EVALUATION OF ANTICANCER ACTIVITIES IN INDIAN SEAWEEDS

Thesis

Submitted to the University of Goa

For the award of

DOCTOR OF PHILOSOPHY

IN

MARINE SCIENCES

By

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Under the guidance of

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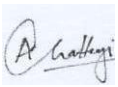
February, 2011


Dedicated to my PARENTS

CERTIFICATE

I here by certify that the thesis entitled **"EVALUATION OF ANTICANCER ACTIVITIES IN INDIAN SEAWEEDS",** submitted by Ms. RASHMI C. VINAYAK for the degree of DOCTOR OF PHILOSOPHY IN MARINE SCIENCES to the University of Goa is the result of the research work carried out by me under the guidance of **Dr. ANIL CHATTERJI**, National institute of Oceanography, Goa.

I further declare that the results of this investigation have not been previously submitted for any degree or fellowship in any university or institution.



(Dr. ANIL CHATTERJI)

Place: NIO, Goa

Date: 7/1/2013

DECLARATION

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ABBREVATIONS

ANOVA Analysis of variance between groups

BSA Bovine serum albumin

bFGF Basic fibroblast growth factor

^{13}C Carbon 13

DMEM Dulbecco's modified eagle’s medium

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

EDTA Ethylene diamine tetra acetic acid

EGF Epidermal Growth FactorELISA Enzyme-linked immunosorbent assay

FBS Fetal bovine serum

FGF Fibroblast growth factor

g Gram (s)

^{1}H Proton

H_2O Water

HeLa Carcinoma of cervix

HEK293 Human embryonic kidney

Hep-2 Human epidermoid larynx carcinoma

HUVEC Human umbilical vein endothelial cells

Inhibitory concentration (50 %) (IC_{50})

i.p. intraperitoneal

IL-1 Interleukin-1

Lethal concentration (50 %) (LC_{50})

MCF-7 Human breast adenocarcinoma cell line

$[M + Na]^+$ Molecule plus sodium ion

m/z Mass to charge ratio

µg Microgram (s)

mg Milligram (s)

NCI National Cancer Institute

nm Nanometer (s)

$OH^{\cdot}$ Hydroxyl radical

PBS Phosphate buffered saline

PDGF Platelet-derived growth factor

ROS Reactive oxygen species

Superoxide anion ($O^{2\cdot-}$)

SME *Stoechospermum marginatum* extract

TBE Tris/Borate/EDTA

Tumor necrosis factor-alpha TNF-α

Tumor necrosis factor-beta TNF-β

VEGF Vascular endothelial growth factor

Vero African green monkey Chlorocebus kidney epithelial

UV Ultraviolet

LIST OF PUBLICATIONS

1. **Vinayak, Rashmi C**.; A.S., Sabu; and Chatterji, Anil (2010) “Bio-prospecting of a few brown seaweeds for their cytotoxic and antioxidant activities,” *Evidence based complementary and alternative medicine*: doi:10.1093/ecam/neq024. **(Published)**

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5. **Vinayak, Rashmi** C.; P, Shilpa.; Chatterji, Anil.; Salimath, Bharathi P (2012)“Anti-proliferative and angio-uppressive effect of *Stoechospermum marginatum* (C.Agardh) extract *in-vitro* and *in-vivo*. **(Manuscript under preparation).**

Chapter – 1

General Introduction

1.1. BACKGROUND AND SIGNIFICANCE

The oldest description of human cancer was found in Egyptian papyri written between 3000 and 1500 BC. The father of the modern medicine, Hippocrates, who studied many types of cancer, is credited with naming cancer as *karkinos*, the Greek name for crab which in English translates to carcinoma. Cancer looked like a crustacean because there is a hard central body to a cancer and the claw-like cancer extension appeared as the legs of the crab. When karkinos was translated into Latin in the first Century, the term "cancer" meaning crab in Latin, was used.

Clinically, cancer is the name given to a large family of diseases, maybe a hundred or more that vary in age of onset, rate of growth, state of cellular differentiation, diagnostic detectability, invasiveness, metastatic potential, and response to treatment and prognosis (Ruddon, 1995). The language of cancer is to be found in the resulting deregulation of crucial biochemical pathways that control proliferation, the cell cycle, survival/apoptosis, angiogenesis, invasion and metastasis. Carcinogenesis mainly involves three stages i.e. initiation, promotion and progression. Initiation occurs because of random or carcinogen-induced DNA damage (Zaridze, 2002). Usually the mechanisms responsible for DNA repair protect the cell from the initial mutation; however, if the DNA damage is located at the gene that regulates DNA repair or cell proliferation, the risk of becoming transformed can be increased. Genes in tumor cells are considered less stable and more sensitive to additional mutations. A population of mutated cells is formed during tumor promotion by continuous and unrestrained stimulation of cell growth. Hormones and reactive oxygen species are some of the substances involved in human cancer promotion (Zaridze, 2002). The single primary tumor acquires further changes of basic behavior allowing its progeny to be able to disregard signals which regulate cell proliferation, cell

differentiation or cell death. (Yokota, 2000). Once a single abnormal cell has undergone an initial mutation, further growth and expansion of the tumor cells over normal cells continues during the stages of progression. Mutations of oncogenes, tumor suppressor genes, and DNA mismatch-repair genes are further accumulated. Eventually, proteins responsible for cell adhesion, migration, invasion and metastasis are abnormally altered leading to the formation of secondary tumors growing at new sites of the body because of the spread of primary tumor cells through the circulatory system (Yuspa *et al.,* 1994; Yokota, 2000; Zaridze, 2002).

1.1.1. Cancer Statistics

According to the Global Cancer Statistics, 2002 (Parkin *et al.,* 2005), there were 10.9 million new cases of cancer, with 6.7 million cancer deaths, and 24.6 million people alive with cancer worldwide. More than half of these cases have originated from the developing world population itself. Although estimates vary it is estimated that by the year 2020 there will be almost 20 million new cases. Worryingly, it is not only in the number of new cases that will increase, the proportion of new cases from the developing world will also rise to around 70%. The magnitude of the problem of cancer in the Indian Sub-continent in terms of sheer numbers is the most alarming. From the population census data for India in the year 1991, about 609,000 new cancer cases were diagnosed in the country. This figure had increased to 806,000 by the turn of the century. The estimated age standardized rates per 100,000 were 96.4 for males and 88.2 for females. The most common cancers found in males were cancers of the lung, pharynx, oesophagus, tongue and stomach while among females cancers of the cervix, breast, ovary, oesophagus and mouth were common (Rao and Ganesh, 1998). Cancer incidence in Indian men is about half to one third of the incidence recorded in USA and Europe. Incidence rates in Indian

women are about half the experience of USA and European women. A global comparison shows that India has high incidence rates of cancer of oral cavity, pharynx, and cervix.

1.1.2. Causes of cancer

In many cases, the causes of cancer are not clear, but both external and internal factors play an important role and among them cigarette smoking is a major causal factor. Other than that, heredity, lifestyle, viruses, exposure to ultraviolet (UV) light and carcinogenic chemicals, hormones and immunology have been implicated in cancer directly or indirectly (Zaridze, 2002).

1.1.3. Cancer Prevention

The best way to reduce deaths from cancer is to follow preventive measures. Medical doctors generally agree that about one-third of all human cancers are directly related to cigarette smoking. (Ruddon, 1995) For smokers, the risk of cancer is much higher than that of the nonsmokers. Excluding the UV rays of sunlight which cause skin cancer, the next most common cited cancer-causing factor is diet. The National Cancer Institute and the American Cancer Society recommend a diet low in fat, high in natural fiber, and rich in fruits and vegetables. Chemoprevention on the other hand is simple prevention with the use of drugs. The word "drugs" is used to include dietary supplements, hormones, and vitamins etc., as well as real drugs such as aspirin and other synthetic agents used for therapeutic purposes. The number of chemopreventive agents is increasing day by day (Kellof *et al.,* 1992).

1.1.4. Cancer treatments

Clinically, weapons to fight cancer are surgery, radiotherapy and chemotherapy (Grella *et al.*, 2003). However, the oldest and the most common treatment for cancer is still surgery, and remains the principal modality of treatment for many of the cancers seen today (Shiu, 2003). Reconstructive surgery is done when one part of the body is replaced with another part. Frequently, hormonal therapy is used to keep cancer cells from getting the hormones they need to grow as a follow-up to surgery (Hellman *et al.*, 2005). In case of radiation therapy, ionizing radiations are used to treat cancer (Ward, 1994) by delivering photon beams and particle beams. Approximately 50 % of all cancer patients are treated with radiotherapy, either as a primary treatment with curative intent or for palliation of cancer related symptoms (Vink *et al.,* 2007). High-energy X-rays are used to damage cancer cells and stop them from growing and spreading. It can be used to shrink a tumor before surgery, but it is often used after surgery. Like surgery, it is a local treatment which affects the cells only in the treated area. Alternatively, chemotherapy is a type of cancer treatment that uses drugs to kill cancer cells. Unlike surgery and radiation therapy it is systemic and works throughout the body. A single drug or a combination of drugs may be used, often after surgery to kill any hidden cancer cells that remain in the body.

1.1.5. Anticancer agents

Classical anti-cancer drugs are grouped as chemotherapy, hormonal therapy and immune therapy (Espinosa *et al.,* 2003). The treatment of cancer has traditionally used agents that interfere with the cell division process (Donald and Hogg, 2004). More recently, research and novel therapies have targeted the growth signals that drive the proliferation and survival of the cancer cells and the tumor vasculature. DNA-interactive drugs in clinical use represent one of the most important

drug classes in cancer therapy (Grella *et al.,* 2003). In general, there are three major types of the above mentioned clinically important drugs:

- The intercalators, which insert between the base pairs of the double helix and determine a significant change of DNA conformation being accompanied by unwinding and elongation of the duplex;
- The alkylators, which react covalently with DNA bases and;
- The DNA strand breakers, which generate reactive radicals that produce cleavage of the polynucleotide strands.

1.1.6. The need for new anti-cancer drugs

The clinical application of new anti-neoplastic drugs has been hindered by their low therapeutic index and lack of efficacy in humans (Sanchez *et al.*, 2001). Thus, an approach to improve old and new anti-cancer has been to manipulate the pharmacokinetic behavior of drug: absorption, metabolism and excretion. Problems such as drug-drug interactions, poor efficacy and high toxicity in humans in contrast to action in laboratory animals, or to variation in these problems among subjects within a population, frequently have their origin in drug metabolism issues. At one time, the treatment of cancer focused on systemic, non-specific, high dose chemotherapy, whereas now the goal is to find a drug that balances minimal adverse events with maximal anti-tumor activity (Abou-Jawde *et al.,* 2003). Initial steps in the development of such cancer treatments have lead to the creation of several new anticancer agents. Improved systemic drug therapy is particularly important for the treatment of patients with advanced metastatic cancer, for whom surgery and radiation therapy can no longer be curative (Workman, 2001). Advanced technology and an understanding of the genetic changes that transform a normal cell into a cell

uncontrolled by the normal feedback mechanism have facilitated the creation of a new generation of targeted treatments and cancer vaccines (Abou-Jawde *et al.,* 2003).

1.1.7. Natural products in cancer chemotherapy

Earlier, many of the cancer patients were treated by surgery; however only those patients whose cancer was localized (non-metastasized) could opt for it. For this reason, patients having cancer in metastatic (spreadable) stage had to opt for chemotherapy. Very few clinically useful anticancer drugs (anti-neoplastics) have been developed by rational design (5-fluorouracil is one of the exceptions). Many of the anticancer drugs in current use are natural products or are derived from natural products. A recent review by Butler lists seventy natural products or natural product derivatives from plants, micro-organisms and marine environment currently undergoing clinical trials in the United States, Europe, Japan, and Korea (Butler, 2005). Drugs from plants (natural product drugs) have played a dominant role in pharmaceutical care for the treatment of various diseases; especially cancer (Table 1.1) (Newman *et al.,* 2003). The introduction of anticancer drugs such as the Vinca alkaloids (vinblastine and vincristine) has wrought modern day miracles. Vincristine (Oncovin®), an antimitotic agent isolated from periwinkle (*Catharanthus roseus*) is used in combination with other agents for the treatment of a wide variety of cancers, including leukemia, bladder cancer, testicular cancer and lymphomas such as Hodgkin's disease (da Rocha *et al.,* 2001). Teniposide (Vumon®), a chemical analog of the natural product podophyllotoxin, shows activity against Hodgkin's disease and other malignant lymphomas, pediatric refractory neuroblastoma, and brain tumors in adults (Butler, 2005). The alkaloid camptothecin was first isolated from the tree *Camptotheca acuminata*. It has good activity against various cancers in the laboratory (Dancey and Eisenhauer, 1996) but is too

insoluble for clinical use. However, various water-soluble analogs of camptothecin (e.g., topotecan) have been developed, and have found significant clinical use. The most important member of the clinically useful natural anticancer agents is paclitaxel (Taxol®)*, which was first discovered in the bark of western yew (*Taxus brevifolia*). Taxol was approved by the United States Food and Drug Administration (FDA) for refractory ovarian cancer in December 1992 and for refractory breast cancer in April 1994.

Table 1.1. Plant derived anticancer agents in clinical trials.

Compound	Cancer use	Status
Vincristine	Leukemia, lymphoma, breast, lung, pediatric solid cancers and others	Phase III/IV
Vinblastine	Breast, lymphoma, germ-cell and renal cancer	Phase III/IV
Paclitaxel	Ovary, breast, lung, bladder, and head and neck cancer	Phase III/IV
Docetaxel	Breast and lung cancer	Phase III
Topotecan	Ovarian, lung and pediatric cancer	Phase II/III
Irinotecan	Colorectal and lung cancer	Phase II/III
Flavopiridol	Under experimental stage	Phase I/II
Acronyciline	Under experimental stage	Phase II/III
Bruceantin	Under experimental stage	Preclinical/phase I
Thalicarpin	Under experimental stage	Preclinical/phase I

1.1.8. Marine natural products as anticancer agents: Their sources, collection and screening

High diversity and limited resources generate a relentless competition among marine habitants. As most sessile marine invertebrates contain only a primitive immune system to defend them,

these organisms developed an impressive arsenal of chemical weapons through millions of years in their everyday fight for survival. These bioactive compounds are necessarily highly potent substances, since they become immediately diluted by large volumes of sea water.

In the last few decades, huge efforts were made by both pharmaceutical companies and academic institutions, to screen marine biotopes for new compounds that may help to battle medical challenges like cancer. This systematic investigation of marine environments is reflected in the large number of novel compounds reported in the literature. The isolation of new anticancer agents derived from marine sources has been based on the collection of marine macro organisms, such as algae, sponges, tunicates and bryozoans (Table 1.2).

The progress in scuba-diving techniques and deep-water collection instruments has been pivotal in the collection programs implemented by academic and pharmaceutical groups. A major advance in the study of marine compounds has been the changes introduced in the nature of the studies performed with isolated products. Nowadays, the compounds are systematically tested for relevant biomedical properties with high-throughput screening that offers the potential to readily screen hundreds of thousands of extracts in parallel against numerous therapeutic targets.

Over 16,000 new marine compounds have been described; patents for the biological activity of more than 300 substances are applied for, and over a dozen marine anticancer molecules are in different phases of clinical trials (Table 1.3). Although, the development of marine compounds as therapeutic agents is still in its infancy, as in contrast to terrestrial natural products, these compounds lack an ethno-medical history of several thousand years. Thus, marine ecosystems potentially stock new compounds of a non-estimable extent which may become clinical relevant.

Table 1.2. Marine organisms as producers of anticancer drugs.

Phylums	Major subgroups	Habitat	Selected cytotoxic compounds	Comments
Algae and marine phanerogams	Chlorophyta (blue algae), Phaeophyta (brown algae), Rhodophyta (red algae)	Ubiquitous	Halomon	Very abundant, easy to collect
Porifera	Calcarea, Hexactinellida, Demospongiae	Ubiquitous	Arabinosyl cytosine, halichondrin B, spongistatin	Highly prevalent, easy to collect; sponges are one of the dominant sources of biologically active products
Bryozoa		Tropical reef habitats	Bryostatin 1	Small size, filter-feeding organism. Contains symbiotic microorganisms
Mollusca	Sea hares	Indian ocean	Dolastatins	Concentrate metabolites obtained from their highly specialized diets, which are based on other marine living organisms (seaweed, sponges) and incorporate them as their own defense mechanism.
Chordata (Tunicates)		Regions with freely flowing water	Didemnins, ectenaiscidins	Sessile, filter-feeding organism; life either solitary or in colonies

Table 1.3. Marine-derived Compounds and Analogues in clinical trials.

Compound	Source	Target	Clinical phase	Company
Bryostatin	*Bugula neritina*	Protein kinase C	II solid tumors	Aphiosis
Halichondrin B (E7389)	*Hakichindrua okadai*	microtubules	II Metastatic breast cancer	Eisai
Kahalalide F	*Bryopsis sp.*	Unknown	II in prostate cancer	Pharma Mar
HTI-286 (Hemiasterlin Derivative)	*Hemiasterella minor*	Microtubiles	II NSCLC	Wyeth
Soblidotin*	*Dolabella auricularia*	Microtubiles	I NSCLC	Daiichi Pharmaceuticals
Cemadotin*	*Dolabella auricularia*	Microtubiles	II NSCLC, Prostrate cancer	Knoll Pharmaceutical company
Synthadotin*	*Dolabella auricularia*	Microtubiles	II NSCLC, Prostrate cancer	Genzyme Corporation
Didemins	*Tridid*emnum *Solidum*	Palmitoyl thioesterase	II non-Hodgkin's lymphoma	Pharma Mar
Aplidin	*Aplidium albicans*	Ornithin decarboxylase	II medullary thyroid	Pharma Mar

**Dolastatin analogues*

1.2. APOPTOSIS: A HISTORICAL OVERVIEW

Hippocrates of Cos, the father of Western medicine (ca. 460-370 BC) used the term, apoptosis for the first time in the textual translation, "the falling off of the bones" describing structural changes (bone erosion) related to tissue and cell death (Degli- Esposti, 1998). Another notable use of the word apoptosis was in textual translation the "dropping of the scabs" denoted by Galen (129-201 AD). This use of the term apoptosis relates to wound healing and inflammation (Hetts, 1998). The term programmed cell death was introduced in 1964, proposing that cell death during development is not of accidental nature but follows sequential, controlled steps leading to defined self-destruction (Lockshin and Williams, 1964). In 1972, the term apoptosis was re-introduced into modern scientific writing by Kerr *et al*. (1972) and had been coined to describe the morphological processes leading to non-inflammatory cell suicide, as opposed to chaotic, unstructured cell death coined necrosis (Kerr *et al.,* 1972).

Substantial down-regulation and up-regulation of apoptosis leads to cancer and neurodegenerative diseases, respectively. Thus apoptosis is critical to the development and maintenance of homeostasis in multicellular organisms. Apoptosis has been documented to play a crucial role in embryogenesis, metamorphosis and morphogenesis (Steller, 1995; Renehan *et al.,* 2001).

1.2.1. Morphological features of apoptosis

Apoptosis is initiated in normal cells by a variety of stimuli such as, ligation of cell surface receptors, a lack of survival signals, developmental death signals, and DNA damage as a result of defects in DNA repair mechanisms or treatment with cytotoxic drugs and/ or irradiation. These

stimuli leads to the activation of several apoptotic pathways with characteristic morphological and biochemical changes (as listed in Table 1.5) that are used in the identification of apoptosis (Kerr *et al.,* 1972; Zhao *et al.,* 2001). The apoptotic bodies resulting from the plasma membrane blebbing are phagocytosed thus preventing inflammation (Savill, 1996). These phenotypic characteristics result from specific cascading events and are thus proof of the existence of distinct pathways for apoptosis. Apoptotic pathways are being delineated, for the purpose of identifying novel apoptosis targets. Inducers or inhibitors of the apoptotic pathways can be used to control the substantial up-regulation and down-regulation of apoptosis and consequently influence disease states. An important distinction between necrosis and apoptosis is the fact that cells that had undergone apoptosis formed apoptotic vesicles that are phagocytosed by macrophages or neighboring cells preventing inflammation (Fig. 1.1) (Ren and Savill, 1998).

Table 1.4. Morphological and biochemical characteristics of apoptosis versus necrosis.

	Differences		Similarity / variables
	Apoptosis	**Necrosis**	
Nuclei	Dense condensation of chromatin	Irregular chromatin clumping	Damage occur in both
Cytoplasmic organelles	Morphologically intact	Disrupted	Secondary damage in apoptosis
Cell membrane	Cell membrane blebbing	Loss of integrity	Changes seen in both
Cell volume	Cells shrink	Cells swell	Occasionally no change
In tissues	Individual cells affected	Groups of cells affected	Superficial epithelial cells are apoptotic.
Tissue response	None	Inflammation	N/A
Nuclear DNA damage	DNA fragmentation (+-200bp), ladder visible on gel	Random DNA fragmentation, smears on gel	Takes place in both
Mitochondrial DNA damage	Occurs late	Occurs early	N/A
Cell membrane	Intact	Loss of function	N/A
Cell internal milieu	Na+/K+ pump intact and ATP required	Defective Na+/K+ pump and no ATP	N/A

Adapted from: Studzinski, 1999

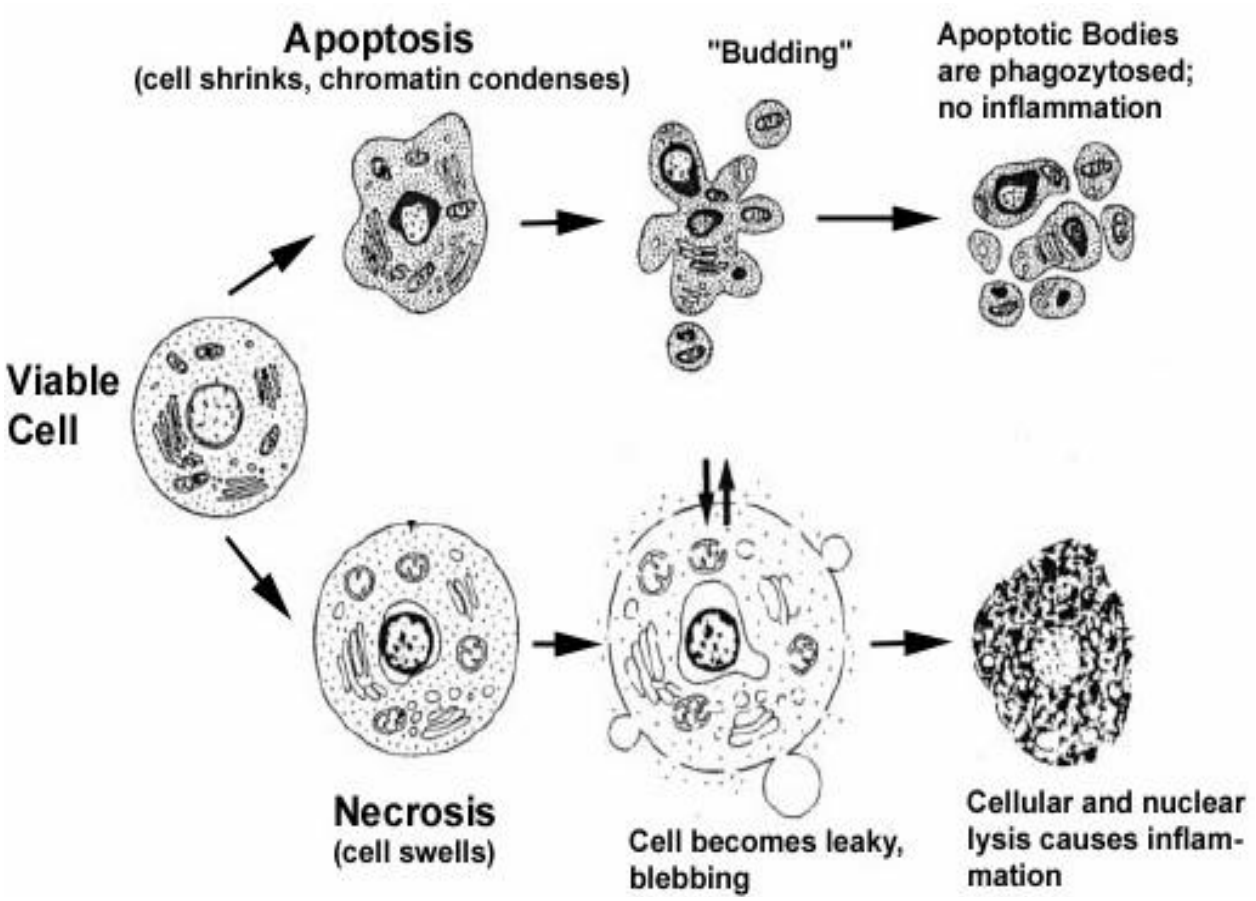


Figure 1.1. Hallmarks of apoptotic and necrotic cell death. Apoptosis includes cellular shrinking, chromatin condensation and margination at the nuclear periphery which ultimately results in membrane-bound apoptotic bodies/ vesicles that are phagocytosed without triggering inflammatory responses. The necrotic cell swells, disruptes and releases its contents into the surrounding tissue causing inflammation (Van Cruchten and Van Den Broeck, 2002).

1.2.2. Physiological significance of apoptosis

The development and maintenance of biological systems depend on the refined interaction between cells. Throughout development, many cells undergo apoptosis which contribute to the sculpturing of the organs and tissues (Meier *et al.*, 2000). Also, adult organs maintain homeostasis by constantly undergoing physiological cell death which is balanced with proliferation. Holistically, the apoptotic processes are of widespread biological significance playing vital roles in development, differentiation, homoeostasis, regulation and functioning of the immune system, as well as the removal of defective or harmful cells.

Consequently, the abnormal or excessive up-regulation and down-regulation of apoptosis is implicated in a variety of pathological conditions. The abnormal or excessive down-regulation and/or the termination of apoptosis result in the formation of malignant tumors and other diseases such as Autoimmune Lymphoproliferative Syndrome (ALPS) (Sneller *et al.*, 1997). Conversely abnormal or excessive upregulation of apoptosis leads to other disease states such as Alzheimer's disease (Jellinger, 2001) and Parkinson's disease (Merad-Boudia *et al.*, 1998). Apoptotic cell death is a common discernible occurrence in all kinds of protozoans (Solomon *et al.*, 1999) and eukaryotes (Frohlich and Madeo, 2000; Ameisen, 2002). Insight into the origin and evolution of apoptosis has been made possible by the identification of crucial apoptosis components in the nematode worm, *Caenorhabditis elegans* (Hengartner and Horvitz, 1994).

1.2.3. Molecular mechanisms of apoptosis signaling pathways

Apoptosis pathways can be initiated via different stimuli—that is, at the plasma membrane by death receptor ligation (extrinsic pathway) or at the mitochondria (intrinsic pathway) (Fig. 1.2).

Stimulation of death receptors results in receptor aggregation and recruitment of the adaptor molecule Fas-associated protein with death domain (FADD) and caspase-8. Upon recruitment, caspase-8 becomes activated and initiates apoptosis by direct cleavage of downstream effector caspases. Mitochondria are engaged via the intrinsic pathway, which can be initiated by a variety of stress stimuli, including ultraviolet (UV) radiation, γ-irradiation, heat, DNA damage, the actions of some oncoproteins and tumour suppressor genes (that is, p^{53}), viral virulence factors, and most chemotherapeutic agents. Mitochondrial membrane permeabilisation is regulated by balance of opposing actions of proapoptotic and antiapoptotic Bcl_2 family members (Bax, Bak, Bcl2 and Bcl-XL, Mcl-1). Following mitochondrial permeabilisation, mitochondrial pro-apoptotic proteins like cytochrome c, Smac/Diablo, Omi/$HtrA_2$ (caspase dependent), AIF, and Endo G (non-caspase-dependent) are released via transmembrane channels across the mitochondrial outer membrane (Ghavami *et al.*, 2009).

1.2.4. Final pathway- Caspases

The final pathway that leads to execution of the death signal is the activation of a series of proteases termed caspases. Not all caspases are involved in apoptosis and the caspases that have been described to be involved are caspase -3, -6, -7, -8 and –9 (Friedlander, 2003). The intrinsic and extrinsic apoptotic pathways converge to caspase-3, which cleaves the inhibitor of the caspase-activated deoxyribo-nuclease, and becomes active leading to nuclear apoptosis. The upstream caspases that converge to caspase-3 are caspases –9 and –8 in the intrinsic and extrinsic pathways, respectively. The downstream caspases induce cleavage of protein kinases, cytoskeletal proteins, DNA repair proteins, inhibitory subunits of endonucleases, and finally destruction of housekeeping cellular functions. Caspases also affect cytoskeletal structure, cell

cycle regulation, and signaling pathways, ultimately leading to the morphologic manifestations of apoptosis, such as DNA condensation and fragmentation, and membrane blebbing (Friedlander, 2003).

1.2.5. Targeting apoptosis pathways in cancer therapy

Inducers of apoptosis have been used in cancer therapy. Intrinsic and extrinsic pathways can be activated separately, but activation of caspases seems central to most apoptotic pathways. Several studies have attempted to induce apoptosis by interfering with a part of an apoptotic pathway that triggers the TRAIL receptor, the Bcl-2 family of proteins, caspases, and intrinsic apoptotic pathways. Most of these therapies are still in the preclinical and clinical stages of development (Table 1.6).

Apoptosis is frequently impaired in many human tumors and is also an important phenomenon in chemotherapy induced tumor-cell killing. Therefore, modulation of apoptosis by targeting proapoptotic and antiapoptotic proteins may be an important way of treating cancer. Although the use of apoptosis inducers has been introduced in cancer therapy, non-tumor cytotoxic effects still occur in normal cells because of the similarity of genes that evoke apoptosis in normal cells and tumor cells. Therefore, the development of more selective apoptosis inducers is needed to minimize side effects and maximize efficacy. Several inhibitors of intracellular antiapoptotic proteins have attracted efforts to develop them for pharmaceutical application because they play a key role in cell survival by modulating signaling pathways. Much work still needs to be done to elucidate the network of agents involved in the modulation of apoptosis.

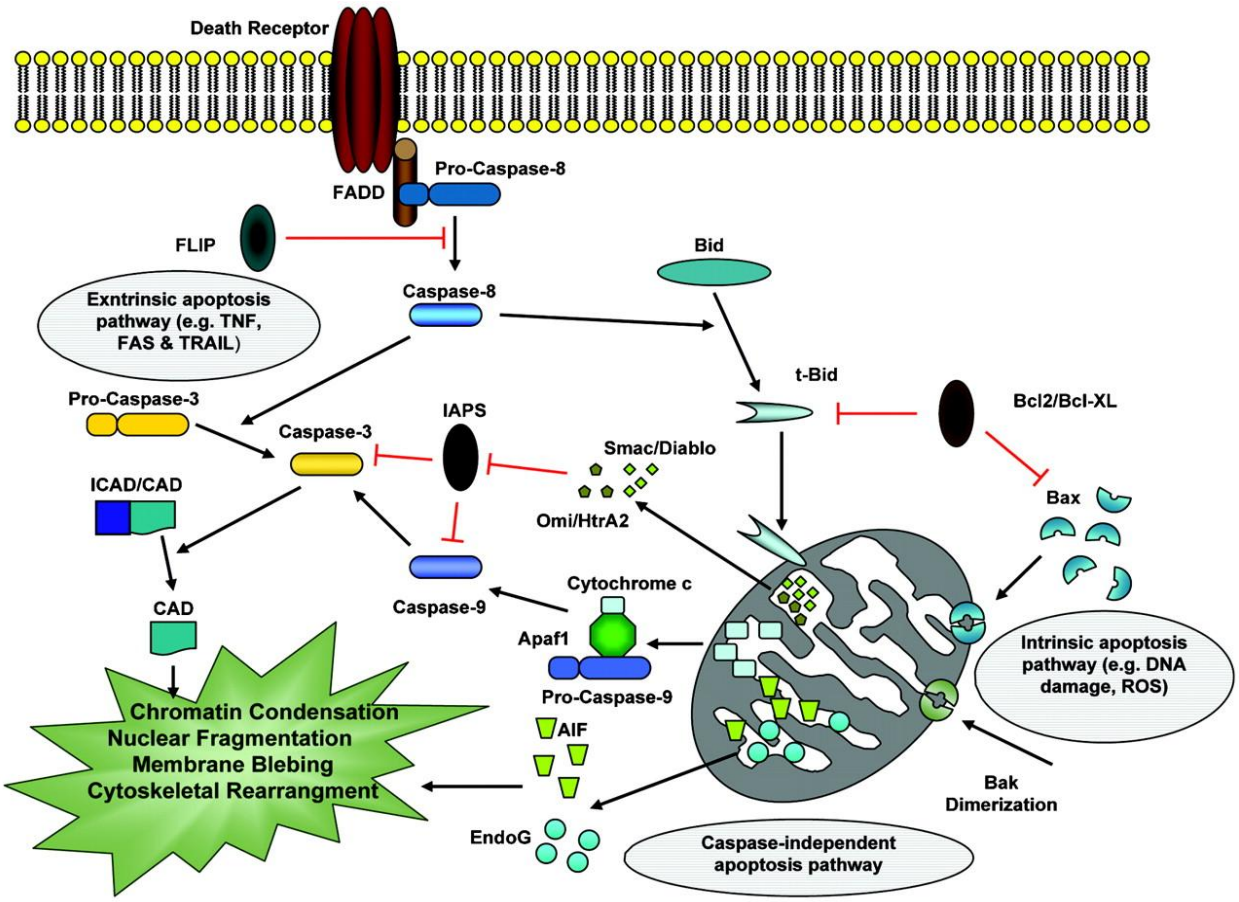


Figure 1.2. The molecular mechanisms of apoptosis. The figure shows a diagramatic relationship between mitochondria, caspases, and Bcl-2- related proteins that are involved in the mechanism of apoptotic cell death at the moleculat level. CAD, caspase activated DNase; FAS, fibroblast associated antigen; ICAD, inhibitor of CAD; ROS, reactive oxygen species; TNF, tumour necrosis factor; TRAIL, TNF related apoptosis inducing ligand (Ghavami *et al.*, 2009).

Table 1.5. Apoptosis-inducing anticancer drugs under clinical evaluation.

Drug	Target	Type	Status
G3139	BCL2	Antisense	Phase I, II/III
TLK286	Activated by GST p1-1	Small molecule	Phase I, II/III
Bortezomib	Proteasome IkB NF-kB	Small molecule	Phase I, II, III
STI571	Tyrosine kinase BCR/ABL c-kit, PDGF	Small molecule	Phase I, II, III
SAHA	Histone deacetylase	Small molecule	Phase I, II
ONYX-015	Mutated p53	Gene therapy	Phase I, II/III
INGN201	p53	Gene therapy	Phase II, III
TRAILR1	TRAILR	mAb	Phase I
17-AAG	HSP90	Small molecule	Phase I
R115777	FTI	Small molecule	Phase II,III
ZD1839	EGFR1 tyrosine kinase	Small molecule	Phase I, II, III

1.3. FREE RADICALS AND DISEASES

A free radical is generally defined as a molecule or an atom with an unpaired electron. By virtue of their unpaired electron, free radicals tend to be unstable and highly reactive (Yoshikawa *et al.*, 1997). It is believed that reactive oxygen species or free radicals (Table 1.6) are closely involved in various biological reactions and are generated by cellular respiration, ionizing radiation of biological molecules, cytochrome P450 metabolism of xenobiotic compounds and phagocytic cells like neutrophils and macrophages, as part of the cellular defense system (Palmer and Paulson, 1997; Abe and Berk, 1998; Klaunig and Kamendulis, 2004; Zangar *et al.*, 2004; Pathak *et al.*, 2005). Since most molecules formed under physiological conditions do not have unpaired electrons, free radicals abstract an electron from a stable compound and transform it into a new free radical. Therefore, free radical reactions usually occur as chain reactions. The most-studied free radical chain reaction in living systems is lipid peroxidation, which is believed to be an important cause of cell membrane destruction and cell damage (Yoshikawa *et al.*, 1997). Lipids, with the major class being triglycerides, exist in fat storage cells of plants, and animals, as well as occur in biological membranes in the form of phospholipids. Besides lipids, enzymes, nucleic acids and proteins are also important target molecules of free radicals. ROS also serve as secondary messengers for various physiological and pathological stimuli, such as inflammatory cytokines, angiotensin, growth factors, ionizing radiation and others (Abe and Berk, 1998), and thus induce MAPK family (Irani *et al.*, 1997), ERK5 or BMK1 (big MAP kinase) (Abe *et al.*, 1997) and protein kinase C (PKC) (Dalton *et al.*, 1997). They can regulate protein phosphorylation by modifying the phosphorylation/ dephosphorylation cascade and play a role in modulating several biochemical events that control cell growth, differentiation and apoptosis. Despite various negatives associated with free radicals, they are naturally produced by some

systems within the human body and show beneficial effects. Under normal circumstances, antioxidant defense systems within the body can easily handle the free radicals that are produced naturally. However, when free radical generation exceeds the capacity of the antioxidant defense systems, it can promote mutations, chromosomal aberrations or carcinogenesis at lower concentrations, and cytotoxicity and cell death at high concentrations (Palmer and Paulson, 1997; Abe and Berk, 1998; Klaunig and Kamendulis, 2004; Zangar *et al.,* 2004; Pathak *et al.,* 2005).

Table 1.6. Reactive oxygen species and free radicals.

$O_2^{\bullet -}$	Superoxide radical
$HO^{\bullet}$	Hydroxyl radical
$HOO^{\bullet}$	Hydroperoxyl radical
$RO^{\bullet}$	Alkoxyl radical
$ROO^{\bullet}$	Alkylperoxyl radical
$NO^{\bullet}$	Nitric oxide
$NO_2^{\bullet}$	Nitrogen dioxide
ClO	Hypochloride ion
$Fe^{4+}O$	Ferryl ion
$Fe^{5+}O$	Periferry ion
H_2O_2	Hydrogen peroxide
$^1O_2$	Singlet oxygen
O_3	Ozone

Adapted from: Yoshikawa *et al.* (1997).

1.3.1 Antioxidants

Antioxidants are defined as: any substance that when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate. An oxidizable substrate might be a lipid, DNA, protein or any molecule found *in-vivo*. The body is endowed with complex antioxidant systems, which include exogenous antioxidants derived from the diet and endogenous antioxidants formed in the body. The mechanisms by which various antioxidants protect against oxidative stress differ and antioxidants can be divided into following three main groups (Young and Woodside, 2001):

1. Antioxidant enzymes - catalyse the breakdown of free radical species
2. Chain breaking antioxidants - small molecules such as vitamin C
3. Metal binding proteins – prevent metal ions to form free radical

1.3.2 Antioxidant mechanism

Ingold (1968) classified all antioxidants into two groups, namely primary or chain breaking antioxidants and secondary or preventive antioxidants. The primary or chain breaking antioxidants can react with lipid radicals to convert them into more stable products, while the secondary or preventive antioxidant can reduce the rate of lipid oxidation by a variety of mechanisms (Gordon, 1990). However, it is noteworthy that certain kinds of substances possess more than one mechanism of antioxidant activity (McClements and Decker, 2000).

1.3.2.1. Primary (chain-breaking) antioxidant

A primary antioxidant, also known as “chain-breaking” antioxidant, is a substance that can accept free radicals and further delay the initiation step or interrupt the propagation step of

autoxidation (Reische *et al.*, 1998). Primary antioxidants (AH) can react with lipid and peroxyl radicals and convert them into more stable radicals or non radical products as shown in the following equations.

$R\bullet + AH \rightarrow RH + A\bullet$

$RO\bullet + AH \rightarrow ROH + A\bullet$

$ROO\bullet + AH \rightarrow ROOH + A\bullet$

The antioxidant radicals (A•) produced by this process are much less reactive than lipid or peroxyl radicals, and therefore do not promote oxidation as lipid or peroxyl radicals do. These antioxidants radicals, in fact, can also terminate the lipid oxidation reaction by reacting with peroxyl radicals, alkoxyl radicals and other antioxidants as shown in the following equations (Mc Clements and Decker, 2000).

$RO\bullet + A\bullet \rightarrow ROA$

$ROO\bullet + A\bullet \rightarrow ROOA$

$A\bullet + A\bullet \rightarrow AA$

1.3.2.2. Secondary antioxidant

Secondary antioxidants can retard lipid oxidation through a variety of mechanisms, including chelation of transition metal ions, oxygen scavenging, replenishing hydrogen to primary antioxidants, absorbing UV radiation and deactivation of reactive species (Gordon, 1990; Reische *et al.*, 1998). The main difference between primary and secondary antioxidants is that

secondary antioxidants do not transform or convert free radical species into more stable products, but usually only delay oxidation by interfering with the prooxidant system, such as metals, radiation *etc.* Many of them show antioxidant activity only if a minor prooxidative component is present in the system. For instance, sequestering agents are only effective in presence of metal ions, and reducing agents such as ascorbic acid are effective in presence of tocopherols or other phenolic antioxidants (Gordon, 1990).

1.3.3 Types of antioxidants

1.3.3.1. Synthetic antioxidant

Some of the most used synthetic antioxidants are phenolic compounds such as butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ) and propyl gallate (PG). They are used widely in the food industry because of their effectiveness and generally being less expensive than natural antioxidants. Concerns regarding toxicological effects and carcinogenic potential of synthetic antioxidants have prompted the need for natural alternatives in the last few decades (Thompson and Trush 1988a and 1988b; Thompson and Moldeus, 1988). Since about 1980, natural antioxidants have appeared as a healthier and safer alternative to synthetic antioxidants (Yanishlieva, 2001).

1.3.3.2. Natural antioxidant

Due to the increasing concerns regarding safety issues of using synthetic antioxidants, research has focused on the development and utilization of antioxidants from natural sources. The empirical use of natural compounds as antioxidants is very old and popularly consists of smoking and spicing in the home for preservation of meat, fish and their treatments (Yanishlieva, 2001).

Natural antioxidants are found in almost all plants, microorganisms, fungi, and even in animal tissues (Pokorny, 2001). The majority of natural antioxidants are phenolic compounds, and the most important groups of natural antioxidants are the tocopherols, flavonoids and phenolic acids. The mechanisms of these natural antioxidants on autoxidation control or rancidity prevention may be different. However, their presence in live plants may be for the sake of protecting tissues from injurious damage. Furthermore, the beneficial effects of consuming plant food have been ascribed, at least in part, to the presence of antioxidants in the plant and are associated with lowering the risk of most cardiovascular diseases and cancer, among other degenerative diseases of aging (Cuppett *et al.,* 1997).

1.3.3.3. Dietary antioxidants

Dietary antioxidants include the micronutrients vitamin C (ascorbate), vitamin E, beta-carotene and the polyphenols. They are known to act as effective antioxidants in biological systems such as plasma, lipoproteins, and cultured cells (Frei *et al.,* 1989; Food and Nutrition Board, 2000; Packer *et al.,* 2002). Beta-carotene and vitamins E and C have been well studied, possess strong antioxidant activities and are well absorbed, with a relatively high bioavailability. For example, vitamin C effectively inhibits lipid and protein oxidation in human plasma exposed to various (patho)physiologically relevant types of oxidative stress, such as activated polymorphonuclear leukocytes, reagent or myeloperoxidase-derived hypochlorous acid, cigarette smoke, or redox-active iron or copper ions (Frei *et al.,* 1989; Packer *et al.,* 2002). Vitamin E is the most abundant lipid-soluble antioxidant in human lipoproteins and tissues and acts as a chain-breaking antioxidant against lipid peroxidation (Packer *et al.,* 2002). ß-carotene, lycopene, lutein, and other carotenoids and oxy-carotenoids are efficient singlet oxygen quenchers and, thus, may be

important in protecting the eye and skin against UV-induced oxidative damage (Food and Nutrition Board, 2000; Mayne, 2003). These small-molecule dietary antioxidants interact with each other in an "antioxidant network" by complementing antioxidant enzymes and metal-binding proteins present in cells and extracellular fluids.

1.3.3.4 Endogenous antioxidants

In addition to dietary antioxidants, the body relies on several endogenous defense mechanisms to help protect against free radical-induced cell damage. They are primarily constituted by the antioxidant enzymes and metal-binding proteins. Cells contain several antioxidant enzymes, which are superoxide dismutase (SOD), glutathione peroxidase (GPX), alpha lipoic acid (ALA), catalase (CAT) and coenzyme Q10 (CoQ_{10}). Metal binding proteins include proteins such as iron-binding proteins transferrin and lactoferrin, and the copper-binding protein caeruloplasmin that bind free transition metal ions that otherwise participate in the formation of free radicals (Halliwell and Gutteridge, 1999). The antioxidant enzymes – glutathione peroxidase (GPX), catalase (CAT), and superoxide dismutase (SOD) – metabolize oxidative toxic intermediates and require micronutrient cofactors such as selenium, iron, copper, zinc, and manganese for optimum catalytic activity. It has been suggested that an inadequate dietary intake of these trace minerals may compromise the effectiveness of these antioxidant defense mechanisms (Nike, 1987).

1.3.4. Phytochemicals

The fact that the diets in industrialized societies, which are deficient in fruits and vegetables, can effectively double the risk of developing many different types of cancer has focused renewed attention on the beneficial properties of these foods. Plant foods are rich in micronutrients, but

they also contain an immense variety of biologically active secondary metabolites providing color, flavor and natural toxicity to pests and sometimes humans. The chemistry and classification of such substances is still a matter for much research and debate, but this has not prevented attempts to isolate and exploit substances that have variously been termed as "protective factors", "phytoprotectants" and "nutraceuticals".

1.3.4.1. Phenolic compounds

Many plant-derived substances, collectively termed "phytonutrients," or "phytochemicals," are becoming increasingly known for their antioxidant activity, with the most active dietary antioxidants belonging to the family of phenolic and polyphenolic compounds (Shahidi, 2000). The term "phenolic" or "polyphenol" can be defined chemically as a substance which possesses an aromatic ring bearing one or more hydroxyl substituents, including functional derivatives e.g. esters, methyl ethers, glycosides etc. These compounds are abundantly found in vegetables, fruits, spices and herbs (Vinson *et al.,* 1998; Kahkonen *et al.,* 2001; Hu *et al.,* 2003). The phenolic compounds which are commonly found in food materials can be classified into three groups including simple phenols and phenolic acids, hydroxycinnamic acid derivatives and flavonoids (Ho, 1992). Phenolic compounds are ubiquitous in plant foods and therefore are an integral part of the human diet. They are closely associated with the sensory and nutritional quality of fresh or processed plant foods. The antioxidant activities of phenolic compounds have been recognized for decades. Recent *in-vitro* studies also confirm that many polyphenols exhibit antioxidant and free radical scavenging properties (Kahkonen *et al.,* 2001; Cheung *et al.,* 2003; Shon *et al.,* 2003). Research and development of the practice of using natural substances or food ingredients containing phenolic antioxidants will continue to be of great interest to the food

industry. In addition, other biological activities, besides being antioxidants, phenolic compounds have also become well known in recent years. The most important biological activity of phenolic compounds is probably their inhibitory effect on mutagenesis and carcinogenesis (Ho, 1992).

1.3.5. Measuring antioxidant activity

Antioxidants are used in a wide variety of food products, and their activity may vary depending on various factors such as temperature, food composition, food structure and availability of oxygen. In other words, the activity of antioxidant can vary significantly in different systems. In practice, measuring the formation of oxidation products is a much more sensitive method of monitoring oxidation (Gordon, 2001). However, the assessment of antioxidant activity by monitoring the formation of oxidation products is not a trivial task. Since a complex mixture of oxidation products is formed and the relative amounts of these products depend on a variety of variables including temperature, metal ion content and other components, such as water, deciding which factors to monitor is an important and difficult decision.

The most thoroughly investigated natural anti-oxidants in fruits and vegetables are flavonoids, polyphenols, carotenoids, vitamins A, B, C, and E, tocopherols, calcium, and selenium (Sun *et al.* 2002; Decker *et al.* 2005; Ozgen *et al.,* 2006). Wu *et al.* (2004) suggested that since there are hundreds of anti-oxidant compounds in foods, the total anti-oxidant capacity of a given food may be the integrated action from different compounds instead of that from any single compound. There are several assays that can be used to evaluate the anti-oxidant capacity of a known sample. These assays may measure total phenolic compounds, metal ion chelating ability, the reducing capacity of the sample, or the *in-vitro* ability to quench stable radicals. Foods will often

have varying concentrations of anti-oxidants and because of this several different anti-oxidant capacity assays need to be performed in order to have a more accurate estimation of the total anti-oxidant capacity of tested food (Decker *et al.,* 2005).

1.4. TUMOR ANGIOGENESIS

Angiogenesis is the physiological process of developing new blood vessels from pre-existing ones. It can be primarily observed during embryogenesis, whereas in adults, vessel formation is quite inactive and only takes place in wound healing, ovulation or menstruation. In the last decades, it was revealed that pathological angiogenesis plays a key role in a variety of diseases, like diabetes, rheumatoid arthritis, proliferative retinopathies or cancer (Folkman, 1995). The angiogenic process commences when endothelial cells in the vessel wall are exposed to angiogenic factors from the surrounding tissue (Rak *et al.,* 1993). The endothelial cells respond by a series of characteristic morphological and functional changes in which the cells proliferate, enzymatically degrade the basement membrane, migrate toward the angiogenic stimulus, form and join capillary sprouts to create a capillary loop and a functional lumen, and finally deposit a new, initially fragmented and leaky basement membrane (Folkman, 1984). (Fig. 1.3)

Whether prompted by physiological or pathological stimuli, the multistep process of neovascularization appears to be remarkably similar. While the actual angiogenic process is similar in normal and tumor tissue, there seem to be some generic differences in regulation of the angiogenic process, as well as both the architecture and function of the resulting vessels. In physiological situations, angiogenesis is turned off once the process is complete. In contrast, angiogenesis in cancer is not self-limiting - once tumor-induced angiogenesis is turned on it continues indefinitely (unless the tumor mass is dormant) until the tumor is eradicated or the host succumbs to the disease (Rak *et al.,* 1993). Secondly, tumor angiogenesis results in a wide variety of structural and functional anomalies, including tortuosities, dilatations, arterial-venous shunts, and trifurcations which are not found in normal blood vessels (Rak *et al.,* 1993). In

addition, tumor-associated blood vessels have poorly developed basement membranes and lack collaterals, innervations, and pericytes which may lead to vessel leakiness, a common characteristic of tumor vessels. These abnormalities in blood vessel architecture result in disturbances in blood flow which may account for various features of tumor tissue such as necrosis and high interstitial pressure which is thought to have negative implications for drug delivery (Rak *et al.*, 1993).

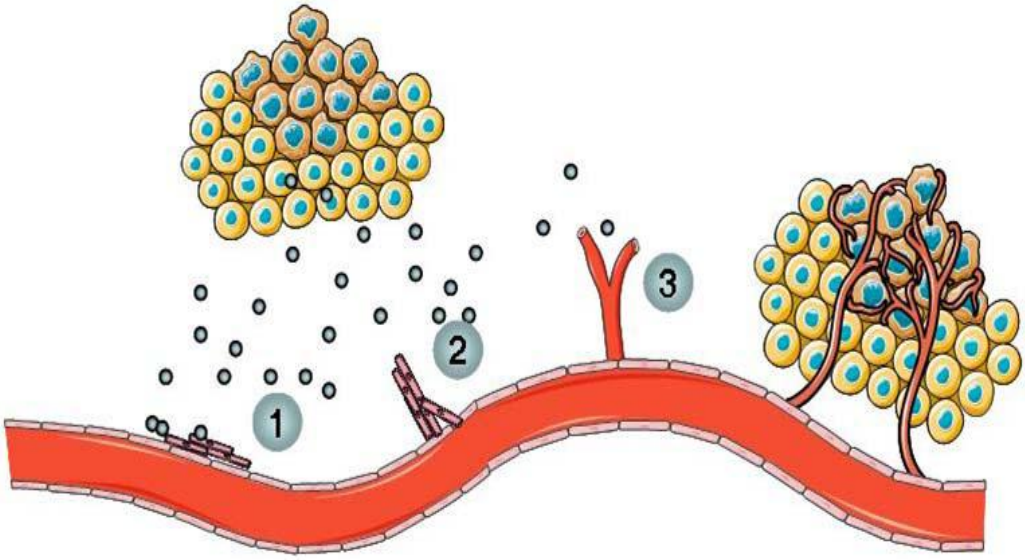


Figure 1.3. Angiogenesis cascade. The physiological process of angiogenesis can be separated into distinct steps. (1) Tumor cells activate and stimulate endothelial proliferation by secretion of endothelial growth factors. (2) Endothelial cells migrate directional to the growth factor gradient and (3) form tubes. Finally, the newly established vessels pervade the tumor tissue and supply tumor cells with oxygen and nutrients.

1.4.1. The angiogenic 'switch'

In 1971, Folkman (1971, 1972) proposed that solid tumor growth is angiogenesis dependent. As such, "once tumor formation has occurred; every further increase in the tumor cell population must be preceded by an increase in new capillaries which converge upon the tumor" (Folkman, 1984). Avascular tumors remain small, restricted in their growth by a poor supply of oxygen and nutrients which are obtained only by diffusion (Denijn and Reuiter, 1993).

Small tumors, unable to induce angiogenesis, stop expanding and reach a steady state, in which the number of dying cells counterbalances the number of proliferating cells (Folkman, 1995). This restrains the increase in tumor volume that results from continuous proliferation, the hallmark of cancer. Thus, tumor masses that are not angiogenic generally are prevented from enlarging to a size that is clinically detectable and the cancer remains dormant (Uhr *et al.,* 1997). Tumor dormancy is defined as a population of tumor cells that is not increasing in size (Uhr *et al.,* 1997). Tumor dormancy results either because the cells are in cell-cycle arrest, or because there is a balance between proliferation and death in the population of tumor cells. In this regard, the interrelationship between tumor angiogenesis and cell death has been gaining attention.

Holmgren *et al.,* (1995) demonstrated that tumor growth can be restricted by apoptosis of tumor cells, due to the inhibition of angiogenesis. When tumor bearing mice were treated with various inhibitors of the angiogenic process, tumor growth was impaired, concomitant with reduced vascularization and increased apoptosis, but without any change in cell proliferative index. Thus, it is clear that angiogenesis or lack thereof, plays a central role in the dormancy of certain tumors.

After months or years in steady state, a tumor may abruptly induce angiogenesis and be released from dormancy. The switch of a tumor from an avascular to a vascular phenotype, often referred to as the 'angiogenic switch', occurs during the early stages of tumor development, suggesting that regulation of angiogenesis is a potentially rate limiting step in the pathway to many solid tumors (Folkman *et al.*, 1989; Folkman, 1995). Solid tumor growth *in-vivo* beyond 1-2 mm in diameter is, almost without exception, associated with the recruitment of new blood vessels (Folkman, 1985, 1990). Once the angiogenic process is turned on, the tumor mass may expand rapidly and begin to invade the surrounding tissue (Folkman, 1995).

1.4.2. Mechanisms of the angiogenic switch

The switch to the angiogenic phenotype is thought to depend on a net balance between angiogenesis activators and inhibitors exported by the tumor cells, mobilized from the extracellular matrix, or released by infiltrating inflammatory cells such as macrophages (Folkman, 1995). In some tissues, the relative absence of angiogenesis stimulators may keep the switch "off', while in others, angiogenesis activators are present but held in check by relatively higher levels of angiogenesis inhibitors. As such, either reducing the overall concentration of inhibitors, or increasing the levels of activators, can change the balance and activate the switch leading to the growth of new blood vessels. Thus, the angiogenic switch is controlled by a regulatory mechanism in which the sum of the levels of activator and inhibitor signals either maintains the endothelial cells in either a state of quiescence or promotes angiogenesis (Fig. 1.4).

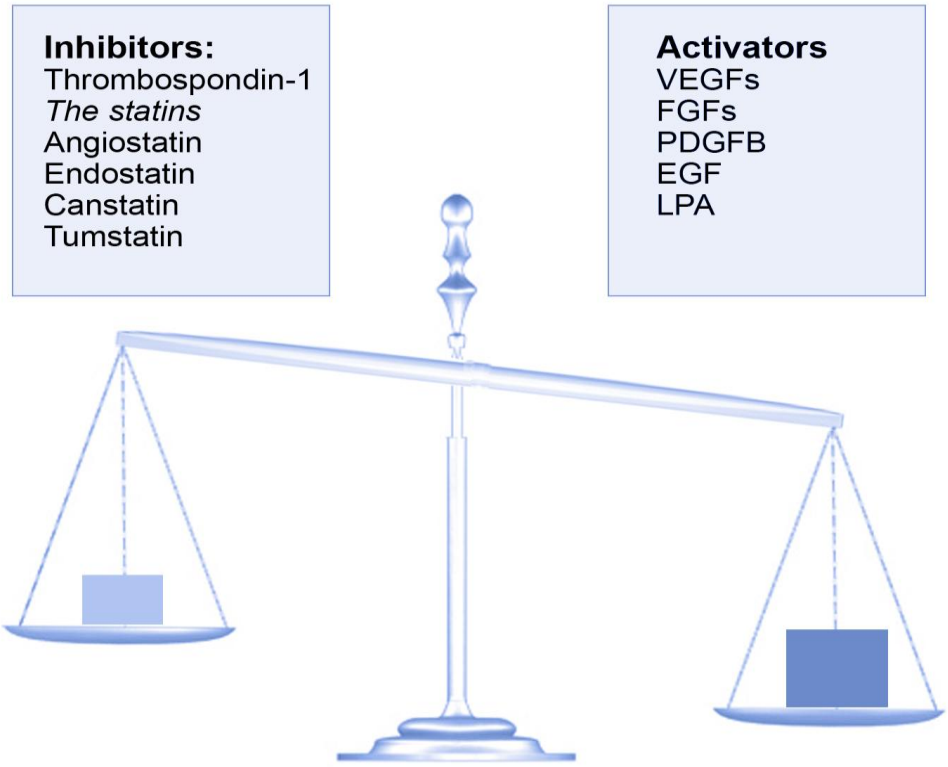


Figure 1.4. The balance hypothesis for the "Angiogenic switch". A balance of activators and inhibitors closely interact to regulate the angiogenic switch only a few of which are listed above. Activators are mainly receptor tyrosine kinase ligands such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), platelet-derived growth factor B (PDGFB) and epidermal growth factor (EGF), but can also be of various origins such as lysophosphatic acid (LPA). Inhibitors include thrombospondin-1 and the statins (Camerliet *et al.*, 2000).

1.4.3. Angiogenesis: Activators and inhibitors

Angiogenesis is a complex process which involves the endothelial cells breaking their contacts, migrating through and breaking down the extracellular matrix, and organizing a new vessel. This process is controlled by proteins which can be divided into activators and inhibitors. Among all the angiogenesis-inducing factors identified to date, the best characterized include acidic fibroblast growth factor (aFGF) (Maciag *et al.,* 1984b), basic fibroblast growth factor (bFGF) (Shing *et al.,* 1984), platelet-derived endothelial cell growth factor (PD-ECGF) (Folkman, 1992), transfoming growth factor-a (TGF-α) (Folkman, 1992) and vascular endothelial growth factor (VEGF) (Ferrara and Henzel, 1989). Of these, the best studied are bFGF and VEGF.

- In 1982, the first angiogenesis stimulator, namely basic fibroblastic growth factor (bFGF) was discovered, followed by the discovery of acidic fibroblastic growth factor (a FGF). Both proteins are members of a family of growth factors and are characterized by their high affinity binding to heparin. Both have been also found to be strongly chemotactic and mitogenic for endothelial cells (Glick *et al.,* 1991). bFGF may function as an angiogenesis factor by inducing or interacting with a direct acting growth factor such as VEGF.
- Platelet-derived endothelial cell growth factor (PD-ECGF) is a 45,000 D polypeptide which stimulates DNA synthesis in endothelial cells and also acts as a chemotactic factor. It was reported to induce synthesis of fibroblast growth factors (FGF) in endothelial cells (Ishikawa *et al.,* 1989).
- Transforming growth factors (TGF) were originally isolated from virally transformed rodent fibroblasts (Mizel *et al.,* 1980; Todaro *et al.,* 1980). Two structurally distinct TGFs, TGF-α and TGF-β have been purified. TGF-α, a 50-amino acid polypeptide with a

molecular weight of 5,500 D has been implicated in angiogenesis and is secreted by transformed fibroblasts, macrophages and several tumor cell types. TGF-α has been shown to stimulate endothelial cell proliferation and angiogenesis (Schreiber *et al.*, 1986). Unrelated to TGF-α but bearing a similar name, the TGF-β family of proteins consists of at least three members TGF-β 1, 2, 3 (Massague, 1990). Paradoxically, although TGF-β shows an inhibitory effect on endothelial cell proliferation, it can induce angiogenesis *in-vivo* (Roberts *et al.,* 1986).

- Another activator of angiogenesis, epidermal growth factor (EGF) shows a structural homology to TGF-α; however its angiogenic regulatory properties are less clear (Shreiber *et al.,* 1986).
- Tumor necrosis factor alpha (TNF-α) is a cytokine, which is produced mainly by activated macrophages in association with an immunological or inflammatory response (Vikkula *et al.,* 1996). The effects of TNF-α on the angiogenic process are controversial; TNF-α has been shown to be inhibitory for endothelial cell proliferation *in-vitro,* yet *in-vivo,* TNF-α has been shown to induce neovascularization (Frater-Schroder *et al.,* 1987). These conflicting results have been attributed to a dose-dependent effect of TNF-α on angiogenesis.
- One of the most potent angiogenic activator is vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF). VEGF is a highly conserved, 34-42 kDa homodimeric glycoprotein, which shares homology with platelet derived growth factor (PDGF) (McGregor *et al.,* 1993). Initially identified by its ability to elicit vascular permeability, VEGF has also been shown to act as a survival factor for newly formed blood vessels (Alon *et al.,* 1995). Consistent with its role as a potent angiogenic

factor, receptors for VEGF were found to be expressed on activated endothelial cells (Quinn *et al.,* 1993). Several experiments have already demonstrated the pivotal importance of VEGF to the process of blood vesse1 formation.

The gene for human VEGF, located on chromosome fragment 6p21.3 (Vincenti *et al.,* 1996) is organized into 8 exons. The promoter region of the gene has been shown to contain a single transcription start site and potential binding sites for Sp-1, AP-1 and AP-2 (James *et al.,* 1995). As a result of alternative splicing, at least 4 transcripts encoding $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ have been detected and seem to express identical biological activities. Yet these four isoforms do display some differences. For example, $VEGF_{121}$ and $VEGF_{165}$ are readily diffusible, secreted proteins, whereas the two larger isoforms, $VEGF_{189}$ and $VEGF_{206}$ are found bound to heparin containing proteoglycans in the cell surface or in the basement membrane.

VEGF was originally purified on the basis of its affinity for heparin, yet the affinity of $VEGF_{165}$ is substantially lower than that of other typical heparin-binding growth factors such as bFGF, and the $VEGF_{121}$ isoform does not bind heparin (Dvorak *et al.,* 1995). The heparin binding ability of $VEGF_{165}$ may be required under conditions in which oxidizing damage and free radicals are produced such as during hypoxia or inflammation (Gitay-Goren *et al.,* 1996). In these conditions, heparin may restore the activity of damaged $VEGF_{165.}$ The relative insensitivity of $VEGF_{121}$ oxidative damage may explain why this particular isoform does not require heparin for its activity (Gitay-Goren *et al.,* 1996). Recently, two other related molecules, namely VEGF-B (Olofsson *et al.,* 1996) and

VEGF-C (Joukov *et al.,* 1996) have been uncovered. Both VEGF-B and VEGF-C are angiogenic molecules although their precise role in tumor angiogenesis i**s** not yet known.

Although an increase in the production of positive angiogenic factors is necessary for expression of the angiogenic phenotype, this alone is not sufficient. Most cells also produce inhibitors of angiogenesis which must be down regulated before the cells can develop into angiogenic tumors. A number of endogenous ad-angiogenic factors (Table 3) have since been identified. They include thrombospondin- 1 **(**Bagavandoss and Wilks, 1990; Good *et al.,* 1991; Iruela-Arispe, 1991; Grossfeld *et al.,* 1997; O'Reilly *et al.,* 1997), angiostatin (Cao *et al.,* 1997; Bergen *et al.,* 1999; Sack, 1999), endostatin (Bergen *et al.,* 1999; Hayes *et al.,* 1999), interferon (Sidky and Borden, 1987), tissue inhibitors of metalloproteinases (Murphy, 1993; Johnson, 1994; Anand-Apte, 1997), cytokines **(**IL-4, IL-2) and proteolytic breakdown products of several proteins including prolactin (Clapp, 1997), and collagen XVIII (O'Reilly, 1997). Among them, TSP-1, angiostatin and endostatin are some of the most potent ones. Endogenous inhibitors of angiogenesis can be divided into those present physiologically and those present pathophysiologically.

- Of several naturally occurring angiogenesis inhibitors, thrombospondin- 1 (TSP-1) has recently been identified as an angiogenic inhibitor which functions as a physiologic regulator of vessel growth that is produced constitutively by normal cells but downregulated during tumorigenesis (Vogel *et al.,* 1993). TSP-1 has also been show to have both in *vitro* (Bagavandoss, 1990; Vogel, 1993) and *in-vivo* inhibitory effects on angiogenesis (Iruela-Arispe, 1997).

- Two angiogenic inhibitors were first identified as cancer cell products. Angiostatin, a fragment of plasminogen (O'Reilly *et al.*, 1996), and endostatin (O'Reilly, 1997), a C-terminal fragment of collagen XVIII has been found to suppress vessel growth by directly inhibiting endothelial cell proliferation. Angiostatin was found in the urine or blood of mice carrying a large tumour burden. Endostatin was isolated from cultures of malignant endothelial cells and was not produced by normal counterparts.
- Another major inhibitor of angiogenesis is interferon-α**.** It acts by antagonizing mitosis and migration of endothelial cells, and by blocking bFGF production by parenchymal cells (Sidky and Borden, 1987).

1.4.4. Angiogenesis inhibitors as a strategy in the treatment of cancer

Besides the classical chemotherapy which targets tumor growth directly by inhibiting tumor cell proliferation, the inhibition of pathological angiogenesis as therapeutic approach has gained particular importance in cancer treatment (Carter, 1999; Kelland, 2005). As tumors can only grow to a size of 1-2 mm^3 without being connected to blood vessels to supply them with oxygen and nutrients, they secrete endothelial growth factors including VEGF (vascular endothelial growth factor) and FGF (fibroblast growth factor) to stimulate vessel growth into the tumor tissue. Tumor angiogenesis allows exceeded growth of the tumor and the formation of metastases. Some cancer types promote angiogenesis to a very high extent, and as such are extremely difficult to treat due to their strong vascularization, which optimizes tumor progression and metastasis. The inhibition of vessel growth starves the tumor and suppresses metastasis, and is therefore an excellent contact point for cancer therapy. Several compounds that

affect the production of angiogenic factors, their binding to receptors on endothelial cells, and receptor signaling are already in clinical trials (Table 1.7).

Table 1.7. Angiogenesis inhibitors in clinical trials.

Drug	**Mechanism**	**Phase of clinical trial**
Drugs that block activators of angiogenesis and their receptors		
SU5416	Blocks VEGFR signaling	I, I/II, II, III
SU6668	Blocks VEGFR, FGFR, PDGFR signaling	I
IFN-α	Inhibition of bFGF and VEGF production	II/III
Anti-VEGF Ab	Monoclonal Ab to VEGF	I, II, III
Angiozyme	Inhibition of VEGFR synthesis	I/II
IMC-1C11	Monoclonal Ab to VEGFR	I
Drugs that inhibit endothelial-specific integrin or survival signaling		
EMD121974	Small-molecule blocker of integrin	I, I/II
Vitaxin	Inhibitor of αvβ3 integrin	I, I/II
Drugs that inhibit endothelial cells		
Thalidomide	Inhibit endothelial cells proliferation	I/II, II, III
Squalamine	Inhibits Na+/H+ exchanger	I, II
TNP-470	Inhibition of MetAP-2	
Endostatin	Inhibition of endothelial cell activities	I,II
Angiostatin	Inhibition of endothelial cell activities	I
Drugs that block matrix breakdown		
Marimastat	Inhibitor of MMPs	III
COL-3	Inhibitor of MMPs	I/II
Neovastat	Inhibitor of MMPs	III
BMS-275291	Inhibitor of MMPs	II/III
Drugs with other mechanisms of action		
CAI	Inhibitor of Ca^{2+} influx	I, II
Interleukin-12	Upregulation of IFNγ and IP-10	I/II
IM862	Unknown mechanism	I, II, III
PI-88	Inhibitor of heparanase	II

In light of the problems associated with conventional chemotherapy, several features of the tumor associated vasculature make anti-angiogenic therapy an attractive anticancer therapeutic agent.

- Firstly, unlike conventional chemotherapy, anti-angiogenic therapy generally has low toxicity. Because it is directed primarily at features specific to proliferating tumor-associated capillary endothelial cells and not the normal resting endothelium, its side effects should theoretically be limited. Clinical trials have demonstrated that anti-angiogenic therapy does not cause bone marrow suppression, gastrointestinal symptoms, or alopecia which is often seen in cancer patients receiving conventional chemotherapy (Folkman, 1995).
- Secondly, drug resistance has not been found to be a significant problem in animal and clinical studies and proliferating endothelial cells have developed little or no resistance to angiogenesis inhibitors (Folkman, 1995). This is because endothelial cells are genetically stable and, as a result, their capacity to develop drug resistance should be minimal. In contrast, tumor cells are genetically unstable and this feature allows them to develop acquired tumor cell resistance to therapeutic agents (Kerbel, 1991).
- Thirdly, the accessibility of endothelial cells to pharmacological agents in the blood makes them particularly attractive. Targeting the tumor vasculature is also a strategy that might circumvent the problem of limited penetration frequently encountered when delivering high molecular weight drugs to solid tumors with elevated interstitial pressures (Jain 1995; Rak *et al.*, 1995).
- Fourthly, the effect of vascular damage of tumor growth may be amplified by the fact that the viability of numerous tumor cells depends on a much smaller number of functional

capillaries. There are approximately between 10 to 100 tumor cells for every endothelial cell in a tumor (Pearlman *et al.,* 1995). Therefore when an angiogenic inhibitor halts the growth of endothelial cells, the effect on the tumor cell population will be greatly amplified.

- Finally, a combination of anti-angiogenic therapy and cytotoxic therapy can be more curative in tumor bearing animals than either therapy alone (Teicher *et al.*, 1994). Folkman (1996) proposed that for therapeutic purposes tumor may be classified into two distinct cell populations namely; a tumor cell population and an endothelial cell population. Because each of these populations may stimulate growth of the other, combination treatment that selectively targets the tumor and endothelial cell populations i.e. cytotoxic chemotherapy and anti-angiogenic therapy, may have a synergistic effect, *in-vivo.* It is plausible that angiogenesis inhibitors may eventually be used to augment conventional chemotherapy in the near future.

Aside from the attractive elements of the use of anti-angiogenic therapy in cancer treatment, problems do exist with this type of approach. The central problem is to achieve selectivity by targeting the tumor associated endothelium and not the normal endothelium (Rak *et al.,* 1995). However, even if complete selectivity cannot be achieved, this does not exclude anti-angiogenic therapy as a useful means to control cancer. An example of this is the widespread use of chemotherapy and radiation in the treatment of cancer, despite their obviously limited selectivity. Another problem is that complete killing of tumor populations may not be possible because small tumors, i.e. those less than l mm in diameter, can survive in the absence of a vasculature (Rak *et al.,* 1995). On this note, anti-angiogenic therapy may be useful as a means to control tumor

growth and stabilize the disease, as opposed to attempting to achieve complete tumor killing**,** as is the goal of conventional chemotherapy.

1.5. OBJECTIVES AND SCOPE OF THE CURRENT INVESTIGATION

Marine floras, such as seaweeds are considered as an extremely important resource of the oceanic biomass. They are taxonomically diverse, largely productive, biologically active and chemically unique offering a great scope for discovery of new anticancer drugs. The marine seaweeds are rich in medicinally potent chemicals predominantly belonging to polyphenols and sulphated polysaccharides. The chemicals have displayed an array of pharmacological properties especially antioxidant, immunostimulatory and antitumour activities. The phytochemicals possibly activate macrophages, induce apoptosis and prevent oxidative damage of DNA, thereby controlling carcinogenesis. In spite of vast resources enriched with chemicals, the marine seaweeds are largely unexplored for anticancer lead compounds and continue to be the subject of concentrated effort. A common strategy of antineoplastic drug discovery in the past several years had been to discover the way by which an effective anticancer agent worked and use this knowledge in a mechanism based drug discovery program. In this study, several marine seaweeds were collected and screened from the coasts of Goa and Malwan (Maharashtra) for lead molecules with antioxidant, anti-angiogenic and/ or proapoptotic activity by activity guided purification.

The main objectives of this investigation were:

1. Preliminary screening of Indian seaweed extracts for antioxidant, cytotoxic/ pro-apoptotic and anti-angiogenic activities using *in-vitro* and *in-vivo* models.
2. Activity guided fractionation and identification of lead molecules.
3. Enumeration of the mechanism of action of the purified compound from *Stoechospermum marginatum* (C. Agardh) Kutzing for its anti-angiogenic, cytotoxic/ pro-apoptotic activities in *in-vitro* and *in-vivo* model systems.

Chapter – 2

Screening for cytotoxic and pro-apoptotic activity in seaweeds collected from the coasts off Goa and Malwan (Maharashtra)

2.1. INTRODUCTION

Cancer presents a medical challenge, inflicting the second highest disease-related mortality in the Western world, right after cardiovascular diseases. It is a genetic disease characterized by uncontrolled cell growth in the absence of cell cycle regulation. Aberrant cell cycle regulation can arise as a consequence of DNA damage. Under normal physiological conditions the uncontrolled growth of damaged cells is restricted by apoptosis. However these cells can escape the regulatory mechanisms of apoptosis as a result of secondary mutations to genes that regulate apoptosis. This DNA damage can be a result of several environmental factors such as stress, smoking, pollution, diet, toxins and endogenous processes such as errors in replication of DNA and chemical instability of certain DNA bases (Thompson, 1995).

The estimated number of new cancer cases is 17 million in 2020 from just under 13 million today, as the population ages and the disease cuts an ever-wider path through emerging economies. The main types of cancer leading to overall cancer mortality each year are: lung (1.3 million deaths/ year), stomach (8, 03, 000 deaths), colorectal (6, 39, 000 deaths), liver (6, 10, 000 deaths), breast (5, 19, 000 deaths) (Danaei *et al.,* 2005). The most frequent types of cancer worldwide among men are: lung, stomach, liver, colorectal, oesophagus and prostate cancer, while breast, lung, stomach, colorectal and cervical cancer are prevalent among women (Danaei *et al.,* 2005).

Cancer cells can be distinguished from normal cells by their uncontrolled proliferation. Thus, targeting the proliferation of cells should repress tumor growth without harming healthy, non-proliferating cells. The importance of mitosis and cell division in proliferation of cancer cells

makes them a prime target for anticancer drugs. The fight against cancer has not been successful so far, particularly in the development of therapies for rapidly growing tumors. Orthodox cancer treatments include chemotherapy, radiotherapy, and surgery (McWhirter *et al.*, 1996). Surgical removal of tumors and radiotherapy has shown benefit in many cancer types but is restricted to local and regional tumors only. However, chemotherapy with cytotoxic compounds is often accompanied by complications, including severe toxic side effects and the development of resistance of cancer cells to chemotherapeutic agents (Curtis *et al.*, 1992). Multi-drug approach provides a glimmer of hope but still leaves much to be desired, as the ideal anti-cancer drug must be selective and cytotoxic to cancer cells. Therefore, effective treatment of patients with advanced or metastatic cancer needs cytotoxic drugs, capable of reaching every organ in the body. Also, investigation of new anticancer drugs is required to improve chemotherapy for the inoperable advanced case. To reduce toxic-side effects and to improve convalescence, it is important to achieve a selectivity of cytotoxic compounds for tumor cells. Therefore, further development of cytotoxic/antiproliferative compounds is of urgent need, to overcome their neurotoxicity and the development of resistances by cancer cells, both of which commonly occur with these types of drugs.

It is an extraordinary challenge to find drugs for the effective treatment of various types of cancer. Most anti-cancer drugs are derived from natural sources, including marine, microbial origin and plants. The secondary metabolites from plants show promise for cancer chemo-preventive effects in constituents of certain culinary herbs, fruits, spices, teas, and vegetables, in which their ability to prevent the development of cancer in laboratory animals has been demonstrated (Ho *et al.*, 1994; Huang *et al.*, 1994).There are now four structural classes of plant

derived anticancer agents on the market in the U.S., represented by the *catharanthus* (Vinca) alkaloids (vinblastine, vincristine, and vinblastine), the epidophyllotoxins (etopside and tenopside), the taxanes (paclitaxel and docetaxel), and the campothecins derivatives (campothecin and irenotecan) (Suffness, 1995; Potmesil and Pinedo, 1995; Chabner *et al.*, 1996; Anon, 1997). These chemotherapeutic drugs have diverse mechanisms of action. However, their ability to induce programmed cell death (PCD) is the unifying event for the mechanisms of chemoprevention (Hadfield *et al.*, 2003).

Many claims have been made for the effectiveness of seaweeds on human health. *Laminaria* and *Sargassum* species have been used in China for the treatment of cancer. Inhibition of cancerous tumors in animals seems to be caused by long – chain polysaccharides. The antitumor active fucoidan or fucan from the brown seaweeds, *Laminaria* species and *Sargassum thunbergii* tested in mice with Ehrlich carcinoma has also been reported (Ito and Sugiura, 1976; Chida and Yamamoto, 1987; Zhuang *et al.*, 1995). Antitumor effect of an extract from *Sargassum kjellmanianum* has also been reported (Lizima- Mizui *et al.*, 1981; Yamamoto *et al.*, 1984). *Laminaria angustata*, a brown seaweed commonly eaten in Japan inhibited breast carcinogenesis (Reddy *et al.*, 1984). Hence, seaweeds have generated a great deal of interest in the screening for plant metabolites and other natural products in modern drug discovery to find potential anticancer preventive agents.

In the present study, we have screened 15 seaweeds for their cytotoxic activity by Brine shrimp and MTT assays. Amongst the seaweeds screened, the seaweed *S. marginatum* belonging to the family dictiyotaceae was selected for detailed investigation for its propaoptotic activity by cell

morphology study, Giemsa staining, Annexin V staining, Propidium iodide staining and DNA fragmentation assay.

2.2. MATERIALS AND METHODS

2.2.1. Animals, Cell lines and Chemicals

Swiss albino mice (8-10 weeks old) were obtained from the animal house, Department of Studies in Zoology, University of Mysore, Mysore, India. EAT cells/ mouse mammary carcinoma cells were maintained in the laboratory of Molecular oncology, University of Mysore, Mysore, India and routinely used for *in-vivo* transplantation. Human Epidermoid Larynx Carcinoma (Hep-2), Carcinoma of cervix (HeLa) and African green monkey Chlorocebus kidney epithelial (Vero) cell lines were obtained from the National Center of Cell Sciences (NCCS), Pune, India. Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Penicillin-Streptomycin and trypsin-EDTA were purchased from Invitrogen, USA. Potassium dichromate ($K_2Cr_2O_7$) reagent was purchased from Qualigen's Fine Chemicals Pvt. Ltd. (Mumbai). MTT, Propidium Iodide and Annexin V were from Sigma-Aldrich Chemicals (USA). All other reagents were of highest analytical grade.

2.2.2. Collection and identification of Seaweeds

Fourteen species of seaweeds (Table 1.01) were collected from the coasts of Goa and Malwan (Maharashtra) in India during the lowest tide and then transported immediately to the Aquaculture Laboratory of the National Institute of Oceanography (NIO), Goa. They were identified by comparing with the voucher specimens deposited in the herbarium of NIO, Goa. Samples were gently rinsed with fresh water to remove salt, sand and epiphytes, epizoones,

animal castings, calcareous and other adhering detritus matters. These cleaned up seaweeds were shade dried at room temperature (30 ^{o}C) for a span of one week to prevent photolysis and thermal degradation, under a continuous stream of air flow. The completely dried seaweed materials were weighed and ground coarsely in a mechanical grinder and then stored at –20 ^{o}C.

2.2.3. Processing and preparation of methanol extract from seaweed samples

Dried and powdered seaweed samples were successively extracted thrice with of methanol by keeping it at room temperature for 24 hrs. The overall organic extract (1.5 L) obtained was filtered and evaporated to dryness under pressure to get a semisolid residue using a rotary evaporator (Roteva, India). This was designated as the methanolic extract (ME) and stored at –20^{o} C until further study. To avoid batch to batch variabily, bulk quantities of the crude extract (5 L) from different species of seaweeds were prepared at one time.

Methanolic extract of the seaweeds were dissolved in 1 % DMSO [final concentration of the DMSO did not exceed 1 % (v/v) and did not adversely affect the cell viability] prepared in serum free medium and filtered by 0.3mm syringe filter and stored.

2.2.4. *In-vitro* culture of cancer cell lines

EAT (Mouse Mammary Carcinoma) cells which were routinely maintained in Laboratory of Molecular Oncology, University of Mysore, Mysore, in Swiss albino mice were drawn in aseptic condition and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich, USA) supplemented with 10 % Fetal Bovine Serum (FBS, Sigma Aldrich, USA) and 1 % Penicillin-Streptomycin. Hep-2 (Human Epidermoid Larynx Carcinoma) and HeLa (Carcinoma

of cervix) cell lines (NCCS, Pune, India) were grown and maintained in Minimal Essential Medium (MEM, GIBCO-. BRL, Gaithersburg, MD) supplemented with 10 % FBS, 1 % Penicillin-Streptomycin and Gentamycin. Vero (African green monkey Chlorocebus kidney epithelial) cell line was also procured from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich, USA) supplemented with 10 % Fetal Bovine Serum (FBS, Sigma Aldrich, USA) and 1 % Penicillin-Streptomycin (Himedia, India). All the cell lines were incubated in a humidified atmosphere of 37 °C and 5 % CO_2. When cells reached confluency, they were passaged by trypsinizing with trypsin/EDTA and used for the experimentation or frozen using freezing mixture (90 % FBS and 10 % DMSO).

2.2.5. Hatching of Brine shrimp

Dried cysts were hatched (1 g cyst per liter) in sterile filtered seawater (0.22 μm) at 27–30° C with adequate aeration, under a continuous light regime. Approximately 12 hrs after hatching the phototropic nauplii were collected with a pipette from the lighted side and concentrated in a small vial.

2.2.6. Brine shrimp lethality test

The toxic effect against *Artemia salina* nauplii was tested according to the method of Sam (1993) with minor modifications. Twenty brine shrimp were transferred to each well using adequate pipette. Each test consisted of exposing groups of 20 nauplii to various concentrations (50, 100 and 500 μg ml^{-1}) of the ME's of individual seaweeds. The toxicity was determined after 6, 18 and 24 hrs of exposure by counting the number of survivors and calculating the percentage of

mortality. Potassium dichromate ($K_2Cr_2O_7$) and Milli-Q water were used as positive and negative control, respectively. Larvae were considered dead if they did not exhibit any internal or external movement during the observation. Mortality below 50 % was considered non-cytotoxic; mortality higher than 50 % but below 75 % was considered mildly cytotoxic; while mortality higher than 75 % was considered as highly cytotoxic.

2.2.7. MTT assay

The MTT assay activity was conducted by observing viable cells incubated with various concentrations of different seaweed crude extracts at 37 °C for 48 hrs. The MTT assay was used to assess the effects of seaweed crude extract on cell viability. The assay relies on production of a colored formazan by the action of mitochondrial enzymes on MTT. The elicited cells were plated with concentration of cells ranging from 10 to 15 x 10^4 cells ml^{-1} following an overnight incubation for 48 hrs with seaweed crude extract. The cells were then incubated with 50 µl of MTT 1mg ml^{-1} at 37 °C for 4-5hrs.Then the MTT was discarded and an equal amount of DMSO (Di-methy sulfoxide) was added. An Elisa reader measured the degree of formazan produced.

The cytotoxicity / growth inhibition was estimated by the following formula:

$$\frac{C - R}{C} \times 100 = \% \text{ of cytotoxic activity or growth inhibition}$$

Where C = Control

R = Reading

2.2.8. Wright's - Giemsa staining

Earlich Ascites Tumor cells were harvested from EAT bearing mice and diluted to the concentration of 1 x 10^6 cells. These cells were treated with seaweed extracts and/ or PBS for 2 hrs at 37 °C at a concentration of 100 µg ml^{-1}. After the incubation they were smeared on a clean glass slide and fixed in methanol and acetic acid (3:1) and stained with Wright's Giemsa stain (Srinivas et al. 2003). The cells were then viewed under Leitz-DIAPLAN florescent microscope and photographed for morphological changes indicating apoptosis.

2.2.9. Annexin V apoptosis detection assay

Apoptosis was induced in a cell suspension of EAT cells by addition of *S. marginatum* extract (SME; 0.1 mg ml^{-1}). Non-induced cells were kept for a zero time control. Cells were incubated for the desired time at 37 °C in a 5 % CO_2 atmosphere. Cells were then washed twice with PBS. Cells were then suspended in PBS at a concentration of 0.5 – 1 x10^6 cells per ml. 50 µl of the cell suspension (induced or non-induced) was then placed onto the slide and left at room temperature for 10 minutes, to allow the cells to absorb on to the plate. Excess liquid was removed carefully by touching a tissue to the side of the circle. Cells were then washed three times with 50 µl of 1x binding buffer each. Excess liquid was blot dried with a tissue as in the earlier step.50 µl of the staining solution (Annexin V) was then placed on the slide and a Petri dish covered with aluminum foil was placed on it. It was then incubated for 10 minutes at room temperature. After staining, the spot of application was washed for five times with 50 µl of 1x binding buffer each as in the earlier step. This removed excess label from the cells. A 35 ml of 1x binding buffer on

this spot was applied and a 24 x 50 mm cover slip was placed on it. The results were observed using a fluorescence microscope and then photographed.

2.2.10. Propidium Iodide (PI) staining for apoptosis detection

Cells were seeded in a 6 well plate at 2.5 x 10^5 cells/ well with 2 ml of cell culture media. Cells were allowed to grow for 24 hrs and then treated with *S. marginatum* extract (SME; 0.1 mg ml^{-1}). Non-induced cells were kept for a zero time control. After incubation for the desired time at 37 °C in a 5 % CO_2 atmosphere the cells were recovered from the wells and added to the tubes. The tubes were then centrifuged at 5000 rpm in a microcentrifuge (i.e at a low setting) to remove the supernatant. To this, 100µl of 500 ng ml^{-1} Propidium Iodide (PI) was added to gently break up the cell pellet and incubated in the dark at 4^0 C for 15 minutes. 20 µl of the suspension was then taken on a microscopic slide and excess liquid was removed by carefully touching a tissue to the side of the spot of application. The cells were the washed three times with 50 µl of 1x binding buffer each. Again, the excess liquid was blotted out with a tissue as in the earlier step and a cover slip was then placed on it. 35 µl of 1x binding buffer was placed on the spot and covered with a 24 x 50 mm cover slip. Cells were then viewed under a fluorescent microscope and photographed.

2.2.11. DNA fragmentation assay

DNA extraction and agarose gel electrophoresis were performed by the method described by Chaudhary *et al.* (2001). EAT cells were either untreated or pretreated with *S. marginatum* extract (SME; 100 µg ml^{-1}) for 2 hrs at 37^0 C. After 2 hrs of treatment the DNA was extracted from the cell lysates as follows. Both the attached and floating cells were collected, washed with

PBS and centrifuged at 15000 rpm for 5 minutes to collect the cell pellet. This was then resuspended in 0.5 ml of lysis buffer [50 mM Tris-HCl pH 8.0] and 0.5 % SDS, transferred to a micro fuge tube and incubated for 1 hr at 37° C. The cell lysate was subjected to 8 M potassium acetate precipitation and left for l hr at 4º C. To the supernatant of each tube 0.5 ml of phenol: chloroform: iso-amyl alcohol (25:24:1) was added, mixed and centrifuged at 13,000 rpm for 1 minute to separate the DNA containing upper aqueous phase. Phenol – chloroform extraction was repeated twice, followed by chloroform extraction alone. To the resulting aqueous phase, two volumes of ice-cold absolute ethanol and 1/10 volume of 3 M sodium acetate were added and incubated for 30 minutes on ice to precipitate DNA. DNA was pelleted by centrifuging at 13,000 rpm for 10 minutes at 4° C. The supernatant was aspirated and the pellet was washed with 1 ml of 70 % ethanol.

After repeating the above centrifugation step and removing the last traces of the supernatant fraction, the pellet was allowed to dry at room temperature for approximately 30 minute and resuspended in 50 µl of Tris – EDTA buffer. The DNA was quantified by UV – Vis spectroscopy and 10 µg of DNA was electrophoresed in a 1.2 % agarose gel containing ethidium bromide in a mini gel tank containing Tris – boric acid – EDTA buffer for 2 hrs under 90 Volts. Then the gel was visualized under UV illuminator and documented using UVP-BioDoc-IT^{TM} system.

2.2.12. Statistical analysis

All experiments were conducted in triplicates (n=3) and expressed as means ± SD using STATISTICA software (Statsoft, 1999)

2.3. RESULTS

2.3.1. Collection and identification of seaweeds collected from the coasts of Goa and Malwan (Maharashtra)

The seaweeds *Amphiroa fragilissima* (Delile) Trevisan, *Asparagopsis taxiformis* (Linnaeus) Lamouroux, *Bryopsis plumosa* (Hudson) C.Agardh, *Caulerpa peltata* J.V. Lamouroux, *Caulerpa racemosa* (Forsskal) J. *Agardh Caulerpa taxifolia* (Vahl) C. Agardh, *Chlorodesmis fastigiata* (C. Agardh) Ducker, *Codium elongatum* (Turner) C. Agardh, *Dictyopteris australis* Lamouroux, *Dictyopteris deliculata* Lamouroux, *Padina gymnospora* (Kützing) Sonder, *Padina tetrastromatica* Hauck, *Sargassum marginatum* (C. Agardh) J. Agardh, *Stoechospermum marginatum* (C. Agardh) Kutzing, *Spatoglossum asperum* J. Agardh, *Spatoglossum variabile* Figari and De Notaris were collected from in and around the coasts of Goa and Malwan, Maharashtra, India for experimentation (Tables 2.1, 2.2 and 2.3).

The voucher specimens of these seaweeds were deposited in the herbarium of National Institute of Oceanography, India and identified with the help of a taxonomist by comparing with available voucher specimens available there (Figs. 2.01, 2.02, 2.03). The identification was mainly based on morphological criteria. Color and morphological differences between different genera/ species and taxonomic characteristic were carefully studied. Taxonomic identification key was followed to identify the seaweed specimen. The taxonomic descriptions of the specimen to be identified were also referred from the books, monograph, reference herbaria etc.

Table 2.1. List of brown seaweeds evaluated for the experiments.

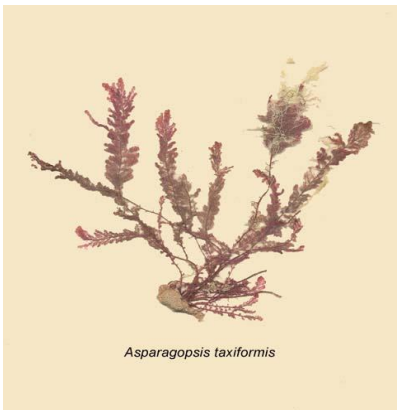
Sr.No	Botanical name	Family	Perceived activities	References	Collected from
BROWN SEAWEEDS					
1	*Dictyopteris australis* Lamouroux	Dictyotaceae	Diuretic, Pytotocis, Antimicrobial, Hypotensive	Bhakuni and Rawat, 2005; Leila and Shameel, 2009	Malwan, Maharashtra
2	*Dictyopteris delicatula* Hering ex J. Agardh	Dictyotaceae	Antioxidant, Antiproliferative, Antifeedant, Antibacterial and Antitumor activity	Hay *et al.*, 1998; Costa *et al.*, 2010	Anjuna beach, Goa
3	*Padina gymnospora* (Kutzing) Sonder	Dictyotaceae	Antibacterial, Antifungal, Antiviral and Antioxidant activities	Premanathan *et al.*, 1992; de Souza *et al.*, 2007	Marvel beach, Goa
4	*Padina tetrastromatica* Hauck	Dictyotaceae	Spasmolytic, Antifertility, Antibacterial, Hypotensive, Antioxidant, Antiamoebic, Anticoagulant, Antiviral and Antifertility activities	Kandhasamy and Arunachalam, 2008; Shanmugam and Mody, 2000; Prmanathan *et al.*, 1992; Naqvi *et al.*, 1980	Baga beach, Goa
5	*Sargassum marginatum* (C. Agardh) J. Agardh	Sargassaceae	Cytotoxic, Atiproliferative and Antimicrobial activities	Ara *et al.*, 1999; Bhakuni and Rawat, 2005	Marvel beach, Goa
6	*Spatoglossum asperum* J. Agardh	Dictyotaceae	Cytotoxic, Anti-implantation and Hypotensive activities	Ara *et al.*, 1999; Bhakuni and Rawat, 2005	Malwan, Maharashtra
7	*Spatoglossum variabile* Figari and De Notaris	Dictyotaceae	Antioxidant and Antibacterial activity	Venkateshwarulu *et al.*, 2007	Anjuna beach, Goa
8	*Stoechospermum marginatum* (C.Agardh) Kutzing	Dictyotaceae	Spasmolytic, cytotoxic, antimicrobial, Nematicidal, Antibacterial, Antifungal, Antiprotozoal, Antiviral, Antifertility, Hypoglycemic, Effect on cardio vascular system, Effect on isolated tissues, Effect on CNS and gross behavior, Diuretic activities	Lakshmi *et al.*, 2006	Marvel beach, Goa

Table 2.2. List of green seaweeds evaluated for the experiments.

Sr.No	Botanical name	Family	Perceived activities	References	Collected from
GREEN SEAWEEDS					
9	*Bryopsis plumosa* (Hudson) C.Agardh	Bryopsidaceae	Antimicrobial, Antiprotozoal,Antiviral, Antifertility, Antitumoral and Antioxidant activities	Garg, 1994; Ara *et al.*, 1999; Sabina *et al.*, 2005	Malwan, Maharashtra
10	*Caulerpa peltata* J.V. Lamouroux	Caulerpaceae	Antifungal properties, Anticoagulant, Larvicidal activities and Ability to lower blood pressure	Thangam and Kathiresan, 1996; Shanmugam *et al.*, 2001	Marvel beach, Goa
11	*Caulerpa racemosa* (Forsskal) J. Agardh	Caulerpaceae	Hypotensive, Cytotoxic,Antileishmenial, Antinociceptive, Anti-inflammatory, Antitumor, Antibacterial, Antifungal, Antiprotozoal, Antifertility, Antiviral, Effect on cardiovascular tissues, Effect on isolated tissues, Effect on CNS and gross behavior activities	Ayyad *et al.*, 1994; Garg, 1994; Ara *et al.*, 1999; Sabina *et al.*, 2005; de Souza *et al.*, 2009; Kandhasamy and Arunachalam, 2008; Lakshmi *et al.*, 2006	Malwan, Maharashtra
12	*Caulerpa taxifolia* (Vahl) C.Agardh	Caulerpaceae	Antiproliferative, Antiviral, Antimicrobial, Diyretic, Hypotensive and Nematicidal activities	Garg, 1994; Fischel *et al.*, 1995; Nicoletti et al., 1999; Bhakuni and Rawat, 2005; Rizvi *et al.*, 2006; Subba Rangaiaha *et al.*, 2010;	Malwan, Maharashtra
13	*Chlorodesmis fastigiata* (C.Agardh) Ducker	Udoteaceae	Antimicrobial, Antiproliferative and Anifouling activity	Paul and Fenical, 1985; Birrel *et al.*, 2008	Anjuna beach, Goa
14	*Codium elongatum* (Turner) C. Agardh	Codiaceae	Antibacterial, Antifungal, Antiprotozoal, Antiviral, Antifertility, Hypoglycemic, Effect on cardiovascular system, Effect on isolated tissues, Diurectic, Anti-inflammatory and mild Hypotensive effect	Garg, 1994; Lakshmi *et al.*, 2006	Anjuna beach, Goa

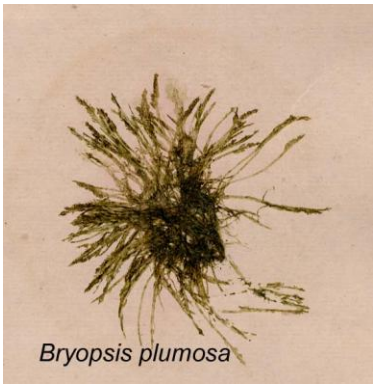
Table 2.3. List of red seaweeds evaluated for the experiments.

Sr.No	Botanical name	Family	Perceived activities	References	Collected from
RED SEAWEEDS					
15	*Amphiroa fragilissima* (Linnaeus)	Corallinaceae	Oxytocic, Spasmogenic and Antifouling activities	Naqvi *et al.*, 1980; Wahidullah *et al.*, 1987; Ambiye *et al.*, 1991	Anjuna beach, Goa
16	*Asparigopsis taxiformis* (Delile)	Bonnemaisoniaceae	Antiprotozoal, Antifouling, Anticyanobacterial, Piscicidal and Crusacean cytotoxicity and Nematicidal activities	Rizvi *et al.*, 2006; Manilal *et al.*, 2010	Anjuna beach, Goa



Amphiroa fragilissima ***Asparagopsis taxiformis***

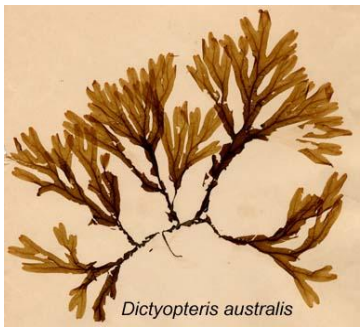
Figure 2.1. Photographs of red seaweeds selected for experimentation.



Bryopsis plumosa ***Caulerpa racemosa*** ***Caulerpa peltata***

Caulerpa taxifolia ***Chlorodesmis fatigiata*** ***Codium elongatum***

Figure 2.2. Photographs of green seaweeds selected for experimentation.



Dictyopteris australis

Dictyopteris delicatula

Padina gymnospora

Padina tetrastromatica

Sargassum marginatum

Spatoglossum asperum

Spatoglossum variabile

Stoechospermum marginatum

Figure 2.3. Photographs brown seaweeds selected for experimentation.

2.3.2. S. *marginatum* extract (SME) inhibits proliferation of HeLa and Hep-2 cells, and is lethal to brine shrimps *in-vitro*

Methanolic extracts of 16 seaweeds were used to study their cytotoxic effect on *Artemia salina* by brine shrimp lethality assay and their effect on the viability of HeLa and Hep-2 cell lines using MTT assay. *Stoechospermum marginatum* extract was the most cytoxic to brine shrimps even at low concentrations when compared to other seaweed extracts (Tables 2.4, 2.5, 2.6) Amongst the seaweed extracts tested against Hela cell line, *Stoechospermum marginatum* (LC_{50} 13.80 ± 0.15 µg ml^{-1}) displayed significantly ($p < 0.001$) superior activity followed by *S. asperum* (LC_{50} 23.65 ± 2.99 µg ml^{-1}), *S. variabile* (LC_{50} 30.67 ± 2.98 µg ml^{-1}), *C. taxifolia* (LC_{50} 33.98 ± 0.95 µg ml^{-1}), *C. peltata* (LC_{50} 35.23 ± 3.54 µg ml^{-1}), *A. taxifolia* (LC_{50} 37.24 ± 1.4 µg ml^{-1}), *D. delicatula* (LC_{50} 42.62 ± 1.56 µg ml^{-1}), *D. australis* (LC_{50} 43.37 ± 9.25 µg ml^{-1}), *A. fragilissima* (LC_{50} 49.18 ± 2.61 µg ml^{-1}), *C. fastigiata* (LC_{50} 50.193 ± 0.98 µg ml^{-1}), *C .elongatum* (LC_{50} 53.02 ± 1.04 µg ml^{-1}), *P. tetrastromatica* (LC_{50} 58.57 ± 9.68 µg ml^{-1}), *C. racemosa* (LC_{50} 67.16 ± 2.55 µg ml^{-1}), *B. plumosa* (LC_{50} 115.19 ± 1.88 µg ml^{-1}), *Sargassum marginatum* (LC_{50} 218.07 ± 12.97 µg ml^{-1}) (Figure 2.4). In the case of Hep-2 cell line, the cytotoxic activity of the seaweed extracts tested varied significantly ($p < 0.001$) and the highest activity was displayed by *Stoechospermum marginatum* (LC_{50} 21.21 ± 1.026 µg ml^{-1}), followed by *A. taxifolia* (LC_{50} 28.23 ± 1.4 µg ml^{-1}), *A. fragilissima* (LC_{50} 34.72 ± 2.61 µg ml^{-1}), *S. asperum* (LC_{50} 35.95± 0.024 µg ml^{-1}), C. *taxifolia* (LC_{50} 37.38 ± 7.35 µg ml^{-1}), *C. elongatum* (LC_{50} 38.87 ± 2.99 µg ml^{-1}), *P. tetrastromatica* (LC_{50} 41.09 ± 2.81 µg ml^{-1}), *D. australis* (LC_{50} 42.65 ± 2.36 µg ml^{-1}), *S. variabile* (LC_{50} 44.94 ± 3.21 µg ml^{-1}), *C. fastigiata* (LC_{50} 44.42 ± 7.11 µg ml^{-1}),*C. peltata* (LC_{50} 45.24 ± 1.55 µg ml^{-1}), *P. gymnospora* (LC_{50} 46.56 ± 2.1 µg ml^{-1}), *D. delicatula* (LC_{50} 46.86 ± 46.86 µg ml^{-1}), *C. racemosa* (LC_{50} 53.65 ± 16.08 µg ml^{-1}), *B. plumose*

(LC_{50} 89.63 ± 12.02 µg ml^{-1}), *Sargassum marginatum* (LC_{50} 141.82 ± 15.00 µg ml^{-1}) (Figure 2.5). *S. marginatum* extract (SME) was found to be the most potently cytotoxic extract and was further tested with Vero cells (derived from the kidney epithelial cells of an adult African green monkey) for *in-vitro* cytotoxicity against normal cells. SME was less cytotoxic to Vero cell line (Fig. 2.6) when compared to the cancer cell lines and its LC_{50} activity was 70.14 ± 3.12 µg ml^{-1}.

2.3.3. *S. marginatum* extract (SME) induces apoptosis in Giemsa, Annexin V and Propidium iodide staining assay of EAT cells

We further examined whether SME induced apoptotic cell death in EAT cells by Giemsa, Annexin V and Propidium Iodide apoptosis detection assay. The treated cells showed apoptotic morphology at a concentration of 100 mg $ml^{-1}$by all three staining procedures (Figs. 2.7, 2.8 and 2.9).

2.3.4. *S. marginatum* extract (SME) induces DNA fragmentation in EAT cells by apoptosis

In the control, no fragmentation was observed in DNA agarose gel electrophoresis. However, SME (100 µg ml^{-1}) treated EAT cells showed the fragmented laddering pattern of DNA, indicating the characteristics of apoptotic cells (Fig. 2.10).

Table 2.04. Cytotoxicity activity of brown seaweed extracts using brine shrimp lethality assay.

Brine shrimp lethality assay for Brown seaweeds							
Sample	**Concentration**	**6 h**	**Cytotoxicity**	**18 h**	**Cytotoxicity**	**24 h**	**Cytotoxicity**
($K_2Cr_20_7$)	50 µg	1.66 ± 2.58	NCT	68.33 ± 6.83	MCT	100.00 ± 0.00	HCT
	100 µg	10.00 ± 4.47	NCT	68.33 ± 2.58	MCT	100.00 ±0.00	HCT
	500 µg	100.00 ± 0.00	HCT	100.00 ± 0.00	HCT	100.00 ± 0.00	HCT
Dictyopteryis australis	50 µg	41.66 ± 6.83	NCT	100.00 ± 0.00	HCT	100.00 ± 0.00	HCT
Dictyopteryis delicatula	50 µg	25.00 ± 4.47	NCT	26.66 ± 2.58	NCT	41.66 ± 2.58	NCT
Padina tetrastromatica	50 µg	11.66 ± 2.58	NCT	20.00 ± 0.00	NCT	25.00 ± 4.47	NCT
Sargassum marginatum	50 µg	10.00 ± 0.00	NCT	11.66 ±2.58	NCT	15.00 ±0.00	NCT
Spatoglossum asperum	50 µg	0	NCT	43.33 ± 6.83	NCT	58.33 ± 9.31	MCT
Spatoglossum variable	50 µg	1.66 ± 2.58	NCT	68.33 ± 2.58	MCT	71.66 ± 13.66	MCT
Stoechospermum marginatum	50 µg	8.33 ± 2.58	NCT	73.33 ± 2.58	MCT	86.66 ± 5.16	HCT
Dictyopteryis australis	100 µg	53.33 ± 5.16	MCT	100.00 ± 0.00	HCT	100.00 ± 0.00	HCT
Dictyopteryis delicatula	100 µg	28.33 ± 6.83	NCT	30.00 ± 4.47	NCT	51.66 ± 2.58	MCT
Padina tetrastromatica	100 µg	20.00 ± 0.00	NCT	25.00 ± 4.47	NCT	36.66 ± 2.58	NCT
Sargassum marginatum	100 µg	23.33 ± 2.58	NCT	23.33 ± 2.58	NCT	23.33 ± 2.58	NCT
Spatoglossum asperum	100 µg	6.66 ± 2.58	NCT	86.66 ± 2.58	HCT	93.33 ± 2.58	HCT
Spatoglossum variable	100 µg	33.33 ± 5.16	NCT	91.66 ± 2.58	HCT	93.33 ± 2.58	HCT
Stoechospermum marginatum	100 µg	16.66 ± 2.58	NCT	90.00 ± 4.47	HCT	91.66 ± 9.31	HCT
Dictyopteryis australis	500 µg	90.00 ± 4.47	HCT	100.00 ± 0.00	HCT	100.00 ± 0.00	HCT
Dictyopteryis delicatula	500 µg	30.00 ± 4.47	NCT	31.66 ± 2.58	NCT	56.66 ± 6.83	MCT
Padina tetrastromatica	500 µg	23.33 ± 2.58	NCT	31.66 ± 5.16	NCT	41.66 ± 6.83	NCT
Sargassum marginatum	500 µg	25 ± 7.75	NCT	31.66 ± 2.58	NCT	36.66 ± 6.83	NCT
Spatoglossum asperum	500 µg	30.00 ± 0.00	NCT	96.66 ± 5.16	HCT	100.00 ± 0.00	HCT
Spatoglossum variable	500 µg	100.00 ± 0.00	HCT	100.00 ± 0.00	HCT	100.00 ± 0.00	HCT
Stoechospermum marginatum	500 µg	60.00 ± 8.94	MCT	100.00 ± 0.00	HCT	100.00 ± 0.00	HCT

Footnote: NCT: Non- cytotoxic activity; MCT: Moderately cytotoxic, HCT: Highly cytotoxic

Table 2.05. Cytotoxicity activity of green seaweed extracts using brine shrimp lethality assay.

Brine shrimp lethality assay for Green seaweeds							
Sample	**Concentration**	**6 h**	**Cytotoxicity**	**18 h**	**Cytotoxicity**	**24 h**	**Cytotoxicity**
($K_2Cr_20_7$)	50 µg	1.66 ±2.58	NCT	68.33 ± 6.83	MCT	100.00 ± 0.00	HCT
	100 µg	10.00 ± 4.47	NCT	68.33 ± 2.58	MCT	100.00 ± 0.00	HCT
	500 µg	100.00 ± 0.00	HCT	100.00 ± 0.00	HCT	100.00 ± 0.00	HCT
Caulerpa racemosa	50 µg	0	NCT	0	NCT	0	NCT
Caulerpa peltata	50 µg	0	NCT	0	NCT	0	NCT
Caulerpa taxifolia	50 µg	0	NCT	0	NCT	0	NCT
Codium elongatum	50 µg	0	NCT	0	NCT	0	NCT
Chlorodesmis fastigiata	50 µg	0	NCT	0	NCT	0	NCT
Bryopsis plumosa	50 µg	5 ± 0	NCT	8.33 ± 2.58	NCT	10 ± 0	NCT
Caulerpa racemosa	100 µg	0	NCT	0	NCT	0	NCT
Caulerpa peltata	100 µg	0	NCT	1.6 ± 2.58	NCT	1.6 ± 2.58	NCT
Caulerpa taxifolia	100 µg	0	NCT	0	NCT	3.3 ± 2.58	NCT
Codium elongatum	100 µg	0	NCT	0	NCT	0	NCT
Chlorodesmis fastigiata	100 µg	0	NCT	0	NCT	0	NCT
Bryopsis plumosa	100 µg	5 ± 5.16	NCT	21.66 ± 6.83	NCT	25 ± 4.47	NCT
Caulerpa racemosa	500 µg	0	NCT	0	NCT	0	NCT
Caulerpa peltata	500 µg	0	NCT	6.66 ± 2.58	NCT	6.6 ± 2.58	NCT
Caulerpa taxifolia	500 µg	0	NCT	1.66 ± 2.58	NCT	5 ± 0	NCT
Codium elongatum	500 µg	0	NCT	1.66 ± 2.58	NCT	3.33 ± 5.16	NCT
Chlorodesmis fastigiata	500 µg	0	NCT	0	NCT	0	NCT
Bryopsis plumosa	500 µg	8.33 ± 2.58	NCT	98.33 ± 2.58	HCT	100.00 ± 2.58	HCT

Footnote: NCT: Non- cytotoxic activity; MCT: Moderately cytotoxic, HCT: Highly cytotoxic

Table 2.06. Cytotoxicity activity of red seaweed extracts using brine shrimp lethality assay.

Brine shrimp lethality assay for Red seaweeds							
Sample	**Concentration**	**6 h**	**Cytotoxicity**	**18 h**	**Cytotoxicity**	**24 h**	**Cytotoxicity**
($K_2Cr_20_7$)	50 µg	1.66 ± 2.58	NCT	68.33 ± 6.83	MCT	100.00 ± 0.00	HCT
	100 µg	10.00 ± 4.47	NCT	68.33 ± 2.58	MCT	100.00 ± 0.00	HCT
	500 µg	100.00 ± 0.00	HCT	100.00 ± 0.00	HCT	100.00 ± 0.00	HCT
Amphiroa fragilissima	50 µg	23.33 ± 2.58	NCT	26.66 ± 2.58	NCT	31.66 ± 5.16	NCT
Asparagopsis taxiformis	50 µg	28.33 ± 2.58	NCT	33.33 ± 6.83	HCT	35 ± 4.47	HCT
Amphiroa fragilissima	100 µg	28.33 ± 2.58	NCT	28.33 ± 2.58	NCT	33.33 ± 5.16	MCT
Asparagopsis taxiformis	100 µg	33.33 ± 2.58	MCT	35 ± 4.47	MCT	36.66 ± 6.83	MCT
Amphiroa fragilissima	500 µg	40.00 ± 4.47	MCT	45 ± 4.47	MCT	53.33 ± 2.58	MCT
Asparagopsis taxiformis	500 µg	38.33 ± 2.58	MCT	55 ± 4.47	MCT	58.33 ± 2.58	MCT

Footnote: NCT: Non- cytotoxic activity; MCT: Moderately cytotoxic, HCT: Highly cytotoxic

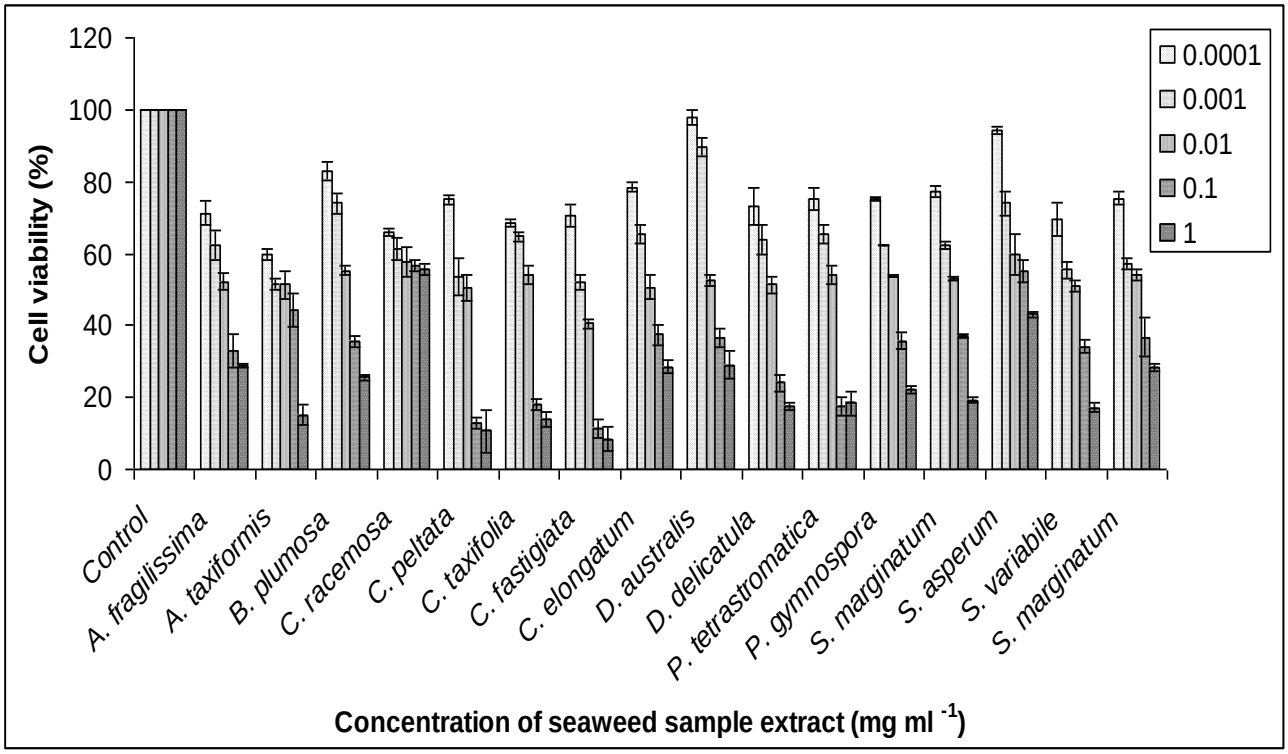


Figure 2.4. Effect of 16 seaweed extracts on the viability of HeLa cells *in-vitro*. HeLa cells were plated in 96 well plates and incubated for 48 hrs. Seaweed extracts of 0.001, 0.001, 0.01, 0.1, 1 mg ml^{-1} concentrations were added to the wells in triplicates and incubated for another 48 hrs. The cells were trypsinized after 2 days and processed for MTT assay. Values are presented as means ± SD (n=3).

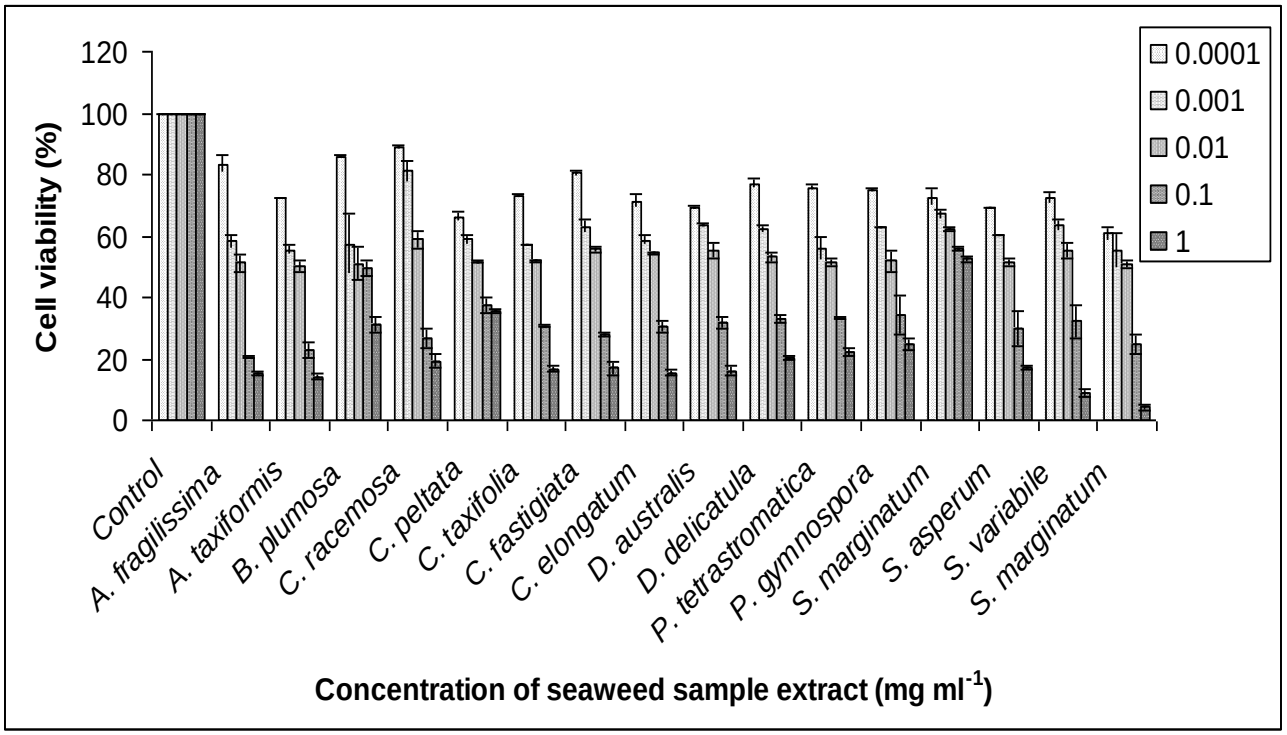


Figure 2.5. Effect of 16 seaweed extracts on the viability of Hep-2 cells *in-vitro.* Hep2 cells were plated in 96 well plates and incubated for 48 hrs. Seaweed extracts of 0.001, 0.001, 0.01, 0.1, 1 mg ml^{-1} concentrations were added to the wells in triplicates and incubated for another 48 hrs. The cells were trypsinized after 2 days and processed for MTT assay. Values are presented as means ± SD (n=3).

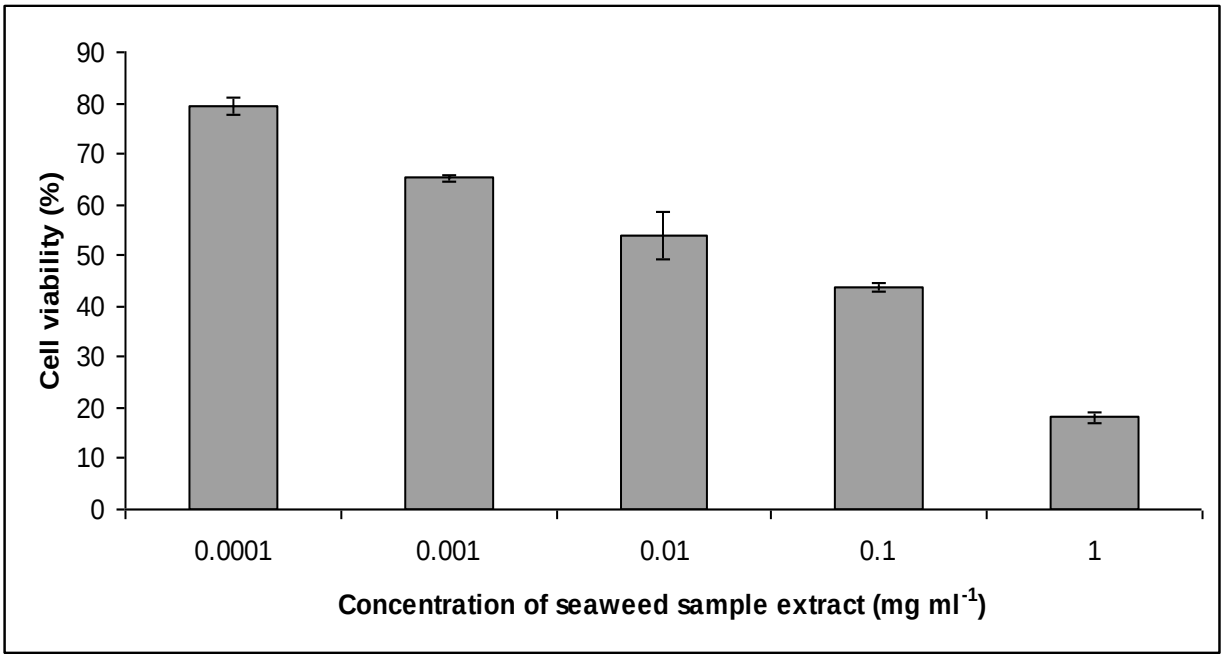


Figure 2.6. Effect of *S. marginatum* extract (SME) on the viability of Vero cells *in-vitro*. Vero cells were plated in 96 well plates and incubated for 48 hrs. *S. marginatum* extract (SME) of 0.0001, 0.001, 0.01, 0.1, 1 mg ml^{-1} concentrations was added to the wells in triplicates and incubated for another 48 hrs. The cells were trypsinized after 2 days and processed for MTT assay. Values are presented as means ± SD (n=3).

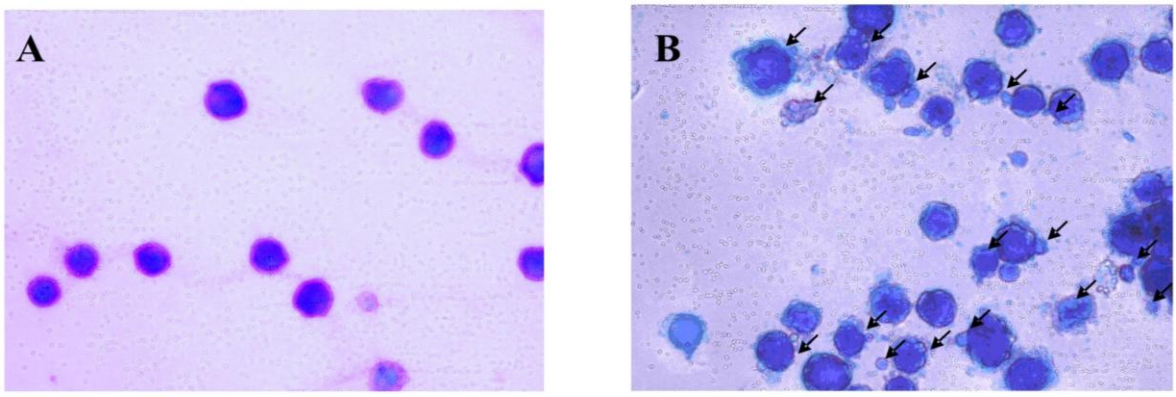


Figure 2.7. Effect of *S. marginatum* extract (SME) on EAT cells showing characteristic apoptotic morphology when stained with Wright's Giemsa stain. (A): Control, (B): SME treated at the concentration 100 µg ml^{-1}. Arrows indicate membrane blebbing, apoptotic bodies and condensed chromatin.

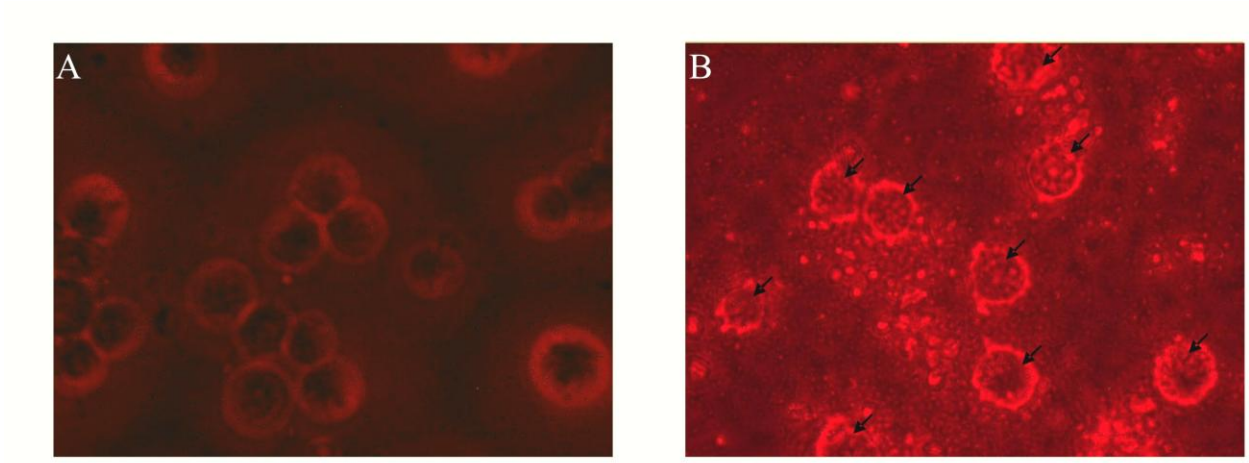


Figure 2.8. Effect of *S. marginatum* extract (SME) on EAT cells showing characteristic apoptotic morphology when stained with Annexin V stain. (A): Control, (B): SME treated at the concentration 100 µg ml^{-1}. Arrows indicate membrane blebbing.

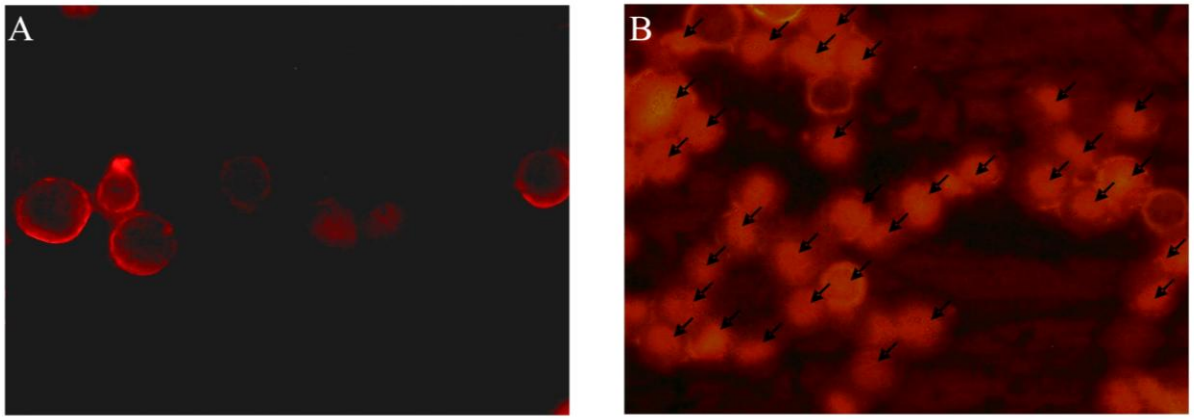


Figure 2.9. Effect of *S. marginatum* extract (SME) on EAT cells showing characteristic apoptotic morphology when stained with Propidium iodide stain. (A): Control, (B): SME at the concentration 100 µg ml^{-1}. Arrows indicate condensed chromatin.

Figure 2.10. *In vitro* effect of *S.marginatum* extract (SME) on DNA fragmentation of Ehrlich Ascites Tumor cells (EAT). DNA run and detected on 2% agarose gel electrophoresis. Lane 1: DNA from control EAT cells, Lane 2: DNA from SME treated EAT cells at the concentration 100µg ml^{-1}.

2.4. DISCUSSION

Natural products play an important role in chemotherapy, having contributed considerably to approximately 60 available cancer chemotherapeutic drugs. The need to develop more effective anti-cancer drugs has prompted investigators to explore new sources of pharmacologically active compounds, especially from the marine sources.

Cancer cell cultures have been useful models to evaluate gene expression and to establish *in-vitro* experiments aiming at the control of tumor cell growth (Correa *et al,* 2002). Consequently, in our studies I screened different seaweed extracts for cytotoxicity where, *S. marginatum* was found to be the most cytotoxic extract when a brine shrimp lethality assay was performed (Tables 2.3, 2.4 and 2.5). This was also confirmed for its cytotoxicity in Hep 2 and HeLa cancer cell lines *in-vitro* by performing MTT assay (Figs. 2.4 and 2.5). However, SME was less cytotoxic to the normal L929 mouse fibroblast cell line (Fig. 2.6). In the present investigation, SME markedly reduced the cell viability in a concentration-dependent manner. The suppression of cell proliferation induced by this extract may be due to the induction of cell death. Thus, the inhibitory activity of SME provides evidence for the *in-vitro* cytotoxicity.

Apoptosis is a major form of cell death that is essential for normal development and for maintenance of homeostasis (Du and Clemetson, 2002; Ande *et al.,* 2008; Zhang and Wu, 2008). The regulation of apoptosis in both normal and malignant cells has become an area of extensive study in cancer research (Johnstone *et al.,* 2002). Apoptosis also seems to be a reliable marker for the evaluation of potential agents for cancer prevention. A wide variety of natural compounds appear to possess significant cytotoxic as well as chemo preventive activity. Hence, the possible

roles of apoptosis in the present study were first examined with Giemasa and then with Annexin V and Popidium Iodide staining of treated and untreated EAT cells. There was characteristic nuclear fragmentations of nuclei in treated EAT cells whereas; the untreated control cells did not show any nuclear fragmentation in case of Giemsa staining (Fig. 2.7). The apoptotic cells displayed the characteristic features of intense fluorescence of condensed nuclear chromatin and formation of membrane blebs in the treated EAT cells stained by Annexin V (Fig. 2.8) and Propidium iodide (Fig. 2.9) staining; while, the untreated control cells showed no such feature. The intense fluorescence of condensed nuclear chromatin, nuclear fragmentation and formation of membrane blebs clearly demonstrates the role of apoptosis in EAT treated cells.

Apoptotic cells often produce a unique ladder composed of nucleotide fragments at an interval of 180-200 base pairs, which can be visualized by DNA-agarose gel electrophoresis. It is generally assumed that the toxicity of antitumor drugs is the consequences of their ability to cause genomic DNA damage in cancer cells (Jamieson and Lippard, 1999). In the present study, DNA ladders appeared in SME treated EAT cells after exposed to the concentration of 100 μg ml^{-1} for 48 hrs. However, the control EAT cells did not show any DNA fragmentation (Fig. 2.10). In general, cytotoxic drugs induce a massive breakage of DNA into oligonucleosome fragments. The degradation of DNA down to oligonucleosomal fragments is a late event of apoptosis. Thus, SME induces DNA damage in EAT cells and thereby causes apoptosis.

Our results in this chapter, strongly suggest that SME extract exhibited significant cytotoxicity toward brine shrimps, Hep-2 and HeLa cells, and also contains a bioactive compound capable of killing carcinoma cells by apoptosis through the activation of the apoptotic pathways. Thus, *S.*

marginatum should be considered as a functional food ingredient and pharmaceutical. This study shows the potential of the methanolic extract of *S. marginatum* as an anticancer agent to inhibit cell viability and trigger apoptosis. However, the identification of the individual compounds and *in-vivo* experiments are needed to understand the mechanism of action.

Chapter – 3

Screening for anti-oxidant activity in seaweeds collected from the coasts of Goa and Malwan (Maharashtra)

3.1. INTRODUCTION

Antioxidants help organisms to deal with oxidative stress, caused by free radical damages. Free radicals are chemical species containing one or more unpaired electrons that are highly unstable and are known to cause damage to other molecules by extracting electrons from them in order to attain stability. Reactive Oxygen Species (ROS) formed *in-vivo*, such as superoxide anion, hydroxyl radical and hydrogen peroxide, are highly reactive and potentially damaging transient chemical species. ROS are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signaling and immune functions. ROS are also regulated by endogenous superoxide dismutase, glutathione peroxidase and catalase but due to over-production of reactive species, induced by exposure to external oxidant substances or a failure in the defense mechanisms, damage to cell structures, DNA, lipids and proteins (Valko *et al*., 2006) occur, which increases risk of more than 30 different disease processes (Aruoma, 1998; Waris and Ahsan, 2006). The most notorious among them being neurodegenerative conditions like Alzheimer's disease (AD) (Smith *et al*., 2000) and Parkinson's disease (PD) (Bolton *et al*., 2000). Other neurodegenerative diseases significantly associated with oxidative stress include multiple sclerosis, Creutzfeldt–Jacob disease and meningoencephalitis. Valko *et al*., (2007) have done an extensive review on the effect of free radicals and antioxidants in normal physiological functions and human disease. The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone (Halliwell and Gutteridge, 1999). Besides DNA, ROS also attack other cellular components involving polyunsaturated fatty acid residues of phospholipids (Siems *et al.,* 1995). Interestingly the body posses defence mechanisms against free radical-induced oxidative stress, which involve preventative mechanisms, repair mechanisms, physical defenses and antioxidant

defenses. Enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) etc. Non-enzymatic antioxidants are ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), carotenoids, flavonoids, etc. These entire agents act by one or more mechanisms like reducing activity, free radical-scavenging, potential complexing of pro-oxidant metals and quenching of singlet oxygen. It is possible to reduce the risks of chronic diseases and prevent disease progression by either enhancing the body's natural antioxidant defenses or by supplementing with proven dietary antioxidants (Stanner *et al.,* 2004). This is one of the reasons why discovery and synthesis of novel antioxidants is a major active area in recent years.

Over the last two decades, there has been an increasing interest in replacing synthetic antioxidants such as butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT), the most commonly used antioxidants in lipid-containing food, with natural alternatives, because of their safety and toxicity problems (Howell, 1986; Amarowicz *et al.,* 2000). In recent years, the use of natural antioxidants present in food and other biological materials has attracted considerable interest due to their presumed safety, nutritional and therapeutic value (Ajila *et al.*, 2007). Nutraceuticals are supposed to hold the key to a healthy society in the coming future.

The formation of cancer cell in human body can directly be induced by free radicals. Further more, ionizing radiation, which causes free radicals, is well documented as a carcinogen. Therefore, radical scavenging compounds such as vegetables and fruits can indirectly reduce cancer formation in human body. It has been reported that, increased consumption of fruits and vegetables, which are rich with free radical scavenging activity, leads up to a doubling of

protection against many common types of cancer formations (Chu *et al.,* 2002; Cooke *et al.,* 2002; Nandita and Rajini, 2004).

The plant kingdom is an abundant source of phytochemicals with important properties such as antioxidant activity. Several natural antioxidants have already been evaluated and isolated from various terrestrial plant materials, such as cereal crops, vegetables, oilseeds, herbs and spices (Ramarathnam *et al.,* 1995; Wettasinghe and Shahidi, 1999; Shon *et al.,* 2003; Cai *et al.,* 2004). In contrast to the study of the antioxidative activities of higher plant-derived natural antioxidants, less attention has been given to the study of marine algae for these properties (Matsukawa *et al.,* 1997; Yan *et al.,* 1998). Interestingly, several marine algae have been used in China for thousands of years in traditional system of folk medicines, as antiviral, anticancer, anti-hypertension agent (Jiang and Zhang, 1994). It has been proved that marine organisms are a rich source of structurally novel and biologically active secondary metabolites (Takamatsu et al. 2003). Several studies of aquatic plants led to the findings that some of the marine organisms also contain antioxidants (Johnson *et al.,* 1999). Recent years, several algal species have also been reported to prevent oxidative damage by scavenging free radicals and active oxygen and hence able to prevent the occurrence of cancer cell formation. Therefore, algal species as alternative materials to extract natural antioxidative compounds have attracted much attention of biomedical scientists recently.

Seaweeds as well-balanced, harmless, natural sources with a high degree of bioavailability of trace elements are strongly advised for fast grown children and pregnant women (Booth, 1964). In contrast to their use as a source of food, marine algae are widely used in the life science as the

source of important compounds with diverse structural forms and biological activities. Over the years marine algal species offer the biological diversity for sampling in discovery- phase of new drug development (Munro *et al.,* 1987, 1999). Therefore, it is clearly documented that, pre-clinical pharmacological research with new marine compounds continues to be extremely active in recent history (Mayer and Gustafson, 2003).

There are some evidences that seaweeds contain compounds with a relatively high antioxidant and antiproliferative activities. Alternatively, antioxidant activity is one of the most important activities of marine bioactive compounds and several seaweeds reportedly rich in antioxidant compounds such as carotenoids, phenolics, terpenoids and sulphated polysaccharides have been characterized (Kuda *et al.,* 2005; Duan *et al.,* 2006). Therefore, extraction of bioactive natural compounds from seaweeds is desired, but little has happened in this area to systematically study their potentiality. Some of the active components identified from the marine seaweeds were; phylopheophytin in *Eisenia bicyclis* (Cahyana *et al.*, 1992), phlorotannins in *Sargassum kjellamanianum* (Yan *et al.,* 1996) and fucoxanthin in *Hijikia fusiformis* (Yan *et al.*, 1999), a low molecular weight sulphated polysaccharide from *Laminaria japonica* (Wang *et al.*, 2008), and mycosporin like amino acids (MAA's) from red seaweeds (Nakayama *et al.,* 1999). The activity has been ascribed to their ROS scavenging ability, quenching singlet and triplet oxygen, chelating ability and reducing power (Karawita *et al.*, 2005). The antioxidative potential of various seaweed extracts have been reported globally (Jimenez – Escrig *et al.*, 2001; Ismail and Hong, 2002; Heo *et al.*, 2005). However, not much data is available on the antioxidant potential of seaweed extracts available in India (Chandani *et al.*, 2007; Ganesan *et al.*, 2007; Kumar *et al.*,

2007; Pandima Devi *et al.*, 2008). A further study in this area is anticipated to elevate their value in the human diet as food and pharmaceutical supplements.

The objective for the present study was to investigate the antioxidant properties of the selected seaweed extracts by *in-vitro* free radical scavenging assays such as, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, reducing power assay, and ferrous ion chelating assay. Additionally, the total polyphenolic content (TPC) of the seaweed extracts was also determined.

2.2. MATERIALS AND METHOD

3.2.1. Chemicals

Ascorbic acid (AA), Butylated hydroxytoluene (BHT), α-tocopherol, dimethyl sulphoxide (DMSO), *1, 1-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent were purchased from Sigma Co. (St. Louis, USA). Ethylene diamine tetra acetic acid (EDTA), $FeSO_4 \cdot 7H_2O$ was purchased from Fluka Co. (Buchs, Switzerland). All other chemicals and solvents used were of the highest purity grade commercially available.

3.2.2. Collection and identification of seaweeds.

The seaweeds were collected and identified from in and around the coasts of Goa and Maharashtra, India in the same manner as mentioned in Chapter 2.

3.2.3. Processing and preparation of methanol extract from seaweed samples

The methanolic extract was obtained in the same manner as mentioned in Chapter 2 and designated as SME for future reference.

3.2.4. DPPH Radical scavenging assay

The scavenging effects of seaweed samples for DPPH *(1, 1-diphenyl-2-picrylhydrazyl hydrate) was determined spectrophotometrically according to the method of Duan *et al.* (2006). Briefly, a 2.0 ml aliquot of test sample (in methanol) was added to 2.0 ml of 0.16 mM DPPH methanolic solution. The mixture was vortexed for one minute and then left to stand at room temperature for 30 minutes in dark. The absorbance was read at 517 nm and percentage of radical scavenging effect was calculated using the equation as depicted below.

$$\text{Scavenging effect (\%)} = [1- (A_{\text{Sample}} - A_{\text{Sample blank}})/ A_{\text{Control}}] \times 100$$

Where $\mathbf{A_{Control}}$ was the absorbance of the control [DPPH solution without sample], $\mathbf{A_{Sample}}$ the absorbance of the test sample [DPPH plus test sample], and the $\mathbf{A_{Sample\ blank}}$ the absorbance of the sample only [sample without DPPH solution]**.** Natural antioxidant like ascorbic acid (AA) was used as the positive control.

3.2.5. Total polyphenolic content (TPC)

The total polyphenolic content was determined by the Folin-Ciocalteu method as described by Sellappan and Akoh (2002). Seaweed extracts (0.5 ml) or gallic acid standard solutions were mixed with 2.5 ml of Folin-Ciocalteu's Reagent (FCR-1:10 dilution) and left to stand for 8

minutes at room temperature causing the FCR to react with the oxidizable substances or phenolates. Then, 2.0 ml of Na_2CO_3 (7.5 % in solution in water) was added to neutralize the residual reagent. The absorbance was measured at 760 nm using a UV-vis spectrophotometer after incubating at room temperature for 2 hours. Results were expressed as milligrams of gallic acid equivalents (GAE)/ gram of dry seaweed extract.

3.2.6. Reducing Power assay

Total reducing power was determined as described by Zhu *et al.* (2002) with minor modification. Briefly, 0.2 ml of the sample solution was mixed with 0.2 ml of phosphate buffer (0.2 M, pH 7.2) and 0.2 ml of 1 % potassium ferricyanide. The mixture was then incubated at 50^o C for 20 minutes and 0.2 ml of trichloroacetic acid (10 %) was added. Finally, 0.125 ml of the mixture and 0.125 ml distilled water was dispensed into a 96-well micro plate and 0.02 ml of 0.1 % $FeCl_3$ was added. The absorbance of the mixture was measured at 655 nm (Bio-Rad, Micro plate reader, Model 680). BHT was used as the positive control for this assay.

3.2.7. Ferrous ion chelation assay

The ferrous ion chelating activity was determined by the method of Decker and Welch (1990). To the sample solution (0.1 ml), distilled water (0.1 ml) and (0.025 ml) of 0.5 mM $FeCl_2$ were added. The absorbance was measured immediately at 562 nm (Abs. 1). To this, 0.025 ml of 2.5 mM ferrozine was added and incubated for 20 minutes at room temperature. The absorbance was measured again (Abs. 2) as described above. Ethylene diamine tetra acetic acid (EDTA), a strong chelator was used as the positive control. The activity was calculated with the equation as mentioned below:

Ferrous ion chelating activity [%] = [1-(sample $_{Abs2}$ - sample $_{Abs1}$) / (control $_{Abs2}$ – control $_{Abs1}$)] x 100

3.2.8. Statistical Analysis

All experiments were conducted in triplicates (n=3) and expressed as mean ± SD. One-way ANOVA test using STATISTICA software (Statsoft, 1999) was utilized to compare the mean values of each treatment and *P*-values < 0.001 was considered highly significant. The relationships between TPC and DPPH scavenging activity and TPC and metal chelation assay were determined using regression analysis.

3.3. RESULTS

3.3.1. DPPH radical scavenging activity

The radical-scavenging activity of the methanol extract of brown, green and red seaweeds were assessed and expressed as percentage reduction of the initial DPPH* absorption by the tested compound. All of the extracts of seaweeds and the synthetic antioxidant used as the positive control possessed the ability to scavenge DPPH radical to various degrees in a dose dependent manner.

Amongst the brown seaweeds, *Stoechospermum marginatum* (IC_{50} 0.56 ± 0.011 mg ml^{-1}) displayed significantly ($p < 0.001$) higher activity followed by *P. tetrastromatica* (IC_{50} 0.61 ± 0.005 mg ml^{-1}), *C. implexa* (IC_{50} 0.66 ± 0.002 mg ml^{-1}), *S. asperum* (IC_{50} 0.98 ± 0.006 mg ml^{-1}), *S. variabile* (IC_{50} 1.01 ± 0.003 mg ml^{-1}), *D. australis* (IC_{50} 1.60 ± 0.013 mg ml^{-1}), and

Sargassum marginatum (IC_{50} 2.87 ± 0.128 mg ml^{-1}) (Fig.3.1). All of the green seaweed extracts showed the ability of scavenging DPPH radical and the most potent activity was found in the extract of *C. peltata* (IC_{50} 1.024 ± 0.012 mg ml^{-1}), followed by *C. taxifolia* (IC_{50} 1.270 ± 0.020 mg ml^{-1}), *C. racemosa* (IC_{50} 1.473 ± 0.022 mg ml^{-1}), *C. fastigiata* (IC_{50} 3.082 ± 0.006 mg ml^{-1}) and *C. elongatum* (IC_{50} 3.105 ± 0.048 mg ml^{-1}) (Fig. 3.2). The scavenging effect of two red seaweed extracts on DPPH radical was also measured and the results are as shown in Figure 3.3. The most potent activity was found in the extract of *A. fragilissima* (IC_{50} 2.89 ± 0.061 mg ml^{-1}) when compared to *A. taxiformis* (IC_{50} 3.38 ± 0.042 mg ml^{-1}). However, none of the extracts showed comparable activity to the positive control, ascorbic acid (IC_{50} 0.07 ± 0.002 mg ml^{-1}).

3.3.2. Total Polyphenolic Content (TPC) of seaweed extracts

The TPC of the ME's of brown (Table 3.1), green (Table 3.2) and red seaweeds (Table 3.3) were determined using Folin–Ciocalteu reagent and it varied from (7.315 ± 0.352 to 38.929 ± 0.628) gallic acid equivalents (GAE) mg g^{-1}.

3.3.3. Reducing power activity

The ME's of brown, green and red seaweeds were able to reduce Fe^{3+} to Fe^{2+} in a concentration dependent manner as a function of reducing power. Results obtained showed that the reducing power in ME's of brown seaweeds at all concentrations of 0.1, 0.5 and 1.0 and 2.0 mg/ml decreased in the following order: *BHT > P. tetrastromatica > C. implexa > S. asperum > S. variabile > Stoechospermum marginatum > D. australis > Sargassum marginatum* (Fig. 3.4). In the case of green seaweeds it decreased in the order of *BHT > C. peltata > C. racemosa > C. taxifolia > C. fastigiata > C. fragile* (Fig. 3.5). In red seaweeds, it decreased in the order of *BHT*

> *A. fragilissima* > *A. taxiformis* (Fig. 3.6)**.** In continuation with the antioxidant activity, the reducing power of methanol extracts also increased with increasing concentration.

3.3.4. Ferrous ion chelating activity

A reasonably significant ferrous ion-chelating efficacy was demonstrated by most of the seaweed extracts in a dose dependent manner. Amongst all the brown seaweeds, ferrous chelating efficacy was significantly highest ($p < 0.001$) in *D. australis* and decreased in the order of *D. australis* (IC_{50} 0.93 ± 0.029 mg ml^{-1}) > *S. asperum* (IC_{50} 1.19 ± 0.020 mg ml^{-1}) > *Stoechospermum marginatum* (IC_{50}1.30 ± 0.413 mg ml^{-1}), > *S. variabile* (IC_{50}1.38 ± 0.102 mg m^{-1}) > *P. tetrastromatica* (IC_{50} 1.76 ± 0.146 mg ml^{-1})> *D. delicatula* (IC_{50} 2.46 ± 0.247 mg ml^{-1}) > *Sargassum marginatum* (IC_{50} 9.17 ± 0.413 mg ml^{-1}) (Fig. 3.7),while in green seaweeds the ferrous chelating efficacy was the highest in *C. peltata* (IC_{50}1.297 ± 0.027 mg ml^{-1}) followed by *C. fragile* (IC_{50}1.645 ± 0.062 mg ml^{-1}), *C. taxifolia* (IC_{50}1.697 ± 0.021 mg ml^{-1}), *C. fastigiata* (IC_{50} 9.130 ± 0.521 mg ml^{-1}) and *C. racemosa* (IC_{50} 9.425 ± 1.034 mg ml^{-1}) (Fig. 3.8). Ferrous chelating efficacy in red seaweeds was highest in *A. fragilissima* (IC_{50}1.27 ± 0.018 mg ml^{-1}) followed by *A. taxiformis* (IC_{50} 2.37 ± 0.172 mg ml^{-1}) respectively. EDTA (positive control) a strong chelator demonstrated the best ferrous chelating efficacy (IC_{50} 0.042 ± 0.0008 mg ml^{-1}) (Fig. 3.9).

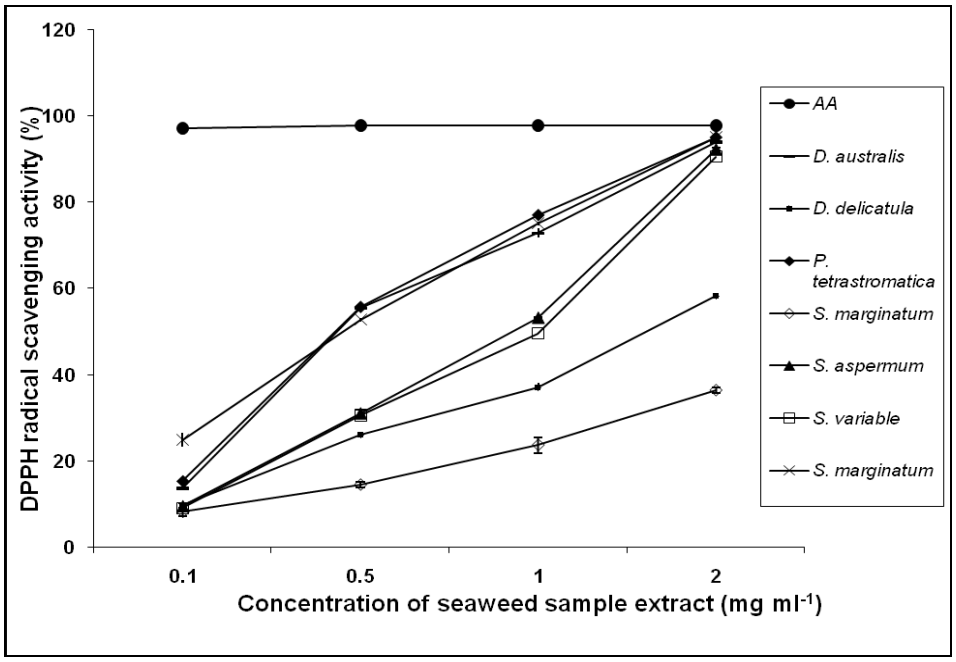


Figure 3.1. DPPH radical scavenging activity (%) of the total methanolic extracts derived from 7 brown seaweeds (at the concentrations 0.1-2 mg ml^{-1} dry seaweed extract). AA: Ascorbic acid. Values are presented as means ± SD (n=3).

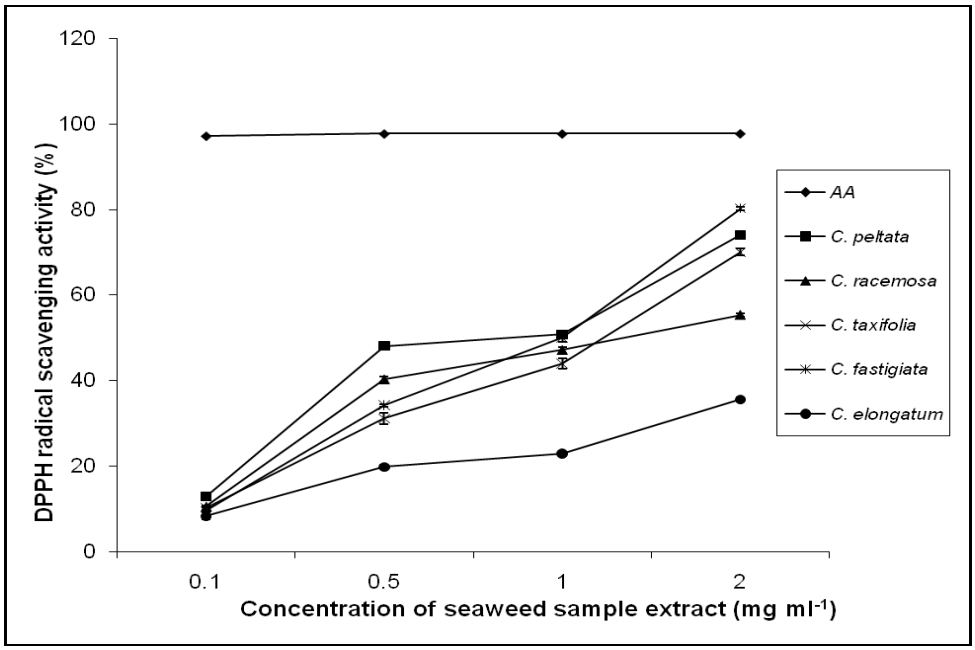


Figure 3.2. DPPH radical scavenging activity (%) of the total methanolic extracts derived from 5 species of green seaweeds (at the concentrations 0.1-2 mg ml^{-1} dry seaweed extract). Values are presented as means ± SD (n=3).

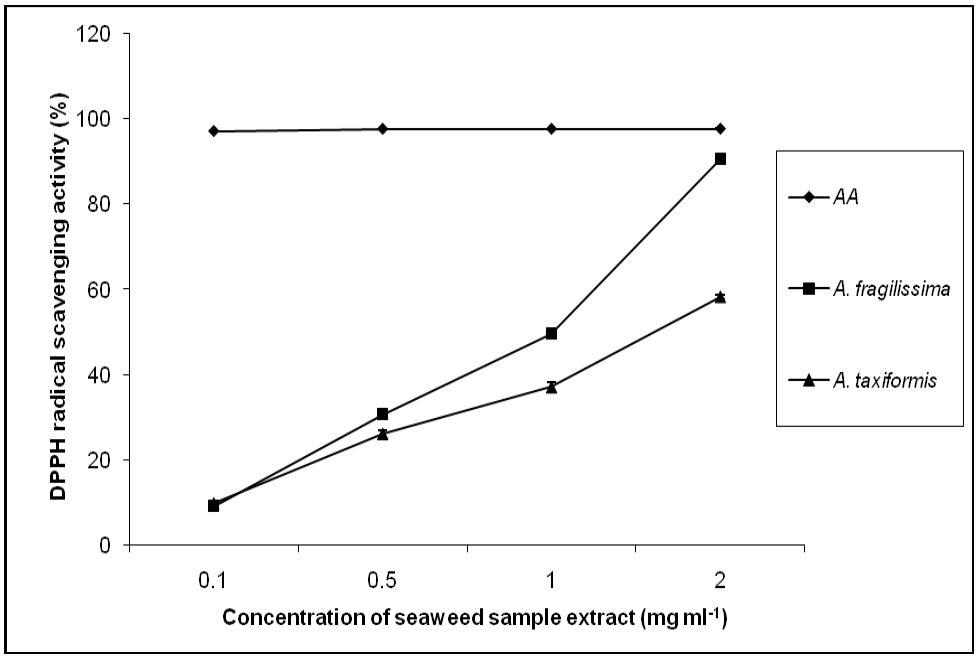


Figure 3.3. DPPH radical scavenging activity (%) of the total methanolic extracts derived from 2 red seaweeds (at the concentrations 0.1-2 mg ml^{-1} dry seaweed extract). Values are presented as means ± SD (n=3).

Table 3.1. Total phenolic content of 7 brown seaweeds expressed as GAE; mg.g^{-1}of methanol extract

Brown seaweed species	GAE; mg.g^{-1} of total methanolic extract (n=3)
Dictypopteris delicatula	21.34 ± 0.428
Dictyopteris australis	13.37 ± 0.140
Padina tetrastromatica	25.29 ± 0.445
Sargassum marginatum	13.19 ± 0.325
Spatoglossum asperum	14.13 ± 0.046
Spatoglossum variable	14.85 ± 0.093
Stoechospermum marginatum	20.04 ± 0.382

Table 3.2. Total polyphenolic content of 5 green seaweeds expressed as GAE; mg.g^{-1} extract

Green seaweed species	GAE; mg.g^{-1} of total methanolic extract (n=3)
Caulerpa racemosa	23.122 ± 0.487
Caulerpa peltata	38.929 ± 0.628
Caulerpa taxifolia	24.087 ± 0.654
Chlorodesmis fastigiata	7.315 ± 0.352
Codium elongatum	7.405 ± 0.280

Table 3.3. Total polyphenolic content of 2 red seaweeds expressed as GAE; mg.g^{-1} extract

Red seaweed species	GAE; mg.g^{-1} of total methanolic extract (n=3)
Amphiroa fragilissima	8.220 ± 0.214
Asparagopsis taxiformis	8.521 ± 0.284

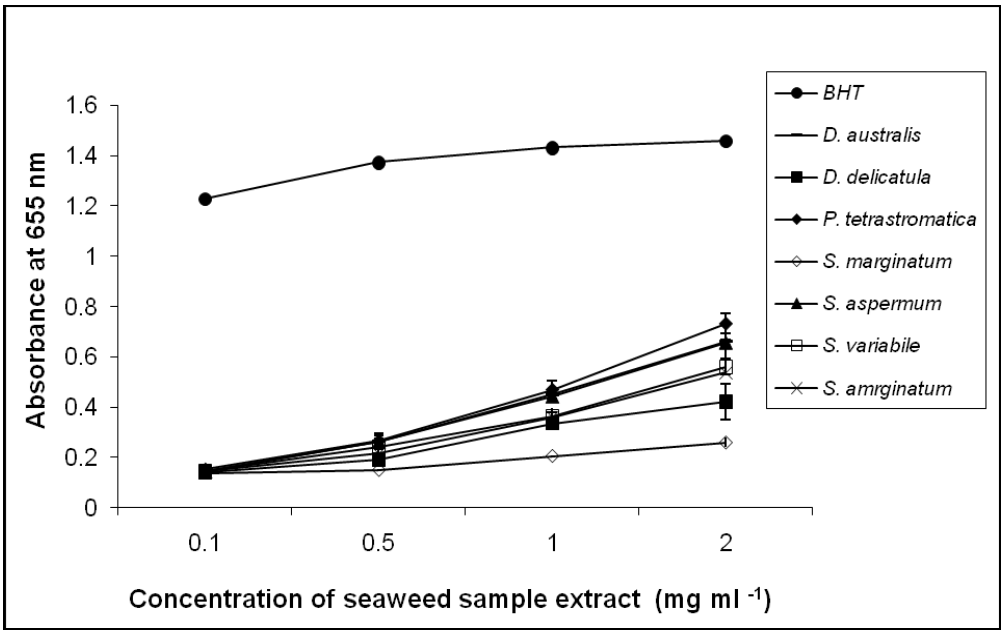


Figure 3.4. Reducing power of total methanolic extracts derived from 7 species of brown seaweeds. (at the concentrations 0.1-2 mg ml^{-1} dry seaweed extract). AA: Ascorbic acid. BHT: Butylated hydroxy toluene. Values are presented as means ± SD (n=3).

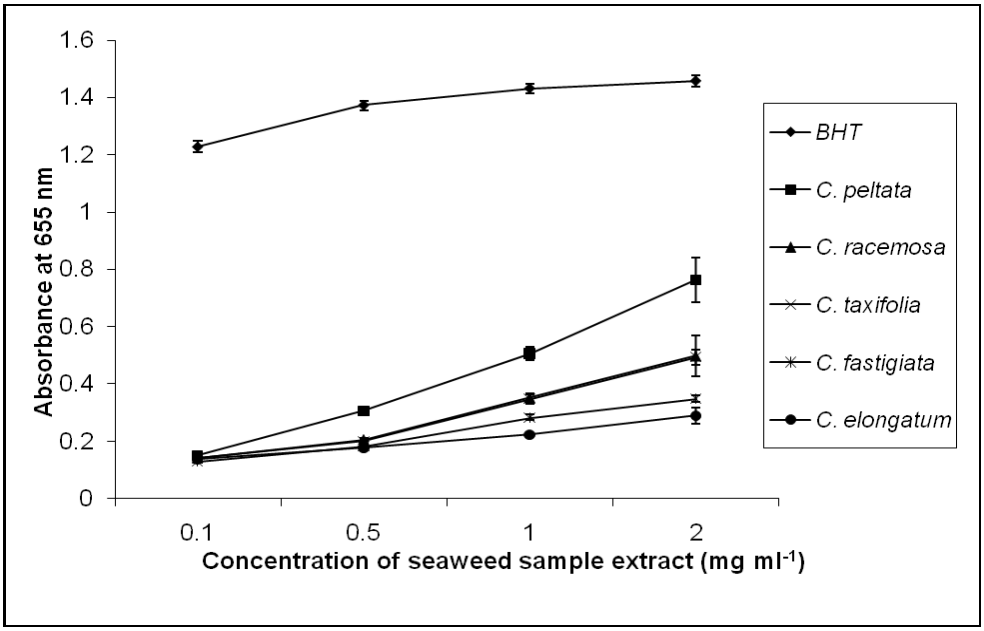


Figure 3.5. Reducing power of total methanolic extracts derived from 5 species of green seaweeds (at the concentrations 0.1-2 mg ml^{-1} dry seaweed extract). BHT: Butylated hydroxy toluene. Values are presented as means ± SD (n=3).

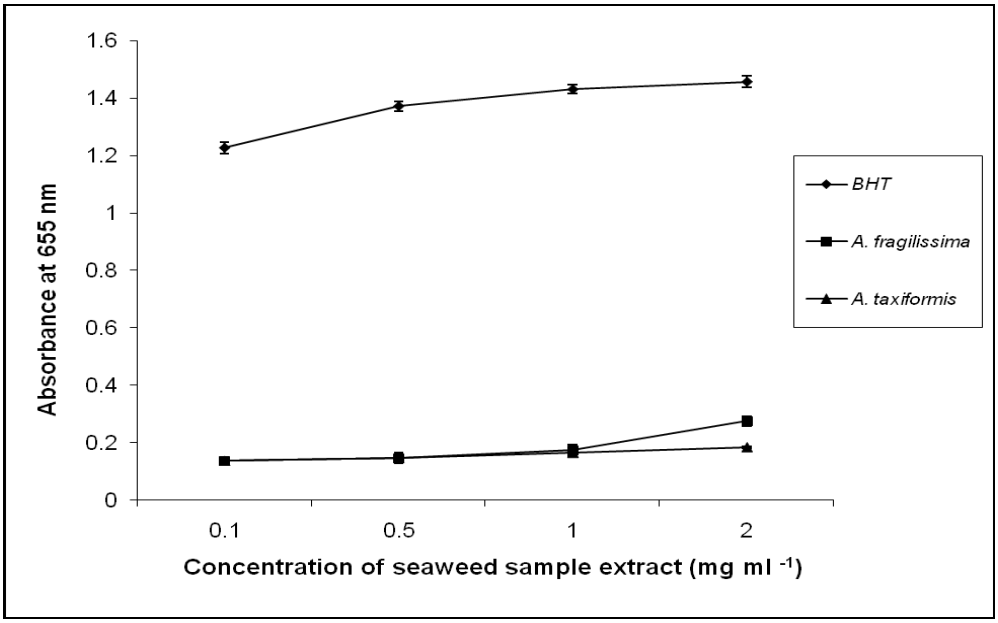


Figure 3.6. Reducing power of total methanolic extracts derived from 2 species of red seaweeds (at the concentrations 0.1-2 mg ml^{-1} dry seaweed extract). BHT: Butylated hydroxy toluene. Values are presented as means ± SD (n=3).

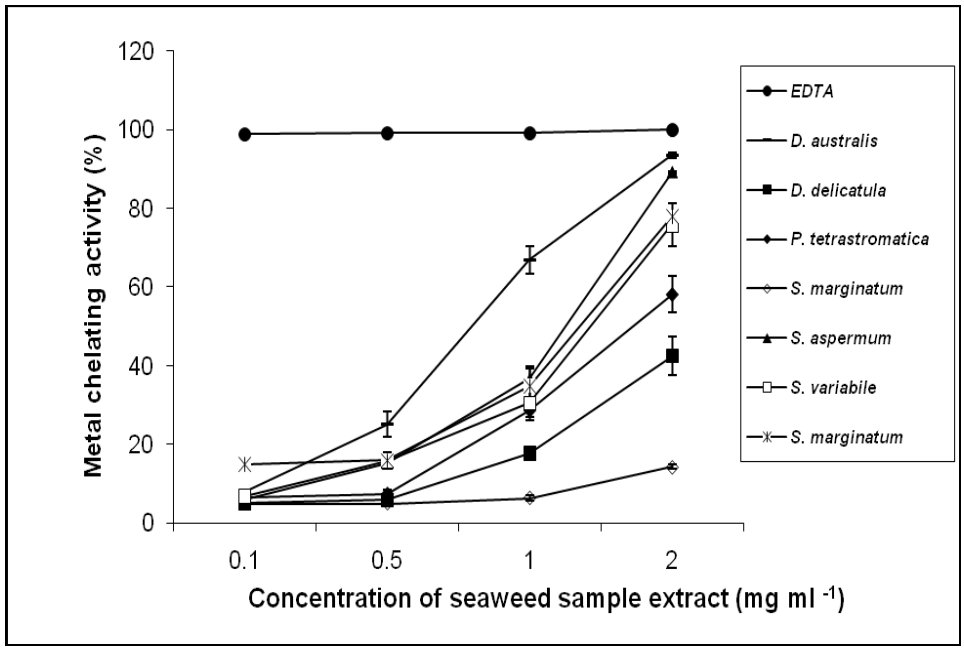


Figure 3.7. Ferrous ion-chelating activity (%) of total methanolic extracts derived from 7 brown seaweeds (at the concentrations 0.1-2 mg ml^{-1}dry seaweed extract). EDTA: Ethylene diamine tetracetic acid. Values are presented as means ± SD (n=3).

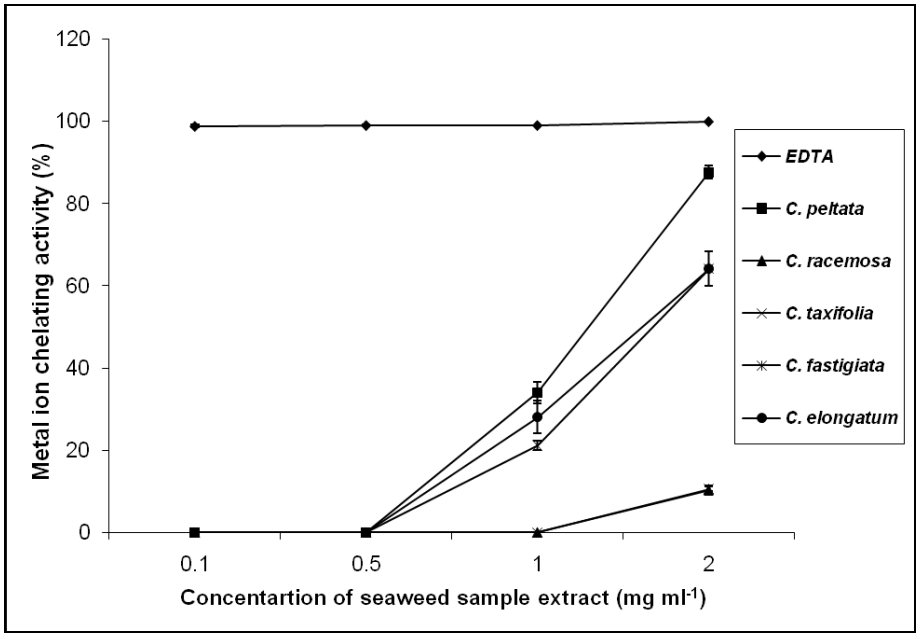


Figure 3.8. Ferrous ion-chelating activity (%) of total methanolic extracts derived from 5 green seaweeds (at the concentrations 0.1-2 mg ml^{-1}dry seaweed extract). EDTA: Ethylene diamine tetracetic acid. Values are presented as means ± SD (n=3).

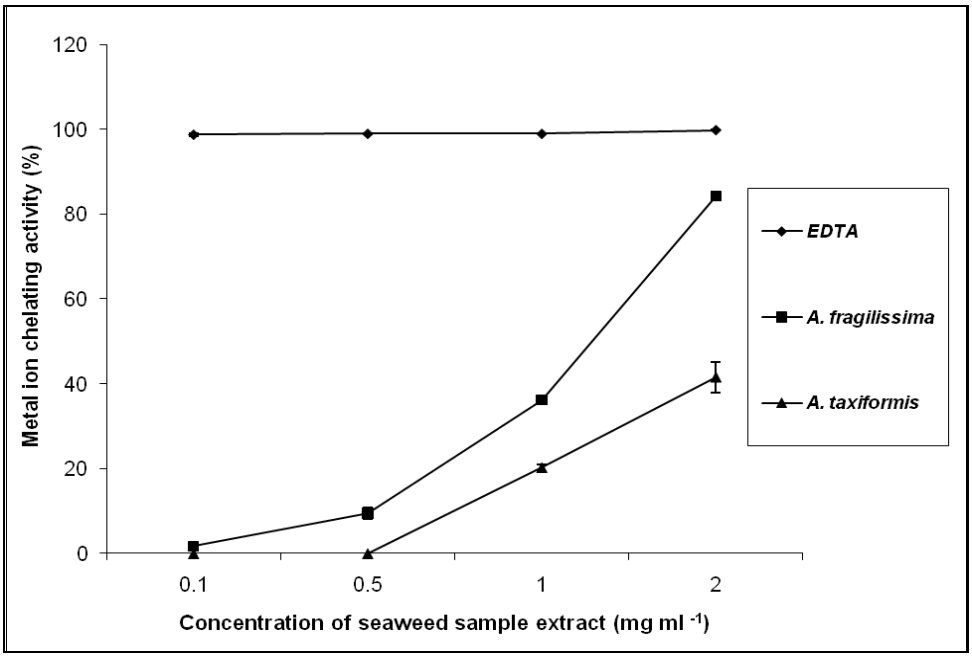


Figure 3.9. Ferrous ion-chelating activity (%) of total methanolic extracts derived from 2 red seaweeds (at the concentrations 0.1-2 mg ml^{-1}dry seaweed extract). EDTA: Ethylene diamine tetracetic acid. Values are presented as means ± SD (n=3).

3.4. DISCUSSION

Reactive Oxygen Species (ROS) such as hydroxyl ($OH^{\cdot}$), superoxide anion ($O2^{\cdot-}$) and peroxyl radicals ($LOO^{\cdot}$) are continuously produced in cells as by-products of metabolism, such as oxidative and reductive processes in mitochondria, oxygen (O_2) derived from respiration, immune responses to foreign antigens, and external exposure to radiation and various chemicals (Cheeseman *et al*., 1993). Superoxide dismutases, catalase, glutathione peroxidase and glutathione reductase, as well as various antioxidant chemicals such as; vitamin C, vitamin E, uric acid and bilirubin form the body's first line of antioxidant defense system. They help to maintain a balance between the generation of ROS and their eradication, which is very crucial for maintaining cellular functions at an optimum level and thus has important health implications. Polyphenolic antioxidants essentially have similar functions as the endogenous antioxidants. Therefore, in the present study I focused on the evaluation of antioxidant activity from brown, green and red seaweed extracts, which were prepared by methanolic extraction process. Their antioxidant effects were evaluated in three different assay systems including free radical (DPPH) scavenging assay, reducing power assay and metal chelation assay. The TPC of the seaweeds was also evaluated.

DPPH is a stable radical with a maximum absorbance at 517 nm. It has been widely used for evaluating reducing substances of plant and microbial extracts comparative to other methods (Hu and Kitts, 2000). DPPH is also considered as a good kinetic model for peroxyl radicals (Rackova *et al*., 2007). It can readily undergo reduction by a hydrogen donating antioxidant to form diphenyl picryl hydrazine (DPPH-H), and this alteration is visibly evident as a discoloration from purple to yellow (Hu and Kitts, 2000; Shon *et al*., 2003). In the present study, the active extracts

were able to reduce the stable radical DPPH to the stable, yellow colored DPPH-H. It is a known fact that, lipid autoxidation initiates a chain reaction in lipophilic radical perpetuating lipid peroxidation. Also, DPPH assay is considered as a good kinetic model for lipid peroxidation and peroxyl radicals. (Rackova *et al*., 2007). The seaweed ME's were not as much capable of scavenging radicals as ascorbic acid, a natural antioxidant and these differences were statistically significant (*p<0.001*). The major scavenging activity was demonstrated by the brown seaweeds followed by green seaweeds and then red seaweeds. The brown seaweed *Sargassum marginatum* showed the lowest DPPH free radical scavenging activity while *Soechospermum marginatum* had the highest (Fig. 3.1). In addition to *Stoechospermum marginatum*; *P. tetrastromatica, D. delicatula, S. asperum* also demonstrated relatively high DPPH radical scavenging activities. Amongst the green seaweeds tested, *C. peltata* (IC_{50} 1.024 ± 0.012 mg ml^{-1}) had the maximum DPPH free radical scavenging activity while *C. elongatum* (IC_{50} 3.105 ± 0.048 mg ml^{-1}) showed the lowest (Fig. 3.2). The extracts of *C. taxifolia* and *C. racemosa* showed comparable radical scavenging activity to *C. peltata.* The extracts of red seaweeds *Amphiroa fragilissima* and *Asparigopsis taxiformis* displayed radical scavenging activity of IC_{50} 2.898 ± 0.061 mg ml^{-1} and IC_{50} 3.386 ± 0.042 mg ml^{-1}, respectively (Fig. 3.3). The study revealed that all the seaweeds showed better radical scavenging activity when compared with the extract of *Palmaria palmata*, IC_{50} 12.5 mg ml^{-1} (Yuan *et al*., 2005) and *Kappaphycus alvarezii,* IC_{50} 3.03 mg ml^{-1} (Kumar *et al*., 2008) except *C. fastigiata* and *C. elongatum*. It is assumed that not only the content but also properties of polyphenolic compounds contribute to differential activities.

Polyphenols constitute a major group of chemical substances acting as primary antioxidants or free radical terminators; generally defined by the presence of more than one phenol unit or

building block per molecule. They are abundantly found in plants and possess the ability to scavenge free radicals, inhibit lipid peroxidation and up-regulate certain metal chelating reactions (Rice-Evans *et al*., 1996; Sugihara *et al*., 1999; Kuda *et al*., 2005). A number of studies have focused on the biological activities of phenolic components from plants with potential antioxidant and free radical scavenging activities (Ragan *et al.*, 1978; Liao *et al*., 2008). The major active compounds in different seaweed extracts have been reported to be phlorotannins and fucoxanthin (Yan *et al*., 1996, 1999). In fact in brown seaweeds, polyphenols form up to 5–15 % of their dried weight (Mandal *et al*., 2009) and display broad range of biological activities. In the present study, the TPC of the tested brown seaweeds varied significantly (p<0.001) and decreased in the following order: *P. tetrastromatica* > *Stoechospermum marginatum* > *Spatoglossum variabile* > *S. asperum* > *D. australis* > *Sargassum marginatum*. The TPC of all the seaweeds were expressed as mg gallic acid equivalent (GAE) g^{-1} of seaweed extract (Table 3.1) ranging from (13.19 ± 0.32 to 25.29 ± 0.445). Further analysis revealed that, there was a positive correlation (R^2 = 0.4, p < 0.005) between the TPC of brown seaweeds and the DPPH radical scavenging activity; although not very strong. This suggested that not only phenolic constituents but other components also contributed to the scavenging. This may be also explained due to the fact that the properties of polyphenolic compounds vary greatly depending on the number of phenolic groups they have and hence react differently to the Folin–Ciocalteu reagent (Singleton *et al.*, 1999). The TPC of the green seaweeds investigated in this study are presented in Table 3.2. The content of phenolic compounds varied significantly (p < 0.001) from 7.315 ± 0.352 to 38.929 ± 0.628 mg gallic acid equivalent (GAE) g^{-1} dry seaweed extract. Green seaweeds contain high amounts of polyphenols such as catechin, epicatechin, epigalloctechin gallate and gallic acid, as reported in one of the species of seaweed *Halimada* (Chlorophyceae)

(Yoshie *et al.*, 2002). Statistical analysis revealed that, there was a significantly high correlation between the antioxidant potential and TPC (R^2= 0.88, $p < 0.05$) which suggested that in this case the TPC of green seaweeds was directly contributing to the DPPH scavenging activity. The TPC of the red seaweeds was also investigated and presented in Table 3.3. The content of phenolic compounds varied significantly ($p < 0.001$) in both *A. fragilissima* and *A. taxiformis* extracts and found to be 8.220 ± 0.214 and 8.521 ± 0.284 mg GAE g^{-1} dry seaweed extract respectively. Researchers have isolated and identified a number of polyphenolic compounds such as catechins (flavan-3-ols), flavonols and flavonol glycosides in methanol extracts of Japanese red and brown algae (Yoshie *et al*., 2000; Yoshie-Stark *et al*., 2003). Several red seaweeds like *Porphyra tenera* (Takano *et al*., 1979), *Porphyra yezoensis* (Nakayama *et al*., 1999) as well as P. *palmata* (Sekikawa *et al*., 1986; Karsten and Wiencke, 1999) have been reported to contain the alcohol- and water-soluble mycosporine-like amino acids (MAAs) which function as UV-absorbing sunscreens in addition to acting as antioxidants (Dunlap *et al*., 1998). It has also been noted that phenolic acids such as caffeic acid are present in red seaweeds such as *P. yezoensis* and *Chondrus sp*. (Yoshie-Stark *et al*., 2003). As a result, the TPC of the methanol extract of the red seaweeds possibly outlines a mixture of both MAA's and phenolic acids.

The ME's of selected brown, green and red seaweeds were investigated for their reducing ability (the $Fe^{3+} \rightarrow Fe^{2+}$ transformation) as reducing capacity has been depicted as a significant indicator of potential antioxidant activity of a compound or sample (Meir *et al*., 1995). Reductones are known to prevent the formation of peroxide by reacting with certain precursors of peroxide (Halliwell, 1991). In this assay, the presence of reductones (i.e. antioxidants) in the ME's of the selected marine seaweeds caused the reduction of the Fe^{3+}/ ferricyanide complex to the ferrous

form giving a Pearl's Prussian blue color, which was measured at 655 nm to monitor the amount of Fe^{2+} present in the reaction mixture. The extracts could neutralize the free radicals by donating an electron and converting them to a more stable product ceasing the radical chain reaction in the process to various degrees. All the extracts showed significantly ($p < 0.001$) higher activities than the control but lower activities than the commercial antioxidant tested (Figs. 3.4, 3.5 and 3.6). Higher absorbance indicated higher reducing power. Similar results were reported in methanol extracts of higher plants (Kumaran and Karunakaran, 2007). All concentrations exhibited the OD value < 1.0. This was also supported by the findings of Kuda *et al*. (2005). Ordinarily, most non-enzymatic antioxidative activities, such as scavenging free radicals and inhibition of peroxidation, are mediated by redox reactions, irrespective of the stage in the oxidative chain in which the antioxidant action is assessed (Zhu *et al.*, 2002). In my results, the seaweed extracts exhibited reducing power, enumerating a possible role in the antioxidative activity observed.

Complex (red colored) formed by the quantitative interaction of ferrozine with ferrous ions was disrupted in the presence of chelating agents present in the reaction mixture, implying that they have the ability to chelate ferrous ion and in turn offer protection against the oxidative damage through their chelating properties. By measuring this color reduction, the chelating effect of the coexisting chelator was determined, for both the seaweed extracts and the standard (EDTA). The ferrous ion chelating abilities between the brown seaweed extracts and EDTA are shown in Figure 3.7. Both the extracts and EDTA showed statistically significant differences ($P < 0.001$). The highest metal chelating activity was demonstrated by *D. australis*. The metal chelating activity was also concentration dependent. Nevertheless, a very poor correlation of ferrous ion

chelating activity with TPC (R^2 = 0.077454, p > 0.05) was observed. The chelating ability of the ME's of green seaweeds and the EDTA displayed statistically significant differences (p < 0.001) and is depicted in Figure 3.8. The highest metal chelating activity was shown by *C. peltata* and the lowest by *C. racemosa*. Besides, a very poor correlation of metal ion chelating ability with TPC of the seaweed ME's was also documented (R^2 = 0.13, p > 0.05). Amongst the red seaweeds, the highest metal chelating activity was shown by *A. fragilissima* (IC_{50}1.278 ± 0.018 mg m^{-1}) than *A. taxiformis* (IC_{50} 2.376 ± 0.172 mg m^{-1}) (Fig. 3.9). A very poor correlation of ferrous ion chelating activity with TPC of all brown (R^2 = 0.08, p > 0.05) and green seaweeds (R^2 = 0.13, p > 0.05) was observed. This consistent poor correlation between TPC and metal chelating activity in brown and green seaweeds indicated that the phenolic compounds might not be the main chelators of ferrous ions. The metal ions chelating capacity of the phenolic compounds depends mainly on the accessibility of properly oriented functional groups (Van Acker *et al*., 1996)). Also it can no longer bind metals when the phenolic group is conjugated with a carbohydrate group, as in naturally occurring phenolic glycosides (Hider *et al*., 2001). Metal-binding capacities have been displayed by dietary fibers previously. This is supported by the various reports on the inhibitory effects on ferrous absorption of algal dietary fibers, such as carageenan, agar and alginate (Harmuth-Hoene and Schelenz, 1980). Transition metals, such as iron help superoxide anion (O^{-}) (Fenton reaction) and hydrogen peroxide to convert into extremely reactive hydroxyl radical ($OH^{\cdot}$) (Haber–Weiss reaction) that cause severe damages to membranes, proteins and DNA (Fisher and Naughton, 2003). They also decompose lipid hydroperoxides into peroxy and alkoxyl radicals and accelerate lipid peroxidation (Halliwell, 1991). In the long run, this process can bring about cellular death, carcinogenesis and mutagenesis. An extract with higher iron chelating ability would thus not only inhibit metal

dependent oxidative events, but would also combat ROS-mediated diseases (Finefrock *et al.*, 2003).

The seaweed ME's investigated in this study have revealed potent antioxidant activities. The antioxidative constituents possibly play a complimentary role by delaying or preventing the oxidation of cellular oxidizable substrates and selectively inhibiting the ROS cascade of events (Fig. 3.10). All the above data imply a protective role for seaweeds and may prove to be of pharmacological importance, which needs to be explored further.

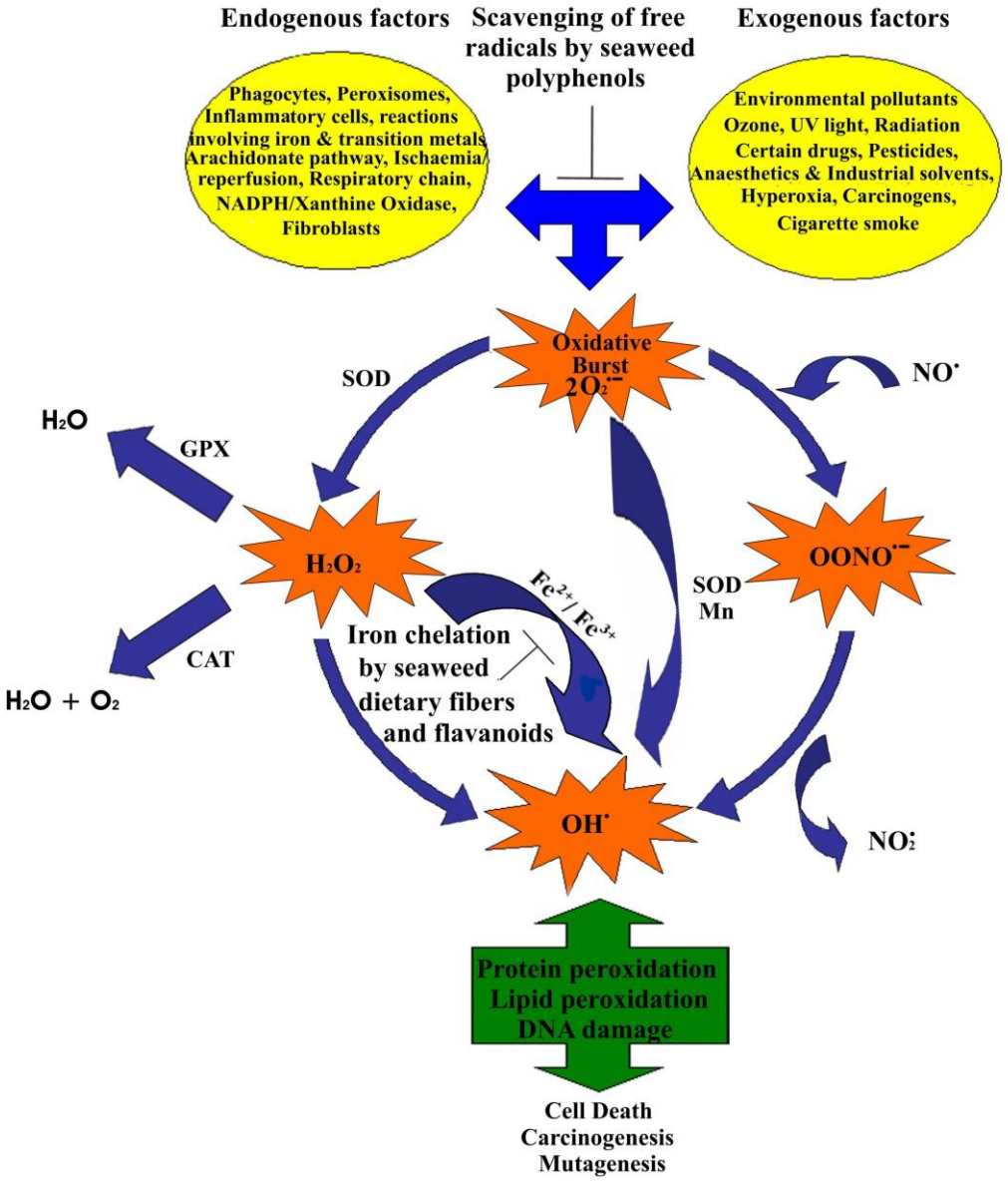


Figure 3.10. A hypothetical figure showing seaweeds and their role in the prevention of ROS-mediated cascade of events. SOD, along with CAT and GPX, forms the first line of the body's antioxidant enzyme defense mechanisms. Various endogenous and exogenous factors give rise to oxidative burst, a phenomenon where superoxide anion radical is the predecessor to majority of ROS and moderator of oxidative chain reactions, which perpetuates the production of secondary ROS. In the long term, this can lead to protein peroxidation, lipid peroxidation and DNA damage within the cell bringing about cell death, carcinogenesis and mutagenesis. The seaweed extracts inhibit these occurrences by preventing the production of ROS at key stages and impeding the inception of cancer and other diseases.

Chapter – 4

Screening for anti-angiogenic activity in Seaweeds collected from the coasts of Goa and Malwan (Maharashtra)

4.1. INTRODUCTION

Angiogenesis or neovascularization normally plays an important role in several vital processes such as wound healing, restoration of blood flow to tissues after injury, and menstruation (Nussenbaum and Herman, 2010). However, when the body is unable to control angiogenesis, due to proangiogenic factors dominating over the anti-angiogenic factors, they cause diseases such as age-related macular degeneration, rheumatoid arthritis, or psoriasis (Gupta and Zang, 2005). Most importantly, angiogenesis promotes growth, proliferation, invasion, and metastasis of solid tumors. (Folkman, 1971, 1990, 1995; Hanahan and Folkman 1996; Pepper, 1997) This in turn is known to secrete a variety of angiogenic factors, such as basic Fibroblast Growth Factor (bFGF) and Vascular Endothelial Growth Factor (VEGF), which can stimulate angiogenesis as well (Kerbel, 1991,1998; Hanahan and Folkman, 1996; Rak *et al.* 2002; Yabushita *et al.,* 2003). This in itself is a multi-step process that begins with the degradation of the basement membrane by activated endothelial cells that migrate and proliferate, leading to the formation of solid endothelial cell sprout into the stromal space. In due course, vascular loops are formed and capillary tubes develop with formation of tight junctions and deposition of new basement membrane (Carmeliet, 2000). This is critical for tumor growth and metastasis, as these newly formed blood vessels provide tumor cells with the required nutrients and oxygen. Each step of this process represents a potential target for the inhibitory action of angiosuppressive drugs, allegedly leading to the desperately needed breakthrough in cancer therapy and other proangiogenic diseases (Keshet and Ben-Sasson, 1999).

In spite of the recent trends of pharmaceutical industry towards fast growth of synthesized *de novo* drugs, plants still remain a traditional source of medicinal compounds; with upto 40 % of

modern drugs directly or indirectly linked to natural compounds. Several terrestrial, plant-derived agents, such as, resveratrol, catechins genistein, curcumin, in addition to others, such as diallyl sulfide, S-allyl cysteine, allicin, lycopene, capsaicin, 6-gingerol, ellagic acid, ursolic acid, silymarin, anethol, and eugenol have already proved their chemopreventive abilities (Dulak *et al.,* 2005). As a result, several angio-supressive drugs are at present in different phases of clinical trials (Kerbel *et al.,* 2000). These drugs have been suggested to act via suppression of cancer cell proliferation, inhibiting growth factor signaling pathways, inducing apoptosis, as well as inhibiting angiogenesis (Dorai *et al.,* 2004). Discovery of new potential therapeutic anti-cancer drugs with all these properties would therefore be a valuable addition to the already growing pharmaceutical drug development initiatives.

Marine plants such as seaweeds are believed to be beneficial for human health due to the myriad bio-protective activities that they possess (Smith *et al.,* 2004) and have found much use in industries and medicine for a variety of purposes (Santoso *et al.,* 2004). A number of studies have focused on screening natural products from marine and freshwater algae for their bioprotective activity, whereas there is only a handful of data available with respect to seaweeds and their angiosuppressive potential (Dias *et al.,* 2005; Ye *et al.,* 2005; Sugawara *et al.,* 2006; Cumashi *et al.,* 2007). It has also been noted that some natural algal polysaccharides, suppress angiogenesis (Matsubara *et al.,* 2003, 2005a).

Therefore, in the present study, I screened 14 marine seaweed extracts for their anti--proliferative activity against EAT (Ehrlich Ascites Tumor cells) and consequently selected a promising

seaweed namely; *Stoechospermum marginatum* (C. Agardh) Kutzing for further study on its angio-suppressive efficacy using both *in-vitro* and *in-vivo* assays.

4.2. MATERIALS AND METHODS

4.2.1. Collection and identification of Seaweeds

The seaweed samples were collected and identified from in and around the coasts of Goa and Maharashtra, India in the same manner as mentioned in Chapter 2.

4.2.2. Processing and preparation of methanol extract from seaweed samples

The methanolic extract was obtained in the same manner as mentioned in the Chapter 2 and designated as SME for future reference.

4.2.3. Animals, *in-vivo* tumor model, cell lines and Chemicals

Swiss albino mice (6-8 weeks old) were obtained from the animal house, Department of Zoology, University of Mysore, Mysore, India. EAT cells (mouse mammary carcinoma) were routinely maintained in the laboratory of Molecular Oncology, Department of Studies in Biotechnology, University of Mysore, Mysore, India and routinely used for in-vivo transplantation. HUVEC (Human Umbilical Vein Endothelial Cells) and Endothelial Growth Medium (EGM-2).were obtained from Cambrex Bioscience, Walkersville, USA. BeWo (choriocarcinoma) and HEK-293 (Un-transformed Human Embryonic Kidney) cells were obtained from the National Center for Cell Science, Pune, India. 3[H] thymidine (Specific activity: 20Ci/mmol) was obtained from BRIT/BARC Vashi Complex, Sector 20 Vashi, Navi

Mumbai – 400703. Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Penicillin (100 U/ml)-Streptomycin (0.1 mg/ml) and Trypsin-EDTA were purchased from Invitrogen, USA. The antibodiy PECAM-1 and ABC reagent for CD 31 staining were procured fom Santa Cruz Biotechnology, California USA. Matrigel was purchased from Chemicon International, CA, USA. Fertilized eggs were from a government poultry farm in Bangalore, India. All other chemicals and reagents were of highest analytical grade available.

4.2.4. *In-vitro* culture of EAT, BeWo, HUVEC and HEK-293 cells.

BeWo (Choriocarcinoma) cells were cultured in DMEM Ham's F-12 medium with 10 % FBS, 1 % Penicillin (100 U/ml) -Streptomycin (0.1 mg/ml) and Gentamycin (0.1 mg/ml). EAT and HEK 293 cells were maintained in DMEM with 10 % FBS and 1 % Penicillin-Streptomycin. HUVEC cells were cultured in EGM-2 medium with 2 % FBS, 0.04 % hydrocortisone, 0.1 % long R3-human Insulin like growth factor (IGF-1),0.1 %ascorbic acid, 0.4 % human Fibroblast Growth Factor (bFGF), 0.1 %VEGF, 0.05 % gentamycin and 0.05 % amphotericin-B according to the manufacturer's protocol. The cells were incubated at 37 ºC in a humidified atmosphere of 5 % CO_2. When the cells reached confluency, they were passaged by trypsinization using 0.025 % trypsin / 0.01 % EDTA. For the experiments, cells from passages 2-5 were used.

4.2.5. Thymidine incorporation assay

^{3}[H] thymidine incorporation assay was carried out as described previously (Naderali *et al.*, 2004). In brief, the tumor cells were plated onto 12-well culture plates at 25 x 10^3 cells/ well and incubated at 37 °C in 5 % CO_2 for 48 hrs in DMEM medium in their respective media. After incubation, seaweed extract at different concentrations was added to the wells in triplicates

leaving three wells as control prior to the addition of ^{3}[H] thymidine (1 µci/ ml) to all the wells. After 48 hrs of incubation, the cells were washed with PBS and then the cells were fixed with 10 % ice-cold trichloroacetic acid to precipitate the DNA. The radioactivity was measured in scintillation solution using liquid scintillation spectrometry. The concentration of the sample was then plotted against the percentage cell survival and the concentration of the extract required to inhibit cell growth by 50 % (IC_{50}), was determined.

4.2.6. Matrigel tube formation assay

The Matrigel tube formation assay has widely been used as an *in-vitro* measurement of endothelial cell differentiation and as such it was performed according to the manufactures instruction. In brief, Matrigel (50 µl) was added to each well of a 96 well plate and incubated at 37 ºC for 1 hr allowing the gel to polymerise. Human Umbilical Vein Endothelial Cells (HUVECS) were suspended in endothelial growth medium and cells (1×10^{4}) were seeded in to each well containing 150 µl of EGM. The cells were incubated with or without the test sample (SME) at 37 ºC and 5 % CO_2. All the conditions were performed in triplicates. After 16 – 20 hrs of incubation, the tube formation was photographed at 40x magnification by using an inverted microscope (Olympus, Germany).

4.2.7. Migration of HUVEC's in oil drop assay

The effect of SME on HUVEC cell migration using oil drop assay was performed as described by Cai *et al.* (2000). To measure migration on vWF bound to type I collagen (vWF/collagen), acidic bovine type I collagen (Vitrogen 100) in 0.012 M HCl was neutralized with an equal volume of 0.012 M NaOH and then adjusted to a final concentration of 1.5 mg ml^{-1} by the

addition of sterile PBS, on ice at 4 °C. A 50 ml aliquot of the neutralized collagen was pipetted and evenly spread using a glass pipette spreader on a 35 mm tissue culture dish. The collagen was polymerized by incubation for 60 minutes at 37 °C in an incubator without CO_2. A 25 ml solution of 100 mg ml^{-1} vWF in 0.1 % BSA/ PBS was then pipetted evenly on top of the collagen gel and allowed to perfuse through the collagen matrix for 60 minutes at 37 °C, after which the excess was aspirated off the dish. vWF collagen-coated dishes were then air-dried in the laminar flow hood for 60 minutes. The dishes were washed twice with sterile nanopure H_2O and air-dried again for 30 minutes, after which they were ready for use in the migration assay.

To each well 1 ml of mineral oil was added and then a 10 ml aliquot of EC, resuspended in HUVEC complete medium at a density of 1.5 x 10^6 cells ml^{-1}, was pipetted through the oil onto the matrix surface. The light mineral oil used for the oil-drop migration assay was a biologically inert, hydrophobic, non-polar structure consisting of saturated aliphatic and alicyclic hydrocarbons. The density of the mineral oil, 0.84 g ml^{-1}, was less than the aqueous drop of cell suspension. As a result, the drop of EC suspension fell through the oil and readily settled down onto the matrix substratum. EC under oil were incubated at 37 °C in an atmosphere of 5 % CO_2 for 60 minutes, to allow for attachment and complete cell spreading. Afterwards, the oil was aspirated off the dish, leaving a circle of spread, confluent HUVEC in the center of the well. HUVEC were washed twice with complete medium, and then incubated in the same medium for 15 minutes allowing any remaining oil to float off the surface of the dish. HUVEC were then washed twice more with M199 supplemented with Penicillin/ Streptomycin to remove any residual oil. To the confluent HUVEC circles, 1 ml of HUVEC-SFM was added, to which 20 ng ml^{-1} b FGF and 10 ng ml^{-1} EGF were added. Inhibitory extract was added at 0 time. Migration

was allowed to proceed to 36 hrs; intermittently images were recorded and captured at 0, 12, 24 and 36 hrs. The images were recorded at higher magnifications and encaptured using phase contrast optics (Olympus, Germany). For evaluating the effect of *S. marginatum* extract on endothelial cell migration and proliferation, *S. marginatum* extract was added to the migration assay at 0 time at a final concentration of 0.1 mg/ well.

4.2.8. Migration of HUVECs in wound-healing assay

An *in-vitro* wound-healing assay was performed to measure unidirectional migration by HUVECs. HUVECs were seeded at 4×10^4 cells per well into a 12-well plate, incubated for 48 hrs at 37 °C in a humidified atmosphere of 5 % CO_2, washed with PBS twice, and then incubated in EGM-2 with 1 % FBS. After 24 hrs incubation, the monolayers of HUVECs were scratch-wounded to approximately 1 mm depth in a straight line using Eppendorf (1 to 200 µl) pipette tips (Axygen Scientific, Central Avenue, CA, USA). For stimulation, VEGF (10 ng ml^{-1}) was added, with or without *S. marginatum* extract at 0.1mg ml^{-1}, and incubation continued for 3, 6, 18, 24, 36 and 42 hrs. The images were taken at the time of the wounding and at different time intervals thereafter using a phase-contrast microscope (Olympus, Germany).

4.2.9. Chorio-allantoic membrane assay (CAM)

CAM assay was followed according to the method of Gururaj *et al*. (2002). In brief, the fertilized eggs were incubated at 37 °C in a humidified and sterile atmosphere for 10 days. A window was made under aseptic conditions on the eggshell to check for proper development of the embryo. The window was resealed and allowed to develop further. On the 12^{lh} day, saline, recombinant VEGF (50 ng per egg) or the extract (100 µl/ egg) was air dried on sterile glass cover slips. The

window was reopened and the cover slip was inverted over the CAM. The window was closed again, the eggs were returned to the incubator for another two days. The windows were opened on the 14th day and inspected for changes in the micro-vessel density in the area under the cover slip and photographed.

4.2.10. Corneal Neovascularization (CNV) assay

The rat corneal micro-pocket assay was performed as described previously (Sarayba *et al.*, 2005). In brief, hydron polymer (poly hydroxyl ethyl-methacrylate was dissolved in ethanol to a final concentration of 12 %. A 5 µl aliquot of this mixture was then pipetted onto Teflon pegs. Aliquots of 10 µl of 12 % Hydron/Ethanol alone (group 1), with 1µg of cytokine VEGF (group 2) and VEGF + 5 µg seaweed extract (group 3) was added to each pellet and allowed to dry under a laminar flow hood at room temperature for 2 hrs. The pellets were incubated at 4 °C overnight. All procedures were performed under sterile condition. Male Wistar rats weighting 300-350 g were anaesthetized with *i.p* with a combination of ketamine (87 mg kg^{-1}) and xylazine (13 mg kg^{-1}). A drop of 0.5 % proparacaine was instilled to the eye and the globe was proptosed using a pair of 0.3 mm tissue forceps. Using a surgical microscope, a paracentral linear incision 1 mm from the center of the cornea, 1.5 nm in length and 50 % of the corneal depth was made with a No. 11 surgical blade to create a corneal micropocket. The incision was bluntly dissected through the stroma to the limbal area using a curved iris spatula. A single pellet was advanced into the lamellar pocket to the limbus using corneal forceps. Postoperatively, gentamycin ointment was applied to the anterior surface of the operated eye. The rats were observed for 24-72 hrs for the occurrence of non-specific inflammation and for localization of the pellets. On day

7, the rats were anaesthetized with ketamine and the corneas were observed under sterio-binocular microscope with CCD camera and photographed.

4.2.11. Record of body weight, ascites volume, cell number and peritoneal angiogenesis of EAT hearing mice treated with *S. marginatum* methanolic extract

The crude extract of the seaweed *S. marginatum* selected for the study was tested for its effect on EAT cell growth *in-vivo*. EAT cells (5×10^6) were injected intraperitoneally *(i.p)* into mice (2 groups of mice, 6 in each group) and growth was recorded everyday from the day of transplantation. To verify whether the seaweed extract inhibited tumor growth and angiogenesis mediated by EAT cells *in-vivo*, the extract (l00 µg of SME) was injected into the peritoneum of the EAT bearing mice everyday from the 6^{th} day of transplantation. The body weight of the mice was monitored from the 1^{st} day till the 12^{th} day. On the 12^{th} day, the animals were sacrificed and the volume of the ascites formed both in untreated and treated mice were recorded. The pelleted cells were counted by trypan blue dye exclusion method using a hemocytometer. The animals were dissected to observe the effect of the extract on peritoneal angiogenesis. All the experiments were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CSPCA), Government of India.

4.2.12. Immunohistological analysis (H and E staining)

To determine whether the SME inhibited the microvessel density, the effect of the extract was verified on the angiogenic response induced by cytokine VEGF in EAT bearing mice. EAT bearing mice were treated regularly with the extract from the 6^{th} day of transplantation. On the 12^{th} day, the animals were sacrificed and the peritoneum from treated or untreated mice was

fixed in 10 % formalin. Sections (5 µm) were made from paraffin embedded peritoneum using automatic microtome (SLEE Cryostat) and stained with hematoxylin and eosin. Microvessel counts and the image photography were done using Leitz-DIAPLAN microscope with an attached CCD camera.

4.2.13. Immunohistolgical analysis (CD 31 staining)

The effect of SME on proliferating endothelial cells was determined by staining the paraffin sections of the peritoneum of treated or untreated mice with anti-CD 31 anti-body (Naderali *et al.,* 2004). Peritoneum sections were processed as per the protocol supplied by the manufacturer (Santa Cruz Biotechnology, CA, USA). In brief, sections were dewaxed in xylene thrice for 5 minutes each. The sections were rehydrated in descending concentrations of ethanol (100 % ethanol for 5 minutes, 95 % for 2 minutes and 80 % for 2 minutes) and washed in distilled water. Anti-gen retrieval was done by heating the sections at 95º C for 15 minutes in a humidified atmosphere. The sections were treated with 3 % H_2O_2 in PBS to block endogenous peroxidase activity. They were blocked in blocking serum for 30 minutes to reduce the non specific binding and then were incubated with anti-CD 31 (PECAM-1) anti-bodies for 2 hrs. Following PBS washing, slides were incubated with secondary anti-body (biotinylated rabbit anti- mouse IgG) for 1 hr at room temperature. The slides were washed in PBS for 5 minutes and incubated with the substrate (100µl/ section) followed by ABC reagent for 45 minutes (2 ml histo buffer + 20 µl Avidin solution + 20 µl Biotin solution). After incubation, the slides were washed in PBS for 5 minutes. Antigen and antibody complexes were detected using substrate (DAB, 100 µl/ section) for 5 minutes. The sections were washed thrice for 2 minutes in tap water and twice for 2 minutes in distilled water. Subsequently, the slides were counter stained with 2 % hematoxylin

for 5-7 minutes and washed again in tap water thrice for 5 minutes each. The slides were washed successfully for 2 minutes each in 50 % ethanol, 80 % ethanol and absolute alcohol. After xylene wash, the slides were mounted using Entellan mountant solution and the sections were scored using DIAPLAN light microscope and photographed.

4.2.14. Survival studies

A mouse assay was performed on EAT mice treated with or without *S. marginatum* extract (4 mg/kg body weight) every day after the 6^{th} day of the transplantation. About 12 EAT bearing animals were used in the study; 6 served as a control and the remaining 6 were treated with *S. marginatum* extract. The mice were weighed everyday starting after the first day of tumor transplantation and weighing continued for the duration of their lifespan. Mice were assesses for their mortality twice daily, in the early morning and late afternoon. Mice were euthanized upon reaching the criteria for morbidity. Deaths occurring overnight were recorded the next morning.

4.2.15. Statistical analysis

All experiments were conducted in triplicate (n=3) and expressed as means ± SD using STATISTICA software (Statsoft, 1999)

4.3. RESULTS

4.3.1. SME inhibits *in-vitro* proliferation of EAT and BeWo but not HEK-293 cells

The preliminary screening of SME demonstrated potent anti-proliferative ability against EAT cells (Fig. 4.1) and was therefore selected for further anti-angiogenic studies. BeWo cells and HEK-293 cells were used to verify if SME inhibits the proliferation of tumor/normal cells *in-*

vitro. SME extract inhibited proliferation of BeWo cells in a dose dependent manner. However, no potent effect was seen in case of untransformed normal HEK-293 cells. As shown in Figure 4.2A, inhibition of 80.55, 83.54, 90.79, 95.43, 96.36 % on the proliferation of BeWo cells was recorded, while in HEK 293 cells it was found to be 4.62, 5.76, 5.85, 6.71, 7.40 % (Fig. 4.2B) inhibition at 0.005, 0.025, 0.050, 0.075, 0.1 mg ml^{-1} concentrations of SME.

4.3.2. SME inhibits *in-vitro* tube formation of HUVEC's induced by VEGF

Tube formation assay was performed *in-vitro* to substantiate the effect of SME on the formation of blood vessels by HUVEC's. In the positive control group stimulated with VEGF (10 ng), the HUVEC's adhered to the matrigel surface within 20 to 24 hrs and formed a branching anastomising network of capillary like tubes with multi-centric junctions over 24 hrs. However, treatment with SME prevented VEGF stimulated tube formation of HUVEC's, in a dose dependent manner (Figure 4.3). A complete inhibition was obtained at 5 mg $ml^{-1}$of SME. At the latter concentration, most of the cells appeared as unorganized cell aggregates.

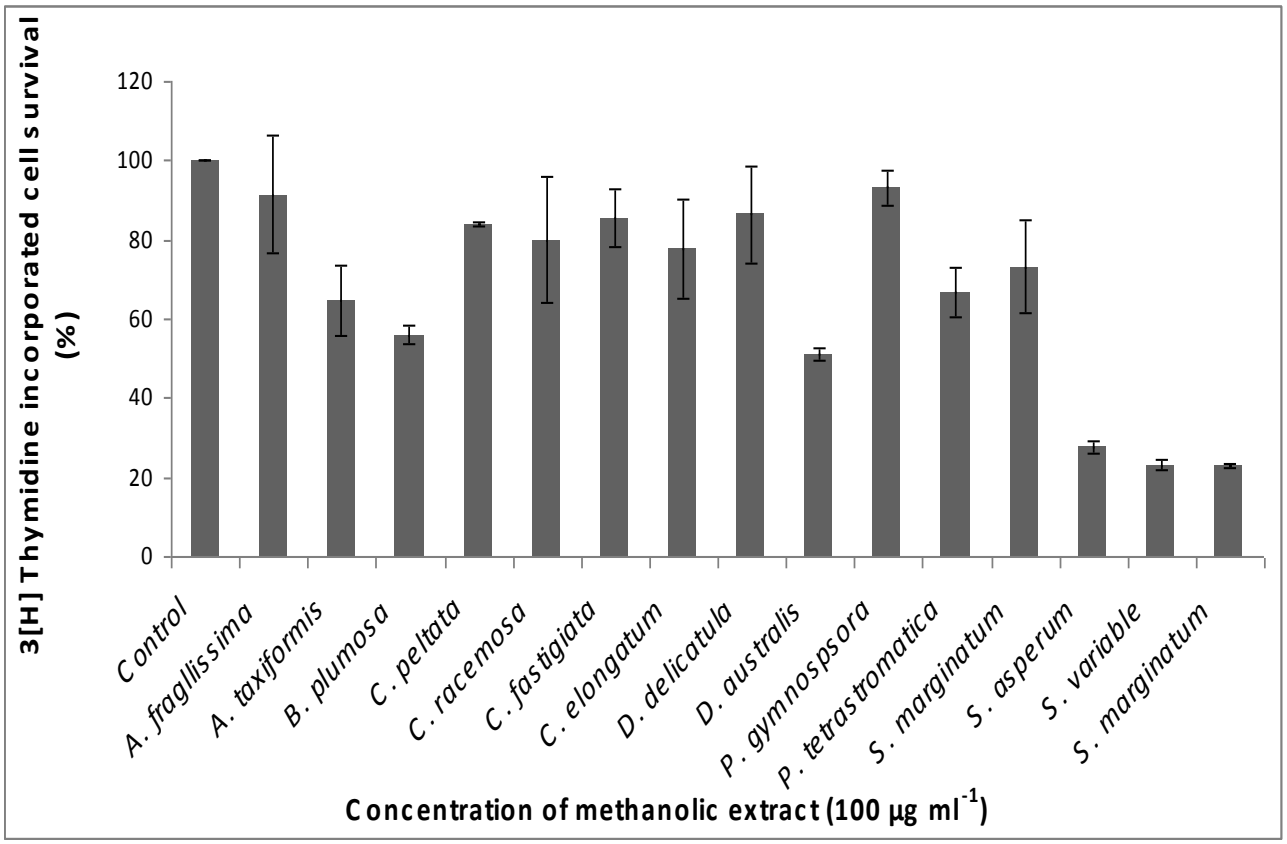
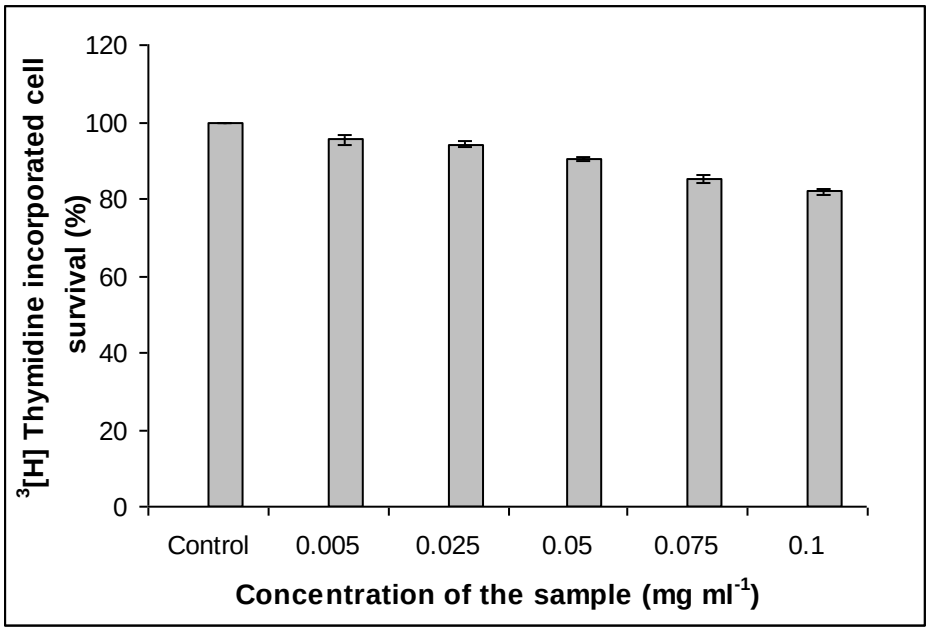


Figure 4.1. Effect of 15 seaweed methanolic extracts on proliferation of EAT cells *in-vitro*. EAT cells were plated in 12 well plates and incubated for 48 h. Seaweed extracts of 100 µg ml^{-1} concentration were added to the wells in triplicates prior to the addition of ^{3}[H] thymidine and incubated for another 48 h. The cells were trypsinized after 2 days and processed for scintillation counting. Values are presented as means ± SD (n=3).

A



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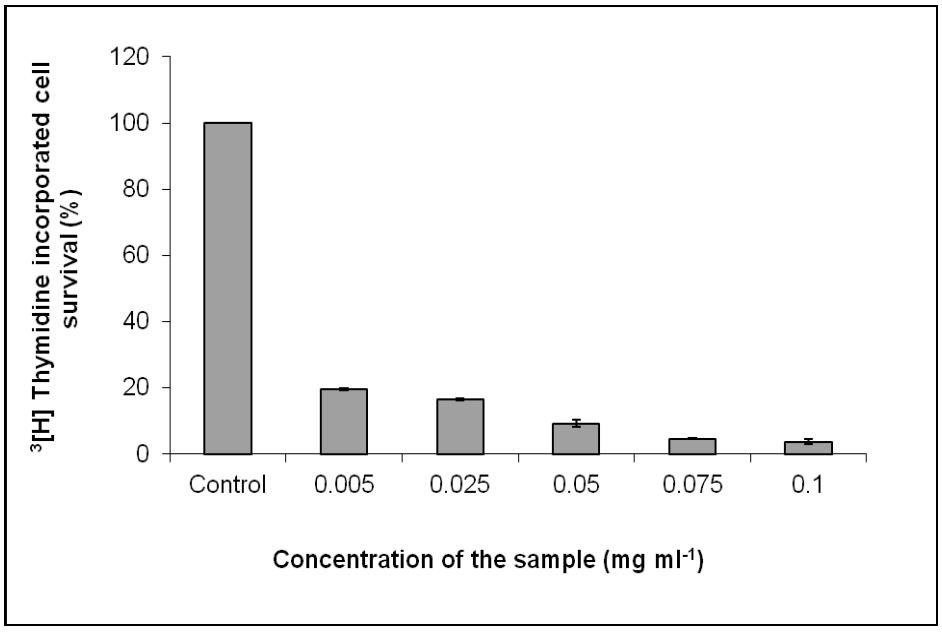

Figure 4.2. Effect of SME on proliferation of HEK-293 and BeWo cells *in-vitro*. (A) HEK-293 and (B) BeWo were plated in 12 well plates and incubated for 48h. SME in concentrations of 0.005, 0.025, 0.05, 0.075 and 0.1 mg ml^{-1} were added to the wells in triplicates prior to the addition of ^{3}[H] thymidine and incubated for another 48 h. The cells were trypsinized after 2 days and processed for scintillation counting. Values are presented as means ± SD (n=3).

4.3.3. Effects of SME on HUVEC migration in wound healing and oil droplet assay

To ascertain if inhibition of cell spreading resulted in an inhibition of cell migration, oil droplet and wound healing assays were performed using HUVEC cells as described in material and methods section. Cell spreading and migration in HUVECs were increased by VEGF in control wells while, SME at 100 μg/ml inhibited its migration and spreading (Figs. 4.4 and 4.5) in both wound healing assay and oil droplet assay.

4.3.4. Anti-angiogenic effect of SME on CAM and CNV

The CAM assay and rat cornea assays form a part of the conventional angiogenesis assays used commonly for *in-vivo* validation of the anti--suppressive efficacy of anti-angiogenic molecules. Results shown in Figure 4.6A and Figure 4.6B indicated that SME had a direct effect on inhibition of angiogenesis in *in-vivo* model system. When compared to the extensive angiogenesis seen in VEGF treated CAM and rat cornea, angiogenesis at the site of the application of SME was significantly reduced.

4.3.5. *In-vivo* treatment of SME extract inhibits growth of EAT cells in peritoneal angiogenesis assay

The treated as well as control animals were observed for the degree of vascularization occurring in the peritoneal cavity. The results in Figure 4.7A indicates that control EAT bearing mice had a gradual increase in body weight of 8 to 10 g when 5 x 10^6 EAT cells were injected on day zero. In the mice treated with/ without SME, a significant decrease was observed in the groups which were treated with SME when compared to the control, indicating the effect of SME in preventing the growth of tumor cells. In a fully grown ascites tumor, a volume of 5-6 ml of ascites is usually

generated during the tumor growth period of 12 days. In SME treated mice, the volume of ascites was about 3 ml (Fig. 4.7B). The numbers of viable cells in fully grown EAT bearing mice is 3 x10^8/ mouse while this number was reduced in SME treated mice to 0.72 x 10^8/ mouse (Fig. 4.7C), indicating considerable reduction when compared to the control. These results indicate the potential anti-tumor activity of SME. In a fully grown ascites tumor *in-vivo*, there is extensive peritoneal angiogenesis. However, in SME treated mice, a significant decrease in peritoneal angiogenesis also observed *in-vivo* (Fig. 4.7D).

4.3.6. SME inhibits formation of MVD and proliferation of endothelial cells

Results on H and E staining indicate that there was a reduction in the number of newly formed microvessels in the peritoneum of SME treated peritoneum of EAT bearing mice compared to the control (Fig. 4.8A). The proliferation of endothelial cells can be indicated by using CD31 marker. In our results of CD31 staining, there was a marked reduction in the number of proliferating endothelial cells in the peritoneum of SME treated EAT-bearing mice (Fig. 4.8B), corroborating the results shown in the inhibition of peritoneal angiogenesis *in-vivo.*

4.3.7. SME increases the survivability of EAT bearing mice

Further, the effect of SME on survivality of EAT bearing animals was tested. Upon intraperitoneal transplantation of 5×10^6 cells/ mice, the EAT-bearing mice survived for 13 days, with tumor cells increasing with increasing days. The animals succumbed to the tumor burden 13 days after tumor transplantation. SME treatment (4 mg kg^{-1} body weight/dose, every day) extended the survival time of EAT-bearing mice from 13 days up to 1 month (Fig. 4.9).

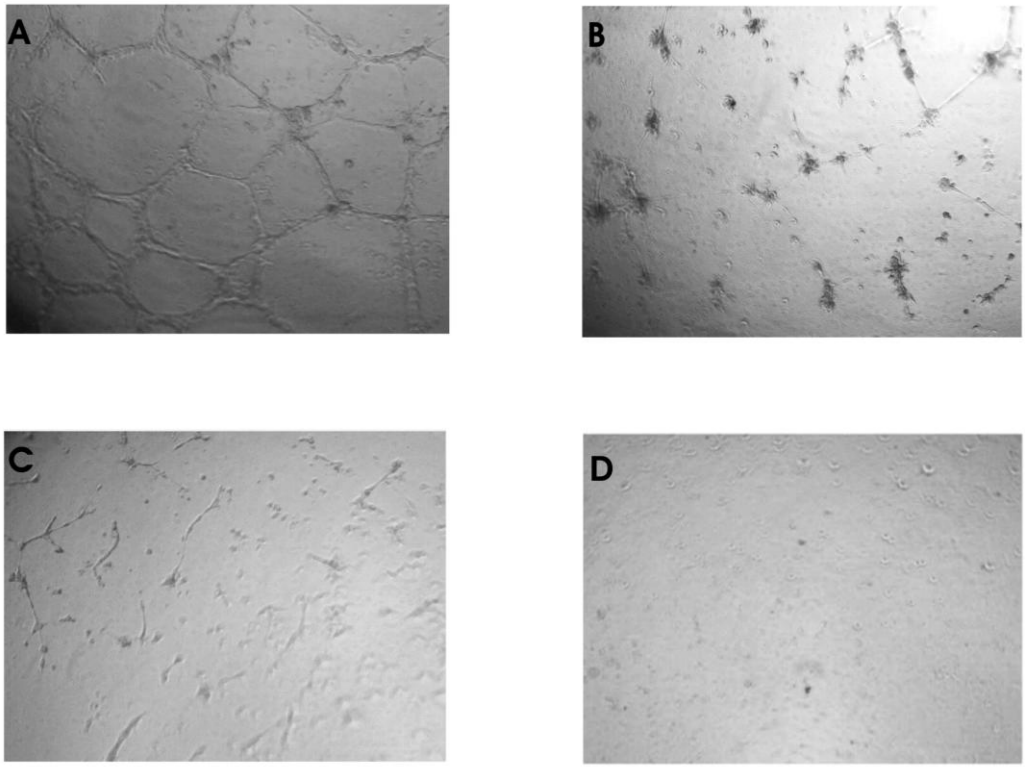

Figure 4.3. Inhibitory effect of *S. marginatum* extract (SME) on VEGF induced tube formation *in-vitro*. HUVEC's were seeded into the matrigel layer in 96-well plate. (A) VEGF alone (+ ve control), (B) without VEGF (-ve control), (C) VEGF + SME (5 μg/ well), (D) VEGF + SME (10 μg/ well). The experiment was repeated thrice with similar results (values are means ± S.D; n=3). Three replicate fields of triplicate wells were digitally photographed.

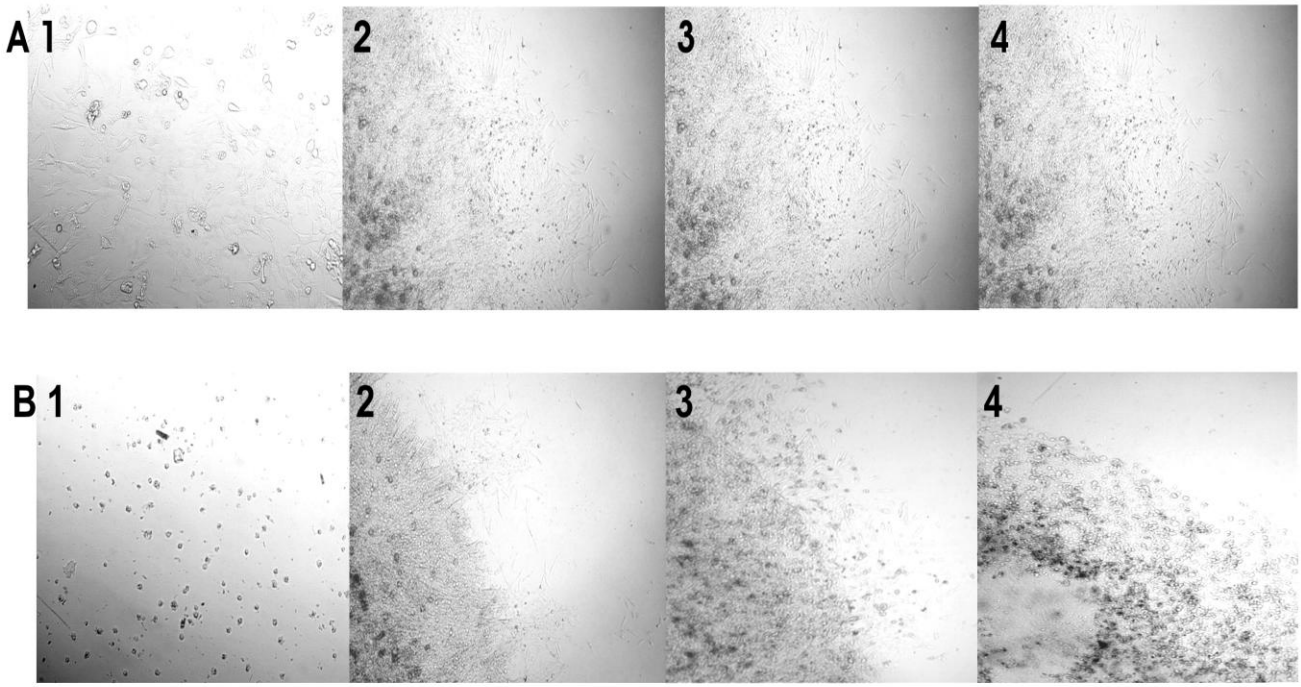

Figure 4.4. Effect of *S. marginatum* (SME) on HUVEC cell migration in an *in-vitro* oil drop assay. The representative phase-contrast images show the cells migrating. A) Cells migrating in control wells at 1) 0h, 2) 12h, 3) 24h, 4) 36h. B) Cells migrating in SME (100 µg/ well) treated wells at 1) 0h, 2) 12h, 3) 24h, 4) 36h.

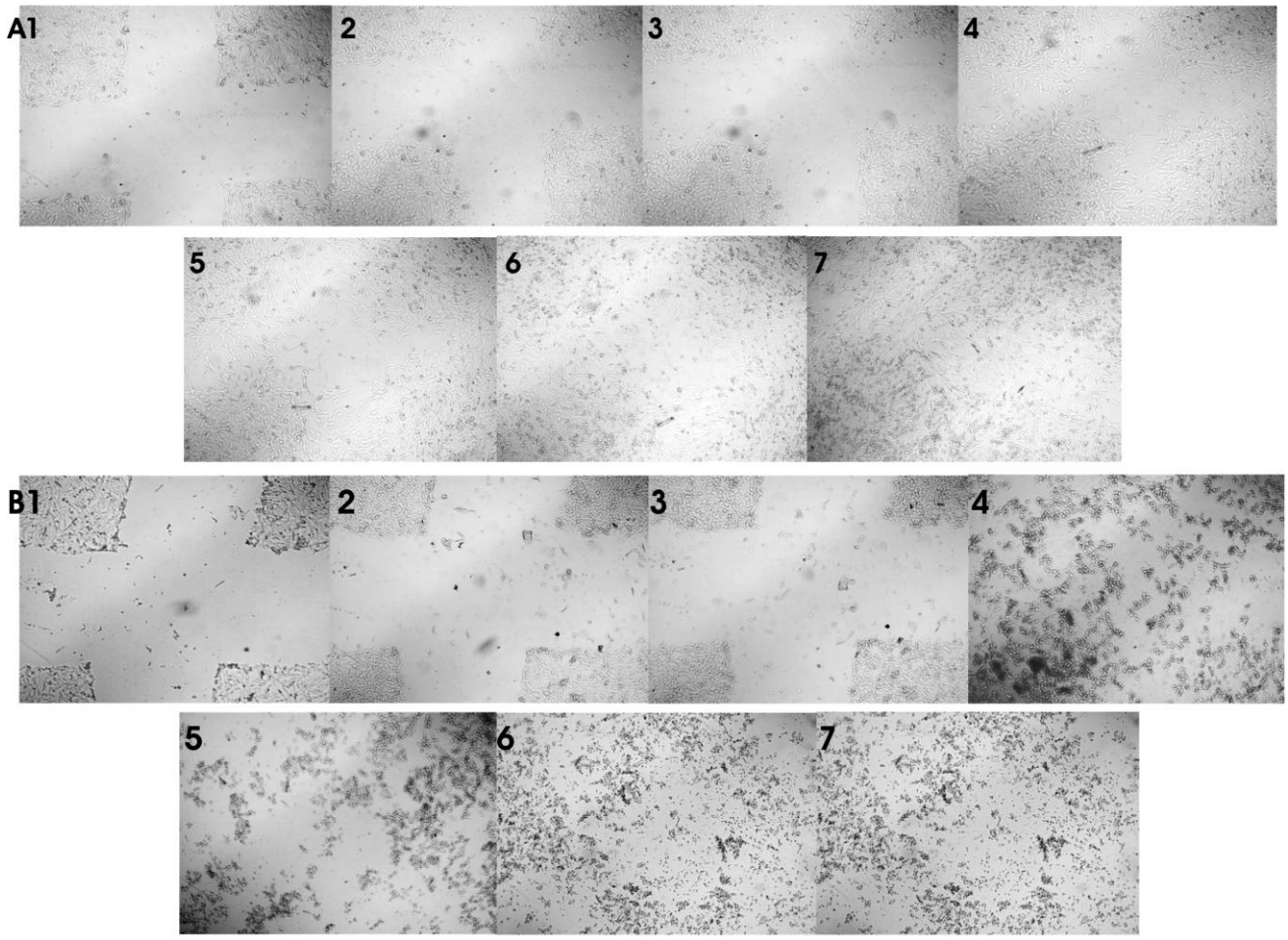

Figure 4.5. Effect of S. marginatum extract (SME) on HUVEC cell migration in an *in-vitro* scratch wound healing assay. The representative phase-contrast images show the migrating of cells into the wounded area. A) Wound closure in control wells at 1) 0h, 2) 3h, 3) 6h, 4) 18h, 5) 24h, 6) 36h and 7) 42h. B) Wound closure in SME (100 µg/ well) treated wells at 1) 0h, 2) 3h, 3) 6h, 4) 18h, 5) 24h, 6) 36h and 7) 42h.

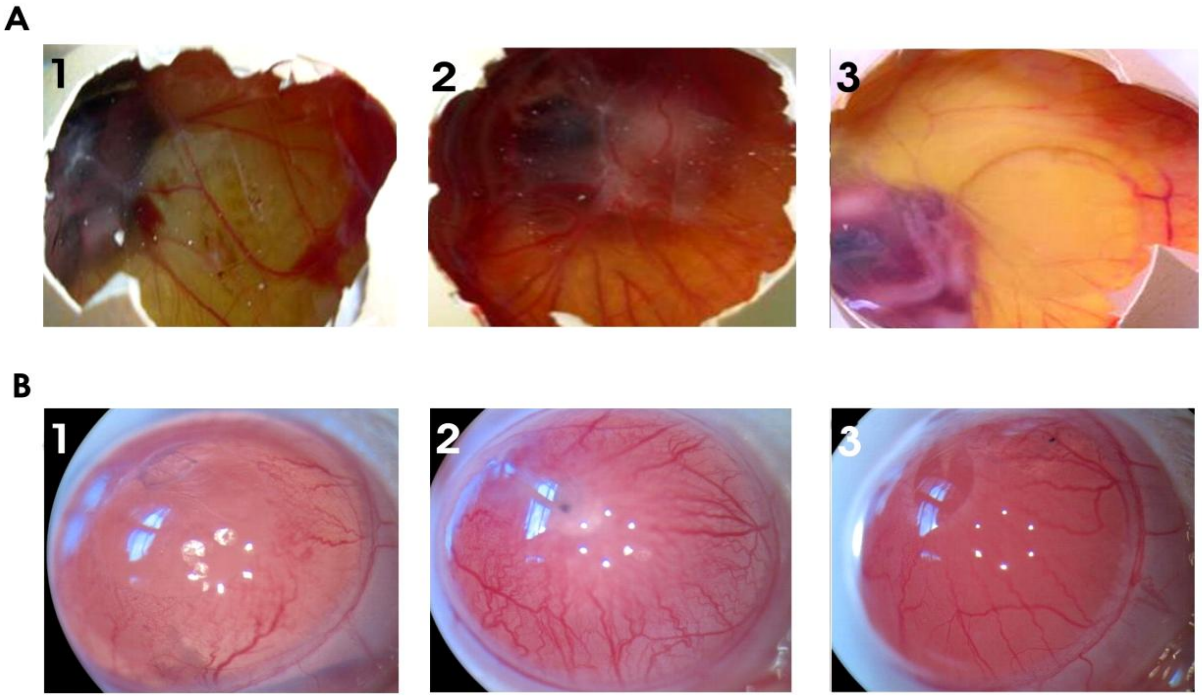

Figure 4.6. Effect of SME on blood vessel regression in the chick CAM and rat cornea assays *in-vivo*. A) Photographs of VEGF-induced neovascularization observed in CAM: (1) Saline (– control), (2) VEGF alone (+ control), (3) VEGF + SME (100 µg) was applied to the CAM of 11-day-old chicken embryos. After 48h of incubation, the treated area was inspected for changes in neovascularization. The arrows indicate the treated area. The data shown represent the result of an experiment that was done using a maximum of six eggs in each group. All photographs were taken at 40 × magnification. B) Photographs of VEGF-induced neovascularization observed in rat corneas: (1) hydron polymer + VEGF (1 µg) (+ control), (2) hydron polymer alone (– control), and (3) hydron polymer + VEGF + SME (100 µg). After 7 days of incubation, the corneas were photographed at 40 × magnification.

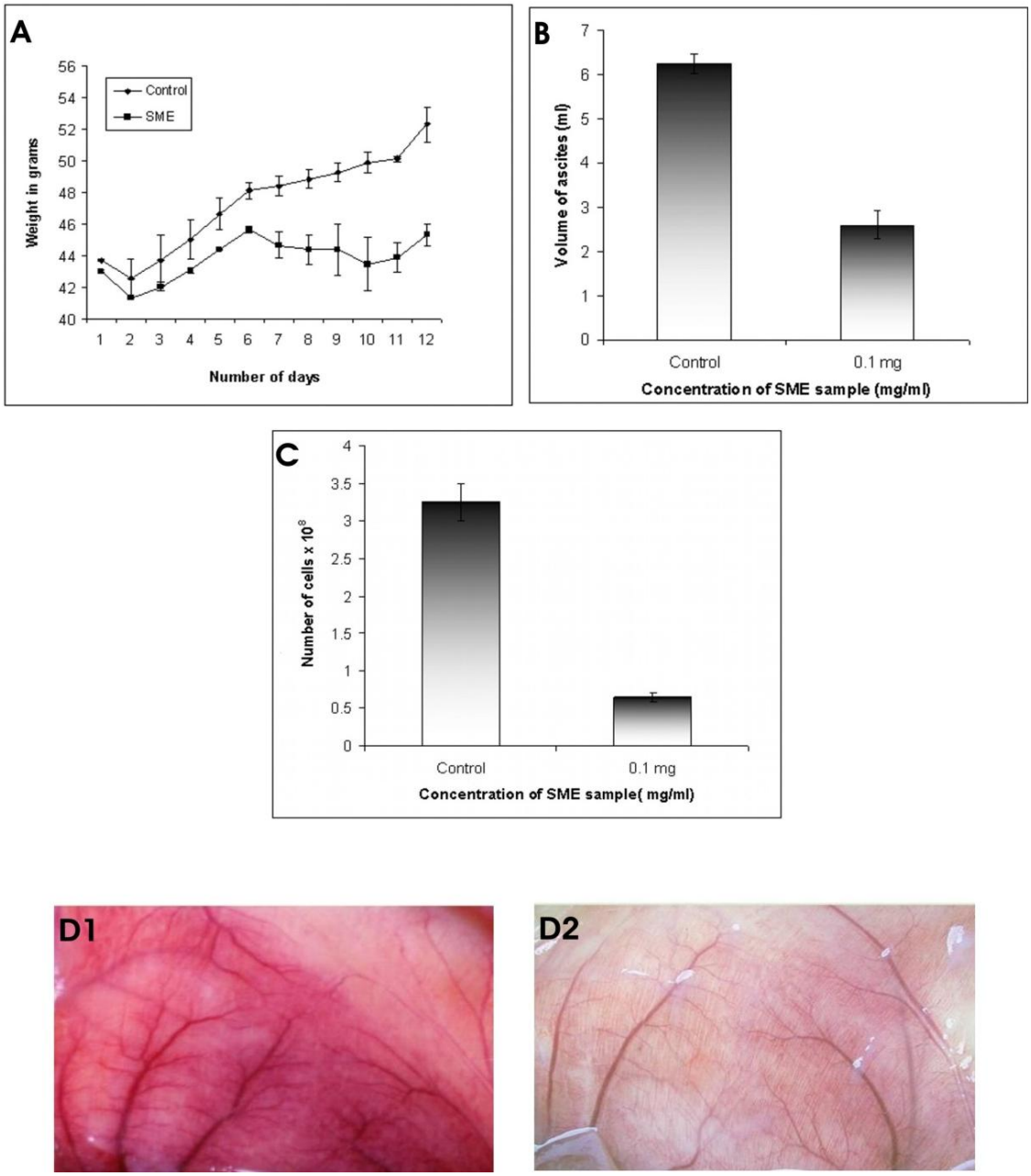

Figure 4.7. ***In-vivo*** **inhibition of tumor growth and angiogenesis by** ***S. marginatum*** **extract (SME).** (A) Body weights of EAT-bearing untreated mice or mice treated with SME were recorded. From the 6th day onwards, SME (4 mg kg^{-1} body weight) was administered *(i.p)* for six doses every day; the animals were sacrificed on the 12th day. (B) EAT cells were collected along with ascites fluid and measured, (C) Cells were counted with a haemocytometer, (D) The peritoneum of the animal was photographed: (1) Control, (2) SME treated. At least six mice were used in each group and the results obtained are an average of three individual experiments and means of ± SD (n = 6 per group).

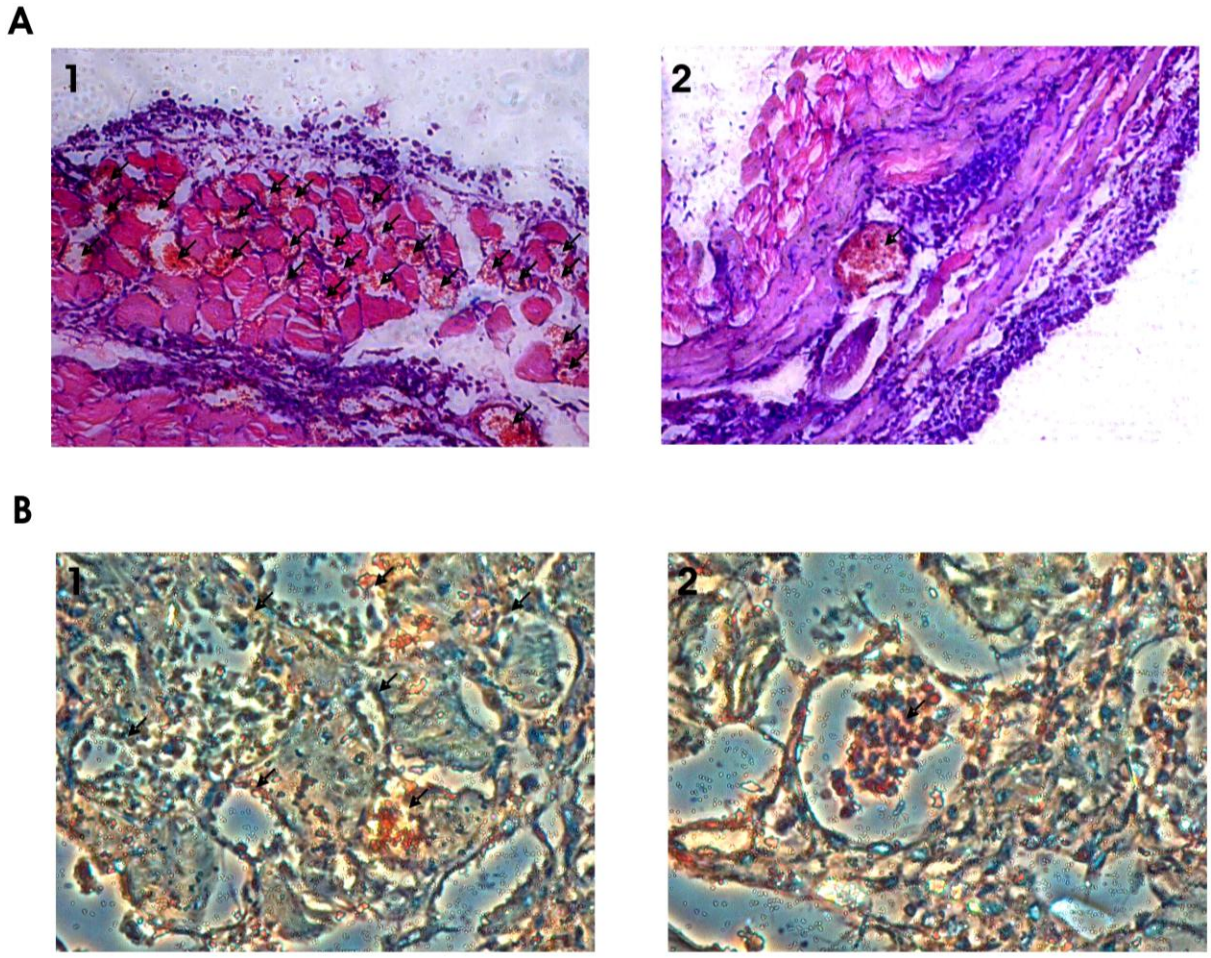

Figure 4.8. ***S. marginatum*** **extract (SME) inhibits MVD and proliferation of endothelial cells in mouse peritoneum.** A) The peritoneums of control (1) as well as SME-treated (2) EAT-bearing mice were embedded in paraffin and 5 μm sections were made using a microtome. The sections were stained with hematoxylin and eosin and observed for microvessel density (40X). Arrows indicate the microvessels. B) Paraffin sections (5 μm) of peritoneum of control (1) and SME (2) mice were immunostained with anti-CD31 (PECAM) anti-bodies. Arrows indicate the stained activated endothelial cells.

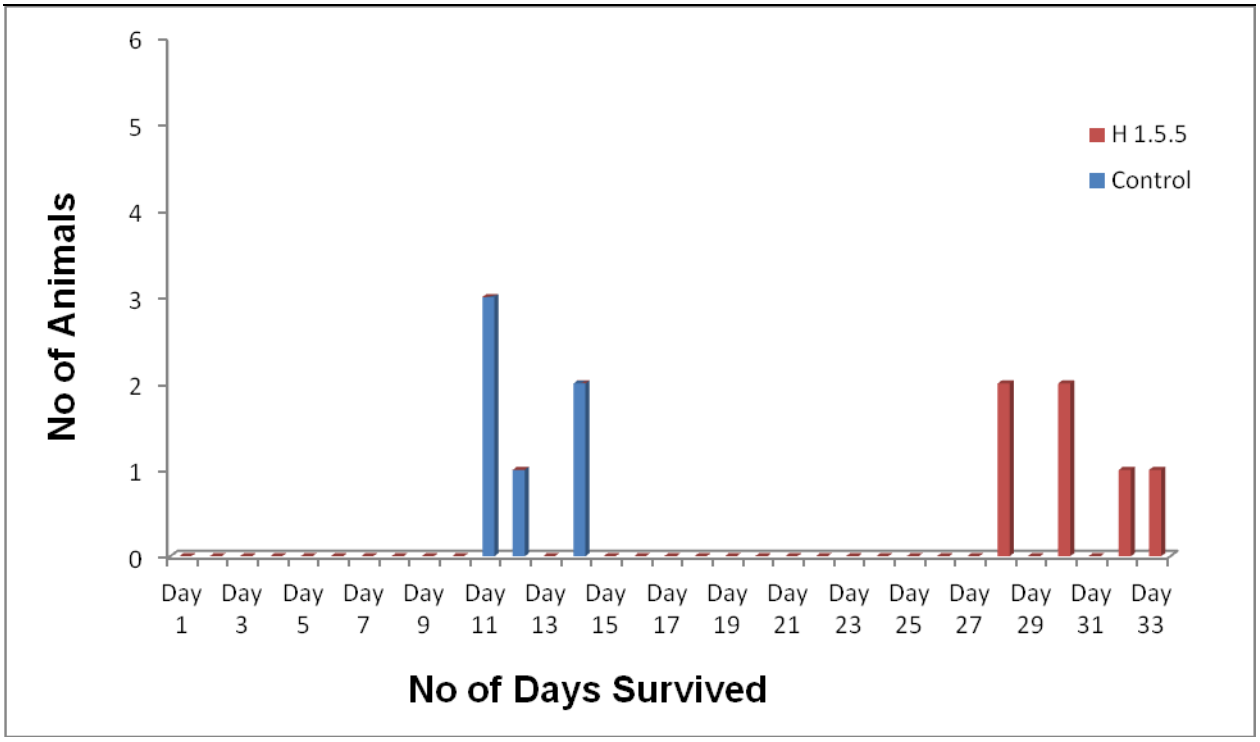

Figure 4.9. Effect of *Stoechospermum marginatum* extract (SME) on the survivability of a tumor bearing mouse *in-vivo*. A mouse survivability assay was performed on EAT bearing mice treated with SME (4mg kg^{-1} body weight) every day after the 6^{th} day of transplantation. Group 1: control tumor bearing mice which is untreated, Group 2: Tumor bearing mice treated with H 1.5.5. At least 6 mice were used in each group and the results obtained are means of ± SD (n=6 per group).

4.4. DISCUSSION

Recently, the hypothesis that anti--angiogenic compounds can be used in combination with cytotoxic drugs for tumor therapy has been proposed (Klement *et al.,* 2000). Since, anti--angiogenesis has become one of the major issues of cancer treatment, search for chemicals expressing anti-invasive and anti-angiogenic properties rather than direct cytotoxic effect on cancer cells have gained huge momentum in recent years of modern drug development. Already, several angiogenesis inhibitors have been developed to date, whose efficacy has been evaluated in different *in-vitro* and *in-vivo* assays and their clinical evaluations are in progress (Kerbel and Folkman, 2002). Also, chemotherapeutic agents have shown anti--angiogenic properties both *in-vitro* and *in-vivo,* (Browder *et al.,* 2000; Miller *et al.,* 2001) leading to the idea of chemotherapy with an anti--angiogenic setup (Hanahan *et al.,* 2000; Miller *et al.,* 2001). Keeping all these facts in mind, we have attempted to screen for potent anti--proliferative activity in marine seaweed extracts *in-vitro* and reported an anti-angiogenic effect of a promising seaweed *S. marginatum* both *in-vitro* and *in-vivo,* for the first time.

The anti-proliferative effect of crude SME was assessed using EAT and BeWo and HEK 293 cell lines. The methanolic extract showed strong inhibition of proliferation of the tumor cell lines. The anti-proliferative effect of SME at the concentration of 100 mg ml-1on the EAT is shown in the Figure 3.1 while its effect on BeWo and HEK 293 cells is displayed in Figure 3.2A and 3.2B. Our results demonstrate that SME strongly inhibits cell proliferation of EAT and BeWo cells *in-vitro,* while having no effect on non transformed HEK 293 cells.

New capillary formation is required for the initial steps of angiogenesis, which involves processes such as endothelial cell activation, proliferation, and migration. To investigate the inhibitory effect of SME, VEGF-induced tube formation in HUVECs was used as a model. The cells were treated with different doses of SME (5-100 μg/ml) for 24 h, and total numbers of tubes formed were counted. Scoring of the total number of tubes showed that SME caused 80 to 95% decrease in total number of tubes (at 0.005 and 0.010 mg/well) as compared to control (Fig. 3.03). It is a well known fact that endothelial cells differentiate and form capillary-like structures when seeded on matrigel, a reconstituted extracellular matrix preparation of EHS mouse sarcoma. This development entails cell-matrix interaction, intercellular communication and cell mobility like *in-vivo* tumor angiogenesis. In this assay system, SME suppressed human endothelial cell tube formation indicating that it inhibits endothelial cell proliferation and consequently angiogenesis *in-vitro*.

VEGF promotes many of the events necessary for angiogenesis including endothelial cell migration through a variety of signaling pathways (Folkman and Shing, 1992). I therefore evaluated the effects of SME on the migration of HUVECs, and found it inhibited VEGF induced HUVEC migration by both wound healing assay and oil droplet assay methods (Figs. 3.04 and 3.05). These results indicate that the inhibitory effects of SME on VEGF induced migration may be attributed to inhibitions of endothelial proliferation.

CAM assay is based upon angiogenesis taking place on the chorioallantoic membrane of fertilized chicken eggs. Therefore, the methanolic extract of *S. marginatum* (SME) was studied for its anti-angiogenic activity by means of CAM assay. SME was able to inhibit capillary

development on the CAMs at100 μg/egg concentration. SME treated CAMs presented avascular zones, indicating that new vessels were not formed (Fig. 3.06A).

Topical application of angiogenic inhibitors is a beneficial route for prevention and treatment of CNV because it is non-invasive and has minimal systemic adverse effects. The present study established the effectiveness of SME by means of topical application in CNV. In fact, topical application of 5μg/eye significantly prevented VEGF induced CNV as confirmed by morphological observation (Fig. 3.6B).

Our preliminary results demonstrated clearly that SME has potent anti-angiogenic effect on Ehrlich ascites tumor (EAT) cells *in-vivo*. Its treatment in EAT bearing mice brought about a decrease in the body weight, ascites volume and cell number *in-vivo* (Fig. 3.7). It is a well known fact that, VEGF in malignant ascites formation is allied to play a pivotal role in ascites fluid accumulation, tumor growth, and microvessel density (Kim *et al.,* 1993; Borgstrom *et al.,* 1996; Mesiano *et al.,* 1998) Therefore, the anti-tumor effects of SME may be correlated with its *anti--angiogenic* activity and this assumption is also supported by the reports of Folkman (1990). An extended duration of survival in EAT bearing mice was also observed in the survivability assay, which may associated with the inhibition of tumor cell growth (Fig. 3.9). Several research reports have already illustrated the anti--tumor potential of seaweeds in a variety of tumor cell types (Yamamoto *et al.,* 1974; Ito and Sugiura, 1976; Yamomoto *et al.,* 1977; Kashiwagi *et al.,* 1980; Yamamoto et al 1984; Iizima-Mizui *et al.,* 1985; Chida and Yamamoto, 1987; *Noda et al.,* 1990; Ohigashi *et al.,* 1992; Zhuang *et al.,* 1995; Riou *et al.,* 1996). All these data provide undeniable proof that inhibition of angiogenesis could be at least one of the many mechanisms of

action instigated by SME and may be cause for the repression of VEGF-like factors or secretion of such factors in the process.

Hematoxylin-eosin staining method is usually used to examine vascular invasion and is characterized by the presence of at least one tumor cell cluster which is clearly evident in decorated vascular spaces, where endothelial cells are stained brown as described by Birner *et al.* (2001). In the present study, peritoneum from mice with and without SME treatment was fixed in formalin. Sections (5 μm) were made from these paraffin embedded peritoneum and stained with Hematoxylin-eosin. The results were expressed as the highest number of microvessels in any single 40 x field. Final MVD was the mean score obtained from the hot spot areas counted using Leitz Diaplan light microscope, Germany, attached to CCD camera. In this study, the average MVD was significantly higher in control (with an average count of 21) with vascular invasion than in test SME (with an average count of 1), suggesting that angiogenesis is closely related with microvessel density of tissue and clinical aggressiveness of tumor (Fig. 3.8A).

Further evidence for the anti-angiogenic potential of SME comes from the current results on inhibition of the extent of proliferating endothelial cells in the peritoneal lining of tumor-bearing mice. A significant decrease in peritoneal angiogenesis and levels of microvessels in the CD31 stained sections of peritoneal wall confirm the anti-angiogenic activity of SME with an average count of 1, while in the control sections an average count of 5 was recorded (Fig. 3.08B). An earlier study has established that the density of microvessels is almost doubled in tumors from patients with metastasis (Vartanian and Weidner, 1994). While, the presence of vascular invasion

which is not a consistent finding is linked to increased incidence of lymph node and distant metastasis and a corresponding decrease in survival (Minsky *et al.*, 1999).

The overall activity of SME may be attributed to the abundant presence of several cytotoxic compounds such as fucoidans, laminarins and terpenoids in this seaweed, stated to possess anti-cancer properties. Based on the results of the present investigation, it may be summarized that methanolic extract of *S. marginatum* possesses potent anti-proliferative and anti-angiogenic activities *both in-vitro* and *in-vivo* and is of immense therapeutic potential.

Chapter – 5

Isolation, purification and characterization of pro-apoptotic/ anti-angiogenic compound from *Stoechospermum marginatum* (C. Agardh) Kutzing

5.1. INTRODUCTION

Globally, more than 12 million cancer cases have newly been diagnosed in 2007 and the number is estimated to rise over 20 million by 2020; with more than half of these cases originating from the developing world population (Garcia *et al.*, 2007). In developing countries like India, the most common cancers found in males are cancers of the lung, pharynx, oesophagus, tongue and stomach; whereas among females, cancers of the cervix, breast, ovary, oesophagus and mouth are the most prominent (Rao and Ganesh, 1998). Thus far, the battle against cancer has not been very successful, primarily in terms of developing new treatments for rapidly metastasizing tumors. It has posed an extreme challenge to find novel drug leads as a panacea for fighting all types of cancer. Despite a number of drugs available to fight cancer, the search for novel drug leads for combating cancer through research has taken great precedence in the pharmaceutical industry.

The marine biodiversity is a vast source of unique natural products with potential therapeutic usefulness in oncology. Over the past decade, various marine sources have yielded > 2000 new compounds with several biological activities. Infact, due to the patronage of the U.S. National Cancer Institute, some of these marine derived compounds have been tested in clinical trials as potential cancer chemopreventive agents and are in advanced stages of development, while others are still in preclinical stages (Mayer and Gustafson, 2008). They include, Bryostatin 1 (Phase II solid tumors), Halichondrin B (Phase II metastatic breast cancer), Kahalalide F (Phase II in prostate cancer), Hemiasterilin (Phase II NSCLC), Didemins (Phase II non-Hodgkin's lymphoma), Aplidin (Phase II medullary thyroid), Ecteinascidin 743 (Phase II), Squalamine (Phase II), Cemadotin (Phase II NSCLC, breast, lung, ovarian cancer), Synthadotin (Phase II

NSCLC, prostate cancer), Soblidotin (Phase I NSCLC), E7389 (halichondrin B derivative) (Phase I), NVP-LAQ824 (Psammaplin derivative; Phase I), Discodermolide (Phase I), HTI-286 (Hemiasterlin derivative; Phase I), LAF-389 (Bengamide B derivative; Phase I), KRN-7000 (Agelasphin derivative; Phase I), Curacin A (Preclinical), DMMC (Preclinical), Salinosporamide A (Preclinical), Laulimalide (Preclinical), Vitilevuamide (preclinical), Diazonamide (Preclinical), Eleutherobin (Preclinical), Sarcodictyin (Preclinical), Peloruside A (Preclinical), Salicylihalimides A and B (Preclinical), Thiocoraline (Preclinical), Ascididemin (Preclinical), Variolins (Preclinical), Lamellarin D (Preclinical), Dictyodendrins (Preclinical) and ES-285 (Spisulosine; Preclinical). These agents are characterized to fight cancer by their distinctive pharmacological properties and mechanisms of action and represent potential candidates for the treatment of malignant disease, either to be used as single agents, or as part of a complimentary regimen. The continuation of preclinical and clinical studies is required in order to assess the exact role of theses new class of compounds in the treatment of patients suffering from cancer. It is anticipated that marine-derived anticancer drugs will represent valuable tools in the oncological armamentarium.

For centuries, seaweeds have been of botanical, industrial, and pharmaceutical interest. Being rich in minerals, vitamins, trace elements and bioactive substances, seaweeds are being hailed as the healing food for the modern era. This has been claimed by their vast beneficial effects on human health. Prior to the 1950s, the medicinal properties of seaweeds were restricted to traditional and folk medicines only (Lincoln *et al.,* 1991). However, since then seaweeds have gained importance as a natural source for compounds with pharmacological properties including

cancer chemopreventive properties (Mayer and Lehmann, 2000). This has been supported by the vast number of studies directed in this area of research.

Kahalalide F, a small natural peptide (produced by *Bryopsis sp* and assimilated by the grazer *Elysia rufescens*) showing anticancer and antitumour properties (Hamann and Scheuer, 1993; Hamann *et al.,* 1996) both *in-vitro* and *in-vivo* is in phase II clinical trials for use as a likely candidate in therapeutics for the treatment of melanoma, non-small lung cancer and hepatocarcinoma (Scheuer *et al.,* 2000). Several sulphated macroalgal polysaccharides with cytotoxic properties have been implicated in cancer inhibition with fudoidan being of prime importance. These long chain polysaccharides found in brown seaweeds such as *Laminaria species* and *Sargassum thunbergii* have demonstrated antitumour, anticancer, antimetastatic and fibrinolytic properties in mice (Coombe *et al.,* 1987; Maruyama *et al.,* 1987), in addition to antiproliferative activities (Religa *et al.,* 2000). Antitumour properties have been noted in Translam, a 1→3:1→6-*β*-D glucan produced by enzymatic action on laminaran (laminarin) (Saito *et al.,* 1992). Ulvan, a cytotoxic or cytostatic compound known to target normal or cancerous colonic epithelial cells has been reported by Kaeffer *et al.,* 1999 from *Ulva sp*. Another compound Chondriamide A showing cytotoxicity against human nasopharyngeal and colorectal cancer cells has been isolated from *Chondria atropurpurea* (Palermo *et al.,* 1992).

Terpenes have proved themselves to have an exceptionally wide range of cytotoxic and antitumour activities. Examples include (*S*)-12-hydroxygeranylgeraniol and (*S*)-13-hydroxygeranylgeraniol derivatives from *Bifurcaria bifurcata* which are toxic towards fertilized sea urchin eggs (Valls *et al.,* 1995; Culioli *et al.,* 2001); caulerpenyne from *Caulerpa taxifolia*

which is cytotoxic towards several human cell lines and as such has anticancer, antitumour and antiproliferative properties (Fischel *et al.,* 1995; Parent-Massin *et al.,* 1996; Barbier *et al.,* 2001); the hydroquinone diterpene, mediterraneol, from *Cystoseira mediterranea* which is an inhibitor of mitotic cell division (Francisco *et al.,* 1985); and the meroterpenes, usneoidone E and Z, from *Cystophora usneoides* which have antitumour properties (Urones *et al.*, 1992).

There remains a great deal of interest in screening of metabolites and other natural products derived from marine seaweed as potential anticancer and cancer preventive agents in modern drug discovery. The present study is based upon a preliminary screening of marine seaweeds found along the West coast Goa and Malwan (Maharashtra), India. Among the seaweeds screened for antiangiogenic activity, the seaweed *Stoechospermum marginatum* belonging to the family *Phaeophyceae* has been selected for comprehensive research study based on its maximum antitproliferative activity against HUVEC cell line *in-vitro*.

Stoechospermum marginatum is an economically important brown seaweed found abundantly along the West coast of Goa and Malwan (Maharashtra). It is also synonimically called as *Dictyota maculata J. Agardh, Stoechospermum maculatum (J. Agardh) J. Agardh, Stoechospermum patens J. Agardh* and *Zonaria marginata C. Agardh.* It is mainly used industrially for the production of agar, alginate and carrageenan and also as a fertilizer. However its pharmacological properties are yet to be explored systemically and the reports are very few. It is known to posses antispasmodic (Naqui *et al.,* 1980), antimicrobial (De Silva, 1982), antifouling (Bhosale *et al.,* 2002), antiviral (Adhikari *et al.,* 2006), cytotoxic (Ara *et al.,* 1999) and nematicidal activities (Abid *et al.,* 1993).

This alga was studied by the three major research groups at the Annamalai University (AU), Indian Institute of Chemical Technology (IICT) and National Institute of Oceanography (NIO). Several phthalate esters and lipids such as ethy palmitate, fucosterol and 24-ketocholesterol were isolated from this alga by Wahidullah *et al.* (1988, 1995). Earlier, they had reported a new spatane diterpene, named stoechospermol (Wahidullah, 1980) from this source. This compound and 8 more new spatane diterpenes were subsequently reported from a Sri Lankan collection of the alga (Gerwick *et al.*, 1981). The major metabolites were (in order of abundance) a tetrol, the corresponding monoacetate, and some diterpenes as relatively minor constituents. Rao *et al*, 1994) working on the alga collected from the Gulf of Mannar reported five spatane diterpenes, two of them present as mixtures of R and S conformers. Subsequently, the IICT group who took up the investigation of the alga from the same region reported an isomeric epoxide (17, 18-epoxy, 5(*R*), 16-dihydroxyspat 13(14)-ene) as a new compound (Venkateswarlu *et al.*, 1995).

As part of the screening, different seaweeds for pro-apoptotic/ anti-angiogenic properties, we found the methanolic extract of *Stoechospermum marginatum* to exhibit promising properties against HUVEC cell lines in an *in-vitro* assay system. Purification of the above extract over silica gel and alumina gel led to the isolation of an active metabolite, which has been reported here.

5.2. MATERIALS AND METHODS

5.2.1. Chemicals

Solvents methanol, hexane, ethyl acetate, acetone, chemical silica gel and alumina gel were of highest analytical grade and obtained from Merck Research Laboratory, Mumbai, India. The

structure elucidation of the active fraction was carried out using various spectroscopic techniques such as IR, UV-Vis, NMR and HRMS.

5.2.2. Collection of the seaweed *Stoechospermum marginatum*

The seaweed *Stoechospermum marginatum* (C. Agardh) Kutzing was collected and identified from in and around the coasts of Goa and Maharashtra, India in the same manner as mentioned in Chapter 2.

5.2.3. Preparation of extract and purification of the active compound H 1.5.5 from *Stoechospermum marginatum*

Shade dried seaweed powder (1 kg) was extracted in 4 liters of methanol at room temperature for 24 hrs. The extraction was repeated thrice and the total organic extract thus obtained were pooled, filtered and evaporated to dryness under pressure using a rotary evaporator (Roteva, India) to get the residual extract (140 gms). The product thus obtained was designated as the original methanol extract and stored at –20 ^{0}C until further analysis. Polarity based partial fractionation of the methanol extract was done using solvents such as hexane, ethyl acetate, and water. All the solvent extracts were subjected to rotary evaporation to obtain the residue. The residual fractions of each solvent extract were tested for antiproliferative activity using HUVEC cells *in-vitro* and the data was recorded. The hexane extract which exhibited positive activity, was further subjected to silica gel column chromatography (Merck 60-200 mesh size) and eluted with Hexane: acetone (100:0, 95:5, 90:10, 50:50 and 0:100). The sub fractions collected were subjected to thin layer chromatography using Hexane: acetone. They were then pooled based on the TLC bands obtained. The obtained fractions were evaporated to dryness and a known

concentration (100 µg/ ml) of each fraction was tested for activity. The fraction H 1.5 (Hexane: Acetone – 0:100) that showed the maximum effect was further subjected to purification (silica gel column chromatography, Merck 60-200 mesh size) and eluted with Hexane: acetone (50:50 to 0:100) to obtain seven subfractions; with H 1.5.5 being the active component. This was again subjected to alumina gel (Grade 3) to remove unwanted fatty acids and pigments. The active compound was then identified and characterized.

5.2.4. Spectral studies

The ID NMR experiments were performed on a Bruker Avance spectrometer at 300 MHz for ^{1}H and ^{13}C experiments respectively. All spectra ('H NMR, ^{13}C NMR) were recorded using 5 mg of the sample and chemical shifts were given in ppm. The UV spectra were recorded with a Graihicord UV-240 Shimadzu UV-visible spectrophotometer using 5 mg of the sample. The IR spectra were scanned as KBr pellets with a Nicolet FTIR spectrophotometer (Model impact 410). The HRMS analyses (mass measurements) were performed in an Applied Biosystem instrument (QTOF).

5.3. RESULTS

5.3.1. Bioassay guided extraction, fractionation and purification of the active component from *S. marginatum*

The brown seaweed *S. marginatum* was collected from Marvel beach, Dona Paula, Panaji, in the southern province of India. The seaweed was washed with fresh water, shade dried, powdered and stored until further use.

The shade dried and powdered seaweed *S. marginatum* (1 kg) in a glass carboy was extracted with 4 liters of methanol (AR Grade) by keeping it overnight for 24 hrs. This was repeated for 2 more times successively. From 1 kg of powdered *S. marginatum*, 140 gms of methanol extract was obtained. This was in turn partially fractionated to obtain Hexane (28 gms), Ethyl acetate (12 gms) and Aqueous extracts (85 gms) by using different polarity based solvents. As the hexane fraction showed positive antiproliferative activity against HUVEC, it was selected for further purification. A known volume of all the solvent fractions was dissolved in 0.1 % DMSO and made up to a concentration of 1mg/ ml at each step of bio-analysis. An aliquot of 100 µl containing 100 µg of each sample was tested for antiangiogenic activity using HUVEC cell line and the activity profile of each fraction and sub-fraction is depicted in Figure 5.2. Flow chart (Fig. 5.1) presents the extraction and purification protocol utilized to obtain the active component from *S. marginatum* and showed 96.81 ± 0.33 % inhibition against HUVEC. The active fraction H 1.5.5 was subjected to TLC to test the purity of the fraction. A single band was obtained from the active component as can be seen in the Figure 5.03. The residue was further purified on alumina gel to give a colorless oil product (0.01 g) possessing anti-angiogenic activity.

5.3.2. Nuclear magnetic resonance of the fraction H 1.5.5

The monoacetate mixture, H 1.5.5 recognized also to be composed of side chain diasteroisomers, was isolated by repeated Silica gel elution as an oil as 1.4% of the extract and showed the following spectral features: IR ($CHCL_3$) 3377, 2925, 2854, 1701, 1463, 1375, 1045, 943, 910, 761. HRMS (70 eV, 180 ^{0}C), *m/z* (relative intensity) 375.2018 ($C_{20}H_{32}O_5$), 359.2156 ($C_{20}H_{32}O_4$), 283.2053 (C_{19} H_{27} O_2), 149.0209 (C_{10} H_{13} O). ^{1}H NMR ($CDCL_3$, 300 MHz) *3.75(d, J=4), 2.28*

(ddd, J=13), 2.92(m), 0.87(d, J=6), 0.94(s), 5.33(s), 4.93(s), 4.50(d, J=6), 5.77(m), 5.75(m), 3.47 (s), 1.31(s). ^{13}C NMR ($CDCL_3$, 300MHz) *13.0 q, 14.4 q, 22.67 q, 27.87 q, 35.0 t, 36.3, 36.8, 37.5, 42.5, 42.9, 43.1, 46.8, 69.8 t, 73.11 s, 74.3 d, 80.0 d, 108.8 t, 131.1 d, 136.1 d, 149.4 s*. The ^{1}H NMR spectrum and ^{13}C NMR spectrum of the compound is shown in Figures 5.4 and 5.5.

5.3.3. Spectral analysis of the fraction H 1.5.5

The IR spectra scanned with a Nicoiet FTIR spectrophototometer is shown in the Figure 5.6. The UV spectra recorded with a Graihicord UV-240 Shimadzu UV-visible spectrophotometer in methanol is shown in Figure 5.7. The HRMS analyses (mass measurements) were performed in an Applied Biosystem instrument (QTOF) (Fig. 5.8).

Based on all these spectroscopic data, the compound was identified to be similar to the 5(*R*), 15, 18 (*R* and *S*) – 19-tetrahydroxyspata -13, 16-(*E*)-diene, an active component present in the seaweed *S. marginatum.* The proposed molecular structure is depicted in Figure 5.9.

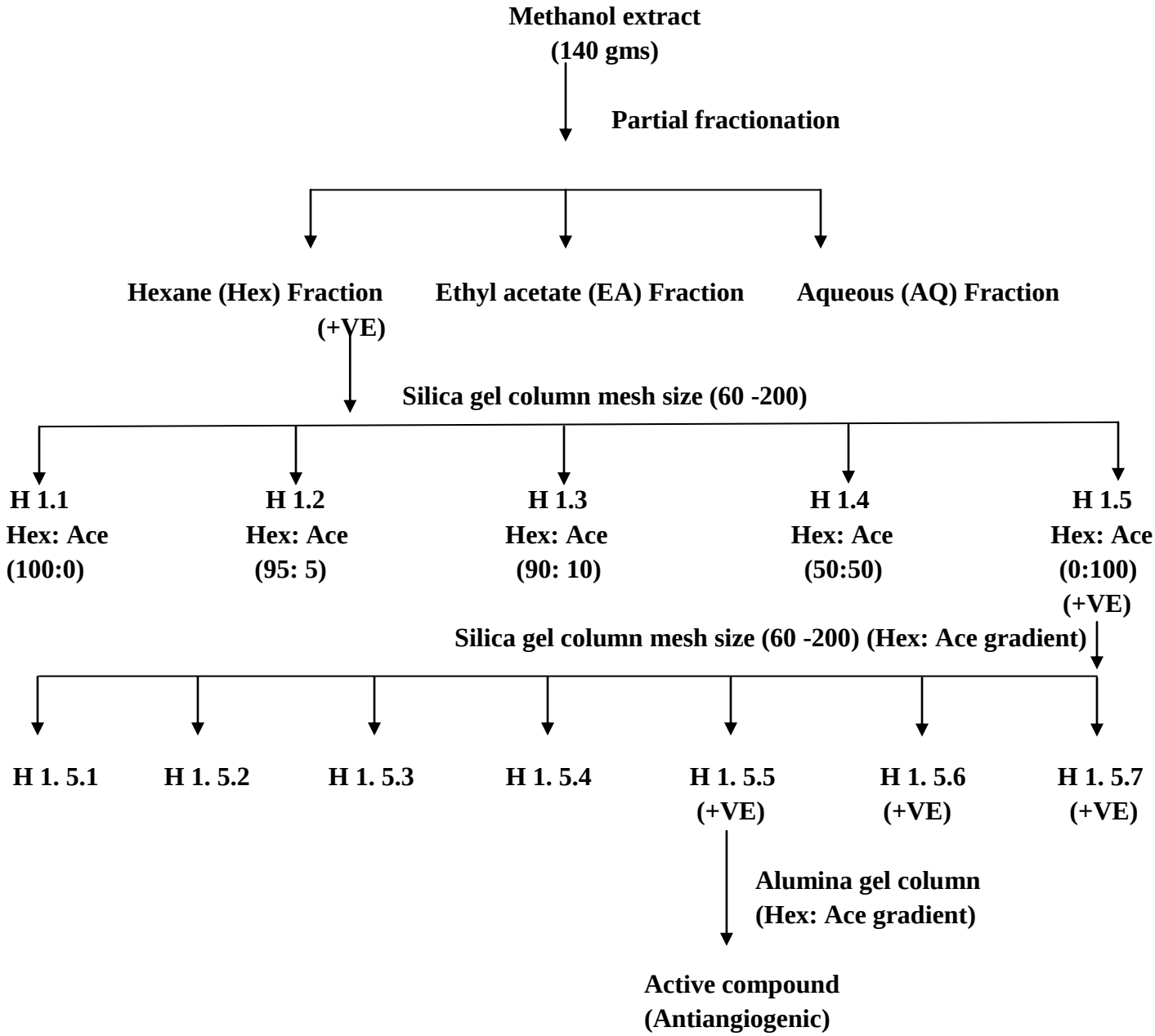

Figure 5.1. General scheme followed for extraction and purification of active metabolite from the seaweed *Stoechospermum marginatum*.

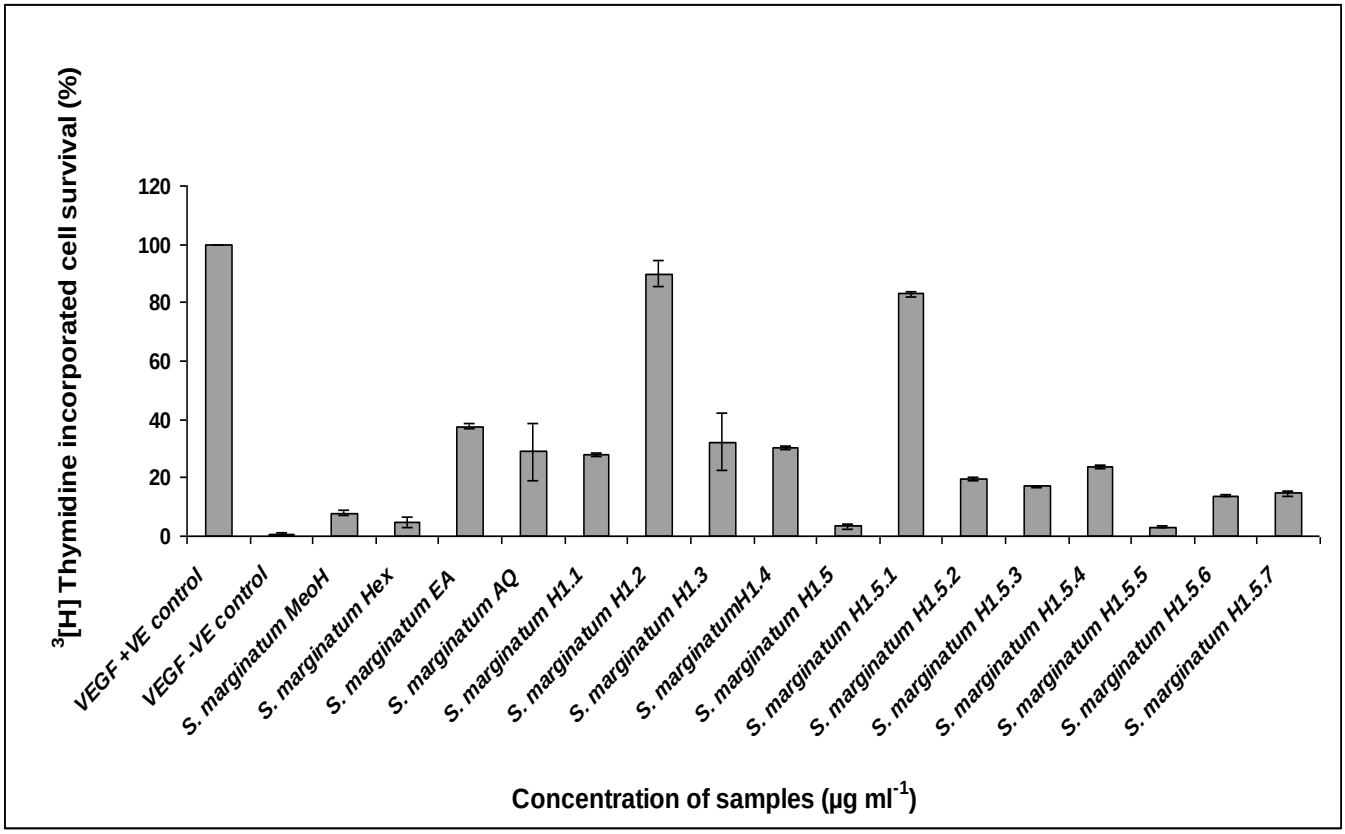

Figure 5.2. Anti-proliferative activity of *S. marginatum* fractions and subfractions (100µg ml^{-1}) on HUVEC cells *in-vitro.*

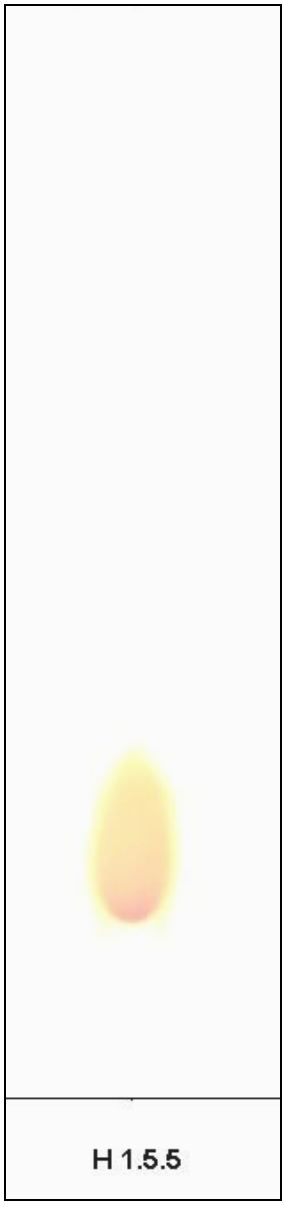

Figure 5.3. Thin Layer Chromatography (TLC) profile of the active anti-angiogenic component from *S. marginatum*

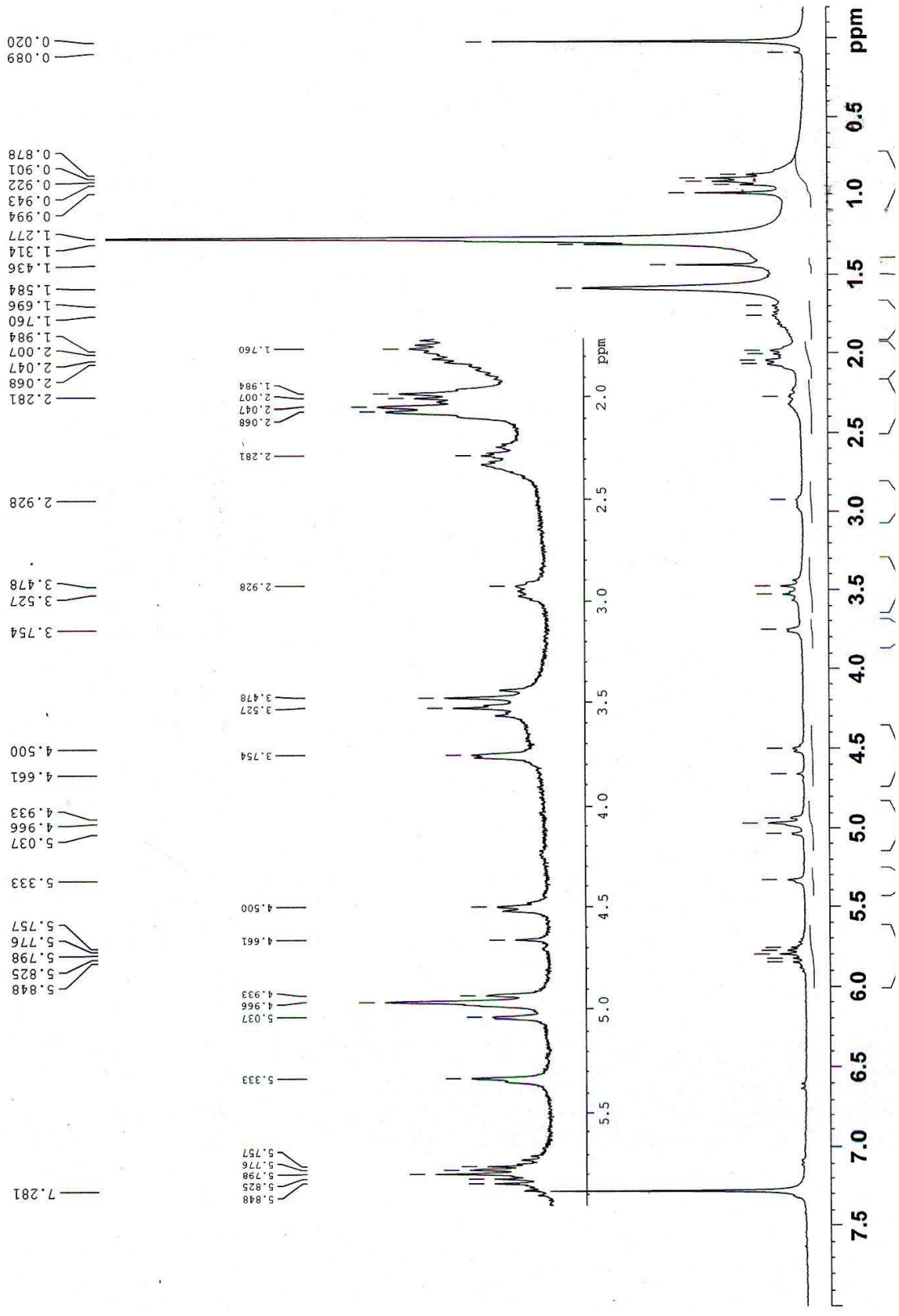

Figure 5.4. ^{1}H NMR spectrum of the active component H 1.5.5 from the seaweed *S. marginatum*

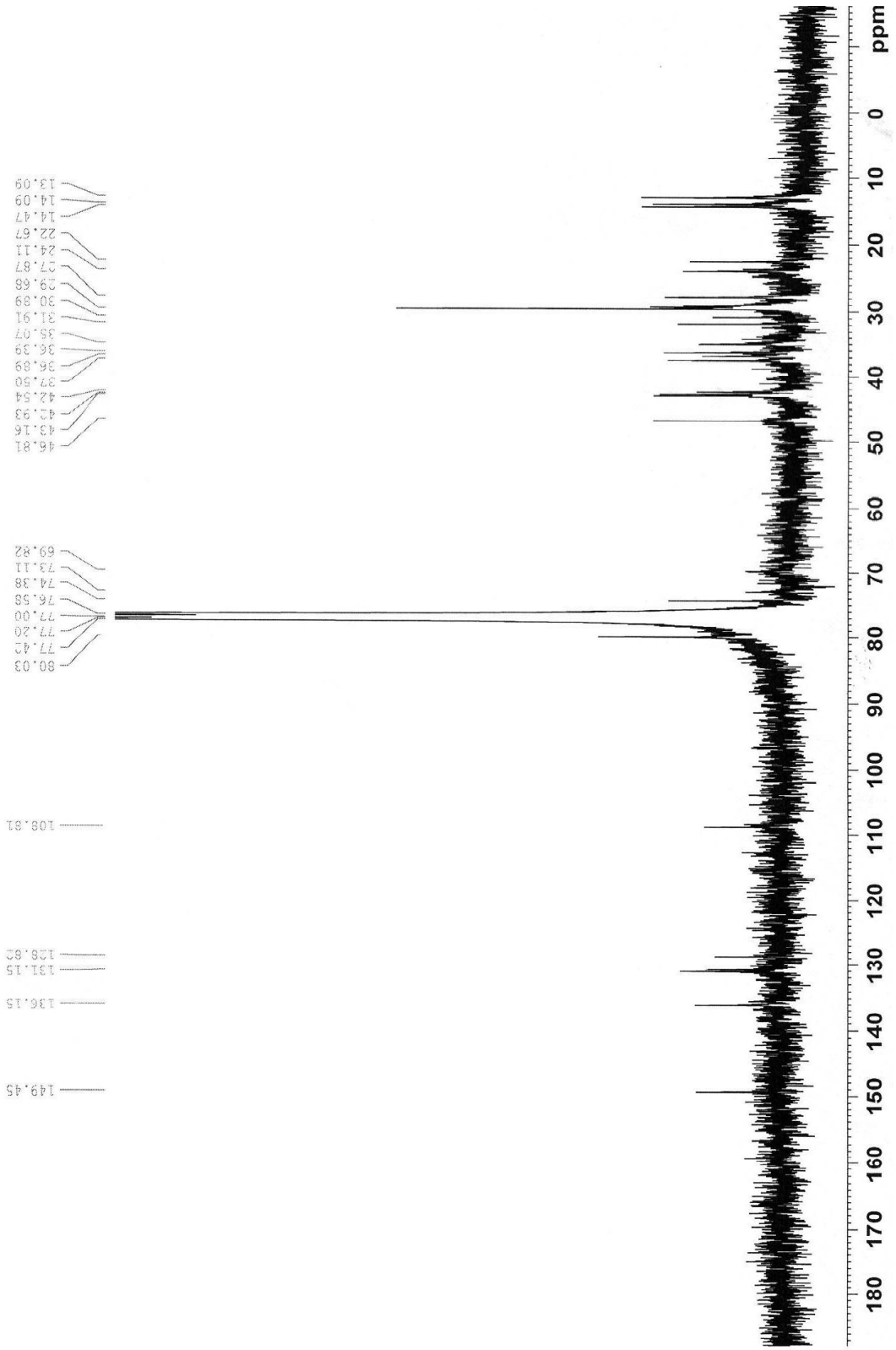

Figure 5.5. ^{13}C NMR spectrum of the active component H 1.5.5 from the seaweed *S. marginatum*.

Table 5.1. ^{1}H and ^{13}C NMR data of H 1.5.5 fraction.

Signal assignment	*Chemical shift "C:*	*Signal assignment (H's at C No's)*	*Chemical shift ^{1}H: a $_{ppm}$ Hz*
C-1	*37.5*	*C-1*	
C-2	*35.0 t*	*C-2*	
C-3	*27.87 t*	*C-3*	
C-4	*46.8*	*C-4*	
C-5	*69.8 t*	*C-5*	*3.75(d, J=4)*
C-6	*73.11 s*	*C-6*	*2.28 (ddd, J=13)*
C-7	*74.3 d*	*C-7*	*2.92(m)*
C-8	*43.1*	*C-8*	
C-9	*42.9*	*C-9*	
C-10	*42.5*	*C-10*	
C-11	*14.4 q*	*C-11*	*0.87(d, J=6)*
C-12	*13.0 q*	*C-12*	*0.94(s)*
C-13	*149.4 s*	*C-13*	*5.33(s)*
C-14	*108.8 t*	*C-14*	*4.93(s)*
C-15	*36.3*	*C-15*	*4.50(d, J=6)*
C-16	*36.8*	*C-16*	*5.77(m)*
C-17	*131.1 d*	*C-17*	*5.75(m)*
C-18	*136.1 d*	*C-18*	
C-19	*80.0 d*	*C-19*	*3.47 (s)*
C-20	*22.67 q*	*C-20*	*1.31(s)*

[a]Some of the assignments are interchangeable, M + Na* = 359.2156

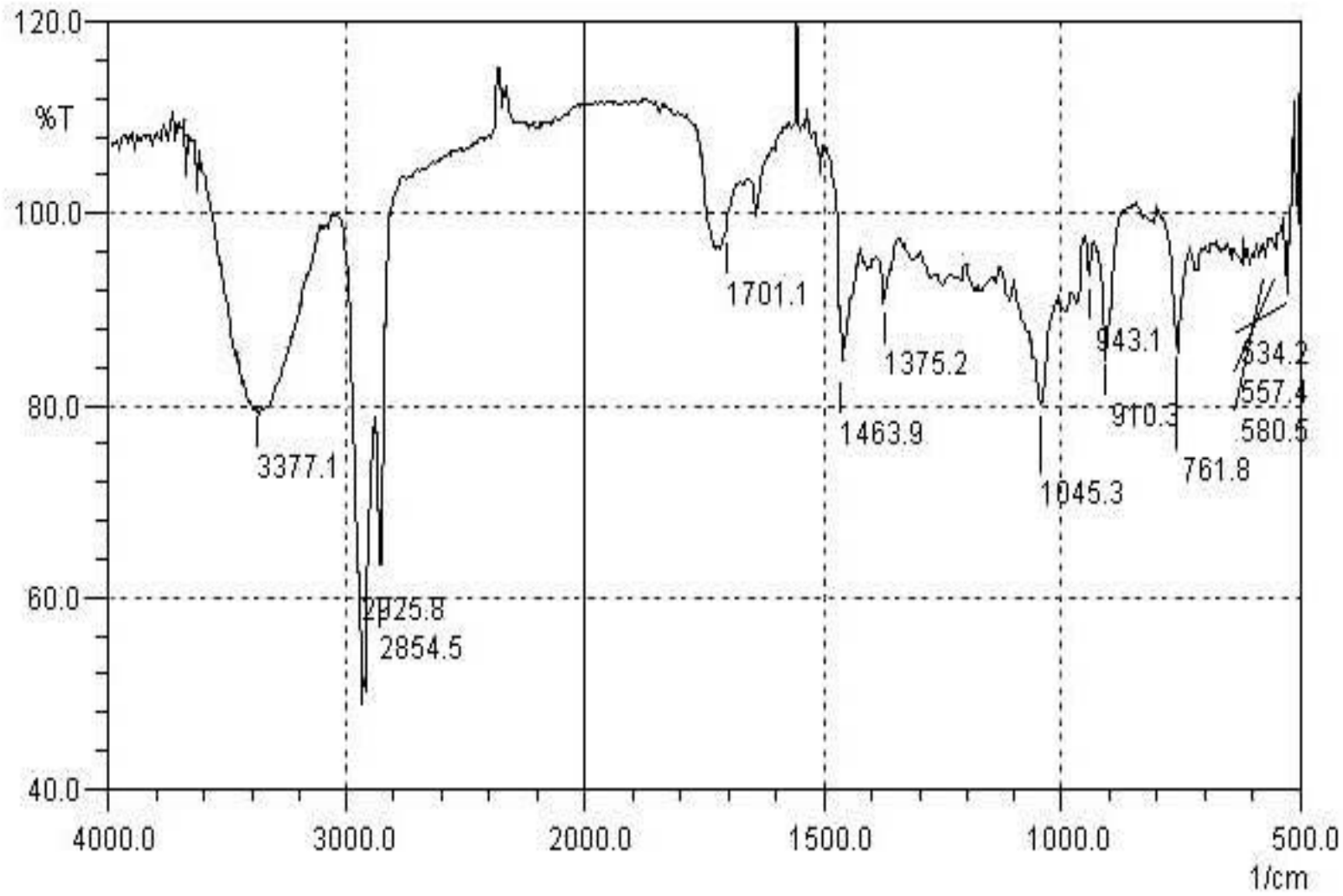

Figure 5.6. Infrared spectrum (IR) of the active component H 1.5.5 from the seaweed *S. marginatum*.

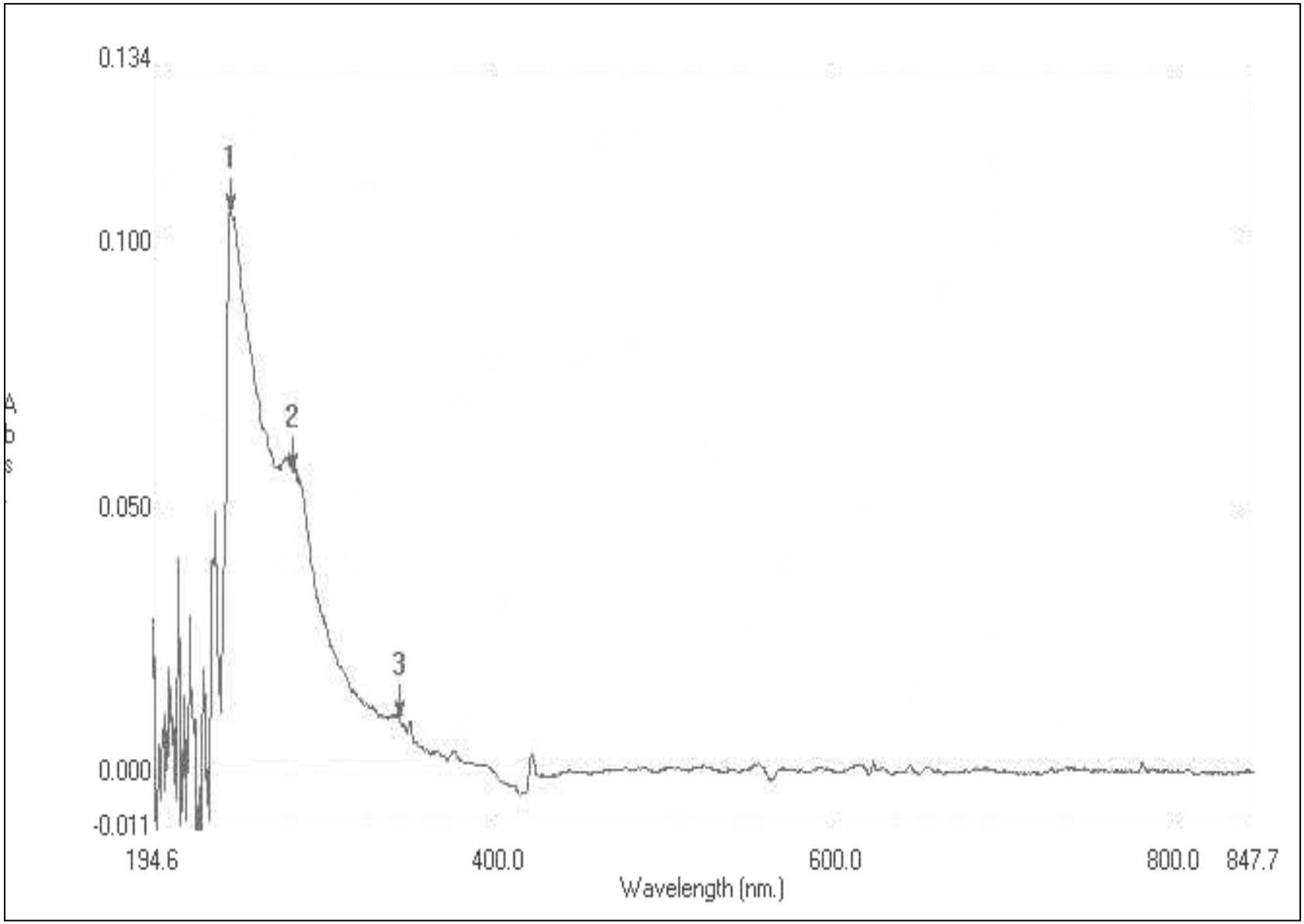

Figure 5.7. UV-Visible spectrum of the active component H 1.5.5 from the seaweed *S. marginatum.*

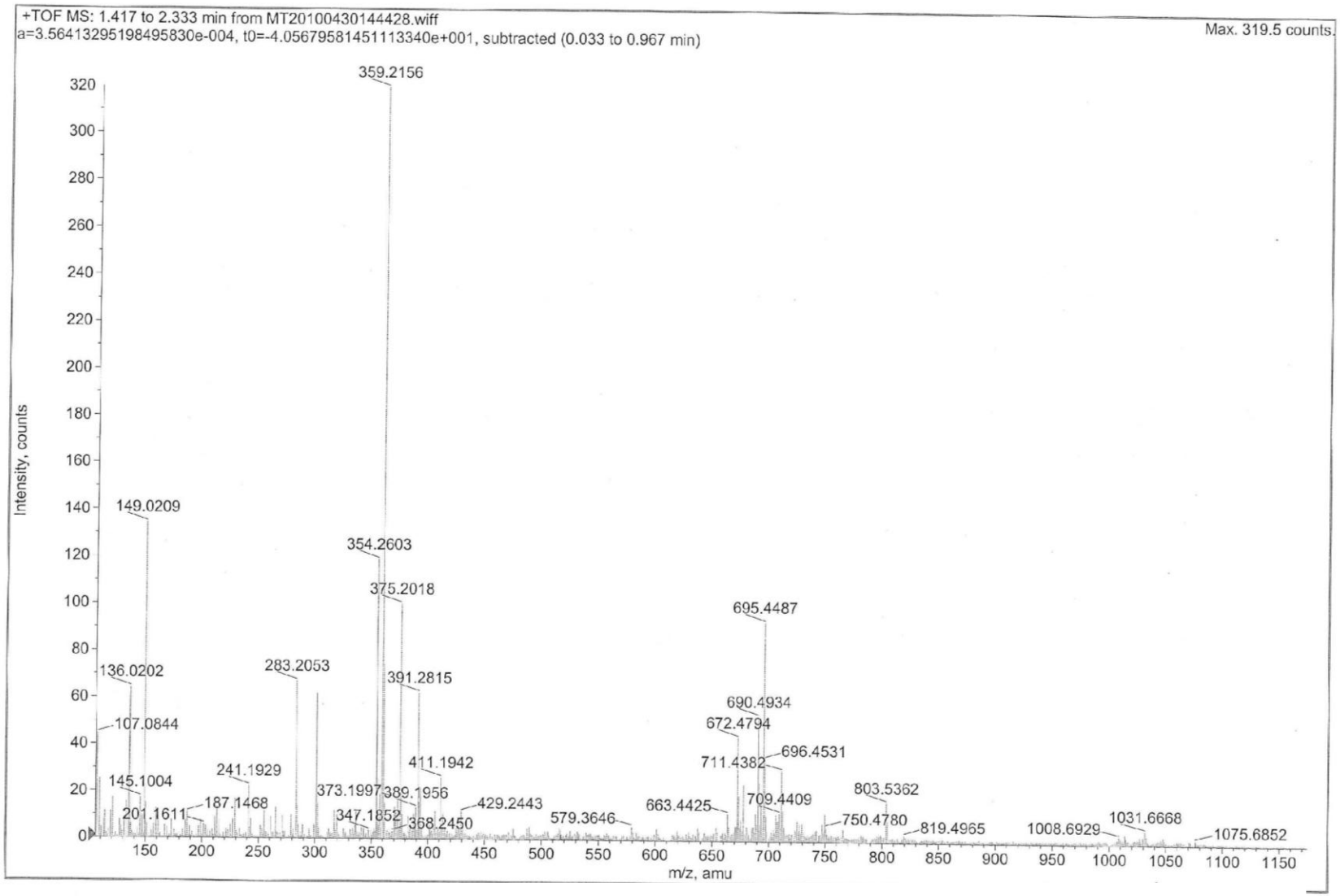

Figure 5.8. Mass spectrum of the active component H 1.5.5 from the seaweed *S. marginatum.*

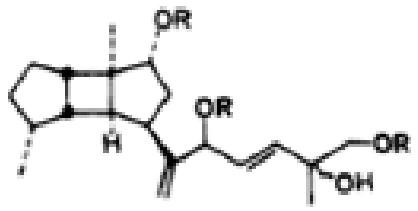

Figure 5.9. Molecular structure of the active component H 1.5.5 [5(*R*), 15, 18(*R* and *S*), 19-tetrahydroxyspata- 13, 16(*E*)-diene] from the seaweed *S. marginatum*

5.4. DISCUSSION

The development of new blood vessels is a complex multistep process. Endothelial cells resting in the parent vessels are activated by an angiogenic signal and stimulated to synthesize and release degradative enzymes allowing endothelial cells to migrate, proliferate, and finally differentiate to give rise to capillary tubules. The growth and metastasis of solid tumors in general, and cancer in particular, are dependent on their ability to initiate and sustain new capillary growth, i.e. angiogenesis. Any of these steps may be a potential target for pharmacological intervention.

With the goal of finding a potent anti-angiogenic drug, I initiated a screening program in my laboratory designed to test a wide variety of seaweed extracts for anti-angiogenic activity. Several agents having antibiotic (Glombitza *et al.,* 1979), antimicrobial (Mc Gonnell *et al.,* 1979), cytotoxic (Gerwick *et al.,* 1980) and icthyotoxic (Gerwick and Fenical, 1981) activities have already been detected and isolated from extracts of marine algae. As part of my research, extracts of seaweeds collected from the coastal waters of Marvel beach, Panaji, Goa, India were preliminarily screened for their pro-apoptotic/ anti-angiogenic activity and the seaweed displaying the most potent activity was selected for further studies. Thus far, we have carried out experiments in order to characterize and identify the chemical structure of the active compound responsible for its pro-apoptotic/anti-angiogenic activities. In this chapter, I report the isolation of an active compound by bio-assay guided fractionation from brown seaweed *Stoechospermum marginatum,* which strongly inhibited the proliferation of HUVEC cells *in-vitro* (Fig. 5.2).

The compound showed UV absorption above 210 nm. Peaks in the IR evidence (vmax cm^{-1}: 3500-3200) indicated the presence of hydroxy functions. The mass spectrum showed peaks at

m/z 359. Since the peak at m/z 359 is an M + Na peak, the molecular weight from the mass spectra was calculated to be 336. Meanwhile, the consideration of ^{1}H NMR (Fig. 5.4) and ^{13}C NMR, allowed the tentative formulation of the compound as C_{20} H_{32} O_4. The structure of the side chain in the structure was followed simply by analysis of ^{1}H NMR and ^{13}C NMR data. Its ^{1}H NMR spectrum had strong signals in the region δ 1.0-2.3, indicative of several methylene groups. The signals in the region δ 3.0-4.0 were indicative of the presence of CHOH moieties. ^{1}H NMR spectrum showed signals due to four olefinic protons [δ 5.73 (2H, *d*), 5.35 (IH, *br s*) and 4.93 (lH, *br s*)], three hydroxy groups [2.60 3H, *br m*)]. The terminal olefin was positioned at C-13, C-14 on the basic of the measurable allylic coupling of the C-7 ring juncture proton with one proton at C-14. The other disubstituted olefin was assigned as E (J 15.5 Hz) and placed at C-16 and C-17 on the lack of UV absorption of the metabolite. The structure of the compound was found to be tentatively similar to that of the mixture of monoacetates whose structure has been established to be 5(*R*), 15, 18 (*R* and *S*) – 19- tetrahydroxyspata -13, 16-(*E*)-diene by Gerwick *et al.* (1981) and is possibly stereoisomeric at C-5 and/ or C-15. The remaining spectral features, requiring one tertiary, one secondary, and one primary alcohol, were also assigned as in the compound 5(*R*), 15, 18 (*R* and *S*) -19- tetrahydroxyspata -13, 16-(*E*)-diene (Fig. 5.09).

Spatanes are 5-4-5 membered tricyclic diterpenes, which have not been reported from terrestrial sources. Some of these classes of diterpenes show cytotoxic (Gerwick *et al.,* 1980) and anti-bacterial (De Silva *et al.,* 1982) activities. However, no reports are available on pro-apoptotic/ anti-angiogenic activities of this compound. In the present study, the fraction H 1.5.5 rich in this compound was found to possess potent anti-proliferative activity against HUVEC cells *in-vitro*. This study is, in fact, the first report of its kind for this compound. Further, its

proapoptotic/antiangiogenic effects have been studied in detail and recorded in the next chapter. With additional characterization of this compound, and taking into account the possibility of analogue synthesis, there are high prospects for diterpenoids from *S. marginatum as* potential anti-cancer/ anti-angiogenic agents.

Chapter – 6

Enumerating the mechanism of action of the anticancer compound isolated from the seaweed *Stoechospermum marginatum* (C. Agardh) Kutzing

6.1. INTRODUCTION

Cancer is one of the most serious threats to human health in the world and chemotherapy is still the standard treatment method. Most of the anticancer drugs currently used in chemotherapy are cytotoxic to normal cells and cause immunotoxicity which affects not only tumor development, but also aggravates patient's recovery. The discovery and identification of new anticancer drugs with low side effects on immune system has become an essential goal in many studies of immunopharmacology (Xu *et al.*, 2009). With this aim, many attentions have been paid to natural compounds in plants, marine organism and microorganisms. Regarding the low side effects of plants and other natural compounds, scientists are interested in working on them to find new medications. Finding anticancer agents from plant sources started in the earliest 1950s with the discovery and development of vinca alkaloids, vinblastine and vincristine and the isolation of the cytotoxic podophyllotoxins (Cragg and Newman, 2005). These and several other promising phytochemical chemopreventive compounds have been found to work by various mechanisms of action targeting initiation, promotion and progression of carcinogenesis.

The ocean, which is called the 'mother of origin of life', is also the source of structurally unique natural products that are mainly accumulated in living organisms. Several of these compounds show pharmacological activities and are helpful for the invention and discovery of bioactive compounds, primarily for deadly diseases like cancer, acquired immuno-deficiency syndrome (AIDS), arthritis, etc., while other compounds have been developed as analgesics or to treat inflammation, etc. The lifesaving drugs are mainly found abundantly in microorganisms, algae and invertebrates, while they are scarce in vertebrates. Modern technologies have opened vast areas of research for the extraction of biomedical compounds from oceans and seas.

Marine algae are one of the natural resources in the marine ecosystems such as the oceans and seas. They are abundantly found in the intertidal zones and clear tropical waters. They contain various biologically active compounds which have been used as source of food, feed and medicine. The bioactive compounds found in seaweed await a major breakthrough for a variety of applications in medical field. Until now, more than 2400 marine natural products have been isolated from seaweeds of subtropical and tropical populations (Manilal *et al.,* 2009). Recent findings evidenced that seaweeds contained antiviral (Matsuhiro *et al.,* 2005), antibacterial (Xu *et al.,* 2003), antifungal (Li *et al.,* 2006) and anti-tumoral (Harada *et al.,* 1997; Hu *et al.,* 2004; Kezia *et al.,* 2008) potentials, among numerous others. The epidemiological data are supported by rodent model studies demonstrating protective effects of dietary kelps and other red and green algae against mammary, intestinal and skin carcinogenesis (Yuan and Walsh, 2006). Many studies have focused on water soluble antitumor active substances from various marine algae, however most anticancer agent have not been used clinically because of their undesirable side effects on normal cells (Harada *et al.,* 1997). According to existing literature, more than ten new experimental anti-tumor agents derived from marine sources have entered clinical trials, including bryostatin-1, aplidine, ecteinascidin-743 (ET- 743), Kahalalide F, as well as derivatives of dolastatin such as TZT-1027 and LU 103793 (Song *et al.,* 2008).

In the previous chapters, I have tested the *in-vitro* antioxidant/ pro-apoptotic/ anti-angiogenic activities of *S. marginatum* a brown alga which are found in many part of the world such as Indian Ocean, Pakistan, South Africa, Srilanka etc. In this chapter, I have enumerated the anticancer potential/ mechanism of action of an isolated compound (H 1.5.5 fraction) from this alga by cytotoxic, proapoptotic and anti-angiogenic assays both *in-vitro* and *in-vivo*.

6.2. MATERIALS AND METHODS

6.2.1. Animals, *in-vivo* tumor model, cell lines and chemicals

Swiss albino mice (6-8 weeks old) were obtained from the animal house, Department of Zoology, University of Mysore, Mysore, India. EAT (Mouse Mammary Carcinoma) cells which were routinely maintained in Swiss albino mice at the Laboratory of Molecular Oncology, University of Mysore. They were drawn in aseptic condition and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich, USA) supplemented with 10 % Fetal Bovine Serum (FBS, Sigma Aldrich, USA) and 1 % Penicillin-Streptomycin. Carcinoma of cervix (HeLa), Human Epidermoid Larynx Carcinoma (Hep-2) and African green monkey Chlorocebus kidney epithelial (Vero) cell lines were obtained from the National Center of Cell Sciences (NCCS), Pune, India. Human Umbilical Vein Endothelial Cells (HUVEC) and Endothelial Growth Medium (EGM-2) were obtained from Cambrex Bioscience, Walkersville, USA. Minimum Essential Medium (MEM), Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Penicillin-Streptomycin and Trypsis-EDTA were purchased from Invitrogen, USA. Poly-2 hydroxyl ethylmethacrylate was purchased from Sigma Aldrich, USA. Matrigel was purchased from Chemicon International, CA, USA. All other reagents were of highest analytical grade commercially available.

6.2.2. *In-vitro* culture of HeLa, Hep-2, EAT and HUVEC cells

Hep-2 (Human Epidermoid Larynx Carcinoma) and HeLa (Carcinoma of cervix) cell lines (NCCS, Pune, India) were grown and maintained in Minimal Essential Medium (Invitrogen, USA) supplemented with 10 % FBS, 1 % Penicillin-Streptomycin and Gentamycin. Human Umbilical Vein Endothelial Cells were cultured in EGM-2 medium with 2 % FBS, 0.04 %

hydrocortisone, 0.1 % long R_3-Human Insulin Like Growth Factor (IGF-1), 0.1 % ascorbic acid, 0.4 % human fibroblast growth factor (b FGF), 0.1% VEGF, 0.05 % gentamycin and 0.05 % amphotericin-B according to the manufacturer's protocol. EAT cells were maintained in DMEM with 10 % FBS and 1 % Penicillin-Streptomycin. The cells were incubated at 37º C in a humidified atmosphere of 5 % CO_2. When the cells reached confluency, they were passaged by trypsinization using 0.025 % trypsin/ 0.01 % EDTA. For the experiments, cells from passages 2-5 were used.

6.2.3. Processing of sample for experimentation

10 mg of the compound H 1.5.5 [5(*R*), 15, 18 (*R* and *S*) – trihydroxylspata -13, 16-(*E*)-diene] was dissolved in 1 % DMSO and made up to 10 ml with sterile distilled water to make a final concentration of 1 µg μl^{-1} and used for subsequent experimentation.

6.2.4. MTT assay

The effect of H 1.5.5 fraction on cells was determined with MTT (3-(4, 5–dimethylthiazol-2-yl)-2-5- diphenyltetrazolium bromide) assay (Arulvasu *et al.*, 2010). Briefly, the cells were plated at a density of 1×10^5 cells/ well in 200 µl of culture medium containing 0.0001, 0.001, 0.01, 0.1 and 1.0 mg/ ml of H 1.5.5 fraction in 96-well microtiter plates. A stock solution of methanolic extract was prepared in DMSO and diluted with the culture media to achieve final concentrations of 0.0001, 0.001, 0.01, 0.1 and 1.0 mg ml^{-1}. The concentration of DMSO remained within the maximum permissible concentration of 0.1 % in both control and treated samples were ensured. Each concentration of H 1.5.5 was repeated in 3 wells. After incubation for the desired period of time at 37º C in a humidified incubator, cell viability was assessed. MTT assay (50 µl, 5 mg ml^{-1}

in phosphate – buffered saline stock, diluted to a working strength of 1 mg ml^{-1} with media) was added to each well and incubated for 2 hrs, after which the plate was centrifuged at 600 g for 5 min at 4º C. The MTT solution was removed from the wells by aspiration. After removal of the medium, 0.1 ml of buffered DMSO was added to each well and plates were shaken. The absorbance was measured at 570 nm in an automated plate reader and percentage of growth inhibition was calculated using the following standard equation.

$$\text{Inhibition \%} = \frac{\text{Absorption control} - \text{Absorption test}}{\text{Absorption test}} \times 100$$

6.2.5. Matrigel tube formation assay

The Matrigel tube formation assay has been widely used as an *in-vitro* measurement of endothelial cell differentiation and was performed by using *In-vitro* Angiogenesis Assay Kit (Chemicon). In brief, Matrigel (50 µl) was added to each well of a 96 well plate and incubated at 37º C for 1 hr allowing the gel to polymerise. Human Umbilical Vein Endothelial Cells (HUVEC's) were suspended in endothelial growth medium and (1×10^4) cells were seeded in to each well containing 150 µl of EGM. The cells were incubated with or without the test sample (SME) at 37º C and 5 % CO_2. All the conditions were performed in triplicates. After 16 – 20 hrs of incubation, the tube formation was photographed at 40 x magnification by using inverted microscopy (Olympus, Germany).

6.2.6. Quantification of VEGF

The quantification of VEGF was carried out by Enzyme Linked Immunosorbent Assay (ELISA) Kit (Merck) and VEGF was estimated in the ascitic fluid collected from both untreated and EAT cells *in-vitro*. In brief, 100 µl of ascitic fluid from DBRE treated and untreated EAT bearing mice were coated onto 96 well microplates using coating buffer (50 mM Na_2CO_3, pH. 9.6) and incubated overnight at 4º C. Subsequently, the wells were washed and blocked using 5 % skimmed milk in PBS for 2 hrs at 37º C. After washing, anti-VEGF165 antibody was added and the plates were incubated for 2 hrs at 37º C. The plates were washed 100 µl/ well of goat anti-rabbit IgG conjugated to alkaline phosphatase (1: 5000). After 1 h at 37 ºC, plates were washed and developed using 100 µl of p-nitro-phenyl phosphate (PNPP). The reaction was terminated by addition of 0.1N NaOH and absorbance was read at 405 nm in micro plate ELISA reader (Medispec). The experiment was carried out in triplicates.

6.2.7. Corneal Neovascularization (CNV) assay

The rat corneal micropocket assay was performed as described previously (Sarayba *et al.*, 2005) In brief, hydron Polymer (poly-2 hydroxy ethyl-methacrylate was dissolved in ethanol to a final concentration of 12 %. A 5µl aliquot of this mixture was then pipetted onto Teflon pegs. Aliquots of 10 µl of 12 % Hydron/ Ethanol alone (group 1), with 1µg of cytokine VEGF (group 2) and VEGF + 5 µg seaweed extract (group 3) was added to each pellet and allowed to dry under a laminar flow hood at room temperature for 2 hrs. The pellets were incubated at 4° C overnight. All procedures were performed under sterile condition. Male Wistar rats weighting 300-350 g were anaesthetized with *i.p.* with a combination of ketamine (87 mg kg^{-1}) and xylazine (13 mg kg^{-1}). A drop of 0.5 % proparacaine was instilled to the eye and the globe was

proptosed using a pair of 0.3 mm tissue forceps. Using a surgical microscope, a paracentral linear incision 1 mm from the center of the cornea, 1.5 nm in length and 50 % of the corneal depth was made with a No. 11 surgical blade to create a corneal micropocket. The incision was bluntly dissected through the stroma to the limbal area using a curved iris spatula. A single pellet was advanced into the lamellar pocket to the limbus using corneal forceps. Postoperatively, gentamycin ointment was applied to the anterior surface of the operated eye. The rats were observed for 24 - 72 hrs for the occurrence of non-specific inflammation and for localization of the pellets. On day 7, the rats were anaesthetized with ketamiine and the corneas were observed under steriobinocular microscope with CCD camera and photographed.

6.2.8. Annexin V Apoptosis detection assay

Apoptosis was induced in a cell suspension of EAT cells by addition of H 1.5.5 fraction (0.005 to 0.025 mg ml^{-1}). Non-induced cells were kept for a zero time control. Cells were incubated for the desired time at 37 °C in a 5 % CO_2 atmosphere. Cells were then washed twice with PBS and suspended in it at a concentration of 0.5–1 $x10^6$ cells per ml. 50 µl of the cell suspension (induced or non-induced) was then placed onto the slide and left at room temperature for 10 minutes, to allow the cells to absorb on to the plate. Excess liquid was removed carefully by touching a tissue to the side of the circle. Cells were then washed three times with 50 µl of 1x binding buffer each. Excess liquid was blot dried with a tissue as in the earlier step.50 µl of the staining solution (Annexin V) was then placed on the slide and a Petri dish covered with aluminum foil was placed on it. It was then incubated for 10 minutes at room temperature. After staining, the spot of application was washed for five times with 50 µl of 1 x binding buffer each as in the earlier step. This removed excess label from the cells. A 35 ml of 1 x binding buffer on

this spot was applied and a 24 x 50 mm cover slip was placed on it. The results were observed using a fluorescence microscope and then photographed.

6.2.9. Propidium Iodide (PI) staining for apoptosis detection

Cells were seeded in a 6 well plate at 2.5 x 10^5 cells/ well with 2 ml of cell culture media. The cells were allowed to grow for 24 hrs and then treated with H 1.5.5 fraction (0.005 to 0.025 mg ml^{-1}). Non-induced cells were kept for a zero time control. After incubation for the desired time at 37° C in a 5 % CO_2 atmosphere the cells were recovered from the wells and added to the tubes. The tubes were then centrifuged at 5000 rpm in a micro-centrifuge (i.e at a low setting) to remove the supernatant. To this, 100 µl of 500 ng ml^{-1} PI was added to gently break up the cell pellet and incubated in the dark at 4º C for 15 minutes. 20 µl of the suspension was then taken on a microscopic slide. Excess liquid was removed by carefully touching a tissue to the side of the spot of application. The cells were then washed three times with 50 µl of 1x binding buffer each. Again, the excess liquid was blotted out with a tissue as in the earlier step and a cover slip was then placed on it. 35 µl of 1x binding buffer was placed on the spot and covered with a 24 x 50 mm cover slip. Cells were then viewed under a fluorescent microscope and photographed.

6.2.10. DNA Fragmentation assay

DNA extraction and agarose gel electrophoresis were performed by the method described by Chaudhary *et al.* (2001) with brief modifications. EAT cells were either untreated or pretreated with H 1.5.5 fraction for 2 hrs at 37º C. After 16 hrs of treatment the DNA was extracted from the cell lysates as follows. Both the attached and floating cells were collected, washed with PBS and centrifuged at 15000 rpm for 5 minutes to collect the cell pellet. This was then re-suspended

in 0.5ml of lysis buffer [50 mMTris-HCl, pH 8.0] and 0.5 % SDS, transferred to a micro-fuge tube and incubated for 1 hr at 37° C. The cell lysate was subjected to 8 M potassium acetate precipitation and left for l h at 4º C. To the supernatant each tube 0.5 ml of phenol: chloroform: iso-amyl alcohol (25:24:1) was added, mixed and centrifuged at 13,000 rpm for 1 minute to separate the DNA containing upper aqueous phase. Phenol – chloroform extraction was repeated twice, followed by chloroform extraction alone. To the resulting aqueous phase, two volumes of ice-cold absolute ethanol was added and incubated for 30 minutes on ice to precipitate DNA. DNA was pelleted by centrifuging at 13,000 rpm for 10 minutes at 4° C. The supernatant was aspirated and the pellet was washed with 1 ml of 70 % ethanol. After repeating the above centrifugation step and removing the last traces of the supernatant fraction, the pellet was allowed to dry at room temperature for approximately 30 minutes and re-suspended in 50 μl of Tris – EDTA buffer. The DNA was quantified by UV – Vis spectroscopy and 10 μg of DNA was electrophoresed in a 1.5 % agarose gel containing ethidium bromide in a mini gel tank containing Tris – boric acid – EDTA buffer for 2 hrs under 90 Volts. Then the gel was visualized under UV illuminator and documented using UVP-BioDoc-ItTM system.

6.2.11. Statistical analysis

All experiments were conducted in triplicate (n=3) and expressed as means ± SD using STATISTICA software (Statsoft, 1999)

6.3. RESULTS

6.3.1. Effect of H 1.5.5 fraction on the growth of HeL, Hep-2 cells and Vero cells

H 1.5.5 fraction inhibited the growth of the HeLa and Hep-2 cancer cell lines in a concentration-dependent manner with a LC_{50} of 1.143 ± 0.52 µg ml^{-1} against HeLa (Fig. 6.01) and a LC_{50} of 1.989 ± 0.90 µg ml^{-1} against Hep-2 respectively (Fig. 6.02) in the MTT assay. However, H 1.5.5 fraction had no potent cytotoxic activity against Vero cells and showed a LC_{50} of 64.65 ± 1.42 µg ml^{-1}.

6.3.2. Effect of H 1.5.5 fraction on tube formation

Since the angiogenic process is also characterized by endothelial cell differentiation, *in-vitro* angiogenesis assays were carried out with HUVECs treated with H 1.5.5 at 1, 5, 10, 15, 20 and 25 µg/ well on a matrigel matrix. In the positive control group stimulated with VEGF (10 ng), the HUVEC's adhered to the matrigel surface within 20 to 24 hrs and formed a branching anastomising network of capillary like tubes with multi-centric junctions over 24 hrs. As shown in Figure 6.03, a spontaneous differentiation of HUVECs into capillary-like structures was observed in control cells. HUVEC cells treated with 1, 5 and 10 µg/ well also formed tubes although the differentiation was not totally completed. However, when HUVECs cells treated at 15, 20 and 25 µg/ well with H1.5.5 were plated on the matrix they failed to differentiate. Cells remained rounded and were not able to form tubes as H 1.5.5 fraction inhibited VEGF stimulated tube formation of HUVEC's, in a dose dependent manner.

6.3.3. Effect of H 1.5.5 fraction on HUVEC migration

Next, I evaluated the effects of H 1.5.5 fraction on endothelial cell migration, which represents an important step in the formation of new capillaries. To this point an *in-vitro* wound-healing assay using HUVEC's was done. Wounds were created in confluent cell monolayers with a 1.0-mm wide tip, after which control cells spontaneously migrate into the empty space. After time intervals (0 to 42 hrs), the extent of wound closure and the inhibition of cell migration by the compound were recorded by clicking digital pictures. H 1.5.5 fraction time-dependently inhibited HUVEC migration (Fig. 6.04).

6.3.4. VEGF quantification by Enzyme-Linked Immunosorbent Assay (ELISA)

As quantified by Elisa, the concentration of VEGF in culture supernatant of H 1.5.5 fraction treated EAT cells was 4812, 4016, 3925, 1232, 163 ng ml^{-1} at the concentrations of 5, 10, 15, 20, 25 µg ml^{-1}, while it was 5400 ng ml^{-1} in the control group after 24 hrs incubation and was statistically significant ($p < 0.001$) (Fig. 6.05).

6.3.5. Effect of H 1.5.5 fraction on Corneal Neovascularization (CNV)

The rat cornea assays forms a part of the conventional angiogenesis assays used commonly for *in-vivo* validation of the angio-suppressive efficacy of anti-angiogenic molecules. Results shown in Figure 6.06 indicate that H 1.5.5 had a direct effect on inhibition of angiogenesis in *in-vivo* model system. When compared to the extensive angiogenesis seen in VEGF treated rat cornea, angiogenesis at the site of the application of H1.5.5 was significantly reduced.

6.3.6. Effect of H 1.5.5 fraction on apoptosis of EAT cells by Annexin V staining

Many chemical compounds can inhibit the growth of tumor cells, but not all of them can trigger apoptosis. To determine whether apoptosis was induced by the compound present in H 1.5.5 fraction, I performed Annexin V staining of the treated EAT cells. The treated cells showed apoptotic morphology in a dose dependent manner (Fig. 6.07).

6.3.7. Effect of H 1.5.5 fraction on apoptosis of EAT cells by Propidium Iodide staining

Staining of nuclei with the DNA binding fluorescent dye propidium iodide (PI) showed that the number of apoptotic cells that took up the dye increased with increasing concentration (Fig. 6.08).

6.3.8. Effect of H 1.5.5 fraction on DNA fragmentation of EAT cells

H 1.5.5 fraction induced apoptotic cell death in EAT cells. In DNA agarose gel electrophoresis, a ladder of fragmented DNA was detected after 12 h of incubation with 5, 15, 25 mg $ml^{-1}$of H 1.5.5 fraction treated EAT cells, as shown in Figure 6.09.

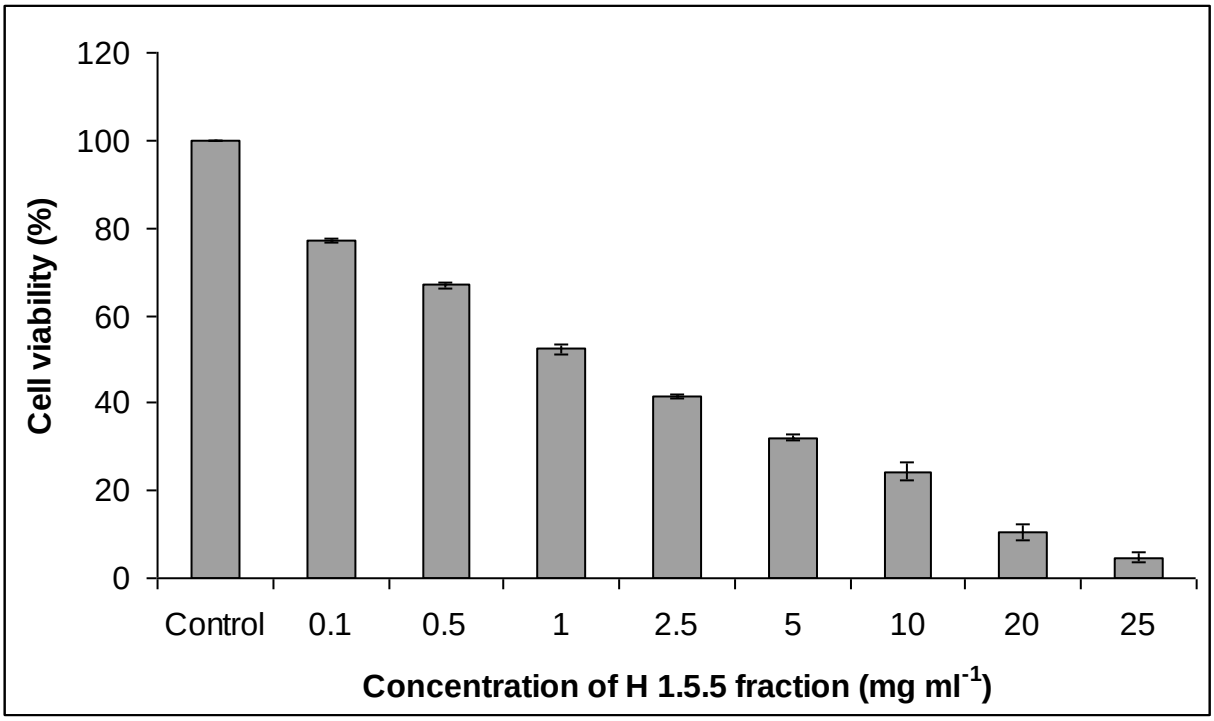

Figure 6.1. Effect of *S. marginatum* extract (SME) on the viability of HeLa cells *in-vitro*. HeLa cells were plated in 96 well plates and incubated for 48h. Seaweed extracts of 0.0001, 0.001, 0.01, 0.1 and 1 mg ml^{-1} concentration were added to the wells in triplicates and incubated for another 48 h. The cells were trypsinized after 2 days and processed for MTT assay. Values are presented as means ± SD (n=3).

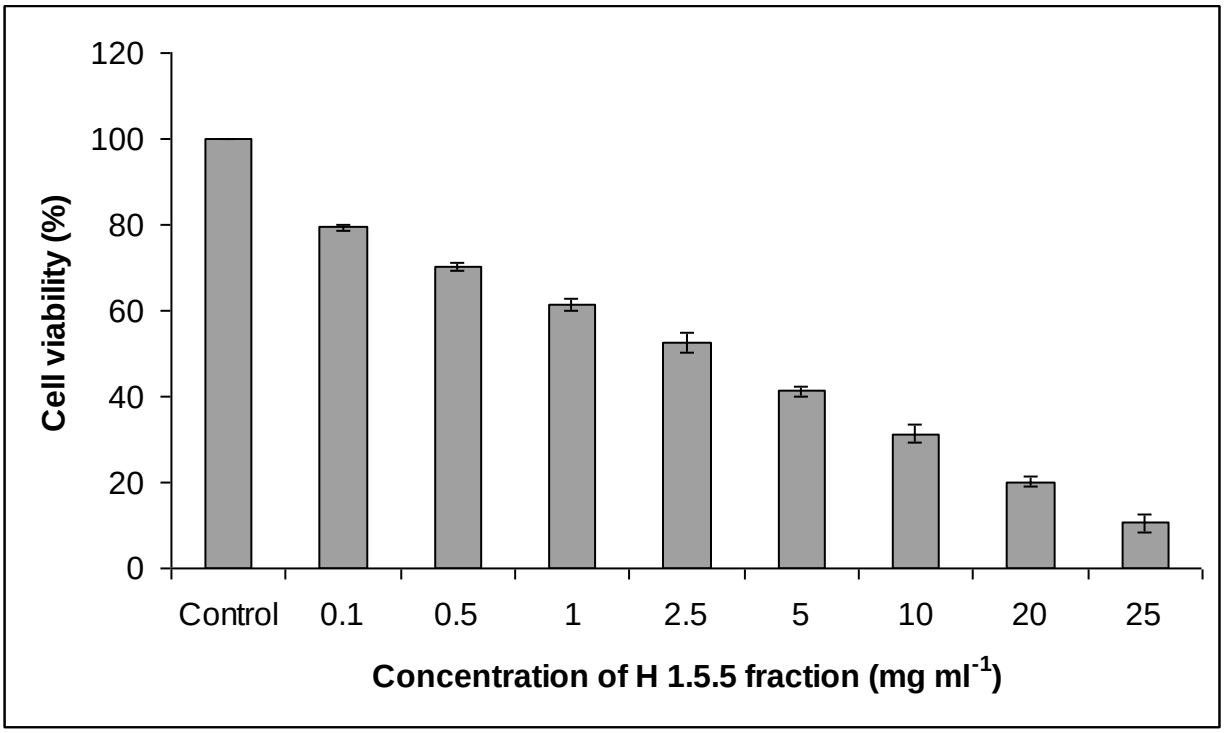

Figure 6.2. Effect of *S. marginatum* extract (SME) on the viability of Hep-2 cells *in-vitro*. Hep 2 cells were plated in 96 well plates and incubated for 48h. Seaweed extracts of 0.0001, 0.001, 0.01, 0.1 and 1 mg ml^{-1} concentration were added to the wells in triplicates and incubated for another 48 h. The cells were trypsinized after 2 days and processed for MTT assay. Values are presented as means ± SD (n=3).

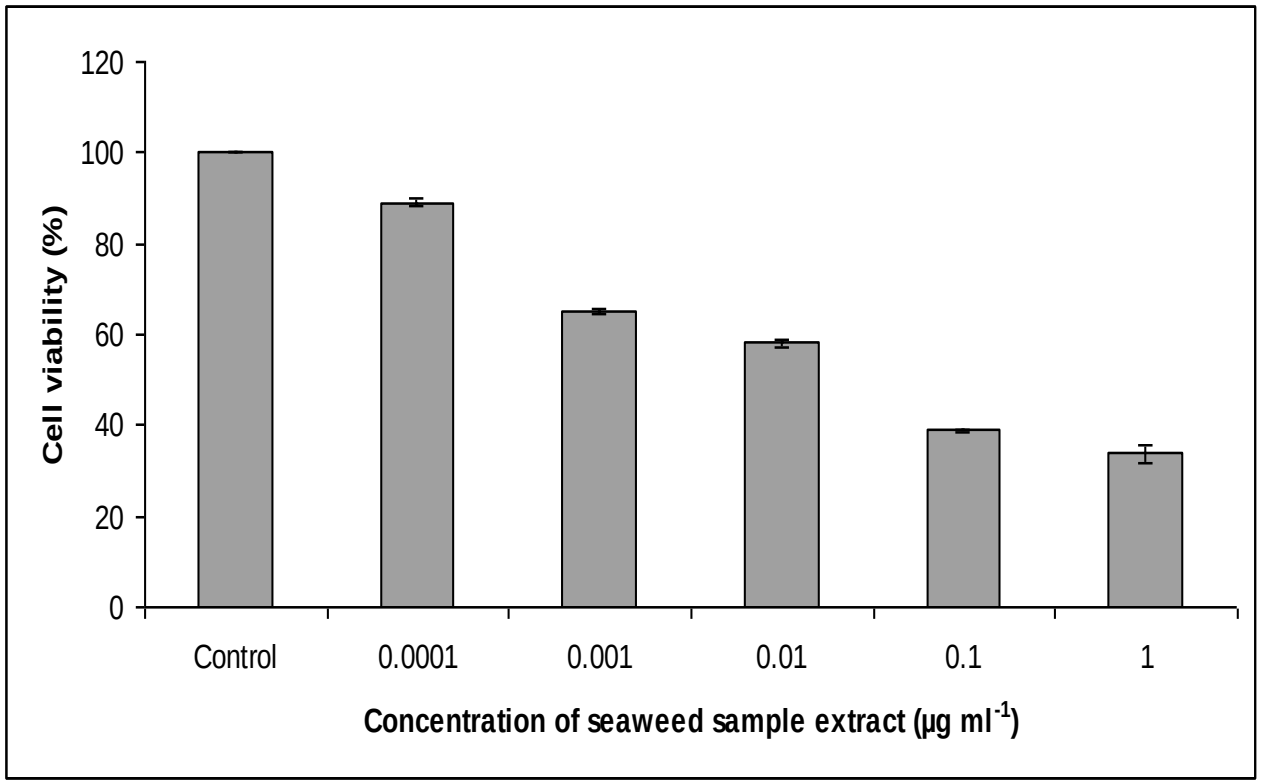

Figure 6.3 Effect of *S. marginatum* extract (SME) on the viability of Vero cells *in-vitro*. Vero cells were plated in 96 well plates and incubated for 48h. Seaweed extracts of 0.0001, 0.001, 0.01, 0.1 and 1 mg ml^{-1} concentration were added to the wells in triplicates and incubated for another 48 h. The cells were trypsinized after 2 days and processed for MTT assay. Values are presented as means ± SD (n=3).

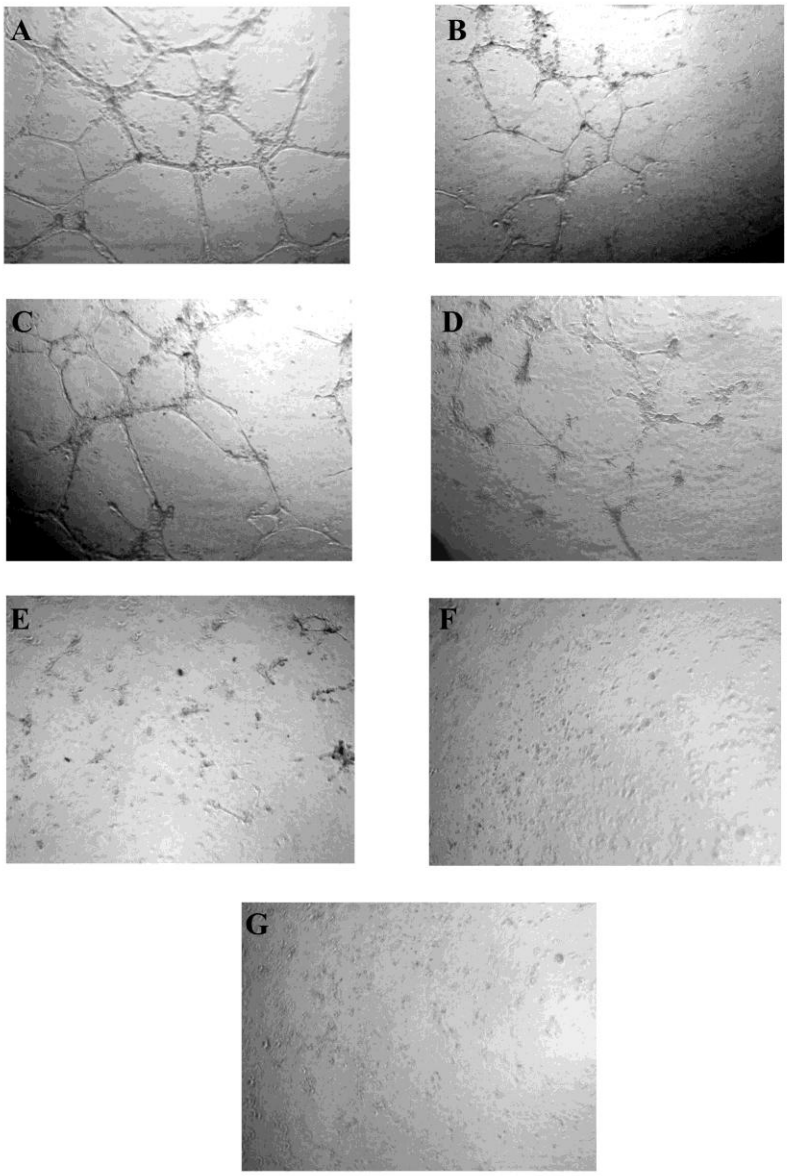

Figure 6.4. Inhibitory effect of H 1.5.5 fraction on VEGF induced tube formation *in-vitro*. HUVEC's were seeded into the matrigel layer in 96-well plate. (A) VEGF alone (+ve control), (B) without VEGF (-ve control), (C) VEGF + H 1.5.5 fraction (1 μg), (D) VEGF + H 1.5.5 fraction (5 μg), (E) VEGF + H 1.5.5 fraction (10 μg), (F) VEGF + H 1.5.5 fraction (15 μg), (G) VEGF + H 1.5.5 fraction (20 μg), (H) VEGF + H 1.5.5 fraction (25 μg) . The experiment was repeated thrice with similar results (values are means ± S.D., n=3) Three replicate fields of triplicate wells were digitally photographed.

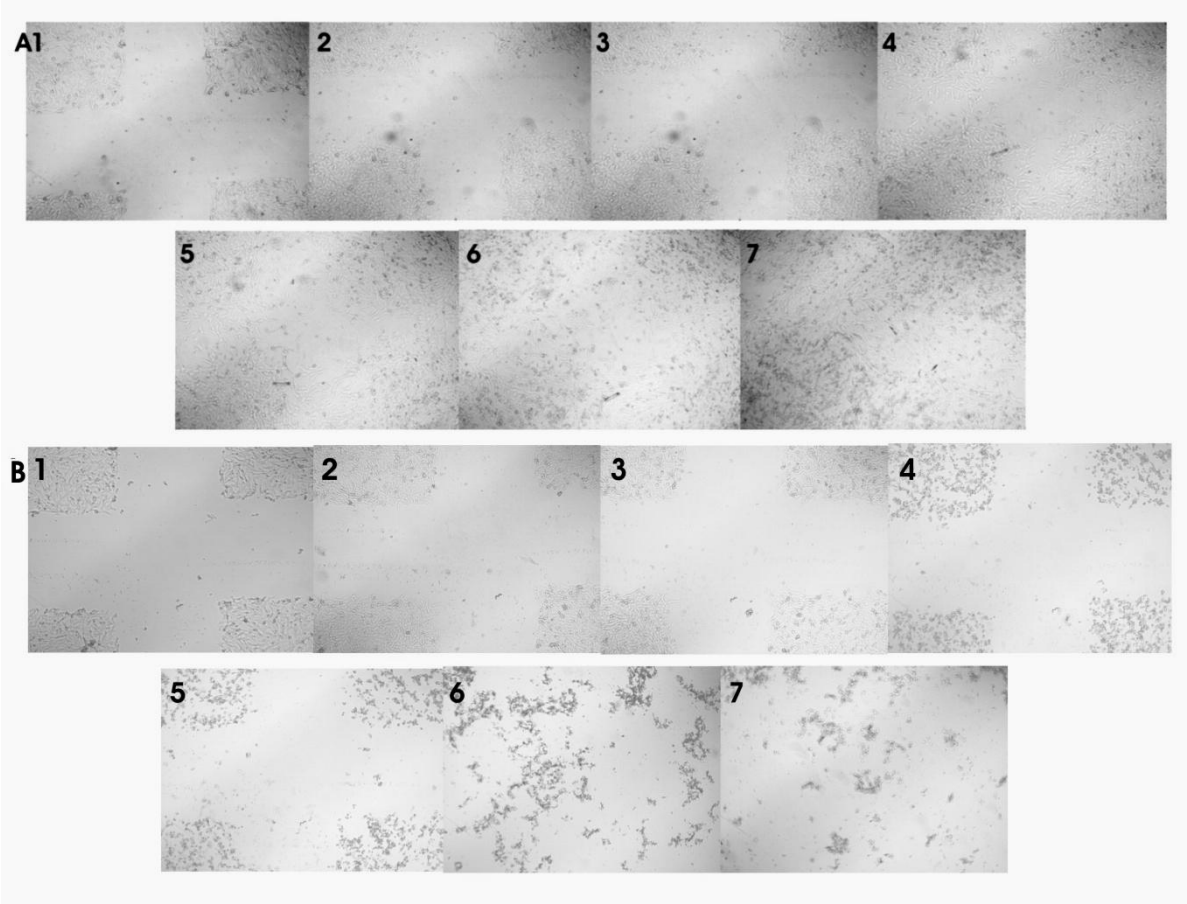

Figure 6.5. Effect of H 1.5.5 fraction on HUVEC cell migration in an *in-vitro* wound healing assay. Representative phase-contrast images show cells migrating into the wounded area. A) Wound closure in control wells at 1) 0h, 2) 3h, 3) 18h, 4) 24h, 5) 36h and 6) 42h. B) Wound closure in H1.5.5 fraction (5µg/ well) treated wells at 1) 0h, 2) 3h, 3) 18h, 4) 24h, 5) 36h and 6) 42h.

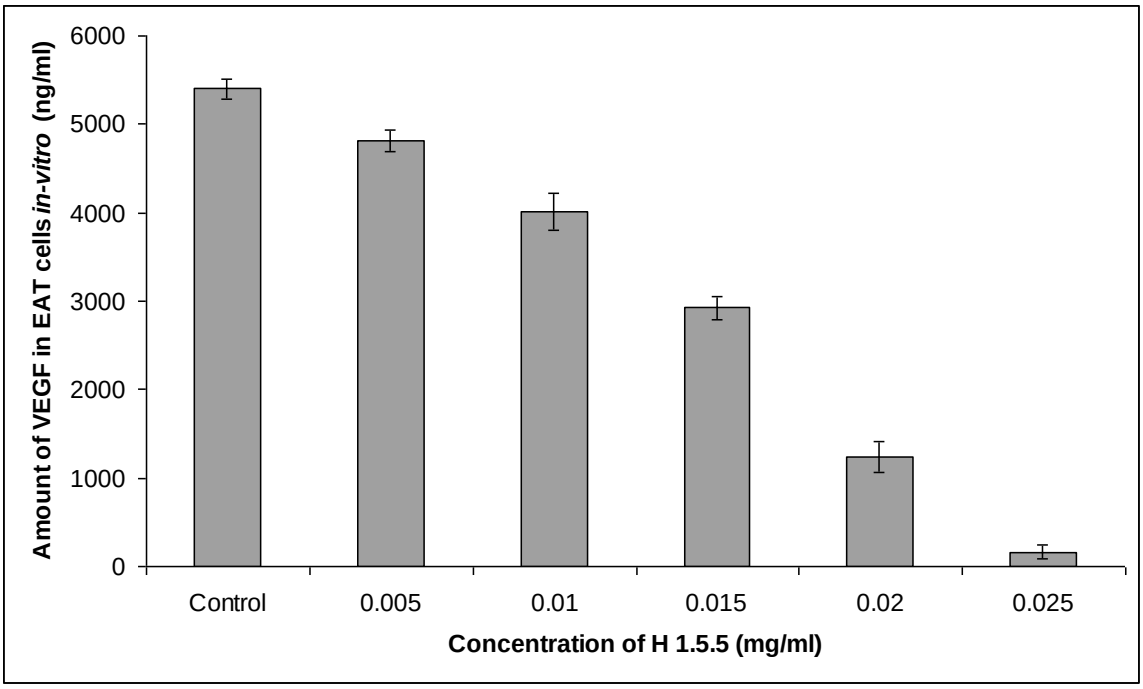

Figure 6.6. Effect of H 1.5.5 fraction on production of VEGF by EAT cells *in-vitro*. EAT cells were grown to confluency and were treated or untreated with H 1.5.5 (0.005, 0.01, 0.015, 0.02, 0.025 µg ml^{-1}) and after 24 hrs the media was collected. Elisa was carried out using the media to quantitate VEGF using anti-VEGF 165 antibodies.

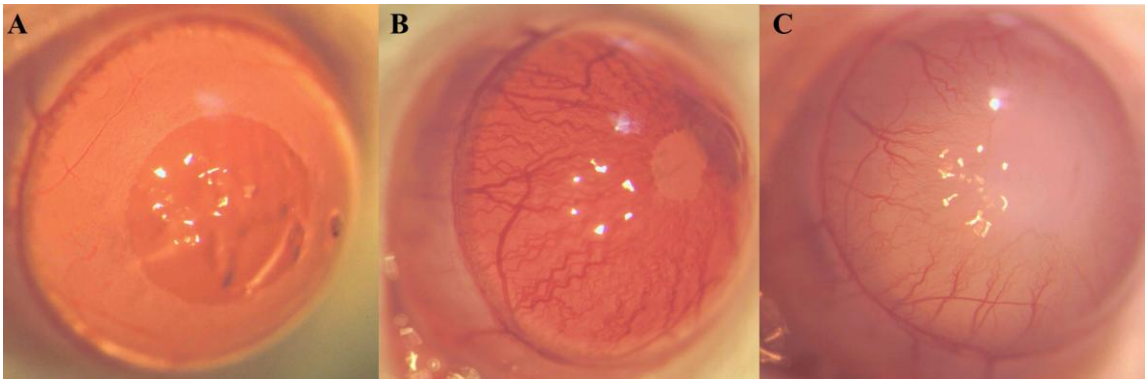

Figure 6.7. Effect of H 1.5.5 fraction on blood vessel regression in the rat cornea assay. Photographs of VEGF-induced neovascularization observed in rat corneas: (A) hydron polymer alone (– control), (B) hydron polymer + VEGF (1 µg) (+ control), and (C) hydron polymer + VEGF + H 1.5.5 fraction (1 µg). After 7 days of incubation, the corneas were photographed at 40 X magnification

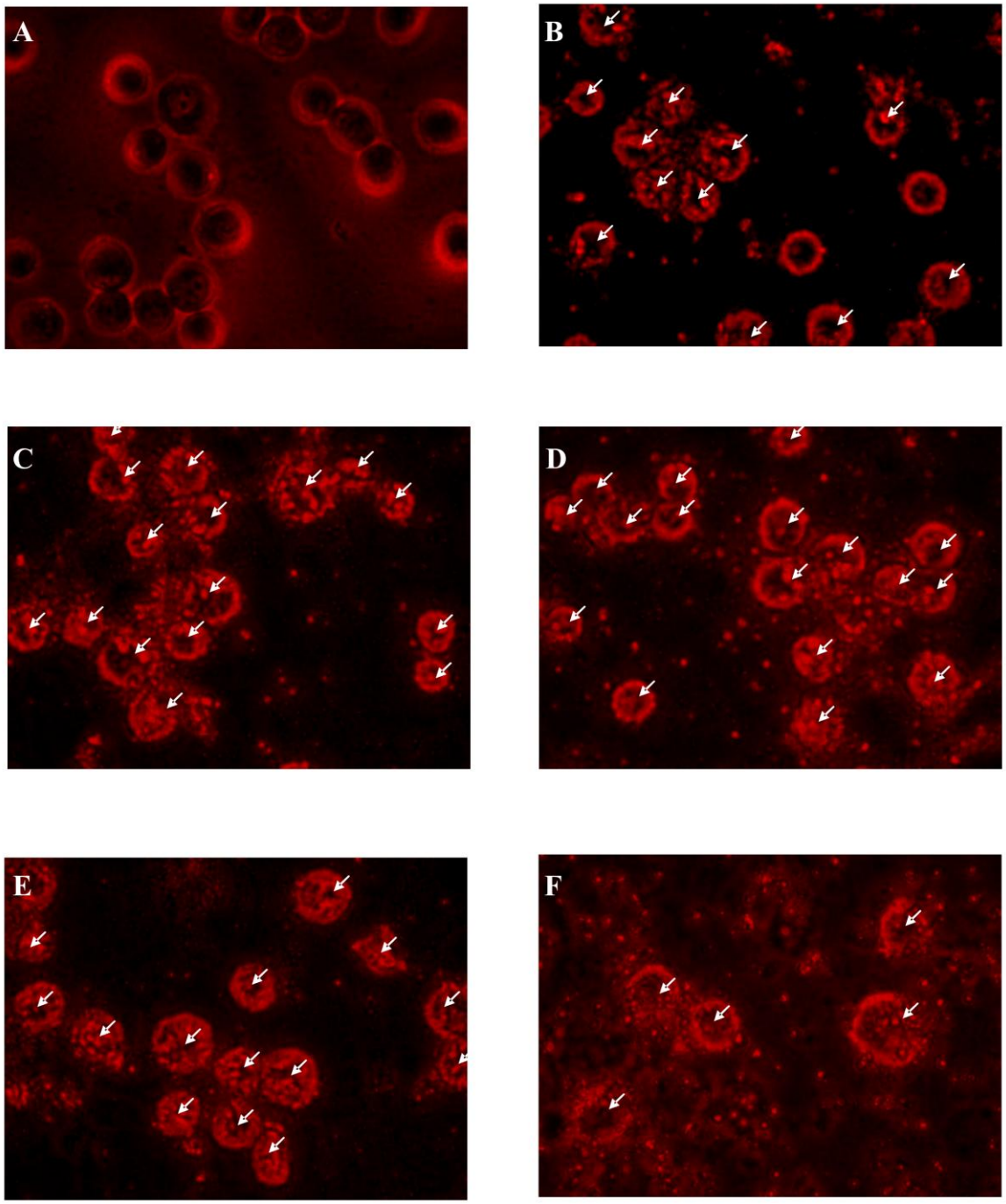

Figure 6.8. Effect of H 1.5.5 fraction on EAT cells showing characteristic apoptotic morphology when stained with Annexin V stain. (A): Control, (B to F): H 1.5.5 fraction treated at the concentration 5, 10, 15, 20, 25 µg ml^{-1}. Arrows indicate membrane blebbing, apoptotic bodies and condensed chromatin.

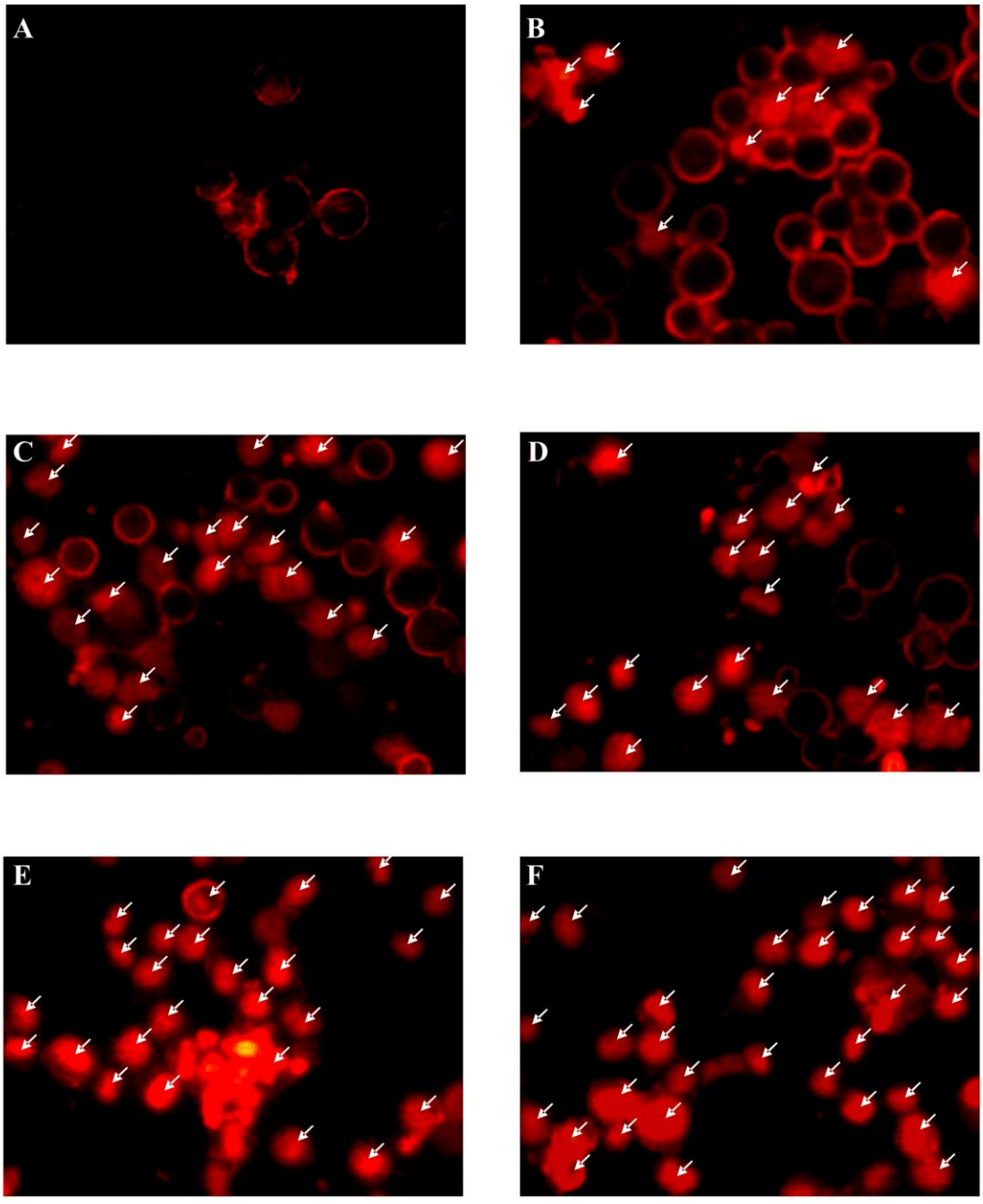

Figure 6.9. Effect of H 1.5.5 fraction on EAT cells showing characteristic apoptotic morphology when stained with Propidium Iodide stain. (A): Control, (B to F): H 1.5.5 fraction treated at the concentration 5, 10, 15, 20, 25 µg ml^{-1}. Arrows indicate membrane blebbing, apoptotic bodies and condensed chromatin.

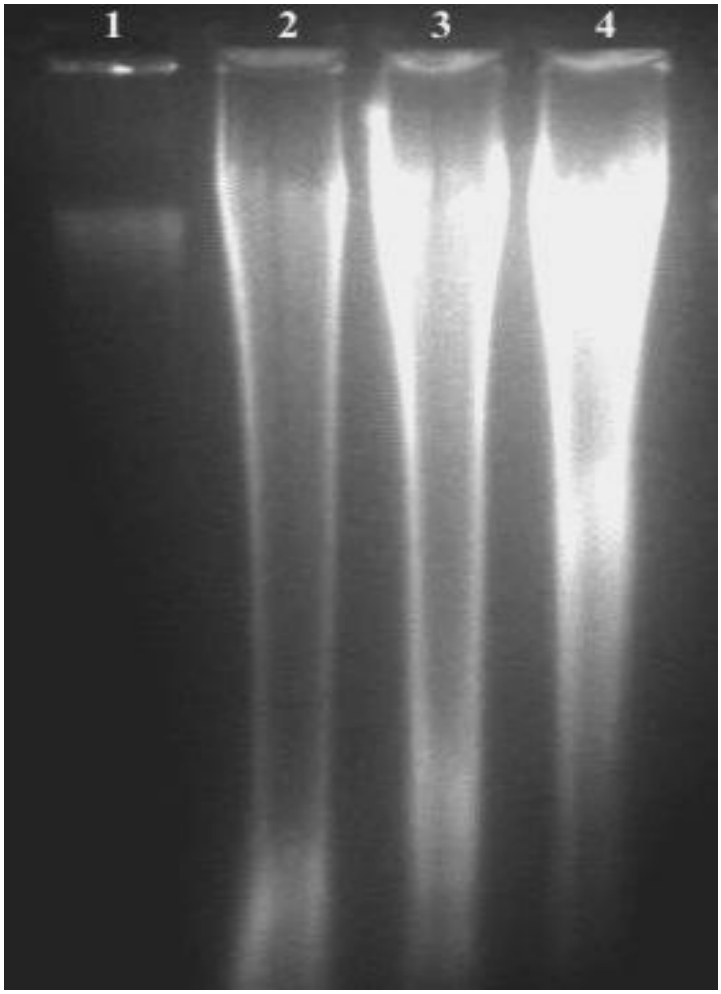

Figure 6.10. ***In-vitro*** **apoptotic effect of H 1.5.5 fraction on the DNA of Ehrlich ascites tumor cells (EAT).** DNA run and detected on 2 % agarose gel electrophoresis. Lane 1: DNA from control EAT cells, Lane 2, 3 and 4: DNA from H1.5.5 fraction treated EAT cells at the concentration 5, 10, 25µg ml^{-1}respectively.

6.4. DISCUSSION

Tumor-induced angiogenesis, the formation of neovessels from preexisting ones, is critical for supporting tumor growth and progression not only by providing the necessary blood supply but also by allowing metastatic cells into the circulation (Belotti *et al.,* 1996). Angiogenesis is an exciting target for novel anticancer therapies because of the many advantages that it may offer, including accessibility to tumors, independence of tumor cell resistance mechanisms and broad applicability to many tumor types (Sccapaticci, 2002). In fact, many therapeutic efforts have been done so far for the treatment of cancer, a deadly disease. Among them, anti-angiogenic therapy and targeted cancer therapy are two important strategies for selective cancer eradication. The potential use of natural and synthetic angiogenesis inhibitors is currently being studied intensively by many laboratories (Minamiguchi *et al.,* 2001, Mohan *et al.,* 2002). Inhibitors of angiogenesis block any of the steps in the angiogenic cascade, including proliferation and attachment of endothelial cells to the extracellular matrix proteins, migration and invasion through the matrix, which is required for the capillary sprouting and morphogenesis in a thin tube meshwork and differentiation and stabilization (Folkman and Klagsbrun, 1987; Folkman, 1992; Folkman, 1995). In recent years, anti-angiogenic treatments have also been shown to synergize with traditional chemotherapeutic and radio-therapeutic regimens. Combining anti-angiogenesis with targeted anti-proliferative and pro-apoptotic agents might also be synergistic.

The present chapter demonstrates that a compound isolated from H 1.5.5 fraction of *S. marginatum* seaweed inhibits several essential steps of the angiogenic/ pro-apoptotic process necessary for the development of cancer.

To determine whether H 1.5.5 fraction could inhibit cancer cell growth, the effect of H 1.5.5 fraction on the growth of HeLa, HEP-2 and Vero cells by MTT assay was evaluated. As shown in Figures 6.1, 6.2 and 6.3, H1.5.5 fraction inhibited the growth of tumor cells in a dose-dependent manner but was less cytotoxic to Vero cells; a normal cell line. SargA, a polysaccharide extracted from the brown marine alga *Sargassum stenophyllum*, caused graded inhibitions viability of cultured B16F10 cells (Dias *et al*., 2005). A fucosterol identified from the hexane fractions of methanolic extract of brown alga *Sargassum angustifolium* and red alga *Chondria dasyphylla*, were also reported to have cytotoxic activity in Caco-2 and T47D cell lines at 4.5, 18, 36, and 72 μg/ml respectively (Khanavi *et al*., 2012). The anti-proliferative activity of phlorotannins derived from brown algae *L. japonica Aresch* extracts collected from Quingdao, China on the human hepatocellular carcinoma cell (BEL-7402) and on murine leukemic cells (P388) by MTT assay were studied by Yang *et al*., (2010) and the inhibitory effect of phlorotannin extract on BEL-7402 and P388 cells was found to be IC_{50}120 mg/mL and >200 mg/mL, respectively. However, the concentartions used were much higher than the ones used in the present study.

The final event during angiogenesis is the organization of endothelial cells in a three-dimensional network of tubes. Therefore, anti-angiogenic activity of H 1.5.5 fraction was first detected using the *in-vitro* tube formation assay for endothelial cells. When plated on matrigel, HUVECs underwent rapid reorganization (visible within 1–2 hrs) and subsequently formed capillary-like structures. In contrast, the addition of H 1.5.5 fraction in a culture medium for 12 hrs caused a dose-dependent inhibition of Matrigel-induced network formation (Fig. 6.4). There was slight inhibition at lower concentrations of H 1.5.5 fraction; however, tube formation was

incomplete as compared with the controls. Treatment with higher doses of H 1.5.5 fraction resulted in a significant reduction in mean tube length; the tubes were less extensive, thinner and foreshortened. At the highest dose, H 1.5.5 fraction inhibited the formation of vessel-like structures. These findings further confirmed that H 1.5.5 fraction can modify endothelial cell functions at indicated concentrations and exposure times at which cell proliferation is not affected and suggest that it might indeed prevent the process of angiogenesis. Various other effective chemopreventive compounds of natural or synthetic origin have also been shown to inhibit the angiogenic cascade at the endothelial cell level (Singh and Agarwal, 2003).

Cell migration is a key step shared by both angiogenesis and tumor progression (Gourley and Williamson, 2000; Quiney *et al.,* 2006). Figure 6.5 shows the effects of H 1.5.5 fraction on endothelial cell (HUVEC) and migration, as determined by wound healing assay. These results clearly show that H 1.5.5 fraction is a potent inhibitor of endothelial cell migration 5 µg/ well of H 1.5.5 fraction was able to completely suppress this VEGF-induced migration of human umbilical endothelial cells.

It has been shown that VEGF, when actively secreted from tumor cells, could potentially trigger tumor angiogenesis; while, reduction of VEGF weakens its stimulation of tumor angiogenesis. I examined the effect of H 1.5.5 on VEGF secretion in the medium in which EAT were grown *in-vitro*. The ELISA analysis showed that VEGF level in the conditioned medium of EAT decreased in a dose dependent manner after treatment with 5, 10, 15, 20 and 25 mg ml-1of H 1.5.5 fraction (Fig. 6.6). The current results indicate the anti-angiogenic activity of H 1.5.5 fraction in this assay system.

The effect of H 1.5.5 on rat corneal angiogenesis *in-vivo* was examined by determining the ability of H 1.5.5 to block vascular endothelial growth factor induced angiogenesis. A vascular endothelial growth factor containing polymer disk induced 100 % angiogenesis in the implanted rat corneas. In contrast, H 1.5.5 (1 µg) completely inhibited this vascular endothelial growth factor induced angiogenesis response in all the rats' *in-vivo* (Fig. 6.7). It was observed that inhibition by H 1.5.5 fraction of the mentioned essential steps of *in-vitro* angiogenesis agrees well with the effect on *in-vivo* angiogenesis in the rat cornea model.

A polysaccharide called Sarg was isolated by Dias et al., (2008) from the brown seaweed *Sargassum stenophyllum*, collected from Santa Catarina State, Brazil. An anti-vasculogenic effect of the polysaccharide, Sarg was studied by both *in vivo* and *in vitro* assays and was demonstrated to effectively inhibit vasculogenesis as well as developmental angiogenesis in chick embryos and could trigger concomitantly with vasculogenesis a specific change in the morphogenetic pattern. However, H.1.5.5 had a more potent effect than sarg both *in vitro* and *in vivo.*

Apoptosis also seems to be a reliable marker for the evaluation of potential agents for cancer prevention. A wide variety of natural compounds appear to possess significant cytotoxic as well as chemo preventive activities. Therefore, the ability of H 1.5.5 fraction to cause apoptosis in EAT cells by AnnexinV and Propidium Iodide staining was determined. An early biochemical change characteristic of cells undergoing apoptosis was the loss of plasma membrane asymmetry, such that high levels of phosphatidylserine become exposed on the outside cell

surface. Apoptotic cells were recognized by staining with Annexin V, which bound to phosphatidylserine with high affinity (Fig. 6.8), indicating that H1.5.5 induced loss of membrane symmetry preceding commitment to apoptotic death in this system.

Gradual fragmentation of inter-nucleosomal DNA is a hallmark of apoptosis. Since, Propidium Iodide is based on the principle that apoptotic cells, among other typical features, are characterized by DNA fragmentation and, consequently, loss of nuclear DNA content, it was used to determine the apoptotic effect of H 1.5.5 on EAT cells. The PI binds to double stranded DNA by intercalating between base pairs of H 1.5.5 treated Eat cells, but is excluded from cells with intact plasma membranes of untreated EAT cells (Fig. 6.9).

The induction of apoptosis is known to be an efficient strategy for cancer therapy. The present results showed that H 1.5.5 fraction had a role in inducing apoptosis of EAT cells and exhibited a progressive increase of the random fragmentation of the DNA in a dose dependent manner (Fig. 6.10).

A fucoidan from *F. vesiculosus* reportedly inhibited the proliferation and induced apoptosis inhuman lymphoma HS-Sultan cell lines (Aisa *et al.,* 2005). Apoptotic bodies were observed in HS-Sultan 24 hr after treatment with fucoidan (100µ/ml) in fucoidan-treated cells but not in control cells (without fucoidan).To quantify the percentage of apoptosis caused by fucoidan, HS-Sultan cells were subjected to annexin-V assay. The percentage of annexin-V-positive cells was much less in control cells to that of cells treated for 48 hr with fucoidan. However the concentrations used were much higher than that of H 1.5.5 used in the present study.

The present studies clearly show that H 1.5.5 fraction from *S. marginatum* seaweed is another natural anti-angiogenic compound with a broad spectrum of targets. Its effects on tumor cells suggest that H 1.5.5 fraction also behaves as an anti-tumor compound. The differences observed between the effects of H 1.5.5 fraction on endothelial versus tumor cells open a window for its potential therapeutical application as either an anti-angiogenic or an anti-tumor drug. Therefore, more studies for final application of this alga could be important in the field of natural anti-cancer investigation as a source of single agent or combined therapy with other chemotherapeutics to support the struggle against cancer. In summary, data shown here indicate for the first time that H 1.5.5 fraction is a compound that interferes with key events in angiogenesis and cell survival, making it a promising drug for further evaluation in the treatment of cancer- related pathologies.

SUMMARY

7. SUMMARY

Cancer is the second most leading cause of death in India. Although the treatment options over the past few decades have substantially improved, the cure rate for patients with advanced cancer remains low. New anti-cancer drugs are still very much needed to improve treatment outcome of patients. Based on these concerns, many therapeutic efforts have been done so far for the treatment of cancer. Rapid advances and recent researches on the mechanisms responsible for the neoplastic disease have resulted in the identification of novel molecular targets. Among them, anti-angiogenic therapy and targeted cancer therapy are two important strategies for selective cancer eradication. In recent years, anti-angiogenic treatments have been shown to synergize with traditional chemotherapeutic and radio-therapeutic regimens. Combining anti-angiogenesis with targeted anti-proliferative and pro-apoptotic agents have also been found to be synergistic.Thus, to find the potential therapeutic anticancer drugs with potent and selective cytotoxic, pro-apoptotic and antiangiogenic properties would be valuable.

Cell-based, target-based, molecular mechanism-based screening methods add immensely to drug discovery programs and help in the identification of more selective anticancer drugs.Therefore, in this thesis an attempt was made to evaluate/ screen potential cytotoxic/ pro-apoptotic, antioxidant, anti-angiogenic activities in marine seaweeds collected from the coasts of Goa and Malwan (Maharashtra), using *in-vitro* and *in-vivo* models.

- Firstly, the cytotoxicity of 15 methanol extracts of seaweeds collected from the coast of Goa and Malwan (Maharashtra) was tested against brine shrimps and cell lines (HeLa, Hep-2 and Vero) *in-vitro*. The preliminary of methanol extracts showed that

Stoechospermum marginatum was the most cytotoxic in brine shrimp lethality assay at all concentrations tested when compared to other seaweed extracts. The extract of *S. marginatum* also showed potent cytotoxic activity against HeLa and Hep-2 cells. However, *S. marginatum* demonstrated more selectivity to HeLa and Hep-2 than the Vero cells indicating its potential for biopharmaceutical use. Giemsa, annexin v and propidium iodide staining exemplified the pro-apoptotic activity of *S. marginatum* extract against EAT cells. Further, *S. marginatum* extract confirmed its proapototic activity by inducing DNA fragmentation of EAT cells in DNA fragmentation assay. Based on the above mentioned results, the potential role of *S. marginatum* as a chemopreventive was therefore suggested.

- Next, the methanolic extracts of 14 species of marine algae were screened for antioxidant properties using 1, 1-diphenyl-2-picrylhydrazyl (DPPH), reducing power and metal chelation assays. The total phenolic contents of the seaweed extracts were also evaluated. Majority of the seaweeds demonstrated antioxidant activities to various degrees. Among the various seaweeds tested, *Stoechospermum marginatum* exhibited the highest, while *Asparigopsis taxiformis* demonstrated the lowest DPPH radical scavenging activity. The total phenolic content of the extracts varied significantly. Statistical analysis indicated a significant association between the DPPH scavenging activity and total phenolic content as well as between reducing power and total phenolic content of the seaweeds tested. The metal chelating activity was highest for *Dictopteris australis* and was comparable to the positive control EDTA at the highest concentration. A poor correlation between the total phenolic contents and the metal chelating activities was observed, indicating that

phenolic compounds might not be the main chelators of metal ions. The methanol extracts of all the seaweeds demonstrated antioxidant activities in a dose dependent manner and consecutively increased with increasing concentration of the extract. Overall, from the results of this study it could be concluded that the seaweeds are a good source of natural antioxidants.

- Further, the effects of 15 seaweed extracts was evaluated for the identification of seaweeds endowed with the most potent chemo preventive activities on the proliferation of EAT cells. Most extracts amongst brown seaweeds inhibited the proliferation of tested EAT cell line whereas extracts from green and red seaweeds were much less effective. *S. marginatum* extract (SME) showed the most effective activity and as a result was selected for further anti-angiogenic studies. The extract potently inhibited cell proliferation of BeWo cells *in-vitro* in a dose-dependent manner, but did not have much effect on the proliferation of normal HEK-293 cells. Further, SME inhibited tube formation and migration of HUVEC cells in *in-vitro* wound healing and oil droplet assays. SME was also able to suppress VEGF-induced angiogenesis in the chorio allantoic membrane, rat cornea, and tumor induced angiogenesis in the peritoneum of EAT bearing mice *in-vivo*. A decrease in the microvessel density count and CD31 antigen staining of treated mice peritoneum provided further proof of its anti-angiogenic activity. Altogether, the data underlines that the VEGF mediated angiogenesis was the target for the anti-angiogenic action of SME at the molecular level.

- As a step towards isolation of the bioactive compound responsible for the above mentioned activities, 1 kg of shade dried seaweed (*S. marginatum* seaweed) was used for the preparation of methanolic extract by keeping it overnight. This methanolic extract was then subjected to activity guided fractionation using different solvents based on polarity to yield three different fractions (Hexane, Ethyl acetate and water). The bioactive hexane fraction was further subjected to column chromatography to yield five sub-fractions. The fifth sub-fraction was bioactive and was again subjected to column chromatography to yield seven sub-fractions. All the fractions and sub-fractions were tested for anti-proliferative activity at each step of purification against HUVEC cells *in-vitro* and the most active fraction H 1.5.5, thus obtained was structurally characterized using NMR, IR, UV and MS spectras. This compound was found to be described previously in the chemical literature and featured same as that of 5 (*R*), 15, 18 (*R* and *S*) -19- tetrahydroxyspata -13, 16-(*E*)-diene, present as one of the main constituent in *S. marginatum*. It was for the first time that it's anti-proliferative of this compound was reported.

- It was previously reported that 5 (R), 15, 18 (R and S) -19- tetrahydroxyspata -13, 16-(E)-diene (H 1.5.5 fraction), found in seaweed *S. marginatum*, was bioactive. However, the cytotoxic / pro-apoptotic and anti-angiogenic effect of this compound was not fully elucidated. In this study, the effects of this compound was investigated using both *in-vitro* and *in-vivo* models. The isolated compound showed very high cytotoxic effects on HeLa and Hep-2 cell lines *in-vitro* but not on Vero cells. It also exhibited anti-angiogenic effect by inhibiting tube formation, migration of endothelial cells in wound healing, and

by inhibiting secretion of VEGF by EAT cells *in-vitro.* The compound further inhibited the angiogenesis in rat cornea assay *in-vivo* and was also confirmed to induce apoptosis in EAT cells by annexin v, propidium iodide staining and DNA fragmentation assay. Taken together, the underlying data suggests that the compound 5 (*R*), 15, 18 (*R* and *S*) -19- tetrahydroxyspata -13, 16-(*E*)-diene is a potent anti-angiogenic and pro-apoptotic compound having great potential in cancer therapy and may be of possible interest for drug development.

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