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Antimicrobial Peptide from Marine Sponge *Clathria indica*, (Dendy,1889)

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Abstract: Marine sponges are potential of new antibiotic. Sponges are believed to have developed secondary metabolites that are active against different strains of microorganisms as part of their defenses and survival in the marine environment. In the present study marine sponges were collected by SCUBA diving from the depth of 20-25m in the gulf of manner region. In antibacterial activity among the various strains maximum diameter of (12 mm) zone of inhibition was recorded in *Escheirchia coli* strain and minimum zone of inhibition of (6mm) was observed in *Salmonella typhi, Proteus mirabilis and Lactobacillus vulgaris* strain. In antifungal activity maximum zone of inhibition was observed in *Fusarium* sp. (+++) and minimum zone of inhibition was recorded in *Aspergillus fumigatus (*+). TLC plate with fractions of *Clathria indica* and developed in B: A: W as the solvent system and sprayed with ninhydrin reagent showing pink colored indicated the presence of amino acids and peptides. On the basis of TLC observations on further confirmations with ^{1}H NMR peptide presented in between (δ 6-8ppm fractions) subjected to studies by using chromatography technique. This resulted in the identification of antimicrobial peptide.

Key words: Sponges, Antibacterial, Antifungal, TLC, NMR

INTRODUCTION

The Ocean covers 71% of the surface of the earth and contains approximately half of the total global biodiversity. In the last 25 years marine invertebrates have provided key structure and compounds that proved their potential in several fields, particularly as new therapeutic agent for a variety of diseases. Sponges have been traditionally known as a source of novel bioactive metabolites like terpenoids, alkaloids, macrolides, polyether, nucleoside derivatives and many other organic compounds. Synthetic analogues of the c-nucleoside spongouridine and spongothymidine isolated from Caribbean sponge led later to the development of cytosine Arabinoside, an anticancer compound [1], more recently the attention has been directed to the search of bioactive peptides from marine sponges. One of the first novel peptides isolated from sponges were the cell growth inhibitory tetradecapeptide discodermins [2]. A review of cytotoxic peptides from sponges [3].

Geodiamolides A-G initially isolated from the Caribbean sponge *Geodia* sp and the similar peptides Jaspamide are also a group of cytotoxic peptides in which three amino acids from a cyclic peptide with a common polypeptide unit. Arenastatin-A [4]. Thronellamides A-F is also a group of cytotoxic compounds isolated by [5] with novel amino acid residues and complex bicyclic macrocyclic rings. Discobahamin-A and B are two bioactive antifungal peptides evaluated as inhibitors of the growth of *Candida albicans,* isolated from the Bahamian deep water marine sponge *Discodermia* sp. [6]. The depsipeptides Halicylindramides A-C and [7]. With antifungal and cytotoxic properties (against P388), were obtained from the Japanese marine sponge *Halichondria cyclindrata*.

Three new antifungal cyclic peptides with unprecedented amino acids, micro schlerodoamins C-E were isolated from two species of sponges, *Theonella* sp. and *Microscleroderma* sp. from the Philippines [8]. Further antifungal cyclic peptides from sponges are the Aciculitins A-C, [9] and the Theonegramide [10]. Other examples are the Haligramides-A and B two new cytotoxic hexapeptides from the sponge *Haliclona nigra* [11] and Mozamides A and B form a Theonellid sponge collected in Mozambique [12].

Papuamides A and B inhibited the infection of human T-Lymphoblastoid cells by HIV-I sub (RF) *in vitro* with an EC50 of approximately 4mg/ml. Another anti HIV candidate from the sponge *Sidonops microspinosa* is the microspinasamide [13]. The origin and role of bioactive peptides inside the sponges in many cases is unclear [14].

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Other examples of bioactive peptides isolated from microorganisms living in association with sponges are the bicyclic glycopeptides. Theopalauamide [8] and the cytotoxic halogenated hexapeptide cyclocinamide-A with a potent *in vitro* selective activity against colon-38 tumor cells [15]. The present study was carried out to study the antibacterial peptide from the marine sponge *Clathria indica.*

MATERIALS AND METHODS

Collection and Identification: *Clathria indica* sponges were 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 India (Fig.1). The specimen was 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.

Extraction and Fractionation Methodology: Sponge (*C. indica*) sample was collected from the Gulf of manner regions and cleaned from the associated marine organisms like sea grass, Brittle stars etc and other extraneous matter.Then *C. indica* species was transferred to a flask containing methanol for extraction and left over night. Extraction of the organisms with different solvents of increasing polarity with petroleum ether, chloroform, ethyl acetate, butanol and methanol. Shown in (Table 1, 2).

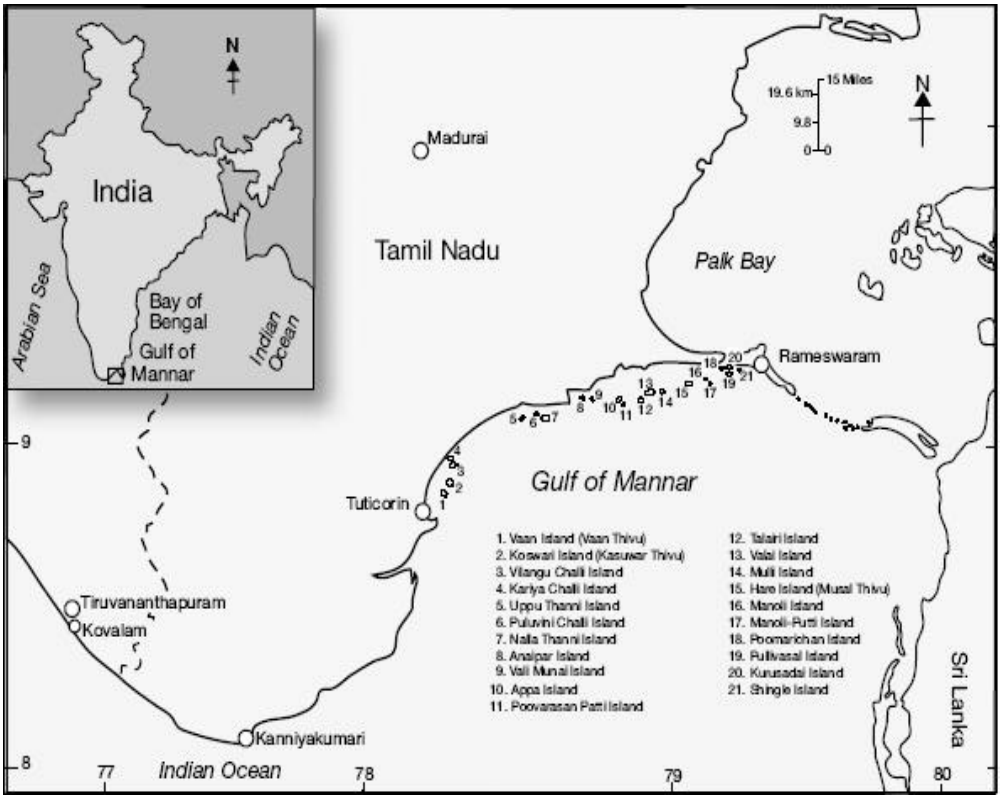

Fig. 1: Showing the Study Area

Table 1: The Yield of Different Fractions From 1.4001 gm (crude residue) of *C.indica* Petroleum ether fractions:

FRACTIONS	EMPTY BOTTLE (gm)	EMPTY BOTTLE+SAMPLE (gm)	SAMPLE WEIGHT (gm)
11-13(Mixture)	52.7210	53.0014	0.2804
14-27(Mixture)	52.7210	53.4480	0.727
28-34(Mixture)	36.9641	36.9934	0.0293

Table 2. Butanol Fractions:

FRACTIONS	EMPTY BOTTLE gm	EMPTY BOTTLE+SAMPLE gm	SAMPLE (gm)
B17+18+19+20+21+22+23+24.	52.7198	52.7218	0.002

Microbial Strains Used: Antibacterial activity of Sponge (*C. indica*) was determined against 9 different bacterial strains viz, *Staphylococcus aureus, Salmonella typhi, S.paratyphi, Klebsiella oxytoca, Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Lactobacillus vulgaris* and *Vibrio choleriae* and 5 fungal strains viz, *Aspergillus niger, Aspergillus fumigatus, Candida albicans, Fusarium solani* and *Fusarium* sp. These clinical strains were obtained from the department of Medical Microbiology (Raja Muthiah Medical College hospital) Annamalai University, Annamalai Nagar.

Anti Microbial Assay: The spectrum of antibacterial and antifungal activity was studied by using the techniques of [16]. Antibacterial and antifungal activity was expressed in terms of diameter of Zone of inhibition was measured in mm using Vernier caliper or a scale and recorded.

Thin Layer Chromatography (TLC): The extract and fractions were applied in diluted form to the thin layer chromatographic plate (Silicagel H 254) with a capillary tube and then placed in a chamber containing solvent system for developing. Since peptides are known to be polar compounds usually Butanol: Acetic acid: Water (5:4:1) is used as the developing solvent system. After development, the plates were sprayed with a detecting agent spraying of chromatograms with visualizing agent is the most frequent method used for detecting the components. In this case ninhydrin was used as detecting agent as peptides were the compounds of interest.

Nuclear Magnetic Resonance (NMR): NMR is usually observed when nuclei of certain atoms are placed in a static magnetic field and exposed to a second oscillating magnetic field. Nuclei of the atoms, which are considered to spin experience this phenomenon, depending upon whether they posses spin of 1/2 or in multiples of ½ i.e. they, act like tiny bar magnets. One such nucleus is the proton, the one of concern to us.

RESULTS

Antimicrobial Assay: Antibacterial activity of crude sample of sponges (*C.indica*) is used for present study. The positive control Amphicillin was also used. Investigation against a range of nine different bacterial strains were used of which one gram-positive bacteria (*Staphylococcus aureus)* and eight gram-negative bacteria (*Salmonella typhi, Salmonella paratyphi,*

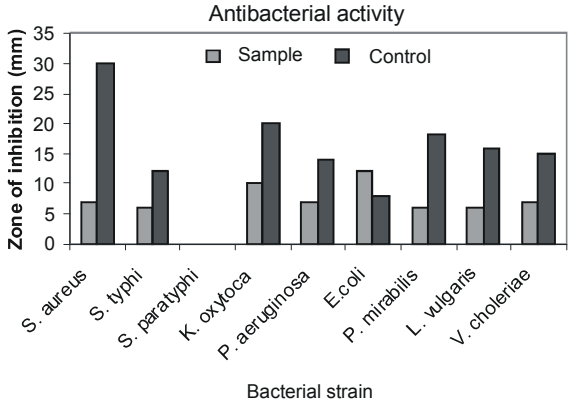

Fig. 2: Antibacterial activity of antibacterial agents from (Amphicillin)

Klebsiella oxytoca, Pseudomonas aeruginosa, E.coli, Proteus mirabilis, Lactobacillus bulgaris and *Vibrio choleriae).*

Antibacterial Activity of *C.indica*: The zone of inhibition in different bacterial strains against *C.indica* is shown in (Fig. 2). Among the various strains maximum diameter of (12 mm) zone of inhibition was recorded in *E. coli* strain and minimum zone of inhibition of (6mm) was observed in *S. typhi, P. mirabilis and L. vulgaris* strain. The extract of antibacterial agent of Amphicillin showed activity against all the bacterial strains tested. The maximum activity showed showed against *S. aureus* (30mm), the minimum activity observed against *S. typhi* (12mm). shown in (Fig. 2).

Antifungal Activity of *C.indica*: The n-butanol fractions were subjected to antifungal testing against different strains of fungi are *Aspergillus niger, Aspergillus fumigatus, Candida albicans*, *Fusarium solani*, *Fusarium* sp. Both, the fraction and the identified peptides mixture exhibited/ showed maximum zone of inhibition was observed in *Fusarium* sp. (+++) and minimum zone of inhibition was recorded in *Aspergillus fumigatus (*+) shown in (Table 3).

Thin Layer Chromotography: All the four fractions, petroleum ether, ethyl acetate, n-butonal and aqueous layers were spotted on TLC plates and developed in different solvent systems. Since our main interest was to isolate peptides, the plates were developed either in Butonal: Acetic acid: Water (B:A:W) CHCL3: MeOH mixtures in different proportion depending on the Rf value

Table 3: Antifungal activities shown against crude extract of *C. indica*

Compound	*A.niger*	*A.fumigatus*	*Candida albicans*	*Fusarium solani*	*Fusarium* sp
1(100mcg)	++	+	++	-	+++
2(100mcg)	++	+	++	-	+++
3(100mcg)	-	-	++	-	-
4(100mcg)	-	-	++	-	+
Standard	-	-	-	-	-

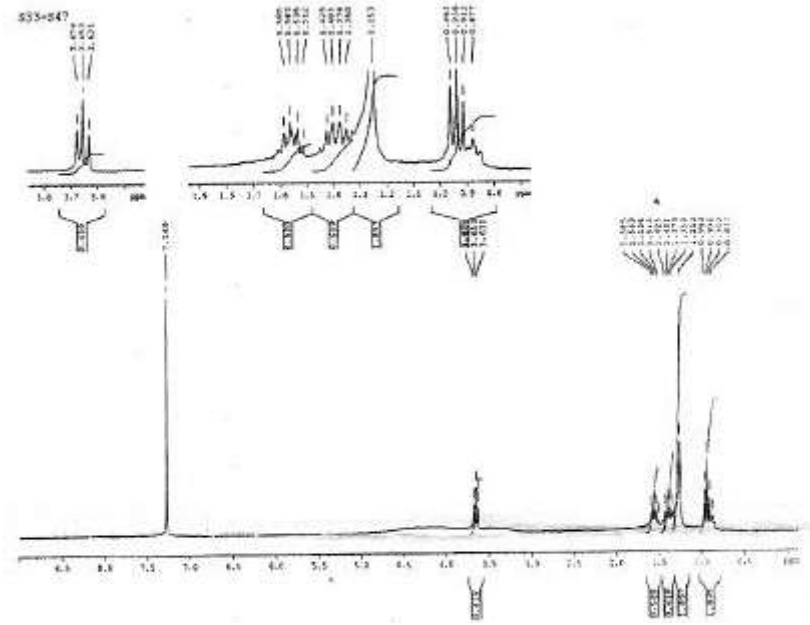

Fig. 3: Shows on various peaks between δ 6-8 ppm rang. These peaks are peptides with a several amino acids, each sight corresponding to a peptide bond

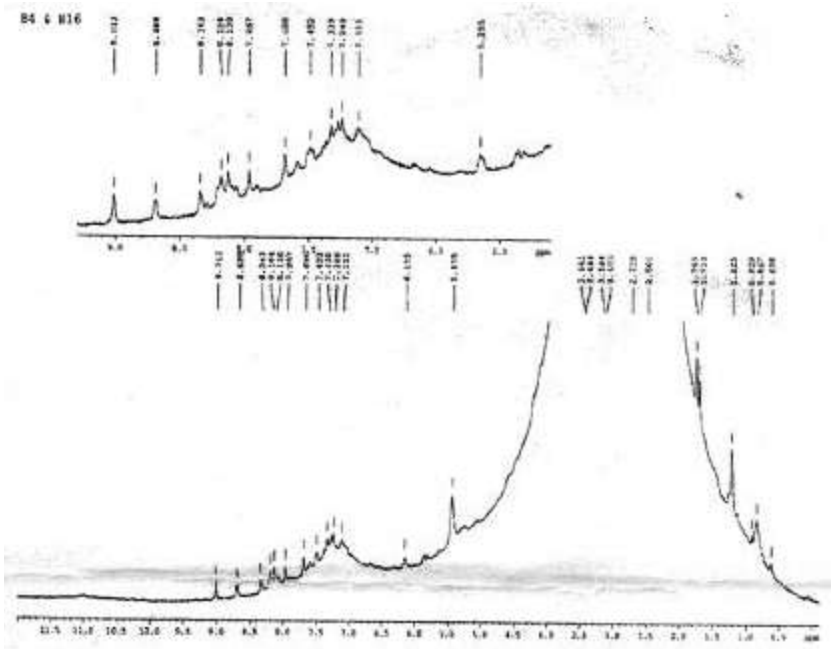

Fig. 4: Butanol samples Shows on various peaks between δ 6-8 ppm rang. These peaks are peptides with a several amino acids, each sight corresponding to a peptide bond

of the spot. Ninhydrin (2% alcoholic solvent) was used as the spraying agent as it is well known to be specific for amines. Primary amine gives intense pink coloration whereas secondary amines are lighter in colour, tertiary one being undetectable. After spraying the plates were heated at 100°C in an oven till the spots were visible. After spotting the fraction on TLC plate and spraying with ninhydrin, it was observed that petroleum ether fraction yielded some pink spots. As it is well known that peptides are known to yield pink spots with ninhydrin and since petroleum ether fraction had yielded pink spots it was taken up for further investigation leading to the isolation of peptides. Hence, petroleum ether fraction was chromatographed first on Sephadex LH 20 followed by final purification on silica gel.

NMR: Repeated chromatography of n-Butonal fractions yielded almost pure compound ^{1}H NMR of this compound showed it to be a peptide (signal in the origin δ 6-8 of ^{1}H NMR spectrum of a mixture B1 and B16, compounds which looked like single spots on TLC and were showing pink spots when sprayed with ninhydrin. Both had the same Rf values and were isolated from the n-butonal fraction by separated column chromatography over Sephadex LH20. They were mixed together and subjected to NMR analysis.

The presence of peptide is evident by the absorption observed in the region 'between' δ 6-8. (Fig. 3 and 4). It shows several peaks in this region indicating it is to a peptide with a several amino acids, each sight corresponding to a peptide bond.

DISCUSSION

In recent years, great attention has been paid to study the bioactivity of natural products of their potential pharmacological utilization. Among these 50 have found widespread use in the prevention and treatment of bacterial disease in animal and man [17]. The first attempt to locate antimicrobial activity in marine organisms was initiated around 1950s [18,19] Since this time, a large number of marine organisms from a wide range of phyla have been screened for anti microbial activity [20,21] Many of these organisms have been antimicrobial properties, although most of the antibacterial agents that have been isolated from marine sources have not been active enough to complete with classical anti microbial obtained from microorganisms [21].

Naturally occurring bacterial cell-wall proteins elicit in *Suberite domuncula* in increase the lytic (lysozyme) activity, [22]. Based on earlier cytological and functional observations these 25-µm large cells are the gray cells [23]. Sponges are filter feeders that are exposed to large amounts of bacteria present in their surrounding aqueous milieu. The characteristic cell wall component of gram-positive bacteria, peptidoglycan(PPG), was used as a model molecule to study the responsiveness of cells from the marine demosponge *S. domuncula* toward gram-positive bacteria [24]. Bacteria associated with sponges and their possible interaction with the host. Bacteria play important role in the production of antibacterial metabolites [25,26].

The α-Proteobacterium MBIC3368 strain SB89 was also recovered from the Mediterranean sponge *Aplysina aerophoba,* which displayed antimicrobial activity against various Gram-positive and Gram-negative bacteria [27]. A c DNA clone was isolated that resembles human perforin and displayed antibacterial activity. Human perforin is found in lytic granules of cytotoxic natural killer and T-cells [28]. The sponge *Tethyalyncurium* synthesizes a pore-forming protein [29], whish is of similar size to other pore-forming toxins,e.g. the *Escherichia coli* hemolysin [30].

The present study indicated that antibacterial activity the maximum zone of inhibition was observed *E. coli* (12mm) and minimum zone of inhibition of 6mm was observed in *S. typhi, P. mirabilis and L. vulgaris* strain. The inhibited growth of antibiotic resistant strains tested. It is an interesting finding that sponge (*C.indica*), being marine animal has the ability to dispose the bacteria upon infection. As the bacteria is a human pathogen, it is important that sea water should be free from this type of bacteria. In conclusion in the present study indicates that the good source of antibacterial activity agents and would replace the existing inadequate and cost effective antibiotics.

Material no single analytical technique is capable of completes characterization of peptide structure. As a consequence, structure elucidation is usually performed by using various techniques, of which ^{1}H NMR spectrometry the most powerful method.The primary technique in ^{1}H NMR peptide peak with the information available depending strongly on instrumentation and ionization methods.

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