.5 McFarland standard turbidity approximately 10^8 CFU/mL. Bacterial cell suspensions (100 µL of target strain) were introduced into the Muelle-Hinton agar plates and spread thinly on the plates using a sterile cotton swab. The discs of 6 mm diameters were impregnated with 30 µL (150 µg/mL) of venom sample and ampicillin (50 µg/disc) used as positive controls or references. The discs were then placed on inoculated Mueller-Hinton agar plates, which were incubated at 37 ˚C for 24 h under aerobic conditions. Antibacterial activity was expressed in terms of diameter of zone of inhibition was measured in millimeters (mm) using vernier caliper or a scale and recorded after 24 h incubation. Tests were performed in triplicate.

2.10. Analysis of active potential free amino acids

The potent antibacterial venom peptides of free amino acids present in the purified venom were analyzed through high performance thin layer chromatography (HPTLC) techniques. The silica gel G-60 F254 precoated TLC aluminium plates (20 × 10 cm; 0.25 mm layer thickness) (Merck, Darmstadt, Germany) were used as stationary phase with *n*-butanol:acetic acid:water (4: 1: 2) as mobile phase. About 6-10 µL of working standard (amino acids) and venom samples were spotted (band width 6 × 10 mm) on TLC plates in individual tracks, using Linomat V automated TLC sampler III (CAMAG, Switzerland). The sample applicator consisted of Linnomat V with nitrogen flow providing delivery through the syringe (10 s/mL). The plate was developed in the developing chamber (CAMAG) with saturated solvent system for 30 min until the solvent (mobile phase) to reach the maximum distance (8 cm). The TLC plate was dried by using air drier for 5 min and followed freshly prepared 0.3% ninhydrin in *n*-butanol solution was sprayed on the surface of the plate. After drying, the plate was heated at 100 ˚C for 3 min to colour developments. The presence of amino acids was determined by the R_f values and images were analyzed using CAMAG TLC scanner with Wincats software (version 4×). All the tracks in these plates were scanned by user-defined wavelength at 490 nm for amino acids determination.

2.11. Statistical analysis

All the methods analysis were replicated three times and results presented with standard deviations from the mean values were used for error analysis.

3. Results

3.1. Purification and protein concentration of E. schistosa venom

The concentration of protein present in the venom of the sea snake was estimated at 457 and 786 µg/mg in crude and purified venom respectively. The results of the present study revealed that the elution profiles of high purity of peaks and protein concentration of antibacterial venom from *E. schistosa* were presented in Figure 1.

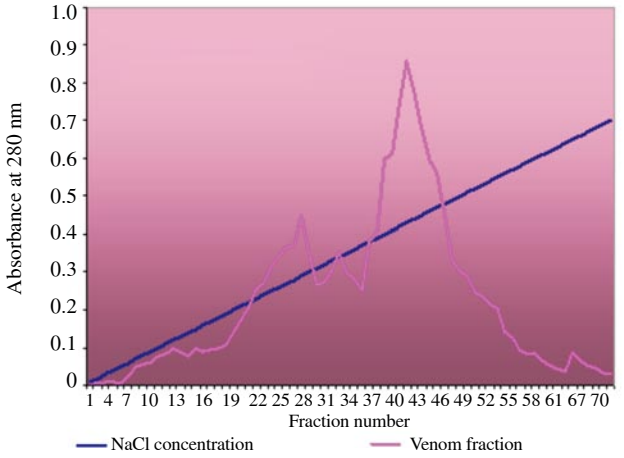

Figure 1. Purification of antibacterial peptides from *E. schistosa* venoms.

3.2. Antibacterial assay

The antibacterial activities of purified venom of *E. schistosa* against 10 bacterial human pathogenic strains were summarized in Table 1. MIC values of *E. schistosa* venom and standard antibiotics were given in Table 2.

Table 1

Antibacterial activity of *E. schistosa* venom and standard antibiotic against pathogenic bacterial strains (mm).

Human pathogenic bacteria	Venom	Ampicillin
S. pyogenes	8.6	12.7
S. aureus	13.8	28.0
S. typhi	8.8	13.4
S. flexneri	14.6	15.5
K. pneumonia	11.2	14.8
V. cholera	9.2	16.0
V. parahaemolyticus	10.3	23.0
E. coli	11.6	18.0
P. aeruginosa	7.5	11.5
S. boydii	16.4	19.0

Table 2

MIC values of standard antibiotic and *E. schistosa* venom against pathogenic bacterial strains (µg/mL).

Human pathogenic bacteria	MIC	
	Venom	Ampicillin
S. pyogenes	150	200
S. aureus	150	100
S. typhi	150	200
S. flexneri	150	150
K. pneumonia	150	150
V. cholera	150	150
V. parahaemolyticus	150	100
E. coli	150	100
P. aeruginosa	150	200
S. boydii	150	100

3.3. Determination of molecular weight

In the case of molecular weight determination, the electrophoretic profile of sea snake venom showed intensive bands at 56 and 44 kDa in crude venom and clear single band were observed at 44 kDa in purified venom sample (Figure 2).

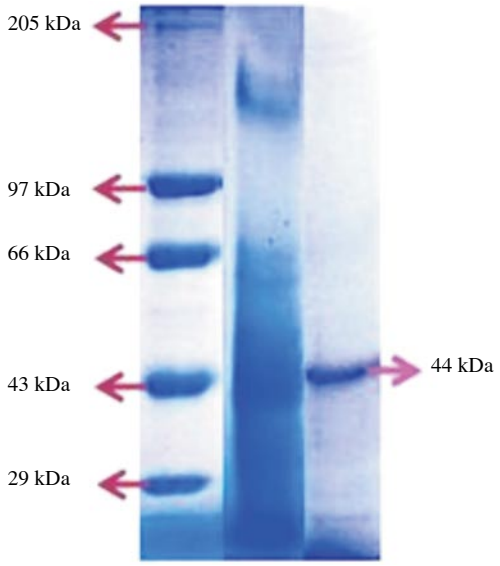

Figure 2. SDS-PAGE of *E. schistosa* venom under reducing conditions using 10% gradient gel.

Lane 1: Marker proteins; Lane 2: Crude venom; Lane 3: Purified venom.

3.4. Free amino acids analysis from sea snake venom

HPTLC based separation of free amino acids of sea snake venom was done by *n*-butanol: acetic acid: water (4: 1: 2) solvent system and the presence of free amino acids in the venom sample was confirmed through HPTLC in comparison with standard amino acids. Figures 3 and 4 showed profiling of free amino acids visual screened by HPTLC fingerprinting totally 13 samples (venom and standard amino acids) were used. This result suggested that the wonderful spots of the chromatograms were visualized under UV at λ_{254} nm. The R_f and migratory distances values of crude and purified *E. schistosa* venom was compared with standard amino acids (Table 3). The results of the present study suggested that the venom of sea snake having free amino acids based on the spectrum value of each band was recorded with a densitometer (490 nm) and clear that the fraction showed only a single spot (Figure 5).

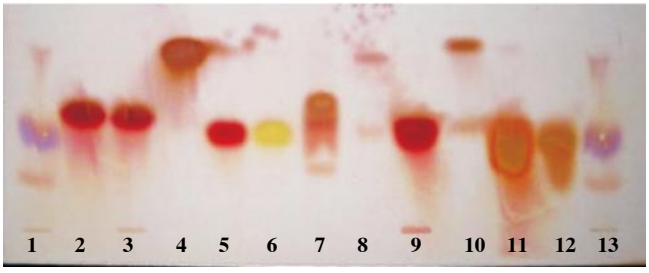

Figure 3. HPTLC chromatogram of purified sea snake venom and standard amino acids: image visible for after derivatization.

1,13 track: Crude and purified venom sample; 2-12 track: Standard amino acids.

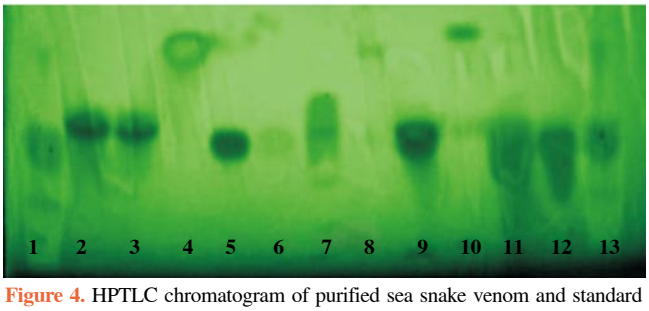

Figure 4. HPTLC chromatogram of purified sea snake venom and standard amino acids (Image visible at 254 nm).

1,13 track: Crude and purified venom sample; 2-12 track: Standard amino acids.

Table 3

The R_f values of *E. schistosa* venom samples and standard amino acids.

Substances name	Distances migration	R_f values
Crude venom	62.1 and 36.7	0.42 and 0.75
Alanine	40.4	0.47
Threonine	39.3	0.45
Leucine	62.1	0.80
Glutamine	36.7	0.41
Proline	38.7	0.44
Cysteine	43.8	0.46
Tyrosine	57.7	0.73
Serine	37.9	0.43
Tryptophan	61.1	0.79
Glycine	25.2	0.23
Asparagine	35.1	0.39
Purified venom	36.7 and 25.2	0.21 and 0.42

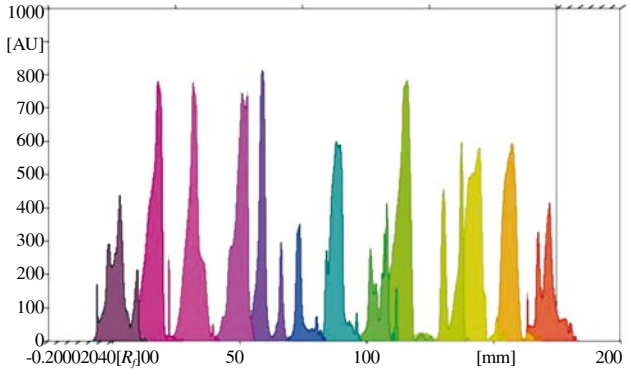

Figure 5. Representative 3D structure of the scanning data of the HPTLC plate shows in Figure 4. The HPTLC profile of crude and purified sea snake venom and standard amino acids and plotted as absorbance of scanning (490 nm) *vs* R_f value.

4. Discussion

Nature has been a source of therapeutic agents for thousands of years and an impressive number of modern drugs have isolated from marine and terrestrial microbes, animals and plants, many based on their use in traditional medicine. Naturally, presents of bioactive molecules of venom may lead to the unknown components with important for the biological applications. The venoms of sea snakes represent a rich source of highly active molecules in a wide range of biochemical and pharmacological aspects. An increasing role has been played by sea snake venom in the making of antibiotics and other drugs for the treatment of some serious diseases.

The results of the present study revealed that the purified sea snake venom obtained from crude venom were subjected into ion-exchange column chromatography, on which 10 fractions were consist of maximum protein substances and the rest of the fractions ignored due to very low protein concentration. With 0.38-0.48 mol/L NaCl in phosphate buffer, the best elution of these fractions of snake venoms was noted with high protein contents and high purity. The maximum amount of protein was found in 39 to 49 fractions with 80% of protein were recovered. The total yield of purified venom was obtained at 90% per gram of venom protein on the dry weight basis. Subsequently, the pooled fractions were desalted and screened for antibacterial activity and it was also possessed a high level of antibacterial activity against the tested all human pathogenic strains.

Many antibacterial screening studies have shown that the Gram-negative bacteria are more sensitive than Gram-positive bacteria[30]. The sea snake venom caused growth inhibition on both Gram-

positive and Gram-negative bacteria. The present study proved that the sea snake venom was potent antibacterial agents against drug resistant human bacterial pathogens and moderate sensitivity percentage were observed against Gram-negative organisms (80%) followed by the Gram-positive (20%) organisms. The results of the present study revealed that the MIC values of *E. schistosa* venom are less than 150 μg/mL, while penicillin is effective to bacterial strains such as *S. aureus*, *V. parahaemolyticus*, *E. coli, S. boydii, S. flexneri, K. pneumonia*, *V. cholera, S. pyogenes*, *S. typhi* and *P. aeruginosa* with MIC values of 100, 100, 100, 100, 150, 150, 150, 200, 200 and 200 μg/mL, respectively.

The antibacterial activity of the purified sea snake venom shows the highest zone of inhibition was observed in *S. boydii* (16.4 mm) and followed by *S. flexneri* (14.6 mm), *S. aureus* (13.8 mm), *E. coli* (11.6 mm), *K. pneumonia* (11.2 mm), *V. parahaemolyticus* (10.3 mm), *V. cholera* (9.2 mm), *S. typhi* (8.8 mm), *S. pyogenes* (8.6 mm), and *P. aeruginosa* (7.5 mm) respectively, at the same time of zone of inhibition of the positive control (ampicillin) was found in *S. aureus* (28 mm), *V. parahaemolyticus* (23 mm), *S. boydii* (19 mm), *E. coli* (18 mm), *V. cholera* (16 mm), *S. flexneri* (15.5 mm), *K. pneumonia* (14.8 mm), *S. typhi* (13.4 mm), *S. pyogenes* (12.7 mm) and *P. aeruginosa* (11.5 mm) respectively.

Earlier, Ferreira *et al.*[31] investigated that the compared four snake venom samples (*Agkistrodon rhodostoma, Bothrops jararaca, Bothrops atrox* and *Lachesis muta*) against 10 Gram-positive and Gram-negative drug resistant clinical bacterial strains to identify them as new sources of potential antibacterial molecules, which is agree with the present work. In addition, antibacterial activity has been widely reported in venoms from different snakes including *Pseudechis australis* (mulga snake), *Bothrops jararacussu*, *Porthidium nasutum* snake venom, have a powerful antibacterial effects against Gram-positive and Gram-negative bacteria [32-34].

The electrophoretic patterns (SDS-PAGE) revealed that the purified sea snake venom sample showed as a single band with the molecular weight of 44 kDa, under reducing conditions. Similarly, Abe *et al.*[35] and Ali *et al.*[36] reported about the molecular weight of snake venom such as *Trimeresurus flavoridis* for 55 kDa and *Eristocophis macmahoni* for 58.7 kDa. Such range is relatively higher than that the obtained from different species of Indian cobra (*Naja naja*) and viper (*Vipera russeli*) venom studied by Chellapandi and Jebakumar[37] who detected molecular weights between ranging from 10-17 kDa. According to recent studies, the molecular weight of venom of snake *Porthidium nasutum* showed a single band of ~15 kDa under non-reducing conditions and in a bidimensional electrophoretical profile was observed a pH of 4.6 and MW of 15 kDa[34]. It was also higher than that of *Piper umbellatum* and *Piper peltatum* untreated toxin migrated as~15 kDa under reducing conditions and corresponding to the mass of its monomeric subunits. In non-reducing condition, the spanning was reported as 28 kDa (homodimer) to 15 kDa (monomer)[38].

In the present study, HPTLC method with digital scanning profiling was used for homogeny. The results revealed that the profiling of free amino acids analyzed for HPTLC fingerprinting. Totally, 13 samples (venom and standard amino acids) were used and the wonderful spots of the chromatogram were visualized under UV at λ_{254} nm. In these results of the free amino acids spectrum of each band in venom sample was recorded with a densitometer (490 nm) and clear that the fraction showed only a single spot. In this case the concentration of amino acids consist in the venom samples were also noticed certain amino acids: such as alanine, threonine, leucine, glutamine, proline, cysteine, tyrosine, serine, tryptophane, glycine and asparagine. The R_f values of purified venom samples correlated with all standard amino acids except for leucine, tyrosine and tryptophane. In our results revealed further confirmed that the isolated venom also consists of basic amino acids which are available as in the active neurotoxin peptides (protein). Bieber *et al.*[39] extensively studied in mojave toxin by laser raman spectroscopy has a molecular weight of 22 kDa and is an acidic protein, while most of the neurotoxins isolated from cobra and sea snake venoms are much smaller in size and are highly basic proteins. When the Raman spectra of Mojave toxin were examined, they showed substantial differences from those sea snake neurotoxins and has been revealed that the rattle snake toxin contains mainly α-helical structure and a tyrosine residue[40]. Recently the proteomic technologies are very useful for improving and developing rapidly[41]. An important goal of proteomic studies of snake venom is discovering molecules that may be used in treatment of diseases or as drugs prototypes. Nevertheless, these techniques may depend on the experimental data generated so far to identify some of these unknown proteins or peptides. Although snake venom peptides and proteins have a limited direct therapeutical use due to their antigenic and 'digestible' structure, their usefulness as prototypes has clear potential[5]. Our data suggested that *E. schistosa* venom is a feasible source for searching antibacterial prototypes and designing new lead antibiotics against human pathogenic bacteria.

Venom of sea snake (*E. schistosa*) had not been previously investigated for the presence of bioactive compounds. Hence, the present investigation has been explored the biomedical potential of this species. In conclusion, the present study indicates that the sea snakes venom would be a good source of significant antibacterial activity against human bacterial pathogenic strains. Therefore, the present work exposed that in recent advances made in in-vitro natural antibacterial compound leads to development of a new antibiotic novel peptide from venom for the biomedical value treatment of bacterial infections and their main role in pharmaceutical application. Further studies we have improved to determine the toxicity, structure of the compound and its *in-vivo* activity in the animal model as well as to elucidate the mechanism underlying its activity.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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