

Antimicrobial activity of marine sponge *Clathria indica* (Dendy, 1889)

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Abstract

Sponges are sessile filter feeders that have developed efficient defence mechanisms against foreign attackers such as viruses, bacteria or eukaryotic organisms. Antimicrobial peptides are known as major components of the innate immune defense system in marine invertebrates. The aim of the present work was to study antimicrobial properties of the Indian sponge *Clathria indica* with special reference to the identification of antimicrobial peptides. Crude methanolic extract and its chloroform, n-butanol and aqueous fractions were tested against 16 human pathogens which include eleven bacteria with four of them being multidrug resistant and five pathogenic fungi. All fractions showed effective antibacterial activity against common and multidrug resistant *Salmonella typhi* and antifungal activity against *C. albicans* and *C. neoformans*. However, they were ineffective against *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Staphylococcus aureus*. Chloroform fraction being the most potent among the fractions tested on chemical investigation was indicative of the presence of peptides as evidenced by ninhydrin positive spots on TLC and presence of peptide bonds by NMR. Its ESI-MS showed presence of several peptides in the range of m/z 850 to 980. Structure of three peptides has been tentatively assigned by ESI-MS/MS or tandem mass analysis, on the basis of the amino acid sequence established. The results clearly show that the sponge *C. indica* represent an interesting source of marine invertebrates-derived antimicrobial peptides in the development of new strategies to treat various infectious diseases.

Keywords: Marine sponge, *Clathria indica*, Antimicrobial peptide, TLC, NMR, ESI-MS/MS.

INTRODUCTION

The indiscriminate use of antibiotics has led to drug resistance in many microbial strains, and the development of new molecules to combat such infections is becoming critically important. The marine environment with its vast resources comprising approximately a half of the total global diversity, is a good source for structurally novel molecules. In particular, sponges (Porifera) are one of the richest sources of both biologically active secondary metabolites and chemical diversity [1, 2]. Until now, more than 5000 different compounds have been isolated and identified from about 500 species of sponges [3] with nearly 800 of them exhibiting antibiotic activity [4]. These natural products belonged to different classes of compounds like terpenoids, alkaloids, macrolides, polyethers, nucleoside derivatives and peptides. More recently attention has been directed to the search of bioactive peptides from sponges, being actually a well established sector in the research of marine natural products.

Peptides have been isolated from a number of marine phyla, and many show high levels of cytotoxicity. Many of the peptides isolated from sponges show interesting biological properties. Kendarimide A, isolated from an Indonesian sponge of *Haliclona* sp. has been shown to reverse multidrug resistance in tumour cells [5]. The lithistid sponge *Scleritoderma nodosum* is the source of the cyclic peptide scleritodermin A [6], which inhibits tubulin polymerization and shows significant cytotoxicity against human tumour cell lines [7].

A very distinctive and massive orange sponge, *C. indica* has not been a subject of published chemical studies. There are eleven other common Indo-Pacific species known in the genus [8, 9]. Except for the identification of an anti-inflammatory polyoxygenated 14 β -steroid clathriol from New Zealand sponge *Clathria lissosclera* [10], N-methylpyrrolidone from *Clathria frondifera* [11], alkaloid clathryimine A from *Clathria basilana* [12] and diethylene glycol dibenzoate from *C. reinwardtii* [13]. The entire chemical knowledge is limited to reports on *Clathria* sp. which affords clathryamides [14], antimicrobial Mirabilin G [15], pteridine alkaloids, pseudoanachynazines A-C [16], an anti-HIV-1RT sulfated sterol Clathsterol [17] and compounds of mixed biogenesis, clathrins A-C [18].

Numerous peptides have been reported from marine sponges to date [19-24]. However, apart from the eurylamides from *Clathria (Thalysias) eurya*, published under its former name *Microcionia eurya*, [25], no other peptides are known from the genus *Clathria* or the family Microcionidae (<http://www.chem.canterbury.ac.nz/research/marinlit.htm>). As part of our continuing search for structurally and pharmacologically interesting secondary metabolites from marine

invertebrates, we decided to undertake exploration of the under investigated marine sponge *C. indica* for its antimicrobial properties. In this work, we report the antimicrobial activity of methanolic extract and its fractions evaluated by agar disc diffusion method, against 16 human pathogens (Table 1) and two antibiotics (streptomycin and ketoconazole) used in therapy. A subsequent chemical investigation of the most active chloroform fraction led to the identification of peptides 1-3, the structure of which have been tentatively assigned on the basis of amino acid sequence established by ESI-MS/MS. We suspect that the peptides may be responsible for the activity observed in the chloroform fraction.

EXPERIMENTAL

Collection and Identification of Sponges

Sponge was collected by scuba diving from a depth of 20-25m in the Gulf of Manner region (Lat 8° 55'– 9° 15' N, Long 78° 0' – 79° 16' E). The specimens were identified based on the skeletal characteristics (size and shape of spicules), external morphology, consistency and pigmentation features of the sponge surface. The organisms were washed extensively with water upon collection and were transported in methanol to the laboratory. A voucher specimen was deposited in the laboratory for records.

Extraction of sponges

The frozen sample of *C. indica* (4kg weight) was thawed, cut into small pieces and then homogenized in liquid nitrogen by crushing the sponge tissues in a mortar and pestle. The minced sample was exhaustively extracted with methanol (3x3 L). The combined organic extract was evaporated at reduced pressure at 35°C, on a rotavapor until the final volume of the aqueous solution was around 200 ml and then successively partitioned with chloroform (3x200 ml) and n-butanol (3x100 ml). The organic extracts (CHCl₃ and n-butanol) were combined separately, and concentrated at reduced pressure to give a viscous crude chloroform extract (13.8 g) and n-butanol extract (9.3 g). The water soluble residues were lyophilized to remove all the water.

Assay of anti-microbial activity

Microbial strains used and culture conditions

Antimicrobial activity of methanolic extract and fractions, (chloroform, methanol, butanol) of the sponge *C. indica* was evaluated separately by using agar disk diffusion technique as described by Bauer et al. [26]. They were tested against 16 different bacterial strains including multidrug resistant, and fungal pathogens. The clinical pathogens listed in Table 1 were used for antibiotic activity.

These clinical strains were obtained from the Department of Medical Microbiology, Goa Medical College, Goa. Cultures were maintained on nutrient agar slants at 4°C and regularly subcultured every month. For bacteria, inocula were made in 5 ml of nutrient broth (Merck) and grown for 24 hours at 37°C. The fungi were inoculated by using either PDB (potato dextrose broth) or SDB (Sabouraud dextrose broth) media. The clinical isolates were grown overnight in Nutrient Broth and spread plated (200 µl) onto Mueller Hinton Agar no.2 plates using a sterile spreader, taking care to distribute the microorganisms as evenly as possible over the agar surface. Antibacterial and antifungal activities were expressed in terms of radii of zone of inhibition in mm.

Chemicals

Streptomycin (Himedia), 10µg/disc and ketoconazole (Himedia), 10 µg/disk were used as reference antibiotics against bacteria and fungi respectively while solvents methanol, chloroform and water were used as negative control.

Sensitivity test: Preparation of discs and diffusion assay

Extracts were prepared for the assay in the following manner. Sterile blanks (6 mm diameter) made of Whatman #1 filter paper, or prepunched (S and S Sterile blanks) were systematically laid out on a clean aluminum foil in such a manner that each extract was provided with three blanks per microorganism. Ten 10 µl of the extract were placed on the blanks by first applying 5 µl with the pipette, allowed to evaporate, then applying another 5 µl, then drying again. The discs were then placed individually using a sterile forceps in appropriate grids which were marked on undersurface of the plated petri plates and kept for incubation at room temperature (27°C±2) for 24 hrs. After incubation overnight at 37°C, plates were observed for zones of inhibition that were recorded in millimeters. The assay was carried out in duplicate.

General experimental procedures

Lyophilization of sample was carried out in a Christ, ALPHA 2-4 LD plus instrument, NMR spectra were recorded at 23 °C on a Bruker Avance AC-300 spectrometer, operating at 300 and 75 MHz for ¹H and ¹³C, respectively, using tetramethylsilane (TMS) as an internal standard. EI-MS and MS/MS were recorded on QSTARXL MS/MS, Applied Biosystems, Switzerland. Sephadex LH-20 (Pharmacia) and silica gel (60-120 mesh) (Qualigens) were used for column chromatography. Culture media Czepak agar and potato dextrose broth were procured from Himedia, Ltd. Mumbai.

Solvents used for column chromatography were purchased from local supplier and were glass distilled prior to use. Precoated kieselgel 60 F₂₅₄ TLC plates (Merck) were used for analytical TLC.

ESI-QTOFXL MS/MS Spectrometry

ESI-MS (electrospray ionization mass spectrum) of the active chloroform fraction was recorded by dissolving the sample in MeOH: H₂O containing trace of 0.1% TFA. The solution was directly infused at the constant flow rate of 10 µl/min into the ion-spray source using an integrated syringe pump. The declustering potential and the collision energy (25– 40 V) were optimized for MS/MS experiments so as to cause fragmentation of the selected molecular ion species as evident by the appearance of the fragment ions and decrease in the intensity of the molecular ion.

Thin layer chromatography (TLC)

A diluted solution of the chloroform extract were applied to the thin layer chromatographic plate (Merck) with a capillary tube and placed in a chamber containing butanol: acetic acid: water (5:1:4) / methanol and chloroform (10:90, v/v) as mobile phase. After development compounds were visualized as rose pink spots on spraying with ninhydrin as detecting agent followed by heating at 100° C till the spots were visible.

RESULTS AND DISCUSSION

Microorganisms have developed resistance to many antibiotics due to the indiscriminate use of antimicrobial drugs, increasing clinical problems in the treatment of infectious diseases. In addition, antibiotics are sometimes associated with adverse effects in the host which includes hypersensitivity, depletion of gut and mucosal microorganisms, immunosuppression and allergic reactions. Therefore need is felt for alternative antimicrobial drugs for the treatment of infectious disease.

Marine organisms represent a rich source from which novel antibacterial and antifungal chemotherapeutic agents may be obtained. However, the research on marine organisms is still in its infancy considering the vast resources that still remain untapped with the majority of organisms yet to be screened for the discovery of useful antibiotics.

In this work crude methanolic extract and the fractions (water soluble, chloroform, butanol) of the sponge *Clathria indica* were tested for antimicrobial activity against 16 clinical isolates which

include seven bacteria, five fungi besides four multidrug resistant bacterial strains. Among the microorganisms chosen were *S. aureus*, a pyogenic bacterium known to play a significant role in invasive skin diseases, including superficial and deep follicular lesions, and *C. albicans*, a fungal microorganism which causes serious systemic infections, especially opportunistic infections in patients infected with HIV [27].

The results of the disc diffusion assay of the crude methanolic extract and the fractions are presented in Table 1. According to these data, crude extract and the fractions were found to be bactericidal against both, wild type strain as well as multidrug resistant strain of *S.typhi* including resistance to reference antibiotic streptomycin used in the present work. The extracts prevented the growth of fungi *C. albicans* and *C. neoformans* but ketoconazole, the standard antifungal agent used showed better potency than *C.indica* extracts, was totally ineffective against *C. neoformans* and *Rhodotorula sp.* The bacteria, *E.coli*, *P. aeruginosa*, and *S. aureus* and MDR *S. pyogenes* were not susceptible to *C.indica* extracts. Selective activity was exhibited by the chloroform fraction on *S. flexineri* and *Vibrio cholerae*, which were insensitive to other extracts. Inhibition effects noted for fungi *Aspergillus fumigatus* and *Rhodotorula sp.* by methanolic extract and chloroform fraction to nearly same extent indicated the active principle to be present in the chloroform soluble fraction. Besides, the chloroform soluble fraction of the *Clathria indica* appeared to be quite promising due to its capacity to inhibit the growth of *S. typhi*, *C. albicans*, *C. neoformans* and all the MDR strains with the exception of *S. pyogens*. Hence, it was selected for further studies. Additionally, most clinically used antibiotics are active at a concentration of 10µg/ml. If a pure substance is not active at 100 µg/ml, it will probably not be clinically useful. Extracts that are active at 100 µg/ml have a good potency level and recommend the determination of the active components by subsequent purification [28]. In the present investigation the crude methanolic extracts as well as fractions were quite active against different microorganisms tested even at 10 µl/ml which indicated that constituent compounds might be of biomedical importance.

Structure determination of constituents of active chloroform fraction

As our main interest was to search for antimicrobial peptides we checked for the presence of this class of compounds in the active chloroform soluble fraction. Its TLC and the presence of doublets in the region δ 6.5 -8.0 in the ^1H NMR (Fig.1) showed that the fraction is rich in peptides.

The ESI-MS profile of the ninhydrin positive chloroform fraction is shown in Fig. 2. It showed presence of several molecular species in the range of m/z 850 to 980. This range was selected as microcionamides A and B identified from organism belonging to the same genus,

Clathria abietina [29] had molecular mass of m/z 875. Three of these peptides with pseudomolecular ions $[M+H]^+$ + 903, 875 and 859 were selected for identification and have been tentatively assigned structures 1-3 as shown in Figures 3a, 4a and 5a respectively.

The fragment ion peaks are labeled according to the nomenclature proposed by Roepstorff and Fohlman [30], subsequently modified by Johnson *et al* [31]. To retrieve the sequence from the mass spectrometric data of the new peptides, we applied the criteria that the mass difference between two adjacent peaks should precisely fit the mass of an amino acid residue.

The amino acid sequence of the three peptides with molecular mass of 902, 874 and 858 amu was established by ESI-MS/MS or tandem mass analysis. The structures assigned are as represented in Figures 3, 4 and 5.

Tandem mass spectrometry

MS/MS at m/z 903 of peptide 1

Positive ESI-MS/MS of peptide 1 provided together with the $[M+H]^+$ pseudomolecular ion many fragment ion peaks containing the N-terminus which were consistent with the amino acid sequence as shown in structures assigned to the peptides 1-3. As evident from ESI-MS/MS peptides fragmented predominantly at the $-CO-NH-$ bonds and gave mainly a, b, and c-series with the exclusion of x, y and z-series. The mass difference between adjacent members of b series (ie. b^2-b^1) gave the complete sequence coverage e.g. peptide 2 (peptide with $M+H-875$), $b^1-b^2 = (201- 88) = 113$; $b^3-b^2 = (347- 201) = 146$ showed the presence sequentially of serine, leucine or isoleucine (isoleucine or leucine was not assigned) and phenylalanine although b-ions deriving from the C-terminal portion were of low intensity, covering the majority of the sequence and the total mass running short by 105 amu. This was taken into account by considering the lipid part as shown. Isoleucine is more probable rather than leucine based on the literature where microcionamide identified from *Clathria abietina* is rich in isoleucine. Similarly, tentative structures were assigned to the other two peptides with molecular mass m/z 902 and m/z 858. The assigned structures are to be confirmed by NMR and chemical analysis.

Marine sponges are potential sources of new antibiotics. Sponges are believed to have developed secondary metabolites that are active against different strains of microorganisms as part of their defense and survival in the marine environment. It has been reported that over 82% of all the sponges studied up to 1985 have antimicrobial properties which in some cases is attributable to peptides. Genus *Discodermia* was found to be a good source of antimicrobial agents. For instance, the discodermins, antimicrobial peptides, were isolated from the sponge *Discodermis kiiensis* [32]. A

novel peptide Polydiscamide A and its derivatives, also constituents of genus *Discodermia*, have been patented for their antibacterial and antitumor properties [33]. Discobahamin A and B, from the Bahamian deep water marine sponge *Discodermia* sp., are two antifungal peptides, inhibitors of the growth of *Candida albicans* evaluated by Gunasekera et al. [34]. Japanese sponge *Halichondria cylindrical* [35] (Li et al. 1996) is known to yield the depsipeptides Halicylindramide D and E with cytotoxic and antifungal properties. Three new antifungal cyclic peptides with unprecedented amino acids, Microsclerodermins C-E were identified from two species of sponges, *Theonella* sp. and *Microscleroderma* sp. from the Philippines [36]. Further antifungal cyclic peptides from sponges are the Aciculitins A-C [37] and the Theonegramide f [38]. New peptides Callipeltins F-I from the sponge *Latrunculia* sp. inhibited the growth of *Candida albicans* (ATCC24433) with a MIC of 10^{-4} M [39].

The present investigation represents a preliminary screening on sponge antimicrobials. We have identified peptides which are presumed to be responsible for the antimicrobial activity observed in the fraction from the most active chloroform fraction of the sponge *Clathria indica*. No data either on chemistry or the biological activity of this sponge belonging to family Microcionidae appear to have been published though there is a solitary reference in the literature [29] on the anti-tuberculosis activity displayed by microcionamides A and B against avirulent strain *Mycobacterium tuberculosis* H37Ra with a MIC values of $5.7\mu\text{M}$ as compared to the positive control rifampicin (MIC values of $1.52\mu\text{M}$). Microcionamides A and B are peptides reported from *Clathria abietina* [29]. Even though we presume that the antimicrobial activity could be due to the presence of peptides identified from the active fraction; the activity being due to the other compounds present in the fraction cannot be ruled out. The work is in progress on the isolation of the peptides for the confirmation of the structure assigned as well as the confirmation of the active principle.

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Table.1. Antimicrobial activity of methanolic extract and fractions of the sponge *Clathria indica*

Pathogens	Fractions				
	Standard Streptomycin	Water	Chloroform	Butanol	Methanol (extract)
<i>Escherichia coli</i>	4-5 static	-	-	-	-
<i>Pseudomonas aeruginosa</i>	1	-	-	-	-
<i>Staphylococcus aureus</i>	1	-	-	-	-
<i>Salmonella typhi</i>	3	1	1	+	+
<i>Shigella flexineri</i>	4 static	-	+	-	-
<i>Klebsiella sp.</i>	-	1	1	-	1
<i>Vibrio cholera</i>	5	-	+	-	-
Fungi: ketoconazole		-	-	-	-
<i>Aspergillus fumigates</i>	1-2	-	0-1	-	1
<i>Rhodotorula sp.</i>	-	-	1	-	1
<i>Candida albicans</i>	2-3	+	1	1	1
<i>Cryptococcus neoformans</i>	-	1	1	1	0-1
<i>A. niger</i>	1-2	-	1	1	-
Multi-drug resistant bacteria: streptomycin		-	-	-	-
<i>Streptococcus pyogenes</i>	2	-	-	-	-
<i>Acinetobacter sp.</i>	Static 4	-	0-1	-	0-1
<i>Salmonella typhi</i>	-	0-1	0-1	0-1	1
<i>Staphylococcus aureus</i>	2	0-1	+	-	-

Figure captions

Fig. 1. ^{13}C NMR spectrum of peptides rich CHCl_3 fraction of *C. indica* in D_2O

Fig. 2. ESI-MS profile of the active Chloroform fraction *Clathria sp.*

Fig. 3. (a) MS/MS spectrum at m/z 903 $(\text{M}+\text{H})^+$ of peptide 1; (b) Structure and Mass Fragmentation of Peptide 1 (isoleucine /leucine not assigned)

Fig. 4. (a) MS/MS spectrum at m/z 875 $(\text{M}+\text{H})^+$ of peptide 2; (b) Structure and Mass Fragmentation of Peptide 2 (isoleucine /leucine not assigned)

Fig. 5. (a) MS/MS spectrum at m/z 859 $(\text{M}+\text{H})^+$ of Peptide 3; (b) Structure of Peptide 3 (isoleucine /leucine not assigned)

Fig. 1

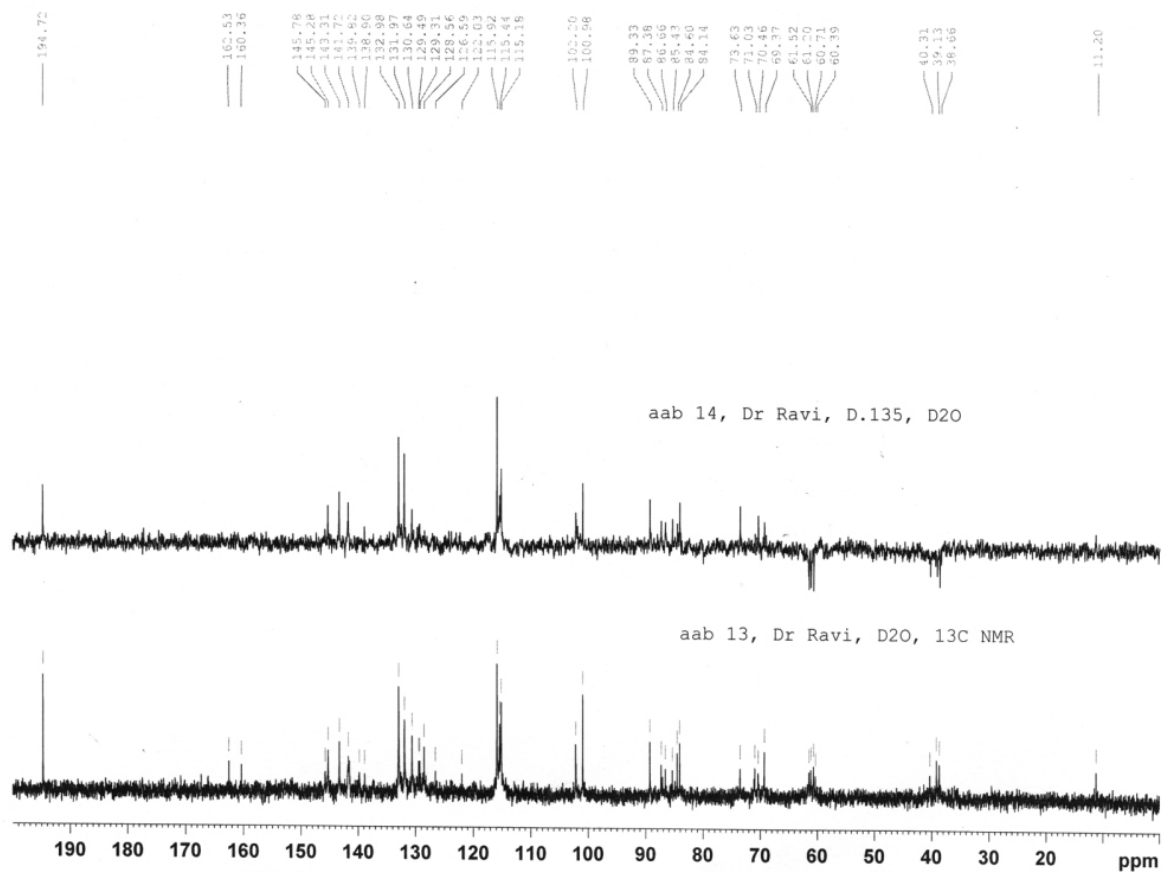


Fig. 2

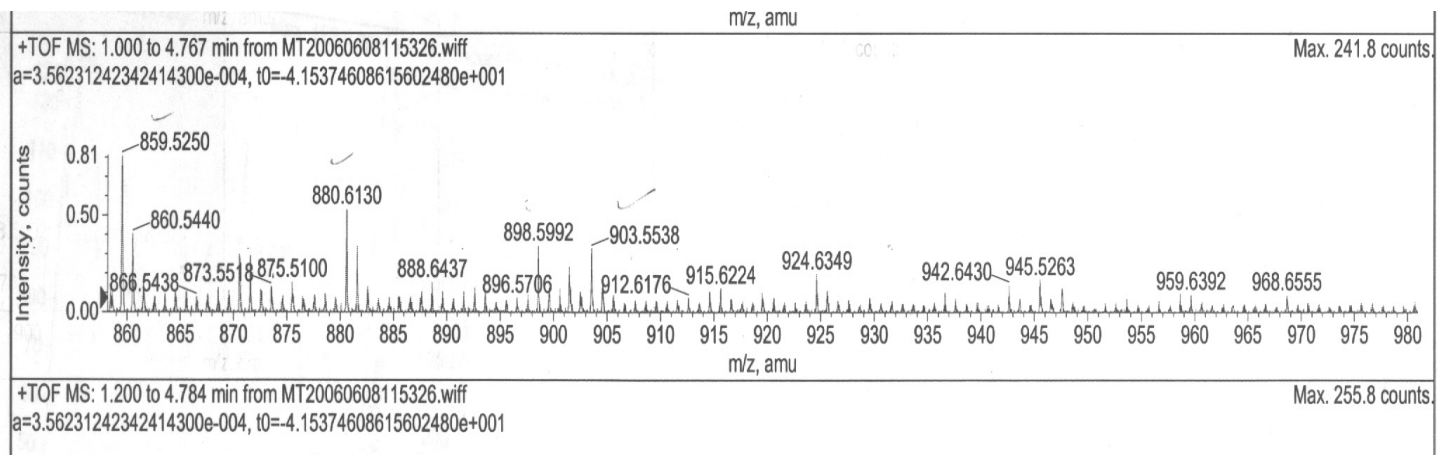


Fig. 3

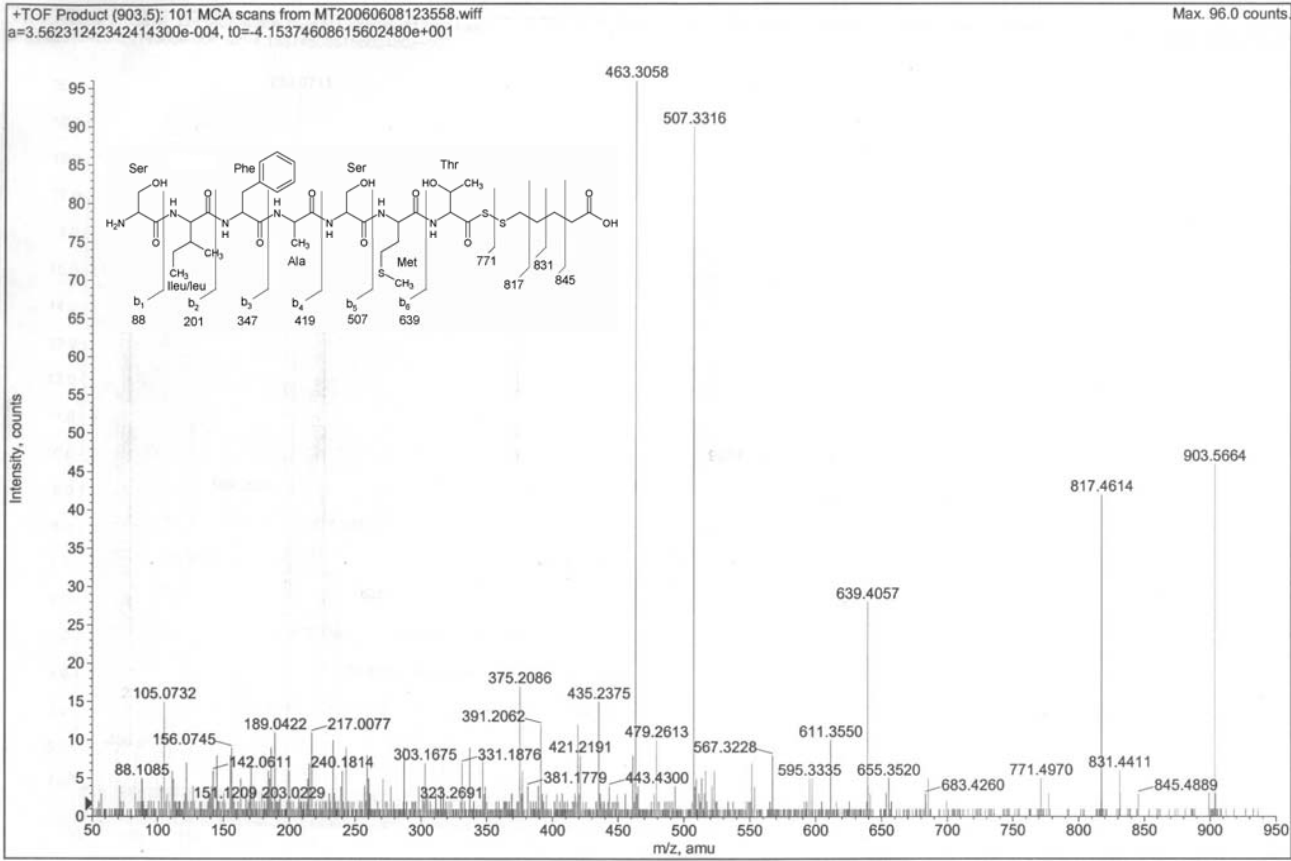


Fig. 4

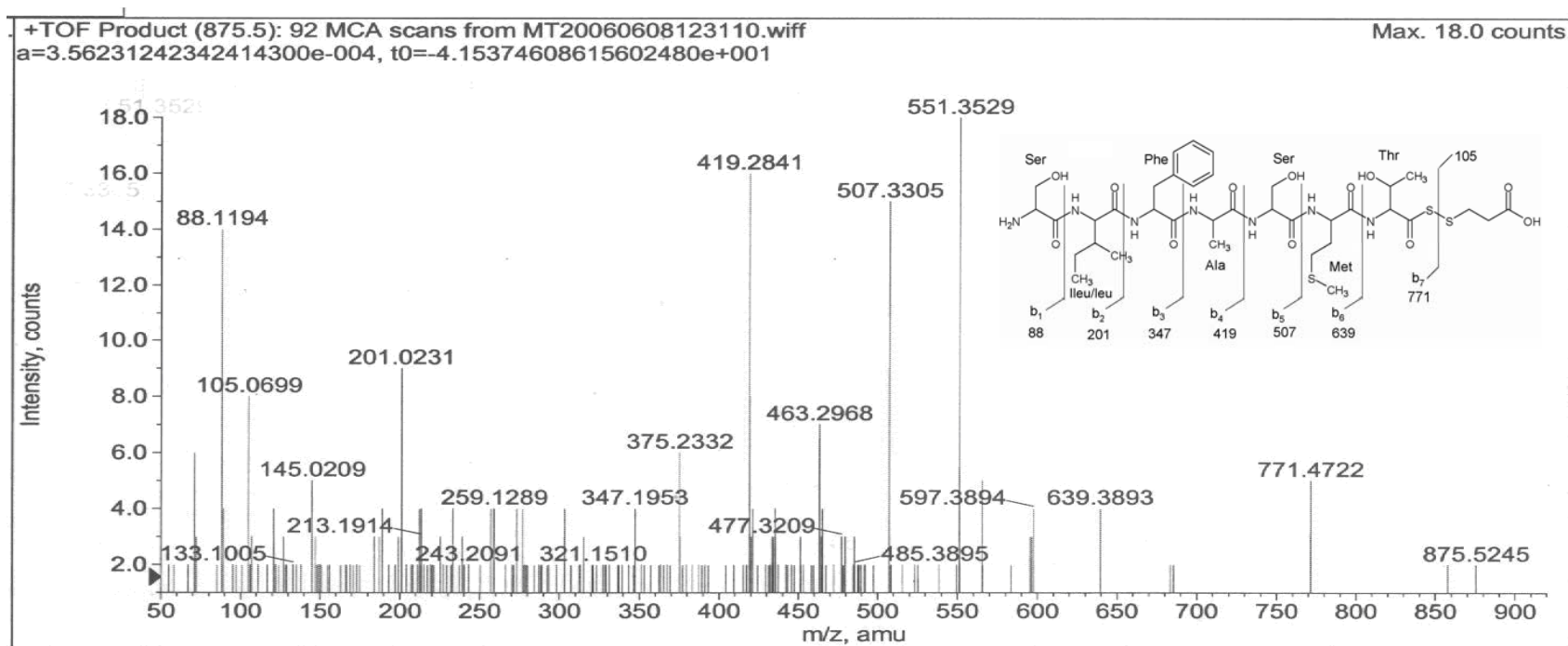
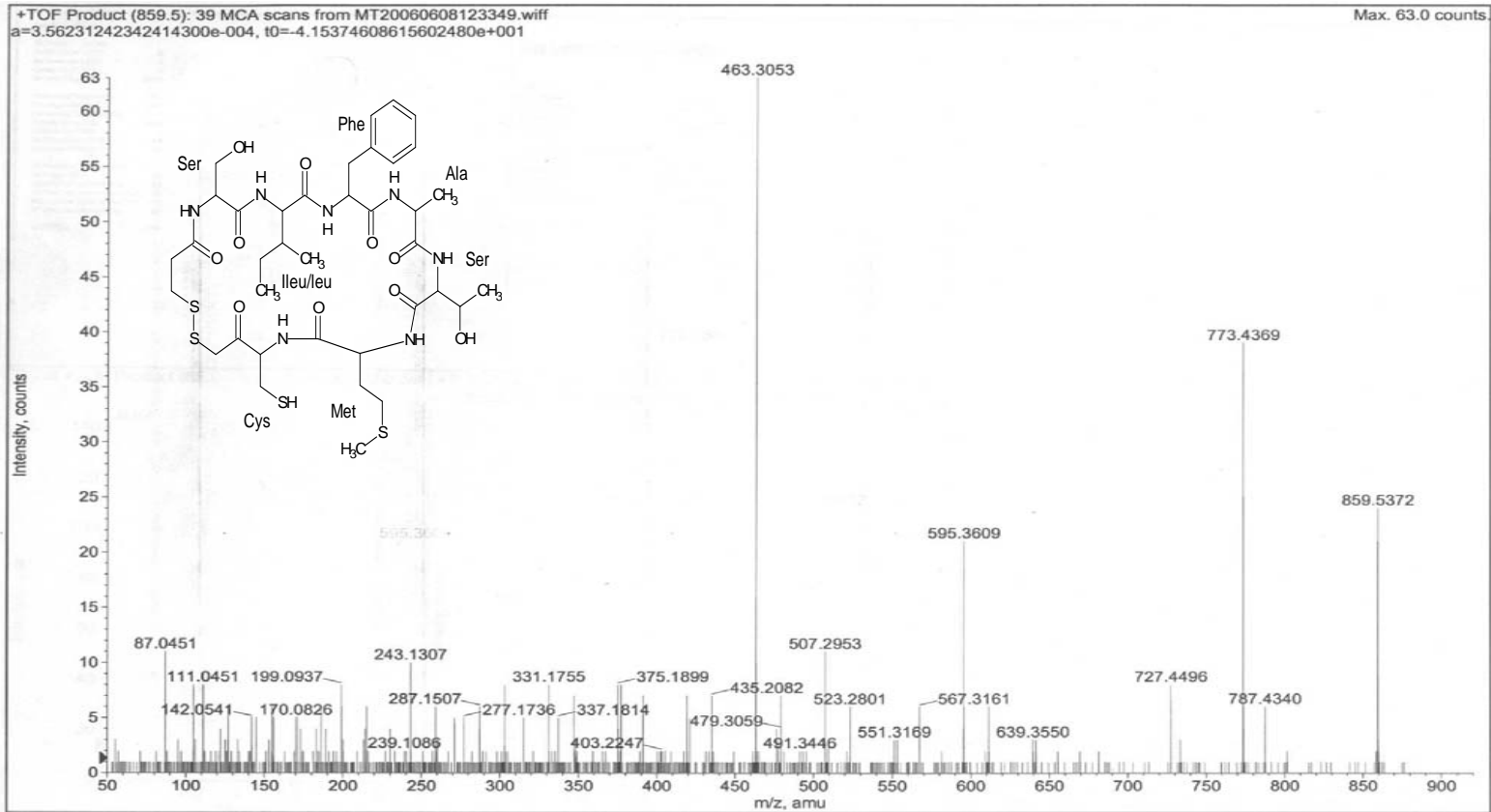


Fig. 5



Abbreviations

TLC Thin layer chromatography

¹H NMR Proton nuclear magnetic resonance

¹³CNMR Carbon 13 nuclear magnetic resonance

m/z Mass-to-charge

ESI-MS Electrospray ionization mass spectrometry

L Litre

CHCl₃ Chloroform

MS/MS Tandem mass spectrometry

MeOH Methanol

H₂O Water

TFA Trifluoro acetic acid

HIV Human Immunodeficiency Virus

TOF Time-of-flight

MDR Multi Drug Resistant

MIC Minimum Inhibition Concentration