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Identification of Cytotoxicity of Marine Sponge *Dendrilla Membranosa* Against L929 Cell Line

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ABSTRACT

Marine sponges are rich sources of pharmacological active compound. Marine sponge, *Dendrilla membranosa* was collected from the Vizhingam coast. The sponge extract was tested against eight human bacterial pathogens. The bioactive compounds present in marine sponge were determined by GC-MS analysis. The cytotoxic effect of the sponge was evaluated by using MTT assay. The extracts showed potent anti-bacterial activity against *Staphylococcus aureus, Bacillus subtilis, Streptococcus pyogens*. The bioactive compound present in marine sponge was identified by GC-MS analysis and the compounds are ethane, butane, formate pentane, Alpha d-galactopyranoside, 2-Ethylhexyl2-ethylhexanoate, Hexahydro hexitol, Styrene, Alpha-d glucopyranoside 1-pentyl-2-proppyl-1-octene, nonadecane, 1,2-benzenedicarboxylic acid, bis-2, Octanoic acid. The test material showed none cytotoxic response to fibroblasts cells. The results of present investigation revealed that, *Dendrilla membranosa* is a potential source of novel anticancer and antibacterial leads.

Keywords : *Dendrilla Membranosa*, GC-MS, Anticancer Activity, Antimicrobial Activity, Antifungal Activity

I. INTRODUCTION

Cancer is a dreadful and some believe it's a non-curable disease [1]. The chemotherapy is the treatment for killing the cancer cells without toxic effect on the host. Researchers found different natural compounds, and are used to improve the efficiency of chemotherapeutic agents by decrease the resistance of cancer cells to chemotherapeutic drugs and alleviate the adverse effects of chemotherapy. Recently scientist focus on marine environment, especially marine sponge to find out novel natural compounds with anticancer properties. Marine sponges are potential sources of many unique metabolites, including cytotoxic and anticancer compounds. Sponges are simple, multicellular, sessile animals with no true tissue layers or organs [2]. Marine sponges are rich sources of pharmacological active compounds (e.g., terpenoids, alkaloids, peptides, and polyketides). These compounds have wide range of biotechnologically relevant properties (e.g., anticancer, antibacterial, antifungal, antiviral, anti-inflammatory and antifouling [3]. In this study, to evaluate the biological effect of methanol extracts of marine sponge (*Dendrilla membranosa*) and find new anticancer and antibacterial metabolites.

II. METHODS AND MATERIAL

2.1. Collection and Identification of Marine Sponge

Sponge was collected by scuba divers from Vizhingam coastal region. The samples were frozen

and stored at -20°C and sent to Vizhingam Research Centre of central marine fisheries research institute (ICAR), Thiruvananthapuram, India for species identification.

2.2. Preparation of Crude Extracts from Sponges

The marine sponge was homogenized in a blender with little water and extracted with MeOH (2×1 l.) at room temperature. The combined methanolic extract was filtered and concentrated under vacuum on a rotary evaporator at low temperature to get crude methanolic extract [4].

2.3. Anti-Microbial Activity of Sponge Extract Against Pathogenic Bacteria

The antimicrobial screening was carried out by Kirby-Baeur agar disc diffusion method [5]. Pathogenic bacteria (gram positive and gram negative) and fungal cultures were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, and Chandigarh. The filter paper discs of uniform size (6mm) are impregnated with specified concentrations of methanolic sponge extract and then placed on the surface of an agar plate that has been seeded with the organisms to be tested. Microbial colonies were allowed to grow overnight at 37° C, then the inhibition zone around the disc was measured.

2.4. Determination of MIC

The MIC was determined by broth dilution method [6]. The multidrug resistant Bacillus megaterium, Staphylococcus aureus and streptococcus pyogens were used for determination of MIC. The 96 well microtitre plates were filled with 0.1 mL of varying concentration of active fractions prepared in Muller Hinton Broth with culture was added to it. The microtitre plates were incubated at 37ºC for 18 hrs. One row served as positive control (antibiotics) and one as negative control (methanol). After incubation, the OD was read at 610 nm in an ELISA reader.

2.5. Identification of Bioactive Compound Present in Sponge by GC-MS Analysis

Mass spectrometry analysis was performed on a Shimadzu GC 17A QP 5000 MS coupled with a mass detector, fitted non – polar DB-5 (Di phenyl Di methyl siloxane). Capillary column of length 25m X 0.25mm Id. GC – MS operation conditions used are initial temperature 60ºC – 300ºC with the injection temperature at 260°C and detector temperature at 300ºC. The injection volume was 0.1µl with helium gas as carrier at the flow rate of 0.6 ml per minute. Relative Retention times (RRts) of constituents were determined using C5 – C30 straight chain alkenes as standards. Individual constituents of the extract were identified by WILEY11 and NIST database matching by comparison of mass spectra with published data and by comparison of their RRts.

2.6. Cytotoxic test

An in vitro cytotoxicity test using Test on extract method was performed with test sample based on ISO 10993 – 5. Extract was prepared by incubating test material with medium containing serum at 37±2ºC for 24 – 26 hours at an extraction ratio of 0.1g/ml. 100% extracts were diluted to get concentrations of 50% and 25% with media. Different dilutions of extract of test sample, negative control and positive control triplicate were placed on sub confluent monolayer of L – 929 cells. After incubation of cells with extracts of test sample and controls at 37± 2ºC for 24±1hour, cell culture was examined microscopically for cellular response. Cellular responses were scored as 0, 1, 2, 3 and 4 according to none, slight, mild moderate and severe based on USP 28. Extracts of negative control gave none cytotoxic response and positive control gave severe cytotoxic response as expected.

2.7.MTT assay

The MTT assay was performed to measure the metabolic activity of cells to reduce yellow coloured tetrazolium salt 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to purple colored formazan. Material extract was prepared by incubating test material with culture medium containing serum at 37 ±2ºC for 24 to 26 h at an extraction ratio of 0.1g/ml. The extract (100 %) was diluted to 50 % and 25 % with culture medium. 100 % extract prepared using HDPE was considered as negative control. Extract and control medium were added to sub confluent monolayer of L-929 cells in triplicate in a 96 well culture plate and incubated at 37 ± 2 for 24 ± 2 h. Extract and control medium was replaced with 200 µl fresh culture medium to which 50 µl MTT (10 mg/ml in serum free C for 2h. After discarding the medium) was added. Cells were incubated at 37 MTT medium, 200 µl of isopropanol was added to all wells and mixed. The colour developed was quantified by measuring absorbance at 570 nm using a microplate reader (Biotek). The MTT Assay of L929 cells after contact with 100%,50% and 25% extract of material showed 91.62%,109.3%109.5%, metabolic activity respectively.

III. RESULTS AND DISCUSSION

3.1. Antimicrobial Activity of Sponge Extract against Pathogen

The antimicrobial activity of the methanol extracts of Dendrilla membranosa was determined against eight bacteria (Bacillus megaterium, Staphylococcus aureus, Streptococcus pyogens, Bacillus subtilis, Escherichia coli, Salmonella typhi, Pseudomonas aeroginosa, Shigella species) by Kirby Bauer Agar disc diffusion method. Among the 8 species of microorganisms, Dendrilla membranosa methanolic extract showed extremely antimicrobial activity against Staphylococcus aureus (18.7mm) and minimum activity against Pseudomonas aeroginosa (7mm) (Table1).

Table 1 : Antimicrobial Activity of Sponge Extract

SI. No.	Microorganisms	Zone of Inhibition (mm)
	Gram Positive bacteria	
1	**1) Bacillus megaterium**	9.3
2	Staphylococcus aureus	18.7
3	Streptococcus pyogens	14.2
4	Bacillus subtilis	7.2
	Gram Negative bacteria	
5	Escherichia coli	12.3
6	**2) Salmonella typhi**	11.7
7	Shigella species	10.5
8	Pseudomonas aeroginosa	7

3.2.MIC

The active antimicrobial extracts of Dendrilla membranosa were tested against Bacillus megaterium, Stapylococcus aereus and Streptococcus pyogens in order to determine the MIC and MBC. The 7µl dilution of crude extract of D.membranosa showed minimum inhibitory concentration (in OD) against Bacillus megaterium (0.007nm), Staphylococcus aereus (0.045nm) and Streptococcus pyogens (0.082 nm). The optical densities of all the tubes were detected at 520nm by using nutrient broth as suitable blank are recorded Table 2.

Table 2 : Minimum Inhibitory Concentrations (MIC) of sponge extract

Dendrilla membronosa Dilution (µl)	Bacillus megaterium OD 520nm	Staphylococcus aereus OD 520nm	Streptococcus pyogens OD 520nm
3	1.980	1.556	1.676
4	1.967	1.432	1.532
5	1.843	1.238	1.438
6	1.501	1.134	1.334
7	0.007	0.045	0.082

3.3. Identification of Bioactive Compound from Sponge by GC-MS Analysis

In the present study, different bioactive compounds were identified from the marine sponge. The chromatogram of extract was presented in the Figure 1. In sponge extract Dendrilla membranosa, 1, 2-benzenedicarboxylic acid, bis-2 was more abundant (24.94%), with retention time of 30.84. The analysis showed the presence of 13 different compounds Table 3.

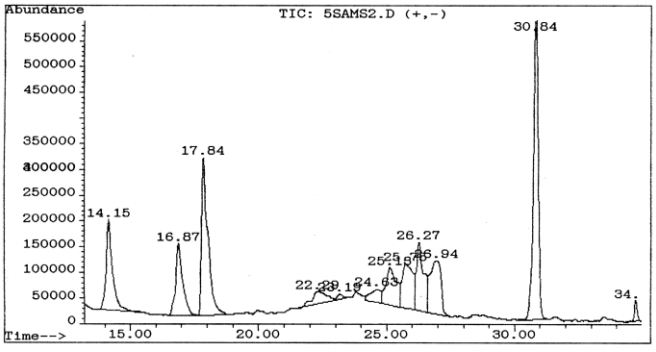

Figure 1 : Gas chromatogram of ethyl acetate crude extract of Dendrilla membranosa

Table 3: Identification of compounds through GC-MS analysis

Number of peaks	Retention time (min)	Compounds	Abundance (%)
1	12.98	Ethane	1.92
2	14.13	Not detected	9.49
3	16.88	Butane	8.38
4	17.83	formate pentane	16.63
5	22.30	Alpha d-galactopyranoside	3.04
6	23.19	2-Ethylhexyl2-ethylhexanoate	0.31
7	24.63	Hexahydro hexitol	2.26
8	25.13	Styrene	6.85
9	25.75	Alpha-d glucopyranoside	7.87
10	26.27	1-pentyl-2-proppyl-1-octene	8.03
11	26.94	Octanoic acid	9.82
12	30.84	1,2-benzenedicarboxylic acid, bis-2	24.94
13	34.77	nonadecane	0.97

3.4 Cytotoxicity of Dendrilla membranosa

The extract of Dendrilla membranosa was prepared by incubating test materials with culture medium containing serum at 37±2^{0}C for 24 – 26 at an extraction ratio of 0.1g/ml. The test material showed none cytotoxic response to fibroblasts cells at the above extraction ratio are recorded in Table 4.

Table 4 : Qualitative evaluation

SI No	Sample	Cytotoxicity scale	Interpretation
1	Negative Control	0	None Cytotoxic
2	Positive Control	4	Cytotoxic
3	Test Sample	0	None Cytotoxic

3.5. MTT Assay

The MTT assay of L929 cells after contact with 100%, 50% and 25% extract of Dendrilla membranosa showed 91.62%, 109.3% and 109.5%.

Table 5 : Metabolic activity

Sample	OD at 570nm	Percentage of Activity
Positive control - Phenol	0.0798	8.00
Negative - HDPE	0.9316	Nil
100% of sample	0.7726	82.93
50% of sample	0.874	93.81
25% of sample	0.948	116.92

IV.CONCLUSION

There is an increasing demand for therapeutic drugs from diverse natural resources. After many years of extensive research, the importance of marine sponge as source of valuable bioactive compounds has been very well established and exploited. Marine sponges are produced potent antimicrobial and anticancer extracellular products. These extracellular products (eg: -terpenoids, alkaloids, peptides, and polyketides) are used as the medicine to cure human disease [7]. As a result, marine sponge metabolites have now become the main focus of drug discovery research.

The antimicrobial activity of the methanol extracts of Dendrilla membranosa was determined against eight bacteria (Bacillus megaterium, Staphylococcus aureus, Streptococcus pyogens, Bacillus subtilis, Escherichia coli, Salmonella typhi, Pseudomonas aeroginosa, Shigella species) by Kirby Bauer Agar disc diffusion method. It revealed that, the extracts showed potent antibacterial activity against Streptococcus pyogens, Staphylococcus aureus, and Escherichia coli. This indicates that marine sponges remain an interesting source of new antibacterial metabolites with better activity then some antibiotics. This is not surprising because the sponges belonging to this genus possess a wide variety of compound with different biological activities [8,9,10]. The extract of sponge Latruniculia apicalis and Haliclona sp. shows antimicrobial activity against terrestrial organisms [11].

The active antimicrobial extracts of Dendrilla membranosa were tested against Bacillus megaterium, Stapylococcus aereus and Streptococcus pyogens in order to determine the MIC. Pettit et al [12] examined the antibacterial activity of the nitrogen heterocyclic sponge constituent cribrostatin 6. It was bacteriostatic for a variety of Gram positive species and was bactericidal for the majority of clinical isolates of Streptococcus pneumoniae, including penicillin-resistant strains. Minimum inhibitory concentration (MIC) ratios were 2 for 75 % of Streptococcus pneumoniae clinical isolates.

In the present study, 12 different bioactive compounds were identified from the marine sponge Dendrilla membranosa by GC-MS and majority of them show antimicrobial, antitumor and anticancer properties. The compounds are Ethane, Butane, formate pentane, Alpha d-galactopyranoside, 2-Ethylhexyl2-ethylhexanoate, Hexahydro hexitol, Styrene, Alpha-d glucopyranoside, 1-pentyl-2-proppyl-1 octene, Octanoic acid, 1,2-benzenedicarboxylic acid, bis-2 and nonadecane. The 1,2 benzenedicarboxylic acid, bis-2 was more abundant (24.94%), with retention time of 30.84. The anticancer properties of marine sponges might be due to the presence of the active secondary metabolites such as alkaloids and quinine derivatives. 1,2 benzenedicarboxylic acid, bis-2 has shown activity as growth inhibitors of several tumor cell lines, in particular the L929 cell line, being 1,2 benzenedicarboxylic acid, bis-2 the most active metabolite.

This investigation reveals a detailed schematic isolation and identification of bioactive compounds from Dendrilla membranosa. This information may

help to develop potential purified bioactive compounds in the pharmaceutical industry for the development of drugs. With the advanced molecular biological tools, target-oriented screens have become available that will accelerate the quest for new sponge-derived drugs.

Conflict of interest statement

We declare that we have no conflict of interest

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