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RESEARCH ARTICLE

IDENTIFICATION OF BIOACTIVE SECONDARY METABOLITES FROM MARINE SPONGE AURORA GLOBOSTELLATA BY HYPHENATED ANALYTICAL METHODS

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ABSTRACT

Two- third of the earth's surface is covered by water especially marine water which provides rich sources of flora and fauna. The marine environment which contains a vast array of organisms with unique biological properties is one of the most underutilized biological resources. There is an ever increasing demand for new lead compounds in the pharmaceutical industry and this has led scientists to search for natural bioactive products. Among various marine species the Sponges (Porifera) are said to possess higher cytotoxic molecules as the secondary metabolites in them. These metabolites are produced by the Sponges and they create surface around them to defend themselves from predators. There is high number of available drugs for cancer, but still there is a growing need to develop more specific compounds for treatment. This study is focused on *Aurora globostellata* its collection, taxonomic identification, extraction, phytochemical analysis, and identification of compounds through GC-HRMS and HR-LCMS. The results obtained from analysis were found to contain higher number of anticancerous compounds, which can be used as a lead molecule for various cancer therapies. These drugs from natural sources can overcome the commercial drugs and can be used for the treatment of cancer.

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INTRODUCTION

Sponges are animals of the phylum Porifera**,** an early branching event in the history of animals separated the Sponges from other metazoans. Based on their phylogenetic position, fossil Sponges are among the oldest known animal fossils, dating from the Late Precambrian. Approximately 5000 living Sponge species are classified in the phylum Porifera, which is composed of three distinct groups: the Hexactinellida (glass Sponges), the Demospongiae, and the Calcarea (calcareous Sponges) (Dembitsky *et al.,* 2002) *(*Dembitsky *et al.,* 2003) Since 1950, over 3000 papers have been published reporting the structures of more than 5500 metabolites from Sponges (Aygun *et al.,* 2000*)* Sponges, as primitive filter-feeders, have a high frequency of bioactive components for their chemical defenses against environmental stress factors such as predation, overgrowth by fouling organisms or competition for space. Studies have shown the highest concentration of antioxidant Sponge metabolites are found in habitats such as coral reefs. (Proksch *et al.,* 1994) Marine Sponges have been considered as a very fertile field for the discovery of bioactive natural chemical substances with respect to the primary and secondary chemical components for the past decades (Higa *et al.,* 1994). Sponges produce high level of chemicals by emitting mucus containing toxins to push back other invading predators. They use this toxin as a poison to prevent themselves without any destruction to their body. (Baker *et al.,* 2007) (Newman *et al.,* 2007,2012). It was proved that marine Sponges produce an enormous array of antitumor, antiviral, anti-inflammatory, immunosuppressive, antibiotic, and other bioactive molecules that have the potential for therapeutic use. Most bioactive metabolites from Sponges are already been proved to be inhibitors of certain enzymes (Frota *et al.,* 2012), (Villa *et al.,* 2010) More than 15,000 marine products have been isolated and tested, in 2011 number of natural products was 1152 out of which 269 compounds have been reported till now. The chemical diversity of Sponge natural products is remarkable, including unusual nucleosides, bioactive terpenes, sterols, cyclic peptides, alkaloids, fatty acids, peroxides, and amino acid derivatives (Blunt JW *et al.,* 2013). The sterols and terpenes are said to be present in higher amount in Sponges which exhibit wide range of biological activities mainly anticancerous activity (Bhakuni, 2005) The study is focused on the Sponge *Aurora globostellata* also

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known as *Rhabdastrella globostellata* and was first described by Henry J. Carter as *Stelleta globostellata* in 1883, named after the globostellate shape of its spicules (Latin globus meaning "sphere" and stellatus meaning "star-shaped"). It is a yellow pot Sponge of the order *Astrophorida,* and is native to many regions of the Indian Ocean . The Sponge *Aurora* has been shown to produce a wide variety of isomalabaricanes, a type of triterpene molecules with notable cytotoxic activity towards certain cancer cell lines (Li, D-D *et al*., 2010). Hence this species is taken for the present research. Bio-discovery is the extraction and testing of molecules for biological activity, identification of compounds with promise for further development, and research on the molecular basis for the biological activity. Many research papers describe the antibacterial, antimicrobial, anticancer, antitumor and antiviral activities of marine Sponge secondary metabolites. Due to the presence of terpenoids in Sponges they are said to exhibit cytotoxicity. (*Stamatios Perdicaris et al.,* 2013)

In this investigation the compounds from marine Sponges are extracted and the bioactive compounds present in them are found by GC-HRMS and HR-LCMS where the compounds with medicinal properties are identified, which can be used as a lead molecule for drug discovery.

The compounds obtained from natural sources will be of fewer side effects when compared with that of the commercial drugs. These compounds provide an insight for drug development in cancer research.

MATERIALS AND METHODS

Collection of sample

Sponge samples were collected as entangled specimens from a bottom trawl fishing net and from low inter tidal pools at Rameswaram coast (9°28′N, 79°12′E) at 4-5 m depth. Samples were collected in bulk depending on the abundance of individual organisms and washed with freshwater to remove adhering debris and associated biota. Collected samples were stored in a refrigerated box and transferred to the lab and stored at -70°C. From the Sponges, tissue samples were incised out and 100 g was taken, air dried and sent for taxonomic identification.

Identification of sample

The taxonomic identification of the organisms was done at Zoological Survey of India, by Dr.G.Sivaleela, Scientist, Marine Biology Regional Centre, Chennai. The Spicules identification was done using nitric acid digestion following standard identification keys (Hooper, Thomas). The Sponges were identified, registered and deposited in National Zoological collections of MBRC/ZSI.

Preparation of Sponge extracts

Prior to the extraction, samples were washed with water, cleaned, air dried, lyophilized and powdered. They were stored for further use (Valentin Bhimba *et al*., 2013). For the extraction of crude bioactives, 100 g of powdered material was exhaustively extracted with 200 ml of ethanol using Soxhlet apparatus and concentrated in a rotary evaporator at reduced pressure (Wang *et al*., 2009). The concentrated extract was used for phytochemical analysis and for isolation of secondary metabolites present in the Sponge sample.

Preliminary screening of Sponges for chemical constituents

Qualitative analysis was carried out using methods described by Harborne (1998).The freshly prepared Sponge extracts were analyzed for the presence of various constituents as described by Chairman *et al*., 2012

GC-HRMS Analysis

GC-HRMS and HR-LCMS analysis is done for finding both the volatile and non volatile compounds present in the Sponges. Gas chromatograph analysis was carried out on a Agilent 7890 (FID Detector, Head space injector, combipal auto sampler) and Mass spectrometer AccuTOF GCV model (EI/CI source, Time of Flight Analyser, Mass range-10-2000 amu, mass resolution-6000). The analysis was carried out at SAIF, IIT, Bombay. The fragmentation patterns of mass spectra were compared with those stored in the spectrometer database using National Institute of Standards and Technology Mass Spectral database (NIST-MS).

HR-LCMS Analysis

High Resolution Liquid Chromatograph Mass Spectrometer 1290 Infinity UHPLC System, 1260 infinity Nano HPLC with Chipcube, 6550 iFunnel Q-TOF. (Mass range 50-3200amu, resolution-40000 FWHM, Ionisation method–API:ESI positive and negative, APCI positive and negative, UHPLC PDA Mass spectrometer). The analysis was carried out at SAIF, IIT, Bombay.

RESULTS

There is an increasing demand for therapeutic drugs from diverse natural resources. After many Years of extensive research, the importance of marine organisms as source of valuable bioactive compounds has been very well established and exploited. As a result, the ocean and metabolites of marine organisms including associated microorganisms has now become the main focus of Drug discovery research (Fenical *et al*., 1994) A conceptual progress in marine environment occurred with the study of Thakur and Anil (2000, 2003) suggesting that marine Sponges and their symbionts (bacteria and fungi) produce various bioactive compounds against invading predators. The discovery of nucleosides spongothymidine and spongouridine in the marine Sponge Cryptotethya crypta, the synthesis of Ara C, the first marine derived anticancer agent and the antiviral drug Ara A was possible (Proksch *et al*., 1994)

Taxonomic classification of Sponges

In the present study the Sponges collected from Rameswaram coast was taxonomically identified from Zoological survey of India. Based on the spicules arrangement the Sponge species were identified using identification keys (P.A.Thomas).

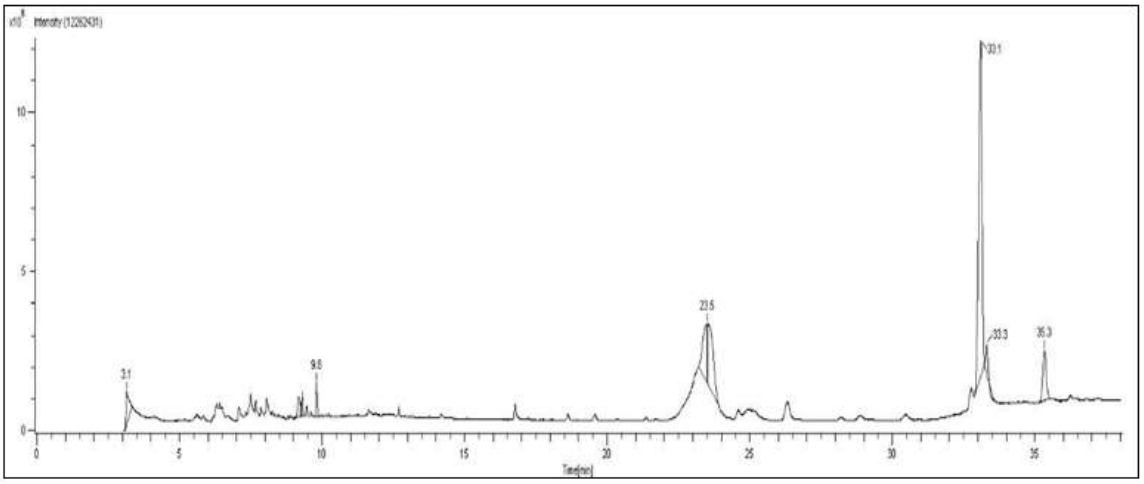

Fig.1. GC-HRMS spectral analysis

GC-HRMS Spectral analysis of the Sponge *Aurora globostellata*

Compound name	Molecular formula	Retention time
1,3-Di (propen-1-yl) adamantane	$C_{10}H_{16}$	9.2
Piperidine,3,4- didehydo -2,2,6,6-tetramethyl-4-(4-hydroxyphenyl)	$C_5H_{11}N$	9.2
Ethyl oleate	$C_{20}H_{38}O_2$	9.3
9-octadecanoic acid ethyl ester	$C_{20}H_{38}O_2$	9.3
Nonadecanoic acid, ethyl ester	$C_{21}H_{42}O_2$	9.8
Octadecanoic acid, 9-methyl-,ethyl ester	$C_{21}H_{42}O_2$	9.8
Squalene	$C_{30}H_{50}$	16.8
Geranylgeraniol	$C_{20}H_{34}O$	16.8
Dodecanoic acid,1,2,3-propanetriyl ester	$C_{39}H_{74}O_6$	23.5
Dodecanoic acid,1-(hydroxymethyl)-1,2-ethanediyl ester	$C_{27}H_{52}O_5$	23.5
Cholestrol	$C_{27}H_{46}O$	24.6
17-(1,5-Dimethylhexyl)-10,13-dimethyl-tetradecahydro-1H-cyclopenta(a)phenanthren-3-ol	$C_{27}H_{44}O_2$	24.6
β-sitosterol	$C_{29}H_{50}O$	32.8
γ-Sitosterol	$C_{29}H_{50}O$	32.8
Cholest-5-en-3-ol,24-propylidene-,(3β)-	$C_{30}H_{50}O$	33.3
Stigmasta-5,24(28)-dien-3-ol,(3β)-	$C_{29}H_{48}O$	33.3
24-methyl-5,28-stigmastadien-3β-ol	$C_{30}H_{50}O$	35.3
Gorgost-5-en-3-ol, (3β)-	$C_{30}H_{50}O$	35.3

Nearly 7 species were identified and all 7 of them belong to class demospongiae. The Sponges *Axinella Durissima* (NZC/MBRC/S.256), *Cliona celata* (NZC/MBRC/S.257), *Sigmadocia fibulata* (NZC/MBRC/S.258), *Sigmadocia pumila* (NZC/MBRC/S.259), *Fasciospongia cavernosa* (NZC/MBRC/ S.260), *Aurora globostellata* (NZC/MBRC/S.261), *Oceanapia sagittaria* (NZC/MBRC/S.262) were identified and deposited in National Zoological Collections of MBRC/ZSI. Out of the seven species *Aurora Globostellata* is taken for the present study due to the abundance of this species and this species was found to have high cytotoxicity. The Sponge *Aurora globostellata* belongs to Class- *Demospongiae*, Order-*Astrophorida*, Family-*Ancorinidae*, Genus-*Aurora*, Species-*Aurora Globostellate*.

The Sponge specimens was cut into small pieces, air dried and powdered.

The extraction was done using ethanol as solvent. The crude bioactives was extracted using soxhlet apparatus, the extract was then evaporated under reduced pressure to yield viscous dark gum which is stored in vials for future use. The qualitative analysis was done using Harborne (1998) method, from the analysis the Sponge *Aurora globostellata* was found to contain steroids, triterpenoids, alkaloids, saponins, flavanoids, aromatic acids and tannins (Regina R. Monaco *et al*., 2014). The GC-HRMS chromatogram of ethanolic extract of Sponge *Aurora globostellata* was found to have 6 major peaks, the compound list is given in the table. The active fraction on the basis of spectral data by GC-HRMS were found to be mixture of fatty acids, steroids and flavanoids which were observed on different retention time. The results showed that the main constituents of the Sponge *Aurora globostellata* exhibits antibacterial, anti-inflammatory and anticancerous activity.

Among the 18 compounds, most of them were found to possess anticancerous activity. The compound Squalene found in the Sponge *Aurora globostellata* is a triterpene that contains six isoprene units. It is also present in olive oil at concentrations between 0.2 – 0.7 %. It is a key intermediate in the biosynthetic pathway to steroids in plants and animals (McCance and Widdowson's., 1976). Due to the presence of Squalene compound in olive oil there are reduced risks for several cancers for those people with olive oil intake (Alvaro L. Ronco *et al*., 2003) The compound geranylgeraniol is a diterpene

found in linseed oil, *Cedrela toona* wood oil, sucupira branca fruit oil and more recently, annatto seed oil, was found to suppress the viability of human DU145 prostate carcinoma cells via cell cycle arrest at the G1 phase and the initiation of apoptosis (Murakoshi *et al*., 1992)

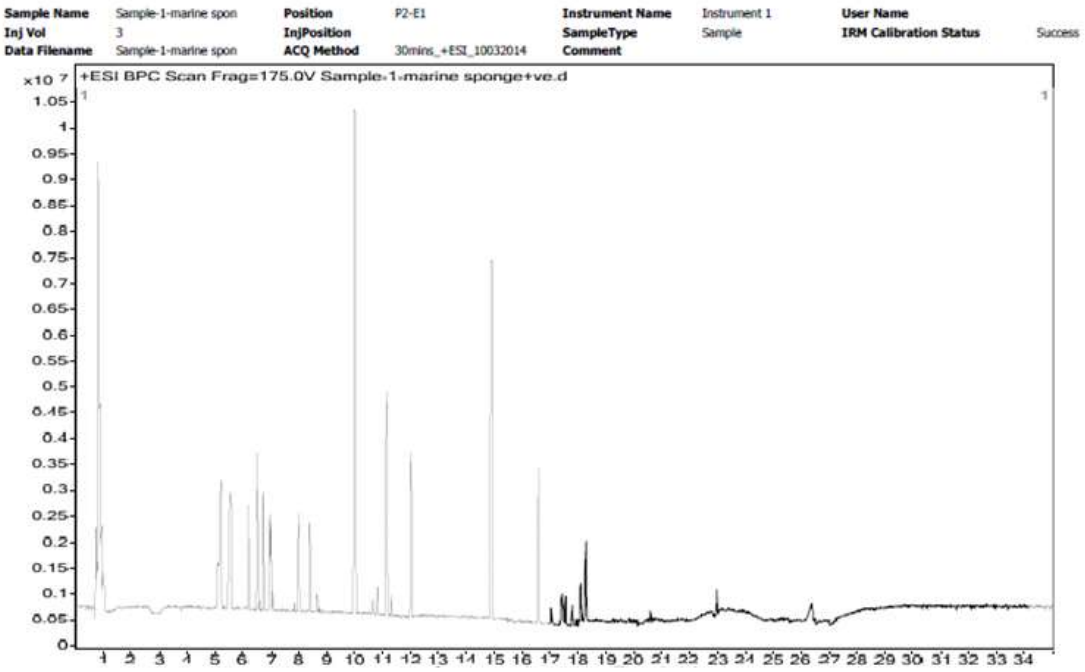

Fig.2. HR-LCMS spectral analysis

HR-LCMS Spectral analysis of the Sponge *Aurora globostellata*

Compound name	Molecular formula	Retention time
Mandelic acid	$C_8 H_8 O_3$	0.687
Caldine	$C_6 H_{17} N_3$	0.744
Leucine	$C_6 H_{13} N O_2$	0.779
Triparanol	$C_7 H_{13} N O_2$	0.786
Citrulline	$C_6 H_{13} N_3 O_3$	0.816
1,4-Methylimidazoleacetic acid	$C_6 H_8 N_2 O_2$	0.843
Methyl N-(a- methylbutyryl)glycine	$C_8 H_{15} N O_3$	0.854
L-Glutamic acid n-butyl ester	$C_9 H_{17} N O_4$	0.854
Triparanol	$C_7 H_{13} N O_2$	0.86
Barbital	$C_8 H_{12} N_2 O_3$	0.865
p-Cymene	$C_{10} H_{14}$	0.872
DL-2-Aminooctanoic acid	$C_8 H_{17} N O_2$	0.887
N-heptanoyl-homoserine lactone	$C_{11} H_{19} N O_3$	0.919
Arecoline	$C_8 H_{13} N O_2$	0.932
Iproniazid	$C_9 H_{13} N_3 O$	0.934
Pyroglutamic acid	$C_5 H_7 N O_3$	0.934
1,4-Methylimidazoleacetic acid	$C_6 H_8 N_2 O_2$	1.06
3-methyl-tetradecanedioic acid	$C_{15} H_{28} O_4$	4.167
Carbidopa	$C_{10} H_{14} N_2 O_4$	4.664
Metaproterenol 3-O-sulfate	$C_{11} H_{17} N O_6 S$	5.148
Phenylethylmalonamide (PEMA)	$C_{11} H_{14} N_2 O_2$	5.205
Dexfenfluramine	$C_{12} H_{16} F_3 N$	5.535
N-(2-fluro-ethyl) arachidonoyl amine	$C_{22} H_{36} F N O$	5.908
Ile Val Val	$C_{16} H_{31} N_3 O_4$	6.209
Oxybutynin	$C_{22} H_{31} N O_3$	6.269
Val His Phe	$C_{20} H_{27} N_5 O_4$	6.279
Indoleglycerol phosphate	$C_{11} H_{14} N O_6 P$	6.594
Nafronyl	$C_{24} H_{33} N O_3$	7.009
Cephalotaxine	$C_{18} H_{21} N O_4$	7.851
Guanidino-Acetic Acid	$C_3 H_7 N_3 O_2$	7.994

The compound nonadecanoic acid is a fatty acid which is an intermediate in the biodegradation of n-icosane, which has been shown to inhibit cancer growth. The compound Piperidine is an alkaloid which acts as an inhibitor and was shown to have cytotoxicity against MCF-7 and A-549 cell lines.

This study suggested that compounds from marine Sponge extracts can be used to develop a good anticancer-agent, since marine Sponge *Aurora globostellata* was found to contain higher amount of cytotoxic compounds.

The nonvolatile compounds from the marine Sponge *Aurora globostellata* was analyzed using HR-LCMS and the chromatogram of active fractions are given. From the HR-LCMS analysis *Aurora globostellata* was found to contain aminoacids, fatty acids, vitamins, steroids and flavanoids as the metabolites in them. Majority of the compounds possess biological activity which can be used as a source in production of drugs for many diseases.

Glycocyamine (guanidinoacetic acid - GAA) is the biochemical precursor of creatine, which is phosphorylated and plays an important role as a high-energy carrier in the muscle. It can be administered in liquid solutions, such as sports drinks, it could be hypothesized that GAA could easily enhance creatine biosynthesis with clear physiological effects yet to be determined. Cephalotaxine is an alkaloid and also inhibitors of nucleic acid associated enzymes and proteins, and agents affecting structural proteins and cytoplasmic enzymes. Nafronyl is a drug used in the management of peripheral and cerebral vascular disorders as a vasodilator (Setoue *et al*., 2008)

p-Cymene is a volatile hydrocarbon that occurs in anise, coriander, cumin, mace, oregano and eucalyptus, which has high medicinal values. Many aminoacids are found to be

present in the HR-LCMS analysis when compared with the GC-HRMS.

DISCUSSION

Marine Sponges have been excellent sources of natural products that are biologically active which include the enzyme inhibitors, cell division-inhibitors, antiviral, antifungal, antimicrobial, antiinflammatory, antitumour, cytotoxic or cardiovascular properties. The marine environment is a rich source of useful compounds with new chemical structures and significant pharmacological effects. For example, the arabinose-nucleosides from Sponge *Cryptotethya crypta*, are used clinically with antiviral and anticancer activity; manoalide obtained from Sponge *Luffariella variabilis* is a candidate with anti inflammatory activity. (Baby Joseph *et al*., 2011) Some of the Sponge-derived bioactive compounds presently available in the market are Ara-A (antiviral), Ara-C (anticancer) and Manoalide (phospholipase A2 inhibitor), while IPL512602 (antiinflammatory), KRN 7000 (anticancer), LAF389 (anticancer), Discodermolide (anticancer) and HTI286 (anticancer) are under clinical trial (Jimenz, *et al*., 2000; Nishimura *et al*., 2003). The presence of steroids and several novel molecular entities potentially were able to target COX-1, COX-2 and the NF-κB pathway. In this study the extracts of marine Sponge *Aurora globostellata* exhibited broad spectrum of anticancerous activity.

Ethanol was better solvent for extracting the bioactive metabolites due to the solubility of the compounds in ethanol. The differences with the compounds isolated from our sample differs from those obtained in the previous studies may be due to the time of sample collection, place of collected material and method of extraction. Phytochemical studies, GC-HRMS and HR-LCMS analysis indicated the presence of fatty acids, steroids, alkaloids and terpenoids as the major components in the ethanol extract which has been given in table. A large number of triterpenoids are known to exhibit cytotoxicity against a variety of tumor cells, as well as anticancer efficacy in preclinical animal models. Thus the Steroids, terpenoids and fattyacids might be the active compounds responsible for anticancerous activity in *Aurora globostellata*. The results of compounds obtained from HR-LCMS showed the presence of more aminoacids, and few alkaloid compounds. The cytotoxic compounds were found to be less when compared with the compounds from GC-HRMS analysis.

Conclusion

In our study, the ethanolic extract of the Sponge *Aurora globostellata* has shown to have strong cytotoxic activity. Almost all the volatile and nonvolatile compounds of the Sponge *Aurora globostellata* are found from the GC-HRMS and HR-LCMS analysis. Further the compounds can be isolated by bioassay guided fractionation *in vitro* assay. The presence of steroids, alkaloids and terpenoids in combination can act as bioactive compounds for cancer. The compound squalene which is present in marine Sponge *Aurora globostellata* was found to have high medicinal properties, and it also plays a vital role in cholesterol synthesis. Many researchers have found that those Sponges which contain triterpenoids were shown to have cytotoxic activities. The Sponge *Aurora globostellata* was also found to have higher amount of triterpenoids and isolation of the triterpenoids through column chromatography or by TLC based purification methods may come up with the bioactive compound. This can act as a good lead molecule for discovering drugs for the treatment of various types of cancer. The isolated compound can be tested for cancer against various cell lines through MTT assay. The compounds from *Aurora globostellata* can be docked using bioinformatics software and tools, against various targets for cancer. These protein-ligand interaction energies can be compared with the existing chemical drugs presently used for cancer. The Sponges are gold mines due to the presence of bioactive compounds in them, which can lead a long way in drug discovery.

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