BIOPROSPECTING OF NOVEL POTENT ANTICANCER METABOLITES FROM CHOSEN MARINE SPONGES

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For the award of the degree of

DOCTOR OF PHILOSOPHY

By

MOSES SAMUEL RAJAN
Reg. No: 4488

Through

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PALAYAMKOTTAI

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Dr. B. Xavier Innocent, ***M.Sc., M.Phil., Ph.D***
Associate Professor and Head
Research Department of Zoology
St. Xavier's College (Autonomous)
Playamkottai, Tirunelveli - 2

CERTIFICATE

This thesis entitled **"Bioprospecting of Novel Potent Anticancer Metabolites from Chosen Marine Sponges"** submitted by **Moses Samuel Rajan** (Reg. No. 4488) for the award of degree of Doctor of Philosophy in Pharmacology - Zoology Interdisciplinary of Manonmaniam Sundaranar University is a record of bonafide research work done by him and it has not been submitted for the award of any degree, diploma, associateship, fellowship of any University/ Institution.

Place :

Date :

Supervisor and Guide

Dr. V. Aldous. J. Huxley, ***M.Sc., M.Phil., Ph.D***
Assistant Professor and DST- SERB young Scientist
Biotech Research Laboratory, Department of Zoology
Thiru. Vi. Ka. Government Arts College
Thiruvarur - 3

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Place :

Date :

Co-Guide

Moses Samuel Rajan (Reg. No. 4488)
Research Scholar,
Department of Zoology,
Thiru. Vi. Ka. Government Arts College
Thiruvarur - 3

DECLARATION

I hereby declare that the thesis entitled "**Bioprospecting of Novel Potent Anticancer Metabolites from Chosen Marine Sponges",** submitted by me for the degree of Doctor of Philosophy in Pharmacology - Zoology Interdisciplinary is the result of my original and independent research work carried out under the Guidance of Dr. B. Xavier Innocent, Associate Professor and Head, Research Department of Zoology, St. Xavier's College (Autonomous), Playamkottai, Tirunelveli - 2, and it have not been submitted for the award of any degree, diploma, associateship, fellowship of any University/Institution.

Place :

Date :

(Moses Samuel Rajan)

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MOSES SAMUEL RAJAN

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List of Abbreviation

MSMs	-	Marine Secondary Metabolites.
LD_{50}	-	Lethal Dose 50.
MTCC	-	Microbial Type Culture Collections.
NS	-	Normal Saline
WHO	-	World Health Organisaiton.
MIC	-	Minimum Inhibitory Concentration.
DPPH	-	1,1-diphenyl-2-picryl hydroxide
NMR	-	Nuclear Magnetic Resonance Spectroscopy
IR	-	Infra red
UV	-	Ultra violet
HPLC	-	High Performance Liquid Chromatography
P	-	Probability
CNS	-	Central Nervous System
GABA	-	Gamma Amino Butyric Acid
ED_{50}	-	Effective Dose 50
IC_{50}	-	Inhibitory Concentration
OECD	-	Organisation of Economics Cooperation Development
mg	-	Milligram
min	-	Minute
sec	-	Second
g	-	Gram
rpm	-	Revolution per Minute
v/v	-	Volume by Volume
NSAID	-	Non Steroidal Anti-inflammatory Drug
SEM	-	Standard Error Mean
MTCC	-	Microbial type Culture Collections
COSY	-	Correlation Spectroscopy
HMQC	-	Heteronuclear Multiple Quantum Coherence

PREFACE

Marine bioprospecting is a rapidly expanding enterprise that entails the investigation of the marine environment in search of novel biomolecules (Newman *et al .,* 2003; Mayer *et al .,* 2007). There is enormous potential for industrial development of marine resources, not only as pharmaceuticals, but also as nutritional supplements, molecular probes, cosmetics, fine chemicals, agrichemicals and paint ingredients. Researchers must also pay attention to the growing awareness of sustainable use of marine resources and to overcoming challenges, such as supplying sufficient biomass without disrupting ecosystems and modernizing coarse collection methods. The first commercialized marine biomolecule of medical interest was cephalosporin, isolated from the marine fungus *Cephalosporium acremonium* in 1948, which functioned as a template for the development of the early antibiotic agents (Newton and Abraham 1955). Since then, only a handful of drugs of marine origin has reached the market; however, there is currently an influx of promising drug candidates in phase II/III trials, such as the anticancerogenic compounds Ecteinascidin-743, Aplidine and Kahalalide F (Haefner 2003). Moreover, marine natural products that fail in clinical trials may still be introduced into the market as tools for biomedical research (Folmer *et al.,* 2007).

Secondary metabolites and host defence Secondary metabolites are assumed to have evolved from primary metabolites. Their biological roles have been debated, though the prevailing view is that they offer evolutionary advantages to the host organisms (Firn and Jones 2000). Secondary metabolites with adaptive characteristics would contribute to the survival of new strains (Faulkner 2000b). A widely accepted definition of secondary metabolites is “substances that are formed in organisms but that do not participate in those metabolic processes which are necessary for the life and development of the organism” (Samuelsson 2004). While secondary metabolism has different functions than primary metabolism, they can‟t be sharply distinguished

from one another. The genes coding for enzymes involved in secondary metabolisms are different, but the precursors are the same (Cavalier-Smith 1992).

Secondary metabolites in marine organisms, especially in invertebrates, are often attributed a defensive function, but reports of how they function in an ecological context is scarce (Hay and Fenical 1988;). The composition and type of compounds involved in the chemical defence can vary dramatically among geographic regions, habitats and between individuals in a local habitat, and even within a single individual (Hay 1996). It is currently assumed that tropical plants and invertebrates have more dynamic chemical defences than do their temperate counterparts, as the intense competition and predation in these species rich tropical areas have led to the evolution of a wider range of secondary metabolites (Bolser and Hay 1996). In the marine environment, secondary metabolites appear to be most common and most ecologically important in tropical benthic organisms subject to high rates of attack by consumers on coral reefs (Bolser and Hay 1996; Faulkner 2002). However, secondary metabolites also play important roles in temperate and polar benthic communities (Toth and Pavia 2007). Chemically defended organisms often produce multiple secondary metabolites, which opens up the possibility of synergistic or additive effects among various metabolites (Hay 1996). Marine secondary metabolites, such as terpenes, alkaloids and polyphenolics, can differ fundamentally from terrestrial secondary metabolites. Incorporation of halogen is a very characteristic feature, and many marine organisms possess chemical skeletons and structures not found in terrestrial organisms (Faulkner 2002). Secondary metabolites can have a wide range of activity, more commonly functioning as antifeedants, antifoulers, microbial pathogens, gamete attractants and trail markers. Marine secondary metabolites involved in the host defence are often active at minute concentrations; the compounds dilute rapidly when released into the surrounding water and must be extremely potent if they are to have the intended effect.

Over the last forty years, sponges (phylum Porifera) have been identified as an excellent source of unique marine natural products, having a higher incidence of biologically active compounds than any other single marine phylum. The high

incidence of bioactive compounds in these primitive filter feeders is likely related to their chemical defence against environmental stress factors. There is a strong correlation in sponges between the absence of physical defence mechanisms and the presence of unconventional biomolecules. Sponges in particular, and sessile marine invertebrates in general, are evolving as economically important sources of compounds.

The past decade has seen a dramatic increase in the number of lead compounds from diverse marine life entering preclinical and clinical trials. The largest group of new chemical entities of natural product origin has anticancer indications, followed by NCEs with antibacterial indications. Hitherto, NCI has conducted the most extensive screening of marine organisms for anticancer activity and, when investigated for antileukemic activity the sponges proved to be the largest source of NCEs with a hit rate of 8.7% (Cragg *et al.,* 2006). However there is a rapidly evolving recognition that a significant number of marine natural products are actually produced by symbiont microbes.

Table – 1; Examples of sponge-derived compounds for treating cancer in preclinical/clinical trials

Metabolite	**Disease area**	**Source sponge**
Halichondrin B	Cancer	*Halichondria okadai*
Mycaperoxide B	Cancer	*Mycale sp.*
KRN7000	Cancer	*Agelas mauritianus*
LAF389	Cancer	*Jaspis digonoxea*
Laulimalide	Cancer	*Cacospongia mycofijiensis*

The present study was indented to explore novel anticancer bioactive secondary metabolites from marine sponge collected from South peninsular coast of India was carried out with the following objectives.

- To screen active cancer drug from marine sources, especially sponges using different assay systems
- To determine the toxicity dose of sponge extract which is require to control and treat cancer
- To identify and characterize the bioactive leads from sponge, which is responsible for anticancer activity

REVIEW OF LITERATURE

Introduction

The term “pharmacognosie” was used for the first time, in 1811, in Lehrbuch der Materia Medica by Johann Adam Schmidt. The word is derived from the two Greek words pharmakon and gnosis, which mean “drug” and “knowledge”, respectively (Samuelsson 2004). The common and original definition of pharmacognosy is the scientific study of drugs derived from nature. However, the focus of pharmacognosy has shifted numerous times: the classical botanical aspects have successively been deemphasized in favour of a more modern pharmacognosy, dealing more with natural product chemistry and pharmacology (Bruhn and Bohlin 1997; Bohlin *et al.,*2007). An expanded model of pharmacognosy that incorporates a broad range of areas has recently been proposed (Larsson 2007).

Natural product chemistry

The rich structural diversity and complexity of natural products have resulted in numerous new drugs and inspired chemists to produce synthetic analogs with enhanced bioactivity (Faulkner 2000a). Although most of the drugs currently in development are results of either semi- or complete synthesis, natural products continue to play a significant role in the discovery and development of new pharmaceuticals, as was recently highlighted (Faulkner 2000a). Scientists have only scratched the surface of many unconventional natural product sources (Tulp and Bohlin 2004). Of all drugs developed between 1981 and 2006, 28% were either natural products or derived from them. Another 20% can be categorized as natural product mimics (NM). This label is somewhat controversial, as it can be interpreted as exaggerating the roles natural products play in NM development. However, one can argue that nature played an inspirational role in the initial development stage. Immunosuppression, antiinfection, oncology and metabolic diseases are regarded as the predominant therapeutic areas of natural product-derived drugs (Butler 2004).

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In the 1990s the pharmaceutical industry in practice selected lead compounds exclusively on the basis of Lipinski‟s rule of five, although the rule is not directly

applicable to natural products (Lipinski *et al.,*1997; Macarron 2006). As a consequence of the rigorous application of this rule, natural products were deprioritized or even eliminated from the drug discovery process. The concept of the discovery process has since then gradually changed and a renewed interest in natural products has resurged. Natural sources offer excellent opportunities for finding leads for novel targets (Tulp and Bohlin 2002). Today there is a growing awareness that natural productstend to occupy a wider chemical space than do synthetic compounds – a fact the scientific community is starting to take into account in the early drug discovery process (Feher and Schmidt 2003; Larsson *et al.,*2007).

Marine natural products

The ocean represents a unique resource providing a diverse array of natural products. The greatest biodiversity is found in ecosystems, such as rocky coasts, kelp beds and coral reefs, where species diversity and population density are exceedingly high (Haefner 2003). In fact, 34 of the 36 phyla of life are represented in the marine environment, implying chemical evolution along many separate lineages. Only a fraction of marine organisms have so far been investigated, but even so over 12,000 novel compounds have been discovered (Donia and Hamann 2003), with hundreds of additional compounds being discovered annually (Faulkner 2002). Properties in the marine environment force aquatic organisms to produce molecules that can differ substantially in structural terms from terrestrial substances. Physical conditions, such as lack of light, low temperature, high salinity or extreme pressure force marine organisms to produce secondary metabolites to overcome these obstacles.

Despite four decades of intense research, marine pharmacognosy is still considered a relatively young field comparedtoterrestrial pharmacognosy. Originally, pharmacognosy dealt exclusively with the study of drugs derived from terrestrial plants and animals. However, in the 1950s, marine organisms were identified as an excellent source of new biologically active compounds (Blunt *et al.,*2007). It was not until 20 years later, however, that systematic investigation of the marine environment was initiated. There are several profound

differences between the marine and terrestrial environments for which scientists must account, such as the sources of the natural products in question. Marine invertebrates and microorganisms have yielded substantially more bioactive natural products than seaweeds have, unlike the terrestrial environments, where plants are considerably richer in secondary metabolites (Proksch 1994; Faulkner 2002).

Several sessile invertebrates have intimate, symbiotic relationships with microorganisms and several natural products isolated from marine invertebrates are suspected to be of microbial origin; however scientific evidence for this is often scarce and incomplete (Proksch *et al.,*2002). The last decade of marine natural product research has witnessed a gradual shift of focus from invertebrates and other macroorganisms to microorganisms.

Cancer

Cancer, after cardiovascular disease, is the second leading cause of death in the world (Jemal *et al.,* 2007). Worldwide about 10 million people per year are diagnosed with cancer and more than 6 million die of the disease and over 22 million people in the world are cancer patients (Steward and Kleihues, 2003). It accounts for about 23 and 7% deaths in USA and India, respectively. The world‟s population is expected to be 7.5 billion by 2020 and approximations predict that about 15.0 million new cancer cases will be diagnosed; with deaths of about 12.0 million cancer patients (Brayand *et al.,* 2006). The prevalence of cancer in India is estimated to be around 2.5 million, with about 8, 00,000 new cases and 5, 50,000 deaths per annum (Nandakumar, 1990-96). According to 1991 Indian census data, about 609000 cancer cases have been observed. This number had drastically increased to 806,000 by the end of the last century; with 96.4 and 88.2% age standardized rates for males and females; out of 100,000 cases analyzed (Rao *et al.,*1998).

During last one decade, about 70% cancer cases have been diagnosed and treated with survival of a few patients (Dinshaw *et al.,* 1999). It is believed that in near future the number of cancer patients will increase in the developing and under developed countries, which may rise up to 70%; a serious issue for all of us. The

magnitude of cancer problem in the Indian Sub-continent (sheer numbers) is increasing due to poor to moderate living standards (Wynder *et al.,*1974) and inadequate medical facilities. Most frequently observed cancers in Indian population are of lungs, breast, colon, rectum, stomach and liver (Murthy *et al.,* 2004). Nowadays, India is growing with a good progress rate and probably will become a developed country within a few decades resulting into its participation in the world development. Therefore, it is important to study the status of cancers in India so that advance measures may be taken to control this havoc in near future. In view of these facts, attempts have been made to study the status of cancers in India including its causes, preventive measures, effect on Indian economy and comparison with global scenario.

Cancer scenario in India

Based on the increasing trends of cancer patients during the last few decades, the numbers of cancer patients have been predicted by the end of 2015 and 2020 in India. These compiled data show that the number of male, female and the total cancer patients in 2004 were 390809, 428545 and 819354 respectively. The number of male and female cancer patients increased continuously up to 2009, with 454842, 507990 and 962832 cases for male, female and total cancer patients, respectively. Similarly, 462408 male cancer patients and 517378 female cancer patients were recorded, with a total number of 979786 patients in 2010. Thus, it is clear from this Figure that the number of cancer cases has increased gradually with time. Moreover, a prediction of cancer patients in 2015 and 2020, respectively, has also been made (Parkin *et al.,*2000).

Predominant cancer types of India

Lung cancer was rare in the beginning of the last century (Parkin *et al.,*2000) but later on it was diagnosed in various patients. Banker *et al.,* (1955) reported about 9210 consecutive autopsies of lung cancer patients in 1970, which were 14.4% of all cancer types. But, nowadays, it has become almost epidemic resulting in greater

number of deaths than those caused by colorectal, breast and prostate cancers (Khuri *et al.,* 2001). The data collected by the National Cancer Registry Program of the Indian Council of Medical Research; from six different parts of the country including both rural and urban areas; showed varying degrees of incidence in different areas (ICMR, 1988- 89).The most common forms of malignancies in males during 1989 in Bombay, Delhi, and Bhopal were cancers of trachea, bronchi and lungs. These cancers were also reported in other cities in the order of Madras > Bangalore > Barshi. These sorts of cancers were rare in females except in Bombay and Bhopal, where they ranked at sixth and seventh positions of malignancies, respectively. Efforts have also been made to find out the total number of cancer cases in five metro cities of India (New Delhi, Bombay, Chennai, Bhopal and Bangalore) during 2008.

Breast cancer is the most common malignancy type diagnosed in women in developed countries and the second most common type diagnosed in developing countries. Breast cancer has been described as an alarmingly health problem in India (Yeole *et al.,* 2003). According to the reports, breast cancers have badly attacked women population in India. A survey carried out by Indian Council of Medical Research (ICMR) in the metropolitan cities viz. Delhi, Mumbai, Bangalore and Chennai; from 1982 to 2005; has shown that the incidences of breast cancer have doubled. Over the years, the incidences of breast cancer in India have steadily increased and as many as 100,000 new patients are being detected every year (Yip *et al.,*2006; Michael *et al.,*2003). A 12% increase has been registered by cancer registries from 1985 to 2001, which represented 57% rise of cancer burden in India (Yip *et al.,*2006; Hadjiiski *et al.,*2006).

Stomach is one of the most essential organs of human body, which frequently gets cancer and stands at fifth position (Parkin *et al.,*1999). South East Asian countries including India were reported to have lower rates of stomach cancers. However, the prevalence of stomach cancer was found to be quite high in Mizoram, North East India. Reports from the National Cancer Registry Programme suggested that stomach remained the leading site of cancer in males in Chennai from 1990 to 1996 with Age Adjusted Rate [(AAR) =13.6/105], followed by Bangalore (9.5/105),

Mumbai (6.4/105), Delhi (3.9/105), Bhopal (3.4/105) and Barshi (1.2/105). In Mizoram, AAR of stomach cancer has been found to be high in both males (39.1/105) and females (14.4/105) as compared to other parts of India. On the basis of the prevalence of stomach cancer Mizoram occupied the first position among Indian states. Moreover, this state comprised fifth position globally (Phukan *et al.,*2004).

Gall Bladder Cancer (GBC) was first diagnosed during laparotomy or laparoscopy procedures, which were expected to confirm the presence of benign gall bladder diseases (Misra *et al.,*1997). Almost 2% gall stone patients were diagnosed with GBC. Gall bladder cancer is the most common abdominal malignancy in northern parts of the country (Singh *et al.,*2004). An incidence rate of 4.5 and 10.1% per 100 000 population of males and females, respectively, has been reported by the Indian Council of Medical Research Cancer Registry in some northern parts of India (ICMR, 1996). The highest incidence of GBCs in India has been reported along the Ganges delta (Kaushik *et al.,*1997) . Gallstones associated with gallbladder carcinoma have been reported in 70-90% of patients with GBC. Approximately, 0.4% of all patients with gallstones have GBC (David *et al.,*1997) .

The most susceptible site of cancer in women in the developing countries is cervix (Parkin *et al.,*1992). During last few decades, it has been observed that the number of cervical cancer cases in women has decreased in India. One case study of Bangalore city supported this observation. In 1982, 32.4% cervical cancer cases per 100,000 populations were reported every year in Bangalore, which decreased to 27.2, 18.2, and 17.0% in 1991, 2001, and 2005 years, respectively. Similarly, in 1988, 25.9 new cases of cervical cancer per 100,000 women population were reported in Delhi, which decreased to 19.1 and 18.9 in 1998 and 2005, respectively. Accordingly, Mumbai recorded 17.9% new cervical cancer cases per 100,000 populations in 1982 followed by 12.7% in 2005. During these 24 years (1982-2005) Chennai recorded a fall of about 50% in cervical cancer cases. According to the reports 41% cases per 100,000 populations were reported in Chennai in 1982, which decreased to 33.4 and 22.0% in 1991 and 2005.

In 2003, Indian Council of Medical Research (ICMR) reported that oral cancer is very common in India (ICMR, 1992). There has been a substantial increase in the incidences of oral sub-mucous fibrosis; especially among youngsters; which further increased the incidence of the oral cancer (Gupta *et al.,*1998). Presently, oral cancer is the fourth common type of malignancy after lung, stomach and liver in males. It is the fifth common cancer after cervix, breast, stomach and lung cancer in females (Park, 1997). Regional Cancer Centre (RCC) Kerala reported about 14% oral cancer patients out of which 17.0 and 10.5% cases were in males and females, respectively (Padmakumary, 2000) . A significant number of oral cancer patients have been reported in Agra, Allahabad, Mainipuri, Varanasi and Moradabad belt of Uttar Pradesh (Wahi *et al.,*1965).

Besides these, some other sorts of cancers have been observed in India. The incidence of esophageal cancer in India is moderately high; associated with diets and lifestyles. According to a data from cancer registries in India, esophageal cancer is the second most common cancer among males and the fourth most common cancer among females (Gajalakshmi, 2001). Colorectal cancer is a disease that usually affects individuals of age 50 years or more (Anthony, 1998). There is a sharp increase in the incidence rate of colorectal cancer after the age of 45 years and 90% of cases are found in persons over the age of 50 years. Head and Neck Neoplasia (HNN) are major forms of cancers in India, which account for nearly 23 and 6% in males and females, respectively (ICMR, 1992). The five year survival of the disease varied from 20 -90% depending on the sub-site of origin and the clinical extent of the disease (Mehrotra *et al.,*2005). India is known to have the world‟s largest reported incidences of HNN in women (Sankaranarayan *et al.,*1998). Nearly 0.2 million head and neck cancer cases are diagnosed in the country annually and approximately 4.5 million globally.

Causes of Cancer

Diet

About 70% colorectal cancer cases are believed to be due to imbalanced diet. The role of diet towards cancer varies greatly according to the type of cancers. As per the International correlation studies, overwhelming positive associations between dietary fat, red meat consumption and colorectal cancer incidence and mortality have been observed. The heavy consumption of red meat is the main cause of several cancers including gastrointestinal tract and colorectal (Bingham*et al.,*2002; Chao *et al.,*2005; Hogg, 2007), prostate (Rodriguez *et al.,*2006), bladder (Garcia -Closas *et al.,*2007), breast (Tappel, 2007), gastric (Hanlon, 2006) and oral cancers (Toporcov *et al.,*2004).

Most probably, it is due to the production of heterocyclic amines (most potential carcinogens) during cooking of red meat. Pyrolysates are produced by charcoal cooking or smoke curing of meat, which exert a cancerous effect on our body cells (Lauber *et al.,*2007). Almost 20% of total mutagencity of fried beef is due to the presence of PhIP (2-amino-1- methyl -6-phenyl-imidazo [4, 5 -b] pyridine), which is the most abundant mutagen by mass in cooked beef. Food kept in plastic containers turns out to be carcinogenic because bios-phenol from the plastic containers gets dissolved and migrates into the food; resulting into the risk of breast (Durando *et al.,*2007) and prostate (Ho *et al.,*2006) cancers. A low intake of fresh fruits and high cooking temperatures in Indian dishes may account for low levels of vitamin C; resulting into higher risks of stomach, mouth, pharyngeal, esophageal. Recently, the case control studies carried out in Asian Indian immigrants to U.K. and U.S.A. found high levels of homocysteine as a risk factor for the breast, ovarian and pancreatic cancers (Wu *et al.,*2002).

Vegetarianism; practiced by a large population of Indians (particularly Hindus); has been associated with lower risks of prostate cancer (Rajaram *et al.,*2000). A comparison of non-vegetarian and vegetarian diets and alcohol and tobacco uses in India was carried out through case control studies. It was observed that vegetarians have a lower risk of esophageal (Roa, 1997), oral (Roa *et al.,*1994)

and breast cancers (Jain *et al.,*1999). Beans, chickpeas and lentils are the principal components of vegetarian diet- a rich source of proteins; and pulses have been significantly associated with reductions in cancer (Jain *et al.,*1999; Mills *et al.,*1989). An increased risk of cancer has been observed with diets with high saturated fats. Middle class people in India and some of the rural areas have a high intake of ghee, which may create an increased cancer risk (Ghafoornissa, 1998; Law, 2000).

The Indian diet containing adequate quantities of vegetables, fruits, and fibre rich grains provides protection against the increased risk of colon and breast cancers (World cancer research fund, 1997). Furthermore, Figure 4 depicts that improper life style and poor dietary habits, which are the key factors for the prevalence of breast and cervical cancers in the female population of Goa. High incidences of throat and food pipe cancers in Andhra Pradesh and Assam were attributed to improper diets (Lammers *et al.,*1998).

Tobacco

The consumption of tobacco is the leading cause of cancers in India. The regular use of tobacco via smoking, chewing, snuffing etc. in some areas of the country, which is responsible for 65 to 85% cancer incidences in men and women, respectively. The various cancers produced by the use of tobacco are of oral cavity, pharynx, esophagus, larynx, lungs and urinary bladder. It has been observed that women in Bangalore are known to have the highest rates of cancers of esophagus in the world (around eight per 100,000). Contrarily, men in Bhopal have the highest rate of tongue cancer in the world (nine per 100,000) (Bobba *et al.,*2003). Smoking is the most notorious factor for the causation of lung cancer (Hammond *et al.,*1958). Approximately, 87 and 85% males and females have been found to have lung cancer due to tobacco smoking in the form of bidi (a thin South Asian cigarette type structure filled with tobacco flake and wrapped in a tendu leaf, tied with a string at one end) (Behera *et al.,*2004) and cigarette in India (Notani *et al.,*1974) . The severe carcinogenic nature of bidi has been proved by the studies of Jussawalla and Jain (Jussawalla *et al.,*1979) and (Pakhale *et al.,*1985) . They observed that the unrefined

form of tobacco used in bidis (WHO, 1999) and the frequency with which a bidi needs to be puffed per minute may be responsible for its relatively higher carcinogenic effects as compared to cigarettes (Bano *et al.,*2009). Bidi smoking at two puffs per minute produces about equal amounts of carcinogens (steam volatile phenols, hydrogen cyanide and benzopyrene) as produced by one puff per minute of unfiltered cigarette (Pakhale *et al.,*1990). Hookah (a special cigar used in India using raw tobacco) smoking causes lung cancer; as reported by Nafae *et al.,*(1973). Recently, Gupta *et al.,*(2001) reported 80 and 33% lung cancers in men and women chain smokers, respectively, as compared to controlled subjects where these numbers were 60 and 20%. Besides, Figure 4 shows that cigarette smoking and Hookah are the main causes of lung cancer in Indian states; especially in Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Manipur, Tripura and some parts of Sikkim. Similarly, bidi and hookah smoking are responsible of oropharyngeal cancers in male population of Haryana. Bidi and cigarette smoking are thought to be etiological factors for the causation of cancers in Andhra Pradesh. In some north- eastern states of India such as Arunanchal Pradesh, Nagaland and Sikkim, high incidences of stomach cancer are attributed to the consumption of smoked meat and chewing of tobacco. High incidences of stomach cancer in Mizoram are the result of the excessive use of tuibur (water filterate of tobacco). Similarly, high incidences of oral cancers in Orissa and Madhya Pradesh are owing to the consumption of beetle leaves and tobacco in different forms. The relatively high incidences of oesophageal cancers in certain parts of Karnataka are because of heavy consumption of tobacco in various forms.

The exceptionally high incidences of oral cancer in some parts of Uttar Pradesh and Gujarat are due to the consumption of Pan Masala, Dohra and Zarda. Similarly, the consumption of Beetal, Nut, Pan Masala, Opium and Bhang (leave and flower powder of female cannabis plant) has been recognized as the major cause of mouth cancer in Rajasthan. Oral cancer being the common malignancy in Allahabad is attributed to the chewing of Dohra; an indigenous preparation of tobacco and slaked lime. The daily consumption of the number of beetle leaves by an individual is

about 15-25 in Allahabad and Varanasi districts, which continuously acts as an irritant to the buccal mucosa (Mehrotra *et al.,*2003) . One of the most important factors responsible for the oropharyngeal malignancy in Agra and Mainpuri belt of Uttar Pradesh is the chewing of beetle nut (Wahi *et al.,*1965). Among various risk factors for the occurrence of esophageal cancer in India, betel quid chewing carries a relative risk of 1.5 to 3.5%. The salted tea made by adding sodium bicarbonate has shown to possess a high methylation activity and may lead to the endogenous formation of nitrosamine (Malkan *et al.,* 1997).

Alcohol

Alcohol consumption has been considered as one of the major causes of colorectal cancer as per a recent monograph of WHO (Baan *et al.,* 2007). Annually, about 9.4% new colorectal cancer cases are attributed to the consumption of alcohol, globally (Parkin *et al.,* 2002). An increased risk of 10% was observed with consumption of more than two drinks per day, which suggests a causative role of alcohol consumption in colorectal cancer (Toriola *et al.,* 2008). Recently, a study revealed that an increased risk of colorectal cancer was limited to consumption of more than 30.0 g of alcohol per day (Longnecker *et al.,*1990). Relationship between alcohol consumption and high risk of oesophageal cancer was first known in 1910 (Tuyns, 1979). However, chronic alcohol consumption has been found to be a risk factor for the cancers of the upper respiratory and digestive tracts, including oral cavity, hypopharynx, larynx and esophagus as well as liver, pancreas, mouth and breast cancers (Tuyns, 1979; Maier *et al.,*1994; Seitz *et al.,*2004). A 10.0 g/day intake of alcohol by a woman increases its relative risk of breast cancer by 7.1% (Doll *et al.,*1981). The mechanism of carcinogenesis due to alcohol consumption is not exactly known, however, it is thought that ethanol being a co-carcinogen might play a crucial role in the carcinogenesis (Poschl *et al.,*2004). The metabolic products of ethanol are acetaldehyde and free radicals. The free radicals are responsible for alcohol assisted carcinogenesis through their binding to DNA and proteins, which destroy foliate leading to secondary hyper proliferation (Anand *et al.,*2000).

Radiation

In the developed and developing countries, the radiations are also notorious carcinogens. About 10% cancer occurrence is due to radiation effect, both ionizing and non-ionizing (Belpomme *et al.,* 2007). The major sources of radiations are radioactive compounds, ultraviolet (UV) and pulsed electromagnetic fields. The main series of cancers induced by exposure to the adequate doses of the carcinogenic radiations include thyroid, skin, leukemia, lymphoma, lung and breast carcinomas. The most common source of ionizing radiation exposure is Radon, which is a radioactive element. Radioactive nuclei of radon, radium and uranium are found to be associated with an increased risk of gastric cancer in rats. High risk of breast cancer among girls at puberty is due to chest irradiation of X- rays (used for diagnostic and therapeutic purposes). The major risk factor for various types of skin cancers viz. basal cell carcinoma, squamous cell carcinoma and melanoma is the exposure to ultraviolet light, which is a non- ionizing radiation (Anand *et al.,* 2000). The underground testing of nuclear weapons may be the major cause of digestive system, liver and kidney cancers, as radiations have been reported in ground water of the nuclear weapon testing area. Moreover, Moses Samuel Rajan et al (2014) a,b.

Miscellaneous pollutants

It is estimated that about 90% cancer is owing to the environmental contaminants (Anand *et al.,* 2000). Various types of cancers are believed to be due to ill effects of the polluted environment. The risk of lung cancers is increased by a number of outdoor pollutants such as poly aromatic hydrocarbons. Long term exposure to PAHs (polyaromatic hydrocarbons) in air was found to increase the risk of deaths associated with lung cancer. Indoor environmental pollutants such as volatile organic compounds and pesticides increase the risk of leukemia and lymphoma, brain tumors, Wilm‟s tumors, Ewing‟s sarcoma and germ cell tumors. An increased risk of cancer has been observed in people using chlorinated water for drinking purposes for a long time. N-Nitroso compounds (mutagenic in nature) are

formed from nitrates present in drinking water and increase the risk of lymphoma, leukemia, and colorectal cancer and bladder cancers (Belpomme *et al.,* 2007). High level of air pollution is responsible for the prevalence of lung cancers in Delhi and some other parts of West Bengal including Calcutta. The low socio-economic conditions related to poor hygiene, poor diet or infections of viral origin are also responsible for various types of cancers (Mehrotra *et al.,*2003).

Preventive measures of cancer in India

As per the proverb, "prevention is better than cure" the prevention strategies are crucial in cancer eradication. This approach offers a great public health concern and inexpensive long term method of cancer control. National Cancer Control Programme (started in 1975-1976 in India) led to the development of Regional Cancer Centers (RCCs), a number of oncology wings in Medical Colleges; supported the purchase of teletherapy machines. District Cancer Control Programme was also initiated but could not result into sustainable and productive activity (National Cancer Control Programme). The education should focus on harmful effects of tobacco and discourage its use. Besides, we should create awareness among public about physical activities, avoiding obesities, healthy dietary practices, reducing occupational and environmental exposures, reducing alcohol uses, immunization against hepatitis B virus and safe sexual practices for avoiding cancer genesis. The same approach should be included in adult education programme. Several state wise programmes like Kerala (Ten year action plan), Tamil Nadu (Kancheepuram Cancer Screening Programme) and opportunistic programmes in social regions have been implemented by some State Governments and Regional Cancer Centers (RCCs) for an early detection of different cancers in India. The predicted results were not materialized in most of the programmes except RCC programme in Trivandrum as the health service system could not support such activities due to deficiencies in health system management and non-availability of human resources (Cytologists /Pathologists) and absence of integration with multi- sectoral groups. Unfortunately, a little population

got aware of cancer havoc, which might be spread to the population of the whole country (National Cancer Control Programme).

As discussed above tobacco is the most notorious agent for cancers, which must be banned to eradicate the prevalence of tobacco related cancers. India should give the highest priority to tobacco control programme due to its acute carcinogenic nature (WHO, 2002). It has been predicted that a ban on tobacco use can prevent up to 30% cancers in India (Central Statistical Organization, 2003 -04). Alcohol consumption is responsible for the occurrence of colorectal cancer. About 25% population is consuming alcohol in India, which must be minimized or avoided to eradicate this havoc. Government needs to impose a ban on the public sale of alcohol. Seminars and public health camps should be conducted to create awareness of alcoholic harmful effects among Indians. Radiations are silent and serious carcinogens that cause a number of cancers and, hence, the strategies that reduce the exposure of people to these notorious radiations should be fully practiced to reduce the incidence of cancers. India being one of the nuclear power nations needs to build safe equipped nuclear plants with greater protection from the hazardous nuclear radiations. Nuclear reactors should be well constructed with good quality shields to provide more protection to the people at work. Nuclear tests should be carried out at safe places away from human populations to avoid exposure to these radiations.

Environmental pollution is a serious issue and has become a challenge for all of us as it is responsible for the genesis of various types of cancers. Air pollution is the most notable cause of lung cancer in the metropolitan cities of India. The harmful gases such as carbon monoxide (CO) and sulphur dioxide (SO2) produced by combustion of fuels in automobiles and several industrial processes, respectively, cause lung cancer, respiratory, digestive, ocular and skin carcinomas. Automobiles that run on compressed natural gas (CNG) should be encouraged; at least in the metropolitan cities of the country to avoid air pollution. The use of chlorofluorocarbons (CFCs), methyl halides, carbon tetrachloride and carbon tetra fluoride is the main cause of the depletion of ozone layer, which protects us from the

harmful UV-rays. The use of such chemicals should be minimized in order to reduce the incidence of skin cancer caused by the harmful effects of UV-rays. The sewage discharged by several industries and municipalities is polluting Indian water resources due to insufficient water treatment plants; leading to various types of cancers. Therefore, these wastes should be treated prior to their discharge to land or river.

Due to over growth of Indian population, farmers are compelled to produce more cereals and vegetables to meet out public requirements. This pressure forces farmers to use excessive fertilizers and pesticides, which are being transported into our body via food and water causing various sorts of cancers. Farmers should be encouraged to use eco-friendly organic manures and biocides to reduce cancer incidences. India is a developing country and gradually adopting modern life styles involving the use of various kinds of chemicals in terms of medicines, cosmetics, cloths, utensils, mobile phones and other luxurious items. The use of such items may cause different sorts of cancers. That is why during past few decades the incidences of cancers have increased. It is urgent to emphasize that Indians should be aware about their life styles particularly the use of synthetic products, fabric dressing, and mode of sex, abuse of drugs and excessive use of mobile phones. Besides, an increased fashion of fast food in this country is also responsible for this havoc. Indians should adopt a healthy food habit having sufficient quantities of vitamins, minerals, proteins, fibers, carbohydrates etc. The healthy and proper foods are important aspects to control different cancers. The consumption of whole grains, vegetables and fruits antagonize the development of some cancers. Briefly, there are no uniform standardized information programs, education and communication (IEC) strategies for cancer prevention in this nation. Besides, limited diagnostic and treatment infrastructures in the country are the serious issues, which must be increased on urgent basis. The government and other NGOs should come forward to initiate the above programs for controlling this havoc so that the present and coming generation of the country may lead healthy life.

Effect of cancer on Indian economy

As per nominal Gross Domestic Product (GDP), the economy of India stands on eleventh position in the world, while it is fourth largest by Purchasing Power Parity (PPP) (CIA- The World Fact book, 2009). Indians are at high risk of acquiring cancers due to high rates of smoking, tobacco use, occupational risks, and unhygienic residential living conditions. The prevalence of cancer in India is affecting the economy of the country. The data on the effect of cancer on Indian economy is not available; however, Popkin *et al.,*(2001) assessed the impact of cancer of diet related health conditions in terms of health spending and on income losses experienced by households (Popkin *et al.,*2001). The estimation of expenditures of cancer patients includes both direct medical and non-medical costs. The direct costs include buying medicine, hospitalization, pathological tests, medical practitioner consultancy, travel, lodging while the indirect costs are loss of income during treatment, premature death and affect on the income of other family members etc. Abegunde *et al.,* (2007) calculated the effect of cancer deaths on Indian economy. Furthermore, they assessed the economic impact of mortality from chronic diseases on Gross domestic product (GDP) (Abegunde *et al.,*2007).

Briefly, Indian economy has been affected by the alarming rise of cancers in the last decade. It is still being affected due to continuous increase of cancer patients. An estimation of the effect of cancer on the Indian economy has been carried out. The economical loss was calculated by considering all the factors viz. both direct medical and non-medical costs. This also shows that the total number of cancer patients in 2004 was 819354 with a total loss of 215.16 million US $. The number of cancer patients and economic loss are continuously increasing, which have become 962832 and 274.10 million US $ by the end of 2009, respectively. Similarly, the total cancer patients in 2010 were 979786 with total economic loss of 270.06 million US $.

Natural medicine and Cancer

A variety of approaches have been employed in cancer chemoprevention. These include changes in diet, supplementation with specific vitamins and minerals, or administration of pharmacologic compounds. Investigators have identified approximately 400 drugs, vitamins, hormones and other agents that might help in preventing cancer. Clinical trials are underway to investigate an increasing number of agents. Most of these trials involve healthy people with a higher-than-average risk of cancer (Dunn and Ford, 2001; Amin and Buratovich, 2007). To sum up, adequate nutrition is a key element of a healthy lifestyle and is associated with a lowered risk for chronic illnesses. The consumption of five portions of fruits and vegetables per day is proposed to sustain optimal health and especially coloured food items are recommended. Data from epidemiological studies consistently show an inverse correlation between the intake of fruits and vegetables and the incidence of several diseases such as cardiovascular, ophthalmological, gastrointestinal or neurodegenerative disorders and some types of cancer (Van Duyn and Pivonka, 2000). It has been postulated that among the different dietary components of fruits and vegetables, secondary plant constituents (such as phytochemicals) play a major role in disease prevention (Stahl and Seis, 2005; Al-Akhras *et al.,* 2007).

The Arabian Gulf region is one of the birthplaces of herbal therapy (Hasan et al, 2000). Herbal medicine occupies a significant part of this region‟s heritage and until recently functioned as the main health care system. Despite the wide variety of herbal species in the region with about 600 species in UAE (Jongbloed, 2003), 1204 in Oman (Ghaznafar, 1994), 2250 in Saudi Arabia (Migahid, 1990), 2088 in Egypt (Tackholm, 1974) and 2367 in Palestine (Zohary, 1973) (most of the species present in the two later countries are also recorded in the Gulf region), only less than 10% of these species have been screened for their medicinal uses. Initially, many medicinal plants and their applications were documented in the Middle East and the Arabian Gulf and in such countries as in the United Arab Emirates (UAE) (El- Ghonemy, 1993), Qatar, Oman (Ghaznafar, 1994), Saudi, Jordan and Egypt (Bolous, 1983). Most of the available literature is based upon information

collected from local informants and as it lacks proper laboratory investigations, most of these reports are of dubious value. Since, medicinal plants represent an important health and economic component to Gulf region biodiversity, it is essential to furnish a complete inventory of the medicinal components of the flora of any country for purposes of conservation and sustainable use. At the present time, the high public demand for unconventional therapies has led many countries in the region to devote time and money to the exploration of the potential of their medicinal flora.

Anticancer potential of sponges

An extract of a sponge *Dysidea herbucea* showed high activity in a cytotoxicity assay. Five constituents have been isolated and identified. Four of them were found to be known diterpenes, ambliofuran, 3 ,7,11,15-tetramethyl-6,10,14-hexadeca~enoiacc id, 2-tetraprenyl- 1,4-benzoquinol, and 4-hydroxy-3-tetraprenylbenmic acid (Walker and Fulkner, 1981). The fifth compound was new and characterized to be 20,24-dimethyldeoxoscalarin-3-one by spectroscopic analysis including COSY and COUX experiments. The relative stereochemistry of this was secured by NOE study. Although the diterpenes are among the classes of compounds found in *Dysideu spp*., the bishomosesterterpene skeleton is typical to the metabolites of some species of *Carrerwongiu* (Phyllospongiu) but not to *D. herbuceu* (Bergquist and Wells, 1983). Except for ambliofuran which was weakly cytotoxic, four other compounds were moderately cytotoxic against P388, A-549 human lung carcinoma, and HT-29 human colon adenocminoma cells. The quinoll was most active at the level of IC, 0.3 pg/mL. Chromatography of an acetone extract of *Hulichondria sp.*, collected in Kerama, Okinawa, yielded nine sesquiteqenes of theonellin (Nakamura, 1984) class (bisabolene skeleton) and dimers. Among the compounds dimers and were cytotoxic, and also showed weak immunosuppressive activity. Recent report on the antitumor effect of some marine carotenoids such as halocynthiaxanthin prompted us to examine carotenoid pigments often encountered during separation of some sponge extracts. Chromatography of an intensely colored

extract from the bright orange sponge *Phakellia stellidem* afforded two red pigments, 19-hexanoyloxymytiloxanthin and 19'-butanoyloxymytiloxanthin.

A methylene chloride-2-propanol extract prepared from the specimen of *Axinella* collected in Palau provided a 101 % increase in life span (at 100 mg/kg) against the PS leukemia with ED_{50} 2.5 μg/ml. By means of P 388 lymphocytic leukemia cell line guided bioassay a new PS inhibitory peptide axinastatin 1 (ED_{50} 0.21 μg/ml) was isolated from this sponge. Extended bioassay directed separation of the more difficult accessible cytotoxic components of this sponge was resulted in the discovery of two cycloheptapeptides; axinastatins 2 and 3. Axinastatin 2 exhibited strong *in vitro* cytotoxicity against the murine leukemia P388 cell line (ED_{50} 0.02 μg/ml), while axinastatin 3 also possessed significant cytotoxic activity (ED_{50} 0.4 μg/ml) against the PS leukemia cell line and showed a higher level of activity than axinastatin 2 against human ovarian, lung, and colon cell lines. As a contiunation of this research marine sponge; *A. carteri* from the Republic of the Comoros was investigated and a cell growth inhibitory cyclopeptide called axinastatin 4, (P 388 lymphocytic leukemia cell line, ED_{50} 0.057 μg/ml) was isolated (. *A. cfcarteri* from the western Indian Ocean yielded a cell (human and murine) growth inhibitory cyclooctapeptide, axinastatin 5 (GI 0.3-3.3 μg/ml). In another study, the alkaloids; debromohymenialdisine, hymenialdisine, and 3-bromohymenialdisine of *A. carteri* collected from tropical region were tested for their cytotoxicity in vitro using L5178y mouse lymphoma cells.

Chapter - I

ISOLATION AND TOXICOLOGICAL CHARACTERIZATION OF CHOSEN MARINE SPONGE EXTRACTS COLLECTED FROM SOUTH PENINSULAR COAST OF INDIA

Introduction

The marine environment is a major sustaining part of ecosystem processes, distinguished by unique biodiversity and being the source of interesting structures. Sponges (phylum Porifera) are a significant component of this environment. They are the most primitive multicellular invertebrates representing the phylogenetically oldest metazoans that evolved 750–570 million years ago (Sipkema *et al.,* 2005). Sponges fascinate scientists from different disciplines that vary from chemical ecology, physiology and morphology to isolation of promising bioactive compounds and association with a wide variety of marine microorganisms in their tissues. Sponges have been considered as a gold mine for the chemist. More than 12,000 compounds have been isolated from marine sources with hundreds of new compounds still being discovered every year, with respect to the diversity of their secondary metabolites, which range from derivatives of amino acids and nucleotides to macrolides, porphyrins, terpenoids to aliphatic cyclic peroxides and sterols, the majority of which are related to sponges and associated microorganisms.

In spite of the developments in medical science, there are no comprehensive cures for AIDS, cancer, arthritis, other inflammatory conditions, and a large variety of viral and fungal diseases. Marine natural products could yield new drugs to cure such diseases. The quest for drugs from the sea has yielded an impressive list of natural products mostly from invertebrates such as sponges that are either in the late stages of clinical trials, or have already entered the market. 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

(anti-inflammatory), KRN 7000 (anticancer), LAF389 (anticancer), Discodermolide (anticancer) and HTI286 (anticancer) are under clinical trial (Thakur and Muller, 2004). Secondary metabolites in sponges are produced in trace amounts and exploitation of sponges from natural resources in bulk is an ethical issue.

Although the number of bioactive compounds isolated from marine organisms is quite considerable, those reached the clinical trials are scanty. Comparatively, more number of secondary metabolites derived from terrestrial sources has reached the clinical trials. This may be due to the failure in the successful collection of concerned source organisms, in bulk. Hence the knowledge on habitat, site of abundance, seasonality and ecofriendly collection of the source organism is of utmost importance in the successful development of marine drugs. Exceeding collection of the source from a specific ecological niche will aid a depletion of the same. Hence an eco-friendly mode of collection is apparently necessary to aid a sustained supply of these marine creatures.

Eventhough multiple theories and findings have been put forth to explain the mechanism of the secondary metabolite synthesis, the mechanism of their chemical ecology and the successful mode of isolation of the parent compound is yet to be elucidated. Owing to the difference in the environment from terrestrial creatures, it is quite reasonable to expect the difference in the secondary metabolites. Though the current study was to develop potent, safe and novel bioactive substances from marine organisms, the knowledge of its toxicity, activity range, potency and it‟s mechanism of action are necessary for the complete development. Hence the toxic influence range, bioactivity and the pharmacological study of the separated compounds were evaluated.

Materials and Methods

Collection of sponges

A diverse variety of sponges was collected (Plate 1) from the peninsular coast of India, especially South Tamilnadu and South Kerala Coast. Based on the previous survey Arokiapuram, Muttom and Vizhinjam Coasts were selected for the collection.

Collection as bycatch

An eco-friendly bulk collection of the sponges by bycatch was carried out at Arokiapuram (near Kanyakumari) coast. Seven species of sponges were collected, identified and taken-up for isolation and bioactivity screening of the secondary metabolites. During November and December, and April and August, the rough sea weather prevails in the Arokiapuram coast leading to the dislodgement of sponges, gorgonids, soft corals and ascidians, which were caught in the fishing nets. Ample collection was carried out in the nets such as Echa vala (Green colour), which was operated during night hours at a depth of 10 „pakam‟ (1 „pakam‟ = 2 mts) and Velameen vala (otherwise it is called othakundu kangoose vala), which was operated at a depth of 16-20 pakam. These bycatches were segregated and collected freshly from the nets in the morning hours when the nets were cleaned (Plate 2). Such freshly disposed specimens were collected and washed in fresh seawater to remove dirt and symbionts and drained the excess water and blotted by a blotting sheet. After recording their colour and pattern for identification, the sponge pieces were separately preserved in methanol. Voucher specimens were packed in tagged plastic vials and kept in the freezer for identification.

Five types of marine sponges were collected from Arockiapuram coast by bycatch. They were coded with MSE2, MSE3, MSE5, MSE7, and MSE9.

Preparation of crude extracts from sponges

The collected sponges were dipped in methanol containers and were squeezed/minced in a tissue homogenizer, depending upon the nature of the sponge

species, *Dendrilla nigra* and *Haliclona exigua* were cut into small pieces and squeezed to prepare the crude extract in a mortar and pestle using methanol as the solvent. *Callyspongia sp.* and *Sigmadocia*, were cut into small pieces and minced in a tissue homogenizer prior to the extraction. In the case of *Axinella donnani* the extract was collected as such from the methanol container and filtered through a Whatman no.1 filter paper fitted with a Buchner funnel, using suction. They were extracted thrice and the combined extract was concentrated in a rotary vacuum evaporator at room temperature. The concentrated crude extract was collected in airtight plastic containers and kept in a refrigerator.

In- vitro antibacterial screening on the isolated MSMs

Antibacterial studies were carried out using 10 gram positive and 10 gram negative bacterial type cultures, obtained from Microbial Type Culture Collections (MTCC), Chandigarh (the bacterial isolates used in this study are given in Table-2). The MTCC type cultures were initially activated in nutrient broth and subsequently purified by agar streak plate method. The antibacterial susceptibility test was conducted only in three sponge extracts and the Minimum Inhibition Concentration (MIC) determination was done.

The agar punch hole method

In the agar punch hole method, initially, nutrient agar spread plates were prepared using 0.1 ml of inoculum containing appropriate bacteria of 18 h culture. The plates were kept as such for 15 minutes for the adhesion of bacteria on the medium. Wells were cut in each plate using a sterile cork borer of 7 mm diameter. Each well was filled with MeOH dissolved extract, up to the brim. Methanol was used as the positive control. After 24 h of incubation at 30 ± 2^{o}C in a B.O.D incubator, the diameter of inhibition zones were measured. The area of the inhibition zone was calculated as follows:

Cross diameter of the inhibition zone = m
Net diameter of the well = n
Net diameter of the inhibition zone, x = m-n
Net radius of the inhibition zone, *r* = x/2
Area of the inhibition zone A = (= 3.14)

Table - 2. Different Bacterial species used in the present study

Bacterial Species	Gram Staining	Source
Bacillus subtilis	Gram positive	MTCC
Bacillus cereus	Gram positive	MTCC
Staphylococcus aureus	Gram positive	MTCC
Streptococcus pyogenes	Gram positive	MTCC
Aeromonas hydrophila	Gram positive	MTCC
Bacillus anthracis	Gram positive	MTCC
Staphylococcus epidermidis	Gram positive	MTCC
Escherichia coli	Gram negative	MTCC
Pseudomonas aeruginosa	Gram negative	MTCC
Vibrio fischeri	Gram negative	MTCC
Vibrio harvay	Gram negative	MTCC
Salmonella typhi	Gram negative	MTCC
Klebsiella pneumoniae	Gram negative	MTCC
Proteus vulgaris	Gram negative	MTCC

MTCC- Microbial Type Culture Collections, Chandigarh

Determination of MIC

As the MSMs are coloured, it may not be possible to determine the MIC values by using the standard Tube Dilution Method. Therefore the MSMs of various concentrations were determined by the agar plates method.

Biotoxicity studies

Brineshrimp cytotoxicity

About 0.1 g of *Artemia salina* cysts were aerated in a 1 lit. capacity glass cylinder (jar) containing filtered seawater. The air stone was placed at the bottom of the jar to ensure complete hydration of the cysts. After 24 h, the newly hatched free-swimming pink-coloured nauplii were harvested from the bottom outlet. Since the cyst capsules floated on the surface, this collection method ensured pure harvest of nauplii. The freshly hatched free-swimming nauplii were used for the bioassay.

The assay system was prepared with 2 ml of filtered seawater containing chosen concentration of methanolic sponge extracts in cavity blocks (Embryo cup). Parallel vehicle control (using 2 % methanol) and negative control wells also kept. In each, 20 nauplii were transferred and the setup was allowed to remain for 24 h, under constant illumination. After 24 h, the dead nauplii were counted with a hand lens. Based on the percentage mortality, the LD_{50} of the test compound was determined, using probit scale

Larvicidal activity

As the larval stages of mosquitoes (*Culex sp.*) were more accessible for control, the early second and final fourth instar larvae were chosen for the experiments. The susceptibility or resistance of the mosquito larvae to the selected concentrations of the extracts was carried out by adopting standard bioassay protocols (WHO, 1981). Observations were made after 24 h of treatment, for the determination of larvicidal activity.

Microalgal lethality bioassay

The microalgae such as *Isochrysis galbana*, *Chlorella salina* and *Nanochloropsis* sp. obtained from CMFRI, Kochi were used for the assay. The stock cultures of algae were maintained as follows: Filtered seawater was boiled, cooled and enriched with Walne‟s medium at a proportion of 1 ml/l Walne‟s A, 0.5 ml/l Walne‟s B and 0.05 ml of Vitamin B1 and B12. 500ml of this enriched medium was taken in 1lit Erlenmeyer flask and inoculated with appropriate algal culture and maintained in optimum light and temperature. The total numbers of cells were counted using a haemocytometer to monitor the growth. Saturated culture of the relevant microalgae was transferred to 20 ml of the fresh enriched medium taken in 100ml capacity Erlenmeyer flasks. Selected concentrations of the extracts as 1%, 2%, 4% and 8% were added into each flask and dissolved by rotating the flasks. Initial hour (0 h) count was recorded for each flask. Algal growth/decline was measured by

counting them in a haemocytometer every 24 h from each flask, for a period of 5 days.

Ichthyotoxicity assay

Fingerlings of marine acclimated *Oreochromis mossambicus* were used for evaluating the ichthyotoxic potential. Five fingerlings were introduced in each experimental and control glass bowls containing 1000ml seawater dissolved with chosen concentrations of the extracts. Immediate reflex changes and mortality were observed continuously for six hours at 1 h interval, for the next 12 h. After 24 h of exposure, the number of dead and live fish was counted. The acute toxicological reflexes were observed and recorded. The mortality percentage was determined and the LD_{50} values were calculated.

Results

Collection and identification of sponges

The successful development of drugs from the sponges completely relies on the continuous availability of the source organism and the cost effective collection methods. In the present study, The Arokiapuram coast was found to be an excellent area for the collection of diverse marine sponges as „bycatch". The landing status of sponges in this coast is more or less same, irrespective of the seasons. However, the trend of frequency and quantity of landings varied according to the fishing area and nets used. The major landing species were: *Callyspongia sp*.and *Sigmodoisia* followed by *Dendrilla nigra*, *Haliclona exigua*, *Axinella donnani* and other species. The identified sponges are presented in Table-3.

Table-3. Identified species of marine sponges used in the present study

Species	Colour Pattern	Area of Collection
Dendrilla nigra	Black	Arokyapuram
Haliclona exigua	Greenish Violet	Arokyapuram
Axinella donnani	Brick red/brown	Arokyapuram
Callyspongia sp.	Pale yellow	Arokyapuram
Sigmodoisia sp.	Light brown	Arokyapuram

(Identified by the renowned sponge taxonomist, Dr. P.A. Thomas, former Principal Scientist, CMFRI, Vizhinjam)

Preparation of crude extract from sponges

The colour of methanolic extract of sponges is presented in Table-4. The sponge methanolic extracts exhibited their characteristic colour. The recovery of the extract was dependent on colour pattern and softness of the sponge body. The yield was very high in the case of *A. donnani* (5.6 g/kg) followed by *H. exigua, D. nigra, Callyspongia sp.* and *Sigmodoisia sp*. at a rate of 5.4 g/kg, 5.2 g/kg, 4.9 g/kg and 3.8 g/kg, respectively (Table-4).

Table - 4. Nature, Quality and yield of methanolic extracts of sponges

Species	Color of Extracts	Code	Quantity of Yield (g/Kg)
Callyspongia sp.	Straw yellow	MSE2	4.9
Axinella donnani	Dark brown	MSE3	5.6
Sigmadocia sp.	Light brown	MSE5	3.8
Haliclona exigua	Greenish violet	MSE7	5.4
Dendrilla nigra	Black	MSE9	5.2

Screening for *in-vitro* antibacterial activity

The antibiogram of MSMs of sponges relied only on the active principles but it may change due to the assay method, incubation temperature and culture media. Among these variables, the difference was studied for assay method and incubation temperature. In this experiment, the agar diffusion method was followed and the results are tabulated in (Table-5).

The *D. nigra* extract exhibited 95% antibacterial activity on the tested bacterial species. The extract furnished 65% remarkable activity, 30% moderate activity; also it was found that, the extract showed nil activity on the remaining 5% of the tested organisms. Totally 95% of the species of the tested organisms was inhibited in growth, by the said extract. Also the extract was found 100% effective on the gram positive bacteria as it inhibited the growth of all the selected species; in addition, it displayed the antibacterial activity against 90% of the gram negative ones.

The *A.donnani* extract displayed overall 90% antibacterial activity on the tested bacterial strains. The said extract showed 35% high activity, 55% moderate activity and nil activity in 10% of the tested bacteria. All the tested gram positive bacteria are inhibited in growth by the sponge extract, but only 80% inhibition was observed in the gram negative ones.

Table - 5. Results of preliminary antibacterial efficacy determination of chosen marine sponges

Bacterial species	**Gram staining**	***D.nigra***	***Callyspongia***	***A.donnani***	***H.exigua***	***Sigmodoisia***
		(mm)	**(mm)**	**(mm)**	**(mm)**	**(mm)**
Bacillus subtilis	Gram positive	21	16	18	12	20
Bacillus cereus	Gram positive	18	-	16	14	-
Staphylococcus aureus	Gram positive	12	10	12	-	11
Streptoccus pyogenes	Gram positive	13	11	12	-	12
Aeromonas hydrophila	Gram positive	16	15	15	9	-
Bacillus anthracis	Gram positive	12	-	11	-	-
Staphylococcus epidermidis	Gram positive	16	15	14	12	-
Escherichia coli	Gram negative	14	-	13	8	12
Pseudomonas aeruginosa	Gram negative	14	13	12	10	-
Vibrio fischeri	Gram negative	16	-	14	8	12
Vibrio harvae	Gram negative	14	-	13	-	-
Salmonella typhi	Gram negative	16	15	-	-	-
Klebsiella pneumoniae	Gram negative	16	-	15	-	14
Proteus vulgaris	Gram negative	14	-	12	-	10

The extract of the *Callyspongia sp.* presented antibacterial activity on 50% of the total tested species of bacteria. Of which, high, moderate and nil activity were shown by 20%, 30% and 50%, respectively. The percentage of test gram positive bacteria which got inhibited in growth by the extract is 70%.But the activity of the said extract on the total gram negative species is only 30.

The extract derived from the sponge Sigmadocia revealed that the activity on the total bacterial species tested was just 45%, in which the high, moderate and nil percentage of activity was 10,35 and 55, respectively. Totally 40 % of the tested gram

positive organisms are inhibited in growth, conversely, the percentage of gram negative bacterial species which was subjected to growth inhibition was 50.

The *H. exigua* extract is found to exhibit 45% antibacterial activity over the 20 tested organisms. This extract showed 5% high activity, 25% moderate activity and 15% low activity, over the entire bacterial species. The extracts did not inhibit 55% of the selected bacterial species. As an overall consideration, 50% activity was shown against gram positive organisms and 40 % activity was shown against gram negative organisms. Intersetingly, the *H. exigua* extract produced the maximum activity against *Bacilus cereus,* and slightly low activity over *Bacillus subtilis*, but the antibacterial activity was nil in the case of other tested Bacillus strains.

The bacteriostatic potential of MSMs varied widely based on the species or strain of bacteria tested. Based on the activity range, the inhibitory potential was graded as highly active (>13 mm.), moderately active (10-13 mm.) less active (7-9 mm) and resistant (no inhibition zone). *H. exigua* successfully prevented the growth of Gram positive strains to the extent of 66.66% at 30^0C whereas all the Gram positive bacteria were susceptible at 30^0C. However, the inhibitory potential was feeble towards the Gram-negative strains and only 50% and 25% at 30 and 20^oC respectively were inhibited.

Influence of temperature on antibacterial activity

Table -6. Results of antibacterial activity of marine sponges against gram-positive bacteria

Sponge	Temperature (^{0}C)	*B.subtilis*	*B.cereus*	*S.aureus*	*S.pyogenes*	*A.hydrophila*	*B.anthracis*	*S.epidermidis*
Haliclona (MSE7)	20	-	+	-	-	-	-	+
	30	++	+ ++	-	-	+	-	++
Dendrilla (MSE9)	20	++	++	++	++	++	++	++
	30	+ + +	+ + +	+ +	+ +	+ +	+ + +	+ + +
Axinella (MSE3)	20	++	++	++	++	++	++	++
	30	+ + +	+ + +	+ +	+ +	+ + +	+ +	+ + +
Sigmadocia (MSE5)	20	-	+	-	-	-	-	+
	30	+++	-	++	++	-	-	+ + +
Callyspongia (MSE2)	20	-	+	-	-	-	-	-
	30	+++	-	++	++	++	-	++

+ - active ; ++ - moderatively active ; +++ - highly active

Table -7. Results of antibacterial activity of marine sponges against gram-negative bacteria

Sponge	Temperature (^{0}C)	*E. coli*	*P. aeruginosa*	*V. fischeri*	*V. harveyii*	*S. typhi*	*K. pneumoniae*	*P. vulgaris*
Haliclona (MSE7)	20	-	-	-	-	-	-	-
	30	-	+	+ +	+	+ +	+	-
Dendrilla (MSE9)	20	+	+	+ +	+	+	+	+ +
	30	+++	+++	+++	+++	++ +	++ +	++ +
Axinella (MSE3)	20	+	+	+ +	+ +	+	+	+
	30	+ +	+ +	+++	+ +	-	++ +	+ +
Sigmadocia (MSE5)	20	+	+	-	+	-	-	+
	30	+ +	-	+ +	-	-	++ +	+ +
Callyspongia (MSE2)	20	-	+	-	-	-	-	-
	30	-	+ +	-	-	++ +	-	-

+ - active ; ++ - moderatively active ; +++ - highly active.

The antibacterial activity range of MSMs is presented in Table- 6 and 7 shows that, the secondary metabolites of *A.donnani* and *D. nigra* successfully inhibited the growth of almost all the tested gram positive and gram negative bacteria. It was noted that high activity was obtained only in the higher temperature (30ºC). Interestingly, in the lower temperature (20ºC), the sponges produced meagre activity. The same trend was noted in all the other sponge extracts. The *Sigmadiosia* and *Callyspongia sp.* produced more or less same results in both the temperatures tested. They produced high activity in *B. cereus* and *P. aeruginosa* at 30ºC. Apart from this, the nearly active range was extended against *S. aureus, S. pyogenes, A. hydrophila,V. fischeri, S. typhi, K. pneumoniae* and *P.vulgaris*, which produced an inhibition of a moderate range at 30ºC. Most of the sponge extracts did not produce any inhibition zone at 20ºC. However, the least acitivity was recorded in *Haliclona* extracts at 30ºC.

MIC of highly active MSMs

The MIC value of highly active MSMs indicated that *D. nigra* is a potent antibacterial compound (MIC = 9.0 mg/ml) followed by *A. donnani* (MIC = 12.5 mg/ml). The results of MIC determination are presented Table-8.

Table-8. MIC of higly active MSMs

MSMs	Concentration (mg/ml)	Zone of inhibition (mm)
A.donnani	100	12
	50	0.5
	25	8.5
	12.5	0
D. nigra	100	13
	50	12
	25	11
	12.5	10
	6.5	9

Biotoxicity studies

Determination of brineshrimp cytotoxicity range

Results of Artemia cytotoxicity bioassay are depicted in Fig-1a, b, c and d. The secondary metabolites of the sponge *A. donnani*exhibited high toxicity against *Artemia* nauplii followed by *D. nigra, H. exigua, Callyspongia,* and *Sigmadocia*. The LC_{50} values of *A. donnani, D. nigra* and *H. exigua* accounted for 0.20%, 0.28% and 0.32%, respectively. Temperature had significantly influenced the toxicity of MSMs. The toxicity profile of MSMs considerably decreased at 20 ± 2°C (Table- 9).

Fig-1.a Graphical representation of Artemia cytotoxicity of *D.nigra*

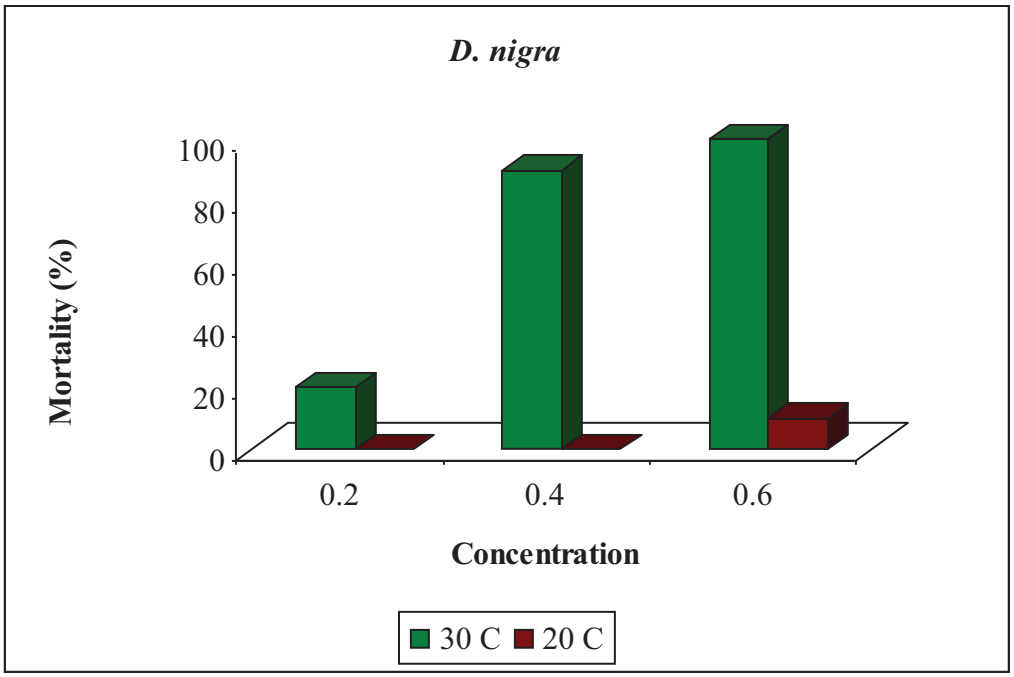


Fig-1.b. Graphical representation of Artemia cytotoxicity of *Sigmadocia sp*.

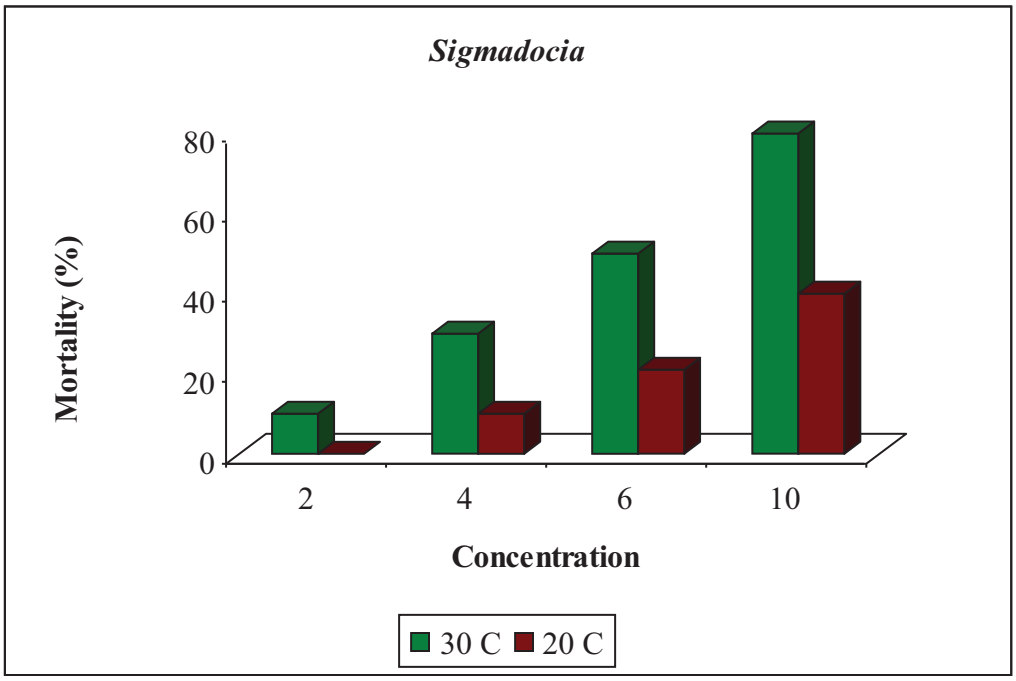


Fig-1.c Graphical representation of Artemia cytotoxicity of *A. donnani*.

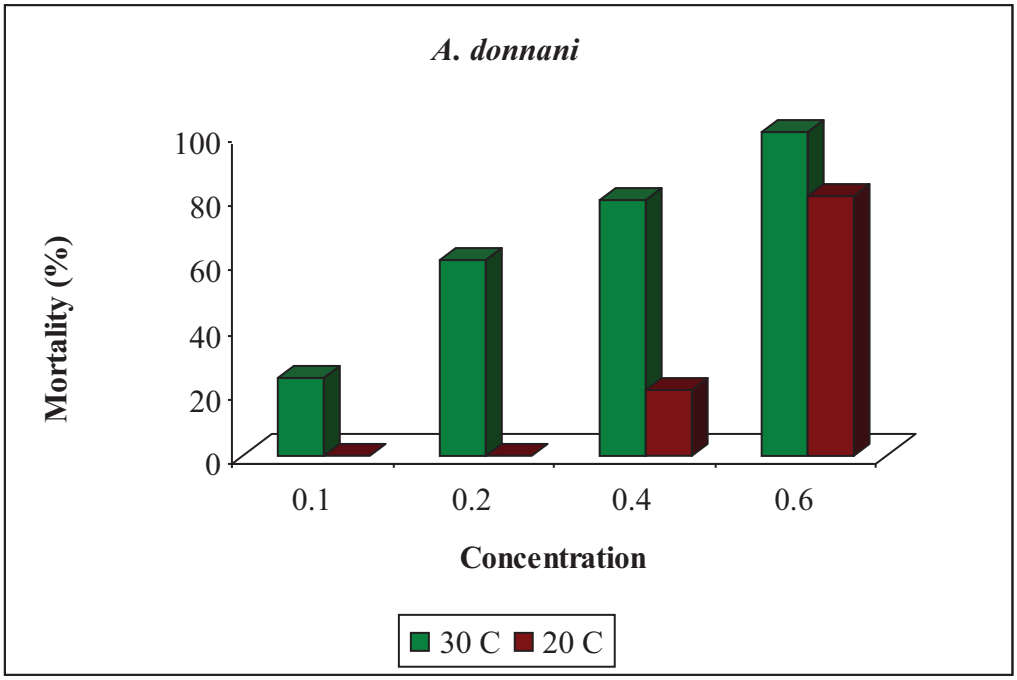
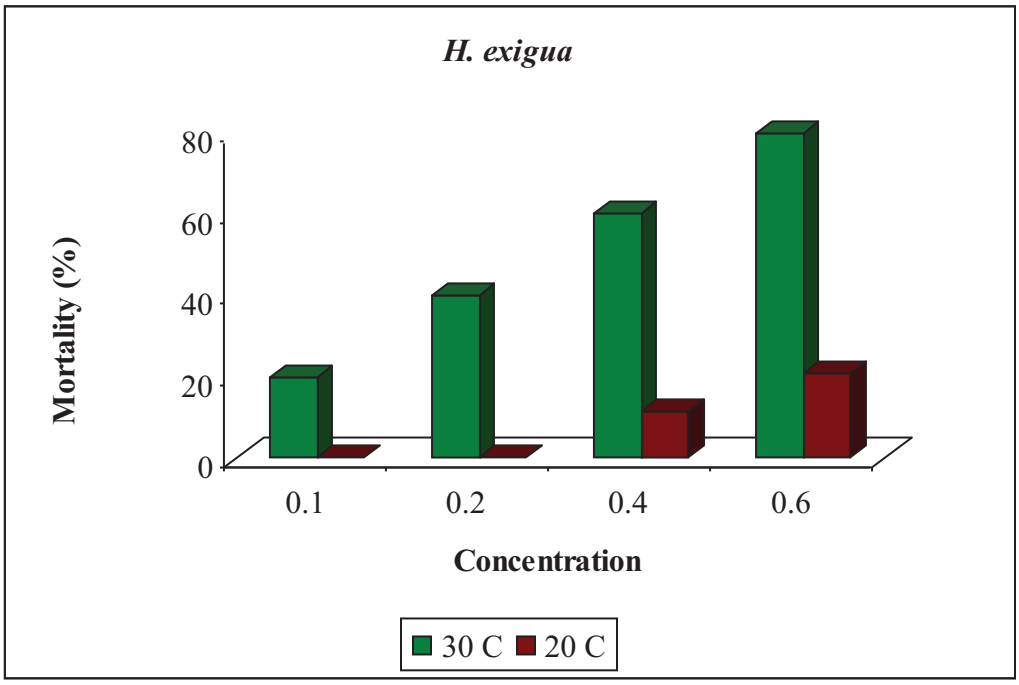


Fig-1.d Graphical representation of Artemia cytotoxicity of *H. exigua*



At this temperature, the toxicity of *Callyspongia sp*. was reduced as it resulted in 60% mortality at 6 mg/ml; while it resulted in 97.5% mortality at the same concentration at 30±2°C. Similarly *H. exigua* showed 40.0% mortality at 6 mg/ml while it produced 100% mortality at 30±2°C.

Table-9. Brine shrimp cytotoxicity profile of sponge extracts at different temperatures.

Sponge extracts	**Concentration**	**Mortality (%)**	
		30±2°C	**20±2°C**
Callyspongia (MSE 2)	1%	10.2±2.6	0
	2%	50.0±7.0	20.0±3.4
	4%	91.6±1.26	40.0±5.6
	6%	97.5±4.8	60.0±7.37
D. nigra (MSE 9)	0.20%	20.0±4.14	0
	0.40%	90.0±3.94	0
	0.60%	100±0.0	10.0±1.26
Sigmadocia (MSE 5)	2%	10.0±1.26	0
	4%	30.0±3.16	10.2±2.5
	6%	50.0±4.93	21.2±2.5
	10%	80.0±5.0	40.0±5.15
A.donnani (MSE 3)	0.1 %	23.8±6.25	0
	0.2 %	60.4±6.46	0
	0.4 %	79.2±8.12	20.0±1.78
	0.60%	100±0.0	80.2±4.74
H. exigua (MSE 7)	0.1 %	20.0±7.87	0
	0.2 %	40.2±1.32	0
	0.40%	60.0±7.0	11.4±3.2
	0.60%	80. ±3.16	20.6±3.0

Mean ± SD n-10 experiments

Micro-algal lethality profile

The results of micro algal lethality bioassay indicated that, the secondary metabolites of the sponges, *D. nigra* and *A. donnani* were extremely toxic whereas, *H. exigua* was less toxic. *D. nigra* effectively prevented the growth of *I. galbana* and

Nanochloropsis sp. at all concentrations. However, it exhibited a lower level of toxicity against *C. salina*. The effect of MSMs on the growth of microalgae is depicted in Table-10. *A. donnani* extract, showed extreme toxicity on the *Nanochloropsis sp*. The toxicity was extended against the growth of *I. galbana* and *C. salina* at all concentrations. It was found that *H. exigua*, slightly influenced the growth of *C. salina* and *I. galbana* at 1.0% level whereas at higher concentrations, the growth declined. A drastic decline in the growth was noticed at all concentrations against *Nanochloropsis sp*. In the case of *Callyspongia sp.* the growth of all the algal species (*C.salina, I.galbana* and *Nanochlorpsis sp.*) declined drastically at all concentrations. It was also noted that the degree of inhibition of the extracts of *Sigmadocia sp.* was notably lesser, when compared with the individual activities of all the other extracts.

Table-10. Microalgal lethality profile of sponge extracts

Sponge extracts	Concentration	Inhibition (%)		
		I. galbana	***Nanochloropsis sp.***	***C. salina***
Callyspongia (MSE2)	1%	60	35	40
	2%	78	45	48
	3%	92	64	63
	4%	98	78	80
D. nigra (MSE9)	1%	92	94	15
	2%	95	98	28
	3%	97	98	34
	4%	100	100	50
Sigmadocia (MSE5)	1%	20	27	22
	2%	30	34	28
	3%	42	45	32
	4%	55	54	43
A. donnani (MSE3)	1%	25	92	-
	2%	34	94	43
	3%	42	97	52
	4%	55	100	61
H. exigua (MSE7)	1%	-	-	-
	2%	21	29	30
	3%	36	41	40
	4%	48	55	44

Larvicidal effect

Larvicidal potentials of MSMs, based on the mortality of second instar larvae are depicted in Fig-2a, b, c, d and e. The results indicated that the second instar larvae were more susceptible than the fourth instar larvae (Table-11).

The secondary metabolites of *H. exigua* had more potent larvicidal activity followed by *D. nigra. Sigmadocia* effectively killed all the second instar larvae at 4 % level whereas D.nigra produced the same degree of activity at 6 % levelThe larvicidal potential of the other three sponge extracts were more or less same and they produced 100% mortality at 10% level. Based on the LC_{50} values, the potency of MSMs were categorized as *H. exigua* > *D. nigra* > *Sigmadocia* in the decreasing order. The activity profile considerably deviated among the different larval stages. Fourth instar larvae produced ,80% mortality but the second instar larvae produced 100% mortality at 4.0% of *H. exigua* extract. administration. Notably the fourth instar larvae produced about 20% and about 10% mortality, at the concentrations that produced 100% mortality on the second instar larvae, exposed to *D. nigra* and *Sigamadoisia*, respectively. Also, the fourth instar larvae exhibited a complete resistance to all the used concentrations of the extracts of *A. donnani* and *Callyspongia*. Moreover, the sponge *H. exigua* at a concentration of 0.8% showed a mortality rate of about 30% in the case of second instar larvae, and at this concentration, the fourth instar larvae exhibited a paramount resistance.

Table-11. Larvicidal profile of MSMs on second and fourth instar larvae of *Culex sp.*

MSMs	Concentration	Mortality (%)	
		Second instar larvae	Fourth instar larvae
Sigmadiosia (MSE5)	10%	100±0.0	10.2±2.71
	6%	61.2±3.0	0
Callyspongia (MSE2)	10%	100±0.0	0
	6%	79.8±1.32	0
A. donnani (MSE3)	10.00%	100±0.0	0
	6.00%	50.0±3.52	0
D. nigra (MSE9)	6.00%	100±0.0	20.4±3.26
	4.00%	80.0±3.05	0
H. exigua (MSE7)	4 .0 %	100±0.0	79.8±5.15
	2.00%	80.0±2.89	60.8±3.05
	1 .0 %	39.8±2.99	9.4±1.95
	0.80%	31.4±5.78	0

Mean ±SD n=10 experiments

Fig.-2a. Larvicidal potential of *Callyspongia sp.*

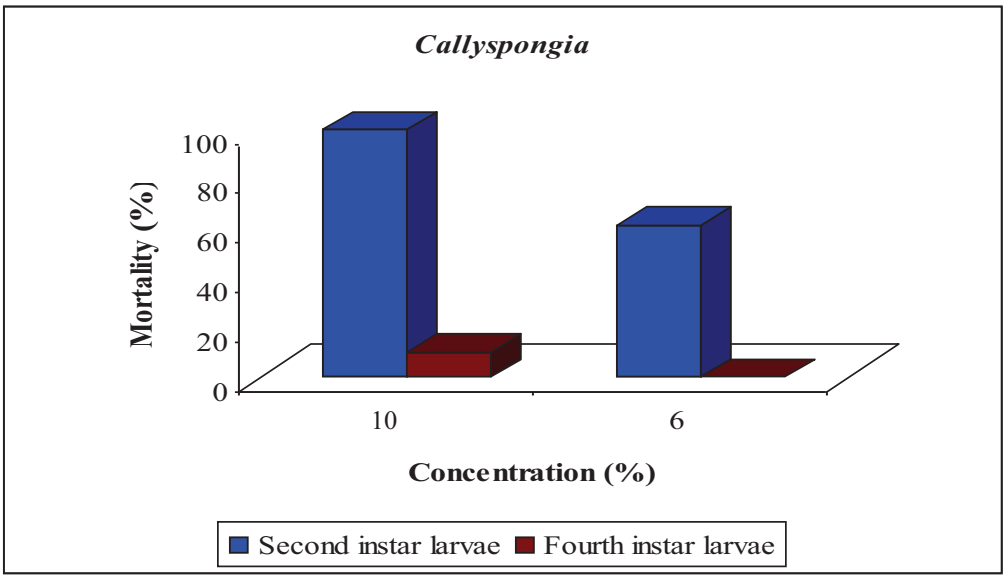


Fig-2b. Larvicidal potential of *D. nigra*

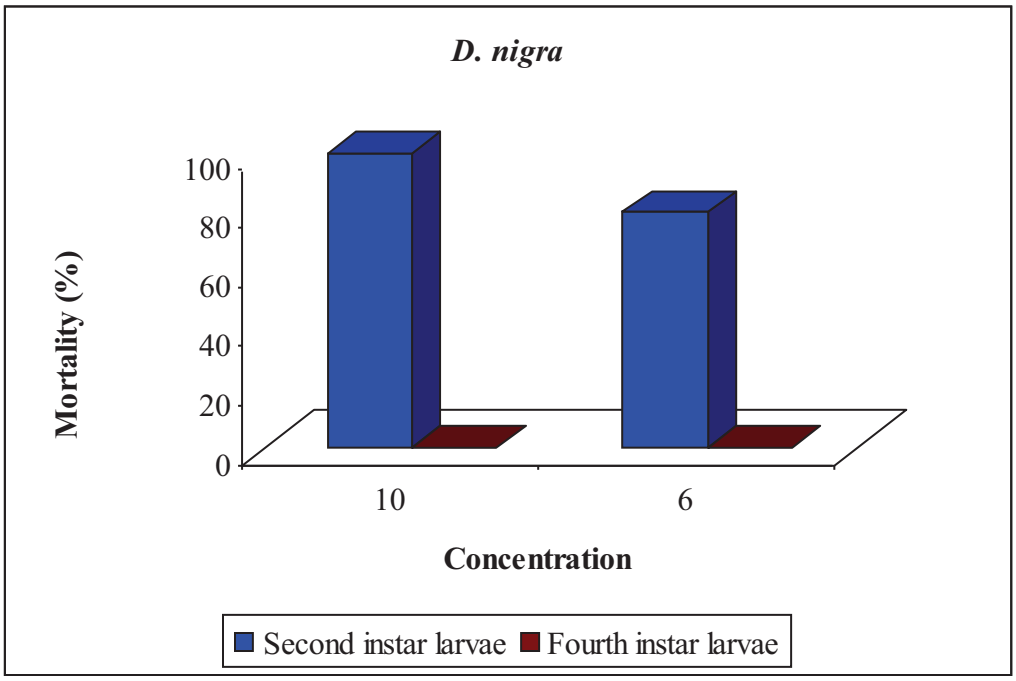


Fig. -2c. Larvicidal potential of *Sigmadocia sp.*

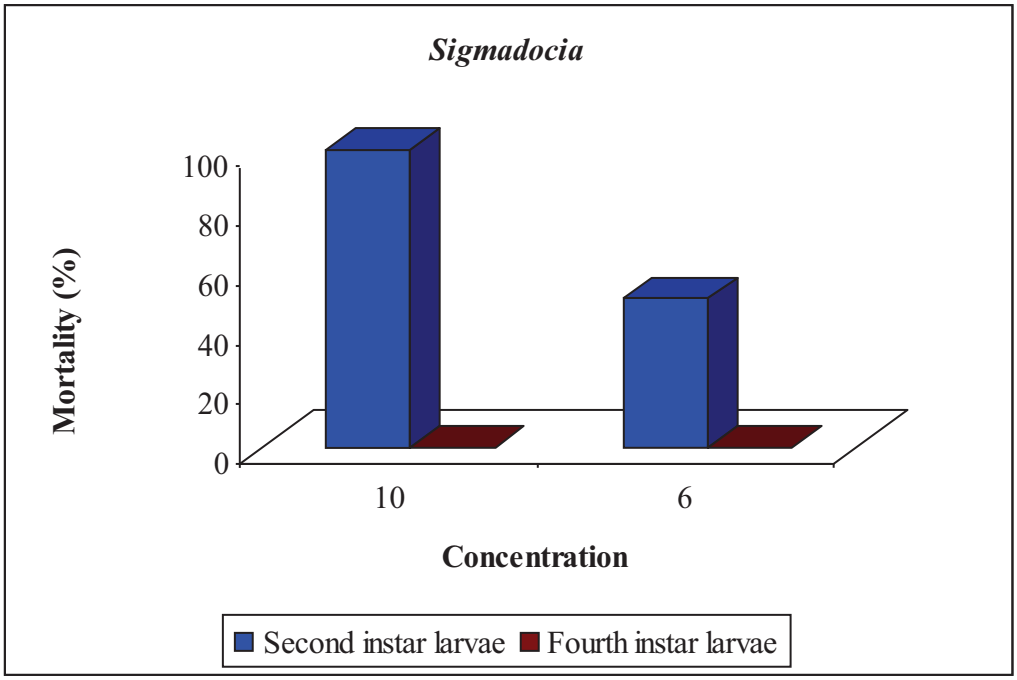


Fig-2d. Larvicidal potential of *A. donnani*

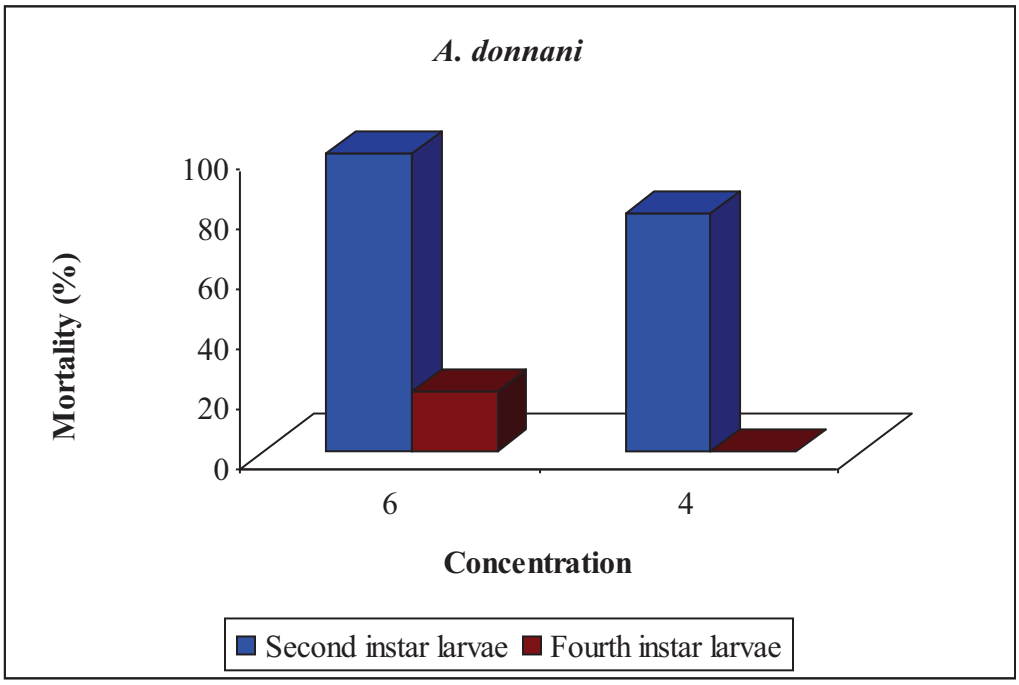
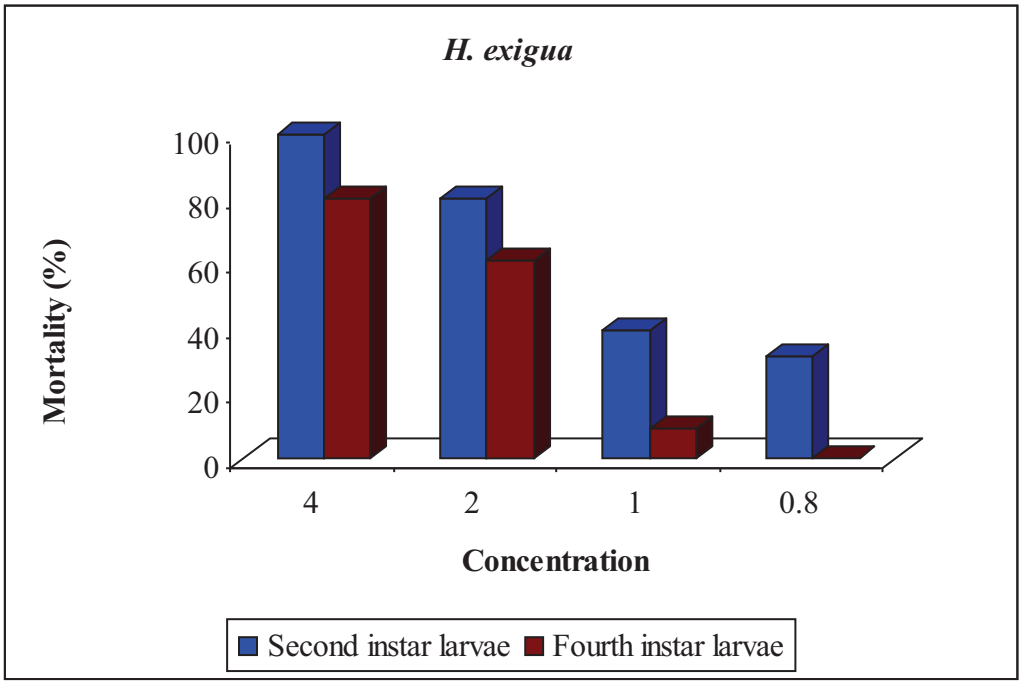


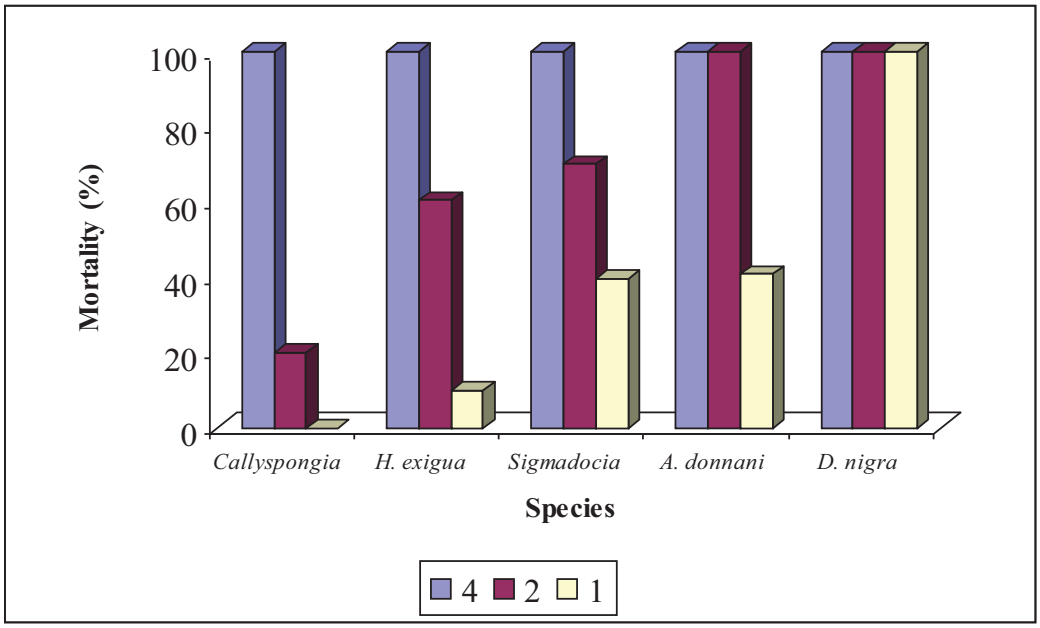
Fig. -2e. Larvicidal potential of *H. exigua*



Ichthyotoxicity

Ichthyotoxicity profile of MSMs is presented in Table-12. *D. nigra* was extremely toxic and killed all the fingerlings (*O. mossambicus*) within a short exposure time of 40 sec. at 4% level, 1 hr at 2% level and 2 hr at 1% level. *A. donnani* was found toxic next to *D. nigra*. It killed all the finger lings in a span of 50 sec. at a concentration of 4%.The ditto effect was observed with the same sponge extract in a duration of 2 hours, at a concentration of 2%.In a duration of 3 hours the mortality percentage was about 40, at 1% concentration of the same extract (Fig-3).

Fig-3 Ichthyotoxicity profile of MSMs of sponge extracts over the *O. mossambicus* fingerlings



Interestingly, the extracts of *H. exigua* produced a differential effect on the same kind of fingerlings. Cent percent mortality was observed in a duration of 1hour at a concentration of 4%, whereas the percentage of mortality was about 60 in a span of 3 hours, at 2% concentration. Moreover at a concentration of 1%, the mortality rate was about 10%, on exposure for 6 hours. Other sponge extracts namely, *Callyspongia sp.* and *Sigmadocia* displayed less toxicity on the fingerlings. Both the extracts

produced 100% mortality at 4% concentration in a duration of 6 and 2 hrs, respectively.

Although all the sponge extracts furnished 100% mortality in a concentration of 4%, the deciding factor of their comparative effectiveness (lethality), is their duration of exposure. Based on the duration of exposure (which is the prime factor to decide the lethality) the sponges are arranged in the decreasing order of their lethality, *D.nigra* > *A.donnani* > *H.exigua* > *Sigamdoisia* > *Callyspongia sp.*extracts.

Table-12. Ichthyotoxicity profile of MSMs of various sponge extracts on *O. mossambicus* fingerlings

Species	**Concentration**	**Mortality (%)**	**Time of death (hr)**
Callyspongia	4%	100	6
	2%	20.0±2.89	6
	1%	0	-
H. exigua	4%	100	1
	2%	60.6±5.6	3
	1%	10.0±3.34	6
Sigmadocia	4%	100	2
	2%	70.2±4.26	3
	1%	39.8±4.0	6
A. donnani	4%	100	50 Sec
	2%	100	2
	1%	40.8±3.96	3
D. nigra	4%	100	40 Sec
	2%	100	1
	1%	100	2

Mean ± SD n=10

Discussion

Collection of the source organism(s)

Over the last three decades, a diverse group of MSMs were chemically elucidated and tested for potent biological activity. However, the number of compounds, which were taken-up for the field/clinical trial is less. In the light of earlier reports, it could be ascribed that the failure of successful collection of concerned source organism in bulk, was the main reason. Moreover, it was reported that over-exploitation of the source organism may pose an imminent threat to the natural bed. Considering this lacuna, in the present study, the area of abundance and eco-friendly collection of the sponges were carried out so as to provide a holistic approach to the MSMs research.

In the present study, an eco-friendly collection of sponges as bycatch in the fishing nets was utilized for bioactivity screening. It is a routine process after fishing to dispose the bycatches from the fishing nets and to clean the nets. All the bycatch collections contained potent biologically active secondary metabolites. When compared to the netting and SCUBA methods, the bycatch collections were more reliable for drug discovery programme due to economy, quantity, diversity and eco-friendly collection of the source organisms. Perusal of literature indicate that utilization of such bycatches for bioactivity screening/chemical elucidation is scanty. However, a *Sigmosceptrella sp.* obtained from trawling operation in the Great Australian Bight for the chemical elucidation and bioactivity screening. A specimen of *Dendrilla cactos* collected during trawling operations in Bass strait, Australia, had yielded two new alkaloids, lamellarin-o lamellarin-p, which were previously reported from tunicates and mollusks.

Extraction solvent system

In the primary antibacterial screening, the methanol and methanol-dichloromethane (1:1) solvent system was found to be more effective in extracting

active principles from marine organisms. This is possibly due to the extraction of both polar and non-polar active principles. According to the earlier reports, these solvents were successfully used in the primary extraction of active principles from marine organisms. Thus development of appropriate solvent systems could be regarded as as important step for systematic drug development. It was reported that among the six different extracts of green alga, *Enteromorpha intestinalis*, the extract obtained with diethylether showed maximum activity against a phytopathogen *Xanthomonas oryzae* followed by ethanol and methanol. The extracts obtained with water, acetone and chloroform were found unsuitable. Also, the organic solvents were efficient in extracting antimicrobial compounds from marine organisms while water extraction was less efficient. Certain reports revealed that, the methanolic extracts are more feasible and effective for pharmacological products extraction. Based on the earlier findings the present study utilized preferred methanolic extraction.

Antimicrobial activity

In the primary antibacterial screening, it was found that the factors such as assay method and incubation temperature, apart from culture media, greatly influenced the antibiotic profile of MSMs. The agar-disc diffusion method was found to be more effective. The possible explanation for this variation is, the pour-plate technique ensures maximum accessibility of the extract to the bacterial seed embedded in the entire well depth rather than the surface-accessibility only ensured in the spread-plate technique. In the light of antibiogram, high antibiotic broad spectrum activity could be ascribed to the extract of the sponge *D. nigra*. Moreover the other sponge extracts appeared to control almost all the gram positive and gram negative bacteria. Interestingly no major difference was detected in the antibacterial activity among sponges *D. nigra* and *A. donnani* against these pathogens. The secondary metabolites of *Sigmadocia* and *Callyspongia sp.* were found to be moderate in antibacterial activity followed by *H. exigua*.

As far the sponges are concerned, the present study provides a new avenue for developing potent bioactive secondary metabolites from the bycatch collections. Methanolic extract of *D. nigra* showed broad spectrum antibacterial activity, although extracts of *A. donnani* and *C. gorgonoides* were found to be active only against Gram positive bacterial strains. Extracts made from *Sigmosceptrella sp.* collected as bycatch during trawling operation in the Great Australian Bight was inhibitory against *M. luteus*, *Serratia marcescens* and *Staphylococcus aureus* Spectrum of antibiotic activity of sponges were apparently narrowed towards Gram positive bacteria. *A. donnani* inhibited all the Gram positive bacteria although the activity was reduced against Gram negative bacteria (25%). Similarly *H.exigua* inhibited 66% of Gram positive bacteria and 12% of Gram negative bacteria. Most of the available reports on antibacterial property of sponges revealed their activity on Gram positive bacteria. Samples of 28 demosponges collected along French coast indicated high antibacterial activity against Gram positive bacteria (77%) than Gram negative bacteria (53%). The dichloromethane-methanol (1:1) extract of the sponge *Phycopsis sp.* collected from the Tuticorin coast, exhibited antibacterial activity. The secondary metabolites were chemically elucidated as Phycopsisenone, a new phenolic derivative.

The bromopyrrole alkaloids found in Agelas dispar showed moderate antibiotic activity against Gram positive bacteria such as B.Subtilis and S.aureaus. The clathramides C and D (derivatives of bromopyrrole alkaloids) showed antifungal activity against Aspergillus niger. The latrunculid marine sponges *Latrunculia sp.* and *Nagombata sp.* contained potent antibacterial discorhabdin R, which was chemically characterized as pyrraloiminoquinone The Korean sponges, *Halichondria okadai* and *Halichondria sp.* contained antimicrobial compounds such as benzoic acid, okadaic acid and dinophysistoxin

Biotoxicity studies

Brineshrimp cytotoxicity

The results of brineshrimp cytotoxicity assay indicated that the sponges *A. donnani* was highly toxic followed by *D. nigra* and *H. exigua*. One of the well-studied species A. donnani reported with vast potential of anti-tumour activity was found to be least active in the present study. Methanolic extract of *A. donnani* was highly toxic to brine shrimp (LC_{50} = 0.2%) followed by *D. nigra* (0.28%) and *Sigmadocia* (5.5%). Methanol soluble extract of the Korean sponge *Petrosia sp.* showed significant activity in the brineshrimp cytotoxicity assay (LD50 = 30 mg/ml). Guided by this assay, further fractionation and purification procedures gave potent cytotoxic polyacetylenes. The Philippine marine sponge, *Plakinastrella sp*. yielded peroxide containing metabolites and the crude methanolic extract were toxic to brineshrimp. Aglestatin A isolated from *Cymbastella sp.* (Axinellida) was highly toxic to brineshrimp. The crude extracts of marine sponges, *Pachastrella sp*. and *Jaspis sp.* collected from the south sea of Korea, exhibited significant brineshrimp cytotoxicity. Guided by this bioassay, fractionation and purification gave Pectenotoxin II and Psammaplin A, which were cytotoxic to human cancer cell lines. The bioactive acetylenic compound isolated from the lipophilic extract of Caribbean sponge *Cribrochalina vasculum* and four new oxygenated diterpenes from another species Caribbean *Myrmekioderma styx* were highly toxic to the brineshrimp*, A. salina*.

In the light of earlier reports, thorectandrols from *Thorectondra sp*., endoperoxides from okinawan sponge *Plakostis lita*, isomalabaricane triterpenes from *Jaspis sp.*, polyacetylenes from *Petrosia sp.*, Hachijodines from *Xestospongia sp.*, and *Amphimedon sp.*, Mycalamides from *Stylinosen sp.*, mycalamide D from *Mycale sp* and Asmarrines from *Raspailia sp.* were reported for potent cytoxicity against cancer cell lines (Simpson 1984)

Micro-algal lethality profile of MSMs

Over the last decade, certain microalgae have been the subject of extensive multidisciplinary research. Several species of microalgae belonging mainly to many families, inhabit inshore coastal waters where they may occasionally bloom and hence impact aquaculture resources. For the many coastal species, tests were conducted with rapidly growing and stationary phase cultures at a range of cellular concentrations and for two different exposure times (24 and 48 h). Five of the coastal species were found to be toxic to *A. salina* nauplii. Allelopathic effects of a filtrate of a culture of microalgae were used to determine the lethality possessed by various test extracts. Scrippsiella trochoidea, *Tetraselmis sp.* and *Isochrysis galbana* were the commonly tested creatures.

The secondary metabolites of *H. exigua* at 1% level, induced the growth of all the algal species (*I. Galbana, Nanochloropsis sp.* and *C.salina*). However, the secondary metabolites of *A.donnani* and *D. nigra* potentially inhibited the growth of almost all microalgae. The extract of *A.donnani* enhances the growth of *C.salina* at a concentration of 1%. Another interesting fact observed was, the extract of *A.donnani* resisted the growth of *Nanochloropsis* sp. at a concentration of 4%. Moreover, the extracts of *D.nigra* produced 100 percent growth inhibition on *I.galbana* and *Nanochloropsis* at a concentration of 4%. The methanol and methylenechloride extract of mollusc *Onchidium verruculatum* induced the growth of *Dunaliella tertiolecta* at a concentration of 10 mg/ml. However the same concentration inhibited the growth of *I. galbana*. The exact mechanism of growth inhibition was not known. However, the organic compounds present in the extract may act as chelators. Naturally occurring organic chelators, are involved in growth regulation of microorganisms.

Larvicidal activity

The results of larvicidal activity indicated that the second instar larvae were more susceptible than the fourth instar larvae. The test compounds might possess

larvicidal and growth regulatory activities when the treatment was effected to different larval stages (Rao et al., 2003). According to the same author the LC_{50} and EC_{50} values of ethylene bis-isobutyl xanthate isolated from a marine alga *Dictyosphaeria favulosa* on mosquito *Aedes aegypti* was 4.91 and 2.32 mg/l, respectively, for 2nd instar larvae and 8.0 and 3.77 mg/l respectively for 4th instar larvae.

In the present study the extracts of the sponges *Sigmadocia, Callyspongia sp.* and *A.donnani* exhibited extreme larvicidal activity (100%) over the second instar larvae, at a concentration of 10 %. On the second instar larvae, the extract of *D. nigra* and *H. exigua* displayed 100 % mortality, at a concentration of 6 and 4%, respectively, revealing that the latter is the most effective (larvicidal) one among all the tested sponge extracts.

The acetone extract of seaweeds *C. scalpelliformis* and *Dictyota dichotoma* exhibited mosquito larvicidal activity against *Aedes aegypti*. The synergistic larvicidal activity of the same extracts with known insecticides such as BHC, DDT and malathion was also reported by scientists. The secondary metabolites of *C. gorgonoides* and *H. scabra* were found to be highly lethal to mosquitoe larvae. The secondary metabolites of *C. gorgonoides* and *H. scabra* were found to be highly lethal to mosquito larvae. Methylicosadienoic acids isolated from the Caribbean sponge *Cymbastrela sp*. (Axinellida) were larvicidal beet army worm*, Spodoptera exigua* and corn rootworm *Diabrotica undecimpunctata*.

Ichthyotoxic activity

Secondary metabolites produced by marine invertebrates, were generally considered to play a role in the survival of an organism. They may be toxic or noxious and prevent predation, infection, and fouling or otherwise mediate ecological phenomena. This mechanism has been proved in several in vitro assays. Ichthyotoxic potential is considered as one of the mechanisms, which may indicate the deterrent property of sponges, to prevent predation. In the present study, the sponges exhibited

ichythyotoxicity at various concentrations. *D. nigra* was highly toxic at 4% level. The *Spongia sp.* reported to produce two classes of terpenoid toxin, the cytotoxic sponginolides and ichthyotoxic kurospongin. Crambines from *Crambe crambe*, were found to be one of the most toxic and widespread species in rocky sublittoral habitats of the Mediterranean Sea due to its antipredation (ichthyotoxic) activity.

In the present study, the moderate toxicity of *H. exigua* observed could be attributed to the presence of toxic substances such as holothurin produced high toxicity against fish larvae. The holothurian *H. leucopilota* toxin was reported to influence the metabolic process of the prawn *Caridina rajadhani.* The lower ichythyotoxicity of seaweeds revealed their dietary choice of herbivorous fish. Based on the LD50 values, *Sigmadocia* and *A. donnani* could be grouped under moderately and nearly toxic compound, respectively. Both of these secondary metabolites were elaborately studied in the shrimp experiments. If the overall bioactivity profile is considered for discussion, it could be inferred that the extract of a single species showed a wide range of bioactivity. For example, *D. nigra* exhibited potent antibacterial antifungal, brineshrimp cytotoxicity, microalgal lethality, insecticidal and anti-predation (ichthyotoxic) activities. Such broad range of bioactivity profiles was also noticed in *A. donnani*. The actual mechanism of such a broad-spectrum of bioactivity exhibited by a single species was not known. As crude extracts only were used, they may contain more than one compound or active principles. Earlier reports on the secondary metabolites such as Puupehenone and its related metabolites isolated from *Hyrtios sp*. showed similar potent antibacterial, antiviral, antifungal, cytotoxic and immunomodulatory activities. Based on these activity profiles, further studies led to the successful isolation of potent antineoplastic compounds from *Hyrtios sp.* Molokaiamine metabolite from *Aplysinella sp.* showed antiviral, antifouling and cytotoxic activities. Manzamine-type alkaloids isolated from the Philippine marine sponge *Xestospongia ashmorica* showed insecticidal, antibacterial and cytotoxicity activities. Cacospongionolide, B a new sesterpene, isolated from *Lascispongia covernosa* showed antimicrobial activity, brineshrimp cytotoxicity and

ichythyotoxicity. The crude extracts of Caribbean sponges *Ircinia campassa*, *Verongula rigida, Agelas conifera* and *Discus oxeata* were found have ichthyotoxic, cytotoxic and antimicrobial activities.

Based on the present findings, the following could be envisaged:

1. Bioassay-guided purification and fractionation of *A. donanni* and *D. nigra* may give-forth potent antimicrobial agents.

2. Microalgal lethality bioassay guided-purification of *H. exigua* extract may provide growth promoting substances.

3. Pharmacological data of *H. exigua* could be used for evaluating potent pharmacologically active compounds, which may lead to the development of novel and safe drugs for human use.

4. The ichthyotoxic potential of the extract of the sponge *H.exigua* is the least, when compared with that of *D.nigra* and *A.donnani*.

5. Among all the kinds of sponge extracts tested, the extract of the sponge *H. exigua* furnished cent percent larvicidal activity at the least concentration (4%),over the second instar larvae. The larvicidal activity of *D.nigra* stands next to the said one, and is followed by the remaining three.

6. Micro algal lethality assay indicated that *D.nigra* inhibited the growth of both *I.galbana* and *Nanochloropsis sp.* to cent percent, at a concentration of 4 %. As a strange observation, it was found that, *H. exigua* enhanced the growth of all the three microalgal species tested.

Chapter - II

IN VITRO ANTICANCER EVALUATIONS OF NOVEL SPONGE EXTRACTS

Introduction

Secondary or primary metabolites produced by marine organisms may be potential bioactive compounds of interest in the pharmaceutical industry. Marine sponges are the excellent source of bioactive compounds such as carotenoids, dietary fiber, protein, essential fatty acids, vitamins and minerals (Sanchez-Machado *et al.,* 2002). Over the past 30 years there has been a marked increase in the number of MNPs reported annually. During the period 1996-2000 there was, however, a decline in the number of new compounds were reported and the same trend still persists. Sponges continue to dominate as a source of novel compounds than the other marine sources (38 % of the total marine bioactive substances are derived from sponges). Sponges with their chemical defense mechanisms are one of the most studied organisms for the isolation of NPAs (Thakur and Anil, 2000). The constant threat from competitors, by way of over growth, poisoning, infection or predation has armed sponges with a store house of potent chemical defese agents (Bakus *et. al*., 1974; Thakur and Muller, 2004). Sponges produce a wide array of secondary metabolites ranging from derivatives of amino acids and nucleosides to macrolides, porphyrins, terpenoids, aliphatic cyclic peroxides and sterols (Faulkner 1993). Also, terpenoids isolated from *Acanthella cavernosa* and analogues of the sesquiterpene avaron from *Dysidea* avara are reported to be promising NPAs on account of the low EC_{50} (< 1µg/ml) values against barnacle cyprids.

Lot of published reviews showed the importance of sponges as potential source of pharmaceutical leads (Nakao *et al.,* 2004). Research on active compound isolation and structural determination are comparatively scanty. Since the functional aspects of a compound are in close relation with its chemical structure, the determination of the chemical structure of the compounds is of utmost importance. Most of the new drugs have been chemical synthesized whose base structure is based on the compounds

derived naturally. In the current study, various bioactive and pharmacological potentials of the sponges namely, *Haliclona exigua, Dendrilla nigra, Axinella donnani, Callyspongia sp.* and *Sigmadocia* were determined. Sponges belonging to the genus *Haliclona* have been a good source for several alkaloids exhibiting diverse pharmacological properties. These include the alkylpyridines from different *Haliclona* sp, ceramides from *H. koremella*, a hexapeptide from *Haliclona* sp etc. The latter two compounds were good inhibitors of the growth and settlement of the green algae *Ulva conglobata* and blue mussel *Mytilus edulis* respectively. In addition, several novel alkaloids have been reported from the species *Haliclona exigua* (Venkateswarlu *et al.,* 2001;) and *H. cribricutis*. In the present study an attempt has been made to purify and also partially characterize the active metabolites of the metabolic extract of the marine sponge *Haliclona* which showed more potency in the pharmacological studies.

Materials and Methods

Chemicals

RPMI 1640 was purchased from Gibco, Caspase-9 assay kit from US Biological, fetal bovine serum, streptomycin penicillin, L-glutamine, HEPES, Trypan blue, MTT [3- (4,5-dimethylthiozol-2-il)-2,5-2,5-dipheniltetrazoliumbromide], cytosine-arabinoside (Ara-C), acridine orange, ethidium bromide, agarose, ethylene diaminetetraacetic acid (EDTA), proteinase K, propidium iodide (PI), Hoescht 33342, annexin- V FITC, RNase A, Caspase-3 assay kit and Cytochrome c oxidase assay kit were purchased from Sigma- Aldrich, All other chemicals and reagents were of analytical grade and purchased locally.

Cell Cultures

K562 (human myelogenous leukemia cell line) and HL-60 (human promyelocytic leukemia cell line) were obtained from National Facility for Animal Tissue and Cell Culture, Pune, India. The cells were cultured and routinely maintained in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum and were incubated at 37 °C in a humidified atmosphere containing 5% CO2 inside a CO2 incubator.

Characters of cell lines

The characteristic features of the cell lines used in this were given in the table:

Table -13. Detailed characteristics of the cell line HL 60 and K 562

No	Cell line and Code	ATCC Code	Characters
1	HL 60 (Human promyelocytic leukemia cell line)	CCL - 240	**Organ:** peripheral blood **Disease:** acute promyelocytic leukemia **Cell Type:** promyeloblast **Protocol:** Cultures can be maintained by the addition of fresh medium or replacement of medium. Alternatively, cultures can be established by centrifugation with subsequent resuspension at 1 X 10(5) viable cells/ml. Do not allow cell concentration to exceed 1 X 10(6) cells/ml. **Interval:** Maintain cell density between 1 X 10(5) and 1 X 10(6) viable cells/ml. **Medium Renewal:** Every 2 to 3 days
2	K562 (Human immortalised myelogenous leukemia line)	CCL 243	**Organ:** blood **Disease:** chronic myelogenous leukemia **Cell Type:** lymphoblast **Protocol:** RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, 90%; fetal bovine serum, 10% **Fluid Renewal:** every 2 to 3 days **Sub culturing:** Cultures can be maintained by the addition or replacement of fresh medium. **Interval:** Start new cultures at 1 X 10 exp5 viable cells/ml. Subculture at 1 X 10 exp6 cells/ml

Extract used

The extracts used in the preliminary screening (Chapter –I) was filtered using filter paper (Wattman No – I) were also used in this anti cancer screening study.

Cell Growth Inhibition Study and Cytotoxicity Study

K562 and HL-60 cells ($1x10^6$) were seeded in 96- well sterile plates and were treated with different concentrations (50, 100, 150 µg/ml) of each extracts for 24, 48 and 72 h. The cell growth inhibition studies were done by trypan blue exclusion method (Sur *et al.,* 1995) and the cytotoxicity studies were performed by MTT assay (Cao and Li, 2002). Briefly, MTT assay is a standard colorimetric assay (an assay which measures changes in colour) for measuring cellular proliferation (Cell growth). It is used to determine cytotoxicity of potential medicinal agents and other toxic materials. Yellow MTT (3-4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple Formosan in the mitochondria of living cells. A solubilization solution (usually either dimethyl sulfoxideor a solution of the detergent sodium dodecyl sulphate in dilute hydrochloric acid) is added to dissolve the insoluble purple Formosan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wave length (usually between 500 and 600 nm) by a spectrophotometer.

This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion is directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent is causing death of cells can be deduced, through the production of a dose response curve.

On the first day of the experiment, one T-25 flask of Human leukemia cell lines was trypsinized and 5 ml of complete media was added to trypsinized cells. Further the cells were centrifuged in a sterile 15 ml falcon tube at 500 rpm in the swinging bucked rotor (400 x g) for 5 min. The media was removed and cells were

resuspended to 1.0 ml with complete media. The cells per ml were counted. The cells diluted to 75,000 cells per ml with complete media. 100 µl of cells were added (7500 total cells) into each well and incubated overnight. On the second day, cells were incubated overnight with sponge extracts with the dose of 10 mg/ml each, in a different experimental setups. On the third day of the experiment, 20 µl of 5 mg/ml MTT was added to each well. One set of wells with MTT was incubated but no cells as the control group. The plates were incubated for 0, 12, 24, 36, 48, 60, 72 hours at 37^{o}C in culture hood. The media was removed carefully and 150 µl MTT solvent was added. The cells were agitated on orbital shaker for 15 min and the absorbance at 590 nm was read with a reference filter of 620 nm.

Detection of Apoptosis by Fluorescence Microscopic Studies

Morphological changes for detection of apoptosis was carrien out in K562 and HL-60 cells (1x106) treated with 100 µg/ml sponge extracts for 24 h were observed using a fluorescence microscope. The untreated control cells and the sponge extracts treated cells were harvested separately, washed with PBS and then stained with acridine orange (100 µg/ml) and ethidium bromide (100 µg/ml) (1:1). The cells were then immediately mounted on slides and observed under a fluorescence microscope for the morphological determination of the cells undergoing apoptosis.

Confocal Microscopic Studies

Leukemic cells (1x106) were treated with 100 µg/ml of sponge extract for 24 h. After 24 h the untreated control cells and treated cells were harvested and washed with ice cold PBS. The cells were then stained with 10 µg/ml of propidium iodide and 10 µg/ml of Hoescht 33342 separately for 5 min. After mounting on slides the cells were observed to see the differences in nuclear morphology between the untreated and the sponge extract treated leukemic cells under confocal laser scanning microscope (Leica TCS-SP2 system, Leica Microsystem) installed with an inverted microscope [Leica DM-7RB] as per Mishell *et al.,* (1980). Images for propidium iodide and Hoescht 33342 were acquired from argon/krypton laser and UV laser line

using 590 nm long pass filter for propidium iodide and 450 nm band pass filter for UV images.

Study of Phosphatidylserine (PS) Externalization

PS externalization was examined after treating the cells ($1x10^6$) with 100 µg/ml of SPONGE EXTRACT for 24 h under confocal laser scanning microscope (Leica TCS-SP2 system, Leica Microsystem, Heidelberg, Germany). The untreated and different extract treated cells were harvested separately, washed with ice cold PBS and annexin V FITC binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl2 2H2O; pH 7.4) respectively and they were then stained with 5 µl of annexin V FITC for 10 min at room temperature. The cells were mounted on slides and the images were captured to observe the cells undergoing early apoptosis.

Detection of Apoptosis by DNA Fragmentation and Agarose Gel Electrophoresis

K562 and HL-60 cells were treated with 100 µg/ml of sponge extract and with standard anti-cancer drug AraC (200 µg/ml) for 24 h. The cells were harvested and washed twice with PBS. The cells were resuspended in 500 µl of lysis buffer (50 mM Tris-HCl, pH -8.0, 10 mM EDTA, 0.5% SDS), 100 µg/ml of proteinase K was added and incubation was done at 50 °C for 1 h and 37 °C overnight respectively. DNA extraction was done by following the general phenolchloroform extraction procedure (Herrmann *et al.,* 1994) and kept at -20 °C overnight. After centrifugation, DNA precipitates were washed with 70% ethanol, dried and evaporated at room temperature and dissolved in TE buffer (pH 8.0) at 4 °C overnight. To detect the DNA fragments, the isolated DNA samples were electrophoresed overnight at 20 V in 1% agarose gel and stained with ethidium bromide. DNA fragmentation was observed in UV transilluminator.

Detection of Apoptosis by Flow Cytometric Analysis

$2x10^6$ cells were treated with 150 µg/ml of extracts (MSE 7) for 48 h. Cells were washed with PBS, fixed with cold methanol by adding methanol drop-wise and kept at -20 °C for 3 min. They were then resuspended in cold PBS and kept at 4 °C

for 90 min. Cells were pelleted down, dissolved in cold PBS, treated with RNase A for 30 min at 37 °C and stained with propidium iodide (20 µl from 50 µg/ml) and kept in dark for 15 min. Cell cycle phase distribution of nuclear DNA was determined on FACS, fluorescence detector equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using Cell Quest software (Becton Dickinson). Analysis of flowcytometric data was performed using Mod-Fit software.

Cytochrome c Oxidase Assay

K562 and HL-60 cells ($1x10^6$) were treated with extract MSE 7 (100 µg/ml) for 24 h. The cells were harvested, lysed and then cytosolic extracts were prepared separately for untreated control and extract treated K562 and HL-60 cells. The cytochrome c oxidase assay was performed according to the instructions provided in the kit. 20 µl of cytosolic extract was added to 950 µl of 1X assay buffer (10 mM Tris-HCl, pH 7.0, containing 120 mM KCl). The reaction volume was made to 1.05 ml with 1X enzyme dilution buffer (10 mM Tris-HCl, pH 7.0, containing 250 mM sucrose) and was mixed by inversion. The reaction was started by the addition of 50µl of Ferrocytochrome c substrate solution and mixed by inversion. The O.D was read immediately at 550 nm.

Hydrogen Peroxide Scavenging assay

The ability of the extracts to scavenge hydrogen peroxide was determined. Hydrogen peroxide solution (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Plant Extracts (1 mg ml-1) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds were calculated as follows:

% Scavenged (H_2O_2) = [(Ao -A1)/Ao] x 100

where Ao was the absorbance of the control and A1 was the absorbance in the presence of the sample of extract and standard.

DPPH Radical scavenging assay

100 µg of extracts were added to 295 µl DPPH solution (4,5 mg DPPH (HIMEDIA) in 100 ml methanol) in each well of 96 well plates. The absorbance at 517 nm was then monitored at 15 seconds interval from 0 to 5 min. Methanol was used as the blank solution. Ascorbic acid was used as a positive control representing 100% radical scavenging activity in each experiment.

Statistical Analysis

All data are represented as arithmetic mean ± S.E.M. Statistical analysis was done by Student‟s t-test. A probability value of less than 0.05 was chosen as the criterion of statistical significance.

Results

Cell Growth Inhibition Study and Cytotoxicity Study

It was clear that all sponge extracts in different concentrations such as 50, 100, 150 µg/ml significantly inhibited the growth of K562 and HL-60 cells compared with that of the control cells after 24, 48 and 72 h of treatment in a concentration-dependent manner were displayed by trypan blue exclusion method (Table - 14).

In the MTT assay, there was significant concentration dependent reduction in the O.D values after treating the K562 and HL-60 cells with 50, 100, 150 µg/ml of sponge extracts for 24, 48 and 72 h compared to the control cells (Table - 15). These observations provided proof for cytotoxic nature of chosen (MSE3 and MSE7) sponge extracts.

Table – 14. The results of cell growth inhibition assay by trypan blue exclusion method

Code	Cell lines	Concentrations of extracts (µg/ml)	Durations		
			24 hrs	48 hrs	72 hrs
Control	HL-60		40	70	90
	K562		38	58	70
MSE2	HL-60	50	38	35	35
		100	32	25	22
		150	35	30	28
	K562	50	30	40	45
		100	25	34	29
		150	24	30	24
MSE3	HL-60	50	36	32	30
		100	30	24	20
		150	28	23	18
	K562	50	30	28	25
		100	24	22	20
		150	22	20	22
MSE5	HL-60	50	48	39	36
		100	38	35	32
		150	30	30	33
	K562	50	40	42	42
		100	38	34	30
		150	34	30	28
MSE7	HL-60	50	28	25	15
		100	23	20	12
		150	22	20	12
	K562	50	26	30	25
		100	25	24	19
		150	22	18	14
MSE9	HL-60	50	40	38	39
		100	38	35	37
		150	35	34	38
	K562	50	40	42	45
		100	38	40	39
		150	34	36	34

Table – 15. The results of MTT cytotoxcity bioassay

Code	Cell lines	Concentrations of extracts (μg/ml)	OD		
			24 hrs	48 hrs	72 hrs
Control	HL-60		0.64	0.58	0.78
	K562		0.58	0.68	0.54
MSE3	HL-60	50	0. 46	0.42	0.38
		100	0.38	0.34	0.28
		150	0.32	0.30	0.24
	K562	50	0.36	0.24	0.22
		100	0.28	0.22	0.20
		150	0.22	0.20	0.18
MSE7	HL-60	50	0. 28	0.24	0.24
		100	0.24	0.24	0.22
		150	0.22	20	12
	K562	50	0.26	0.24	0.23
		100	0.24	0.22	0.16
		150	0.22	0.20	0.19

Detection of Apoptosis by Fluorescence Microscopic Studies

Observations revealed that sponge extract MSE3 and MSE7 treated leukemic cells (K562 and HL-60) were stained with both acridine orange and ethidium bromide compared with that of the untreated control cells, stained with only acridine orange, indicating the fact that the treatment with sponge extract brought about apoptotic changes in the cells like condensation of chromatin and nuclear fragmentation (plate- 3)

Confocal Microscopic Studies

Sponge extract induced apoptotic changes in all tested leukemic cells after 24 h of treatment showing chromatin disintegration and formation of apoptotic bodies whereas the untreated control cells were with intact nuclei (plate - 4).

Detection of Apoptosis by DNA Fragmentation and Agarose Gel Electrophoresis

The gel pattern of the DNA samples isolated from untreated control K562 cells showed intact DNA bands whereas the gel pattern of the DNA samples isolated from sponge extracts MSE 3, MSE5 and MSE7 treated K562 cells showed degraded DNA

bands in the form of ladders (Plate - 5). So, the observations confirmed that the treatment with sponge extract caused apoptosis in human leukemic cells.

Detection of Apoptosis by Flow Cytometric Analysis

Flow cytometric data analysis revealed that after 24 h of treatment with MSE7, 23.16% of K562 and 9.21% of HL-60 cells were in LR quadrant (early apoptotic stage) and 27.65% of K562 and 24.42% HL-60 cells were in UR quadrant (late apoptotic stage) (Plate - 6).

Cytochrome c Oxidase Assay

The cytochrome c oxidase colorimetric assay is based on observations of the decrease in absorbance at 550 nm of ferrocytochrome c caused by its oxidation to ferricytochrome c by cytochrome c oxidase. The cytosolic extracts of control untreated K562 and HL-60 cells showed higher O.D values compared with that of MSE7 extract treated cells indicating decreased oxidation of ferrocytochrome c to ferricytochrome c or in other words, lesser activity of cytochrome c oxidase whereas the decreased O.D values of the cytosolic extracts of sponge extract treated K562 and HL-60 cells implied greater cytochrome c oxidase activity resulting in the increased oxidation of ferrocytochrome c to ferricytochrome c. Treatment with 50, 100 and 150 µg/ml of MSE7 showed concentration-dependent response in all leukemic cell lines (Table -16). It might be plausible that increased cytochrome c oxidase activity in the sponge extract treated K562 and HL-60 cells was associated with the process of cell death.

Table – 16. Cytochrome C oxidase potential of sponge extract MSE7

Code	Cell lines	Concentrations of extracts (µg/ml)	OD at 24 hrs
Control	HL-60		0.150
	K562		0.147
MSE7	HL-60	50	0. 146
		100	0.139
		150	0.132
	K562	50	0.143
		100	0.140
		150	0.138

Hydrogen Peroxide Scavenging assay

The results of H2O2 scavenging activity is shown in Table - 17. The results clearly displayed that the MSE7 produced high percentage of Hydrogen peroxide scavenging activity than MSE3. Here Hydrogen peroxide scavenging activity is directly proportional to the concentration.

Table - 17. The results of H2O2 scavenging activity of sponge extracts

Incubation time	MSE7	MSE3
10	21.4±1.14	22.0±0.70
20	22.0±1.58	25.2±0.83
30	33.4±2.4	27.2±0.83
40	42.2±1.92	35.2±1.30
50	56.0±1.58	35.2±1.30
60	56.2±1.78	36.2±1.30
70	71.0±1.58	44.0±1.0
80	73.8±1.48	46.4±1.67
90	96.4±1.14	56.4±1.40
100	97.8±1.30	66.8±0.85

DPPH Radical scavenging assay

The results of DPPH radical scavenging activity are depicted table 18. The results clearly indicated that the sponge extract MSE7 induced more DPPH scavenging profile than the other groups in all concentrations. Interesingly in highr concentration (90 and 100 µg) It was also noted that the MSE7 produced high activity in low concentrations.

Table – 18. The DPPH radical scavenging activity of marine sponge extracts

Incubation time	Control	MSE3	MSE7
10	47.4±1.51	84.6±1.81	82.8±0.83
20	56.6±1.14	53.4±0.91	92±1.58
30	54.6±0.54	93.7±1.48	86±1.0
40	49.4±0.54	85.0±1.58	85.8±0.83
50	56.4±0.54	95.44±1.63	87.2±1.30
60	57.4±0.54	62.56±0.94	91.6±1.14
70	56.6±0.54	92.46±1.53	83.6±1.67
80	56.8±0.44	91.2±1.30	84.1±0.74
90	45.6±0.54	74.02±0.69	82.2±1.92
100	63.8±1.09	64.96±0.65	96.56±1.12

Discussion

A significant part of drug discovery in the last forty years has been focused on agents to prevent or treat cancer. This is not surprising because, in most developed countries and, to an increasing extent, in developing countries, cancer is amongst the three most common causes of death and morbidity. Treatment for cancer involves surgery, radiotherapy and chemotherapy and often a combination of two or all three is employed. Natural products provide an appreciable percentage of new active lead molecules, clinical candidates and drugs despite competition from different methods of drug discovery. The number of natural product derived drugs present in the total drug launches from 1981 to 2002 was recently analysed (Newman *et al.,* 2003) and it was concluded that natural products are still a significant source of new drugs, especially in the anti-cancer and anti-hypertensive therapeutic areas (Newman *et al.,* 2003). Chemoprevention is a novel approach emphasizing on the prevention or delay of carcinogenesis by means of pharmacological, biological, and nutritional intervention and recently, cancer chemoprevention with strategies using foods and medicinal herbs has been regarded as one of the most visible fields for cancer control (Jo EH *et al.,* 2004).

Practical experience has shown that once a sponge extract is found to possess anti-inflammatory activity, it is better to test whether it possesses any anti-cancer activity because it is now pretty well established that inflammation and cancer go hand in hand. Since the anti-inflammatory activity of sponge extract has already been reported (it is likely that the extract of *Litchi chinensis* leaves may also have anti-leukemic activity. The present investigation confirmed the cytotoxic activity and apoptosis inducing ability of aqueous methanolic extract of *Haliclona exigua* (sponge extract) against three human leukemic cell lines- K562 and HL-60. The anti-proliferative and the cytotoxic activities of sponge extract were supported by the observations in cell growth inhibition studies and in MTT assays respectively. *Haliclona* extract inhibited the growth and the metabolic activities of K562 and HL-60 cells in a concentration dependent manner. In a separate study it was found

that sponge extract possesses potent immunomodulatory activity and that at concentrations used in the present study it did not show any cytotoxic effect in a macrophage cell line, RAW264.7 as observed in MTT assay. This finding reveals that *Haliclona* extract preferentially acts on leukemic cells.

Apoptogenic activity of *Haliclona* extract was investigated by different morphological studies like fluorescence microscopic, confocal microscopic and phosphatidylserine (PS) externalization studies. The process of apotosis is characterized by several morphological changes such as cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies. Fluorescence microscopic images clearly showed nuclear disintegration of *Haliclona* extract treated leukemic cells compared with that of the untreated control cells when stained with acridine orange and ethidium bromide. The untreated control cells showed bright green fluorescence as the live cells with intact membrane excluded ethidium bromide and only acridine orange could enter into them. On the contrary *Haliclona* extract treated cells showed more intense orange-red fluorescence and reduced green fluorescence since apoptotic and necrotic cells could not exclude the dyes and gave a combination of orange-red and green fluorescence. So, the observations indicated that the treatment with *Haliclona* extract was inducing apoptosis in the leukemic cells. Apoptogenic activity of *Haliclona* extract was further evidenced from the confocal microscopic images of the treated leukemic cells when compared with that of the untreated control cells. After *Haliclona* extract treatment, K562 and HL-60 cells showed several signs of apoptosis like chromatin condensation, nuclear fragmentation and formation of apoptotic bodies whereas the untreated control cells were with intact nuclei. Externalization of PS from inner leaflet to outer leaflet of the membrane is the hallmark of early phase of apoptosis. Externally translocated PS binds with annexin V in a calcium dependent manner (Martin *et al.,* 1995). Fluorescence microscopic images of treated K562 and HL-60 cells showed bright green fluorescent rings of externalized PS, supporting the fact that treatment with *Haliclona* extract induced apoptosis in the leukemic cells. Further evidence in

support of the apoptogenic activity of *Haliclona* extract was obtained from the gel patterns of agarose gel electrophoresis. *Haliclona* extract treated cells showed degraded DNA bands in the form of ladders, a typical indication of apoptosis, whereas the untreated control cells showed intact DNA bands when observed in UV transilluminator. Dual staining with annexin V FITC and propidium iodide in dot plot assay made it possible to identify live, early apoptotic and late apoptotic cells (Darzynkiewicz *et al.,* 2001; Wising *et al.,* 2005). Experiments showed increased number of cells in the early and late apoptotic stage after treatment with *Haliclona* extract implying the fact that apoptosis was triggered by the treatment with *Haliclona* extract in K562 and HL-60 cells.

There are two major apoptotic pathways known to date, initiated by either the mitochondria (the 'intrinsic' pathway) or the cell surface receptors (the 'extrinsic' pathway). Mitochondria- mediated apoptosis occurs in response to a wide range of death stimuli, including activation of tumor suppressor proteins (such as p53) and oncogenes (such as c- Myc), DNA damage, chemotherapeutic agents, serum starvation, and ultraviolet radiation (Shi, 2001). In the intrinsic pathway, diverse proapoptotic signals converge at the mitochondrial level, inducing the translocation of cytochrome c into the cytosol. Cytochrome c triggers caspase-9 activation initiating a downstream caspase cascade through the complex formation with Apaf-1, dATP, and pro-caspase-9 in the cytosol, which ultimately lead to the activation of the executioner caspase-3 and finally cell death (Budihardjo *et al.,* 1999; Earnshaw *et al.,* 1999; Kaufmann *et al.,* 2000).

In the present study, the release of cytochrome c into the cytosol was demonstrated in the cytochrome c oxidase assay. The cytochrome c oxidase activity was increased in a concentration- dependent way after treatment with *Haliclona* extract (50, 100 and 150 µl) in K562 and HL-60 cells as represented by the decreased O.D values when compared with that of the untreated control cells. Caspase-9 and caspase-3 assays showed concentration-dependent increase in the activities of caspase-9 and caspase-3 respectively after *Haliclona* extract treatment in K562 and

HL-60 cells indicating that the cytochrome c releasewas a preceding event for the activation of the mitochondria mediated caspase cascades. The involvement of the caspase cascade in the cytotoxic activity of *Haliclona* extract needs to be confirmed using specific caspase inhibitors. These observations in the present work suggest that treatment with *Haliclona* extract induced apoptosis via mitochondria mediated intrinsic pathway in K562 and HL-60 cell populations.

H2O2 and DPPH are the tumor inducing compounds. In the present study the scavenging activity of H2O2 and DPPH were done. Various concentration of sponge extracts were tried. Among these *Haliclona* extract (100 µg) showed good scavenging activity against H2O2 and DPPH. It was followed by *A. donnani* (45 % of scavenging activity at 100 µg levels). The results of DNA fragmentation assay demonstrated that MSEs have the apoptotic effect .To ascertain the mechanism of apoptosis DNA fragmentation is the hall mark event of apoptosis (Zhang and Huang, 2005; Mugalska *et al.,* 2006.)

Apoptosis is characterized by a series of morphological changes, such as chromatin condensation, cell shrinkage ,membrane bebbling ,packing of orgenells ,the formation of apoptotic bodies ,inter nucleosomal DNA fragmentation (Fleisher ,1997). The morphology of apoptotic cell shows a highly condensed chromatin in a fragmented nucleus (Wyllie *et al.,* 1998) .Same trend was noticed in the present study. DNA loss and decrease of DNA accessibility to the die were observed in the present investigation The same characters of apoptotic cells were reported by Zamai *et al.,* (2000). The phytochemical studies of *Sigmadocia* showed that the presence of oil and phytostrols, flavanoids, esters, tannins and steroids (Deepak *et al.,* 1995; Roy *et al.,* 2002).

K562 is an erythroblastic cell line expressing the typical hallmark of CML, the Philadelphia (Ph) chromosome, and the B3A2 bcr-abl (Lozzio and Lozzio, 1975) and it has been extensively studied in order to investigate CML and the metabolic pathways underlying its therapy. K562 cell line has also been the object of

experiments concerning its resistance to apoptosis inducing drugs (Martin *et al.,* 1990). Since the present work confirmed the apoptogenic activity of *Haliclona* extract against multi-drug resistant human myelogenous leukemia cell line, K562, it is plausible that it may lead to identification of a novel anti-leukemic agent against CML. Moses Samuel Rajan et al (2014) a,b.

Chapter - III

ISOLATION AND CHARACTERIZATION OF ACTIVE PRINCIPLES FROM CHOSEN POTENT ANTICANCER MARINE SPONGES

Introduction

Bioactive substances derived from living organisms have found extensive use in the treatment of various diseases. The advent of biotechnology as a powerful and applied branch of biological sciences has accelerated the momentum of search for novel bioactive substances. Among the marine natural organisms, sponges have provided the greatest number of marine natural products and have attracted a considerable synthetic attention. Over the past 30 years there has been a marked increase in the number of MNPs reported annually. During the period 1996-2000 there was, however, a decline in the number of new compounds were reported and the same trend still persists.

Sponges continue to dominate as a source of novel compounds than the other marine sources (38 % of the total marine bioactive substances are derived from sponges). Sponges with their chemical defense mechanisms, are one of the most studied organisms for the isolation of NPAs (Thakur and Anil, 2000). The constant threat from competitors, by way of over growth, poisoning, infection or predation has armed sponges with a store house of potent chemical defense agents (Thakur and Muller, 2004). Sponges produce a wide array of secondary metabolites ranging from derivatives of amino acids and nucleosides to macrolides, porphyrins, terpenoids, aliphatic cyclic peroxides and sterols (Faulkner 1993). Also, terpenoids isolated from *Acanthella cavernosa* and analogues of the sesquiterpene avaron from *Dysidea avara* are reported to be promising NPAs on account of the low EC_{50} (< 1µg/ml) values against barnacle cyprids.

The rich structural diversity and complexity of natural products have resulted in numerous new drugs and inspired chemists to produce synthetic analogs with enhanced bioactivity (Faulkner 2000a). Although most of the drugs currently in development are results of either semi- or complete synthesis, natural products continue to play a significant role in the discovery and development of new pharmaceuticals, as was recently highlighted (Faulkner 2000a). Scientists have only scratched the surface of many unconventional natural product sources (Tulp and Bohlin 2004). Of all drugs developed between 1981 and 2006, 28% were either natural products or derived from them. Another 20% can be categorized as natural product mimics (NM). This label is somewhat controversial, as it can be interpreted as exaggerating the roles natural products play in NM development. However, one can argue that nature played an inspirational role in the initial development stage. Immunosuppression, antiinfection, oncology and metabolic diseases are regarded as the predominant therapeutic areas of natural product-derived drugs (Butler 2004).

In the 1990s the pharmaceutical industry in practice selected lead compounds exclusively on the basis of Lipinski‟s rule of five, although the rule is not directly applicable to natural products (Lipinski *et al.,*1997; Macarron 2006). As a consequence of the rigorous application of this rule, natural products were deprioritized or even eliminated from the drug discovery process. The concept of the discovery process has since then gradually changed and a renewed interest in natural products has resurged. Natural sources offer excellent opportunities for finding leads for novel targets (Tulp and Bohlin 2002). Today there is a growing awareness that natural products tend to occupy a wider chemical space than do synthetic compounds – a fact the scientific community is starting to take into account in the early drug discovery process (Feher and Schmidt 2003; Larsson *et al.,*2007).

Lot of published reviews showed the importance of sponges as potential source of pharmaceutical leads (Nakao *et al.,*2004). Research on active compound isolation and structural determination are comparatively scanty. Since the functional aspects of a compound are in close relation with its chemical structure, the

determination of the chemical structure of the compounds is of utmost importance. Most of the new drugs have been chemical synthsised whose base structure is based on the compounds derived naturally. In the current study, various bioactive and pharmacological potential of the sponge *H. exigua* was determined. Sponge belonging to the genus *Haliclona* has been a good source for several alkaloids exhibiting diverse pharmacological properties. These include the alkylpyridines from different *Haliclona sp*, ceramides from *H. koremella*, a hexapeptide from *Haliclona sp* etc. In addition, several novel alkaloids have been reported from the species *Haliclona exigua* and *H. cribricutis*. In the present study an attempt has been made to purify and also partially characterize the active metabolites of the metabolic extract of the marine sponge, *Haliclona exigua* (MSE7) which shows more anticancer potency against leukemia cell lines.

Materials and Methods

Extracts used

H. exigua was collected and extracted based on the earlier method used in Chapter I was the condensed extract was purified and the active principles were identified.

Purification by HPLC

Crude extracts which showed comparatively high apoptotic induction against human leukemia cell lines *H. exigua* (MSE7) extract were dissolved in ethyl acetate and filtered through 0.8-µm nitrocellulose membranes, The ethyl acetate extracts were rotary evaporated at 32°C, and the dried extracts were resuspended in 50% (v/v) acetonitrile: water in 1 µL/ mL of ethyl acetate. This solution was fractionated on a reverse-phase C^{18} analytical HPLC column (5 µm, 250-4, Waters, USA) mounted with a guard column by injecting 200- µL samples, eluting for 5 min with water, then for 40 min with a linear gradient of increasing acetonitrile to 100% (v/v), and maintaining 100% (v/v) acetonitrile elution for an additional 15 min. A flow rate of 0.75 mL min-1 was maintained throughout the sample run. The elutions were recorded at 450 nm graphs were collected using an automated fraction collector for further structural determination.

Structural Analysis using NMR spectroscopy

H^1 NMR and C^{13} NMR were performed in Biogeno laboratories, Agamadabad, India using the following procedure.

Hydrogen NMR (H^1) and Carbon NMR (C^{13})

1. Solvent: Chloroform (CDC^{l3}) (Deuterated solvent)
2. Few drops of a internal standard (Tetramethylsilane (TMS) added with solvent

3. Residual water in the solvent is removed by the addition of activated 4 armstrong sieves
4. The solvent is then neutralized with anhydrous granular K2CO3.
5. The purified compound as the result of HPLC elucidation is dried to remove any solvent residues.
6. The dry NMR tube is placed with kimwipe pipet filter.
7. Approximately 10 mg of sample is collected from the pipet is dipped inside the NMR tube with deuterated solvent.
8. The NMR tube is caped tightly and placed in Varian Y-3 NMR) Spectrometer. In case of Carbon NMR, the NMR tube is caped tightly and placed in Varian XL-300 Spectrometer
9. The chemical shifts were recorded and tabulated at 400 Hz frequency.

Fourier Transform Infra-red Spectroscopic analysis of compounds

For FTIR spectroscopy, the sample is ground using an agate mortar and pestle to give a very fine powder. A small amount is then mixed with nujol to give a paste and several drops of this paste are then applied between two sodium chloride plates (these do not absorb infrared in the region of interest). The plates are then placed in the instrument sample holder ready for scanning (Nicolet 6700 FT-IR Spectrometer, Thermo Scientfic).

GC MS Analysis

The crude plant extract *Sigmodicia* was subjected to centrifugation at about 10,000 rpm for about 30minutes to remove the particulates. The clear supernatant was aspirated using a pipette and transferred into a clean vial and labeled. Then the supernatants were subjected to gas chromatography analysis using a Varian Cp 3,800 model gas chromatography equipped with two flame ionization detectors and connected with Cp-ware (Polyethylene glycol)(60m x0.25nm) and Cp-5 (100%

dimethyl polysiloxane) capillary column (50 mts x0.25 nm),(film thickness0.2µm). The operating conditions were as follows;

- Carrier gas-Nitrogen
- Pressure -17psi for Cp ware column and 16 psi for Cp 5 columns
- Oven temperature –programmed from 60 ^{0}C to 240 ^{0}C at 5^{0}c/ minute.
- Detector temperature-300$^{0C.}$

The peak area calculations were done by star work station and peak identification by comparison with authentic, wherever available calculations of Kovats Retention index was done.

The Kovats index system has been widely used in the analysis of food flavors, pesticides and essential oil analysis. Kovats retention index is defined and calculated by following equation (Douglas, 2000).

1= 100 N + 100 log‟‟R (N+n) – log‟‟R(N).

Where t‟‟R (N) = adjusted retention time of n paraffin hydrocarbon of carbon number eluting before solute A.

T‟‟R (N+n) = adjusted retention time of n paraffin hydrocarbon of carbon number (N+n) eluting after solute A.

T‟‟R (A) = adjusted retention time of solute A.

Mass spectrometry analysis was performed on a Shimadzu GC 17 A QP 5,000 MS coupled with a mass detector , fitted non-polar DB-5 (Diphenyl diphenyl siloxane) capillary column of length 25 m × 0.25 mm id. GC MS operation conditions at initial temperature 60 ^{0} C – 300 ^{0} C. The injection volume was 0.1µl with helium gas as carrier at the flow rate of 0.6ml per minute.

Relative retention times (RRts) of constituents were determined using c5-c30 straight chain alkanes as standards. Individual constituents of the extract were identified by WILEY and NIST database matching by comparison of mass spectra with published data and by their comparison of their RRts

Results

Identification of Secondary Metabolites

H^1 and C^{13} NMR spectral data of the active Fr.2-3 indicated it to be related to bis-1- oxaquinolizidines, already reported from this species, while HRMS studies indicated it to be a mixture of at least 5 compounds (Table 19). Thus, its 1H NMR spectrum had strong signals in the region δ1.0-2.3, indicative of several methylene groups (Fig-4). The signals in the region δ 3.0-4.0 were indicative of the presence of CHOH and CH-N moieties. Its 13C NMR and DEPT.135 spectra (Fig-5) had multiple signals in the region δ 20-45 (t, CH2s), δ 50-55(t, CH2s linked to N atoms), δ 70-95(d, CH linked to O and N atoms). The strong signals at δ 53.4 (t) and 53.2 (t) could be due to the C-4 and C-6. The peaks at δ 77.3(d), 76.8(d), 76.6(d) and 75.9(d) are expected to be from the C-2 (of the 1-oxaquinolizidine rings) and other oxygenated carbons. HRMS data revealed the presence of 5 major compounds, i.e. nor-Araguspongine C (m/z: 465.3686, C27H49N2O4); Araguspongine C (m/z: 479.3912, C28H51N2O4) (Fig-6); dihydroxy Araguspongine (533.3535, C28H50N2O6Na) and mono & dimethyl derivatives of the latter (547.3781, C29H52N2O6Na & 561.3960, C30H54N2O6Na). Absence of vinyl proton signals in the 1H NMR spectrum and the observed double bond equivalence of five, as revealed by the elemental compositions of all above compounds also confirmed the araguspongine-type molecular structures.

Table 19. The Results of HRMS studies of *Haliclona exiguva*

Sl. No.	m/z	Elemental Composition	Calculated Mass	ppm Error	DBE
1.	561.3960 (10.38%)	$C_{30}H_{54}N_2O_6Na$	561.3960	15.3026	4.5
2	547.3781 (12.98%)	$C_{29}H_{52}N_2O_6Na$	547.3717	11.5842	4.5
3.	533.3535 (36.36%)	$C_{28}H_{50}N_2O_6Na$	533.3561	-04.8916	4.5
4.	479.3912 (6.49%)	$C_{28}H_{51}N_2O_4$	479.3843	14.3201	4.5
5.	465.3686 (33.76%)	$C_{27}H_{49}N_2O_4$	465.3686	-00.1800	4.5

Fig.4: 1H NMR spectrum of *Haliclona exiguva*

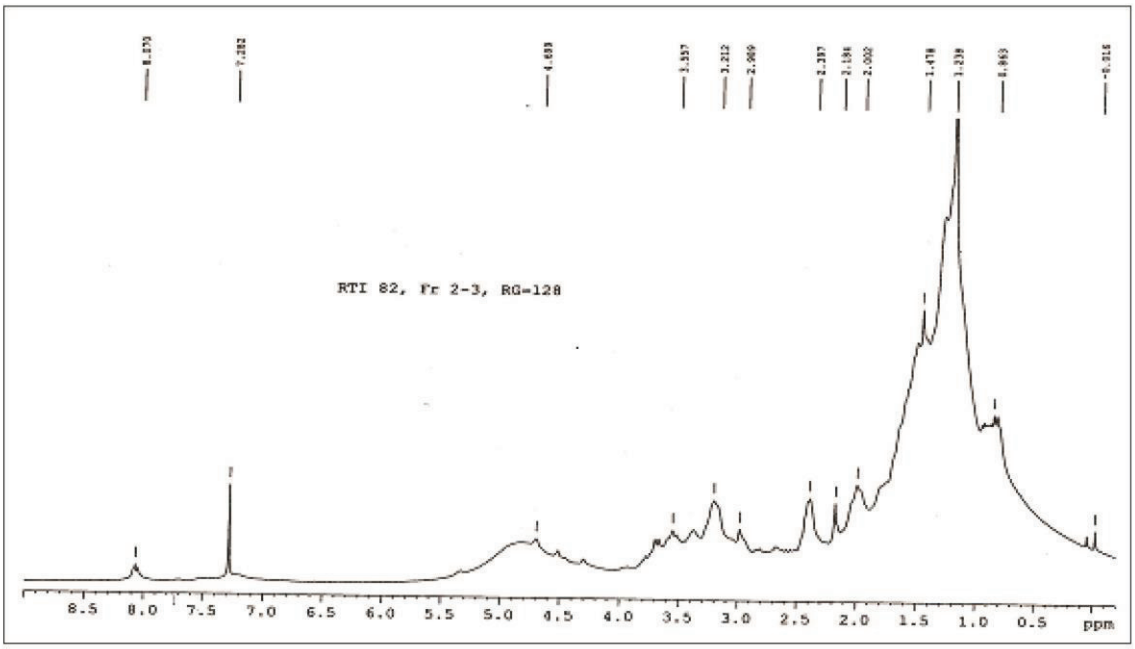


Fig.5 : C13 NMR spectrum of *Haliclona exiguva*

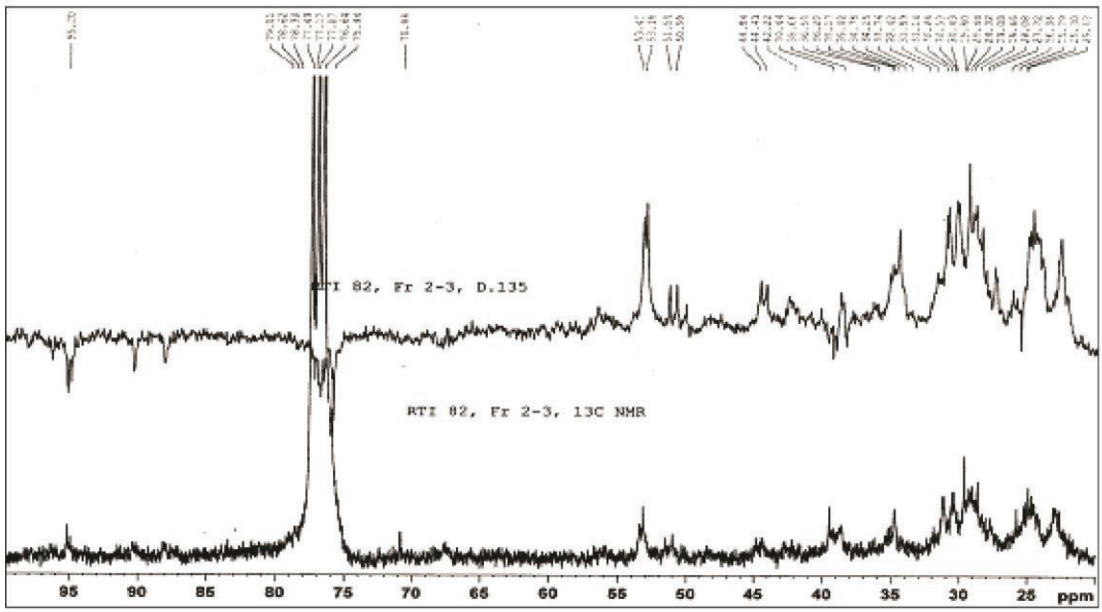
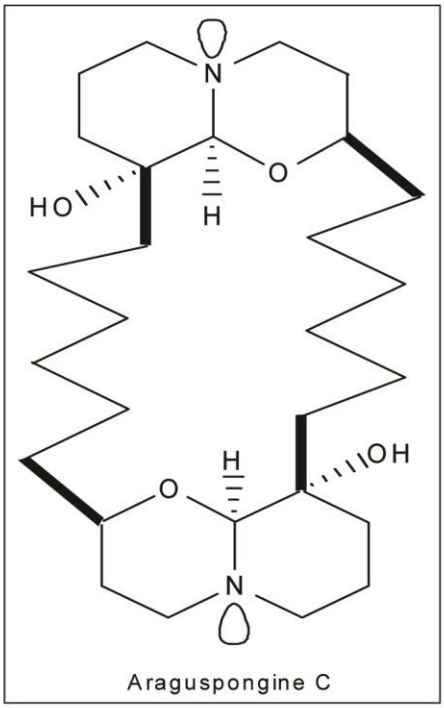


Fig.6: Identified compound from *Haliclona exiguva*



Araguspongine C

Discussion

Marine organisms exhibit a wide range of biological activity, antifertility, antiviral, (Gustafson and Milanowski 2004) antibiotic, antifungal and antimicrobial activities of marine organisms have been reported (Osterhage *et al.,*2000; Torres *et al.,*2002; Schumacher *et al.,*2003; Savoia *et al.,*2004; Shin *et al.,*2004 and Pan *et al.,*2004). Sponges have provided the greatest number of new marine natural products and have attracted considerable synthetic attention. An acetylated tetrahydroxy ceramide was isolated from an acetylated extract of *Fasciospongia cavernosa* collected on the south east coast of India. Three sulfated ceramides, calyceramides A-C with neuraminidase inhibition activity were obtained from a Japanese collection in *Discodermia calyx*. A sponge of the genus Calyx, collected in Sulawesi, Indonesia, yielded a ketosphingolipid, calyxoside with DNA damaging properties. The keto substitution of the calyxoside was located by reductive amination of a penta-acetate derivative and analysis of MS fragmentation, while the relative and absolute stereochemistry was proposed from the CD analysis of the perbenzoyl aglycone. Three glycosphingolipids were obtained from *Aplysinella rhax* collected in New Caledonia. A presumably new species of Haliclona from Queensland contained four unsaturated aminoalcohols with antifungal properties. Plaskoside A was synthesized and found to have optical rotation and spectroscopic data identical to plakoside A from Plakortis simplex. The spectroscopic data were also identical to the previously synthesized diastereomer; the absolute stereochemistry of the cyclopropyl groups remains unknown.

Three furan- containing fatty acid derivatives, plakorsins A-C, and an epoxide, plakortic acid was isolated from Thaiwanese *P. simplex* specimens. The sponge *Spirastrella abata* collected from Korean waters yielded four phosphatidyl cholines, which showed an inhibitory effect on the biosynthesis of cholesterol. *Callyspongia sp.* fallax collected in the Carribbean was found to contain the methoxylated acids. Two antimicrobial lysoplasmanylinositols were isolated from a Japanese *Theonella swinhoei*. Halicholactone from *Halichondria okadai* was synthesized stereo

selectively using chiral (diene)- Fe(CO)3 complexes. Three dithiocyanates, thiocyanatins A,B and C, were isolated from an *Oceanapia* species collected from South West Australia. These compounds have nematocidal activity and their structures were confirmed by synthesis. *Acarnus bicladotylota* collected from the South West coast of India yielded the acetylenic cycloperoxides, peroxyacarnoic acids C and D which were isolated as their methyl esters. A further series of cytotoxic polyacetylenic alcohols, have been isolated from a Korean *Petrosia* species that has previously yielded similar compounds. The absolute stereo chemistry of (-)-adociacetylene B from Adocia sp. was confirmed from a synthesis of both (+) and (-)- isomers employing enzymatic resolution.

The weakly cytotoxic heptapeptide, wainunuamide was isolated from Stylotella aurantium collected in Fiji. The total synthesis of cis, cis- ceratospongamide from the red alga *Ceratodictyon spongiosum* and symbiotic sponge Sigmadocia symbiotica has been reported. Hymenamide C from *Hymeniacidon sp*. has been synthsised using solid support methodology. A total synthesis of phakellistatin 11, isolated from *Phakkelia sp.*, revealed that the synthetic product is much less cytotoxic than the originally isolated sample. The structures of two potent inflammatory peptides, halipeptin A and B, isolated from a member of the genus Haliclona from Vanuatu, were from the member of the genus *Halilcona* from Vauatu were proposed from an analysis of spectroscopic data. Two iron chelating peptides, haliclonamide A and B, isolated from a *Haliclona* species collected in Palau were proposed to have structures with furan and benzene ring respectively on the basis of spectroscopic analysis.

A potent, neurologically- active aminoacid, neodysiherbaine A has been reported as a minor metabolite of a Micronesian *Dysidea*. The relative and absolute stereo-chamistries were determined by asymmetric total synthesis. The enantioselective synthesis of both (+)- and (-)- dysibetaine, isolated from *D. herbacea*, has established the absolute configuration as (S,S). Two polychlorinated thiazoles were isolated from Queensland specimens of *D. herbaceae*. From the same

collection, several polychlorinated dipeptides were reported separately; the absolute stereochemistries were determined by comparision of optical rotation data. The methyl esters are considered to be artifacts of methanolic extraction. An undescribed species of Dysidea collected in the Philippines yielded the praline-derived dysideaprolines A-F together with the enol-ether containing barbaleucamides A and B.

Two antifungal bromopyrroles, 3-bromomaleimde and 3-4 dibromomaleimide were found on *Axinella brevistyla* collected in Japan. (-)- Haliclorensin, isolated from *Haliclona tulearensis*, and assigned the structure, was synthesized by two independent groups and found to be spectroscopically non-identical with the natural product. Subsequently, a re-isolation and re-investigation of the spectroscopic data lead to a revise structure that was confirmed by enantioselective synthesis of both enantiomers. Both enantiomers of stellettadine A from *Stelleta sp*. were synthesized from (S)- and (R)- citronellal. The (S) isomer was found to have a negative rotation similar to the natural compound which had previously been assigned as (R). (-)- Stellattimide B, originally reported from a *Stelletta* species with a (6"S) configuration , has now been established as (1S,4S,8Ar,6" R) by total synthesis. (+)- Batzelladine F, originally isolated from *Batzella sp.,* recently re-assigned as *Monanchora arbuscula*, was originally assigned structure. The structure has been revised on the basis of the enantioselective synthesis of both the revised and putative structures. Mirabilin G was isolated from a South Australian *Clathria* species.

An acetylated tetrahydroxy ceramide was isolated from an acetylated extract of *Fasciospongia cavernosa* collected on the south east coast of India. Three sulfated ceramides, calyceramides A-C with neuraminidase inhibition activity were obtained from a Japanese collection in *Discodermia calyx*. A sponge of the genus Calyx, collected in Sulawesi, Indonesia, yielded a ketosphingolipid, calyxoside with DNA damaging properties. The keto substitution of the calyxoside was located by reductive amination of a penta-acetate derivative and analysis of MS fragmentation, while the relative and absolute stereochemistry was proposed from the CD analysis of the

perbenzoyl aglycone. Three glycosphingolipids were obtained from *Aplysinella rhax* collected in New Caledonia. A presumably new species of *Haliclona* from Queensland contained four unsaturated aminoalcohols with antifungal properties. Plaskoside A was synthesized and found to have optical rotation and spectroscopic data identical to plakoside A from *Plakortis simplex*. The spectroscopic data were also identical to the previously synthesized diastereomer; the absolute stereochemistry of the cyclopropyl groups remains unknown.

The Palauan sponge *Protophlitaspongia aga* yielded 3,4,5,6- tetrahydro-6-hydorxyl methyl-3,6, dimethyl pyrimidine-4-carboxylic acid that was found to inhibit the settling of larvae of the barnacle *Balanus amphitrite*. The wondonins A and B were isolated from an association of *Poecillastra wondoensis* and a *Japsis* species from Korea. Naamine B from *Leucetta chagosensis* has been synthesized.

The sponge *H. exigua* is a rich source for several bis-1-oxaquinolizidine alkaloids, exhibiting diverse biological properties such as cytotoxic, antifungal, antimalarial, antituberculosis and anti-rat brain nitric oxide synthase activities (Venkateswarlu *et al.,*2004;) Araguspongine C, belonging to the bis-1-oxaquinolizidine type of alkaloids was first isolated from *H. exigua*. A number of pharmacological activities have been reported for this compound. However, no reports are available on antifouling activity by this class of compounds. In the present study, a fraction rich in bis-1- oxaquinolizidine alkaloids was found to be active against fouling bacterial strains as well as barnacle cyprids, for the first time.

As several araguspongine analogs have already been successfully synthesized, their use in antifouling paint industry may not be a distant dream. It is obvious from the present studies that the bis-1-oxaquinolizidines in Fr.2-3 significantly inhibited the fish larval growth (Ichthyotoxicity), mosquito larva growth in effective concentration. Apart from this, *H.exigua* also has some influence due to bis-1-oxaquinolizidine alkaloids in the sample. It was concluded that the bis-1-oxaquinolizidine compound has least toxicity than other sponges and has a vast

bioactive and pharmacological potential. This compound may be synthesized and utilized as bio-medicine.

Summary

In the present study, five different species marine sponges were collected as bycatch from the peninsular coast of kerala and tamilnadu of india were extracted with methanol to determine the biotoxicity using various bioassay like brineshrimp cytotoxicity bioassay, larvicidal activity, microalgal lethality bioassay and ichthytoxicity assay were carried out using standard protocol. The results clearly indicated that the extract of *Axinella donnani* (MES 3) and *Haliclona exigua* (MES 7) possess significant activity than the other extracts. Interestingly the extracts of *Sigmadocia sps* produced 100% inhibition over all mosquito larvae even in the lower concentrations. Moreover that it produced 90% mortality in microalgae and 80% mortality in fish larvae. In addition to that the rate of toxicity is very high at the temperature 30^{o} than 20^{o}C. The same trend was noted in all tested extracts. The brineshrimp cytotoxicity profile of MES 3 at 30 and 20`C clearly displayed that the *A. donnani* is more toxic than *H. exigua*, it produced 90% mortality in 4 mg/ml concentration but the other extract produced only 80% mortality even in the higher concentration (8mg/ml) in 30^{o}C. Identical trend was also noted in all other biotoxicity studies. The antimicrobial potential of the extracts with commercial bacterial pathogens were carried out using well diffusion method. The results clearly displayed that all the sponge extracts tested for bioactivity were having antimicrobial potential. But the efficacy is more in gram positive bacteria than gram negative bacteria. The minim minimum inhibitory concentration (MIC) of *A. donnani* and *D. nigra* extracts were 6.5mg/ml and 12.5mg/ml respectively. Based on this preliminary toxity screening the extracts MES3 and MES 7 were used for further studies.

In the second chapter, the chosen extracts MES 3 and MES 7 were subjected to various *in vitro* anticancer bioassays against Human promylocytic leukemic (HL 60) and Human immortalized myelogenase leukemia (K - 562) cell lines. Various studies like cell growth inhibition study, cytotoxic study, morphological study, DNA fragmentation, Cytocrome C oxidase were studied. The result of cell growth inhibition study indicated that MES7 extracts inhibit the growth of both tested cell

lines than the other MES 3 extract. Sponge extracts MES7 and MES 3 at the concentrations of 50, 100, 150 µg/ml significantly inhibited the growth of K 562 and HL 60 cell compared with that of the control cells after 24, 48 and 72 hrs of treatment in a concentration dependent manner. Same trend as also noted in the MTT cytotoxicity bioassay. To determine apoptosis induction in leukemic cells (HL 60 and K 562) by sponge metabolites carried out by florescence and Confocal microscopy studies. Observation revealed sponge extracts treated leukemic cells (HL 60 and K 562) were stained with both acridine orange and ethidium bromide comparing with that of untreated control cells, stained with only acridine orange indicating the fact that the treatment of sponge extracts produced condensation of chromatin and nuclear fragmentation. Likeise , the confocal microscopic studies the spong extract induced apoptotic changes in all leukemic cell fter 24 h of treatment showing chromatin disintegration and formation of apoptotic bodies whereas the untreated control cells where with intact nuclei . The total cellular DNA of cancer cells was exposed to different concentrations of sponge extracts for 24 h. Then it was subjected to gel electrophoresis for the analysis of DNA fragmentation. the extent of DNA fragmentation with sizes in 180 bp ant multiples of 180 bp was detected by agarose gel electrophoresis .the both extracts treatment caused DNA fragmentation in K-562 and HL-60 cells ,whereas DNA fragmentation was not observed in control cells. Flow cytometric analysis was performed to monitor the changes in cell cycle distribution of cancer cells. Cells were treated with extracts and analyzed by flow cytometer. A distinct sub G1 peak was observed, when cells were treated with MSE 7.In agreement with DNA fragmentation results, typical subdiploid apoptotic peaks were observed in HL 60 and K 562 cells at 5 and 101M concentration for MES7 and MES3 respectively. The cytochrome c oxidase colorimetric assay is based on observations of the decrease in absorbance at 550 nm of ferrocytochrome c caused by its oxidation to ferricytochrome c by cytochrome coxidase. The cytosolic extracts of control untreated K562 and HL 60 cells showed higher O.D values compared with that of treated cells indicating decreased oxidation of ferrocytochrome c to ferricytochrome c or in other words,lesser activity of cytochrome c oxidase whereas the decreased O.D values of

the methanolic extract treated K 562and HL 60 cells implied greater cytochrome c oxidase activity resulting in the increased oxidation of c.ferrocytochrome c to ferricytochrome

Finally, to screen and identify the active principles which are associated with anticancer activity of *Haliclona exigua* extracts. The HPLC, NMR and GCMS were carried out to explore the fractions of the extracts with appropriate techniques confirmed the presence of araguspongine-type molecular structures.

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