CYTOTOXIC ACTIVITY OF *SARGASSUM WIGHTII* ON PC-3 CANCER CELL-LINE

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By

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CERTIFICATE

I declare that the dissertation entitled "**Cytotoxic Activity of *Sargassum wightii* on PC-3 Cancer Cell Line**" has been prepared by me under the supervision of Dr. Felix Bast, Assistant Professor, Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

Cytotoxic Activity of *Sargassum wightii* on PC-3 Cancer Cell Line

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Finding novel antitumor compounds with low side effects could be an interesting proposal. Antioxidant and anticancer potentials of seaweed extracts can possibly be explored for developing the new anticancer drugs. Polyphenols are extensively distributed in seaweeds and these are reported to be free radical scavengers. This study was designed to check the cytotoxic potential of the methanolic and hydromethanolic extracts from the brown algae S. wightii. The total phenolic content in the hydromethanolic extract and the methanolic extract of S. wightii was determined by the Folin-Ciocalteu method. The total phenolic content in methanolic extract was found to be greater than hydromethanolic extract. The percentage inhibition or scavenging activity of both the extracts was calculated by using the DPPH assay and was more for the methanolic extract. PC-3 cancer cell-line was used as an experimental model. The methanolic crude extract is significantly cytotoxic against the PC-3 cancer cells. On the other hand the hydromethanolic crude extract was not found to be the significantly cytotoxic against the PC-3 cancer cells.

Mohd Jahid Dr. Felix Bast

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Mohd Jahid

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List of Abbreviations

Sr. No.	Full form	Abbreviation
1	Diphenyl Picryl Hydrazine	DPPH
2	White Spot Syndrome Virus	WSSV
3	Prostate Specific Antigen PSA	PSA
4	Prostate Specific Membrane Antigen	PSMA
5	Ethylene Diamine Tetra Acetic Acid	EDTA
6	National Centre for Cell Science	NCCS
7	Fetal Bovine Serum	FBS
8	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide	MTT
9	PCa Cell Line-3	PC-3
10	Total Phenolic Content	TPC
11	Butylated hydroxyl toluene	BHT
12	World Health Organisation	WHO
13	Over The Counter	OTC
14	Traditional Complementary and Alternative Medicine	TCAM
15	Hydromethanolic Extract	HME
16	Hydromethanol	HM
17	Amylogalactosidase	AMG
18	Cyclosporine A	CsA
19	Kirtan Ras	K Ras
20	Dimethyl sulfoxide	DMSO
21	American Type Culture Collection	ATCC

Chapter – 1

Introduction

Cancer is a global problem and the leading cause of deaths throughout the world. According to World Health Organization (WHO) cancer factsheet, in 2008 about 7.6 million deaths (around 13% of all deaths worldwide) occurred because of cancer malignancy (Jemal et al., 2011). Cancer is a fatal disease in which a cell, or a group of cells display uncontrolled proliferation i.e. division beyond the normal limits, invasion i.e. intrusion and distortion of adjacent tissues and metastasis, spreading from one part to another part in the body through lymph or blood. These three malignant properties of cancers differentiate them from benign tumors. Benign tumors are self-limited and do not invade or metastasize while malignant tumors are not self-limited and metastasize. Cancer is a great tragedy to human that affects people at all ages but the risk to the most types of cancer increases with age.

Since ancient time, nature is an important source of medicinal compounds - a fact which is illustrated by the large number of natural products currently in use in medical practice. These products have been recognized and developed through traditional knowledge of the medicinal properties of plant and animal products. The medicinal products obtained from the plants have been used for medicinal purposes in India, China, Nepal and Europe, since ancient times (Shoeb, 2008). Ayurvedic system is one of the most ancient traditional practice that is widely practiced in India, Sri Lanka and other countries (Chopra & Doiphode, 2002). Oceans are important source of pharmaceutically important organisms, such as bacteria, cyanobacteria, fungi, microalgae, seaweeds, and other halophytes. These constitute most of the oceanic biomass. While the marine ecosystem covers more than 70% of the earth's surface, it represents 95% of the biosphere (Schaufelberger et al., 1991). Life on our planet was born in ocean and a number of biologically active compounds with varying degrees of action such as anti-cancer, anti-microtubule, anti-proliferative, cytotoxic and photo-protective activities have been isolated to date from marine sources. It is well established that with 3.5 billion years of existence on earth and evolving biosynthesis, the marine floras remain nature's best source of chemical entities. The studies have clearly demonstrated that the marine environment is an excellent source of novel

chemicals and these chemicals are not found in terrestrial sources. More than 10,000 compounds have been isolated from marine organisms with hundreds of new compounds are being discovered every year (Kathiresan et al., 2008). Thus, potential antioxidant and anticancer properties of plant extracts or isolated products of plant origin can possibly be explored for developing the anticancer drugs (Kaur & Kapoor, 2002). The National Cancer Institute (NCI) of the United States of America (USA) has screened about 1,14,000 extracts from an estimated 35,000 plant samples against a number of tumor systems (Cragg & Boyd, 1996).

The anticancer activity is one of the most important properties in marine natural products and a number of algae have shown potent cytotoxicity. A number of studies have been done on anticancer activity of marine natural products in the eastern countries of Asia (Xu et al., 2004). Many antineoplastic compounds from algae such as Halomon, had been progressed to the clinical phase (Egorin et al., 1996). For more than a millennium, seaweeds have been integral part of the culinary in many countries. The human consumption of green algae (5%), brown algae (66.5%) and red algae (33%) is high in Asia, mainly Japan, China and Korea (Marinho-Soriano et al., 2006). Now the demand for seaweeds as food has extended to North America, South America and Europe (Manivannan et al., 2009).

Seaweeds such as Ecklonia kuroma and Sargassum fusiforme have been used in traditional Chinese herbal medicine in treatment of tumors (Xu et al., 2004). Seaweeds have gained special attention as a source of natural antioxidants (Yamada & Matsukawa, 1999). Mechanism of antitumor activity of Sargassum fusiforme has been investigated in China (Zandi et al., 2010). Sulfated polysaccharides of Sargassum polycystum, S. oligocystum, S. mcclurei, S. wightii and S. denticaprum have demonstrated considerable antitumor activity (Ly et al., 2005). Chemical constituents of seaweeds are extraordinarily rich in biologically active and medicinally potent chemicals. In the extracts of these species polyphenols, alkaloids and polysaccharides are the most predominant group of compounds showing antioxidant and anticancer activities. Seaweeds contain a good amount of polyphenols such as phenolic acids, flavonoids, anthocyanidins, lignin, tannins, catechin, epicatechin, epigallocatechin and gallic acid (Bandaranayake, 2002). Sulfated polysaccharides present in seaweeds can enhance the innate immune response (Zhou et al., 2005). Alkaloids obtained from

marine algae mostly belong to the phenylethylamine and indole groups (Sithranga et al., 2011). Sargassum wightii is a brown seaweed and also one of the prominent sources of alginic acid. In the present study the crude extracts of S. wightii in hydromethanol and methanol were used to study the cytotoxic activity on PC-3 cancer cell-line.

Chapter – 2
Review of literature

2.1. Need of natural products

While scientists throughout the world have desperately been working for many decades, no potent medicine is available for such an alarming disease like cancer. A limited number of anticancer drugs are currently in clinical trials. Most of the chemotherapeutics have long-lasting side effects such as nausea, vomiting, diarrhea, skin rashes, headache, anorexia, alopecia and renal dysfunction (Cohen & Stadler, 2001). The need for side-effect free, affordable and effective drug to combat this dreaded and alarming disease is therefore imminent. Natural products continue to be a major source of pharmaceuticals and for the discovery of new molecular targets (Schaufelberger et al., 1991).

The medicinal products derived from plant resources have played a crucial role in health care of ancient and modern cultures including Ayurveda (Imhoff et al., 2011). Natural products, especially from the plants, have been used for the treatment of various diseases from time immemorial. The medicines from the plants have been used in Egypt, China, India and Greece from ancient times and an impressive number of modern drugs have been developed from them (Shoeb, 2008). The sources and number of anticancer and anti-infective agents reported in annual reports of medicinal chemistry from 1984 to 1995 indicate that more than 60% of drugs developed and approved for the diseases such as cancer, can mark out their lineage back to natural products (Rocha et al., 2002).

2.2. History of natural products used as therapeutics

Traditionally natural products have played a significant role in the field of drug discovery (Butler, 2005). The first written records on the plants, which have been used for the medicinal purpose, came into the existence about 2600 BC from the Sumerians and Akkaidians. The best acknowledged Egyptian pharmaceutical record is “Ebers Papyrus”, which documented over 700 drugs and represents the history of Egyptian medicine dated from 1500 BC (Newman et al., 2003). “Materia Medica”, the Chinese documentation, describes more than 600 medicinal plants

and it has been well recognized with the first record dating from about 1100BC (Cragg & Newman, 2005). Ayurvedic system is one of the most ancient system and up till now exists as traditional practice widely in India, Sri Lanka and other countries (Chopra & Doiphode, 2002).

Millions of Indians use herbal drugs and formulations regularly in daily life, as spices, home-remedies, health foods and tonics as well as over-the-counter (OTC) as self-medication (Vaidya & Devasagayam, 2007). Globally, there is a positive trend towards holistic health and integrative sciences. (Patwardhan et al., 2005). It is observed that in a number of developed countries a considerable number of people commonly use some form of traditional complementary and alternative medicine (TCAM). In Germany about 75% of people use the TCAM (Kayne, 2010). In Canada 70% people rely on TCAM for the health care (Hollenberg, 2006). In England about 47% of people show their interest in using TCAM (Thomas et al., 2001).

In this context, the natural products extracted from medicinal plants have gained significance in the treatment of cancer. According to the World Health Organization (WHO), 80% of the world's population primarily those of developing countries depend on plant derived medicines for the health-care (Gurib-Fakim, 2006). Approximately 60% of drugs approved for cancer treatment such as vinblastin, vincristin, taxane, etoposide and camptothecins are of natural origin (Atkins et al., 2000). Majority of chemo-preventive and chemotherapeutic drugs for the cancer treatment are from the plant origin (Goldin et al., 2007). Herbal drug formulations for the prevention and treatment of cancer has increased over the last decades and is continuously increasing (Balunas & Kinghorn, 2005).

2.3. Ocean source of biomedical compounds

It is considered that life is originated in the ocean and during the evolution, marine organisms have developed into very sophisticated physiological and biochemical systems. The interesting fact is that the biodiversity in the marine environment far exceeds that of the terrestrial systems but the research involving the use of marine natural products as pharmaceutical agents is still in its infancy and this situation may be due to the lack of ethno-medical history and the difficulties involved in the collection of marine organisms (Jha & Zi-rong, 2004).

The correlation between the ethno- medical usage of medicinal plants and modern medicines discovered from the plants has been studied by Fabricant and Farnsworth (2001).

The oceans are described to cover more than 70% of the world surface and among 36 known living phyla, 34 of them are found in marine environments including more than 300,000 known species of fauna and flora (Butler & Carter-Franklin, 2004). So far during the past decade, over 5000 novel compounds have been isolated from shallow waters to 900-m depths of the sea (Jha & Zi-rong, 2004). The ocean is affluent in the biodiversity and it is found that the micro flora and microalgae alone comprise more than 90% of oceanic biomass (Boopathy & Kathiresan, 2011). So the opportunity to discover new drugs from this vast marine flora is greater and this offers a new scope to researchers to find out different drugs and formulations. It is incessantly recognized that there are a lot of natural products and noble chemical molecules present in ocean. The entities with exclusive biological activities may be valuable as potential drugs with greater effectiveness and specificity for the treatment of human diseases (Haefner, 2003). The novel chemicals produced by the marine organisms have the capacity to withstand extreme variations in ambient surroundings like pressure, salinity and temperature (Boopathy & Kathiresan, 2011).

The marine environment has proved to be an abundant source of structurally novel bioactive agents and several have advanced to clinical development as potential anticancer agents (Taskin et al., 2010). The systematic exploration of marine environments is reflected in the large number of novel compounds reported in the literature over the past decade (Mayer & Gustafson, 2003). Before three to four decades, the collection of marine organisms was restricted to those found in intertidal and shallow sub tidal environments. The introduction of scuba diving has enabled investigators to explore shallow sub tidal environments to a depth of 40 m for 15 min with no decompression stops and now the depths of up to 200 m are easily reached using closed-circuit computerized mixed gas rebreathers (Amador et al., 2003). The research related to drug discovery from marine organisms has been accelerating and involves the interdisciplinary research including biochemistry, biology, ecology, organic chemistry and pharmacology (Haefner, 2003).

2.4. Anticancer compounds from seaweeds

In the field of biomedical science, seaweeds have attracted the interest of the researchers mainly due to their bioactive substances to be used as antioxidant, antimicrobial, antiviral and anti-tumor drugs (Taboada et al., 2010). Marine flora have been used for medicinal purposes in India, China, Nepal and Europe since ancient times. The people of China and Japan consume seaweeds as food. The traditional Japanese diet includes seaweeds that are commonly used as sushi wrappings, seasonings, condiments, vegetables and can thus comprises between 10% to 25% of food intake by most of the Japanese (Skibola, 2004). In Japan and China the prevalence of prostate cancer rate in one year is 10.4 and 0.7 while that in North America and Europe is 117.2 and 53.1 (Parkin et al., 2005). It is estimated that about 90% of the species of marine plants are constituted by algae and about 50% of the global photosynthesis is contributed by algae (Ganesan et al., 2008). According to an approximation, in both inter-tidal and deep water regions of the Indian coast there are about 841 species of marine algae (Oza & Zaidi, 2001). Most algae grow in the inter tidal zone so these have to face the unfavorable environmental conditions such as harsh fluctuations in oxygen concentrations, UV radiation, temperature and therefore, it is not surprising that the bioactive compounds harvested from these algae are primarily antioxidants such as phlorotannins, carotenoids, tocopherols, and ascorbic acid (Zubia et al., 2009). The first record that a drug from algae was used to treat the breast cancer in western medicine dates back to the 1960's. This drug was an important mile stone in the discovery of drugs from the algae, known as ''algasol T331'' (Folmer et al., 2010). It is remarkable that about 6% of the patents published on marine natural products between 1999 and 2003, are algal secondary metabolites (Abad et al., 2008).

The classification of seaweeds may be done as into Rhodophyta (red algae), Phaeophyta (brown algae) and Chlorophyta (the green marine macro algae). Seaweeds are considered the extraordinary source of the vitamins, minerals, proteins and other biologically important substances and therefore the extracts of the seaweeds may have excellent anticancer properties (Mans et al., 2000). Edible seaweed such as Palmaria palmata exhibits the antioxidant and anticancer properties (Yuan et al., 2005). The seaweeds Acanthaphora spicifera,

Ulva reticulata, Gracilaria foliifera, and Padina boergesenii of the Gulf of Mannar region are reportedly exhibiting cytotoxic activity in their alcoholic extracts (Vasanthi et al., 2004). Fucoidans exhibit antitumour, anticancer, antimetastatic and fibrinolytic properties in mice (Religa et al., 2000).

The seaweeds have been reported to contain high amount of polyphenolic compounds such as catechin, epicatechin, epigallocatechin gallate and gallic acid (Yoshie et al., 2002). Sargassum thunbergii is a brown algae and its extract shows the antitumor activity (Zhuang et al.,1995). It was also reported to inhibit the tumor metastasis in the rat mammary adeno carcinoma cell i.e.13762 MAT (Coombe et al., 1987). Ascophyllum nodosum has shown the antiproliferative effect on normal as well as malignant cells i.e. hamster kidney fibroblast CCL39, sigmoid colon adenocarcinoma cells (COLO320 DM) and smooth muscle cells (Vischer & Buddecke, 1991). Stylopoldione is a metabolite of Stypodium sp. and it is a potent cytotoxic metabolite, which halts mitotic spindle formation (Gerwick & Fenical, 1981). The compound Condriamide-A is isolated from Chondria sp. and it shows the cytotoxicity towards the human nasopharyngeal and colorectal cancer cells (Brunelli et al., 2000). The chemical entity Phloroglucinol and its different polymers named as eckol (trimer), phlorofucofuroeckol A (pentamer), dieckol, and 8,8-bieckol (hexamers) are isolated from the brown alga Eisenia bicyclis and these compounds exhibit the antioxidant activity (Shibata et al., 2002). Five different types of carbohydrates such as AMG (Amylogalactosidase), celluclast, termamyl, ultraflo, and viscozyme have been used to hydrolyze the brown alga Eclonia cava to produce enzymatic extracts and these all have shown to be potential natural water-soluble antioxidants with dose dependent radical scavenging activities (Heo et al., 2005).

2.5. Chemical constituents of seaweeds

Seaweeds are extraordinarily rich in biologically active and medicinally persuasive chemical substances. In the extracts of these polyphenols, alkaloids and polysaccharides are the principal groups of compounds and these compounds are thought to be responsible for the antioxidant and antiproliferative activities.

2.5.1. Polyphenols

Polyphenols are extensively distributed in seaweeds and these are reported to exhibit free radical scavenging, antimicrobial and anticancer activities (Pinilla et al.,2005). Plants such as seaweeds contain a good amount of polyphenols such as phenolic acids, flavonoids, anthocyanidins, lignin, tannins, catechin, epicatechin, epigallocatechin, and gallic acid (Bandaranayake, 2002). These polyphenolic compounds have revealed many health benefits such as antioxidant, anticancer and anti-inflammatory activities (Mohsen & Ammar, 2009). A number of studies have revealed a positive correlation between the increased dietary intake of natural antioxidants and decreased cancer mortality as well as longer life expectancy (Math et al., 2011). The phenolic compounds show the metal chelating property and these are the natural metal chelaters with high antioxidant activity (Ferrari, 2004). Previous reports suggest that polyphenols may stimulate *α*-tocopherol production through reduction of the *α*-tocopheroxyl radical (Bors et al.,1990).

A close association has been reported between anti-carcinogenic activity and antioxidant activity in a chemically induced mouse carcinoma system with the low molecular weight polyphenolic compounds (Makita et al., 1996). Earlier reports suggest that polyphenols found in marine algae, the phlorotannins, which are only known to occur in brown algae and are constrained to polymers of phloroglucinol (1,3,5-trihydroxybenzene) (Heemst et al., 1996). Polyphenolic compounds are proposed to inhibit cancer cells by the biotransformation of xenobiotic metabolizing enzymes that alter metabolic activation of potential carcinogens. Some flavonoids are reported to modify hormone production and inhibit aromatase enzyme to prevent the development of cancer cells (Zhao et al., 2007). Cellular protein and mitotic index are diminished by the phenolics and therefore decrease the colony formation during cell proliferation of cancer cells (Gawron & Kruk, 1992). There are many studies which show a strong correlation between the antioxidant activity and the total phenolic content (Wang et al., 2009).

2.5.2. Polysaccharides

The polysaccharides present in the algae are mainly the sulphated polysaccharides (Okai et al., 1998). There are several studies which demonstrate that algal sulfated polysaccharides enhance the innate immune response through tumoricidal activities of macrophages and natural killer cells (Zhou, et al., 2005). Many studies show that sulfated polysaccharides can increase the adaptive immune response (Choi et al., 2009). Some recent studies show that sulfated polysaccharides can increase the proliferative response of T lymphocytes by binding to CD2, CD3 and CD4 in T lymphocytes (Miao et al., 2005). B-1, the sulfated polysaccharide isolated from the culture filtrate of marine Pseudomonas sp., induces apoptosis of human leukaemic cells (U937) (Matsuda et al., 2003). A sulphated polysaccharide glycosaminoglycans hinders with transcription function and consequently induces apoptosis of murine melanoma cells (Berry et al., 2004). Fucoidan is one of the representative sulfated polysaccharides i.e. sulphated L-fucose and it is derived from the cell wall of brown algae (Berteau & Mulloy, 2003). It is reported that fucoidan induces apoptosis in human lymphoma HS-Sultan cell-lines and this induced apoptosis is accompanied by the activation of caspase-3 and down-regulation of extracellular signal regulated kinase pathway (Aisa et al., 2005). Fucoidan can also alter the clinically important phenomena such as angiogenesis and tumor metastasis (Boisson et al., 2007).

2.5.3. Alkaloids

First of all the term alkaloid was proposed by Meissner in 1819 to characterize these “alkali-like” compounds found in plants. Alkaloid chemistry and the anticancer potential of alkaloid have been explored with immense interest by the researchers in terrestrial plants, but the number of studies in marine plants is limited. Alkaloids in marine algae are somewhat rare, when compare with terrestrial plant alkaloids. Structurally the alkaloids obtained from marine algae, mostly belong to the phenylethylamine and indole groups (Boopathy & Kathiresan, 2011). Hordenine was the first alkaloid isolated from marine algae in 1969 (Percot et al.,2009).

2.6. *Sargassum wightii*

Domain	Eukaryota
Kingdom	Chromista
Phylum	Ochrophyta
Class	Phaeophyceae
Order	Fucales
Family	Sargassaceae
Genus	Sargassum
Species	wightii

Fig. 1: Sargassum wightii

Sargassum is a genus of brown algae in the order Fucales and it is a marine subtidal macroalga i.e. seaweed. Many species are distributed throughout the temperate and tropical ocean of the world where they generally inhabit the shallow water and coral reefs. However the genus is known for its free floating species. While many species within the class Phaeophyceae are primarily cold water inhabitant, genus Sargassum appears to be an exception to this general rule. Atlantic Ocean's Sargasso Sea was named after this alga, as it hosts a large amount of Sargassum. Sargassum was named by the Portuguese sailors who found it in the Sargasso sea after that a species of rock rose, that grew in their water wells and that was called sargaco in Portuguese.

In India S. wightii is distributed in Kerala and south east cost such as Rameshwaram, Tamil Nadu. S. wightii is considered a good raw material for the commercial extraction of alginic acid in Kerala.

2.6.1. Morphological features

Sargassum wightii has the appearance of flowering plants and it has slender branching system. The wide blades are with midrib. Blades are usually once divided and with inflated air bladders or vesicles. The air bladders are sub-spherical or ovoid. Stipes bear radially or distichously long primary branches. The primary branches are terete, angular, compressed or with three-side axes and relatively narrow. Homogenous parenchyma can be observed with stipe cross-

section. Holdfast is discoid-conical and no place for haptera. These are the characteristic features of genus Sargassum. The petioles are flat and the leaves, egg shaped. Thallus is monoecious or dioecious and receptacles unisexual or bisexual. These features are species delineating characteristics for S. wightii.

2.6.2. Application

The crude extract of S. wightii contains plant growth regulators such as cytokinin and auxins (Sridhar, 2011). When Penaeus monodon (Giant tiger prawn) was fed with fucoidan of S. wightii -supplemented diet, the innate immunity was enhanced and increased the resistance against White Spot Syndrome Virus (WSSV) infection (Immanuel et al., 2012). One study suggested that the sulphated polysaccharides obtained from S. wightii had therapeutic potential in Cyclosporine A (CsA) - induced liver injury (Josephine et al., 2008). Dioctyl phthalate isolated from the chloroform–methanolic extract of S. wightii is a potent antibacterial compound (Sastry & Rao, 1995).

2.7. Prostate cancer cell-line

While there are several established prostate cancer cell-lines available, most of the research on prostate cancer is centered on LNCaP, DU-145 and PC-3 cell-lines. PC-3 and DU-145 are classical cell-lines of prostate cancer (Hsieh & Wu, 1999). These are derived from metastasis and therefore do not have the original prostate cancer phenotypes. LNCaP is androgen-responsive human prostate cancer cell-line and DU-145, PC-3 and JCA-1 are androgen-nonresponsive. The PC-3 cells have higher metastatic potential in comparison to the DU-145 cells and DU-145 have a moderate metastatic potential (Pulukuri et al., 2005).

2.8. PC-3 cancer cell-line as an experimental model

PC-3 cell-line is derived from a bone metastasis of a grade IV prostatic adenocarcinoma, originated from 62-year-old male Caucasian. PC-3 cell-line is non- responsive to androgen, p53 negative and K-Ras mutated human prostate

cancer cell- line. These exclusive characteristics make this cell-line less prone to the interference of androgens such as testosterone, tumor suppression and Ras gene activation. The PC-3 cell-line expresses Prostate Specific Antigen (PSA). These have low testosterone-5-alpha reductase activity and are prostate specific membrane antigen (PSMA) negative (Ghosh et al., 2005).

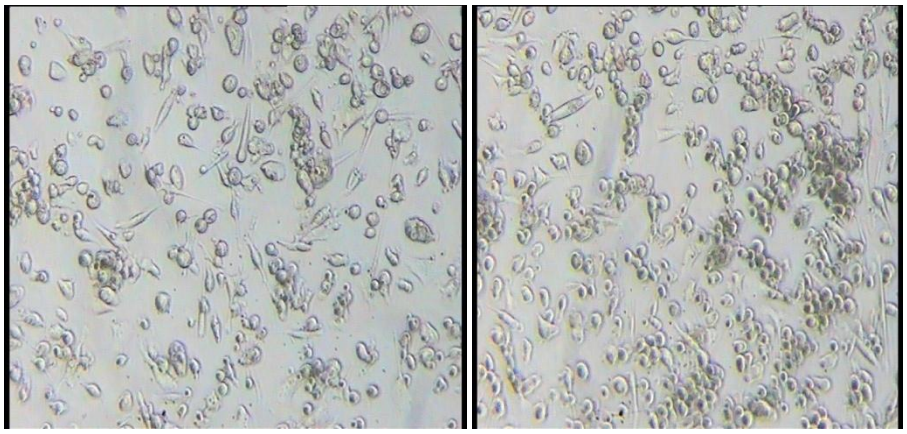


Fig. 2: 10X inverted microscope image of PC-3 cell- line

Chapter -3
Objectives and hypothesis

Following are the objectives of the study -

- To get the crude extract of S. wightii in hydromethanol and methanol
- To determine the total phenolic content in methanolic and hydromethanolic extract
- To determine the percentage scavenging activity of hydromethanolic and methanolic extracts by DPPH antioxidant assay
- To check the cytotoxicity of crude methanolic and hydromethanolic extracts

The polyphenolic compounds are the antioxidants and these scavenge the free radicals. The crude extract of S. wightii contains the polyphenols and other antioxidant compounds. So the crude extracts of methanol and hydromethanol may reduce the viability of the prostate cancer cells.

Chapter-4
Materials and methodology

4.1. Materials

1. Algal sample

The brown algae S. wightii was collected from the cost of Mandapam, Rameshwaram (Tamil Nadu). The sample was identified based on morphology.

2. Cell culture medium

Dulbecco's Modified Eagles Medium (DMEM, Gibco, Invitrogen, India.) was prepared as per the manufacturer's instructions.

3. Cell-lines

PC-3 cell-lines (passage number -17) were obtained from NCCS Pune.

4.2. Methodology

4.2.1. Extraction process

The sample of brown algae S. wightii was collected from the cost of Mandapam, Rameshwaram (Tamil Nadu). Calcareous and sand particles and other associated organisms like polychaetes, arthropods and small bivalves attached to the algae were removed by washing with the seawater and were kept in plastic bags without adding any preservatives. The sample was brought to the laboratory and again washed four to five times thoroughly with freshwater in order to remove the salt contents and other debris on the outer surface of the algal sample. After this the sample was dried in shade. The dried sample was obtained in powdery form by grinding it mechanically in grinder.

4.2.2. Preparation of methanolic and hydroalcoholic extracts

The extraction of S. wightii is done according to the Malik and Singh (1980) protocol. The extraction was done in two different solvents hydromethanol (water: methanol = 1:1) and methanol. One gram sample of powdered S. wightii was centrifuged with 10ml of each hydromethanol and methanol at 4°C and at 3000 rpm for 10 minutes. This centrifugation process was repeated three times. After this the crude extracts were filtered through Whatman filter No1. Further the filtrate was concentrated in the vacuum concentrator (Concentrator Plus, Eppendorf, Germany). The hydromethanolic extract was concentrated at aqueous mode and the methanolic extract was concentrated at alcoholic mode. The weight of dried crude extracts was noted down and kept at -20 °C for the experimental purpose.

4.2.3. Total phenolic content

The total phenolic content of S. wightii in the hydromethanolic extract and the methanolic extract was determined by the Folin- Ciocalteu method (Maurya & Singh, 2010). Gallic acid was used as standard and different concentrations of gallic acid were prepared in the methanol. Dry crude hydromethanolic and methanolic extracts were dissolved in methanol and hydromethanol to prepare

separate 0.5 mg/ml concentrations. 0.5 ml volume of each sample was taken in separate test tubes. Folin - Ciocalteu reagent was diluted 10 times with distilled water and 2.5 ml of this was added to the tubes. After this 2.0 ml of sodium carbonate (7.5%) was added in the tubes. The tubes were covered with parafilm and incubated for thirty minutes at the room temperature. The absorbance was read on spectrophotometer (Shimadzu, Japan) at 760 nm. All the readings were taken in triplicates. The results were presented in the term of percentage dry weight of sample.

4.2.4. DPPH assay

The free radical scavenging activity of S. wightii extract was estimated by the method of Liyana- Pathirana and Shahidi (2005) using diphenyl picryl hydrazine (Xu et al., 2010). Butylated hydroxyl toluene (BHT) was used as standard. Methanol was used to prepare 0.135 mM DPPH solution. 1.0 ml of this solution was taken in test tubes and 1.0 ml of each hydromethanolic and methanolic extracts was added into different test tubes. The tubes were vortexed and covered with aluminium foil. After this the reaction mixture was kept in dark at room temperature for thirty minutes and the readings were taken using the spectrophotometer at 517 nm.

The percentage inhibition or scavenging activity can be calculated by the following formula-

(%) inhibition = [ABSORBANCE control - ABSORBANCE sample) / ABSORBANCE control] × 100

Where ABSORBANCE control = Absorbance of DPPH radicals and Methanol

ABSORBANCE sample = Absorbance of DPPH radicals and standard or Sample extracts

4.2.5. Preparation of culture medium

The Dulbecco's Modified Eagles Medium (DMEM) was prepared as following-

- 45 ml of liquid DMEM was dispensed in 50 ml falcon tube.

- It was mixed with 5 ml filtered foetal bovine serum (FBS) (10%) (Gibco, Invitrogen, India).
- 50 µl of either of the antibiotics penicillin/ streptomycin (Gibco, Invitrogen, India) was added to the medium.
- The sterilized media was filtered through a 0.2 µm filter disc and it was stored at 4°C.

4.2.6. Propagation of PC-3 cell-lines

PC-3 cells were grown in T-25 and T-75 flasks (Orange Scientific, Borosil, India). The flasks had Dulbecco's modified Eagles medium with 1% L-glutamine, 0.2% penicillin/streptomycin and was supplemented with 10% heat inactivated FBS. The culture was maintained at 37°C with humidified 5% CO_2 environment in CO_2 incubator.

4.2.7. Revival of cancer cell-line

The cryovials containing the frozen cells were taken out from the liquid nitrogen and thawed subsequently in order to maintain the viability and quick recovery of the cells. The vials were washed with 70% alcohol and the caps of vials were loosened to release the internal pressure and trapped residual liquid nitrogen. The cap of cryovials was firmly closed and kept in water bath at 37°C. After thawing, the whole content of the cryovial was pipetted out into a sterile centrifuge tube. Then media (at 37°C) was added to that tube and centrifuged at 1200 rpm for 5 minutes. The supernatant was then discarded without disturbing the pellet. The cells were re-suspended in growth media and then transferred to T-25 and T-75 culture flasks. The flasks were incubated at 37°C in humidified 5% CO_2 for 24 hours. The media was changed as soon as its colour changed, till the cells were confluent to about 70% of the flask.

4.2.8. Sub-culturing of the cells

Once the cells were confluent to about 70%, the cells were sub-cultured using the following protocol-

- Old media was removed from the flask by using a sterile pipette.

- 1-2 ml of trypsin-EDTA was added and left the flask for 5 minutes to detach the adhered cells from the surface and swirled the solution across the flask to make sure that trypsin reached to all the cells.
- Fresh culture medium containing FBS was added to flask to inactivate the trypsin.
- Cell suspension was transferred to centrifuge tubes and then centrifuged at 1200 rpm for 5 minutes.
- The supernatant was discarded and the pellet re-suspended into the new media.
- About 10^5 cells was transferred in a new flask and incubated at 37°C in 5% CO_2 environment.

4.2.9. Cell counting

A suitable concentration of cells is needed in cell suspension for the seeding purpose. Cells were counted using the haemocytometer. Cover slip and haemocytometer slide were washed and made clean by using 70% ethanol. The cover slip was fixed over the counting chambers. About 10µl of the cell suspension was loaded in each chamber with the help of micropipette. 10X objective of the microscope was used to focus the cells in haemocytometer. Cells were counted based on the total number of cells within the defined area beneath the cover slip. The total number of cells was calculated by the following formula-

Cells/ ml = Average of Total Number of Cells Counted in Four Corner squares x 10^4

Total number of cell = Cells/ml x Total Volume of Cell Suspension

4.2.10. Cryopreservation of cell-lines

The cryopreservation is needed to keep the cells at lower passage number and to store for further use. For the cryopreservation of the cells, these were centrifuged at 1200 rpm for 5 minutes. After this the cells were pelleted down and the supernatant was discarded. About $2x10^8$ cells were resuspended in sterile cold freezing media (Invitrogen, India) which contained 95% FBS and 5% DMSO. After

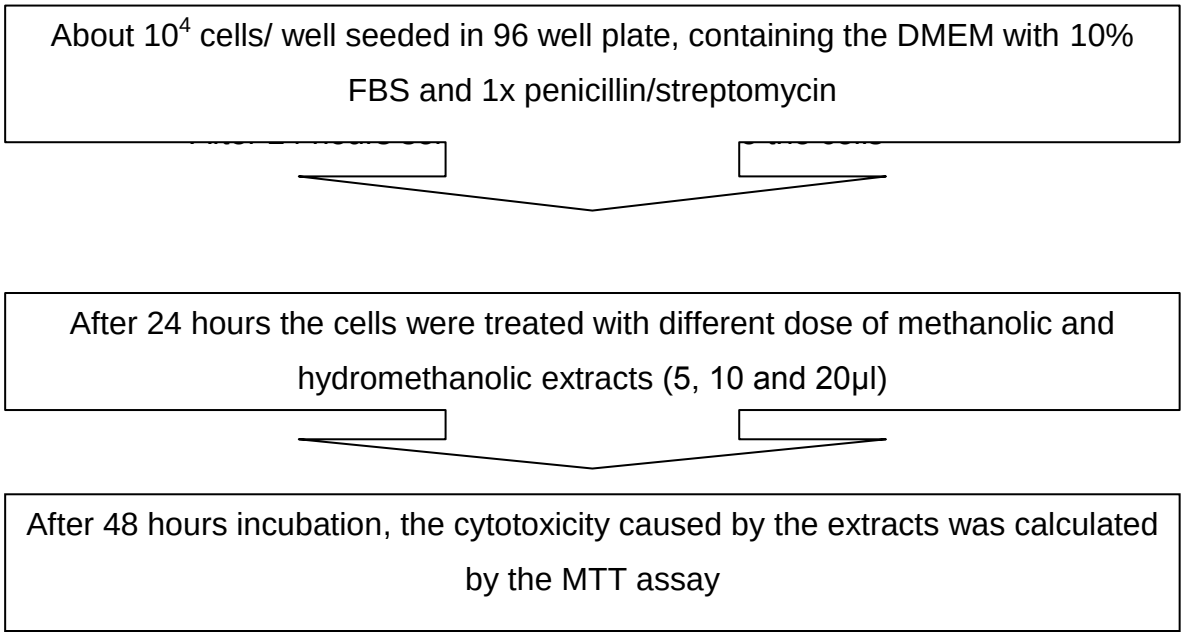
this the cell suspension was aliquoted and stored in cryovials. These cryovials were insulated with propanol freezing canister and kept in -70°C deep freezer for overnight and transferred in liquid nitrogen.

4.2.11. Seeding of cells

About 10^4 cells per well were seeded in the media containing 10% FBS and 1x penicillin/streptomycin in 96 well plate and then incubated for 24 hours at 37°C in order to get the cells to be adhered. After 24 hours, the complete media of cells was replaced with the serum free media (Serum starvation). After 24 hours the cells were given the treatment.

4.2.12. Treatment with extracts

Different dose (5, 10 and 20µl) of 250 µg/ml of methanolic and hydromethanolic extracts were prepared and the cells were treated with these extracts. To calculate the actual cytotoxicity caused by the extracts, methanol and hydromethanol (water: methanol) were used as the vehicle control for the methanolic and hydromethanolic extracts respectively. The Cells were treated with the extract using the following protocol-



4.2.13. Mitochondrial tetrazolium test assay (MTT)

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) is a yellow tetrazolium salt and this colorimetry-based assay was originally described by Mosmann (1983). It is a useful method for the measurement of the in vitro cytotoxicity. This method is widely used for screening and assessment of the anticancer drugs. This assay has been demonstrated as a sensitive, convenient, precise, economical and rapid test by many studies (Hongo et al.,1990). Denizot reported that MTT assay depends on both the number of cells present as well as the mitochondrial activity per cell (Denizot & Lang, 1986). These crystals are solubilized by the addition of a detergent.

The MTT assay was performed by Papageorgiou protocol (Papageorgiou et al., 2008). 100 µl of filtered, sterilized MTT (concentration 5 mg/ml in PBS) was added to each well and plates were incubated for 4 hours at 37^{o}C. To dissolve the formazan crystals, 100µl DMSO was added to each well. The absorption was read on microplate reader at 490 nm. Thus a linear relationship between the cell number and absorbance was established. The results were prepared in six replicates.

4.2.14. Calculation and statistics

All the experiments were performed in a completely randomized block design and performed twice. The data collected from dose response study was subjected to one way ANOVA with Tukey's test.

Chapter -5

Results

5.1. Total Phenolic Content

The methanolic and hydromethanolic extracts of S. wightii showed different amount of total phenolic content in various extracts.

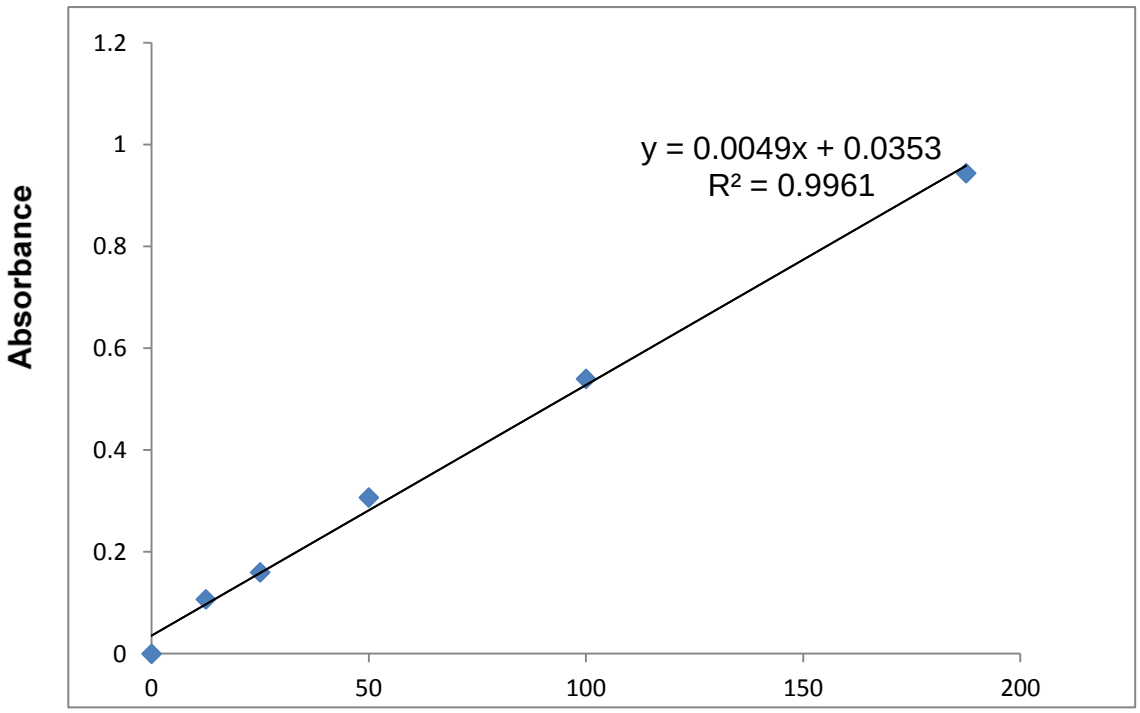


Fig.3: Gallic acid standard curve

The amount of total phenolics in methanolic and hydromethanolic extracts was determined by the Folin-Ciocalteu method. Gallic acid was used as a standard compound and the total phenolic content was expressed as percentage dry weight of sample. The calculation was done by using the standard curve equation:

$$y = 0.0049x + 0.0353, R^2 = 0.9961$$

Where y is absorbance at 760 nm and x is total phenolic content.

The total phenolic content in methanolic and hydromethanolic extracts was 5.34% ± 0.23 and 4.56% ± 1.62 [Mean ± SD (N=6)] respectively.

5.2. Antioxidant assay

The antioxidant activity of the extracts was determined by the DPPH assay.

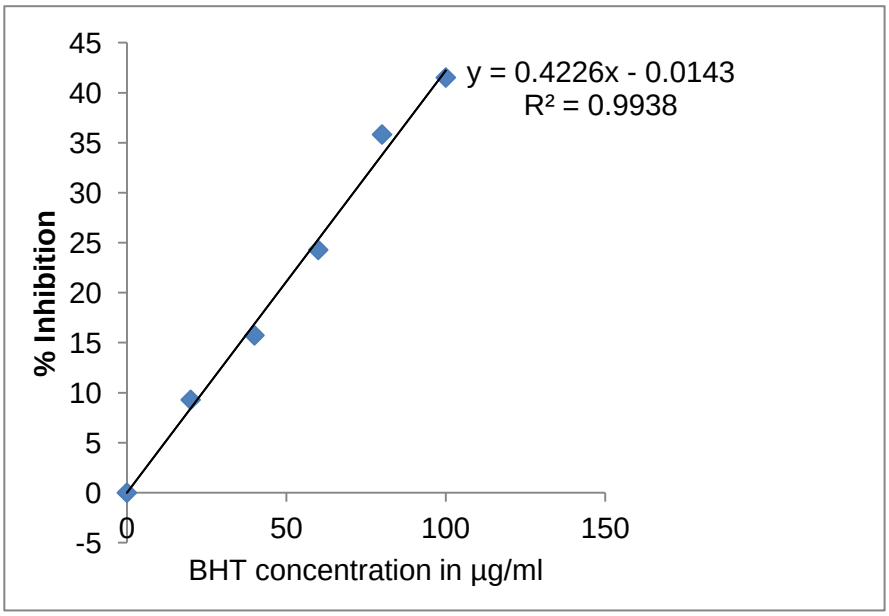


Fig. 4: DPPH scavenging activity

The antioxidant activity of the methanolic and hydromethanolic extracts was determined by using the DPPH. BHT was used as a reference compound. The percentage inhibition or scavenging activity of the methanolic and hydromethanolic extracts was calculated by using the following standard curve equation:

$$y = 0.4226x - 0.0143, R^2 = 0.9938$$

The percentage inhibition or scavenging activity of the methanolic and hydromethanolic extracts was 36.28% ± 1.18 and 22.94% ± 0.79 [Mean ± SD (N=6)] respectively. Thus it was observed that methanolic extract of S. wightii showed more potent antioxidant activity as compared to the hydromethanolic extract.

5.3. Cytotoxic activity of methanolic extract

The percentage viability curve against the different volumes of extract and methanol is plotted.

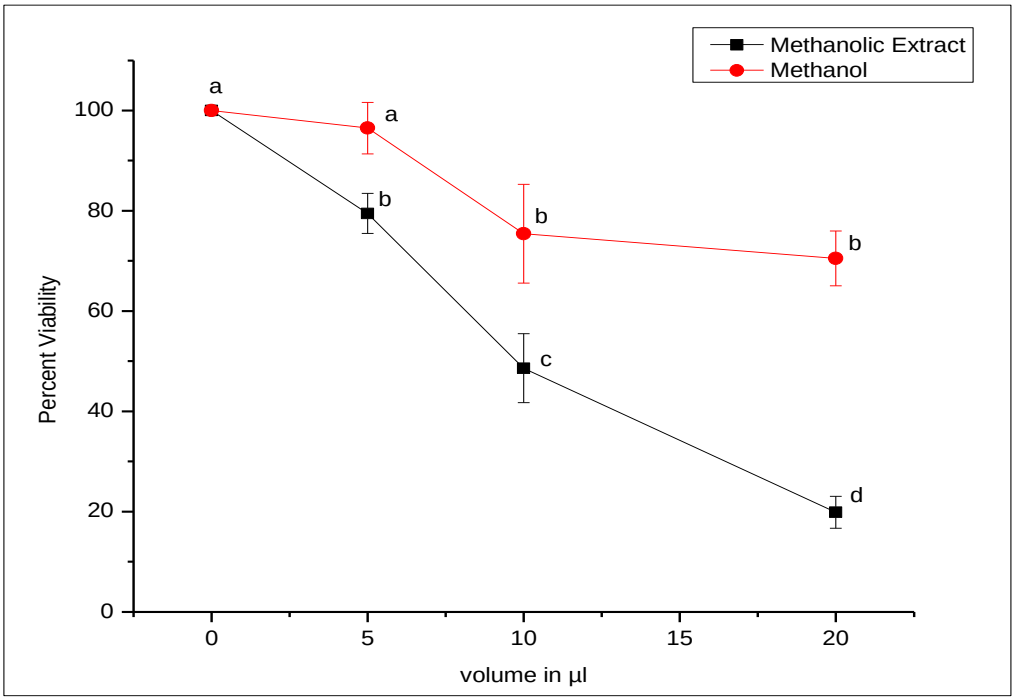


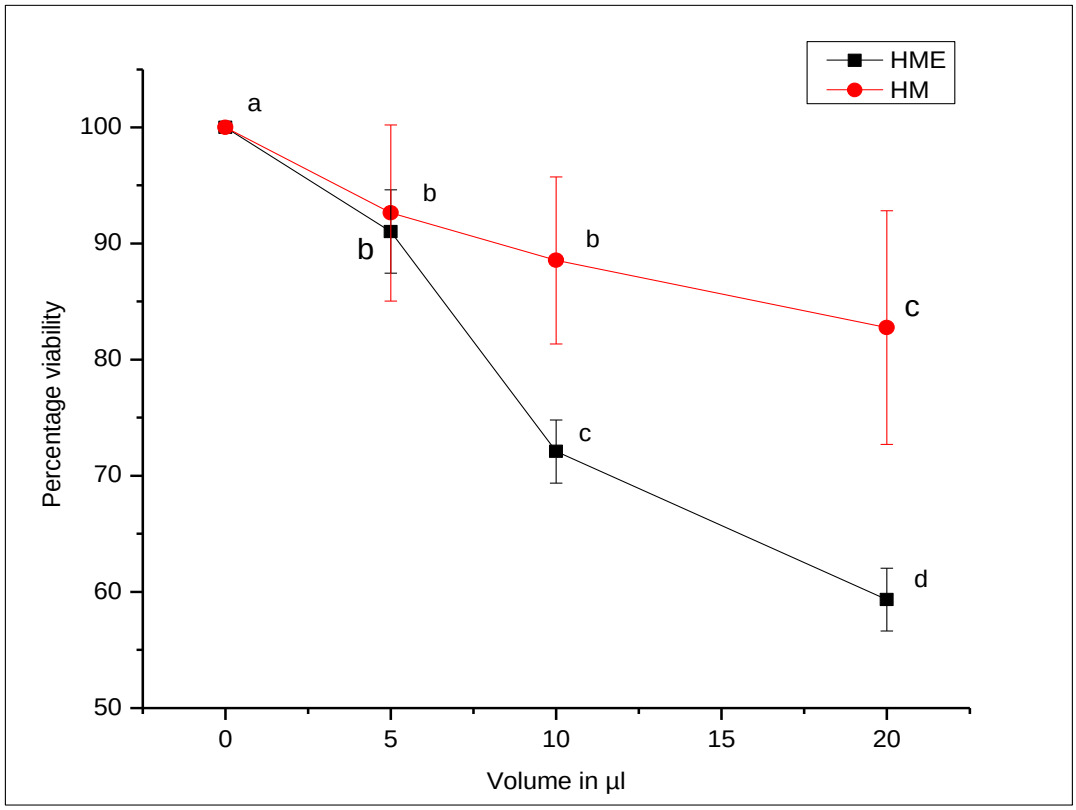
Fig. 5: Cytotoxic effect of methanolic extract

Values are given as mean ± SD (N=6). Means followed by the same letters are not statistically significant according to Tukey's HSD test at $P \leq 0.05$.

In the present study the methanolic extract showed the greater cytotoxic effect in comparison to vehicle control methanol.

5.4. Cytotoxic activity of hydromethanolic extract

The percentage viability curve against the different volumes of extract and hydromethanol is plotted.



Where HME is hydromethanolic extract and HM is hydromethanol.

Fig.6: Cytotoxic activity of hydromethanolic extract

Values are given as mean ± SD (N=6). Means followed by the same letters are not statistically significant according to Tukey's HSD test at $P \leq 0.05$.

In the present study the hydromethanolic extract showed the greater cytotoxic effect in comparison to the vehicle control hydromethanol.

Chapter-6
Discussion

Seaweeds are rich source of structurally novel and biologically active metabolites. Seaweeds are used as food source among the people in various countries. It is reported that many algae have long been used as traditional herbal medicine in the treatment of cancer (Yamamoto et al.,1984) and the algae are rich source of polyphenolic compounds (Ye et al., 2009). Phenolic compounds in seaweed constitute a major class of secondary algal metabolites. Most of the useful characteristics of the phenolic compounds have been endorsed to their antioxidant activity which is a fundamental property important to life (Andreasen et al., 2001, Ainsworth & Gillespie, 2007). Human beings are familiar from a long time with the seaweeds as a rich source of pharmacologically active metabolites with antioxidant, antineoplastic, anti-inflammatory and antiviral effects (Tziveleka et al., 2003).

The antioxidant activity of seaweeds is believed to be mainly due to the redox properties of these compounds and these have a crucial role in adsorbing and neutralizing the free radicals (Zheng & Wang, 2001). Fucoidans obtained from the brown algae, block the generation of reactive oxygen species (Jhamandas et al., 2005). Consumption of seaweed generally increases the endogenous antioxidant enzymes (Mohamed et al., 2011). In the biological systems oxygen produces a large number of free radicals and other different reactive species which are collectively known as ‘reactive oxygen species’ (ROS). Another group of reactive species are termed as ‘reactive nitrogen species’ (RNS) (Devasagayam et al., 2004). In normal healthy human beings, the generation of ROS and RNS are efficiently maintained at balanced level by the various antioxidant defense mechanisms. The seaweeds have also been observed to show the suppression of tumor initiation (Lee & Sung, 2003).

In the present study the crude extracts of S. wightii in methanol and hydromethanol exhibit various levels of total phenolic content. In this study the total phenolic content of methanolic extract was 5.34% ± 0.23 [Mean ± SD (N=6)] and that of the hydromethanolic extract was 4.56% ± 1.62 [Mean ± SD (N=6)] of dry weight of sample. It clearly indicates that there would be such constituents in the crude extract that are more soluble in the methanol in comparison to the

hydromethanol. This is supported by the antioxidant assay- the DPPH assay. The percentage inhibition or percentage scavenging activity of methanolic extract is greater than the hydromethanolic extract. Thus indicating that the constituents present in methanolic extract are better antioxidants in comparison to the constituents present in the hydromethanolic extract.

The methanolic crude extract showed the significant cytotoxic activity against PC-3 cancer cells. The cells of control sample were assumed 100% viable. 5µl extract showed approximately 20% cytotoxicity but 5 µl vehicle control (methanol) did not show the significant cytotoxicity. When 10 µl of methanolic extract was used around 50% cells were dead. Further increasing the dose of methanolic extract to 20 µl it was observed that approximately 80% cells were dead. On the other hand it was found that at 10 µl and 20 µl of vehicle control (methanol) approximately 20% cells were dead. Thus it could be predicted that the cytotoxicity due to crude extract without methanol at 10 µl and 20 µl might be approximately 30% and 60%.

On the other hand 5µl hydromethanolic extract and 5µl as well as 10µl vehicle control (hydromethanol) did not show significant cytotoxicity. But at 10 µl of hydromethanolic extract around 14% cells were dead. It was observed that at 20 µl hydromethanolic extract approximately 40% cells were dead. But at 20 µl of vehicle control (hydromethanol) approximately 15% cells were dead. Thus it could be predicted that the cytotoxicity due to crude hydromethanolic extract is not significant.

It may be predicted that the effective constituents in hydromethanolic extract may not be present in sufficient quantity. This is favored by the finding of total phenolic content and scavenging activity of both the extracts. It was observed that the percentage viability of PC-3 cells was different on administering the different doses of extracts. The percentage viability decreases on increasing the dose of the extracts.

Chapter-7
Summary

The products obtained from the plants have been used for medicinal purposes in India, China, Nepal and Europe since ancient times. Most of the biological activities on our planet take shape in ocean and a number of biologically active compounds with varying degrees of action, such as anticancer, antimicrotubule, antiproliferative, cytotoxic and photo protective have been isolated to date from marine sources. A number of algae and their metabolites have shown potent cytotoxicity. Seaweeds have gained special attention as a source of natural antioxidants. Polyphenols, alkaloids and polysaccharides are the most predominant group of biologically active compounds found in the extracts of the marine algae which have promising use in pharmaceutical and nutraceutical industry owing to the antioxidant and anticancer activities. Polyphenols have antioxidant and antineoplastic activities.

In the present study S. wightii, brown alga is used to check the cytotoxic activity on PC-3 cancer cell- line. The extraction was done in two different solvents hydromethanol and methanol. The crude extracts of methanol and hydromethanol were obtained. The total phenolic content of hydromethanolic extract and the methanolic extract of S. wightii was determined by the Folin- Ciocalteu method. The total phenolic content in methanolic and hydromethanolic extracts was determined as 5.34% ± 0.23 [Mean ± SD (N=6)] and 4.56% ± 1.62 [Mean ± SD (N=6)] respectively of dry weight of sample. This indicates that phenolic constituents in the crude extract are more soluble in the methanol in comparison to the hydromethanol.

The free radical scavenging activity of S. wightii extract was estimated by the DPPH assay. BHT was used as standard compound. The percentage inhibition or percentage scavenging activity of the methanolic and hydromethanolic extracts was 36.28% ± 1.18 [Mean ± SD (N=6)] and 22.94% ± 0.79 [Mean ± SD (N=6)] respectively. This suggests the constituents present in methanolic extract were good antioxidants in comparison to the constituents present in the hydromethanolic extract.

PC-3 cells were grown in Dulbecco's modified eagles medium. The culture was maintained at 37°C with humidified 5% CO_2 environment in CO_2 incubator. MTT assay was used for the measurement of the in vitro cytotoxicity. It was concluded that the methanolic crude extract was significantly more cytotoxic against the PC-3 cancer cells than that of hydromethanolic extract. It was observed that effective constituents in hydromethanolic extract pertaining to cytotoxicity may not be present in sufficient quantity and these were more soluble in the methanol. This is favored by the finding of total phenolic content and scavenging activity of both the extracts.

In conclusion the S. wightii could be a good choice for in vivo antineoplastic assessment. More research on the effect on other cancer cell-lines is warranted to bring out bio-prospecting of this alga in pharmaceutical arena. In addition this study demonstrates that seaweeds can be potential candidate for the future development of antitumor drugs.

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