ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITIES OF SEVERAL MARINE ORGANISMS FROM THE WEST COAST OF PENINSULAR MALAYSIA

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ABSTRACT

Three species of seaweeds (*Padina tetrastromatica*, *Caulerpa racemosa* and *Turbinaria ornata*) and a sponge (*Spheciospongia vagabunda*) were evaluated for their antioxidant and antiproliferative activities in the human breast adenocarcinoma cell line, MCF-7. The crude extracts of the seaweeds and sponge were sequentially extracted with hexane, dichloromethane, ethyl acetate, acetone and methanol. The extracts showed potent 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, nitric oxide, superoxide anion and hydroxyl radical scavenging assays. The crude extracts were also evaluated for their antiproliferative effects on MCF-7 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. None of the extracts showed any toxicity in the normal breast cell line, 184B5. The extracts showed antiproliferative activity in MCF-7 cells with half-maximal inhibitory concentration (IC_{50}) of $60.0 - 130.0$ μg/ml. The superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) activities in the extracts-treated MCF-7 cells decreased in a dose-dependent manner. Bioassay guided fractionation was conducted on the extract showing the most potent antiproliferative effect. MTT assay was carried out on the partially purified fractions and showed IC_{50} of $12.0 - 18.0$ μg/ml. The most potent fractions were then subjected to liquid chromatography-mass spectrometry (LC-MS) analysis. Camptothecin, pseudopelletierine and lycodine accountable for the cytotoxicity were detected. The IC_{50} of camptothecin, pseudopelletierine and lycodine were 2.4 ± 2.03, 4.8 ± 1.84 and 6.4 ± 1.97 μg/ml, respectively. It was hypothesised that apoptosis was a mode of cell death by the cancer cells upon treatment. To test this hypothesis, the cellular mechanisms rendering the decreased cancer cell viability were studied. Treatment of MCF-7 cells with the partially purified fractions and pure compounds caused a collapse in the mitochondrial membrane potential (MMP). Caspase-8, -9 and -3 activities increased

relative to the untreated negative control. Through DNA fragmentation analysis, laddering patterns and fragmentation were observed in the treated MCF-7 cells; further implying that apoptosis was employed as a mechanism of cell death. To further identify and verify the proteins involved in apoptosis, a proteomics approach wasused to study the protein expressions of treated and untreated MCF-7 cells. The methodology involvedtwo dimensional-polyacrylamide gel electrophoresis (2D-PAGE) and Western blotting. The differentially expressed proteins obtained from the 2D gels were analysed usingquadrupole time-of-flight (Q TOF)/LC-MS.A total of 42 proteins were differentially expressed and grouped into 10 biological processes based on their biological functions described in the UniProtKB/Swiss-Prot protein database. Three pro-apoptotic proteins (p53, Bax and PARP-1) were up-regulated while the antiapoptotic protein, Bcl-2, was down-regulated. The fold change in the expression levels of these apoptotic proteins were validated by Western blotting, which were in agreement with the results obtained in 2D-PAGE. In conclusion, exploration of the *in vitro* pharmacological properties of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* revealed new possibilities and may represent a new generation of potential drug candidates for the treatment of breast cancer. However, further research in animal models as well as clinical trials are required to ascertain their anticancer properties *in vivo*, efficacy and safety prior to application in the pharmaceutical industry as natural therapeutic agents.

ABSTRAK

Tiga spesies rumpair laut (*Padina tetrastromatica*, *Caulerpa racemosa* dan *Turbinariaornata*) dan span (*Spheciospongia vagabunda*) telah dinilai untuk aktiviti antioksidan dan antiproliferatif dalam sel adenokarsinoma payudara, MCF-7.Ekstrak mentah dari rumpair laut dan span telah berurutan diekstrak dengan heksana, diklorometana, etil asetat, aseton dan metanol.Ekstrak menunjukkan aktiviti perencatan radikal DPPH, nitrik oksida, anion superoksida dan hidroksil yang tinggi. Aktiviti antiproliferatif oleh ekstrak mentah rumpair laut dan span ke atas sel MCF-7 juga dinilai dengan asai MTT. Tiada ekstrak yang menunjukkan ketoksikan ke atas sel payudara normal, 184B5. Ekstrak-ekstrak menunjukkan aktiviti antiproliferatif ke atas sel MCF-7 dengan nilai IC_{50} antara 60.0 dan 130.0 µg/ml. Aktiviti enzim antioksidan dalam sel MCF-7 yang diuji dengan ekstrak dinilai melalui aktiviti SOD, CAT dan GR yang mana menurun berkadar dengan dos. Ekstrak yang menunjukkan aktiviti antiproliferatif yang tertinggi disubjekkan kepada pencerakinan bioasai secara pemanduan. Fraksi separa purifikasi diuji dengan asai MTT dan menunjukkan aktiviti antiproliferatif yang lebih tinggi daripada ekstrak mentah dengan nilai IC_{50} antara 12.0 - 18.0 µg/ml. Fraksi yang menunjukkan aktiviti tertinggi dipilih untuk analisa LC-MS. *Camptothecin*, *pseudopelletierine* dan *lycodine* yang bertanggungjawab denga aktiviti antiproliferatif telah dikenalpasti melalui LC-MS. Sebatian-sebatian tulen yang dikenalpasti tersebut diuji dengan asai MTT untuk menilai ketoksikan sebatian tersebut ke atas sel MCF-7. Nilai IC_{50} untuk *camptothecin*, *pseudopelletierine* dan *lycodine* adalah 2.4 ± 2.03, 4.8 ± 1.84 and 6.4 ± 1.97 µg/ml, masing-masing. Adalah dihipotesiskan bahawa mod kematian sel adalah secara *apoptosis*.Untuk menguji hipotesis ini, mekanisme selular yang menyebabkan penurunan viabiliti sel telah dikaji melalui penentuan pengubahan MMP, aktiviti *caspase* dan analisa DNA fragmentasi ke atas fraksi-fraksi separa

purifikasi dan sebatian-sebatian tulen yang diasingkan. Rawatan sel MCF-7 dengan fraksi-fraksi separa purifikasi dan sebatian-sebatian tulen menyebabkan penurunan dalam MMP. Aktiviti caspase-8, -9 dan -3 meningkat berbanding dengan kawalan negatif.Melalui analisa DNA fragmentasi, DNA kelihatan seperti tangga akibat fragmentasi DNA telah diperhatikan. Ini menunjukkan bahawa *apoptosis* dipilih sebagai mod kematian oleh sel. Pendekatan proteomik melalui *2D-PAGE* dan *Western blotting* telah digunakan untuk mengkaji profil ekspres protein dalam sel MCF-7 yang tidak dirawat dan yang dirawat dengan fraksi-fraksi separa purifikasi untuk mengenalpasti protein-protein yang terlibat dalam proses *apoptosis*. Protein-protein yang diekspres berbeza dari gel 2D dianalisa menggunakan Q TOF/LC-MS. Sebanyak 42 protein yang diekspres berbeza dan dikumpulkan dalam 10 proses biologikal berdasarkan fungsi-fungsi biologikal mereka yang diterangkan dalam pangkalan data protein UniProtKB/Swiss-Prot. Peningkatan ekspresi dalam tiga protein *pro-apoptotic* (p53, Bax dan PARP-1) dan penurunan ekspresi dalam satu protein *anti-apoptotic* Bcl-2 telah dikesan. Kadar perubahan ekspresi protein-protein *apoptotic* ini disahkan melalui *Western blotting*.Sekata dengan keputusan yang diperoleh dari 2D-PAGE, analisa *Western blotting* juga menunjukkan peningkatan ekspresi dalam protein *pro-apoptotic* dan penurunan ekspresi dalam protein *anti-apoptotic*. Kesimpulannya, penerokaan sifat farmakologi secara *in vitro* untuk *P. tetrastromatica*, *C. racemosa*, *T. ornata* dan *S. vagabunda* mewakili generasi baru agen antiproliferatif yang berpotensi untuk merawat kanser payudara. Walau bagaimanapun, kajian dalam model binatang dan percubaan klinikal adalah diperlukan untuk memastikan ciri-ciri antikanser *in vivo* serta keselamatan dan keberkesanan mereka sebagai agen terapeutik semulajadi dalam industri farmaseutikal.

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LIST OF SYMBOLS AND ABBREVIATIONS

Å	Angstrom(s)
ACN	Acetonitrile
APS	Ammonium persulphate
BPE	Bovine pituitary extract
BSA	Bovine serum albumin
Caspase	Cysteine-aspartic protease
CAT	Catalase
CE	Catechin equivalent
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
cm	Centimetre(s)
CO_2	Carbon dioxide
C. racemosa	*Caulerpa racemosa*
Da	Dalton(s)
DCM	Dicholoromethane
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTT	Dithiothreitol
EA	Ethyl acetate
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionisation
g	Gram(s)
FBS	Foetal bovine serum
FC	Folin-Ciocalteu

FeCl$_3$	Iron (III) chloride
GAE	Gallic acid equivalent
GR	Glutathione reductase
GSH	Glutathione in the reduced form
GSSG	Glutathione disulphide
h	Hour(s)
HCl	Hydrochloric acid
H$_2$O$_2$	Hydrogen peroxide
hEGF	Human epidermal growth factor
HRP	Horse radish peroxidase
IAA	Iodoacetamide
IC$_{50}$	Half-maximal inhibitory concentration
IEF	First dimensional Isoelectric Focusing
IPG	Immobilised pH gradient
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbo-cyanine iodide
k	Kilo(s)
l	Litre(s)
LC-MS	Liquid chromatography-mass spectrometry
M	Molar
mA	Milliampere(s)
m/z	Mass-to-charge ratio
MDA	Malondialdehyde
MEBM	Mammary epithelial basal medium
MEGM	Mammary epithelial cell growth medium
mg	Milligram(s)

min	Minute(s)
ml	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar
MMP	Mitochondrial membrane potential
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
µA	Microampere(s)
µg	Microgram(s)
µl	Microlitre(s)
µm	Micrometre(s)
µM	Micromolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2CO_3	Sodium carbonate
NADH	Nicotinamide adenine dinucleotide
NL	Non-linear
nl	Nanolitre(s)
NaOH	Sodium hydroxide
NBT	Nitroblue tetrazolium
nm	Nanometre(s)
NO	Nitric oxide
PAGE	Polyacrylamide Gel Electrophoresis
PARP-1	Poly-ADP ribose polymerase-1
PBS	Phosphate buffered saline
PMS	Phenazine methosulphate
pNA	p-nitroaniline

P. tetrastromatica	*Padina tetrastromatica*
R_f	Retention factor
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulphate
sec	Second(s)
SOD	Superoxide dismutase
SNP	Sodium nitroprusside
S. vagabunda	*Spheciospongia vagabunda*
TBA	Thiobarbituric acid
TBST	Tris-buffered saline with Tween 20
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFC	Total flavonoid content
TLC	Thin layer chromatography
TMB	3,3',5,5'-tetramethylbenzidine
T. ornata	*Turbinaria ornata*
TPC	Total phenolic content
V	Voltage
v/v	Volume/volume
W	Watt(s)
w/v	Weight/volume

CHAPTER 1

INTRODUCTION

1.1 Research Background

Cancer is the leading cause of death globally, accounting for 8.2 million deaths in 2012, with lung cancer topping the chart, followed by liver, stomach, colorectal and breast cancer (Msyamboza *et al*., 2012). Despite varying estimations, it is generally estimated that by the year 2020, there will be almost 20 million new cases of cancer. According to the World Cancer Report (2014), the worldwide burden of cancer is expected to rise to 22 million new cases annually within the next two decades while global cancer deaths are predicted to rise from an estimated 8.2 million annually to 13 million per year. Adding to the concern, not only the number of new cases will increase, but also the proportion of new cases from developing countries will increase to 70%.

Cancer is the generic term of malignant neoplasm which is a category of diseases in which a group of cells exhibit uncontrolled growth or division surpassing the usual boundaries. Other defining feature of malignant neoplasm is the ability of cells to invade adjacent tissues and organs; and occasionally metastasise or spread to other parts of the body. Metastases are the major cause of death from cancer. Three characteristics of benign tumours that differentiate them from malignant tumours or neoplasm are that benign tumours are self-limited, do not invade or metastasise (Ma *et al*., 2010). The majority of tumours are classified as carcinoma, which are derived from epithelial cells including breast, lung, colorectal, skin and prostate cancers. Sarcoma arises from non-epithelial cells which include connective tissues or mesenchymal cells, such as fibroblasts and osteoblasts. Lymphoma and leukemia are developed from blood-forming

cells known as hematopoietic cells. Tumours derived from totipotent cells found in testicle and ovary is known as germ cell tumour. Blastic tumour or blastoma, on the other hand, is a malignant tumour which has close resemblance to an immature or embryonic tissue (Weinberg, 2007).

Cancer arises from a normal single cell of the body and can differentiate from various cell types. The transformation of a normal cell into a tumour cell, which is generally a progression of a pre-cancerous lesion to malignant tumour involves a multistage process. These alterations are the consequences of the interaction between the genetic materials of an individual and three categories of external agents [as classified by the World Health Organisation (WHO) (2015)], which include physical or environmental carcinogens such as ionising and ultraviolet radiations, chemical carcinogens such as tobacco, arsenic and asbestos, biological carcinogens resulting from viral or bacterial infections. In addition to these external factors, the development of cancer is also attributed to genetic predisposition, unhealthy diet and sedentary lifestyles (Anand *et al*., 2008).

Carcinogenesis is the transformational process of normal cells into cancer cells. Under normal circumstances, the equilibrium between cell proliferation and programmed cell death, normally in the form of apoptosis, occurs by tight modulation of both processes to ensure the integrity of organs and tissues. Carcinogenesis is brought about by the aforementioned factors by damaging DNA which are either usually repaired or used for the induction of senescence and elimination of the cell, depending on the extent of damage. According to Weinberg (2007), the basic requirement for the progression of cancer from *in situ* dysplasia to a malignant tumour is the accumulation of multiple mutations. The disruption of the orderly processes which are required to maintain

proper cell function as a result of DNA mutations ultimately lead to the development of cancer (Rosen, 2013).

The acquisition of the various genetic alterations by cancer cells eventually manifest as six essential properties considered as the "hallmarks of cancer": growth signal autonomy, resistance to anti-growth signals, ability to invade and metastasise, unlimited replication potential; sustained angiogenesis and escape of apoptosis (Hanahan & Weinberg, 2000).

Breast cancer affects 1.3 million women annually and it is the most common cancer in women both in developed and developing countries. Increased life expectancy, increased urbanisation and adoption of western lifestyles lead to the elevation of the incidence of breast cancer in developing countries. It was estimated that over 508 000 women worldwide died of breast cancer in 2011 (Eittah *et al*., 2014).

Despite the rapid advancement in early diagnostic and conventional treatment strategies, the incidence of breast cancer is significantly increasing at an alarming rate globally. Furthermore, breast cancer cells have developed resistance to chemotherapeutic drugs which are regulated by the over expression of the multidrug resistance proteins that pump drugs out of the cells, thus rendering the drugs ineffective (Tomas, 2006). One of the predicaments in the diagnosis and treatment of breast cancer is the occurrence of different subtypes of breast cancers originating from different gene mutations in the luminal or progenitor cell population (Sims *et al*., 2007).

The term "natural product" is commonly regarded as being identical with secondary metabolites. These organic substances are of relatively small molecular weight (< 3000

Daltons) and are structurally diverse (Kinghorn *et al*., 2009). Such compounds tend to be in the right chiral form to elicit bioactivities and this in turn facilitates species survival by either repelling or attracting other organisms (Williams *et al*., 1989).

Nature has been acknowledged as the fundamental source of natural products with an abundance of profound and promising medicinal virtues since ancient times. The stupendous diversity found in plants, animals, marine organisms and microorganisms makes it possible for nature to serve as an attractive source of novel therapeutic candidate compounds (Rocha *et al*., 2001). Natural products have long played a prominent role in the treatment of diseases (Mayer *et al*., 2010). Approximately 62-80% of the world's population still depends on traditional herbal medicines as therapeutic medicines for their primary healthcare (WHO, 2015; Zhang, 2004). Higher plants are commonly used as sources of medicinal compounds with dominant roles in maintaining human health for centuries (Farombi, 2003). Several systems of medicine such as Ayurveda and Unani offer a good foundation for scientific exploration of medicinally important biomolecules from nature. Since then, the term 'modern medicines' is redefined following the rediscovery of Ayurveda. The emerging concept of the combination between Ayurveda and advanced drug discovery programme is now accepted worldwide (Pandey & Madhuri, 2009).

In recent years, the use of traditional medicine information on natural products research has once again gained considerable attention. Natural products lack much of the toxicity found in synthetic chemicals, hence, enhancing their appeal for long term preventive strategies (Zou *et al*., 2005). In defiance of the major scientific and technological breakthroughs in the medical field, drugs derived from natural products still account for an enormous contribution to drug discovery (Rocha *et al*., 2001). In fact, more than 50%

of all modern drugs are of natural product origin which made up a major component of modern pharmaceuticals for the treatment of human diseases (Rosangkima & Prasad, 2004; Mayer *et al*., 2010). The pharmacological properties of natural products are mainly contributed by the existence of chemically important secondary metabolites such as flavonoids, alkaloids and phenolics which are able to elicit a definite physiological response on the human body (Edeoga *et al*., 2005).

Oceans are exceptional reservoirs of bioactive compounds, most of which are novel chemical classes that possess distinctive structural or chemical features not found in terrestrial natural products (Carte, 1996; Guven *et al*., 2010). The diversity of marine organisms has become an inspiration for researchers to identify novel marine natural products that could eventually be developed into therapeutics or pharmaceutical products (Mayer *et al*., 2010). There is a rapid increase in the number of natural products isolated from marine organisms, with hundreds of new compounds being discovered annually (Faulkner, 1990). In fact, many structurally diverse natural products isolated from marine organisms are believed to possess an astounding array of bioactivities, particularly anticancer activities against multiple tumour types, antibiotic, antiviral, antioxidant and anti-inflammatory activities (Schwartsmann, 2000; Schwartsmann *et al*., 2001). More than 3000 new substances have been identified from marine organisms over the past three decades, evidencing the great potential of the ocean as a source of novel chemical classes (Schweitzer *et al*., 1991; Rinehart, 2001).

Over the past few years, bioprospecting of marine natural products has yielded a number of drug candidates for the treatment of various diseases (Haefner, 2003). Most of these molecules are still in the stage of preclinical or early clinical trials but some are already available in the market such as cytarabine, a chemotherapeutic agent isolated

from *Cryptotethya cryta* which is used for the treatment of hematological malignancies. Ecteinascidins 743 or ET743 (Yondelis) isolated from *Ecteinascidia turbinata*, has been approved in 2007 for the treatment of soft tissue sarcoma, and in 2009 for ovarian carcinoma. Concurrently, many other marine natural products are currently being evaluated in different phases of clinical trials (Mayer *et al.*, 2010).

Three macroalgae or seaweeds (*Padina tetrastromatica*, *Caulerpa racemosa* and *Turbinaria ornata*) and a sponge (*Spheciospongia vagabunda*) were selected. *P. tetrastromatica* and *C. racemosa* are used in food preparation. There is some evidence that the algae have antioxidant and anticancer activities. The sponge, *S. vagabunda*, was also procured by a collaborator in the project, Associate Professor Dr. Khoo Kong Soo, in his diving expeditions. Extensive studies are more often than not, conducted in Japanese and Chinese seaweeds. However, little information and knowledge are available pertaining to Malaysian seaweeds. To the best of our knowledge, the present study was the first attempt to evaluate the antiproliferative activities of the marine organisms by determining their mechanisms of action through the proteomics approach. Taking these into account, the potential of these marine organisms to serve as chemotherapeutic agents became an attractive prospect.

1.2   Hypothesis and Objectives

This study aims to determine the efficacy of the crude extracts and partially purified fractions of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* against MCF-7 cells and to elucidate the *in vitro* mechanisms of action. The hypothesis for this study is:

Crude extracts and partially purified fractions of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* induce apoptosis as a mechanism of cell death, and therefore will improve cancer patient outcomes if they were to be used in the treatment of breast cancer.

This hypothesis will be tested through the following objectives:

1. To evaluate the efficacy of the crude extracts and partially purified fractions of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* on MCF-7 cells by determining their antioxidant and antiproliferative properties in human breast adenocarcinoma, (MCF-7) cells.

2. To elucidate the mechanism of action of the crude extracts and partially purified fractions on MCF-7 cells by *in vitro* models.

3. To identify and isolate the bioactive compounds responsible for antiproliferative activity through bioassay-guided fractionation.

4. To ascertain the protein expression profiles of fractions-treated MCF-7 cells via the proteomics approach.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

2.1.1 Pathogenesis of Cancer

Three classes of genes which include oncogenes, tumour suppressor genes and metastasis suppressor genes have been implicated in the development of cancer. Oncogenes are a group of impaired genes whose presence in certain forms and/or overexpression can induce the development of cancer by the expression of proteins that stimulate excessive cell growth and division (Netea-Maier *et al.*, 2015). When a hormone receptor on a recipient cell is activated, signal transduction from the cell surface to the nucleus occurs and leads to some alterations in gene transcription regulation at the nuclear level. The activated transcription factors in turn "switch on" the genes required for cell growth and proliferation. H-*ras*, K-*ras*, N-*ras*, *myc* and L-*myc* are examples of oncogenes involved in signal transduction and serve as transcription factors (Swartling, 2012).

Tumour suppressor genes are the second group of genes implicated in cancer. The functional absence of this group of genes can lead to cancer. As the name suggests, tumour suppressor genes inhibit cell proliferation. Inactivation or inhibition of their function will result in a limitless expansion of cell growth (Rivlin *et al.*, 2011). Factors such as gene mutation, allelic loss of the gene or regional DNA hyper-methylation that terminates gene transcription or predisposes it to allelic loss might bring about the inactivation of tumour suppressor genes (Zhou *et al.*, 2012). Three main characteristics

of tumour suppressor genes are the ability to repress genes essential for continuation of the cell cycle, the ability to couple cell cycle to DNA damage and the ability to enable the cell to initiate apoptosis should the damage be irreparable (Rambold & Lippincott-Schwartz, 2011). Transcription regulators such as p53, p73 and WT1 are several examples of tumour suppressor genes involved in DNA repair, regulating transcription and inducing apoptosis (Marabese *et al*., 2003).

A series of complicated and multistep genetic events is involved in tumour progression and metastasis. nm23 gene is an anti-metastatic gene which produces nm23 protein required for the regulation of cell growth and differentiation (Masoudi *et al*., 2013). This protein supplies nucleoside triphosphates except ATP to the cells, provides GTP to the G-proteins involved in signal transduction and regulates microtubule polymerisation in the mitotic spindle and cytoskeleton (Saha & Robertson, 2011).
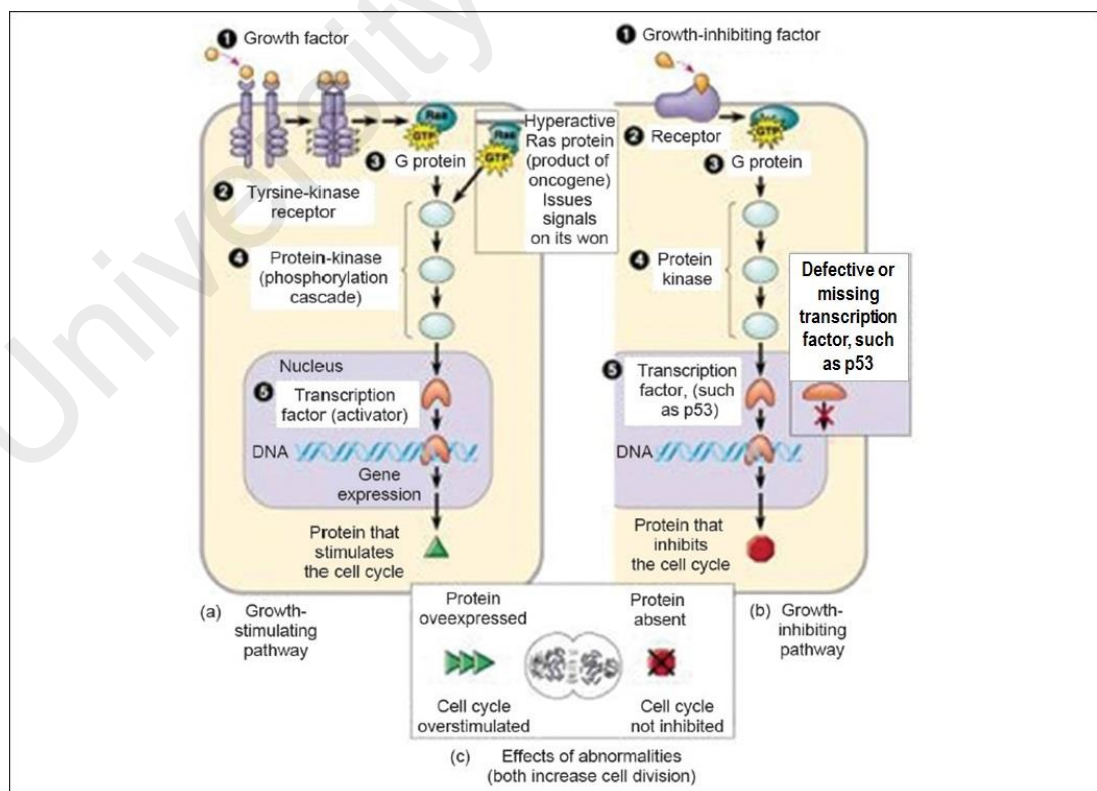
Figure 2.1: Pathogenesis of cancer. Oncogenes produce hormones or "chemical messenger" between cells which encourage mitosis, the process by which depends on

the signal transduction of the recipient tissue or cells. When a hormone receptor on a recipient cell is triggered, the signal is transducted from the cell surface to the cell nucleus, thereby causing some changes in gene transcription regulation at the nuclear level. Oncogenes are often involved in transcription of DNA in protein synthesis. (From "Role of Ayurveda in tumorigenesis: a brief review" by Sharma & Rawai, 2012. *International Journal of Green Pharmacy*, *6*, 93-101).

2.1.2 Pathogenesis of Breast Cancer

BRCA1 and BRCA2 are genes involved in the production of tumour suppressor proteins which ensure the integrity and stability of the cells' genetic materials by facilitating the repair of damaged DNA. Hereditary mutations in BRCA1 and BRCA2 have been related to increased risk of female breast cancer. The vast majority of breast cancer patients do not have a family history of the disease albeit 5-10% of all breast cancer is hereditary, as a consequence of mutations in BRCA1 and BRCA2 (Campeau *et al*., 2008; Tazzite *et al*., 2013).

The activation of steroid hormones such as estrogen and progesterone results in breast cancer pathogenesis. These steroid hormones bind to the estrogen and progesterone receptors in breast epithelial cells to promote cell growth, differentiation and survival (Taneja *et al*., 2010). Likewise, breast cancer development is also driven by signal transduction by receptor tyrosine kinases (RTKs) located on the surface of breast epithelial cells (Pegram *et al*., 2000), particularly, the ErbB family which includes EGFR (ErbB1), HER2 (ErbB2), ErbB3 and ErbB4. These are implicated in breast cancer pathogenesis by responding to growth factor stimulation through hetero- and homodimerisation and subsequent activation of downstream signalling pathways (Hynes *et al*., 2005; Sergina *et al*., 2007). In conclusion, the overexpression and activation of the genes in ErbB family lead to the development of breast cancer.

2.2   Modes of Cell Death

2.2.1   Apoptosis

Apoptosis is defined as a programmed cell death which is controlled by two distinct pathways and occurs by various internal and external stimuli (Li *et al*., 2012). Apoptosis as a mode of cell death is intensively studied among researchers. Understanding apoptosis in disease conditions is crucial as it not only provides insights into the pathogenesis of a disease but also indicates on how the disease can be treated. More often than not, cancer is accompanied by a loss of balance between cell division and cell death. In other words, cells that are supposed to die did not receive the signals to do so. An example of deregulated apoptotic pathway is the down-regulation of the tumour suppressor gene, p53, which is usually related to many human cancers due to the reduction in apoptosis (Bauer & Hefand, 2006; Morton *et al*., 2010). Unrestrained apoptosis may lead to degenerative diseases while too little apoptosis results in malignant cells that will not die, and ultimately, cancer. Despite being a double-edged sword, whereby apoptosis can be the cause of problems as well as the solution, apoptosis plays a pivotal role in the treatment of cancer as it is a popular target for many treatment strategies (Wong, 2011).

The induction of apoptosis in cancer cells is the primary method employed by current cancer therapies such as chemotherapy, suicide gene therapy, γ-irradiation or immunotherapy to exert their anticancer effects (Makin & Dive, 2001; Fulda & Debatin, 2004; Wong, 2011). Apoptosis is an evolutionally conserved, intrinsic program of cell death that occurs in different types of physiological and pathological conditions (Hengartner, 2000). Characteristic features which serve as typical morphological and biochemical hallmarks of apoptosis include cell shrinkage, plasma membrane blebbing,

nuclear fragmentation, chromatin condensation and formation of apoptotic bodies (Hengartner, 2000; Reed, 2000).

Apoptosis is initiated and executed by a series of biochemical events that is tightly regulated by complex signalling networks (Zhang & Yu, 2013). Damage to DNA or to other critical molecules is considered as a common initial event which is subsequently propagated by the cellular stress response (Rich *et al.*, 2000). Examples of apoptotic signal transducers are several stress-inducible molecules which include c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK), ceramide and nuclear factor kappa B (NF-κB) (Davis 2000; Karin *et al.*, 2002). Proteolytic enzymes such as caspases play a vital role as apoptotic effector molecules (Degterev *et al.*, 2003). Activation of caspases in response to an external stimulus such as anticancer chemotherapy can be initiated through the activation of the extrinsic (receptor) mediated pathway or at the mitochondria by stimulation of the intrinsic pathway (Fulda & Debatin, 2004).

It is of utmost importance to tightly regulate apoptotic pathways because an inappropriate activation of apoptosis programs will result in a series of potential detrimental effects on cell survival. The antiapoptotic mechanisms regulating cell death have been implicated in conferring drug resistance to cancer cells (Fulda & Debatin, 2004). Nevertheless, the major mechanism by which cancer cells are eliminated cannot be represented solely or universally by apoptosis alone, as caspase-independent apoptosis or other modes of cell death have to be considered as well as cellular response to cancer therapy (Brown & Wilson, 2003; Brown & Attardi, 2005).

2.2.1.1 Mitochondrial Cell Death Effectors

The toxic proteins released from the mitochondria following its permeabilization can be classified into three categories, namely, direct caspase activators, indirect caspase activators and caspase-independent pro-apoptotic factors. Cytochrome c together with pro-caspase-9 and apoptosis-protease activating factor (apaf-1) forms a complex called apoptosome, whose main function is to cleave and activate caspase-9 (initiator caspase) which in turn cleaves and activates downstream effector caspases-3, 6 and 7 (Pradelli *et al*., 2010).

On the contrary, the indirect caspase activators, Smac/DIABLO and Omi/HtrA2, bind and inhibit the inhibitor of apoptosis proteins (IAPs) which are endogenous caspase inhibitory proteins (Kroemer *et al*., 2007; Brenner & Mak, 2009). Activation of effector caspases is normally depicted as the final step in the apoptosis signalling pathway. The activated effector caspases will subsequently cleave substrates such as poly (ADP-ribose) polymerase (PARP), cytoskeletal and nuclear matrix proteins (Reed, 2000).

The release of the caspase-dependent cytotoxic factors from the mitochondria following mitochondrial outer membrane permeabilization (MOMP) is accompanied by the release of pro-apoptotic caspase-independent proteins, which include apoptosis inducing factors (AIFs) and a mitochondrial endonuclease, endonuclease G (EndoG) (Pradelli *et al*., 2010). These proteins evade the activation of caspases to induce caspase independent programmed cell death (CICD) (Constantinou *et al*., 2009). Following release from the mitochondria, EndoG translocates to the nucleus to trigger DNA fragmentation and CICD (Pradelli *et al*., 2010). The flavoprotein AIF which is required for the regulation of the mitochondrial respiratory chain in physiological conditions is anchored to the mitochondrial inner membrane in the intermembrane space (Millan

&Huerta, 2009; Pradelli *et al*., 2010). AIF is cleaved and activated by calpain or cathepsin proteases upon MOMP and it is released to the cytosol whereby large-scale DNA fragmentation and CICD are induced (Millan & Huerta, 2009). The down-regulation of AIF in metastatic colon cancer is the contributing factor to chemotherapy resistance (Millan & Huerta, 2009).

Circumvention of apoptosis is imputed to acquired resistance of metastatic cancer cells to chemotherapeutic agents and is therefore, the hallmark of cancer cells (Fulda, 2009). Imbalance towards proliferation and reduced cell death brought about by overexpression of antiapoptotic proteins and/or down-regulation or loss of function of pro-apoptotic proteins is typical to all types of cancer cells (Green & Evan, 2002). The development and application of specific molecules which target Bcl-2 family proteins to eventually induce MOMP and inevitably cell death have made a great breakthrough in the treatment of metastatic cancers (Giménez-Bonafé *et al*., 2009; Fulda *et al*., 2010a; Hockenbery, 2010). Another possible mechanism of resistance to cell death in cancer cells is the pro-survival process of autophagy (Dalby *et al*., 2010).

2.2.1.2 Extrinsic Death Receptor Pathway of Apoptosis

The extrinsic death receptor pathway, as suggested by its name, begins when a death ligand binds to a death receptor. Type 1 tumour necrosis factor receptor (TNFR1) is the best known death receptor among others, such as Fas and (TNF-related apoptosis-inducing ligand) TRAIL receptors. These receptors oligomerise upon binding with their ligands, TNF, FasL and TRAIL. Receptors then bind to adaptor proteins such as TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD) through an exposed death domain (DD) (Schneider & Tschopp, 2000). The death effector domain (DED) on FADD binds with the DED on pro-caspase-8 following

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complex formation with the receptor (Reed, 2000). Assimilation of the ligand-receptor-adaptor protein complex constitutes the death inducing signalling complex (DISC) which then initiates the assembly and activation of pro-caspase-8 (O'Brien & Kirby, 2008). The activated form of the enzyme, caspase-8, is an initiator caspase which induces apoptosis by activating and cleaving other downstream effector or executioner caspases-3, -6 and -7 (Kroemer *et al*., 2007; Karp, 2008). Caspase-8 provides a connection between the extrinsic and intrinsic pathways of apoptosis through an alternate mechanism in which caspase-8 cleaves and activates the pro-apoptotic Bcl-2 family protein Bid to truncated Bid (tBid), which subsequently interacts with anti-apoptotic Bcl-2 family proteins on the mitochondrial outer membrane to stimulate mitochondrial-mediated apoptosis (intrinsic pathway of apoptosis) (Billen *et al*., 2008).

2.2.1.3 Intrinsic MitochondrialPathway of Apoptosis

In the intrinsic pathway of apoptosis, caspase activation is associated with permeabilization of the outer mitochondrial membrane, which is a process regulated by pro-apoptotic members of the Bcl-2 family of proteins, mitochondrial lipids, proteins that regulate bioenergetics metabolite flux and components of the mitochondrial permeability transition pore (MPTP) (Green & Kroemer, 2004). The Bcl-2 family of proteins encompasses both pro- and anti-apoptotic members. Pro-apoptotic proteins are further classified as multi-domain or BH3-only proteins (Gogvadze *et al*., 2009).

The Bcl-2 family of proteins are well known for their function as regulators of apoptosis and are categorised into three subfamilies depending on the number of Bcl-2 homology (BH) domains they share. The first subfamily comprises four BH domains (BH1-4) and consists of anti-apoptotic proteins such as Bcl-2, Bcl-xL, Bcl-w, MCL-1 and A1/BFL-1, which are mainly located in the mitochondrial outer membrane (Kroemer *et al*., 2007;

Letai, 2008). The second subfamily consisted of pro-apoptotic proteins with three BH domains (BH1-3), represented by Bax, Bak and Bok. They are the key proteins accountable for MOMP. Bax is cytosolic while Bak resides in the outer membrane under normal physiological conditions. However, translocation of Bax to the outer membrane and conformational alteration in Bak are observed upon activation by pro-apoptotic stimuli (Fulda *et al.*, 2010a). The last subfamily is the BH3-only proteins that, as implied by their names, are characterised by the presence of only the BH3 domain. The BH3-only pro-apoptotic proteins are further subdivided depending on their role as either direct activators of Bax and Bak (tBid and Bim) or as derepressors of anti-apoptotic proteins such as Bik, Bad, Puma and Noxa (Letai, 2008; Brenner & Mak, 2009).

Cell survival and cell death in response to various physiological and pathological stimuli such as oxidative stress, hypoxia, genetic damage and high concentration of cytosolic calcium are controlled by a complex regulatory network formed by the Bcl-2 family of proteins (Lomonosova & Chinnadurai, 2010). Once the intrinsic pathway is activated, the BH1-3 pro-apoptotic proteins such as Bax and Bak undergo conformational changes resulting in oligomerisation and insertion in the outer mitochondrial membrane. As a consequence, permeabilization of the outer mitochondrial membrane followed by egression of apoptogenic factors such as cytochrome c and AIFs which subsequently activate the caspase cascade, leading to cellular demise will occur. This entire process is actively antagonised by the BH1-4 anti-apoptotic members (Kroemer *et al.*, 2007; Lomonosova & Chinnadurai, 2010). Cytoplasmic release of cytochrome c triggers the formation of a complex known as apoptosome.

MOMP is generally considered as 'the point of no return', after which the cells cannot be rescued (Gogvadze *et al.*, 2009). Along with the release of pro-apoptotic factors following MOMP, there is a succeeding loss of mitochondrial membrane potential (MMP) due to uncoupling of the mitochondrial respiratory chain (MRC) (Fulda *et al.*, 2010a). Dissipation of MMP is due in part to opening of the MPTP to a high-conductance state (Gogvadze *et al.*, 2009). A dysregulated influx of solutes due to accumulation of reactive oxygen species (ROS) and/or calcium overload through opened MPTP causes swelling of the mitochondrial matrix and rupture of the mitochondrial outer membrane and ultimately, resulting in cell death (Kinnally & Antonsson, 2007).

Yaacob *et al.* (2014) demonstrated that the induction of apoptosis in MCF-7 and MDA-MB-231 cells by *Strobilanthes crispus* leaves (SCS) was achieved by the activation of the executioner caspase-3/7. It was also shown that the initiator caspase-8 was strongly activated by SCS in both cells. The activation of caspase-9 following the activation of caspase-8 which subsequently led to the activation of the effector caspase-3 has been reported in the same study. In agreement to this, Chong *et al.* (2012) reported that the caspase-9 concentration was increased and cytosolic translocation of cytochrome c in MCF-7 cells was induced by the ethanolic extract of *S. crispus*. In another study conducted by Karimian *et al.* (2014), treatment of the hexane extract of the leaves of *Ferulago angulata* induced apoptosis in MCF-7 cells by triggering both the extrinsic and intrinsic pathways. The results obtained in these aforementioned studies were in defiance of the results reported by Kallio *et al.* (2005) a decade ago, which stated that there was no cleavage of caspase-9 in MCF-7 cells.
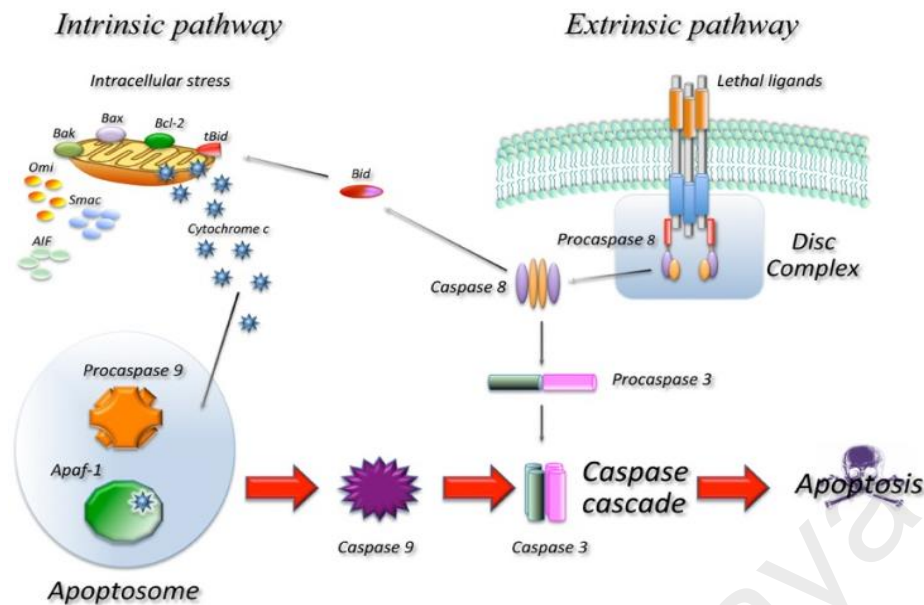
Figure 2.2: Extrinsic and intrinsic pathways of apoptosis. The extrinsic pathway is dependent on death receptor stimulation by the appropriate ligand, leading to death receptor trimerisation and recruitment of death effector domains and pro-caspase-8 that together constitute the death-inducing signalling complex (DISC). Activation of caspase-8 in turn leads to activation of caspase-3 and/or tBid (BH3-only proteins). The intrinsic pathway is depicted by p53 activation of pro-apoptotic Bcl-2 family of proteins in response to DNA damage which leads to MOMP and the release of pro-apoptotic factors such as cytochrome *c*, AIFs, SMAC/DIABLO).
[From "Role of apoptosis in disease" by Favaloro *et al*., 2012.*Aging*, *4*(5), 330-349].

2.2.2   Non-apoptotic Forms of Cell Death

2.2.2.1 Necrosis

Necrosis is usually triggered as a result of complement attack, severe hypoxia, hyperthermia, lytic viral infection and exposure to various toxins and respiratory poisons. In contrast to apoptosis or programmed cell death which is characterised by cytoplasmic shrinkage and nuclear densification (pyknosis), necrosis or accidental cell death, corresponds to swelling of the cell, affecting both cytoplasm and nucleus. There are significant differences in the physiopathology between both types of cell death (Mutaku *et al*., 2002). Other defining features of necrosis include disintegration of plasma and organelle membranes, remarkable organelle swelling (oncosis), pronounced inflammation and severe ATP depletion (Kung *et al*., 2011).

2.2.2.2 Autophagy

Autophagy is a cellular process that is portrayed by a self-cannibalisation mechanism which involves the engulfment of cytoplasmic material and intracellular organelles within double-membrane vesicles, known as autophagosomes (Nikoletopoulou *et al*., 2013). Completion of the autophagosome is followed by fusion with a lysosome to form an autolysosome, in which the captured material is degraded by specific acidic hydrolases (Levine & Kroemer, 2008). Autophagy is a catabolic process which is tightly regulated by autophagy-related proteins (ATG) that are activated downstream of inactivation of mammalian target of rapamycin (mTOR) kinase (Dalby *et al*., 2010; Essick & Sam, 2010). In addition to its cytoprotective role, autophagy also plays a part in promoting cell death during normal development and in disease (Mizushima *et al*., 2008; Mizushima & Levine, 2010). Autophagy is activated by starvation and other environmental and hormonal cues such as growth factor depletion, nutrient deprivation and hypoxia (Kroemer *et al*., 2010). Thus, survival is promoted upon degradation of cytoplasmic components in response to stress conditions. However, excessive autophagy may impose harmful effects in the context of specific diseases (Nikoletopoulou *et al*., 2013). This alleged duality of autophagy has brought about immense controversy pertaining to the role of autophagy in tumourigenesis and chemo resistance; and the complicated reciprocity between apoptosis and autophagy (Dalby *et al*., 2010).

According to several studies, an essential autophagy gene, Beclin-1, acts as a tumour suppressor as its mutation results in defective autophagy related to malignant transformation (Dalby *et al*., 2010; Fimia & Piacentini, 2010). In contrast, autophagy was reported to be used by tumour cells as a means of survival in the harsh conditions

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of the tumour microenvironment (Dalby *et al*., 2010). The contradictory roles of autophagy in cancer are supported by numerous research.

2.3   Crosstalk between Apoptosis and Necrosis

Less effort has been made to study necrosis due to the belief in its accidental, unregulated and uncontrolled nature despite knowing that necrosis occurs under various pathological conditions. Nonetheless, studies of the death receptors provide insights pertaining to regulated necrosis mechanisms. In most cells, activation of Fas and TNFR family of death receptors induces apoptosis instead of necrosis as the default cell death pathway through the recruitment of adaptor proteins such as FADD and upstream caspases such as caspase-8 (Hitomi *et al*., 2008). In certain cell types, if FasL or TNFα are stimulated under apoptosis deficient conditions, necrotic cell death might ensue instead, as a back-up cell death pathway (Vercammen *et al*., 1997; Kawahara *et al*., 1998). There is a dispute for the existence of a regulated cellular necrosis mechanism distinct from apoptosis, which is termed 'necroptosis', since activation of Fas/TNFα receptors may lead to either apoptotic or necrotic cell death (Degterev *et al*., 2005).

Receptor-interacting protein 1 (RIP1) is a death domain-containing kinase which is usually linked to the death receptors but its kinase activity is dispensable for the activation of MAPKs and NF-κB, which are involved in the induction of apoptosis mediated cell death (Chan *et al*., 2003; Degterev *et al*., 2005). In apoptosis deficient conditions as such, the kinase activity of RIP1 is necessary for the activation of necroptosis by death receptor agonists (Holler *et al*., 2000). Necrostatins (Necs) are small molecule inhibitors of necroptosis. Nec-1 was identified as an allosteric inhibitor of necroptosis and more importantly, the RIP1 kinase activity was found to be the target of Nec-1 (Degterev *et al*., 2005; Degterev *et al*., 2008).

Several studies have reported that RIP3 was identified as an important upstream activating kinase that regulates RIP-1-dependent necroptosis (Cho *et al*., 2009; He *et al*., 2009). The formation of a RIP1-RIP3 pro-necrotic complex is triggered by TNF treatment. During death receptor-induced apoptosis, caspase-8 cleaves RIP1 and RIP3, hence supressing their anti-apoptotic and/or pro-necrotic properties (Feng *et al*., 2007; Nikoletopoulou *et al*., 2013).

2.4 Crosstalk between Apoptosis and Autophagy

Both apoptosis and autophagy are well-controlled biological processes that play crucial roles in tissue homeostasis, development and disease. Both pathways are stimulated by similar stimuli. A complex crosstalk between apoptosis and autophagy is implied by the complicated interactions among the components of both the pathways. It was reported that both apoptosis and autophagy are activated in response to metabolic stress (Nikoletopoulou *et al*., 2013). Furthermore, it was suggested that autophagy is triggered as an adaptive response against endoplasmic reticulum (ER) stress. Disturbances in either ER calcium homeostasis or ER function lead to increased autophagy and apoptotic cell death. The influence of autophagy on cell survival in ER stress is dependent upon the type of tissue. ER-induced autophagy offers protection against cell death in colon and prostate cancer cells via the disposal of unwanted polyubiquitinated protein aggregates. Nevertheless, this does not occur in normal human colon cells, but rather, ER-induced apoptosis is triggered (Ding *et al*., 2007). Accumulating evidence shows that apoptosis and autophagy can cooperate, antagonise or assist each other, thus differentially affecting the fate of the cell (Nikoletopoulou *et al*., 2013).

In the process of autophagosome formation, the anti-apoptotic proteins, such as Bcl-2, Bcl-xL, Bcl-w and MCL-1, are reported to have interactions with the novel BH3-only

protein, Beclin-1 (Fulda *et al*., 2010b). Autophagy and autophagic cell death are inhibited by this interaction. It was reported that autophagy is inhibited when caspases inhibit Beclin-1 and the cysteine protease Atg4, involved in the elongation step of autophagy, while apoptosis is induced by calpain-cleavage of Atg5 (which is also involved in elongation) (Levine & Kroemer, 2008; Fimia & Piacentini, 2010).

Autophagy and/or apoptosis can be induced by tumour suppressor p53 through its cytosolic and nuclear effects in response to DNA damage as well as other stressors (Fimia & Piacentini, 2010). In addition, oxidative stress such as an augmented production of ROS can also induce apoptosis and autophagy (Essick & Sam, 2010).

Figure 2.3: Mechanism of crosstalk between apoptosis and autophagy. Apoptosis and autophagy share common stimuli and signalling pathway as well as exhibit some degree of mutual inhibition. Caspase-mediated cleavage of Beclin-1 generates fragments ('N' and 'C') that lose their ability to induce autophagy during sustained exposure to apoptotic stimuli. The C-terminal fragment translocates to the mitochondria and sensitises cells to apoptotic signals. In contrast, autophagy partly inhibits apoptosis by degrading active caspase-8 or preventing activation of Bid by Beclin-1.
(From "The Beclin 1 network regulates autophagy and apoptosis" by Kang *et al*., 2011.*Cell and Death Differentiation*, *18*, 571-580.).

2.5   Free Radicals

Free radicals are molecules or molecular fragments with one or more unpaired electrons (Ibraheim *et al*., 2015). Free radicals are generally less stable and highly reactive as the unpaired electron tends to pair with other electron(s). Numerous metabolic and physiological reactions in biological systems involve oxygen, which is mostly reduced to water. However, incomplete reduction will cause a series of reactive free radicals to be formed (Powers & Jackson, 2008). Free radicals are also generated due to the exposure to environmental factors such as ionising radiations, tobacco, unhealthy diet and trace metals (Pham-Huy *et al*., 2008).

Free radicals obtain electrons from the cellular lipid membrane, thereby initiating a free radical attack on the cell, otherwise known as lipid peroxidation. The carbon-carbon double bonds of polyunsaturated fatty acids of cellular membranes are the targets of reactive oxygen species (ROS) (Dekkers *et al*., 1996). As a consequence, the carbon-hydrogen bonds became weakened, rendering the dissociation of the hydrogen by the free radical. The free radical will then abstract the single electron from the hydrogen bound with the carbon at the double bond. This in turn leaves carbon with an unpaired electron which subsequently becomes a free radical (Halliwell & Gutteridge, 1985). The process continues in a cascade of chain reactions and would lead to detrimental effects. Some of the common free radicals found in the body are reactive oxygen species (ROS), reactive nitrogen species (RNS) and certain sulphur-centred radicals, just to name a few.

2.5.1   Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS), representing the most crucial class of radical species generated in living system, are radicals derived from oxygen. ROS are generated during normal oxidative phosphorylation as a consequence of incomplete electron transfers by

complexes I and III of the mitochondrial respiratory chain (MRC) (Gogvadze *et al*., 2009). Occasionally, under normal biological conditions, oxygen is capable of forming free radicals by capturing electrons from other molecules.

Superoxide anion radical ($O_2^{\bullet-}$) is the single electron reduction product of oxygen. It is considered as a primary ROS and it is generated in many autoxidation reactions by the electron transport chain (Sharma *et al*., 2012). Superoxide anion is a secondary messenger in a variety of cellular processes, one of which is to confer tolerance to various environmental stresses. This, in turn, controls a variety of physiological responses (Sharma *et al*., 2012). It is less reactive and less harmful in physiological systems. Nonetheless, it is able to react with other molecules to form secondary ROS, either directly or through enzymatic or non-enzymatic process. It undergoes dismutation to form hydrogen peroxide spontaneously or by enzymatic catalysis. If it involves the transfer of two electrons, the product is hydrogen peroxide (H_2O_2) which is mainly formed by dismutation of O_2^- or by direct reduction of O_2 (Spiers *et al*., 2015). H_2O_2 is lipid soluble, therefore enabling it to diffuse across cellular membranes. H_2O_2 is the precursor of the most reactive ROS, namely hydroxyl radical ($OH^{\bullet}$) albeit being a stable non-radical. Hydroxyl radical is one of the most potent oxidants known thus far with a very short half-life. It is extremely reactive and attacks most cellular components (Mokudai *et al*., 2012).

2.5.2   Reactive Nitrogen Species (RNS)

Nitric oxide ($NO^{\bullet}$) is a small molecule containing a single unpaired electron and is thus, a radical. Nitric oxide is generated in biological tissues via five electron oxidative reactions by nitric oxide synthases which metabolise arginine to citrulline (Förstermann & Sessa, 2012). It has a short half-life of only a few seconds and is therefore a highly

reactive molecule. Its stability in an environment is inversely proportional with oxygen concentration. The high solubility of nitric oxide in aqueous and lipid media enables it to readily diffuse through the cytoplasm and plasma membrane(Victor *et al*., 2009).

Table 2.1: Chemical and biological sources of ROS and RNS

ROS/RNS	Chemical and biological sources
Superoxide anion ($O_2^{\bullet-}$)	**Chemical** Univalent reduction of O_2 by hydrated electrons. **Biological** Enzymatic reactions, autoxidation reaction of oxyhaemoglobin, mitochondrial electron transport chain, phagocytosis.
Hydrogen peroxide (H_2O_2)	**Chemical** Photolysis of H_2O Dismutation reactions: $OH^{\bullet} + OH^{\bullet} \longrightarrow H_2O_2$ $2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2$ **Biological** Enzyme systems, phagocytosis.
Hydroxyl radical ($OH^{\bullet}$)	**Chemical** Fenton reaction: $Fe^{2+} + H_2O_2 \longrightarrow OH^{\bullet} + OH^- + Fe^{3+}$ **Biological** Radiolysis of H_2O_2, Fenton reaction.
Nitric oxide ($NO^{\bullet}$)	**Chemical** Smoke. **Biological** From vascular endothelial cells catalysed by nitric oxide synthases.

2.6   Antioxidants

Antioxidants are a group of substances which are able to significantly inhibit or delay any oxidative processes when they are present in low concentrations as compared to oxidizable substrates (Halliwell *et al*., 1992). Hence, it is of utmost importance to

maintain the equilibrium between oxidants and antioxidants. This equilibrium cannot be solely maintained by the endogenous antioxidant system, but rather, an exogenous supply of antioxidants is required (Bouayed & Bohn, 2010). Antioxidants can be divided into three main groups: antioxidant enzymes which catalyse the breakdown of free radical species; chain breaking antioxidants which are mostly small molecules like Vitamin C; and metal binding proteins to prevent metal ions from forming free radicals.

Natural antioxidants are potential pharmaceutical and therapeutic agents (Bazzano *et al*., 2001). The pharmacological and physiological actions of phenolic compounds are primarily ascribed to their antioxidant capacity, free radical scavenging ability and modulation of gene expression (Soobrattee *et al*., 2005).

2.7 Oxidative Stress

A system of enzymes comprising of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) contributes a vital antioxidant defence mechanism in the effort to quench and neutralise ROS and ultimately to minimise the effects of oxidants (Fulda *et al*., 2010b). These enzymes work by decreasing the concentrations of the most harmful oxidants in the body. The second line of defence is the presence of antioxidants. Antioxidants serve as the antidote to fight against free radicals (Bagchi & Puri, 1998). Antioxidants are essentially important to shield and protect cellular membranes from lipid peroxidation by free radicals.

Oxidative stress can be defined as the disruption in the balance between oxidants and antioxidants in favour of oxidants (Rahal *et al*., 2014). The potential biological damage caused by the harmful effects of ROS is termed oxidative stress when there is an excessive production of ROS and/or deficiency of enzymatic and non-enzymatic

antioxidants in the biological system (Kovacic & Jacintho, 2001). Oxidation of important biomolecules might occur due to oxidative stress. Cell damage and cell death might even occur in severe oxidative stress. Oxidative stress is thought to play a crucial role in numerous degenerative disorders including cancer, senescence, neurodegenerative diseases, cardiovascular diseases and rheumatoid arthritis (Uttara *et al.*, 2009).

2.8   The Balance between Prooxidants and Antioxidants

Endogenous and exogenous free radical formation due to normal metabolism and environmental oxidants is inevitable (Poljsak *et al.*, 2011). The mitochondrial electron transport chain and the nitric oxide synthase reaction are the most important endogenous sources of oxidants. In addition, free radicals are also formed exogenously through the production of energy from food and oxygen, exposure to microbial infections, ionising radiations and pollutants. Sies (1991) first defined oxidative stress as "a disturbance in the prooxidant to antioxidant balance in favour of the former, leading to potential damage that can culminate in cell death". Excessive amount of ROS will be produced as a consequence of the imbalance between the production and reduction of ROS (the latter is regulated by antioxidant defences) (Poljsak *et al.*, 2013).

Excessively produced ROS are scavenged by the antioxidant networks in cells. Generally, the balance between the production and scavenging of ROS leads to homeostasis. Nonetheless, the balance is somehow shifted towards the formation of free radicals, which leads to the accumulation of cell damage over time. The damaging effects of ROS can be attenuated by antioxidants (Poljsak *et al.*, 2013). However, some studies showed that antioxidant therapies are ineffective and sometimes, the intake of

antioxidants even increased the rate of mortality (Kim *et al*., 2002;Vivekananthan *et al*., 2003; Bjelakovic *et al*., 2004; Miller *et al*., 2005).

Some ROS are such as mitochondrial ROS, NOX, I, II and III complexes of the MRC signalling molecules involved in cellular signalling pathways (De Giusti *et al*., 2013). Hence, oxidative stress is both damaging and beneficial. It is crucial to maintain the balance between ROS and antioxidants, as both extremes, oxidative and antioxidative stress, are damaging (Poljsak *et al*., 2013). In normal physiological conditions, the balance between prooxidants and antioxidants is kept slightly in favour of prooxidants, thus favouring a mild oxidative stress (Dröge, 2002). Mild oxidative stress is usually well tolerated by cells. The cellular repair processes and other protective systems, such as those involving the chaperones, can even be upregulated by this mild oxidative stress (Poljsak *et al*., 2013).

Imbalance between ROS and antioxidants can be divided into increased oxidative stress and increased antioxidative stress. The former is due to a number of exogenous (pollution, smoking, nutrition, psychological and emotional stress) and endogenous (increased mitochondrial leakage, increased O_2 concentration and inflammation) factors (Poljsak, 2011; Poljsak *et al*., 2011; Poljsak & Milisav, 2012). Reduced antioxidant enzyme activities and insufficient intake of antioxidants from food also contribute to increased oxidative stress (Poljsak *et al*., 2013). The primary roles of antioxidants are to neutralise ROS and to decrease oxidative stress. However, an overconsumption of antioxidants is not always beneficial with respect to the development of a disease and its progression (such as cancer) as antioxidants are incapable of distinguishing among radicals with a beneficial physiological role and those that cause oxidative damage to biomolecules (Poljsak & Milisav, 2012). An overdose of some antioxidants could result

in antioxidative stress. The immune system and other defensive mechanisms which are involved in the removal of damaged cells, including those that are precancerous or cancerous, might be compromised when the level of free radicals is decreased upon the administration of antioxidants (Salganik, 2001). Some antioxidants such as beta-carotene, Vitamin C and Vitamin E can act as prooxidants by increasing oxidative stress when they are taken in excessive amounts (Surai *et al*., 2003; Poljsak *et al*., 2013). Hence, the physiological balance between the ROS formation and neutralisation may be interrupted by a high-level of antioxidants (Poljsak *et al*., 2013).

Some of the endogenous defence mechanisms deteriorate with age, while the production of free radicals increases (Sohal & Weindruch, 1996; Allemann & Baumann, 2008). Therefore, the antioxidant defence system must be capable of minimising the levels of harmful ROS on one side while still permitting sufficient ROS to maintain their useful functions, which include cell signalling and redox regulation (Poljsak *et al*., 2013).

2.9 Antioxidant Mechanisms

The mechanisms by which antioxidants protect against oxidative stress may vary. All antioxidants were classified into two groups, namely primary or chain-breaking antioxidants and secondary or preventive antioxidants (Ingold, 1968). However, it is noteworthy that certain substances undergo several mechanisms of antioxidant activities (McClements & Decker, 2000).

2.9.1 Primary (Chain-breaking) Antioxidants

A primary antioxidant, also known as a chain-breaking antioxidant, is defined as a substance which can accept free radicals and further delay the initiation step or interrupt the propagation step of autoxidation (Reische *et al*., 1998). Primary antioxidants can

react with lipid and peroxyl radicals, thereby converting these radicals into more stable radicals or non-radical products as depicted in equation (1).

$$R\bullet \; + \; AH \longrightarrow RH \; + \; A\bullet \tag{1}$$

$$RO\bullet \; + \; AH \longrightarrow ROH \; + \; A\bullet$$

$$ROO\bullet \; + \; AH \longrightarrow ROOH \; + \; A\bullet$$
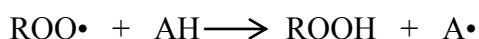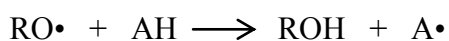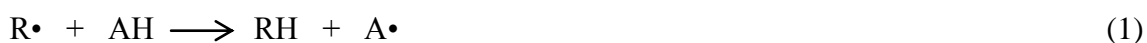
Where AH denotes primary antioxidant and A• denotes antioxidant radicals.

The A• generated by the processes as shown in equation (1) are much less reactive than lipid or peroxyl radicals, and hence do not promote oxidation as lipid or peroxyl radicals do. In contrast, these antioxidant radicals can terminate the lipid oxidation reaction by reacting with peroxyl radicals, alkoxyl radicals and other antioxidants as shown in equation (2) (McClements & Decker, 2000).

$$RO\bullet \; + A\bullet \longrightarrow ROA \tag{2}$$

$$ROO\bullet \; + A\bullet \longrightarrow ROOA$$

$$A\bullet \; + A\bullet \longrightarrow AA$$
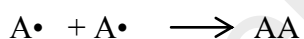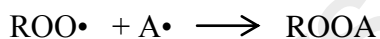
2.9.2 Secondary Antioxidants

Secondary antioxidants differ from primary antioxidants as the former usually only delay oxidation by interfering with the prooxidant system, such as metal and radiation, but do not transform or convert free radicals into more stable products. Lipid oxidation can be retarded by secondary antioxidants through a variety of mechanisms which include oxygen scavenging, absorbing UV radiation, chelation of transition metal ions, replenishing hydrogen to primary antioxidants and deactivation of reactive species (Gordon, 1990; Reische *et al*., 1998). Most of the secondary antioxidants show

antioxidant activity only if a minor prooxidative component is present in the system. For example, reducing agents such as ascorbic acid are effective in the presence of tocopherols or other phenolic antioxidants; and sequestering agents are only effective in the presence of metal ions (Gordon, 1990).

2.10   Phytochemicals in Marine Organisms

The marine environment has always been thought of as a tranquil and peaceful habitat. However, this is not the case for marine organisms. The marine environment is a highly perilous place where predators are abundant and competition is furious. Marine organisms such as seaweeds and sponges nourish themselves through the process of osmosis despite not having roots, leaves or vascular systems like terrestrial plants (Gupta & Abu-Ghannam, 2011). Due to the complications and diversity of the environment, marine organisms have developed a range of complex mechanisms for survival and defence purposes. Marine phytochemicals might be far more powerful than phytochemicals from terrestrial organisms due to their ability to kill prey rapidly. Hence, they might serve as potent anticancer agents (Wu *et al*., 2000).

It is a basic requirement for all living organisms to interconvert and transform prodigious amounts of organic compounds to ensure life, growth, reproduction and survival. For this, an integrated network of enzyme mediated and cautiously regulated reactions are involved. Conjointly, they are referred to as metabolism and the reaction steps involved are termed metabolic pathways (Craney *et al*., 2013). The pathways for modification and synthesis of carbohydrates, proteins, nucleic acids and fats are described as primary metabolism and they are essentially the same in all organisms albeit the varying characteristics among all the organisms (Muto *et al*., 2013). Hence,

processes such as glycolysis, protein digestion and β-oxidation of lipids are categorised as primary metabolism.

On the other hand, secondary metabolism is defined as metabolism concerning compounds with limited distribution in nature which is particular to certain organisms. Such organisms are therefore termed secondary metabolites (Craney *et al*., 2013). Secondary metabolites are basically produced in response to oxidative or climatic stress, infection, for propagation and defence against predators. Phenolic compounds, alkaloids, steroids and terpenoids are some examples of pharmacologically active natural products of secondary metabolism (Iqbal *et al*., 2015). Phytochemicals which are known as secondary metabolites with pharmacological significance are discussed below.

2.10.1 Lipids and Their Derivatives

Lipids are biomolecules which are insoluble in water but soluble in non-polar organic solvents. However, there are some limitations in this classification as most organic compounds fall in this category and many classical fatty acids have significant solubility in water. A more constricted classification of lipids is to classify them as fatty acids and their derivatives while other hydrocarbon-based natural products are treated separately (Fahy *et al*., 2005).

2.10.1.1   Hydrocarbons

Hydrocarbons are made up of a relatively small group of natural products with low polarity or non-polar nature. Aliphatic hydrocarbons are mainly derived through the decarboxylation of fatty acids and usually have an odd number of carbon atoms (Savage *et al*., 1996). Aliphatic and aromatic as well as saturated and unsaturated hydrocarbons are found in marine organisms.

2.10.1.2   Alcohols

Vast varieties of volatile aliphatic alcohols occur in small amounts in marine organisms as a part of essential oils. Straight chain alcohols from C_1 to C_{10} exist either in free or esterified forms. Aliphatic alcohols containing cis-3-hexene-1-ol moiety have characteristic odours and have thus sparked interest in the fragrance industry (Clark, 1990).

2.10.1.3   Aldehydes and Ketones

Low and medium molecular weight aldehydes and ketones occur as a part of volatile oils. Citral, octanal and nootketone are some examples of carbonyl compounds of industrial importance (Moshonas, 1971).

2.10.1.4   Fatty Acids (FAs)

Natural FAs may comprise of 4-30 carbon atoms with the most abundant being those with 16-18 carbons. FAs mainly occur as esters with glycerol and are called fats or oils, depending on whether they exist as solid or liquid state in room temperature. Mixed triglycerides are the main constituents of most natural fats and oils. Fats from marine organisms contain predominantly unsaturated FA esters and tend to be liquids (Bergé & Barnathan, 2005).

2.10.1.5   Terpenoids

Terpenoids form a massive and structurally diverse family of natural products derived from C_5 isoprene units, joined in a head to tail manner, which therefore are also referred to as isoprenoids. Typical structures contain carbon skeletons represented by $(C_5)_n$, and are classified as hemiterpenes (C_5), monoterpenes (C_{10}; example: geraniol), sesquiterpenes (C_{15}; example: farnesol), diterpenes (C_{20}; example: geranylgeraniol),

sesterterpenes (C_{25}), triterpenes (C_{30}; example: squalene) and tetraterpenes (C_{40}; example: phytene) (Figure 2.4) (Gross & König, 2006).
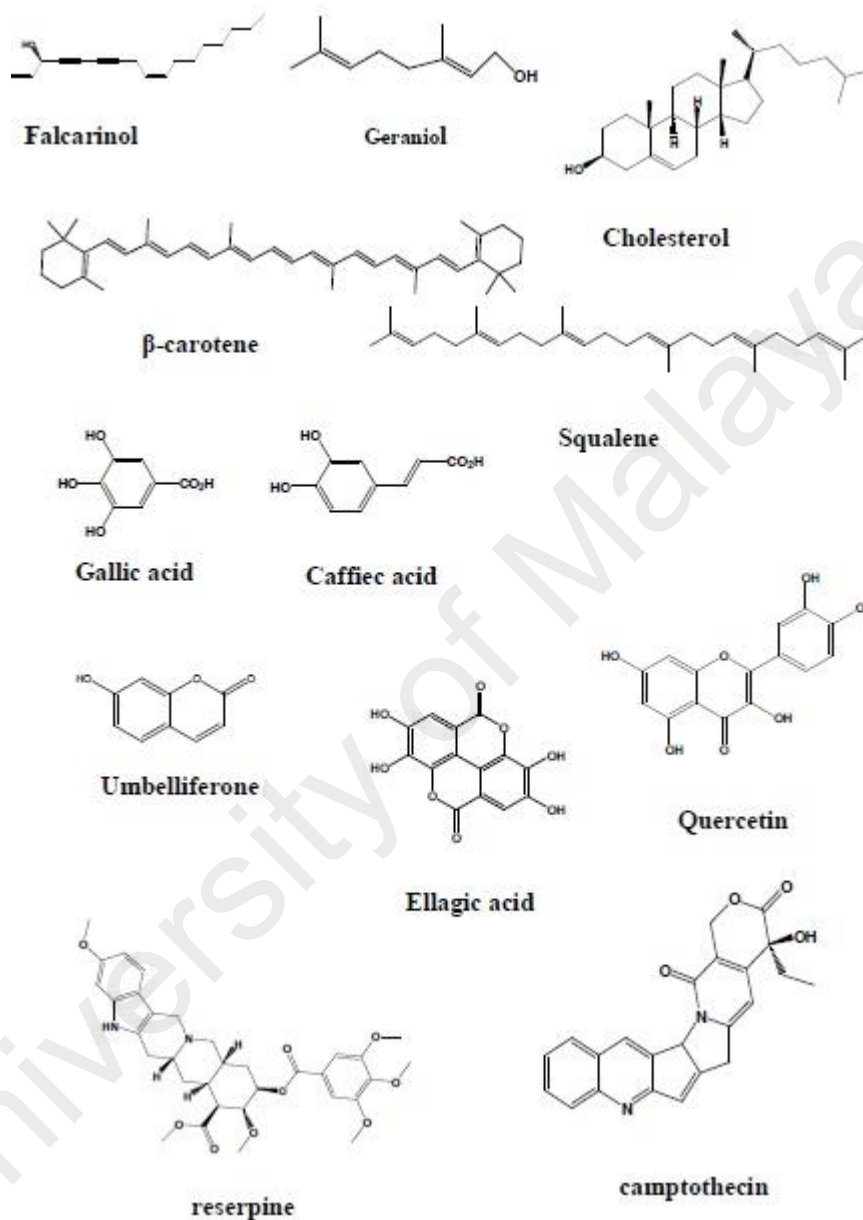
Figure 2.4: Structures of some biologically active phytochemicals.
(From "Terpenoids from marine organisms: unique structures and their pharmacological potentials" by Gross & König, 2006. *Phytochemistry Reviews*, *5*, 115-141).

2.10.1.6   Steroids

Steroids are modified triterpenoids containing the tetracyclic ring system of lanosterol.

Cholesterol (Figure 2.4) exemplifies the basic structure of steroids. Side-chain

modifications lead to a broad range of biologically important natural products (Focken & Hanessian, 2014). A variety of bioactive compounds which are considered as steroids are sterols, steroidal saponins, bile acids, cardio active glycosides, mammalian sex hormones and corticosteroids (Kris-Etherton *et al*., 2002).

2.10.1.7   Carotenoids

Carotenoids are the most common tetraterpenoids which are widely distributed and are derived from lycopene through the cyclisation of end groups. Not only do carotenoids impart bright colour to plants, but are also involved in photosynthesis and antioxidant defence mechanism (Mikami & Hosokawa, 2013).

2.10.2 Aromatics

Compounds with aromatic rings contribute a large part of natural products. Aromatic compounds are produced through several biosynthetic pathways including polyketide and shikimate pathways as well as from terpenoids (Vogt, 2010). Phenols represent the vast majority of aromatic compounds (Pereira *et al*., 2009).

2.10.2.1   Phenolic Compounds

Phenolic compounds contain one or more hydroxyl groups attached directly to an aromatic ring. The hydrogen of the phenolic hydroxyl is labile because of the aromatic ring, making phenols weakly acidic. Compounds having more than one phenolic hydroxyl group attached to one or more benzene rings are termed polyphenols. The term 'phenolics' covers a large and diverse group of chemical compounds. Phenolic compounds can be classified in several ways. Harborne and Simmonds (1964) classified these compounds based on the number of carbons in the molecule (Table 2.2).

Table 2.2: Structure-based classification of phenolic compounds

Structure	Class
C_6	Simple phenolics
C_6-C_1	Phenolic acids and aldehydes
C_6-C_2	Acetophenones and phenyl acetic acids
C_6-C_3	Cinnamic acids, cinnamyl alcohols and cinnamyl aldehydes
C_6-C_1-C_6	Benzophenones
C_6-C_2-C_6	Stilbenes
C_6-C_3-C_6	Flavonoids
C_{18}	Betacyanins
C_{30}	Biflavonoids
C_6, C_{10}, C_{14}	Quinines
Dimers or oligomers	Lignans and neolignans
Oligomers or polymers	Tannins
Polymers	Phlobaphens

2.10.3 Amines and Alkaloids

2.10.3.1   Amines

Amines are compounds containing nitrogen as a part of their side chain. These molecules act as insect attractants as they impart a fishy odour (Brenner, 2002).

2.10.3.2   Alkaloids

Alkaloids present special interest for researchers due to their pharmacological activities. Alkaloids represent the most significant compounds, not only in terrestrial plants, but especially in marine organisms and marine algae (Mayer *et al*., 2010).

Meissner first proposed the term 'alkaloid' in 1819 to characterise these 'alkali-like' compounds found in plants but it was not defined accurately (Swan, 1967; Pelletier, 1970). With time, the definition has changed to a compound which has nitrogen atom(s) in a cyclic ring (Bentley, 1957). The term alkaloid then comprises of numerous

biological amines and halogenated cyclic nitrogen-containing substances; whereby the latter is specific from marine organisms and marine algae, which could not be discovered in terrestrial plants (Güven *et al*., 2010).

Since the 18th century, pure active compounds are used in therapy after isolation of alkaloids instead of plant extracts. Morphine was the first alkaloid isolated from a terrestrial plant in 1805 as reported by Kappelmeier (1912) while hordenine was the first alkaloid extracted from a marine alga in 1969 (Güven *et al*., 1969; Güven *et al*., 1970). Alkaloids in marine algae are classified into phenylethylamine alkaloids, indole and halogenated indole alkaloids and other alkaloids (Güven *et al*., 2010).

The phenylethylamine group of alkaloids is comprised of phenylethylamine (PEA), N-acetylphenylethylamine (N-ACPEA), tyramine (TYR), N-acetyltyramine (N-ACTYR), hordenine (HORD) and dopamine (DOP). PEA is an aromatic amine in which an ethylamine side chain is attached to a benzene ring. The PEA alkaloid group is a precursor of many natural and synthetic compounds. The structure of PEA enables substitutions on the aromatic rings, the α and β carbons and terminal amino groups (Güven *et al*., 2010). PEA acts as a neuromodulator and a neurotransmitter in the human brain, whereby it has been shown to relieve depression in 60% of depressed patients (Barroso & Rodriguez, 1996).
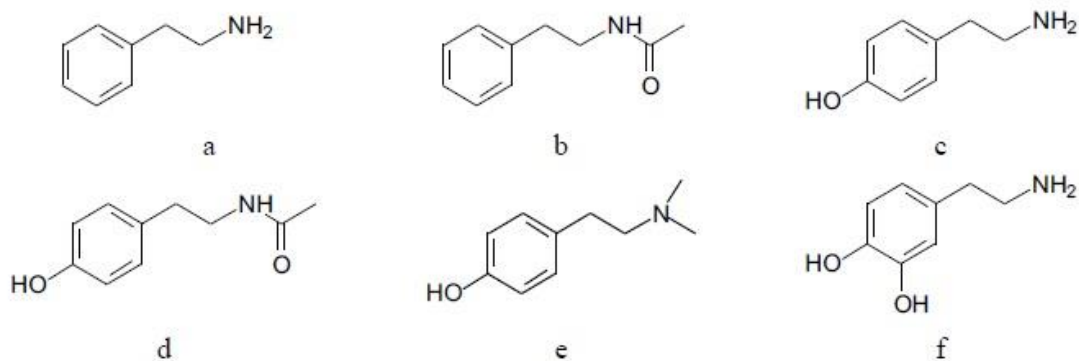
Figure 2.5: Structures of phenylethylamine derivatives: (a) PEA; (b) N-ACPEA; (c) TYR; (d) N-ACTYR; (e) HORD; (f) DOP.
(From "Alkaloids in marine algae" by Güven *et al*., 2010.*Marine Drugs*, *8*, 269-284).

The indole group of alkaloid containing a benzylpyrrole (derived from tryptophan) includes caulerpin (CLP), caulersin (CLS), martensine (MRT), martefragine (MRF), fragilamide (FRG), denticine (DTC) and almazolone (ALM) (Figure 2.6).
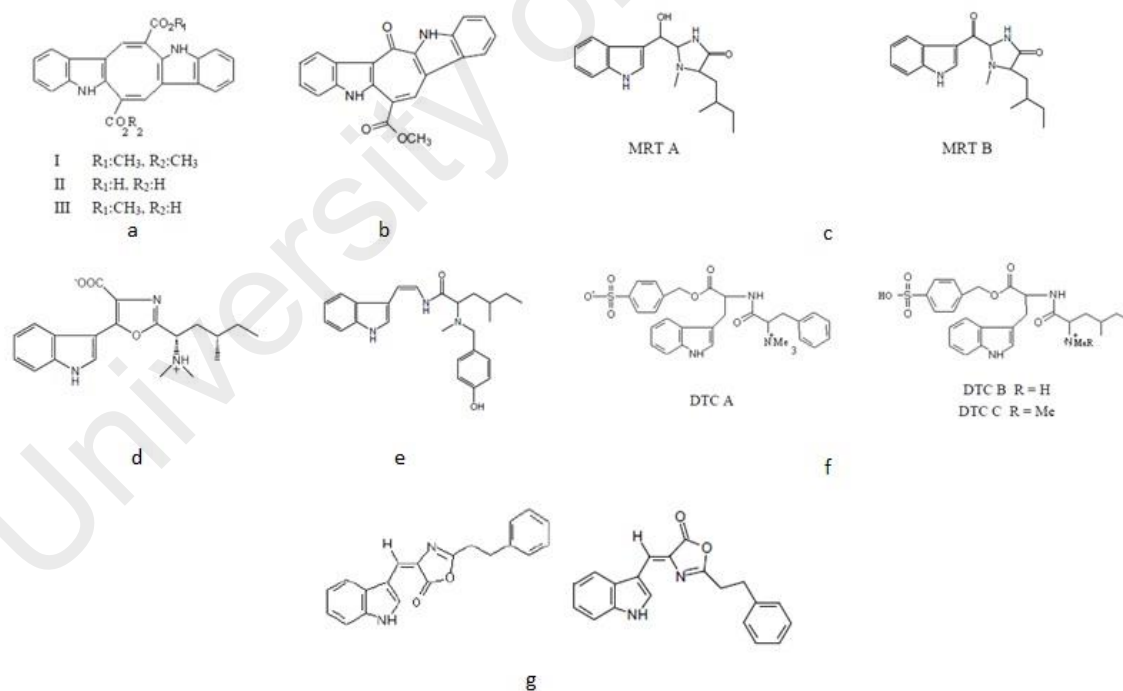
Figure 2.6: Structures of indole group of alkaloids: (a) CLP; (b) CLS; (c) MRT A and MRT B; (d) MRF; (e) FRG; (f) DTC; (g) ALM.
(From "Alkaloids in marine algae" by Güven *et al*., 2010.*Marine Drugs*, *8*, 269-284).

Halogenated indole (HLI) alkaloids were only isolated from marine organisms and algae but not in terrestrial plants. HLI alkaloids are usually isolated from red algae and only one is isolated from green algae. This group of alkaloids contain an indole group replaced by chlorine and bromine atoms. Sulfur-containing bromoalkaloids were also isolated from red algae (Figure 2.7). Antibacterial activities of halogenated alkaloids have been studied in terrestrial and marine bacteria (Güven *et al*., 2010).

a. $R_1 = CH_3$, $R_2 = H$, $R_3 = Br$,
b. $R_1 = CH_3$, $R_2 = Br$, $R_3 = H$,
c. $R_1 = H$, $R_2 = Br$, $R_3 = Br$,
d. $R_1 = CH_3$, $R_2 = Br$, $R_3 = Br$,

e. $R_1 = R_3 = H$, $R_2 = Br$,
f. $R_1 = R_2 = Br$, $R_3 = H$

g. $R_1, R_2 = H$, $R_3, R_4, R_5 = Br$
h. $R_1 = CH_3$, $R_2 = H$, $R_3, R_4, R_5 = Br$
i. $R_1, R_4 = H$, $R_2, R_3, R_5 = Br$

j
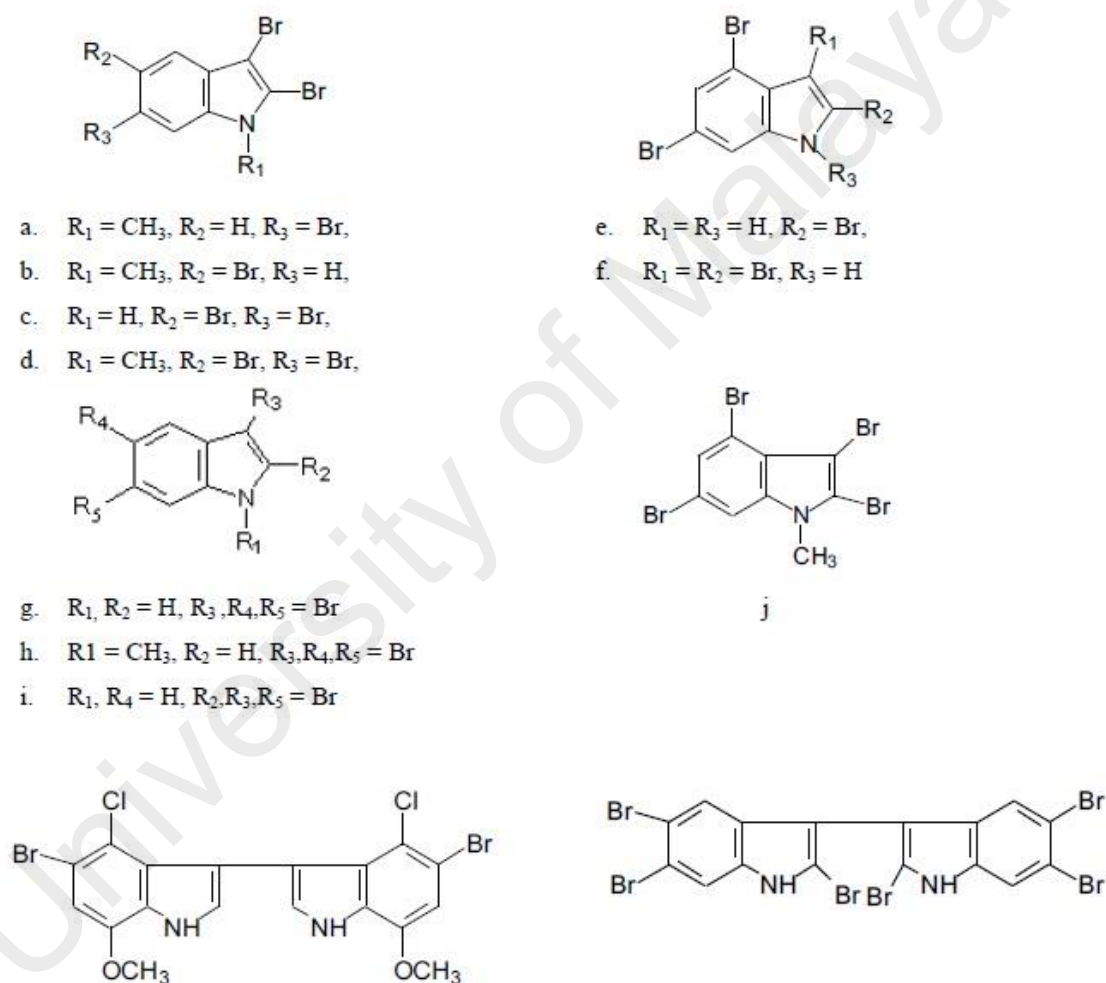
Figure 2.7: Structures of some halogenated indole alkaloids.
(From "Alkaloids in marine algae" by Güven *et al*., 2010. *Marine Drugs*, *8*, 269-284).

2.11   Cancer Therapy

2.11.1 Conventional Cancer Therapy

Despite the rapid advances in medical technology, cancer still remains as the hardest battle to combat by medical practitioners. The development of chemotherapeutic drugs and treatments such as chemotherapy, DNA alkylating agents, radiation and surgery serves as a means to curb cancer. Each of these conventional treatments comes with side effects such as metastasis of cancer cells, compromised immune system and damage to other unaffected organs of the body (Malaker & Ahmad, 2013).

Chemotherapy is administered following surgical resection of the primary tumour mass. Chemotherapy has not drastically changed since the mid-1970s apart from improvements to stability and toxicity of the treatment (Chabner & Roberts, 2005). Some widely used chemotherapeutic drugs are 5-fluorouracil (5-FU) for advanced colorectal carcinoma, anti-metabolites like methotrexate, anti-mitotics such as paclitaxel, DNA alkylating agents such as cisplatin or cyclophosphamide and topoisomerase inhibitors like etoposide and camptothecin (Chabner & Roberts, 2005; Goodwin & Asmis, 2009). While their long track record demonstrates superior utility in the combat against cancer, it is undeniable that chemotherapy belies the serious toxic effects associated with drugs that have such common critical targets and hence, is often used in combination with agents that provide protection to normal cells (Blagosklonny, 2005; Chabner & Roberts, 2005). Severe side effects and dose limitations are some of the prevailing complications arising despite the use of the aforementioned protective drugs for the treatment of metastatic cancer. Currently available drugs generally kill both proliferating cancer and normal cells due their failure in distinguishing the cells (Shanmugaraju *et al*., 2011). Some of the complications of conventional drugs and

treatments include significant heart damage brought about by the usage of chemotherapy drugs.

Radiation, on the other hand, causes acute and chronic side effects. Radiation damages normally dividing cells in its attempt to eliminate cancer cells and this will subsequently lead to acute radiation side effects at the treated site (Lawrence *et al*., 2008). Chronic side effects of radiation include fibrosis, infertility and rarely, the development of a second cancer caused by radiation therapy (Travis *et al*., 2008). Surgery is believed to contribute to metastasis, thereby activating dormant tumour cells. In addition, surgery may contribute to the early peak of relapses observed after removal of the primary tumour (Katharina, 2011). Owing to this, conventional treatments, surgery and semisynthetic drugs show very limited effects and pose severe side effects to humans.

Moreover, conventional cancer chemotherapy has the limitation of multidrug resistance (MDR) which is due in part to the over expression of integral membrane drug transporter proteins which can cause efflux of intracellular anticancer drugs and thereby, reduce the accumulation of drugs (Bai *et al*., 1992). A feasible strategy to overcome MDR is through the development of new anticancer drugs which can serve as modulators of MDR with reversal effects.

2.11.2 Current Cancer Therapy

Increased understanding of the complex molecular and genetic changes related to cancer has brought about the development of targeted therapy. The first breakthrough targeted therapy was imatinib for the treatment of (Ph+) chronic myelogenous leukemia, which paved the way for new targeted molecules against other types of cancers (Chabner & Roberts, 2005). Thereafter, monoclonal antibodies developed against growth factors,

such as Gefitineb, and oncogenic receptor tyrosine kinases, like Herceptin and Cetuximab, were approved for clinical use (Blagosklonny, 2005; Goodwin & Asmis, 2009). Tamoxifen is an oestrogen-receptor antagonist used in endocrine therapy for treating breast cancer (Wang *et al*., 2009b). These targeted therapies are widely used as adjuncts to chemotherapy in efforts to ameliorate the effects of cancer cell-killing (Goodwin & Asmis, 2009; Ficazi *et al*., 2010). In addition, hormone antagonists which are aromatase inhibitors can only be used in postmenopausal patients (Bird & Swain, 2008; Ewer *et al*., 2011; Petit *et al*., 2011).

2.11.3 Complementary and Alternative Cancer Therapies

All of the abovementioned treatments are still being used to date despite the side effects that come with the treatments. Tumours also have the ability to adapt to the selective pressures of conventional therapies, thus gaining resistance to treatment and metastatic capabilities (Don & Hogg, 2004; Letai, 2008; Fulda *et al*., 2010a). That being said, more and more people are resorting to complementary and alternative cancer therapies. According to Cassileth (1999), complementary therapies are used as adjuncts to mainstream cancer therapy such as chemotherapy or surgery for symptom management and to improve the quality of life. Alternative therapies, in contrast, may be perceived literally per se and they include any unproven therapies that are promoted as a cancer treatment or as a treatment to be used in place of mainstream oncology care.

The National Centre for Health Statistics of the United States reported that cancer patients have a high tendency to die from non-cancer causes (Bell, 2010). Consequently, the negative effects on the quality of life as well as the short- and long-term symptoms or cancer-related medical issues were some of the initiating factors for cancer patients to opt for complementary and alternative therapies (Carpenter *et al*., 2008; Goldstein *et al*.,

2008; Wesa *et al*., 2008). An increased prevalence in the use of complementary and alternative therapies is also due to the use of chemotherapy during treatment (Mao *et al*., 2008).

Numerous Chinese herbal medicines are being used as adjuncts to chemotherapy or radiotherapy to improve the efficacy of conventional cancer therapies and to reduce the side effects and complications that arise from conventional cancer treatments, although this practice is highly frowned upon by many practitioners of western medicine (Yin *et al*., 2013). Between 28% and 98% of ethnic Chinese cancer patients in Asia are reported to have consumed herbal medicines as part of their cancer therapy. A number of traditionally used Chinese and Asian herbal medicines such as astragalus, turmeric (curcumin), ginseng and kanglaite are used widely by cancer patients to either "treat" cancer and/or "minimise the toxicity" induced by chemotherapy or radiotherapy (Yoshida *et al*., 1997; Hofseth & Wargovich, 2007; Guo *et al*., 2008; Jurenka, 2009).

2.12 Marine Natural Products and Their Biological Activities

In contrast to the terrestrial environment which has been screened for sources of novel drugs for over 4,000 years, until recently, the marine environment was to a certain extent, inaccessible for such investigations. High diversity and limited resources lead to a persistent competition among marine organisms (Angelini *et al*., 2011). Marine organisms developed an outstanding arsenal of chemical weapons through millions of years in their daily combat for survival as most of them contain only a primitive immune system as a defence mechanism. These bioactive compounds are essentially highly potent substances due to the immediate dilution by large volumes of sea water (Amador *et al*., 2003). During the last few decades, tremendous efforts had been made by both research and pharmaceutical companies in the screening of marine biotopes for

new compounds that may help to battle diseases such as cancer. Marine natural products possess a broad spectrum of bioactivities. Undoubtedly, antimicrobial, antifouling and cytotoxic activities contribute to the enormous portion of bioactivities which are the most essential ones for survival. As reviewed by Mayer *et al*. (2013), a total of 102 marine natural products were reported to possess antibacterial, antifungal, antiprotozoal, antiviral and antituberculosis pharmacological activities.

Chrysophaentin A isolated from an alga, lyngbyoic acid isolated from a cyanobacterium and discorhabdin Z from a sponge, are some examples of marine compounds with antibacterial activity, in which their mechanism of action has been studied. More than half of the mechanisms of action of the compounds still remain undetermined. In addition, sixty marine compounds were reported to possess antidiabetic and anti-inflammatory effects apart from affecting the immune and nervous systems. Furthermore, sixty eight marine metabolites will probably contribute to multiple pharmacological properties upon further studies on the mechanisms of action due to their interaction with a variety of receptors and molecular targets (Mayer *et al*., 2013).

Identification of chemical entities for use in treatment of cancer is the main essence in the discovery of anticancer drugs. To date, the existence of technological and methodological advances in structure elucidation, organic synthesis and development of bioassays have resulted in the isolation and clinical evaluation of various novel anticancer compounds (Simmons *et al*., 2005). Moreover, this enables a deeper comprehension regarding tumour biology and molecular medicine, thus offering new insights into mechanisms responsible for neoplastic disease. Marine floras are rich in medicinally potent phytochemicals which predominantly consist of polyphenols and sulphated polysaccharides (Boopathy & Kathiresan, 2011).

These phytochemicals have exhibited a broad spectrum of pharmacological properties which include antioxidant, antitumour and immunostimulatory activities. Furthermore, they can possibly control carcinogenesis by activation of macrophages, induction of apoptosis and prevention of oxidative damage of DNA (Boopathy & Kathiresan, 2011). Some of the marine floral derivatives with anticancer or cytotoxic activities include Ecteinascidin 743, Dolastatin 10, Bryostatin 1, Didemnin B and Lamellarin D, among others.

The physiological process whereby new blood vessels are formed from pre-existing vessels is termed angiogenesis, which plays a vital role in growth and development, as well as in the formation of granulation tissue and wound healing (Carmeliet & Collen, 1998). However, this is not the case for cancer as angiogenesis is the fundamental step in the transition of benign to malignant tumours. There is an increased demand for nutrients from blood during the tumour development and this can only be met by the formation of new blood vessels through overexpression and release of growth factors such as vascular endothelial growth factor (VEGF) (Chung *et al*., 2010). Angiogenesis also facilitates metastasis by enabling the tumour to spread throughout the body (Shayan *et al*., 2006).

Several anti-angiogenic agents have been discovered from marine sources, for instance, fascaplysin, which is an indole alkaloid isolated from the marine sponge, *Fascaplysinopsis* sp., found to specifically inhibit the cyclin dependent kinase 4 (CDK4), which is involved in the regulation of the G0/G1 phase and G1/S phase transition of the cell cycle. The anti-angiogenic activity of the compound was proven by its ability to block VEGF (Zheng *et al*., 2010). Another example is cortistatin A, which is a steroidal alkaloid isolated from the marine sponge *Corticium simplex*. This

compound has the ability to selectively inhibit proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVECs) (Aoki *et al*., 2006).

Apoptosis can be indirectly influenced by some marine derived compounds which can regulate protein kinases and phosphatases through modulation of signal input/transmission to cell death pathways. For instance, bryostatins are macrocyclic lactones isolated from the marine bryozoan, *Bugula neritica*. Bryostatin 1, the most abundant of this group, which is currently under phase I clinical trials, is able to inhibit the growth of murine P388 leukaemic cells at subnanomolar concentrations through overactivation of protein kinase C (Mayer *et al*., 2013). Another potent cytotoxic marine natural product that induces apoptosis in cultured human cells in picomolar to nanomolar range is aurulide. Sato *et al*. (2011) reported that aurilide selectively binds to prohibitin 1 (PHB1) in the mitochondria, thereby activating the proteolytic processing of optic atrophy 1 (OPA1) and leads to mitochondria-induced apoptosis.

Tubulin is the main component of cellular microtubules which plays a major role in maintaining cell shape during interphase and in the formation of mitotic spindle fibres required for mitosis. Current drugs targeting tubulin are vinca alkaloids and taxol which inhibit microtubule polymerisation and depolymerisation, respectively. Marine natural products that interrupt mitosis constitute probably the most crucial class of anticancer drug leads, as more have been characterised than perhaps any other classes of anticancer or antitumour marine natural products (Hamel & Covell, 2002). Halichondrin B isolated from the marine sponge, *Halinchondria okadai*,is an example of important antimitotic marine natural products. Its synthetic analogue, eribulin mesylate which binds to the vinca domain of tubulin, is now marketed as Halaven™ since 2010 (Bai *et al*., 1991; Mayer *et al*., 2013). A more recent example is leiodermatolide, a polyketide macrolide

isolated from the marine sponge, *Leiodermatium* sp., which displayed potent antimitotic activity and strongly inhibited proliferation in several cell lines such as the human A549 lung adenocarcinoma, PANC pancreatic carcinoma, P388 murine leukaemia, DLD-1 colorectal carcinoma and NCI/ADR-Res ovarian adenocarcinoma at nanomolar concentrations (Paterson *et al*., 2011).

Topoisomerases control the number and topological conformations of supercoils in DNA. They are categorised into two types – type I (topo I) and type II (topo II) topoisomerase. The enzymes Ecteinascidin 743 or trabectidin, marketed as Yondelis®, is an alkaloid isolated from the sea squirt, *Ecteinascidia turbinate*, and features three fused rings. Two ring systems (subunits A + B) inhibit proteins required for DNA replication, such as DNA polymerase and topoisomerases by covalently binding the DNA and resulting in cross links. On the other hand, the third ring (subunit C) projecting from the DNA duplex enables interactions with adjacent nuclear proteins that are involved in DNA repair mechanisms (Dubois & Cohen, 2009).
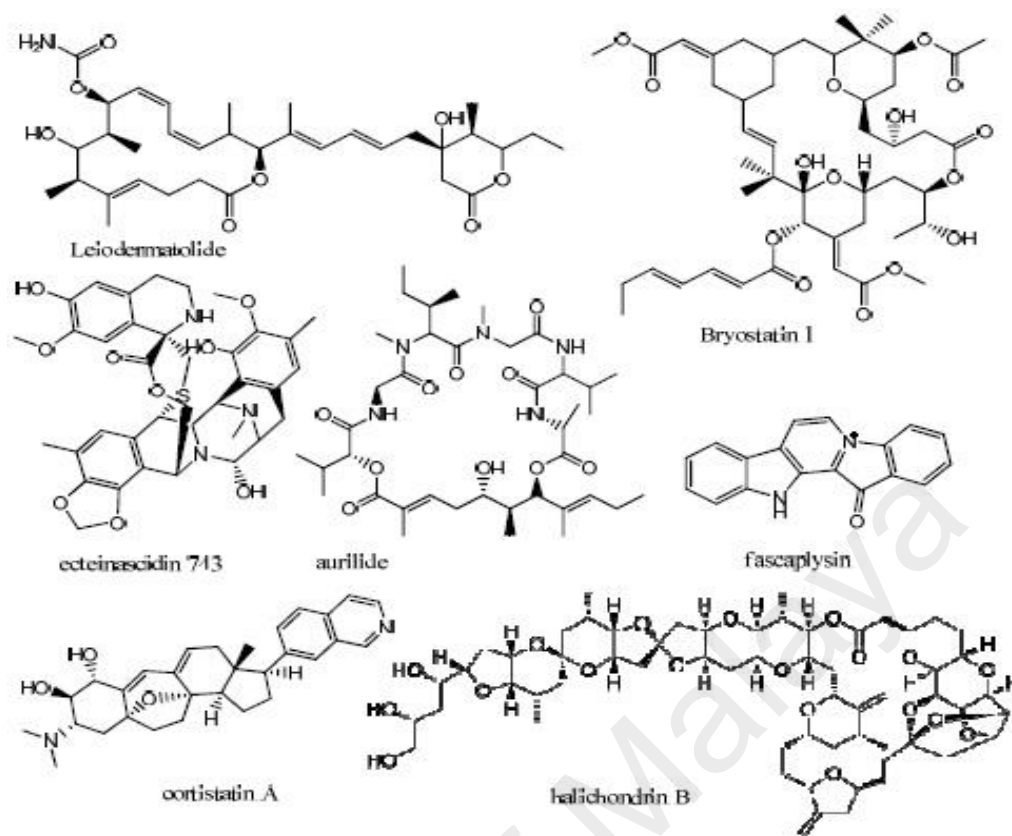
Figure 2.8: Several cytotoxic marine natural products.
(From "Alkaloids in marine algae" by Güven *et al*., 2010.*Marine Drugs*, *8*, 269-284).

2.13   Current Marine-Derived Drugs in Pre-Clinical and Clinical Trials

Marine organisms have been in the limelight for scientists as they have been proven to possess potentially active metabolites which are considerably valuable for further pre-clinical and clinical trials. Among the well-known examples of marine-derived drugs which have been approved by the Food and Drug Administration (FDA) and used in medical treatment are Yondelis® as an antitumour agent for the treatment of advanced soft tissue sarcoma and ziconotide (marketed as Prialt®) as a potent analgesic for severe chronic pain. Halaven™ made an entry into the US drug market in 2010. Adding to cytarabine and vidarabine which are long known in the standard anticancer and antiviral treatments, other drugs such as kahalalide F, plitidepsin (Aplidin®) and jorumycin derivative (Zalypsis®) are currently in clinical trials for treatment of solid tumours and haematological malignancies (Mayer *et al*., 2013; Newman & Cragg, 2014).
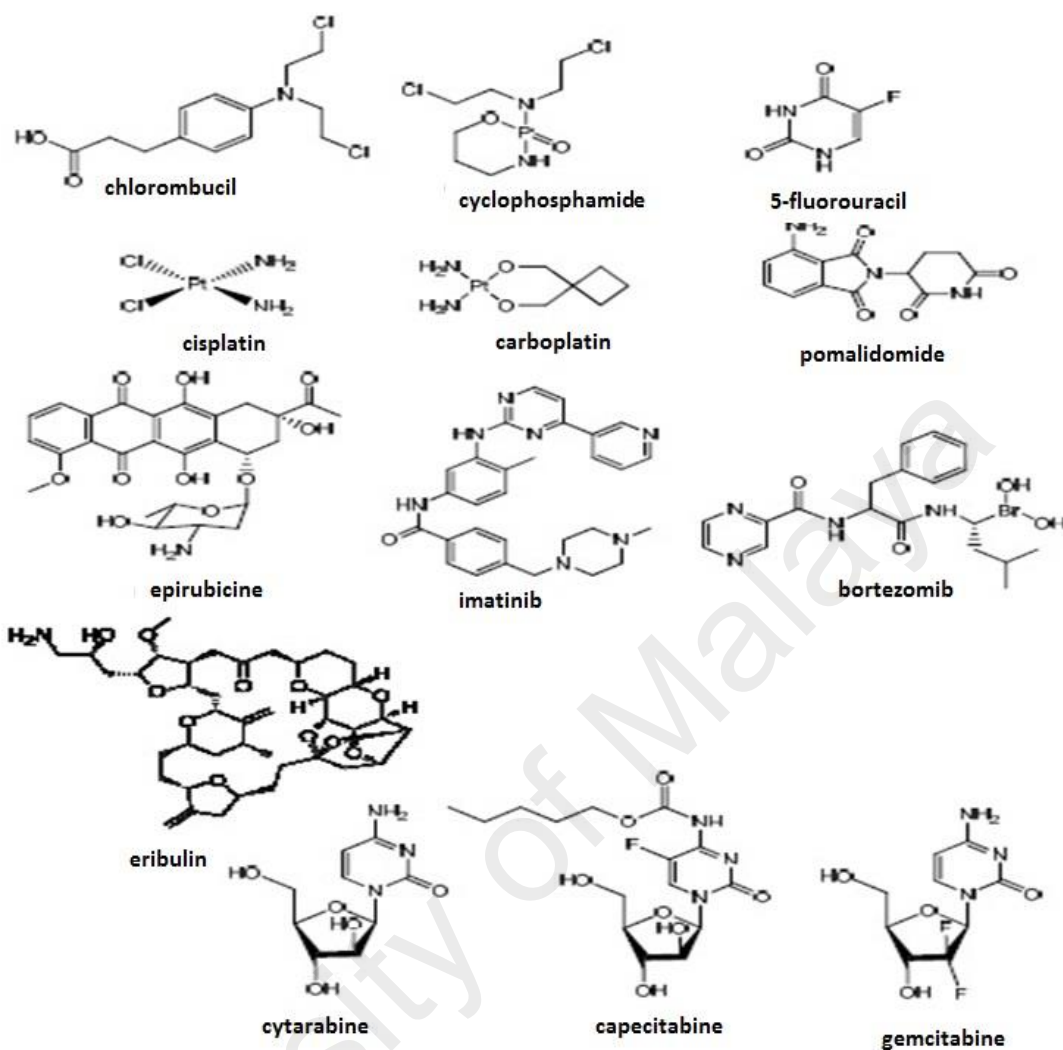
Figure 2.9: Structures of some current anticancer drugs.
(From "Marine pharmacology in 2009-2011: Marine compounds with antibacterial, antidiabetic, antifungal, anti-inflammatory, antiprotozoal, antituberculosis and antiviral activities; affecting the immune and nervous systems, and other miscellaneous mechanisms of action" by Mayer *et al*., 2013. *Marine Drugs*, *11*, 2510-2573)

2.14 Algae

The term algae refers to a large and diverse assembly of eukaryotic organisms which contain chlorophyll and are able to carry out oxygenic photosynthesis. Algae are not only exclusively microscopic and unicellular organisms, but also larger multicellular organisms such as seaweeds, otherwise known as macroalgae (Milledge *et al*., 2014). Brown algae (*Phaeophyta*), green algae (*Chlorophyta*) and red algae (*Rhodophyta*) are

the common groups of macroalgae. Typically, they are found along coastlines down to 50 m and are attached by holdfasts.

2.15 Common Applications

2.15.1 Dietary Supplements

Seaweeds are rich sources of vitamins A, B1, B12, C, D and E, riboflavin, folic acid, pantothanic acid and minerals such as sodium, potassium, calcium and phosphorus. They possess most of the essential amino acids required for the maintenance of a healthy life. In addition, seaweeds contain more than 54 trace elements which are vital for physiological functions in humans, in quantities remarkably exceeding those in vegetables and other terrestrial plants (Dhargalkar & Pereira, 2005).

China and Japan are the main seaweed cultivators, producers and consumers worldwide. The significance of seaweed consumption is well established dating back to 300 BC in China and Japan. Seaweeds are consumed as salad, jelly and soup in other Asian countries such as Malaysia, Singapore, Thailand, Indonesia and Korea (Dhargalkar & Pereira, 2005).

For thousands of years, Asians have been using seaweeds such as kelp, nori and wakami for culinary purposes. Seaweed is also used medicinally in China for the treatment of liver diseases, swelling, cysts, phlegm and enlarged thyroid glands (Liu *et al.*, 2012). During the 18th century, kelp was discovered as a rich source of iodine in the diet and is used to treat enlarged thyroid glands (Clark *et al*., 2000). Since the early 1980s, several types of algae have been marketed as supplements. Spirulina, Chlorella and certain blue-green algae are among the examples of supplements sold worldwide (Branger *et al*., 2003).

2.15.2 Source of Profitable Colloids and its Uses

Seaweeds are versatile products which are used widely in industries. Agar, carrageenan and alginate, which are used as thickening and gelling agents, are the three major commercial phycocolloids extracted from brown and red seaweeds (McHugh, 2003). Seaweed phycocolloids are usually used as emulsifiers in dairy products, textile, leather and pharmaceutical industries. Sodium alginates are known to be able to effectively treat radioactive and heavy metal poisoning. Besides that, seaweed alginates have a soothing and cleansing effect on the human gastrointestinal tract. Natural seaweed resources become precious as these phycocolloids cannot be synthesized chemically due to the formidable chemical barriers (Dhargalkar & Pereira, 2005). There are several artificial products reputed to be suitable replacements for seaweed phycocolloids but none of them have the exact gelling and viscosity properties of seaweed phycocolloids. Hence, it is very unlikely that seaweeds will be replaced as a source of these polysaccharides in the near future (Marinho & Bourret, 2003). On top of that, the phycocolloids in brown seaweeds as well as the carrageenan and agar in red seaweeds aggressively trap metallic ions, thus enabling the excretion of heavy metals from the body (Thinakaran *et al*., 2012).

Sulphated polysaccharides from seaweeds are used in the films that are placed between the bones to be grafted to increase the growth rate of connective tissues (Dimitriou *et al*., 2012). The polysaccharides which play a prominent role in promoting and assisting the healing process of the body are capable of treating arthritis. In the field of modern biotechnology, these polysaccharides are used for the immobilisation of biological catalysts in the industrial process (Dhargalkar & Pereira, 2005).

2.15.3 Source of Bioenergy Production

Unlike terrestrial crops cultivated for biofuel, algae do not require agricultural land for cultivation. Many species of algae grow in brackish or salt water in order to avoid competition for land and fresh water required for food production (Christi, 2007). In addition, the potential biomass yield of algae per unit area is higher than that of terrestrial plants (Tredici, 2010). For instance, brown seaweeds have yields of ~13.1 kg dry weight m^{-2} x $year^{-1}$ as compared to ~ 10 kg dry weight m^{-2} x $year^{-1}$ from sugarcane (Leu & Boussiba, 2014). Thus, algae are considered as among the most potentially significant future sources of sustainable biofuels. Moreover, algae have been regarded as potential sunlight-driven cell factories for the conversion of carbon dioxide to biofuels and chemical feedstock (Christi, 2007; Menetrez, 2012).

2.15.4 Animal Feed

For centuries, seaweeds have been used to feed livestock and have been mentioned as such in Ancient Greece and the Icelandic sagas. In Iceland, where long periods of scarce fodder are common, seaweeds were grazed by sheep on the beaches, or fed to farm animals such as horse, sheep and cattle for 6-8 or even up to 18 weeks of the year. Seaweeds were usually dried and stored in barns. Furthermore, seaweeds are also preserved as silage and used as winter feedstuff for sheep and cattle in the early 1900s (Evans & Critchley, 2013). There were numerous reports of occasional and systematic use of seaweeds to feed livestock in France, Scandinavia and the Scottish islands to ruminants in the 19th and early 20th centuries. To date, the Orkney sheep in Northern Scotland are still grazing on a diet almost exclusively based on seaweeds (Hansen *et al*., 2003).

It has been well recognized that seaweed meals improve fertility and birth rate of animals. Meals prepared from *Gracilaria* and *Hypnea* (red algae) and *Sargassum* (brown algae) are used as fodder for fish and prawn cultures, which have more palatability enriched carbohydrates, minerals and amino acids as well as helping to maintain the water quality in aquaculture. Seaweeds also serve as water purifiers due to their ability to recycle the water polluted by fish waste in aquaculture (Dhargalkar & Pereira, 2005).

2.15.5 Eco-Friendly Organic Manure

Fertility of the soil is impaired by the use of chemical fertilizers which make it acidic, rendering it inappropriate for growing crops. Hence, farmers worldwide are switching over to organic fertilizers. Seaweed manure improves the soil fertility, increases moisture holding capacity and supplies sufficient trace metals. Seaweeds are magnificent fertilizers due to the presence of adequate amounts of growth promoting hormones, nitrogen, potassium, humic acids and micronutrients. Seaweed fertilizers are environmentally friendly as they are biodegradable, non-hazardous, non-toxic and non-polluting to living organisms (Dhargalkar & Pereira, 2005).

2.15.6 Health Boosters

Since ancient times, seaweeds have been used in ointments, anesthetics, vermifuges and for the treatment of goiter, gout, wounds, cough and sexually transmitted diseases (STDs). Sterols present in seaweeds are capable of lowering the blood plasma cholesterol level. In addition, seaweeds modify lipid metabolism in the human body (Dhargalkar & Pereira, 2005; Boopathy & Kathiresan, 2011). All seaweeds provide an unusual level of potassium which highly resembles the human blood plasma level as compared to terrestrial plants. This has gained considerable attention from the medical

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field. Two Japanese surgeons have used a ground-breaking method of mixing seaweed compounds with water to replace whole blood in transfusion and surprisingly, this has been a success in over 100 operations (Boopathy & Kathiresan, 2011).

Seaweeds, especially brown seaweeds, are rich natural sources of biomolecular dietary iodine (Dhargalkar & Pereira, 2005; Boopathy & Kathiresan, 2011). Some seaweeds contain a thousand times as much iodine as cod, an average iodine-containing fish. Essential thyroid hormones such as thyroxine (T_4) and tri-iodothyronine (T_3) are formed from the precursor, di-iodotyrosine (DIT) provided by seaweeds. Thus, consumption of seaweeds lowers the risk and incidence of goiter and glandular diseases (Dhargalkar & Pereira, 2005; Carle *et al*., 2014).

Clinical trials are being conducted to enable diabetic patients to be free from injection by the introduction of insulin secreting "jelly capsule" made of seaweed derived alginic acid. The capsule renders protection from leucocytes and the patient's immune system (Kjaervik, 1993; Dhargalkar & Pereira, 2005). In agreement with this, Kim *et al*. (2008) reported the consumption of seaweed indeed influences the glycaemic control and may be effective in lowering blood lipids and improving antioxidant enzyme activities. In recent anti-diabetic research, exploration of therapeutically active agents such as bromophenols, 2-piperidione, benzeneacetamide, n-hexadecanoic acid, fucoxanthin, dysidine, biologically active insulin and methyl-ethyl ketone derivatives with significant inhibitory potentials against enzymes pathogenic to diabetes from seaweeds and sea corals added a new dimension to anti-diabetic research (Rathinamoorthy & Sasikala, 2011; Fedekar *et al*., 2013;Bhattacharjee *et al*., 2014).

2.15.7 Cosmetics

Some seaweeds contain collagen-like molecules which give rise to skin elasticity and firmness. An impressive similarity between human skin tissue and algal cellular structure has provided solutions to numerous cosmetic and dietetic problems. To date, seaweeds have become key ingredients in the manufacture of cosmetic products such as shampoo, soap, sprays, powders and creams. Seaweeds are found to be naturally moisturizing, revitalizing and nourishing due to the presence of amino acids, minerals and vitamins. Extracts from brown seaweeds, such as *Fucus* spp. are used in massage therapy for the elimination of impurities from the body and simultaneously balance the pH of the skin. Therefore, seaweeds and other marine organisms have become natural alternatives to the chemical based cosmetics due to their natural therapeutic properties (Dhargalkar & Pereira, 2005).

2.16   Chemical Constituents in Seaweeds and Their Uses

The marine biodiversity along with the associated chemical diversity constitute an unlimited reserve of bioactive substances in the field of bioactive products. Marine organisms, particularly marine algae such as seaweeds are potential renewable resources from the marine environment containing structurally diverse bioactive compounds with various biological activities (Etcherla & Rao, 2014). Seaweeds are well known for their high nutritional value as they are a reservoir of proteins, carbohydrates, vitamins, essential minerals, essential fatty acids and fibre content which play an important role in the biochemical reactions in the human body (Chandini *et al*., 2008). Besides, seaweeds also contain a variety of non-nutritional organic and inorganic compounds such as polyphenols, alkaloids, tocopherols, carotenoids, terpenes and ascorbic acid which are involved in the aetiology of chronic diseases through oxidative damage to cells and cellular molecules (Chanda *et al*., 2010).

Generally, seaweeds contain 80-90% of water and their dry weight basis comprises of 50% of carbohydrates, 7-38% of minerals and 1-3% of lipids. However, the protein contents of seaweeds are highly variable, depending on the seasonal and environmental growth conditions (Dawczynski *et al*., 2007). Despite the low protein content, seaweeds comprise mainly of essential amino acids. Seaweeds contribute few calories to the diet due to their low fat abundance and the presence of protein and carbohydrate substances. Recent research showed that seaweeds have the potential to reverse the signs of metabolic syndromes which include type 2 diabetes, obesity, hypertension, hyperlipidaemia and cardiovascular complications (Kumar & Brown, 2013; Kumar *et al*., 2015).

The chemical constituents and the abundance of carbohydrates differ among seaweed species. Carbohydrates play a vital role in providing the energy required for respiration and metabolism (Kokilam *et al*., 2013). Brown seaweeds such as *Padina tetrastromatica* and *Turbinaria ornata* contain a variety of typical carbohydrates which include fucoidan, cellulose, alginates, mannitol and laminaran (Dawczynski *et al*., 2007). On the contrary, the typical carbohydrates found in red seaweeds consist of cellulose, floridean starch, xylan and mannan. The carbohydrates in the seaweeds are mainly dietary fibre and these are indigestible by the human gastrointestinal tract (Ghada & Amany, 2013). The approximate range of the total dietary fibre in seaweeds is 33-50 g/100 g of dried weight (Ruperez & Saura-Calixto, 2001). In addition, the sulphated polysaccharides in the green seaweed, *Caulerpa racemosa*, were found to have anticoagulant, antiviral and antitumour activities (Rahul *et al*., 2014). The composition of the polyunsaturated fatty acids (PUFAs) in seaweeds is superior to those of terrestrial plants despite having low lipid content (Kumari *et al*., 2010). Apart from that, seaweeds are also much sought after for their mineral content such as sodium, potassium, calcium,

magnesium, manganese, iron and zinc for human nutrition as these minerals are not found in edible terrestrial plants due to the geographical origin as well as the seasonal, environmental and physiological variations (Ruperez, 2002). On the other hand, higher concentrations of trace elements which include cadmium, cobalt, iron, nickel and zinc were detected in the sponge, *Spheciospongia vagabunda*, as compared to most other sponge species (Padovan *et al*., 2012). The high iron content has been attributed to the incorporation of iron into spongin fibres (Araüjo *et al*., 2003). Capon *et al*. (1993) suggested that the accumulation of cadmium and zinc in the sponge confers antibacterial properties and defence against fouling organisms and grazers. A recent research by Eltamany *et al*. (2015) reported that three new ceramides isolated from *S*. *vagabunda* exhibited cytotoxicity against liver and breast cancer cell lines.

2.17   Extraction Techniques of Bioactive Compounds from Natural Products

The first step in the utilisation of phytochemicals for the development and preparation of pharmaceutical and nutraceutical products, dietary supplements, food ingredients and cosmetic products involves the extraction of bioactive compounds from natural products (in this case, marine organisms) (Dai & Mumper, 2010). The samples are usually prepared by air-drying, freeze-drying or even oven-drying, followed by grinding, milling and homogenization before the extraction process. The method of freeze-drying is the more preferred method over air-drying and oven-drying as freeze-drying has the ability to retain higher levels of phenolic content in the sample, as compared to the air- and oven-drying (Lee *et al*., 2012). Asami *et al*. (2003) reported a higher total phenolic content level in freeze-dried Marion berries, strawberries and corn compared with those air-dried. The choice of the drying method used governs the quality of the dehydrated product. Air-drying is the oldest method used for the preservation of food, by which the foodstuffs are dried by a high temperature at a low expense, thus extending the shelf life

of the product (Garau *et al*., 2006). Freeze-drying, on the other hand, is an expensive process for the manufacture of a dried product. In spite of the higher cost of freeze-drying, it can prevent the quality deterioration and microbiological reactions while concurrently maintaining the shape and structure of the original product by minimising the volume reduction (Ratti, 2001; Garau *et al*., 2006). However, caution should be taken while studying the medicinal properties of the marine organisms as all the drying processes, including freeze-drying can cause undesirable effects on the constituent profiles of the marine organisms (Abascal *et al*., 2005).

The next critical step after sample preparation is the complete extraction of phenolic compounds. Solvent extractions are the most commonly used procedures for the preparation of extracts due their ease of use, efficiency, economic feasibility and wide applicability. The general techniques of bioactive compound extraction include maceration, hot continuous extraction (Soxhlet), sequential extraction, ultrasound extraction (sonication), supercritical fluid extraction (SFE), counter current extraction and microwave-assisted extraction (Pandey & Tripathi, 2014).

Solvent extraction employs either organic or inorganic solvents. Properties of a good solvent for extraction include low toxicity, ease of evaporation at low heat, preservative action, promotion of rapid physiologic absorption of the extract as well as the inability to cause the extract to dissociate or complex (Pandey & Tripathi, 2014). Basically, the yield of extraction is influenced by a few parameters, which include the type of extraction solvents with varying polarities, extraction temperature and time, sample-to-solvent ratio, chemical nature and physical properties of the samples. Moreover, the optimum recovery of phenolic compounds varies from one sample to the other, depending on the type of natural products and their bioactive compounds (Khoddami *et*

al., 2013). Rahul *et al*. (2014) extracted 50 g of the marine algae *Caulerpa racemosa* powder with petroleum ether, chloroform, ethyl acetate, acetone, ethanol, methanol and water for 48 h by maceration. The water extract produced the highest extractive value (8.5%) while the extractive value of the petroleum ether extract was the lowest (0.15%). In another study by Chairman *et al*. (2012), the ethyl acetate and methanol extracts of 12 species of sponges were tested for antimicrobial activity. The ethyl acetate extract exhibited a more potent antimicrobial effect than methanol.

Besides the selection of the optimal solvent for extraction, there are two other parameters that play a crucial role in the percentage yield of polyphenols extracted, namely, extraction time and temperature (Khoddami *et al*., 2013). Usually, the analyte solubility will be enhanced by increasing the extraction time and temperature. However, phenolic compounds are generally degraded or undergo undesirable reactions such as enzymatic oxidation due to extended extraction time and temperature (Biesaga & Pyrzynska, 2013). In addition, the recovery of phenolics is also affected by the sample-to-solvent ratio. The extraction of phenolic compounds is promoted by increasing the sample-to-solvent ratio but determination of the optimal ratio is advisable so that the solvent input and saturation effects of solvent by the phenolics are minimised (Al-Farsi & Lee, 2008).

Soxhlet, maceration and heated reflux extraction are some examples of the conventional methods used for the extraction of phenolics from solid samples. The Soxhlet and heated reflux extraction are normally performed at 90 °C for several hours whereas maceration is performed at ambient temperature for several days (Castro-Vargas *et al*., 2010; Biesaga, 2011). These conventional methods are relatively cheaper, simple and result in adequately high phenolic extraction rates (Castro-Vargas *et al*., 2010). In spite

of the many positive aspects of these methods, there are substantial disadvantages including long extraction time, the need to use large volumes of hazardous organic solvents as well as interference with and degradation of targeted components due to both external and internal factors such as light, high temperatures, air and enzymatic reactions (Altuner *et al*., 2012). Suresh *et al*. (2012) evaluated the antioxidant properties of the sequential extracts of the brown seaweed, *Sargassum plagiophyllum*, and reported that the sequential extraction procedure was an efficient procedure for the recovery of various products based on the differential solubility, charge distribution and molecular mass of polysaccharides from brown seaweeds.

To overcome the shortcomings of these conventional methods, modern extraction and isolation techniques have been employed as alternative techniques to considerably reduce solvent consumption while accelerating the extraction process. These modern techniques include supercritical fluid extraction (SFE), microwave assisted extraction (MAE) and ultrasonication assisted extraction (UAE) (Gupta *et al*., 2012).

2.18 Physiology and Distribution of *Padina tetrastromatica*, *Caulerpa racemosa*, *Turbinaria ornata* and *Spheciospongia vagabunda*

Padina tetrastromatica (class of Phaeophyceae and family of Dictyotaceae) is also regarded as a taxonomic synonym of *Padina antillarum* (Guiry & Guiry, 2011). Generally, the phaeophyceae members represent the major component of seaweed population of the tropical countries in the world. *P. tetrastromatica* is a striped, yellowish-brown, fan-shaped thallus which turns olive green upon drying (Shaikhi *et al*., 1991). It is foliaceous and can grow up to 5-55 cm long and 1-3 cm wide. This seaweed is irregularly cleft into narrow lobes with involuted apical margins with hairs scattered all over the surface of the thallus and reproductive structures occurring as marginal sori

(Mica, 1966; Sethi, 2012). It can be found growing in shallow and sand-covered rocky areas (Shameel, 1990).

Caulerpa racemosa (class of Bryopsidophyceae and family of Caulerpaceae) is a species of green algae with branches linked to stolons which are attached to the sediments by rhizoids. *C. racemosa* varies morphologically and has many different growth forms that have been identified and named. It can grow to a height of 30 cm with spherical or ovate side shoots branching off the seaweeds, giving rise to the name of sea grapes due to its resemblance. *C. racemosa* is coenocytic, i.e., the entire seaweed is comprised of one giant cell with many nuclei and no cross-walls, which is why any part of *C. racemosa* that is fragmented can regenerate to form an entirely new plant (Verlaque *et al*., 2003). *C. racemosa* is commonly distributed in shallow temperate and tropical seas (Klein & Verlaque, 2008).

Turbinaria ornata is tropical brown algae (class of Phaeophyceae and family of Sargassaceae) which can grow up to 20 cm tall upon reproduction. It contains conical, hard and thick blades with double rows of stiff and spiky spines around the irregular triangular margin of the blade in aerial view. Its holdfast bears one terete and its basal portion is irregular or conical, usually with several unbranched or dichotomously branched root-like structures growing from the basal area of the erect axes. It grows in a variety of habitats which include rocky intertidal, tide pools, intertidal benches, reef flats and deeper water (Russell & Balazs, 2000).

Spheciospongia vagabunda is a tropical sponge (class of Demospongiae and family of Clionaidae) which is irregularly shaped and can grow up to 20 cm wide and 12 cm high. It has a hispid surface with steep-sided conical projections. Its hard and corky texture

makes it not easily compressible and hard to tear. Tightly packed spicules which criss-cross each other or protrude to the surface are also observed. *S. vagabunda* resides in shallow lagoonal reefs (Hooper & van Soest, 2002).

Figure 2.10: *Padina tetrastromatica*.
(From:http://www.niobioinformatics.in/seaweed/system_Padina%20tetrastromatica.htm)

Figure 2.11: *Caulerpa racemosa*.
(From:http://www.forestryimages.org/browse/detail.cfm?imgnum=1357016).

Figure 2.12: *Turbinaria ornata*.
(From:http://www.daunafrika.com/index.php/157-agrobisnis/rumput-laut-alga/alga-coklat).

Figure 2.13: *Spheciospongia vagabunda*.
(From:http://lkcnhm.nus.edu.sg/dna/organisms/details/147).

2.19   Bioactivities of *Padina tetrastromatica*, *Caulerpa racemosa*, *Turbinaria ornata* and *Spheciospongia vagabunda*

The bioactivities and pharmacological properties of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* are summarised in Table 2.3.

Table 2.3: Bioactivities of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda*.

Marine Organisms	Bioactivities	References
P. tetrastromatica	The dichloromethane and ethyl acetate polyphenol fractions of *P. tetrastromatica* promote anti pancreatic tumour potential, as demonstrated by inhibition of cell viability, proliferation and induction of apoptotic cell death.	Aravindan *et al.*, 2013
	Seaweed extract of *P. tetrastromatica* has antioxidant activity with properties of hepatoprotection.	Clara *et al.*, 2014
	The methanolic extract of *P. tetrastromatica* exhibits strong antimicrobial and antioxidant activities.	Thangaraj *et al.*, 2013
	The ascophyllan fraction from *P. tetrastromatica* showed strong free radical scavenging abilities and had good antioxidant effects.	Mohsin *et al.*, 2013
	The antimicrobial activity of *P. tetrastromatica* extract from various organic solvents was studied against three bacterial species.	Ponnanikajamideen *et al*., 2014
	The most antibiotic productive isolate from *P. tetrastromatica* was isolated and observed to inhibit *Pseudomonas aeruginosa* and *Klebsiella pneumonia*.	Ravisankar *et al*., 2013
	Methanolic extract of *P. tetrastromatica* has a strong anti-Hepatitis B virus activity.	Subramaniam *et al*., 2011

Table 2.3, continued. Bioactivities of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda*.

C. racemosa	The *C. racemosa* polysaccharide had strong antitumour activity against both K562 cells *in vitro* and against H22 tumour transplanted in mice *in vivo*.	Ji *et al.*, 2008
	The bioactive compounds obtained from the methanolic extracts of *C. racemosa* showed bactericidal and larvicidal activities.	Nagaraj & Osborne, 2014
	Sulphated polysaccharides extracted from *C. racemosa* caused a significant increase in sPLA2 enzymatic and bactericidal activity as well as increased its edematogenic effect.	Pires *et al.*, 2013
	C. racemosa extract and a secondary metabolite from *C. racemosa* (Caulerpenyne) showed antiproliferative and apoptotic effects on SHSY5Y and Kelly cell lines.	Cavas *et al.*, 2006
	C. racemosa polysaccharide exhibited significant antioxidant and free radical scavenging activities.	Mahendran & Saravanan, 2013
T. ornata	The crude extracts of *T. ornata* have antidiabetic properties due to their strong enzyme inhibitory effects in α-amylase, α-glucosidase and DPP-IV enzymes. Additionally the extracts were capable of scavenging free radicals *in vitro*.	Unnikrishnan *et al.*, 2014
	The phenolic fraction of *T. ornata* exhibited higher antioxidant activity than that in *P. tetrastromatica*.	Kayalvizhi *et al.*, 2014
	An unusual sulphated fucan-like polysaccharide containing amino sugar constituent was isolated from *T. ornata* and it was shown to demonstrate antiproliferative effect on asynchronous cells of a human non-small-cell bronchopulmonary carcinoma line (NSCLC-N6) *in vitro*.	Deslandes *et al.*, 2000
	The acetone extract of *T. ornata* showed antiulcer, wound healing and hepatoprotective activities.	Senthil & Murugan, 2013
	The anti-inflammatory activity of the aqueous extract of *T. ornata* was reported to be due to its potential antioxidant and free radical scavenging properties.	Ananthi *et al.*, 2011

Table 2.3, continued. Bioactivities of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda*.

S. vagabunda	The extracts of *S. vagabunda* showed anticancer activity against HepG2 and MCF-7 cell lines.	Eltamany *et al*., 2014

2.20   Proteomics and Drug Mechanisms

Proteins are the major drug targets and critical role players in drug design. Subtle changes in protein abundance under different conditions can be distinguished by comparative proteomics, for instance, control (untreated) versus drug-treated. Proteomics analysis provides insights for drug mechanisms, identifying drug targets, identifying biomarkers, examining signalling pathways and discovering the reason for drug resistance (Wang *et al*., 2006).

While genome refers to the complete set of DNA and genes of an organism, the term 'proteome' is, by analogy with the term 'genome', addresses the total protein complement encoded by a genome (Wasinger *et al*., 1995). The proteome of the cell includes all the proteins at a certain time, under specific conditions. There are various splice variants and post-translational modifications such as phosphorylation, proteolysis, acetylation, glycosylation, ubiquitination and methylation in the process of protein translation from RNA and DNA, thus making the human proteome to be far more complicated than the human genome. The human cell has up to one million distinct protein isoforms despite only having 23,000 genes (de Hoog & Mann, 2004). Proteomics is gaining importance in many areas including medicine. The mechanism of drug action can be delineated by comparing the cellular proteomes before and after drug treatment (Wang *et al.*, 2006).

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is the gold standard for separation of proteins from complex mixtures of cells, tissues or whole organisms for almost three decades (O'Farrell, 1975). It is considered as a high resolution method for separating proteins in two dimensions based on the isoelectric point (pI) in the first dimension and molecular weight in the second dimension (Gorg *et al*., 1988). Briefly,

proteins are separated in a small gel strip containing an immobilised pH gradient known as IPG strip, in the first dimension, during which, the proteins migrate through the pH gradient until they reach their pI. The IPG strip is then applied to a polyacrylamide gel for the second dimensional separation. In the second dimension, the pI focused proteins are moved out of the strip by an electric current into the gel and proteins are separated according to their sizes due to the sieving action of the gel (O'Farrell, 1975; Gorg *et al*., 2004).

Approximately 2,000 proteins can be detected on a 2D gel and the proteins can be visualised by several staining techniques, namely, Coomassie blue (200-500 ng protein per spot), silver stain (0.1 ng protein per spot), fluorescent stain such as Sypro Ruby (1-2 ng protein per spot) or using radioactive isotopes (Gorg *et al*., 2004). The individual proteins can then be cut from the gel as gel plugs and digested by a proteolytic enzyme such as trypsin to produce peptides that are analysed by mass spectrometry. Apart from 2D-PAGE, other recently developed proteomics technologies which feature high-throughput parallel analysis of the proteome include two-dimensional fluorescence difference gel electrophoresis (DIGE), liquid chromatography mass spectrometry (LC-MS), high performance liquid chromatography (HPLC) and antibody microarrays (Wang *et al*., 2006). The results from proteomics research accelerate the clinical validation of drug candidates and the development of more effective drugs (Sleno & Emili, 2008).

2D-PAGE is widely used in anticancer research. Tang *et al*. (2013) reported 72 differentially expressed proteins which were involved in tumour cell adhesion, apoptosis, angiogenesis and metastasis in the *Phyllanthus* extracts-treated PC-3 cells. Shanmugaraju *et al*. (2011) reported that the anticancer protein against HepG2 cancer

cell line obtained from *Bacillus pumilus* isolated from soil sample was characterised by 2D-PAGE. 2D-PAGE was used in a study carried out by He *et al.* (2014) for the analysis of the protein profiles of human colorectal cancer SW480 cells treated with or without caffeic acid phenethyl ester.

CHAPTER 3

MATERIALS AND METHODS

3.1   Preparation of Sample

3.1.1   Sample Collection and Identification

The three seaweed samples (*P. tetrastromatica*, *C. racemosa* and *T. ornata*) and sponge (*S. vagabunda*) were collected from the coastal regions of Teluk Kemang, Port Dickson, Malaysia. They were identified by Khoo Kong Soo and voucher specimens for the three seaweed samples were deposited at the Department of Chemical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Kampar, Malaysia, under numbers UTAR/FSc/10/001, UTAR/FSc/10/002 and UTAR/FSc/10/003, respectively. The sponge sample was sent to the Naturalis Biodiversity Centre in the Netherlands for identification with specimen number #RMNH POR.8671.

3.1.2   Sample Preparation

The samples were washed, cleaned and cut into smaller pieces before being freeze-dried. The freeze-dried samples were thenfinely pulverized into powderby grinding using Khind BL710 blender (Khind, Malaysia), followed by weighing with a top loading balance (Sartorius AG, Germany) prior to sequential extraction.

3.1.3   Sequential Extraction of the Seaweed and Sponge Samples

Sequential extraction was carried out for the extraction of the powdered materials using solvents of different polarities, namely, hexane (Merck, Darmstadt, Germany), dichloromethane (DCM) (Friendemann Shmidt, Australia), ethyl acetate (EA)(Friendemann Shmidt, Australia), acetone (Merck, Darmstadt, Germany) and

methanol(John Kollin Chemicals, India). Ground materials (25 g) were extracted by stirring with a hot plate stirrer with 500 ml of the solvent (solute to solvent ratio of 1:20) for 24 h at ambient temperature. The same extraction procedure was repeated thrice before changing to a solvent of higher polarity. The extracts were filtered through Whatman No. 1 filter paper (Whatman International Ltd., England) and subsequently concentrated to dryness under reduced pressure at 40 °C with a rotary evaporator (Buchi Labortechnik AG, Switzerland).For preparation of stock solutions, the concentrated crude extracts were then dissolved in 10% dimethyl sulfoxide (DMSO) at a final concentration of 10 mg/ml (Merck, Darmstadt, Germany) and stored at 4 °C until further use.

3.1.4 Determination of Extraction Yield

The extraction yields for each of the seaweed and sponge samples were calculated and expressed as percentage of yield (%). The percentage of yield of each extract was calculated as the final weight percentage of the dried extract obtained with respect to the initial weight of powder used (Dhanani *et al*., 2013) by using the following equation (3).

$$\text{Percentage of yield} = \frac{\text{Final weight of dried extract}}{\text{Initial weight of powder}} \times 100\% \qquad (3)$$

3.2   Cell Culture

3.2.1   Cell Lines

The cancer cell line used in the current study was derived from human breast adenocarcinoma while the normal breast cell line was derived from human breast epithelial cells as shown in Table 3.1.

Table 3.1: Types of human cell lines used

Human Cell Line	
Origin	**Designation**
Human breast adenocarcinoma	MCF-7 (oestrogen receptor-positive, ER+)
Human mammary gland/breast epithelial	184B5

3.2.1.1 Human Breast Adenocarcinoma, MCF-7 (ATCC® HTB-22™)

The human breast adenocarcinoma cell line, MCF-7, derived in 1973 from a malignant pleural effusion of a 69-year-old Caucasian female patient with metastatic breast cancer, is one of the most commonly used culture models for human breast cancers. The wide usage of MCF-7 is due in part to its sensitivity to hormones through the expression of oestrogen receptor (ER), thereby making it an ideal cell line for hormone response studies (Levenson & Jordan, 1997). The cell line was named after an institute in Detroit, the Michigan Cancer Foundation-7, where the cell line was established by Soule and associates in 1973 (Soule *et al*., 1973). Theadherent MCF-7 cell line was routinely maintained in the complete cell culture medium containing Roswell Park Memorial Institute (RPMI) medium(Sigma-Aldrich, Saint Louis, Missouri, USA), supplemented with 10% foetal bovine serum (FBS)(Sigma-Aldrich, Saint Louis, Missouri, USA), 1% (v/v) penicillin-streptomycin antibiotics(Sigma-Aldrich, Saint Louis, Missouri, USA) and 1% (v/v) glutamine (Sigma-Aldrich, Saint Louis, Missouri, USA).

3.2.1.2 Human Non-malignant Breast Epithelial Cell Line, 184B5 (ATCC® CRL-8799™)

The 184B5 human breast epithelial cell line was isolated from normal mammary epithelial tissue obtained from a normal reduction mammoplasty from a 21-year-old woman with no detectable breast epithelial cell pathology. The transformed immortalised cell line was established by Stampfer and Bartley (1985) via the exposure of the rapidly growing primary cultures of normal human mammary epithelial cells to benzo(α)pyrene. The 184B5 cell line was grown as an adherent monolayer cultured in complete cell culture medium containing Mammary Epithelial Basal Medium (MEBM) (Lonza, USA) and supplemented with bovine pituitary extract (BPE), hydrocortisone, human epidermal growth factor (hEGF) and insulin using Mammary Epithelial Cell Growth Medium (MEGM) SingleQuots (Lonza, USA). Both cell lines (MCF-7 and 184B5) were maintained at 37 °C in a humidified incubator with 95% air and 5% carbon dioxide (CO_2) (Kang *et al*., 2005).

3.2.2   Sub-culturing of Adherent Cells

Inverted microscope (CK2 Olympus, Japan) was used to check the morphology, growth and confluence of the cultured cells. Sub-culture was carried out when the cells reached a confluence of approximately 80 - 90%. Spent medium was removed and the cells were washed twice with phosphate buffered saline (PBS) (Sigma-Aldrich, Saint Louis, Missouri, USA), followed by trypsinisation with 1 ml and 3 ml of 0.25% trypsin-EDTA (Sigma-Aldrich, Saint Louis, Missouri, USA) for 25 cm^2 and 75 cm^2 flasks, respectively. Cells were incubated at 37 °C for 5 − 10 min to enable complete detachment of cells. The trypsinised cells were added with complete cell culture medium for the inactivation of trypsin. Cells were then spun down at 1,500 rpm at 25 °C for 5 min. The cell pellet

was resuspended with culture medium and the cell suspension was seeded into cell culture flasks containing fresh medium.

3.2.3 Cryopreservation and Thawing of Cells

For cryopreservation, the cell pellet was obtained by centrifugation at 1,500 rpm at 25 °C for 5 min and resuspended with complete cell culture medium containing 5% DMSO. 1 ml of the cell suspension was aliquoted into 2 ml cryovials and stored at 4 °C for 30 min. The cells were then stored at -20 °C for 4 h before storing overnight at -80 °C. The cryopreserved cells were then stored in the liquid phase of liquid nitrogen until further use.

To resuscitate the cells, the cryopreserved cells were thawed instantly in a pre-heated 37 °C water bath, centrifuged at 1,500 rpm at 25 °C for 5 min and resuspended in culture medium. Cell suspension was then transferred into a cell culture flask containing the complete culture medium and incubated at 37 °C in a humidified incubator with95% air and 5% carbon dioxide (CO_2).

3.2.4 Cell Counting

The Neubauer-improved haemocytometer (Marienfeld, Lauda-Königshofen) was utilised for cell counting. Cells were spun down at 1,500 rpm at 25 °C for 5 min and an adequate volume of culture medium was added to resuspend the cells. 10 µl of the cell suspension was gently mixed with 90 µl of trypan blue dye (Life Technologies, California, USA). The cell mixture (10 µl) was loaded into the counting chamber and placed underneath the coverslip by capillary action. Cells were observed in the haemocytometer under inverted light microscope (CK2, Olympus, Japan) at a magnification of 200X and the 16 corner squares were focused. The viable, unstained

cells falling within the four sets of the 16 corner squares at any position of the upper and

left hand side boundary line were counted using a hand tally counter. The concentration

of cells (cells/ml) was calculated using equation (4).

Cell concentration = $\underline{\text{Total number of viable cells}}$ x Dilution factor (10) x 10^4 (4)
(cells/ml)   4
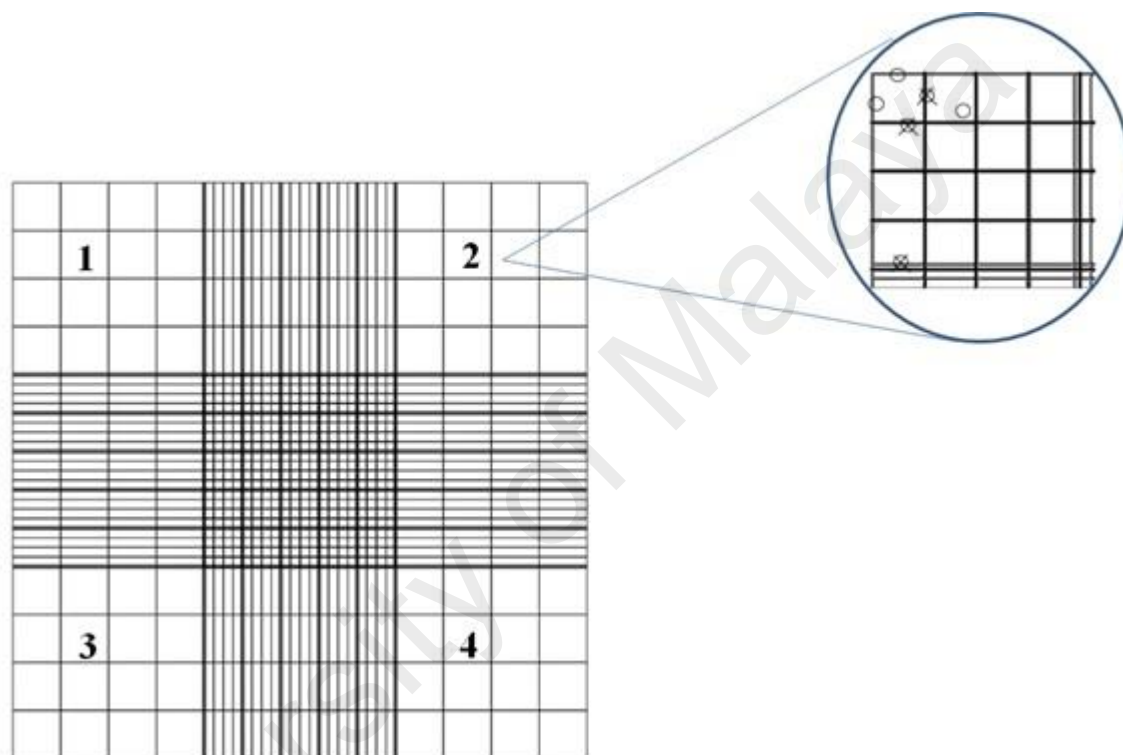
Figure 3.1: Grid layout of the Neubauer-improved haemocytometer. Viable cells in
the four big corner squares labelled as 1, 2, 3 and 4 were counted. Viable cells falling in
the 16 squares in each corner square and at any position of the upper and left hand side
boundary line (as represented by the zoomed in diagram in this figure) were counted.

3.3   Cytotoxicity Assay

3.3.1 Construction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Standard Curve

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a

method developed by Mosmann (1983) which is usually used for the detection of

cytotoxicity or cell viability following exposure to the *in vitro* treatment of compounds.

The principle of the colourimetric method used in the MTT assay is based on the

capacity of the mitochondrial enzyme, succinate dehydrogenase, in living cells to reduce the yellow soluble substrate tetrazolium MTT salt into an insoluble purple coloured formazan crystals. This reduction activity occurs only in metabolically active living cells and thus, the amount of the formazan product is directly proportional to the number of viable cells. In other words, the level of activity is a measure of the cell viability (Mattana *et al*., 2012).

The MTT standard curve was constructed by using the MTT assay according to Bradshaw *et al*. (2008) with slight modifications. A total of six cell concentrations were prepared: 0, 20,000, 40,000, 60,000, 80,000 and 100,000 cells/ml. 100µl of each concentration were added into each well. The plate was incubated for 24 h, after which 10µl of 5mg/ml MTT (Sigma-Aldrich, Saint Louis, Missouri, USA) in PBS was added. The plate was incubated for 4 h in the dark, after which the media was aspirated and 100µl of DMSO was added to dissolve the formazan formed. Absorbance readings were taken at 570 nm using a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA).

3.3.2 Assessment of Cell Proliferation

Cell proliferation was assessed by the MTT assay according to the methods described by Bradshaw *et al*. (2008) with slight modifications. Briefly, 100 µl of MCF-7 and 184B5 cells were seeded at a seeding density of 5000 cells/well in a 96-well microtiter plate in their respective complete culture media. After overnight incubation, 20µl of serially diluted doxorubicin (Sigma-Aldrich, Saint Louis, Missouri, USA) or of extracts were added into each well and further incubated for 48 h. Doxorubicin was used as the positive control while the vehicle control, DMSO, was used as the negative control. After the incubation period, 10 µl of the MTT stock solution of 5 mg/ml in PBS was added and incubated for another 4 h in dark. The spent medium was aspirated and the

formazan crystals formed were dissolved with DMSO. The absorbance readings were measured at 570 nm using a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA). All experiments were performed in triplicate. The percentage inhibition of cell growth was calculated using equation (5).

$$\text{Inhibition of cell growth (\%)} = \frac{A_{595} \text{ (control)} - A_{595} \text{ (sample)}}{A_{595} \text{ (control)}} \times 100\% \qquad (5)$$

The half-maximal inhibitory concentration (IC_{50}) value was determined from the dose response curve.

3.4   Estimation of Phytochemicals

3.4.1   Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) was assessed using the Folin-Ciocalteu (FC) assay as described by Siddhuraju and Becker (2007) with slight modifications in the concentration of sodium carbonate (Na_2CO_3). The FC assay is used extensively for the quantification of the total phenolic content in an extract (Prasain *et al*., 2008). The principle of the FC assay is based on the reduction of phosphomolybdic and phosphotungstic acid complexes to blue chromogens in the presence of phenolic compounds under alkaline conditions (Mehran *et al*., 2014).

The assay involves FC phenol reagent, gallic acid as the standard and the pure flavonoids, quercetin and rutin, as the positive controls(all from Sigma-Aldrich, Saint Louis, Missouri, USA). 1 ml of 10% FC reagent was added to 20 µl of extract or standard. The mixtures were mixed well and incubated for 5 min before adding 700 µl of 10% Na_2CO_3. The solutions were further incubated for 2 h before taking the absorbance readings at 765 nm. Gallic acid in the range of 20-200 mg/l was used in the construction of a calibration curve. The TPC was expressed as milligram of gallic acid

equivalent (GAE) per gram of dried weight (mg GAE/g dried weight) (Iqbal *et al*., 2012). The experiments were performed in triplicate.

3.4.2 Determination of Total Flavonoid Content (TFC)

The aluminium chloride colorimetric method of Chang*et al*. (2002)was used for the determination of total flavonoid content (TFC). This assay was based on the development of acid-stable complexes between aluminium chloride and the C-4 keto group along with either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminium chloride also forms acid-labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids (Rajanandh & Kavitha, 2010).

Quercetin (Sigma-Aldrich, Saint Louis, Missouri, USA)was used as the standard while catechin (Sigma-Aldrich, Saint Louis, Missouri, USA)was used as the positive control. A total of 60 µl of methanol was mixed with 20 µl of extract or standard, 4 µl of 10% (w/v) aluminium chloride, 4 µl of 1 M potassium acetate and 122 µl of Milli-Q$^®$ water. The mixtures were incubated at room temperature for 30 min before measuring the absorbance at 415 nm. A calibration curve was prepared using catechin at concentrations of 12.5-100 µg/ml in methanol. TFC was expressed as milligram of catechin equivalents (CE) per gram of dried weight (mg/g dried weight). All experiments were performed in triplicate.

3.5 Determination of the Antioxidant Potential of the Crude Extracts of *P.tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* using Different Antioxidant Assays

3.5.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity is the most common spectrophotometric antioxidant assay in which the hydrogen or electron donating ability of an extract is measured from the bleaching of a purple methanol solution of DPPH free radical to a yellow-coloured non-radical form of DPPH. This spectrophotometric assay uses the stable DPPH radical as a reagent, during which DPPH absorbs wavelengths of 517 nm and the absorption gradually disappears with time as the DPPH concentration is reduced by the existence of hydrogen-donating antioxidants (Murali *et al*., 2011).

The DPPH radical (Sigma-Aldrich, Saint Louis, Missouri, USA) was used for the evaluation of radical scavenging activity of the extracts as described by Costa *et al*. (2010), with minor changes in the DPPH concentration. The reaction mixtures containing 120 µl of 0.04 mg/ml DPPH solution in methanol were mixed with 20 µl of different concentrations (25-400 µg/ml) of the extracts (with quercetin and rutin being used as positive controls) and shaken vigorously before being incubated in the dark for 20 min. Reduction in the absorbance of DPPH was measured against a blank at 517 nmusing a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA). The radical scavenging activity was calculated using equation (6).

Percentage of DPPH radical scavenging (%) = $(A_{blank} - A_{samples}) / A_{blank}$ x 100 (6)

Where A_{blank} and A_{sample} denote the absorbance of blank and absorbance of samples, respectively.

3.5.2 Superoxide Anion Radical Scavenging Activity

The superoxide anion scavenging activity was assayed according to the method of Shaikhi*et al*. (1991) with slight modifications in the nitroblue tetrazolium (NBT) (Sigma-Aldrich, Saint Louis, Missouri, USA)and phenazine methosulphate (PMS)(Sigma-Aldrich, Saint Louis, Missouri, USA) concentrations. The principle of this assay is based on the generation of superoxide radicals in the PMS-nicotinamide adenine dinucleotide (NADH) (PMS-NADH) system, followed by the oxidation of NADH and subsequently assayed by the reduction of NBT (Elmastas *et al*., 2004).

The reaction mixture containing 25 µl of NBT solution (150 µM NBT in 100 mM phosphate buffer, pH 7.4), 2 µl of PMS solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) and 20 µl of NADH solution (Sigma-Aldrich, Saint Louis, Missouri, USA)(468 µM in 100 mM phosphate buffer, pH 7.4) was added to different concentrations (25-400 µg/ml) of the extracts. The mixture was incubated in the dark for 10 min at 25 ºC and the absorbance read at 560 nmusing a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA). Quercetin and rutin were used as positive controls. All experiments were done in triplicate and results were expressed as percentage inhibition of superoxide anion radical using equation (7).

$$\text{Percentage of superoxide anion radical scavenging (\%)} = (A_{blank} - A_{samples}) / A_{blank} \times 100\% \qquad (7)$$

3.5.3 Nitric Oxide Scavenging Activity

The nitric oxide scavenging activity was conducted using the method developed by Rai *et al*. (2006). The compound sodium nitroprusside (SNP) decomposes in aqueous solution at physiological pH 7.2, producing nitric oxide (NO). Under aerobic conditions, NO reacts with oxygen to produce stable products (nitrite and nitrate). The nitric oxide scavenging assay is based on the competition between the scavengers of nitric oxide with oxygen, which subsequently leads to the reduced production of nitrite ions (Parul *et al*., 2012).

10 µl of 10 mM SNP (Sigma-Aldrich, Saint Louis, Missouri, USA)in phosphate buffer was mixed with 10 µl of different concentrations (25-400 µg/ml) of extracts. The mixture was incubated in the dark at room temperature for 2.5 h. Quercetin and rutin were used as positive controls. After the incubation period, 40 µl of sulphanilic acid reagent (Sigma-Aldrich, Saint Louis, Missouri, USA)(0.33% sulphanilic acid in 20% glacial acetic acid) was added to the mixture and further incubated for 5 min, after which 40 µl of 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride (Sigma-Aldrich, Saint Louis, Missouri, USA)was added, mixed and incubated for another 30 min at 25 ℃. The absorbance of the chromophore formed was read at 540 nmin a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA). All determinations were performed in triplicate and results were expressed as percentage of nitric oxide scavenging by using equation(8).

Percentage of nitric oxide scavenging (%) = $(A_{blank} - A_{samples}) / A_{blank}$ x 100%　(8)

3.5.4 Hydroxyl Radical Scavenging Activity

The 'site specific' hydroxyl radical scavenging assay was determined as described by Halliwell *et al*. (1987). The incubation of deoxyribose with hydrogen peroxide (H_2O_2) and Fe^{3+} causes the degradation of deoxyribose into products that can react to form a thiobarbituric acid-malondialdehyde (TBA-MDA) chromogen. The unchelated iron ions added to deoxyribose-containing reaction mixtures can become weakly associated with the deoxyribose. Any hydroxyl radicals produced from the reaction of the iron ions with H_2O_2 (through the Fenton reaction) will cause an immediate attack of the hydroxyl radicals at the deoxyribose and this 'site specific' attack is not easily prevented by scavengers. Therefore, the hydroxyl radical scavenging ability, which is directly proportional to the amount of hydroxyl scavengers, is measured by a reduction in the absorbance reading at 550 nm (Halliwell *et al*., 1987).

The reaction mixture containing 23.8 µl of 100 mM $FeCl_3$ (Sigma-Aldrich, Saint Louis, Missouri, USA)solution, 23.8 µl of 1.25 mM hydrogen peroxide (H_2O_2) (Sigma-Aldrich, Saint Louis, Missouri, USA)solution, 23.8 µl of 2.25 mM deoxyribose (Sigma-Aldrich, Saint Louis, Missouri, USA)and 23.8 µl of 100 mM ascorbic acid (Sigma-Aldrich, Saint Louis, Missouri, USA)was added to 5 µl of different concentrations (25-400 µg/ml) of extracts. The mixture was incubated at 37 ºC for 1 h after which 100 µl of 0.5% of thiobarbituric acid (TBA) (Sigma-Aldrich, Saint Louis, Missouri, USA)in 25 mM sodium hydroxide (NaOH) and 100 µl of 2.8% trichloroacetic acid (TCA) (Sigma-Aldrich, Saint Louis, Missouri, USA)were added. The resulting mixture was then boiled at 100 ºC for 15 min and subsequently cooled on ice before taking the absorbance readings at 550 nm. Quercetin and rutin were used as positive controls. All determinations were performed in triplicate and results were expressed as percentage of hydroxyl radical scavenging activity as calculated by equation (9).

Percentage of hydroxyl $\qquad$ = $(A_{blank} - A_{samples}) / A_{blank}$ x 100%   (9)
radical scavenging (%)

3.6   Preparation of Cell Extracts for Antioxidant Enzyme Assays

The methanolic extracts of the seaweed and sponge samples were selected for antioxidant enzyme assays. The cell extracts were prepared according to methods described by Pieme *et al.*(2010) with slight modifications in the lysis buffer (Tris/HCl 20mM and 0.2% Triton X-100) and extract concentrations used. Cells were seeded in 25 cm^2 flasks and incubated for 24 h. Extracts at a concentration of 400 µg/ml were added and incubated for another 8, 16 and 24 h. After incubation, the cells were washed with phosphate buffered saline (PBS) and harvested with a cell scraper. Cells were centrifuged at 1,500 rpm for 10 min and pellets were resuspended in 1 ml of lysis buffer and sonicated thrice in ice. Finally, the cells were centrifuged for 15 min at 3,000 rpm at 4 °C and the supernatant stored at -80 °C.

3.7   Antioxidant Enzyme Assays

3.7.1   Superoxide Dismustase (SOD) Assay

The most crucial parameter determining the biological impact of the antioxidant enzymes is activity. Enzymatic assays are often utilised to measure the antioxidant enzyme activities because the expression of the antioxidant enzyme mRNA and protein does not necessarily result in an increase in activity (Czaja *et al.*, 1994). The superoxide dismutase (SOD) activity was determined using the protocol described by Kakkar*et al*. (1984). The principle of the SOD activity assay was based on the inhibition of NBT reduction as described by Ornstein (1964). The principle of this assay is based on the reaction between superoxide and NBTwhich reduces the yellow tetrazolium to a blue precipitate (Weydert & Cullen, 2010).

A total of 500 µl of 0.052 M sodium pyrophosphate buffer (pH 8.3) was added to500 µl of 186 µM PMS, 500 µl of 300 µM NBT and added to 50 µl sample, 500 µl dH$_2$O and 500 µl of 780 µM NADH and incubated at 37 °C for 90 sec. 1 ml of glacial acetic acid was added to stop the reaction. The mixtures were shaken vigorously with 4 ml of n-butanol and incubated for 10 min before being centrifuged. The butanol layer was separated and the colour intensity of the chromogen in the butanol was measured at 560 nm.

3.7.2 Catalase (CAT) Assay

The catalase (CAT) assay was carried out following the protocol described by Sinha(1972). This assay employs a spectrophotometric procedure to measure the removal of peroxides (Lewis *et al*., 2005).

600 µl of 0.1 M PBS (pH 7.1) was added to 350 µl of 0.059 M hydrogen peroxide (H$_2$O$_2$)and 50 µl of sample. The absorbance was then read at 340 nmin a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA).

3.7.3 Glutathione Reductase (GR) Assay

The glutathione reductase (GR) activity was determined according to the method described by Goldberg and Spooner (1987). The principle of the GR assay is based on the catalysis of the reduction of glutathione disulphide (GSSG) to GSH by GR. This enzyme is involved in the maintenance of glutathione in the reduced form (GSH), enabling GSH to play its antioxidant roles (Kaplowitz & Tsukamoto, 1996). Thus, this assay measures the disappearance of GSSG.

855 µl of Milli-Q® water was added to 50 µl of 0.1 M PBS (pH 7.7), 50 µl of NADH, 20 µl of glutathione disulphide (GSSG)(Sigma-Aldrich, Saint Louis, Missouri, USA) and 25 µl of samples. The absorbance was then read at 340 nmusing a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA).

3.8 Isolation of Cytotoxic Compounds from the Partially Purified Fractions of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* through Bioassay Guided Fractionation

Bioassay guided fractionation plays a vital role in the isolation of active compounds or fractions from natural products (Tayarani-Najaran *et al.*, 2013). The methodology integrates the separation processes of compounds in a mixture using various analytical methods with the results obtained from biological testing. Bioassay guided fractionation is initiated with testing an extract to confirm its bioactivities, followed by crude separation of the compounds and eventually testing the crude fractions. Fractions that are active at a concentration threshold are subjected for further fractionation while the inactive fractions are either set aside or discarded (Ode *et al.*, 2011). This process of fractionation and biological testing is repeated until pure compound(s) are obtained. This is then followed by structural identification of the pure compound(s). This method obviates overlooking novel compounds that are usually missed in studies that only identify compounds which are familiar to the investigator (Jamil *et al.*, 2012).

3.8.1 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is a simple and the earliest chromatographic technique used for the preliminary exploration of secondary metabolites from natural

products (Senguttuvan & Paulsamy, 2014). Like any other chromatographic technique, TLC is based on a multistage distribution process. The separation process by TLC involves a suitable adsorbent (stationary phase), solvents or solvent mixtures (mobile phase or eluent) and the sample molecules (analytes).The adsorbent for TLC is coated as a thin layer on a suitable support such as glass plate, polyester or aluminium sheet. The sample is separated by elution with a suitable solvent on this layer. The principle of TLC is the distribution of a compound between a solid fixed phase applied to a glass or plastic plate and a liquid mobile phase, which moves over the stationary phase (Kumar *et al*., 2013).

A small amount of sample is applied to the starting point on the TLC plate just above the solvent level in the TLC chamber. The solvent migrates up through the particles on the TLC plate by capillary action. The compounds in the sample will either remain with the stationary phase or dissolve in the solvent and migrate up the TLC plate as the mobile phase moves over the compounds. The most soluble compounds in the sample will be carried the furthest up the TLC plate by the mobile phase. In contrast, compounds that are less soluble in the mobile phase will remain at the stationary phase (Singhal *et al*., 2009). The affinity of the compounds in the mobile or stationary phase is governed by the physical properties of the compounds, especially their molecular structures and functional groups (Kumar *et al*., 2013).

The most potent extract of each sample with the lowest IC_{50} were chosen for partial purification through column chromatography. The best solvent system for column chromatography was determined by thin layer chromatography (TLC) using 20 x 20 cm TLC silica gel 60G F_{254} glassplates (Merck, Darmstadt, Germany). For the separation of phytochemical compounds, the extracts were spotted manually by using a capillary tube.

The thin end of the spotter was placed in the sample and the sample rises up in capillary action. The TLC plate was touched lightly with the spotter at the starting line. The solvent was allowed to be evaporated and the same place was spotted again. This procedure was repeated until a small and concentrated spot was obtained. Over spotting should be avoided as this would deteriorate the quality of the separation (a phenomenon known as 'tailing'). The spotted plates were then placed in a solvent chamber containing various solvent systems to detect the suitable mobile phase. The following solvent systems, hexane: ethyl acetate; acetone: methanol and ethyl acetate: methanol were tested at ratios of 5: 5; 6: 4; 7: 3; 7.5: 2.5; 8: 2 and 9: 1. All TLC plates were visualised immediately after drying with an ultraviolet (UV) Transilluminator at 254 nm and 365 nm.
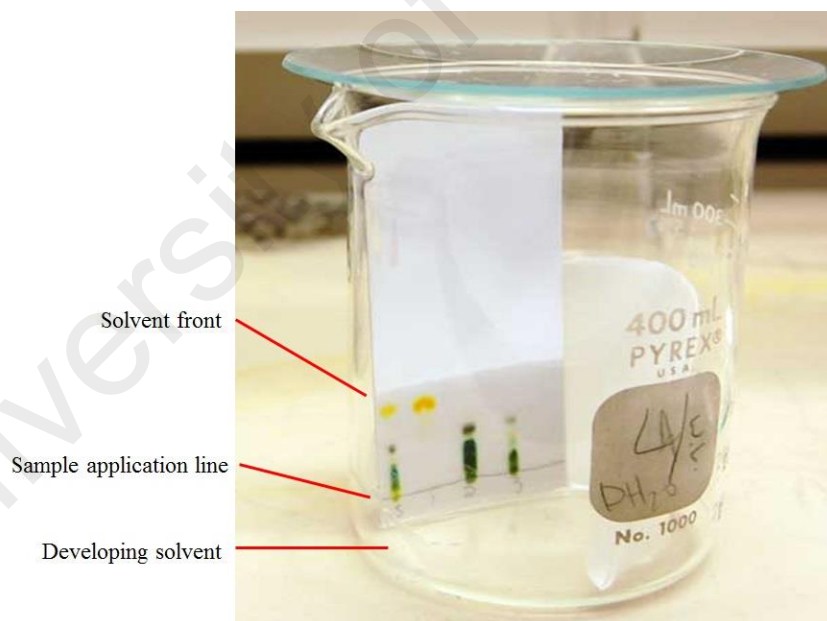
Figure 3.2: The setup of a TLC developing chamber. The samples are applied on top of the sample application line which is located above the level of the developing solvent so that the compound mixtures in the sample will not be dissolved in the solvent. The solvent front is marked after the run is completed for the calculation of R_f value.
(Figure from: http://www.pitt.edu/~ceder/lab5/exp5text.html).

3.8.2   Partial Purification of Extracts by Column Chromatography

Column chromatography is one of the most useful methods for the separation and purification of solids and liquids in small scale experiments. The separation can be

either through adsorption (liquid/solid) or partition (liquid/liquid). A solid adsorbent (stationary phase) is usually placed in a vertical glass column, and the mobile phase is added from the top and allowed to flow down the column by either application of external air pressure or gravity (Baczek *et al*., 2007). Since different compounds in the sample have varying interactions with the mobile and stationary phases, the compounds will be carried along with the mobile phase to varying degrees and a separation will be achieved (Fair & Kormos, 2008).

A glass column (32 cm length and 1.3 cm diameter) was packed with silica gel 60(Merck, Darmstadt, Germany) and hexane (Merck, Darmstadt, Germany) by the dry packing method. The column was equilibrated with hexane overnight to prevent cracking. 10μl of samples were loaded and eluted with pure hexane. Subsequent elutions were carried out using solvents of higher polarities according to Table 3.2. TLC was carried out simultaneously with hexane: EA (7.5: 2.5). The colour of the spots was observed and the retention factor (R_f) values were calculated using equation (10). Fractions with the same $R_f$values were pooled together and concentrated for further analysis.

Table 3.2: The mobile phase (solvent systems) used for the elution of the compounds

Solvent system	Ratio
Pure hexane	

Hexane: Ethyl acetate (EA)	9: 1; 8: 2; 7: 3; 7.5: 2.5; 6: 4; 5: 5; 4: 6; 3: 7; 2: 8; 1: 9
Pure EA	
Acetone: Methanol	9: 1; 8: 2; 7: 3; 6: 4; 5: 5; 4: 6; 3: 7; 2: 8; 1: 9
Pure methanol	

$$\text{Retention factor (Rf)} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}} \qquad (10)$$

Figure 3.3: The apparatus setup for silica gel column chromatography. The column was packed and equilibrated with hexane overnight before loading the sample. Elution was carried out using the best solvent system and the fractions were collected for further biological testing.
(Figure from: http://www.wfu.edu/chemistry/courses/organic/CC/index.htm).

3.8.3 Antiproliferative Activity of Partially Purified Fractions

Following column chromatography, the partially purified pooled fractions were freeze

dried and dissolved in DMSO to a final concentration of 1 mg/ml. The fractions were

then subjected to the MTT assay to determine their antiproliferative actitivies in MCF-7. Fractions with the lowest IC_{50} values were used for further analysis. These fractions (fractions 76 - 80 for *P. tetrastromatica*, fractions 41 - 45 for *C. racemosa*, fractions 21 - 25 for *T. ornata* and fractions 41 - 45 for *S. vagabunda*) were subsequently subjected to liquid chromatography-mass spectrometry (LC-MS).

3.8.4 Liquid Chromatography-Mass Spectrometry (LC-MS) Profiling

Liquid chromatography-mass spectrometry (LC-MS) is now widely used in many clinical and biochemical applications (Pitt, 2009). Electrospray ionisation (ESI) has been developed into a simple and robust ion source, which is capable of interfacing to LC-MS. The coupling of LC-MS with ESI thus demonstrated its application to a wide range of important classes of biological molecules (Fenn *et al*., 1989). Additionally, the development of highly sensitive and accurate assays is made possible by the use of tandem MS and stable isotope internal standards. Rapid scanning speeds allow a high degree of multiplexing and hence, many compounds can be measured in a single analytical run (Pitt, 2009).

The fractions were subjected to LC-MS after testing for antiproliferative activity. Only the fraction with the highest antiproliferative activity was used for each sample. 500 µl of samples were injected and analysed with Agilent 1290 Infinity LC system coupled to Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source. The dimension of the column used was 2.1 mm x 150 mm x 3.5 µm. The binary mobile phase consisted of solvents A (0.1% formic acid in water) and B (100% acetonitrile with 0.1% formic acid). The flow rate was 0.21 ml/min. Data was analysed with Agilent MasHunter Qualitative Analysis B.05.00 software. Compounds were identified by searching METLIN : Metabolite and Tandem MS Database.

The MS parameters were: nebulizer pressure, 45 psi; gas temperature, 300 °C; capillary voltage, 4,000 V; fragmentor voltage, 125 V; gas flow, 10 µl/min. The electrospray ionization (ESI) source was set at positive and negative modes for acquiring all mass spectrometric data. The purified LC-MS fractions of all four samples were tested for toxicity on the human non-malignant breast cell line, 184B5.

3.9 Elucidation of the Mechanisms of Action Leading to the Antiproliferative Effects of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda*

The partially purified fractions were dried in a vacuum centrifuge (Savant Speed-Vac) and subsequently dissolved in DMSO with a final concentration of 1 mg/ml. On the other hand, the pure compounds were directly dissolved in DMSO with afinal concentration of 1 mg/ml. The antiproliferative effects of the partially purified fraction and the pure compounds isolated from *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* were evaluated by determination of the mitochondrial membrane potential (MMP), determination of caspase activities and analysis of DNA fragmentation.

3.9.1 Mitochondrial Membrane Potential (MMP) Analysis by Fluorescence Microscopy

The determination of mitochondrial membrane potential (MMP) in treated and untreated cells was carried out using the JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical Company, Michigan, USA) according to the manufacturer's instructions. Briefly, MCF-7 cells were cultured in a 6-well plate at a density of 2 x 10^5cells/well in 100 µl of culture medium in a CO_2 incubator overnight at 37 °C. Cells were treated with the IC_{50} concentration of partially purified fractions and pure compounds for 48 h. Experiments were carried out in triplicate. A total of 200 µl of JC-

1 staining solution were added into each well and mixed gently. Cells were incubated at 37 °C for 15 - 30 min. The cells were then analysed with an inverted fluorescence microscope with settings designed to detect fluorescein isothiocyanate (FITC) with excitation and emission wavelengths at 485 and 535 nm, respectively.

3.9.2 Quantitative Determination of Mitochondrial Membrane Potential (MMP) ($\Delta\Psi$m)

MCF-7 cells were stained with the cationic dye, JC-1, which exhibits potential-dependent accumulation in mitochondria. The cyanine dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbo-cyanine iodide) has become widely used for microscopic and cytometric estimation and measurement of the MMPdue to its ability to form J-aggregates which are spectrally distinguishable from dye monomers. JC-1 forms J aggregates and exhibits red fluorescence at high membrane potentials. On the contrary, JC-1 exists as a monomer and produces a green fluorescence at low membrane potentials (Salido *et al.*, 2007).

The mitochondrial membrane potential (MMP) of treated and untreated cells were determined using the JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical Company, Michigan, USA) according to the manufacturer's protocol. Briefly, MCF-7 cells were cultured in a black 96-well plate at a density of 2×10^5 cells/well in 100 μl of culture medium in a CO_2 incubator overnight at 37 °C. Cells were treated with 10-50 μg/ml of the partially purified fractions and pure compounds (camptothecin, pseudopelletierine and lycodine) for 48 h. A total of 10 μl of JC-1 staining solutionwere added into each well and mixed gently. Cells were incubated at 37 °C for 15 - 30 min. The plate was then centrifuged at 400 x *g* for 5 min at room temperature. The supernatant was aspirated carefully and 200 μl of Assay Buffer (from the kit) was added

to each well and the plate was centrifuged at 400 x *g* for 5 min at room temperature. The supernatant was aspirated carefully. This step was repeated once. 100 µl of Assay Buffer was added into each well. The absorbance readings for healthy cells were taken at 535 and 595 nm while the absorbance readings for apoptotic cells were taken at 485 and 535 nm. Experiments were carried out in triplicate.

3.9.3   Determination of Caspase Activity

Apoptosis in mammalian cells is initiated by the activation of caspases. The caspase-8, -9 and -3 Colorimetric Assay Kits (BioVision Inc., California, USA) were used for the determination of caspase-8, -9 and -3 activities, respectively. These kits offer a simple and convenient means for assaying the activities of caspase-8, -9 and -3 that recognize the sequence IETD, LEHD and DEVD, respectively. The assay is based on the spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labelled substrates (IETD-pNA, LEHD-pNA and DEVD-pNA). The pNA light emission can be quantified at 400 or 405 nm. The folds increase in the caspase activities can be determined by comparing the absorbance of pNA from an apoptotic sample with an untreated control (negative control) (Lee *et al*., 2012).

MCF-7 cells were treated with the most potent fraction (with the lowest IC$_{50}$) and pure compounds for 8, 16, 24 and 45 h. The assay was carried out with the caspase-3, caspase-8 and caspase-9 Colorimetric Assay Kit (BioVision Inc., California, USA). Treated cells were counted and pelleted (1 x 10^6 cells). Cell pellets were resuspended in 50 µl of chilled lysis buffer [from the kit: 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris (pH 8.0)]. The suspensions were incubated in ice for 10 min and subsequently centrifuged at 10,000 x *g* for 1 min. The supernatants were stored at -80 ℃ for further analysis. Protein concentrations were

quantified using the Bradford assay. 50 µg of protein (as determined by the Bradford assay) was diluted with 50 µl of cell lysis buffer. 10 µl of dithiothreitol(DTT) (Sigma-Aldrich, Saint Louis, Missouri, USA)was added to 1 ml of 2X reaction buffer (from the kit). 50 µl of 2X reaction buffer and 5 µl of substrate were added and incubated for 2 h. Samples were diluted with dilution buffer and absorbance was taken at 405 nm.

3.9.4   DNA Fragmentation Assay

3.9.4.1 Homogenising Samples

Samples were homogenised according to the manufacturer's protocol. Briefly, MCF-7 cells were treated with the most potent partially purified fractions (with the lowest IC_{50}) and pure compounds for 24 and 48 h. Tri Reagent (Life Technologies, California, USA) was used for the extraction of DNA. The growth media was removed from the culture flasks. 5 ml of Tri Reagent was added directly to the cells in the culture flask. The cells were lysed directly in the culture flask by pipetting up and down repeatedly.

3.9.4.2 Phase Separation

The homogenised sample was incubated for 5 min at room temperature to allow complete dissociation of the nucleoprotein complex. 1 ml of chloroform was added and the tube was shaken vigorously for 15 sec; followed by incubation for 2 – 3 min at room temperature. The sample was centrifuged at 12,000 x g for 15 min at 4 °C. The mixture was then separated into a lower red phenol-chloroform phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA). The interphase was kept for DNA extraction.

3.9.4.3 DNA Extraction

Any remaining aqueous phase overlying the interphase was removed. This step is critical for the quality of the isolated DNA. 1.5 ml of 100% ethanol was added and the

sample was inverted several times for mixing. This was followed by incubation for 2 – 3 min at room temperature. The sample was then centrifuged at 2,000 x g for 5 min at 4 °C to pellet the DNA. The phenol-ethanol supernatant was kept for protein extraction.

The DNA pellet was washed with 5 ml of sodium citrate/ethanol solution (0.1 M sodium citrate in 10% ethanol, pH 8.5) and incubated for 30 min at room temperature. The mixture was mixed occasionally by gentle inversion and centrifuged at 2,000 x g for 5 min at 4 °C. Supernatant was removed and discarded. These steps were repeated once. 10 ml of 75% ethanol was added and incubated for 10 – 20 minutes at room temperature. The tube was mixed gently by gentle inversion, followed by centrifugation at 2,000 x g for 5 min at 4 °C. The supernatant was removed and discarded. The DNA pellet was air dried for 5 – 10 min.

A total of 0.5 ml of 8 mM NaOH was added to the DNA. Insoluble materials were removed by centrifuging the sample at 12,000 x g for 10 min at 4 °C. The supernatant containing the DNA was then transferred to a new tube.

DNA samples were chromatographed by 2% agarose gel electrophoresis at a constant voltage of 80 V for 3 h. Image acquisition was performed using the gel documentation system Biospectrum 410, UVP (Fisher Scientific, Loughborough, United Kingdom).

3.10   Proteomics Approaches for Identification of Cancer Relevant Protein Targets in MCF-7 Cells

3.10.1 Preparation of Protein Samples for Proteomics Analysis

MCF-7 cells were cultured in 75 cm^2 flasks and treated with the IC$_{50}$ value concentrations of LC-MS fractions of each marine sample for 48 h. Total proteins were extracted from untreated (negative control) and treated groups. The cells were then pelleted by centrifugation at 6,000 x g for 10 min at 4 °C and subsequently lysed with M-PER Mammalian Protein Extraction Reagent (Thermo Scientific Pierce, Loughborough, UK). The cell lysates were then sonicated thrice for 3 min, with 2 min intervals. The suspensions were centrifuged at 14,000 x g, for 10 min at 4 °C. Supernatants were stored for further processing and analysis.

3.10.1.1 Protein Clean-up by 2D Clean-Up Kit

The 2D Clean-Up Kit (GE Healthcare, Buckinghamshire, UK) was used to purify and precipitate the protein samples. Proteins were precipitated according to the manufacturer's instructions. Briefly, 300 µl of precipitant (from the 2D Clean-Up Kit) was added to 100 µl of protein and mixed well by vortexing or inversion. The mixture was then incubated on ice for 15 min. 300 µl of co-precipitant (from the 2D Clean-Up Kit) was added to the mixture and mixed by vortexing. The mixture was then centrifuged at 12,000 x g for 5 min at 4 °C. The supernatant was removed by decanting or careful pipetting. Without disturbing the pellet, 40 µl of co-precipitant was layered on top of the pellet and incubated in ice for 5 min. The mixture was then centrifuged at 12,000 x g for 5 min at 4 °C. Milli-Q$^®$ water (25 µl) was added onto the pellet and vortexed for 5 – 10 sec. The pellet would disperse, but not dissolve in the water. 1 ml of wash buffer (which was pre-chilled for at least 1 h at -20 °C) and 5 µl of wash additive were added and vortexed until the pellet was fully dispersed. The mixture was incubated at -20 °C for at least 30 min and vortexed for 20 – 30 sec once every 10 min. The mixture was centrifuged at 12,000 x g for 5 min at 4 °C. The supernatant was carefully removed and discarded. The pellet was air dried for less than 5 min and resuspended in

300 µl of rehydration buffer [8 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM dithiothreitol (DTT), 0.5% IPG buffer pH 3-10 NL, 1% bromophenol blue]. The mixture was vortexed for at least 30 sec until the pellet fully dissolved, followed by centrifugation at 12,000 x g for 5 min at 4 °C. The supernatant was stored at -80 °C until further analysis.

3.10.1.2 Determination of Protein Concentration using 2-D Quant Kit

Protein concentration was determined using a 2-D Quant Kit (GE Healthcare, Buckinghamshire, UK) with slight modifications. Briefly, 100 µl of the precipitant (from the 2-D Quant Kit) was mixed with the protein sample or BSA standard and incubated at room temperature for 3 min. Co-precipitant (100 µl) (from the 2-D Quant Kit) was then added into the mixture. After centrifugation at 10,000 x g for 5 min at 4 °C, the supernatant was discarded. Copper solution (20 µl) and 80 µl of Milli-Q$^{®}$ water were pipetted onto the pellet and vortexed until it was fully dissolved. Subsequently, 200 µl of the working colour reagent (mixture of 100 parts of colour reagent A with 1 part of colour reagent B) (from the 2-D Quant Kit) was added into each tube and incubated at room temperature for 20 min. The absorbance reading of each sample was taken at 460 nm using a Model 680 microplate reader (Bio-Rad, Hercules, CA). A standard curve was constructed by plotting the absorbance reading at 460 nm versus the amount of the BSA standard used (BSA ranging from 0 to 12 µg). Protein concentration for each sample was calculated using the constructed standard curve.

3.10.2 2D Gel-Based Proteomics Analysis

3.10.2.1 Sample Loading and Rehydration of Immobilised pH Gradient (IPG) Strip

A total of 250 µg of protein were dissolved in the rehydration buffer (8 M urea, 2% CHAPS, 40 mM dithiothreitol (DTT), 0.5% IPG buffer pH 3-10 NL, 1% bromophenol blue) to a final volume of 250 µl. The total volume of the mixture (250 µl) was pipetted onto the slot of the re-swelling tray. Subsequently, the dried gelside of the IPG strip (13 cm, non-linear pH 3 - 10 gradient) was placed onto the slot containing the protein sample. 2 ml of the cover fluid was overlaid over the strip to prevent evaporation and urea crystallisation. Strip rehydration was conducted at room temperature for 16 - 18 h.

3.10.2.2 First dimensional Isoelectric Focusing (IEF)

Rehydrated strips were transferred onto the channels of the manifold with the gel side facing up. Pre-wetted paper wicks were placed at the acidic and basic ends of each strip. Subsequently, the electrode was placed on top of the paper wicks and the cams were swivelled into closed position. All the channels of the manifold were flooded with the cover fluid to prevent urea crystallisation. The Bio-Rad Protean i12 IEF Cell (Bio-Rad Laboratories, Inc., California, USA) was programmed with the parameters as shown in Table 3.3.

Table 3.3: First dimensional isoelectric focusing (IEF) setting for running 13 cm IPG strips on Bio-Rad Protean i12 IEF Cell. Running conditions: Temperature 20 °C, current 50 µA per strip.

Step	Ramping Mode	Voltage (V)	Time (h:min)	kVh
1	Step and hold	500	1:00	0.5
2	Gradient	1,000	1:00	0.8
3	Gradient	8,000	2:30	11.3
4	Step and hold	8,000	0:30	4.4

3.10.2.3 Equilibration of Immobilised pH Gradient (IPG) Strip

Upon completion of the first-dimensional separation, the IPG strips were equilibrated as follows: step 1 – reduction with 64.8 mM of dithiothreitol-sodium dodecyl sulphate

(DTT-SDS) (Sigma-Aldrich, Saint Louis, Missouri, USA) equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue) for 15 min, followed by step 2 – alkylation with 135.2 mM of iodoacetamide (IAA) – SDS (Sigma-Aldrich, Saint Louis, Missouri, USA) equilibration buffer for another 15 min.

3.10.2.4 Preparation of 12% Sodium Dodecyl Sulphate (SDS) Acrylamide Gel

Resolving gel (12%) was prepared according to the method of Laemmli (1970). The composition of the resolving gel is listed in Table 3.4:

Table 3.4: The composition of resolving gel

Chemicals/Solutions	Volume
Monomer solution	20 ml
4X resolving buffer	13 ml
10% SDS	500 µl
10% ammonium persulphate (APS)	500 µl
N,N,N',N'-Tetramethylethylenediamine (TEMED)	50 µl
Milli-Q® water	16 ml

The gel was overlaid with Milli-Q® water and the gel cassette was allowed to stand at room temperature for 30 min for gel polymerisation.

3.10.2.5 Second Dimensional Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The second dimensional electrophoresis was performed by electrophoresing the protein samples in 12% SDS acrylamide gels (prepared as described in section 3.10.2.4) using

the SE600 Ruby electrophoresis tank (GE Healthcare, Buckinghamshire, UK). The IPG strip was placed onto the surface of the 12% resolving gel. 3 ml of sealing solution (25 mM Tris base, 192 mM glycine, 0.1% SDS, 0.5% agarose, 0.002% bromophenol blue in a final volume of 100 ml) was pipetted into the gel cassette above the IPG strip to seal the IPG strip with the resolving gel. The lower chamber of the tank was filled with 3 l of 1X SDS electrophoresis running buffer while the upper chamber was filled with 1 l of 2X SDS electrophoresis running buffer. The running parameters were programed according to the following settings in Table 3.5:

Table 3.5: The running parameters of the second dimensional SDS-PAGE

Phase	Voltage (V)	Current (mA)	Watt (W)	Duration
1	50	40	25/gel	30 min
2	600	40	25/gel	1 - 2 h

Electrophoresis was stopped when the tracking dye front reached a half centimetre from the bottom of the gel.

3.10.2.6 Protein Staining

After electrophoresis was completed, the gels were fixed with fixing solution (30% ethanol, 10% acetic acid and 60% Milli-Q® water) overnight. The gels were then stained with Silver Stain Kit (Pierce Biotechnology, Inc., Rockford, USA) according to the manufacturer's instructions. After fixation, the gels were washed twice in 10% ethanol for 5 min each wash; followed by washing twice in Milli-Q® water for 5 min each wash. Gels were incubated in the sensitizing solution for 1 min and washed with two changes of Milli-Q® water for 1 min for each wash. Gels were stained with staining solution [containing 1% (v/v) silver stain enhancer] for approximately 5 min and washed twice with Milli-Q® water for 20 sec each wash. Developer solution was then added until

protein spots appeared. Once the desired spot intensity was reached, the reaction was stopped by adding 5% acetic acid in Milli-Q® water and incubated for 10 min with agitation.

3.10.2.7 Image Acquisition and Analysis

Image acquisition was performed using ImageScanner™ III (LabScan6.0, Swiss Institute of Bioinformatics). The fold changes of the differentially expressed proteins were identified byImageMaster™ 2D Platinum 7.0 software (GE Healthcare, Buckinghamshire, UK). The gel images of the negative control and the fractions-treated group were analysed using Progenesis SameSpots software Q1 v2.0 (Nonlinear Dynamics, United Kingdom). Only protein spots showing significant differences ($p<$ 0.05) and maximum fold change of > 1.0 fold were selected for mass spectrometry analysis.

3.10.3 Protein Identification

3.10.3.1 Destaining of Gel Plugs

The 2D gels were washed twice in Milli-Q® water for 10 min each time. The significant protein spots (as mentioned in section 3.10.2.7) were manually excised from the polyacrylamide gel electrophoresis (PAGE) gels and kept in sterile 1.5 ml Eppendorf tubes. The excised spots (gel plugs) were washed with destaining solution (containing 74 µl of Reagent A, 245 µl of Reagent B and 4 ml of Milli-Q® water: for 10 gel plugs). The gel plugs were mixed gently with destaining solution and incubated at room temperature for 15 min. This step was repeated once, after which the destaining solution was removed. Gel plugs were then washed with 200 µl of wash solution [containing 0.16 mg of ammonium bicarbonate in 50% acetonitrile (ACN)] thrice, for 10 min each time.

3.10.3.2 In-Gel Tryptic Digestion

After destaining, the gel plugs were incubated with 150 µl of reducing buffer [containing 10 mM of dithiothreitol (DTT) in 100 mM of ammonium bicarbonate] at 60 °C for 30 min. They were then cooled at room temperature and the reducing buffer was discarded. Gel plugs were then incubated with 150 µl of alkylation buffer [containing 55 mM of iodoacetamide (IAA) in 100 mM of ammonium bicarbonate] at room temperature for 20 min in the dark. The alkylation buffer was then discarded. Washing buffer (containing 50% ACN in 100 mM of ammonium bicarbonate) was added to the gel plugs and shaken twice for 10 min each. The supernatant was then discarded and 50 µl of dehydration buffer (100% ACN) was added to the gel plugs and shaken vigorously for 15 min. The supernatant was discarded and the gel plugs were vacuum dried at ambient temperature for 10 min. In-gel digestion was performed using trypsin (Pierce Biotechnology, Inc., Rockford, USA). A trypsin stock solution was prepared by diluting 1 µg/µl of trypsin in 50 mM of glacial acetic acid. In-gel tryptic digestion was conducted by overnight incubation with a final trypsin concentration of 0.01 µg/µl in 100 mM of ammonium bicarbonate at 37 °C.

3.10.3.3 Peptide Extraction and Desalting

After overnight tryptic digestion, the gel plugs were vortexed and centrifuged at 1,000 rpm for 1 min. 50 µl of 50% ACN was added to the sample and incubated for 15 min at room temperature with shaking. The supernatant was collected. Peptides remaining in the gel plugs were extracted again by adding 50 µl of 100% ACN and followed by incubation at room temperature for another 15 min. The supernatant was collected and pooled into the previous tube. Finally, peptides weredried completely using speed vacuum and stored at -20 °C for further analysis.

Peptides were reconstituted with 10 µl of 0.1% (v/v) formic acid prior to desalting. The C18ZipTip® (Ziptip C18, Millipore, Bedford, MA, USA) was rinsed thrice with 10 µl of the wetting solution [50% (v/v) ACN in Milli-Q® water] and equilibrated thrice with 0.1% (v/v) formic acid. Ten microliters of the reconstituted peptide proteins were aspirated into the pre-equilibrated C18ZipTip® and dispensed. This step was repeated for 15 times. C18ZipTip® was washed three times with the washing solution [0.1% (v/v) formic acid]. Subsequently, peptides were eluted thrice with 4 µL of the 50% acetonitrile containing 0.1% (v/v) formic acid.Samples were then vacuum dried and stored in -20 °C for further analysis.

3.10.3.4 Accurate-Mass Q-TOF LC-MS Analysis

The Agilent 1200 HPLC-Chip/MS Interface was coupled with the Agilent 6520 Accurate-Mass Q-TOF LC/MS platform for LC-MS analysis (Agilent Technologies Inc., California, USA). The columns used were the large capacity chip, 300 Å, C18, 160 nl enrichment column and 75 µm x 150 mm analytical column (Agilent part no: G4240-62010). Peptide samples were reconstituted with 5 µl of Solvent A (0.1% formic acid in water). Samples were spun at 13,200 rpm for 5 min.

1 µlof sample was injected into the C18 column using autosampler at 10 °C (Agilent Technologies Inc., California, USA), followed by an initial wash with Solvent A. The flow rate was set at 4 µl/min from Agilent 1200 Series Capillary Pump (PN: G1382A) to the enrichment column and 0.5 µl/min from Agilent 1200 Series Nanoflow LC Pump (PN: G2226A) to the analytical column. Peptides were eluted from the C18 column with 0 - 50% Solvent B (90% ACN in water with 0.1% formic acid) over 30 min at 0.5

µl/min followed by 50 - 95% Solvent B over 9 min at 0.5 µl/min. The eluted solution was directed into a nanospray ionisation source of the mass spectrometer.

The MS parameters were: positive ion polarity; Vcap: 2,010 V; fragmentor voltage: 175 V; gas temperature: 325 °C; drying gas flow: 5.0 l/min; collision energy: 3.7 V/100 Da, 2.5 V offset. Spectra were recorded in positive ion mode. Agilent ESI Q-TOF was used as the ion source. Precursor selection selected three max precursors per cycle. Precursors m/z of 922.0098 (Z=1), 121.0509 (Z=1), 299.294457 (Z=1) and 1221.990637 (Z=1) (reference ions) were excluded. Mass spectra were acquired in the range of 600 - 4,000 m/z.

3.10.3.5 Protein Identification and Data Processing

The data of MS/MS was analysed using the Agilent Spectrum Mill MS Proteomics Workbench software packages (Rev B.04.00.127) against the database of NCBInr, species of *Homosapiens* (human) using the identity search mode. Fixed modification was set for carbamidomethylation of cysteine. Peptide and protein identification were done by the automatic validation process of the software with the default settings.

3.10.4 Western Blot Analysis

3.10.4.1 Cell Lysate Proteins Preparation and Quantitation

The cell lysate proteins for Western blot analysis were prepared as described earlier in section 3.10.1. Protein samples were cleaned-up and the protein concentrations were quantified according to the manufacturer's instructions as described in sections 3.10.1.1 and 3.10.1.1.2, respectively.

3.10.4.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was conducted using 4-gel Mini-PROTEAN® Tetra Cell (Bio-Rad, Hercules, California, USA). The molecular weight of the enzyme was determined by Precision Plus Protein™ Dual Colour Standards broad range protein ladder (Bio-Rad, Hercules, California, USA), as calibration standards (molecular mass 10 - 250 kDa). A 12% (w/v) polyacrylamide gel was used for the separation of proteins. The formulations of solutions for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) are shown in Table 3.6.

Table 3.6: Formulations of solutions for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Solution	Formulations
Solution A	30% acrylamide/bis solution (Bio-Rad, Hercules, California, USA)
Solution B	1.5 M Tris-HCl, 0.4% sodium dodecyl sulphate (SDS) (w/v), pH was adjusted to 8.8
Solution C	10% (w/v) ammonium persulphate (freshly prepared)
Solution D	0.5 M Tris-HCl containing 0.4% sodium dodecyl sulphate (w/v), pH was adjusted to 6.8
Sample incubation buffer	62 mM Tris-HCl, 2.3% (w/v) sodium dodecyl sulphate, 10% (v/v) glycerol, 5% β-mercaptoethanol and 0.005% bromophenol blue, pH was adjusted to 6.8
Electrophoresis buffer	25 mM Tris-HCl, 129 mM glycine and 0.1% (w/v) sodium dodecyl sulphate

3.10.4.3 Preparation of 12% Resolving Gel and 4% Stacking Gel

Formulations of resolving gel and stacking gel are shown in Table 3.7. Solution mixtures for resolving gel were gently swirled and subsequently poured into the gel cassette until a level which was sufficient for the comb to be inserted. A thin layer of water was then overlaid on top of the gel. The gel was allowed to stand at room temperature for 20 – 30 min for polymerization.

After polymerization of the resolving gel, water was carefully removed. Solution mixtures for stacking gel were layered on top of the resolving gel and the comb was inserted immediately. The gel was allowed to stand at room temperature for 10 - 15 min for polymerization.

Table 3.7: Composition of resolving and stacking gels

Solution	12% Resolving Gel (ml)	4% Stacking Gel (ml)
Solution A	8.0	1.4
Solution B	5.2	-
Solution C	0.1	0.2
Solution D	-	2.5
N,N,N',N'-Tetramethylethylenediamine (TEMED)	0.01	0.01
Milli-Q® water	6.4	6.1

3.10.4.4 Preparation of Protein Samples under Denaturing and Reducing Condition

Protein samples (30 µg) were mixed with sample incubation buffer (according to Table 3.6) at an equal ratio for a maximum volume of 20 µl. Sample mixtures were boiled at 80 °C for 5 - 10 min and subsequently chilled in ice.

3.10.4.5 Running Condition

Gel cassettes were assembled onto the electrophoresis tank according to the manufacturer's protocols (Bio-Rad, Hercules, California, USA). Sample mixtures were loaded onto the wells of the stacking gel and electrophoresis was carried out at a constant voltage of 90 V for approximately 2 h.

3.10.4.6 Western Blotting

Proteins on the SDS-PAGE gel were then electroblotted onto nitrocellulose membranes by tank blotting method using the Mini Trans-Blot Cell and the Criterion Blotter (Bio-Rad, Hercules, California, USA) in Towbin buffer [25 mM Tris-HCl, 129 mM glycine, 20% (v/v) methanol and 0.1% (w/v) sodium dodecyl sulphate] at 100 V for 1 h. The gel sandwich was assembled with the gel and membrane sandwiched between pieces of blot absorbent filter paper as shown in Figure 3.4.
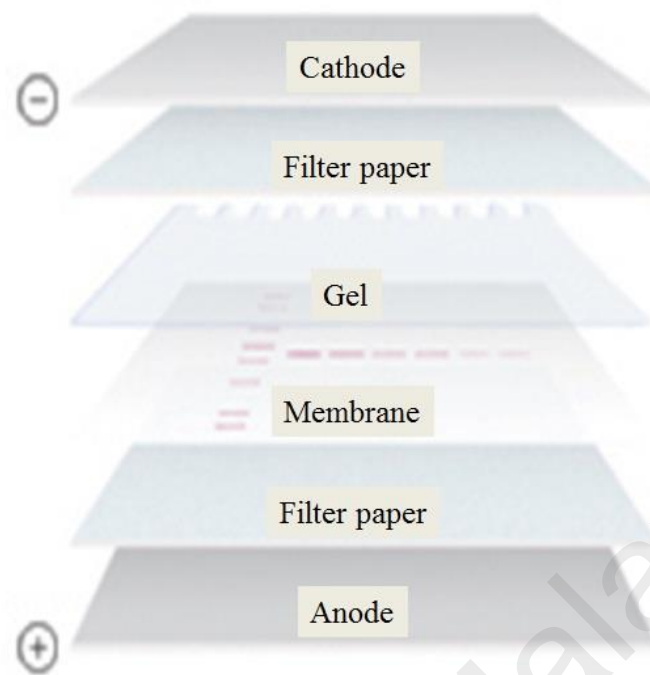
Figure 3.4: Transfer assembly for a tank transfer system.
(Figure from: http://www.bio-rad.com/en-sg/category/western-blotting-membranes-filter-paper).

Following electroblotting, the membrane was blocked with blocking solution [5% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBST)] for 1 h with agitation at room temperature, followed by overnight hybridisation with primary antibodies at 4 °C in 2% BSA blocking solution. The primary antibodies (β-actin, p53, Bax, Bcl-2 and PARP-1) were selected based on the results obtained from 2D-PAGE and Q-TOF LC-MS analysis. p53, Bax and PARP-1 are apoptotic proteins while Bcl-2 is an anti-apoptotic protein. β-actin was used as the internal loading control. All the antibodies were purchased from Pierce Biotechnology, Inc., Rockford, USA. After overnight incubation, the membrane was washed thrice with TBST and further incubated at room temperature for 1 h in 2% BSA blocking solution containing horse radish peroxidase (HRP)-anti mouse conjugated secondary antibody (Bio-Rad Laboratories, Inc., California, USA). The membrane was washed once again with TBST before detection with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Bio-Rad

Laboratories, Inc., California, USA) for 5 min. The antibody dilutions are summarised in Table 3.8.

Table 3.8: Dilutions of primary antibodies for Western blotting analysis

Antibody	Source	Dilution
Polyclonal anti-p53	Rabbit	1: 3,000
Monoclonal anti-Bax	Mouse	1: 100
Monoclonal anti-Bcl-2	Mouse	1: 1,000
Polyclonal anti-PARP-1	Rabbit	1: 1,000
Monoclonal anti-beta actin	Mouse	1: 10,000

3.10.4.7 Stripping of Nitrocellulose Membrane

For repeated hybridization with antibody against beta-actin as an internal loading control, the membrane was stripped in the stripping buffer [0.4 M glycine, 2% (v/v) Tween-20, and 0.2% (w/v) SDS, pH 2.2] at room temperature for 10 - 20 min. After stripping, the membrane was washed twice with TBST and blocked with 5% BSA blocking solution for 1 h before re-hybridizing with the loading control.

3.10.4.8 Image Acquisition and Statistical Analysis

Image acquisition was performed using the gel documentation system Biospectrum 410, UVP (Fisher Scientific, Loughborough, United Kingdom). The band intensities were analysed by ImageJ analyser and software. The fold changes of protein expression were calculated according to equation (11). Changes of 1.5-fold or greater in protein expression ($p < 0.05$) were considered as significant.

$$\frac{\text{Band intensity of treated group}}{\text{Band intensity of untreated group}} \tag{11}$$

3.11   Statistical Analysis

For all experiments, data are reported as mean ± standard error (SEM) ($n = 3$) of data obtained from triplicate experiments using SPSS software. Statistical analyses were performed by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons and the Student's t-test. The correlation between TPC and DPPH radical scavenging activity of each extract was assessed by the Pearson correlation tests and the level of significance was tested by the Student's t-test ($p < 0.05$). A p-value of less than 0.05 or 0.01 was considered significant. SPSS, version 18.0 (Chicago, Ill, USA) and Microsoft Excel 2010 (Roselle, Ill, USA) statistical software was used for the statistical and graphical evaluations.

3.12 Flow Chart of Methodology

The methodology of this study is summarised in the following flowchart.
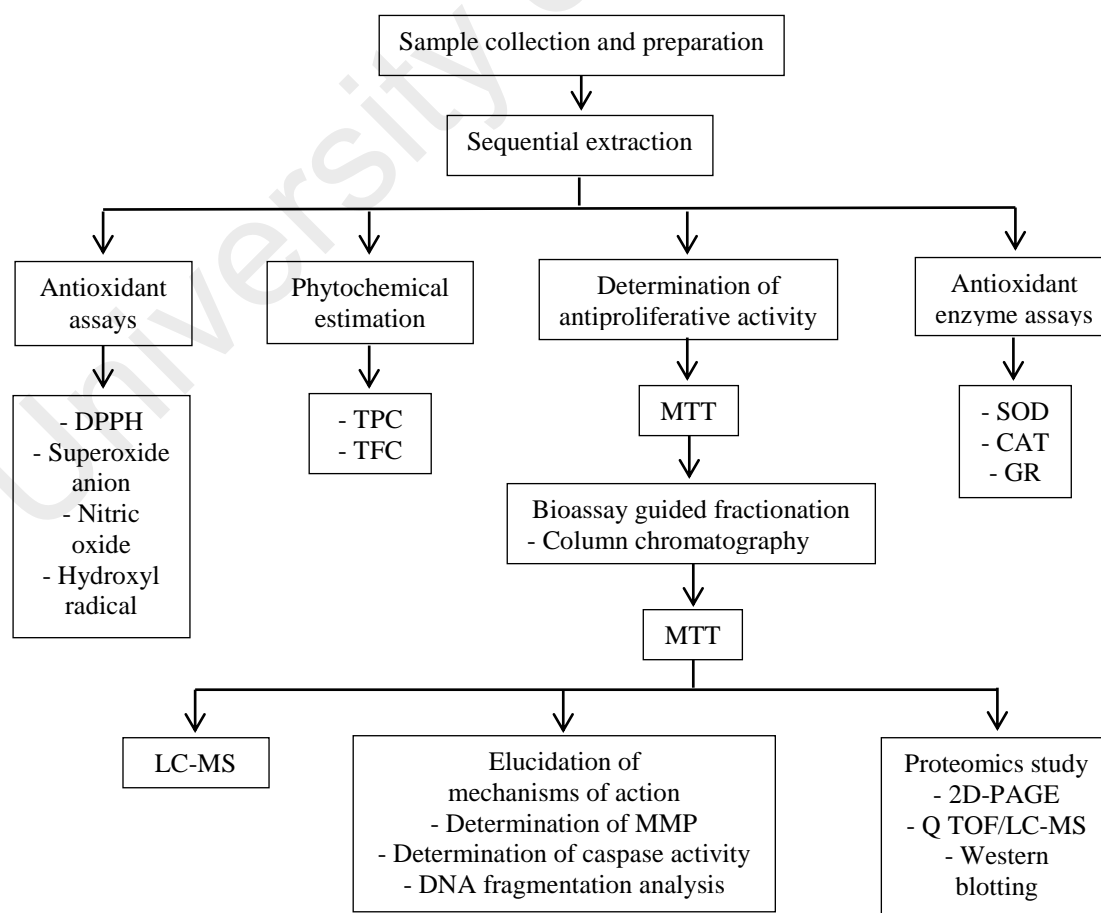
CHAPTER 4

RESULTS

4.1   Assessment of Cell Proliferation

The antiproliferative effects of the different solvent extracts and the cytotoxicity of doxorubicin (a standard chemotherapeutic drug) on MCF-7 cells were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. As shown in Table 4.1, the methanolic extracts of *C. racemosa* and *T. ornata* had the lowest IC_{50} values (60.0 ± 1.47 and 60.0 ± 1.14 µg/ml, respectively) whereas for *P. tetrastromatica*, only the hexane extract showed IC_{50} value (130.0 ± 1.72 µg/ml).

Table 4.1:Antiproliferative activity of different solvent extracts of the seaweeds, *P. tetrastromatica*, *C.* racemosa and*T. ornata* in MCF-7 cells

Extracts	*P. tetrastromatica* IC_{50}(in µg/ml) ± SD	*C. racemosa* IC_{50} (in µg/ml) ± SD	*T. ornata* IC_{50} (in µg/ml) ± SD
Hexane	130.0 ± 1.72	ND (IC_{20} = 40.0 ± 0.98)	240.0 ± 1.89
DCM	ND (IC_{20} = 80.0 ± 1.86)	ND (IC_{20} = 20.0 ± 1.26)	ND (IC_{20} = 60.0 ± 1.57)
EA	ND (IC_{20} = 45.0 ± 1.73)	ND (IC_{20} = 20.0 ± 1.35)	ND (IC_{20} = 40.0 ± 1.28)
Acetone	ND (IC_{20} = 100.0 ± 1.62)	100.0 ± 2.41	480.5 ± 2.27
Methanol	ND (IC_{20} = 200.0 ± 1.21)	60.0 ± 1.47	60.0 ± 1.14

Antiproliferative activity is expressed as IC_{50} (in µg/ml) ± SD (n = 3), which is the concentration of extract at which 50% of cell growth was inhibited relative to cells incubated in the presence of 0.1% DMSO (vehicle control).
ND denotes that IC_{50} values were not detected.

Similar to the brown seaweed, *P. tetrastromatica*, the sponge under study, *S. vagabunda*, also showed IC_{50} only in the hexane extract (as shown in Table 4.2). Among all tested samples, *T. ornata* showed antiproliferative activity in three of the five solvents (hexane, DCM, EA, acetone and methanol) used in extraction. However, the antiproliferative activity of *P. tetrastromatica*, *C. racemosa* and *S. vagabunda* were only shown in one or two solvents used (Table 4.1 and Table 4.2). Thus, the hexane extracts of *P. tetrastromatica* and *S. vagabunda*, and the methanolic extracts of *C. racemosa* and *T. ornata* were chosen for partial purification through column chromatography since these two solvent extracts (the hexane extracts of *P. tetrastromatica* and *S. vagabunda*, and the methanolic extracts of *C. racemosa* and *T. ornata*) showed the best antiproliferative activity for the respective samples.

Table 4.2: Antiproliferative activity of the sponge, *S. vagabunda* in MCF-7 cells

Extracts	*S. vagabunda* IC_{50} (in µg/ml) ± SD
Hexane	80.0 ± 2.18
DCM	ND ($IC_{20} = 220.0 \pm 1.62$)
EA	ND ($IC_{20} = 90.0 \pm 0.93$)
Acetone	ND ($IC_{20} = 55.0 \pm 1.18$)
Methanol	ND ($IC_{20} = 60.0 \pm 0.78$)

Antiproliferative activity is expressed as IC_{50} (in µg/ml) ± SD (n = 3), which is the concentration of extract at which 50% of cell growth was inhibited relative to cells incubated in the presence of 0.1% DMSO vehicle control.
ND denotes that IC_{50} values were not detected.

No cytotoxicity was observed in the extracts-treated non-tumourigenic breast (184B5) cell line (Figure 4.1). In other words, treatment with the crude extracts inhibited less than 50% of the aforementioned cell line. Therefore, there were no IC_{50} values detected. For comparison, the IC_{50} values of the standard anticancer drug, doxorubicin, in MCF-7 and 184B5 cells were also determined under the same experimental conditions. Surprisingly, the drug doxorubicin showed significantly higher cytotoxicity compared to the crude extracts with IC_{50} values of 0.21 ± 1.25 µg/ml and 0.02 ± 1.97 µg/ml in MCF-7 and 184B5 cells, respectively (Figures 4.2 – 4.3).
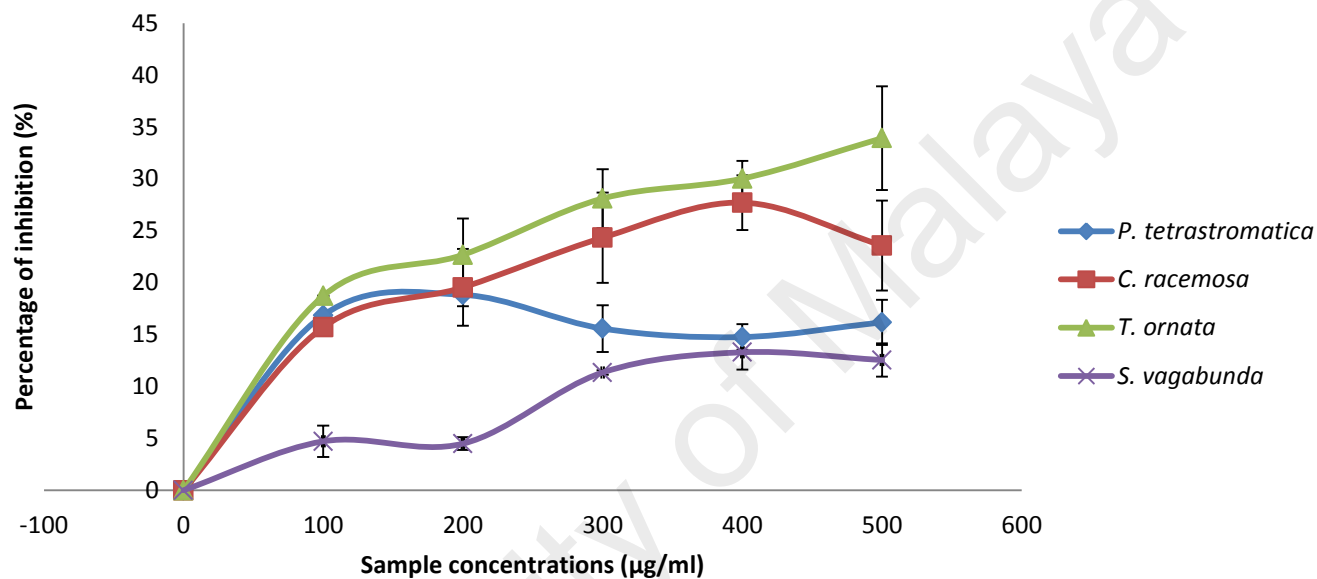
Figure 4.1: Effects of the partially purified fractions of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* on the viability of the human non-tumourigenic 184B5 cell line. The human non-tumourigenic 184B5 cells were treated with different concentrations of the partially purified fractions (100 – 500 µg/ml) for 48 h. Antiproliferative activity is expressed as IC_{50} (in µg/ml) $\pm$ SD (n = 3), which is the concentration of extract at which 50% of cell growth was inhibited relative to cells incubated in the presence of 0.1% DMSO vehicle control. Significance levels were determined using an unpaired, two-tailed Student's t-test, whereby ($p < 0.05$).
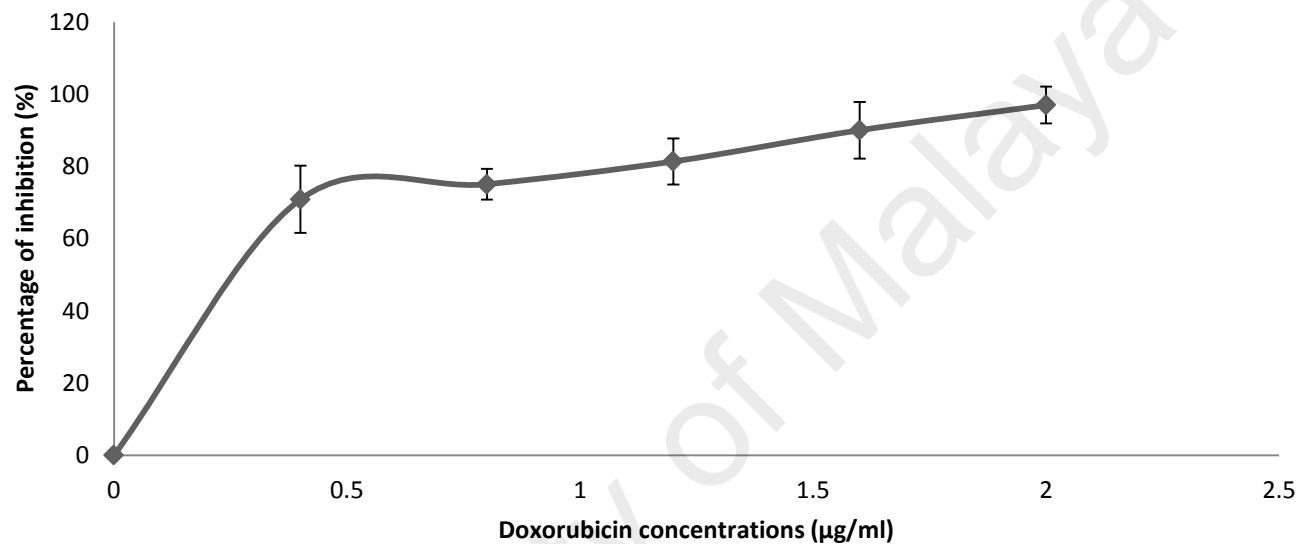
Figure 4.2: Effect of doxorubicin on the viability of the human tumourigenic MCF-7. The human tumourigenic (MCF-7) cells were treated with different concentrations of the standard drug, doxorubicin (0.5 – 2.0 µg/ml) for 48 h. Antiproliferative activity is expressed as IC_{50} (in µg/ml) ± SD (n = 3), which is the concentration of extract at which 50% of cell growth was inhibited relative to cells incubated in the presence of 0.1% DMSO vehicle control. Significance levels were determined using an unpaired, two-tailed Student's t-test, whereby ($p < 0.05$).
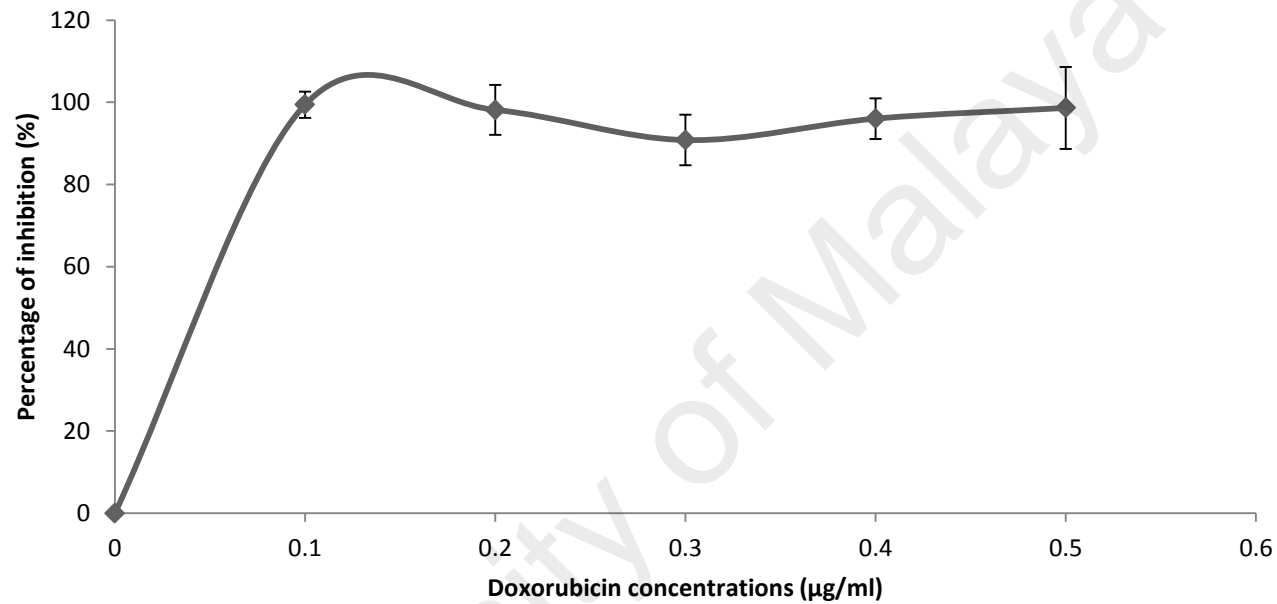
Figure 4.3: Effect of doxorubicin on the viability of the human non-tumourigenic 184B5 cell lines. The human non-tumourigenic (184B5) cells were treated with different concentrations of the standard drug, doxorubicin (0.1 – 0.5 µg/ml) for 48 h. Antiproliferative activity is expressed as IC_{50} (in µg/ml) $\pm$ SD (n = 3), which is the concentration of extract at which 50% of cell growth was inhibited relative to cells incubated in the presence of 0.1% DMSO vehicle control. Significance levels were determined using an unpaired, two-tailed Student's t-test, whereby ($p < 0.05$).

4.2   Determination of Percentage Yield of Extraction

Following sequential extraction of the seaweeds and sponge using five different solvents of increasing polarity, the percentage yield of each solvent (as shown in Tables 4.3 and 4.4) was calculated according to equation (3) in section 3.1.4 of Chapter 3. The methanolic extracts of *P. tetrastromatica* and *C. racemosa* exhibited the highest percentage yield among the five solvents tested, with percentage yields of 0.75% and 8.71%, respectively. DCM showed the highest percentage yield for *T. ornata* (4.08%) while hexane displayed the highest percentage yield for *S. vagabunda* (0.55%). Comparing the seaweeds and sponge in this study, *C. racemosa* showed the highest percentage yield while the sponge, *S. vagabunda* showed the lowest yield of percentage.

Table 4.3: Percentage yield (percentage of the final dried weight of the extracts over the initial weight of the powder) of different solvent extracts of the seaweeds, *P. tetrastromatica*, *C. racemosa* and *T. ornata*

Extracts	*P. tetrastromatica*	*C. racemosa*	*T. ornata*
Hexane	0.04%	0.28%	1.04%
DCM	0.16%	8.40%	4.08%
EA	0.12%	0.04%	0.05%
Acetone	0.32%	0.34%	0.30%
Methanol	0.75%	8.71%	2.45%

Table 4.4: Percentage yield (percentage of the final dried weight of the extracts over the initial weight of the powder) of different solvent extracts of the sponge,*S. vagabunda*

Extracts	*S. vagabunda*
Hexane	0.55%
DCM	0.46%
EA	0.17%
Acetone	0.28%
Methanol	0.31%

4.3   Estimation of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Among the seaweeds tested, the acetone extract of *T. ornata* had the highest TPC with a value of 71.3 ± 2.60 mg GAE/g dried weight (Table 4.5), despite its low percentage yield (Table 4.3). Quercetin and rutin, were used as positive controls for the determination of TPC. The methanolic extracts of *P. tetrastromatica* and *C. racemosa* demonstrated the highest TPC among the five solvents used, with TPC values of 69.5 ± 1.40 and 19.8 ± 2.05 mg GAE/g dried weight, respectively.

The TFC of the different solvent extracts of the three seaweed samplesis shown in Table 4.5. The methanolic extract of *P. tetrastromatica* had the highest TFC (38.4 ± 0.870 mg/g dried weight) while the DCM extract of *C. racemosa* and *T. ornata* had the lowest TFC of 4.6 ± 0.060 and 4.6 ± 0.090 mg/g dried mass, respectively. It is interesting to note that the acetone extract of *T. ornata* demonstrated a relatively low TFC (8.1 ± 0.740 mg/g dried mass) despite having the highest TPC.

The hexane extract of *S. vagabunda* showed a TPC value of 37.5 ± 0.480 mg GAE/g dried weight (Table 4.6). The ethyl acetate extract of *S. vagabunda* showed the highest TFC among the five solvents tested (8.6 ± 1.26 mg/g dried weight). By comparison, the seaweeds, *P. tetrastromatica* and *C. racemosa*, have a relatively higher TPC value compared to the sponge, *S. vagabunda*. In addition, the seaweeds also demonstrated a higher TFC as compared to the *S. vagabunda*.

Table 4.5: Total phenolic content (TPC) and total flavonoid content (TFC) for extracts of the seaweeds, *P. tetrastromatica*, *C. racemosa* and *T. ornata*

Samples	Total phenolic content (TPC) (mg GAE/g)			Total flavonoid content (TFC) (mg/gdried weight)		
	P. tetrastromatica	*C. racemosa*	*T. ornata*	*P. tetrastromatica*	*C. racemosa*	*T. ornata*
Quercetin	980.8 ± 0.570	980.8 ± 0.570	980.8 ± 0.570	ND	ND	ND
Rutin	437.6 ± 0.630	437.6 ± 0.630	437.6 ± 0.630	ND	ND	ND
Catechin	ND	ND	ND	56.7 ± 0.380	56.7 ± 0.380	56.7 ± 0.380
Hexane	45.9 ± 1.28	6.4 ± 0.450	61.5 ± 1.46	14.9 ± 0.740	4.7 ± 0.860	17.5 ± 0.930
DCM	41.4 ± 2.96	7.4 ± 1.15	4.8 ± 1.27	22.3 ± 2.17	4.6 ± 0.060	4.6 ± 0.090
EA	41.3 ± 3.62	16.6 ± 2.20	14.3 ± 0.820	17.8 ± 1.60	16.0 ± 1.68	11.0 ± 0.320
Acetone	33.6 ± 2.79	7.2 ± 2.46	71.3 ± 2.60	31.5 ± 0.520	6.2 ± 2.10	8.1 ± 0.740
Methanol	69.5 ± 1.40	19.8 ± 2.05	33.1 ± 2.47	38.4 ± 0.870	5.0 ± 2.15	4.9 ± 0.180

Quercetin (1 mg/ml) and rutin (1 mg/ml) were used as positive controls for the determination of TPC while catechin (1 mg/ml) was used as a positive control for determination of TFC. Each value is expressed as mean ± SD (n = 3).
ND denotes that TPC and TFC values were not detected.

Table 4.6: Total phenolic content (TPC) and total flavonoid content (TFC) for extracts of the sponge,*S. vagabunda*

Samples	Total phenolic content (TPC) (mg GAE/g)	Total flavonoid content (TFC) (mg/gdried weight)
Quercetin	980.8± 0.57	ND
Rutin	437.6± 0.63	ND
Catechin	ND	56.7± 0.380
Hexane	37.5± 0.48	10.0± 1.60
DCM	31.2± 1.52	9.4± 1.57
EA	15.7± 2.36	8.6± 1.26
Acetone	28.7± 0.57	9.2± 0.880
Methanol	29.9± 1.38	9.3± 2.14

Quercetin (1 mg/ml) and rutin (1 mg/ml) were used as positive controls for the determination of TPC while catechin (1 mg/ml) was used as a positive control for determination of TFC. Each value is expressed as mean ± SD (n = 3).
ND denotes that TPC and TFC values were not detected.

4.4   Antioxidant and Free Radical Scavenging Assays

A number of antioxidant assays such as the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, superoxide anion, nitric oxide and hydroxyl radical scavenging assays,were performed to determine the free radical scavenging ability of the seaweed and sponge extracts.

4.4.1   2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

The hexane extract of the green seaweed, *C. racemosa*, showed the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity among the three seaweeds tested with an IC_{50} of 90.00 ± 0.0027 µg/ml whilst the IC_{50} for the methanol extract of *T. ornata* was highest the with 280.67 ± 1.15 µg/ml (Table 4.7). Table 4.8 shows that the ethyl acetate (EA) extract of *S. vagabunda* had the lowest IC_{50} value of 70.00 ± 1.72 µg/ml.

For the DPPH radical scavenging activity, the *S. vagabunda* again showed a higher DPPH scavenging activity than all the three seaweeds (as shown in Table 4.8). The DPPH radical scavenging activities of the seaweed and sponge samples decreased in the following order: *S. vagabunda*, *C. racemosa*, *P. tetrastromatica* and *T. ornata*.

4.4.2   Superoxide Anion Scavenging Activity

Table 4.7 shows that the superoxide anion scavenging activity was not detected in any of the extracts of *P. tetrastromatica* and *C. racemosa*. As presented in Table 4.7, only the ethyl acetate, acetone and methanolic extracts of *T. ornata* demonstrated strong superoxide anion scavenging activity among the three seaweeds. The IC_{50} values of these extracts were significantly lower than that of the positive controls, indicating their higher potency in scavenging superoxide anions. Therefore, the antioxidant mechanism of these extracts might be mainly due to their superoxide anion scavenging ability.

As shown in Table 4.8, no superoxide anion scavenging activity was detected in the sponge, *S. vagabunda*.

4.4.3 Nitric Oxide Scavenging Activity

The DCM and acetone extracts of *T. ornata*, which showed IC_{50} values of 25.00 ± 0.00 µg/ml, had the highest nitric oxide scavenging activity among the seaweeds tested. The DCM extract of *C. racemosa* also showed a strong nitric oxide scavenging activity with an IC_{50} value of 38.33 ± 2.89 µg/ml (Table 4.7).

Similar to superoxide anion scavenging activity, no nitric oxide scavenging activity was detected in any of the extracts of *S. vagabunda* (Table 4.8).

4.4.4 Hydroxyl Radical Scavenging Activity

The hexane and acetone extracts of *C. racemosa*; and the methanolic extract of *T. ornata* demonstrated strong hydroxyl radical scavenging activity with similar IC_{50} values (20.0 µg/ml), compared to that of the positive control, quercetin. The hexane extract of *P. tetrastromatica* (IC_{50} of 23.33 ± 2.89 µg/ml) also similarly showed strong hydroxyl radical scavenging activity. All three seaweeds demonstrated a stronger activity than the positive control, rutin ($IC_{50} = 101.67 \pm 2.89$ µg/ml) (Table 4.7).

As presented in Table 4.8, the dichloromethane (DCM) extract of the sponge, *S. vagabunda*, also showed a strong hydroxyl radical scavenging activity (IC_{50} of 20.00 ± 2.61 µg/ml), comparable to that of quercetin as well as the three seaweeds tested.

Table 4.7: IC_{50} values in free radical scavenging assays for extracts of the seaweeds,*P. tetrastromatica*, *C. racemosa* and *T. ornata*

	Quercetin	Rutin	*P. tetrastromatica*	*C. racemosa*	*T. ornata*
IC_{50} of DPPH radical scavenging activity (µg/ml)	21.67 ± 2.89	23.33 ± 2.89	171.67 ± 2.89 (EA extract)	90.00 ± 0.00 (Hexane extract)	280.67 ± 1.15 (Methanol extract)
IC_{50} of superoxide anion scavenging activity (µg/ml)	260.00 ± 0.00	223.33 ± 2.89	ND	ND	20.00 ± 0.00 (EA, acetone, methanol extracts)
IC_{50} of nitric oxide scavenging activity (µg/ml)	20.00 ± 0.00	20.00 ± 0.00	ND	38.33 ± 2.89 (DCM extract)	25.00 ± 0.00 (DCM and acetone extracts)
IC_{50} of hydroxyl radical scavenging activity (µg/ml)	20.00 ± 0.00	101.67 ± 2.89	23.33 ± 2.89 (Hexane extract)	20.00 ± 0.00 (Hexane and acetone extracts)	20.00 ± 0.00 (Methanol extract)

Quercetin and rutin were used as positive controls. Each value is expressed as mean ± SD (n = 3).
ND denotes that IC_{50} values were not detected.

Table 4.8: IC$_{50}$ values in free radical scavenging assays for extracts of the sponge, *S. vagabunda*

	Quercetin	Rutin	*S. vagabunda*
IC$_{50}$ of DPPH radical scavenging activity (µg/ml)	21.67 ± 2.89	23.33 ± 2.89	70.00 ± 1.72 (EA extract)
IC$_{50}$ of superoxide anion scavenging activity (µg/ml)	260.00 ± 0.00	223.33 ± 2.89	ND
IC$_{50}$ of nitric oxide scavenging activity (µg/ml)	20.00 ± 0.00	20.00 ± 0.00	ND
IC$_{50}$ of hydroxyl radical scavenging activity (µg/ml)	20.00 ± 0.00	101.67 ± 2.89	20.00 ± 2.61 (DCM extract)

Quercetin and rutin were used as positive controls. Each value is expressed as mean ± SD (n = 3).
ND denotes that IC$_{50}$ values were not detected.

4.5   Evaluation of Antioxidant Enzyme Activities

Treatment of MCF-7 cells with 400 µg/ml of the crude extracts for 8, 16 and 24 h evoked a significant variation in the SOD, CAT and GR activities as compared to untreated negative control (Figures 4.4 – 4.9). All three enzymes exhibited a decrease in activities relative to the negative control in a time-dependent manner. There was a sharp decrease in CAT activity within the first 8 h of treatment.
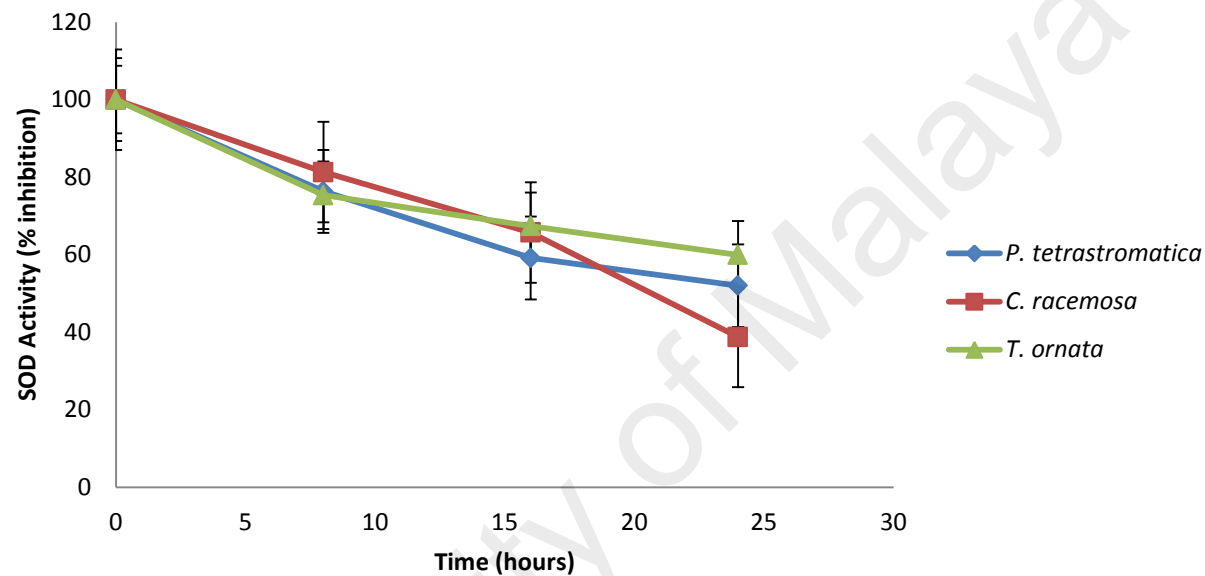
Figure 4.4:Superoxide dismustase (SOD) activity of the methanolic extracts of the seaweeds, *P. tetrastromatica*, *C. racemosa* and *T. ornata*. MCF-7 cells were treated for 8, 16 and 24 h with the extracts and the SOD activity assayed. Untreated MCF-7 cells were used as the control for each time point and set as 100%. Each value is expressed as mean ± SD (n = 3).Significance levels were determined using an unpaired, two-tailed Student's t-test, whereby ($p < 0.05$).
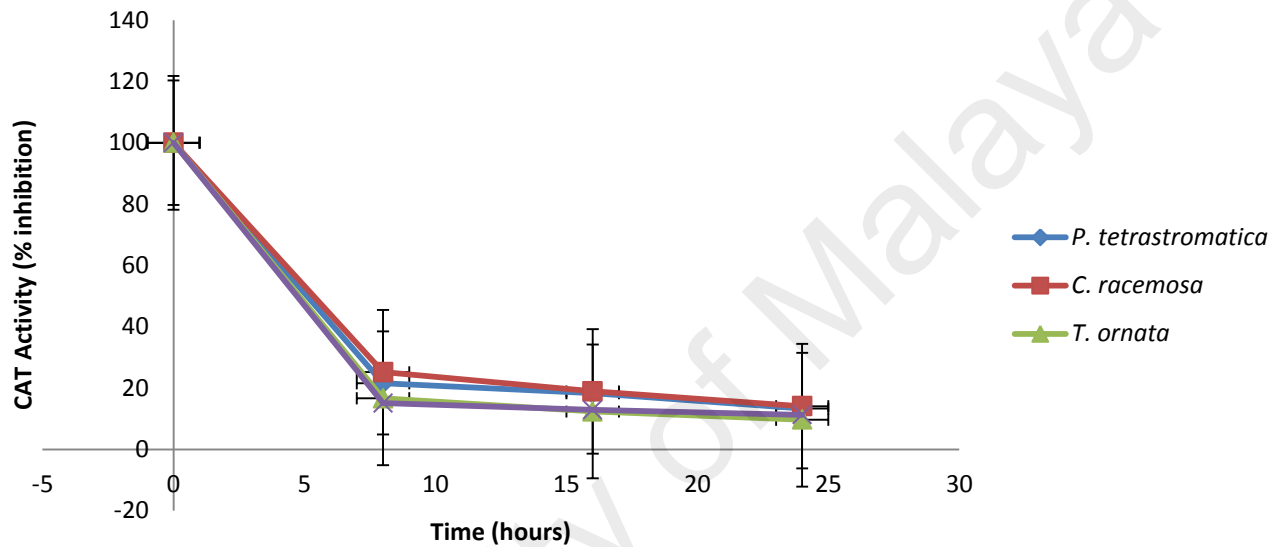
Figure 4.5:Catalase (CAT) activity of the methanolic extracts of the seaweeds, *P. tetrastromatica*, *C. racemosa* and *T. ornata*. MCF-7 cells were treated for 8, 16 and 24 h with the extracts and the CAT activity assayed. Untreated MCF-7 cells were used as the control for each time point and set as 100%. Each value is expressed as mean ± SD (n = 3).Significance levels were determined using an unpaired, two-tailed Student's t-test, whereby ($p <$ 0.05).
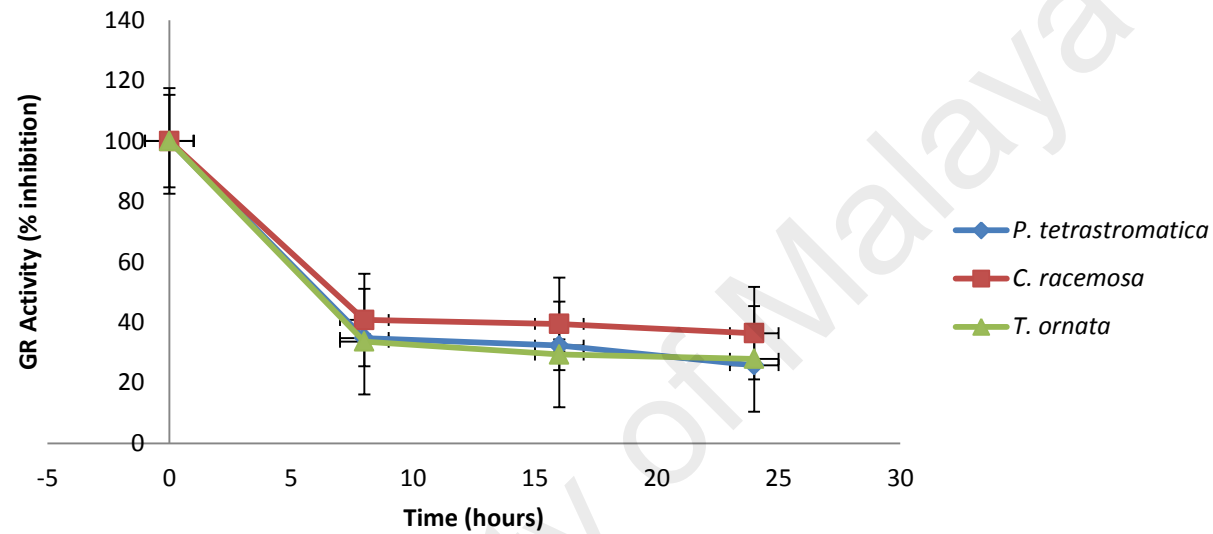
Figure 4.6:Glutathione reductase (GR) activity of the methanolic extracts of the seaweeds, *P. tetrastromatica*, *C. racemosa* and *T. ornata*. MCF-7 cells were treated for 8, 16 and 24 h with the extracts and the GR activity assayed. Untreated MCF-7 cells were used as the control for each time point and set as 100%. Each value is expressed as mean ± SD (n = 3).Significance levels were determined using an unpaired, two-tailed Student's t-test, whereby ($p < 0.05$).
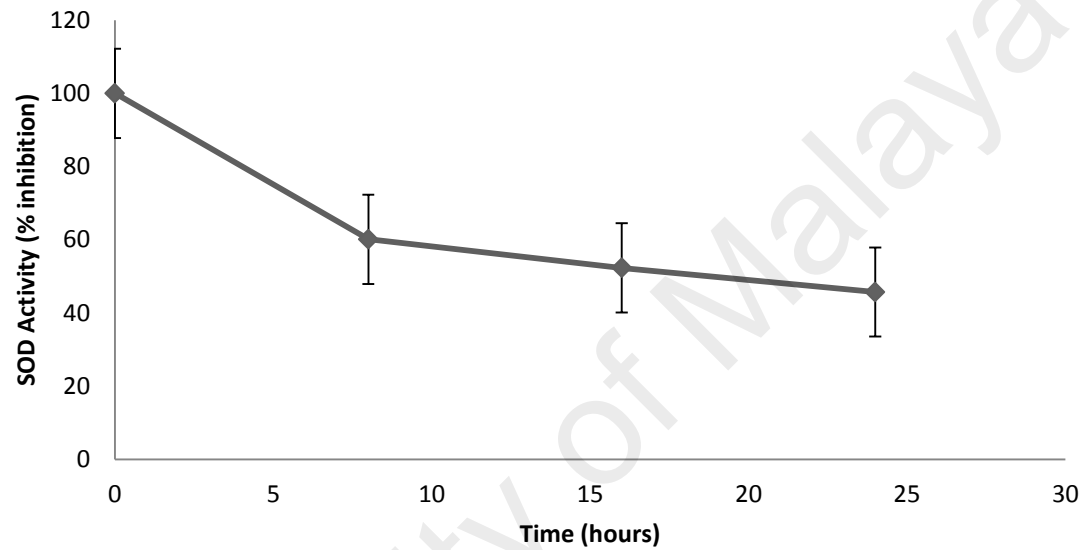
Figure 4.7:Superoxide dismustase (SOD) activity of the methanolic extracts of the sponge, *S.vagabunda*. MCF-7 cells were treated for 8, 16 and 24 h with the extracts and the SOD activity assayed. Untreated MCF-7 cells were used as the control for each time point and set as 100%. Each value is expressed as mean ± SD (n = 3).Significance levels were determined using an unpaired, two-tailed Student's t-test, whereby ($p < 0.05$).
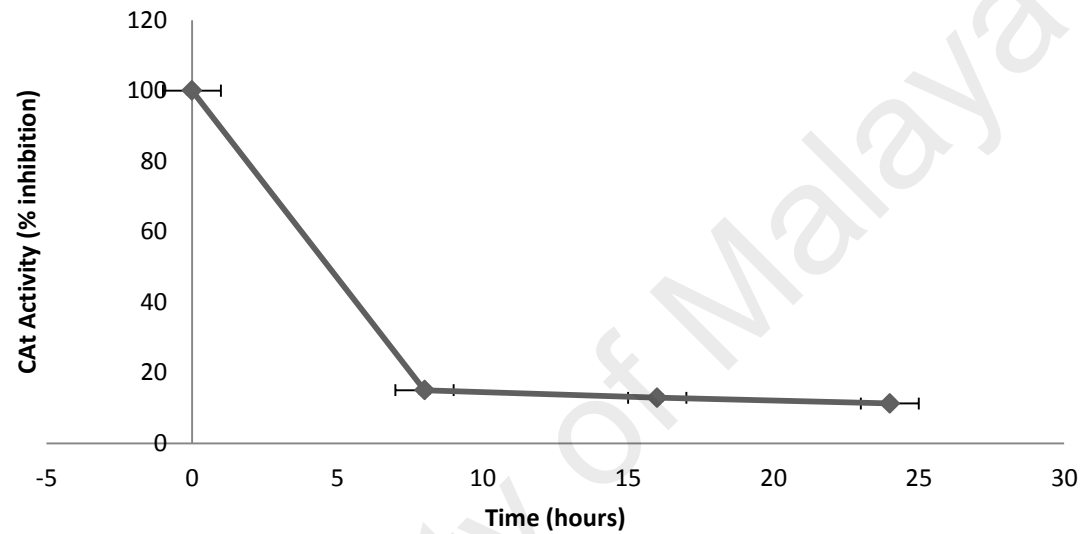
Figure 4.8:Catalase (CAT) activity of the methanolic extracts of the sponge, *S.vagabunda*. MCF-7 cells were treated for 8, 16 and 24 h with the extracts and the CAT activity assayed. Untreated MCF-7 cells were used as the control for each time point and set as 100%. Each value is expressed as mean ± SD (n = 3).Significance levels were determined using an unpaired, two-tailed Student's t-test, whereby ($p < 0.05$).
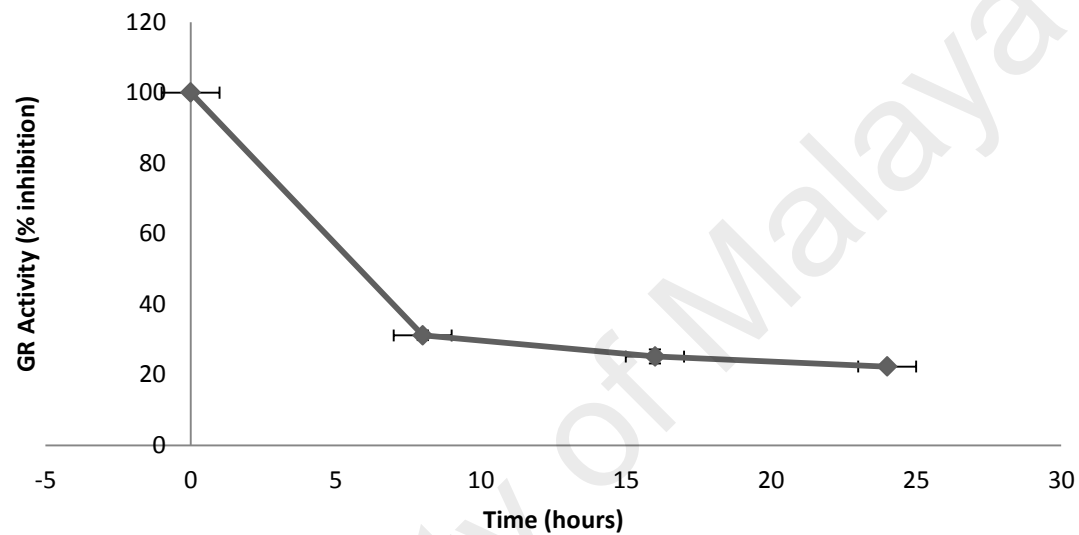
Figure 4.9:Glutathione reductase (GR) activity of the methanolic extracts of the sponge, *S.vagabunda*. MCF-7 cells were treated for 8, 16 and 24 h with the extracts and the GR activity assayed. Untreated MCF-7 cells were used as the control for each time point and set as 100%. Each value is expressed as mean ± SD (n = 3).Significance levels were determined using an unpaired, two-tailed Student's t-test, whereby ($p < 0.05$).

4.6   Correlation between TPC and Different Antioxidant and Antioxidant Enzyme Activities

4.6.1   Correlation between TPC and TFC

The correlation between TPC and TFC; DPPH radical, superoxide anion, nitric oxide and hydroxyl radical scavenging activities; and antioxidant enzyme activities is interesting to note. The Pearson coefficient correlation was used to analyse the aforementioned correlations whereby the degree to which two variables are related was quantified. The two variables are independent of each other and are not experimentally manipulated. Therefore, it only measures the relatability of the two variables and it does not fit a line through the data, unlike line regression analysis, in which the best line passes through most of the data points. The extracts with the highest TPC values were correlated with their TFC values. As presented in Tables 4.7 and 4.8, the methanol extracts of *P. tetrastromatica* and *C. racemosa*; acetone extract of *T. ornata* and the hexane extract of *S. vagabunda* showed the highest TPC value among five solvents tested. Therefore, these extracts were selected for correlational studies.

A correlation between the TPC and TFC of all the seaweed and sponge extracts was carried out. Tables 4.9 and 4.10 show a positive correlation between the TPC of the methanol extracts of *P. tetrastromatica* and *C. racemosa*; acetone extract of *T. ornata* and hexane extract of *S. vagabunda* with their respective TFCs. The Pearson correlation coefficients between the TPC and TFC are 0.86, 0.99, 0.71 and 0.74 for *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda*, respectively. *C. racemosa* showed the highest correlation between TPC and TFC among the seaweed and sponge samples tested in this study.

Table 4.9: Correlation between TPC and TFC of the seaweeds, *P. tetrastromatica*, *C. racemosa* **and** *T. ornata*
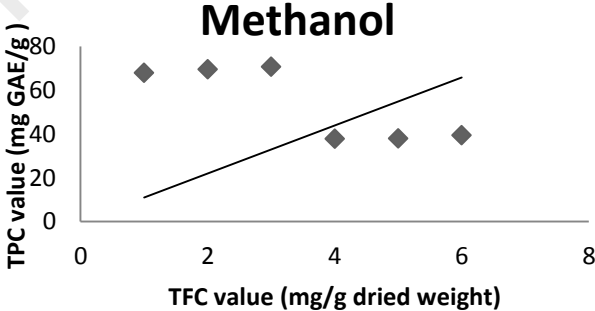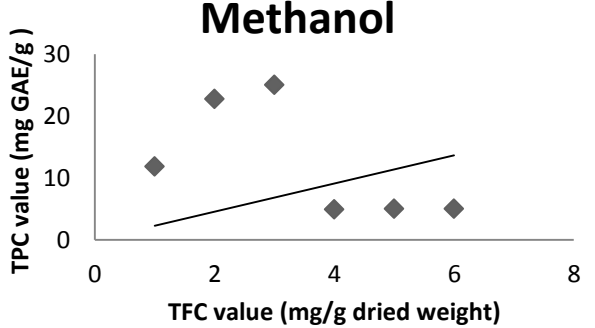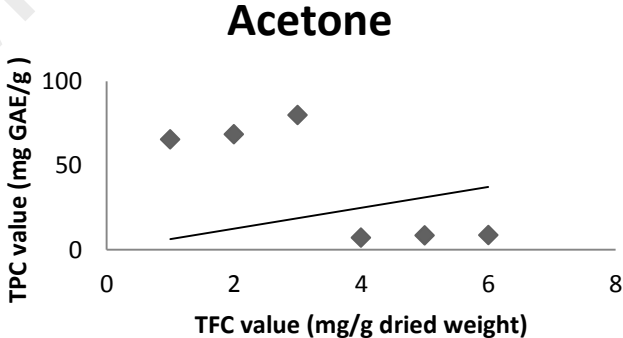
Samples	Pearson correlation coefficient (r)	Correlation between TPC and TFC
P. tetrastromatica	0.86*	
C. racemosa	0.99	

Table 4.9, continued. Correlation between TPC and TFC of the seaweeds,*P. tetrastromatica*, *C. racemosa* and *T. ornata*

Samples	Pearson correlation coefficient (r)	Correlation between TPC and TFC
T. ornata	0.71^{*}	

Each value is expressed as mean $\pm$ SD (n = 3).
* denotes significance at $p < 0.01$.

Table 4.10: Correlation between TPC and TFC of the sponge,*S. vagabunda*

Samples	Pearson correlation coefficient (r)	Correlation between TPC and TFC
S. vagabunda	0.74[*]	

Each value is expressed as mean ± SD (n = 3).
[*] denotes significance at $p < 0.01$.

4.6.2 Correlation between TPC and DPPH Radical Scavenging Activity

The solvent extracts showing the highest TPC value for each sample were tested for correlation with their respective DPPH scavenging abilities. The TPC of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* were found to be positively correlated with the DPPH radical scavenging activity (Tables 4.11 and 4.12). The TPC values of the methanolic extracts of *P. tetrastromatica* and *C. racemosa* were most highly correlated with their percentage of DPPH scavenging (Pearson correlation coefficient of 0.96 and 0.93, respectively). Meanwhile, the Pearson correlation coefficient of the acetone extract of *T. ornata* and the hexane extract of *S. vagabunda* were 0.77 and 0.37, respectively. Hence, the results showed that the TPC of *S. vagabunda* has the lowest correlation with the DPPH radical scavenging activity among the tested marine organisms.

Table 4.11: Correlation between TPC and DPPH radical scavenging activity of the seaweeds, *P. tetrastromatica*, *C. racemosa* **and** *T. ornata*

Samples	Pearson correlation coefficient (r)	Correlation between TPC and DPPH radical scavenging activity
P. tetrastromatica	0.96[*]	
C. racemosa	0.93[*]	

Table 4.11, continued. Correlation between TPC and DPPH radical scavenging activity of the seaweeds, *P. tetrastromatica*, *C. racemosa* **and** *T. ornata*

Samples	Pearson correlation coefficient (r)	Correlation between TPC and DPPH radical scavenging activity
T. ornata	0.71[*]	

Each value is expressed as mean ± SD (n = 3).
[*] denotes significance at p < 0.01.

Table 4.12: Correlation between TPC and DPPH radical scavenging activity of the sponge, *S. vagabunda*

Samples	Pearson correlation coefficient (r)	Correlation between TPC and DPPH radical scavenging activity
S. vagabunda	0.37[*]	

Each value is expressed as mean ± SD (n = 3).
[*] denotes significance at p < 0.01.

4.6.3 Correlation between TPC and Superoxide Anion Scavenging Activity

The Pearson correlation coefficient between the TPC and superoxide anion scavenging activity of *P. tetrastromatica* is 0.74 (Table 4.13). In contrast, the TPC of *C. racemosa*, *T. ornata* and *S. vagabunda* was weakly correlated with their respective superoxide anion scavenging ability (Tables 4.13 and 4.14) with Pearson correlation coefficient of 0.34, 0.55 and 0.33, respectively. Similar to the correlation between TPC and DPPH radical scavenging activity, *S. vagabunda* demonstrated the lowest correlation between TPC and superoxide anion scavenging activity compared to the seaweeds tested.

Table 4.13: Correlation between TPC and superoxide anion scavenging activity of the seaweeds,*P. tetrastromatica*, *C. racemosa* and *T. ornata*
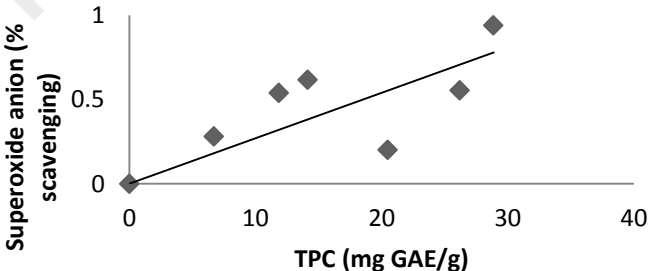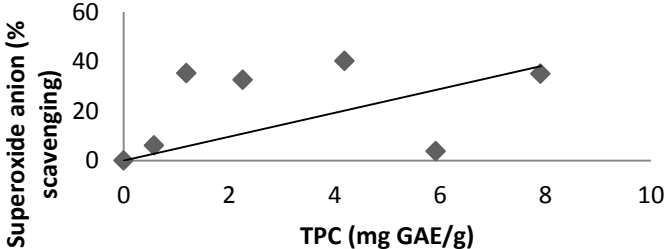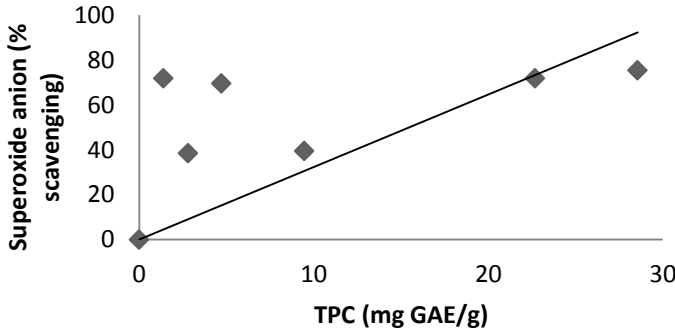
Samples	Pearson correlation coefficient (r)	Correlation between TPC and superoxide anion scavenging activity
P. tetrastromatica	0.74[*]	
C. racemosa	0.34	
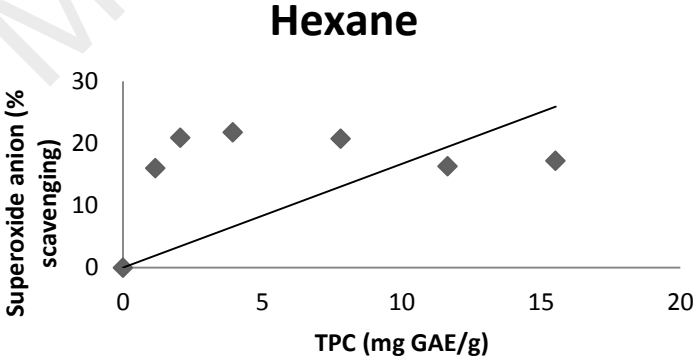
Table 4.13, continued. Correlation between TPC and superoxide anion scavenging activity of the seaweeds,*P. tetrastromatica*, *C. racemosa* and *T. ornata*

Samples	Pearson correlation coefficient (r)	Correlation between TPC and superoxide anion scavenging activity
T. ornata	0.55[*]	

Each value is expressed as mean ± SD (n = 3).
[*] denotes significance at $p < 0.01$.

Table 4.14: Correlation between TPC and superoxide anion scavenging activity of the sponge, *S. vagabunda*

Samples	Pearson correlation coefficient (r)	Correlation between TPC and superoxide anion scavenging activity
S. vagabunda	0.33	

Each value is expressed as mean ± SD (n = 3).
[*] denotes significance at $p < 0.01$.

4.6.4 Correlation between TPC and Nitric Oxide Scavenging Activity

As presented in Table 4.8(a), the TPC of *P. tetrastromatica* was highly correlated with its nitric oxide scavenging activity (Pearson correlation coefficient of 0.96). *C. racemosa* showed only moderate correlation with a Pearson correlation coefficient of 0.64 while a low correlation was demonstrated by *T. ornata* and *S. vagabunda* (Pearson correlation coefficients of 0.17 and 0.47, respectively) (Tables 4.15 and 4.16).

Table 4.15: Correlation between TPC and nitric oxide scavenging activity of the seaweeds, *P. tetrastromatica*, *C. racemosa* **and** *T. ornata*
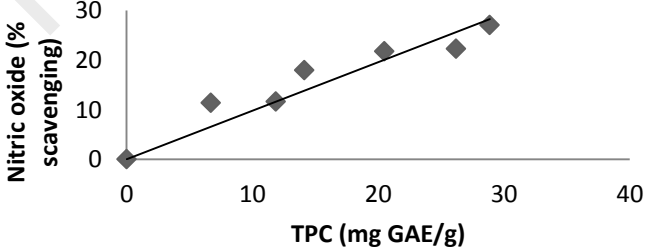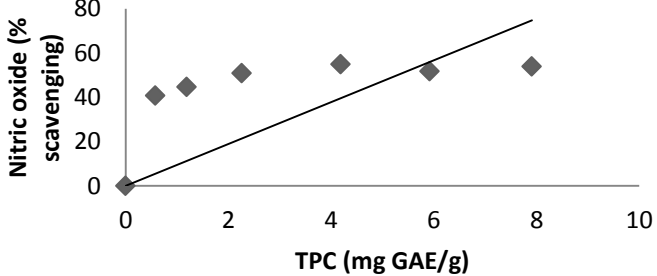
Samples	Pearson correlation coefficient (r)	Correlation between TPC and nitric oxide scavenging activity
P. tetrastromatica	0.96	
C. racemosa	0.64*	
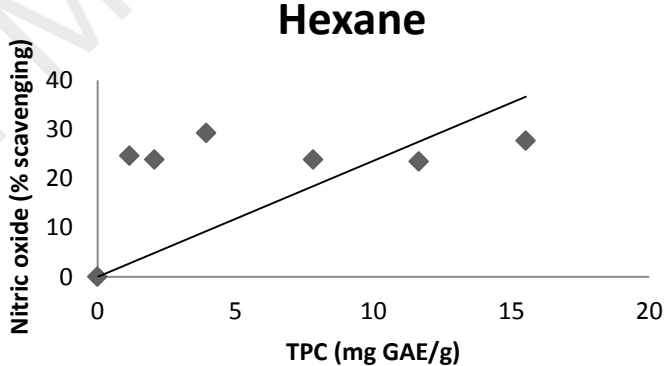
Table 4.15, continued. Correlation between TPC and nitric oxide scavenging activity of the seaweeds, *P. tetrastromatica*, *C. racemosa* and *T. ornata*

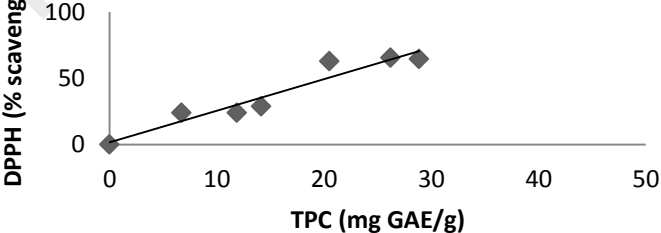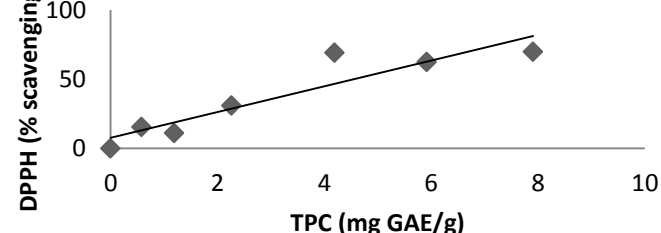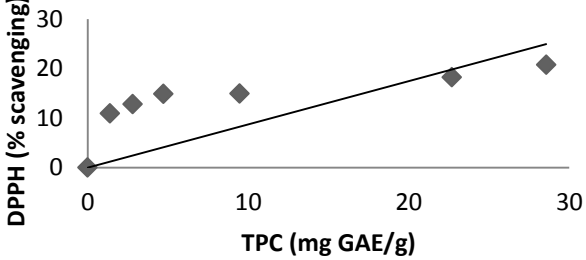| Samples	Pearson correlation coefficient (r)	Correlation between TPC and nitric oxide scavenging activity
T. ornata	0.17[*]	

Each value is expressed as mean $\pm$ SD (n = 3).
[*] denotes significance at $p < 0.01$.

Table 4.16: Correlation between TPC and nitric oxide scavenging activity of the sponge, *S. vagabunda*

Samples	Pearson correlation coefficient (r)	Correlation between TPC and nitric oxide scavenging activity
S. vagabunda	0.47*	

Each value is expressed as mean ± SD (n = 3).
* denotes significance at p < 0.01.

4.6.5 Correlation between TPC and Hydroxyl Radical Scavenging Activity

P. tetrastromatica and *S. vagabunda* showed a relatively higher correlation between the TPC and hydroxyl radical scavenging activity (Pearson correlation coefficients of 0.87 and 0.94, respectively) as compared to *C. racemosa* and *T. ornata* which showed a low correlation (Pearson correlation coefficient of 0.39 and 0.59, respectively) (Tables 4.17 and 4.18). *S. vagabunda* showed stronger correlation between the TPC and the hydroxyl radical scavenging activity than the three seaweeds.

Table 4.17: Correlation between TPC and hydroxyl radical scavenging activity of the seaweeds,*P. tetrastromatica*, *C. racemosa* and *T. ornata*
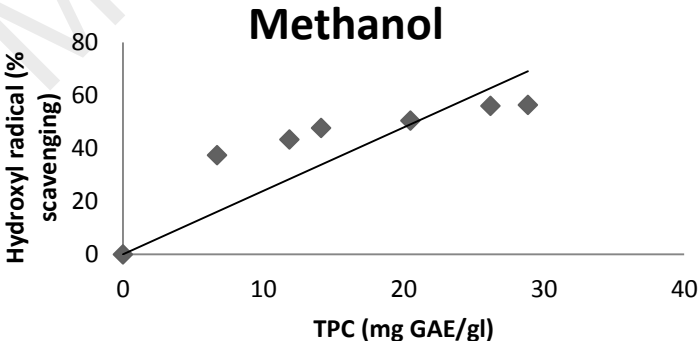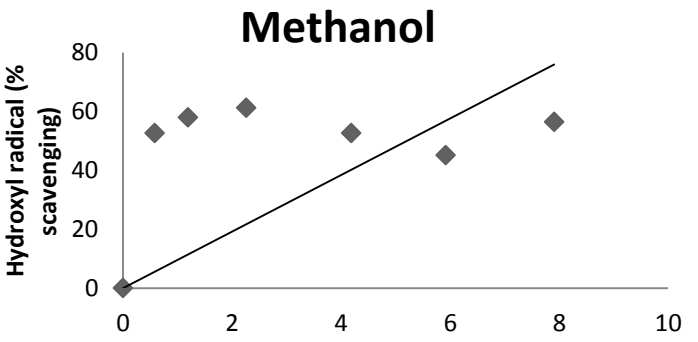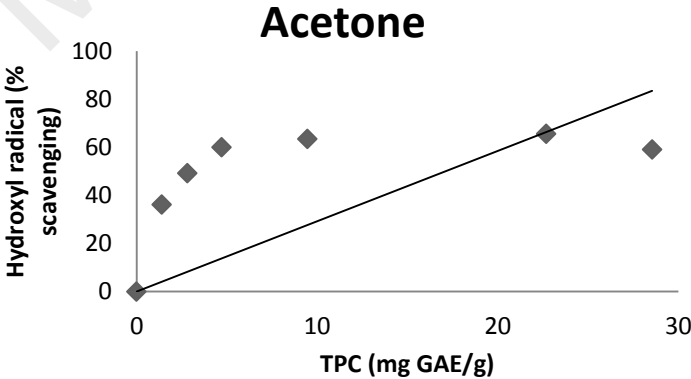
Samples	Pearson correlation coefficient (r)	Correlation between TPC and hydroxyl radical scavenging activity
P. tetrastromatica	0.87*	
C. racemosa	0.39*	

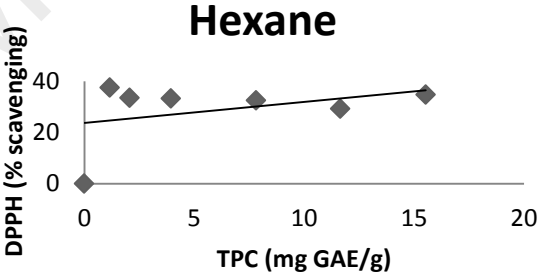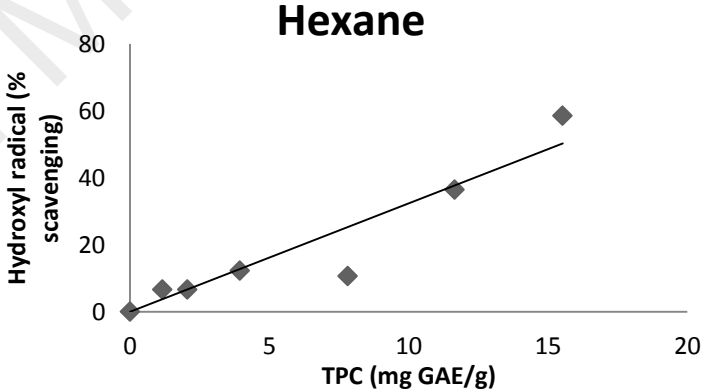**Table 4.17, continued. Correlation between TPC and hydroxyl radical scavenging activity of the seaweeds,** *P. tetrastromatica*, *C. racemosa* **and** *T. ornata*

Samples	Pearson correlation coefficient (r)	Correlation between TPC and hydroxyl radical scavenging activity
T. ornata	0.59*	

Each value is expressed as mean ± SD (n = 3).
* denotes significance at p < 0.01.

Table 4.18: Correlation between TPC and hydroxyl radical scavenging activity of the sponge,*S. vagabunda*

Samples	Pearson correlation coefficient (r)	Correlation between TPC and hydroxyl radical scavenging activity
S. vagabunda	0.94	

Each value is expressed as mean ± SD (n = 3).
* denotes significance at p < 0.01.

4.6.6 Correlation between TPC and Antioxidant Enzyme Activities

In addition to antioxidant activities, the correlation between TPC and the reduction of antioxidant enzyme activities were also studied. The correlation between TPC and the reduction of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) of the four marine organisms were analysed. It is interesting to note that the TPC of the seaweeds (*P. tetrastromatica*, *C. racemosa* and *T. ornata*) and the sponge (*S. vagabunda*) was strongly correlated to the reduction of SOD activities of the respective samples with Pearson correlation coefficients of 0.97, 0.99, 0.98 and 0.93, respectively (Tables 4.19 and 4.20).

Table 4.19: Correlation between TPC and the reduction of the superoxide dismutase (SOD) activity of the seaweeds,*P. tetrastromatica*, *C. racemosa* and *T. ornata*
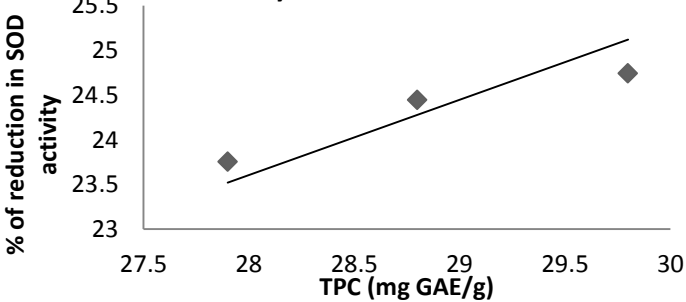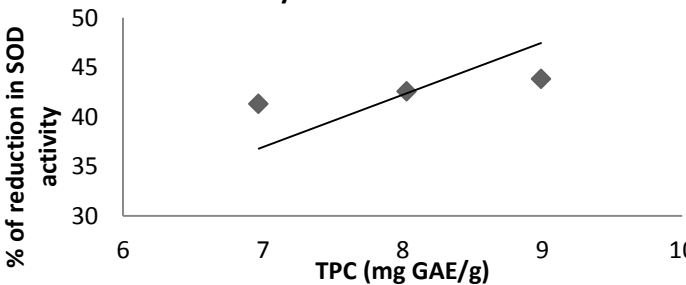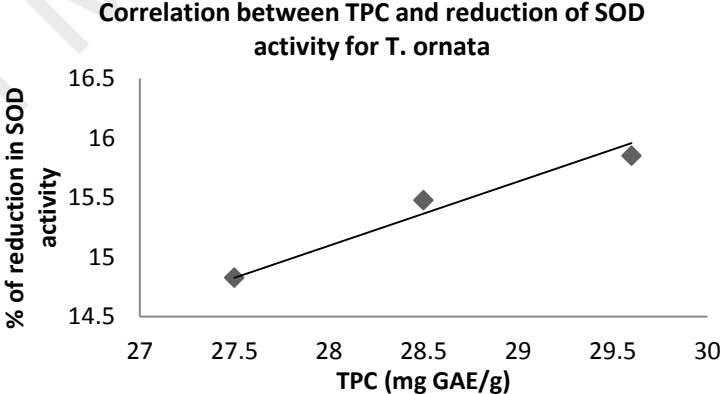
Samples	Pearson correlation coefficient (r)	Correlation between TPC and the reduction in superoxide dismutase (SOD) activity
P. tetrastromatica	0.97*	
C. racemosa	0.99*	

Table 4.19, continued. Correlation between TPC and the reduction of the uperoxide dismutase (SOD) activity of the seaweeds, *P. tetrastromatica*, *C. racemosa* **and** *T. ornata*

Samples	Pearson correlation coefficient (r)	Correlation between TPC and the reduction in superoxide dismutase (SOD) activity
T. ornata	0.98*	

Correlation between TPC and reduction of SOD activity for T. ornata

Each value is expressed as mean $\pm$ SD (n = 3).
* denotes significance at p < 0.01.

Table 4.20: Correlation between TPC and the reduction of the superoxide dismutase (SOD) activity of the sponge,*S. vagabunda*

Samples	Pearson correlation coefficient (r)	Correlation between TPC and the reduction in superoxide dismutase (SOD) activity
S. vagabunda	0.93	

Correlation between TPC and reduction of SOD activity for S.vagabunda

Each value is expressed as mean ± SD (n = 3).
* denotes significance at p < 0.01.

The correlation between TPC and the reductions of CAT and GR activities also showed similar results. The TPC of *P. tetrastromatica*, *C. racemosa, T. ornata* and *S. vagabunda* were highly correlated with their reduction of CAT activities with Pearson correlation coefficients of 0.93, 0.87, 0.98 and 0.97, respectively (Tables 4.21 and 4.22).

Table 4.21: Correlation between TPC and the reduction of the catalase (CAT) activity of the seaweeds, *P. tetrastromatica*, *C. racemosa* **and** *T. ornata*
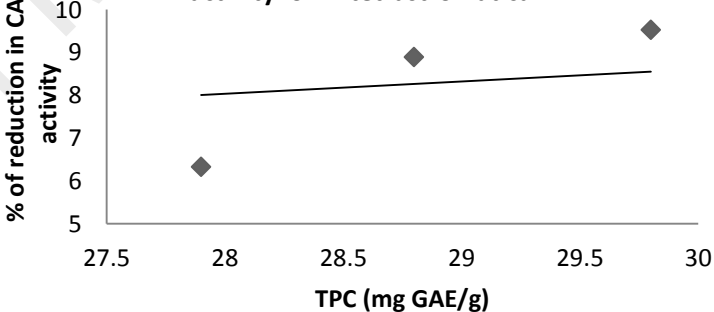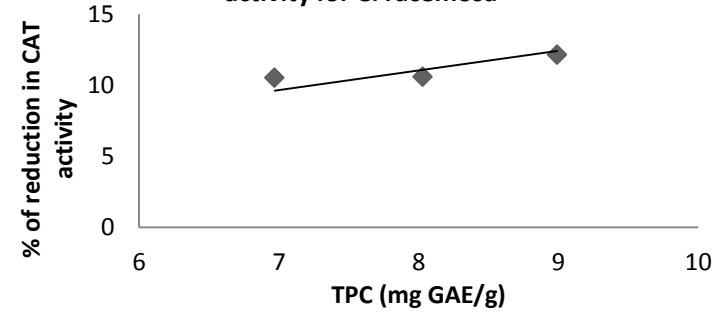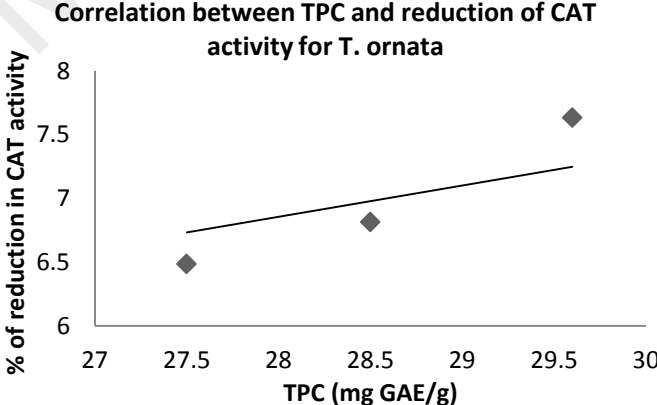
Samples	Pearson correlation coefficient (r)	Correlation between TPC and the reduction in catalase (CAT) activity
P. tetrastromatica	0.93*	
C. racemosa	0.87*	

Table 4.21, continued. Correlation between TPC and the reduction of the catalase (CAT) activity of the seaweeds,*P. tetrastromatica*, *C. racemosa* and *T. ornata*

Samples	Pearson correlation coefficient (r)	Correlation between TPC and catalase (CAT) activity
T. ornata	0.98*	

Correlation between TPC and reduction of CAT activity for T. ornata

Each value is expressed as mean ± SD (n = 3).
* denotes significance at p < 0.01.

Table 4.22: Correlation between TPC and the reduction of the catalase (CAT) activity of the sponge, *S. vagabunda*

Samples	Pearson correlation coefficient (r)	Correlation between TPC and the reduction in catalase (CAT) activity
S. vagabunda	0.97	

Correlation between TPC and reduction of CAT activity for S. vagabunda

Each value is expressed as mean ± SD (n = 3).
* denotes significance at p < 0.01.

The Pearson correlation coefficients for the correlation between TPC and the reduction of GR activities of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* was 0.94, 0.99, 0.99 and 0.91, respectively (Tables 4.23 and 4.24).

Table 4.23: Correlation between TPC and the reduction of the glutathione reductase (GR) activity of the seaweeds, *P. tetrastromatica***,** *C. racemosa* **and** *T. ornata*
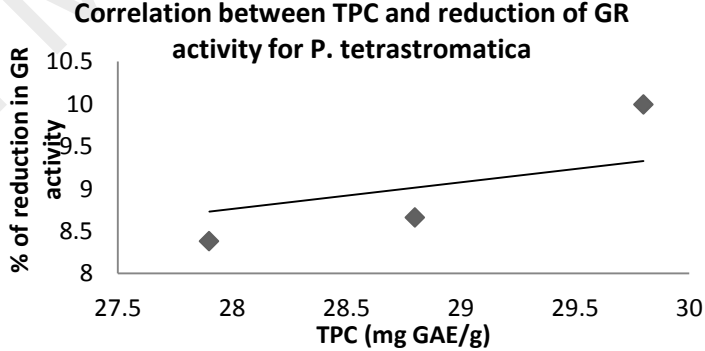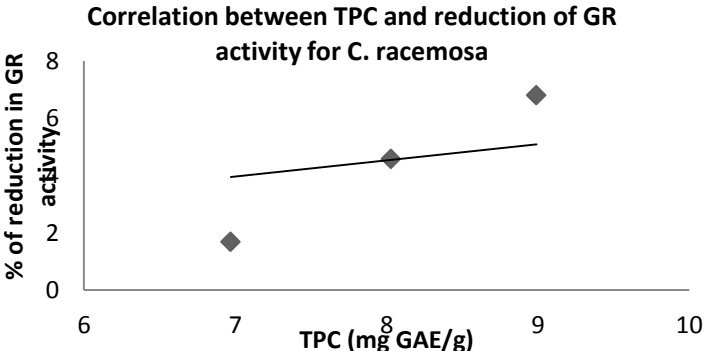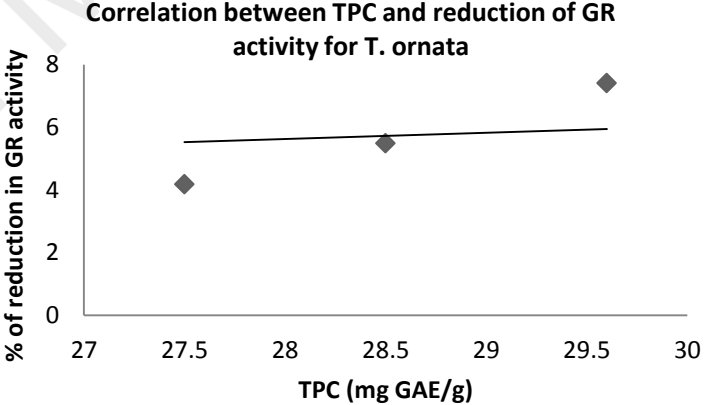
Samples	Pearson correlation coefficient (r)	Correlation between TPC and the reduction in glutathione reductase (GR) activity
P. tetrastromatica	0.94*	
C. racemosa	0.99	

Correlation between TPC and reduction of GR activity for P. tetrastromatica

Correlation between TPC and reduction of GR activity for C. racemosa
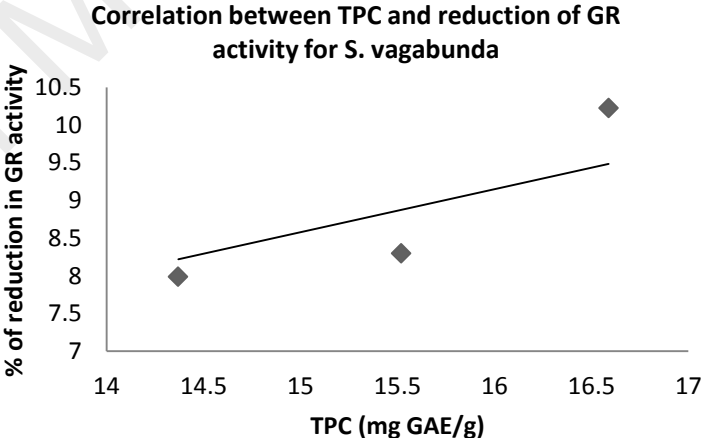
Table 4.23, continued. Correlation between TPC and the reduction of the glutathione reductase (GR) activity of the seaweeds, *P. tetrastromatica*, *C. racemosa* **and** *T. ornata*

Samples	Pearson correlation coefficient (r)	Correlation between TPC and the reduction in glutathione reductase (GR) activity
T. ornata	0.99*	

Correlation between TPC and reduction of GR activity for T. ornata

Each value is expressed as mean ± SD (n = 3).
* denotes significance at p < 0.01.

Table 4.24: Correlation between TPC and the reduction of the glutathione reductase (GR) activity of the sponge,*S. vagabunda*

Samples	Pearson correlation coefficient (r)	Correlation between TPC and the reduction in glutathione reductase (GR) activity
S. vagabunda	0.91*	

Correlation between TPC and reduction of GR activity for S. vagabunda

Each value is expressed as mean ± SD (n = 3).
* denotes significance at p < 0.01.

4.7   Determination of Antiproliferative Activity of Partially Purified Fractions

From the results obtained as shown in Tables 4.1 and 4.2, the most potent crude extracts (hexane extracts of *P. tetrastromatica* and *S. vagabunda* and the methanolic extracts of *C. racemosa* and *T. ornata*) were selected for partial purification through column chromatography. The partially purified fractions obtained from the column chromatographic separation were then subjected to the MTT assay. The antiproliferative activity of the partially purified fractions of the seaweed and sponge samples in MCF-7 cells increased significantly (Tables 4.25 and 4.26).

Table 4.25: Antiproliferative activity of column chromatographic pooled fractions of the seaweeds,*P. tetrastromatica***, *C. racemosa* and *T. ornata***

Samples	Pooled Fractions	IC$_{50}$ (µg/ml)
	P1	13.0 ± 1.68
	P2	12.0 ± 2.01
P. tetrastromatica		
	P3	15.0 ± 1.73
C. racemosa	P1	18.0 ± 1.43
	P1	12.0 ± 2.35
T. ornata		
	P2	22.0 ± 1.96

Antiproliferative activity is expressed as IC$_{50}$ (in µg/ml) $\pm$ SD (n = 3), which is the concentration of extract at which 50% of cell growth was inhibited relative to cells incubated in the presence of 0.1% DMSO vehicle control.
ND denotes that IC$_{50}$ values were not detected.

Table 4.26: Antiproliferative activity of column chromatographic pooled fractions of the sponge,*S. vagabunda*

Samples	Pooled Fractions	IC_{50} (µg/ml)
	P1	ND
	P2	12.0 ± 1.79
S. vagabunda	P3	14.0 ± 2.14
	P4	20.0 ± 1.42

Antiproliferative activity is expressed as IC_{50} (in µg/ml) $\pm$ SD (n = 3), which is the concentration of extract at which 50% of cell growth was inhibited relative to cells incubated in the presence of 0.1% DMSO vehicle control.
ND denotes that IC_{50} values were not detected.

Fractions with the same retention factor (R_f) values were pooled together as one pooled fraction. Through column chromatography, a total of three, one and two pooled fractions were collected for *P. tetrastromatica*, *C. racemosa* and *T. ornata*, respectively. As summarised in Table 4.25, the P2 fraction of *P. tetrastromatica* and the P1 fraction of *T. ornata* showed the highest antiproliferative activity among the three seaweeds with $IC_{50}$of 12.0 µg/ml. The single pooled fraction of *C. racemosa* (P1) showed an IC_{50} of 18.0 ± 1.43 µg/ml.

The column chromatographic separation of the hexane crude extract of *S. vagabunda* resulted in a total of four pooled fractions; whereby no IC_{50} was detected in the P1 fraction while the highest antiproliferative activity was shown in fraction P2 (IC_{50} of 12.0 ± 1.79 µg/ml) (Table 4.26). By comparison, *S. vagabunda* showed similar antiproliferative activity with the brown seaweeds (*P. tetrastromatica* and *T. ornata*) in this study.

No cytotoxicity was observed in the fractions-treated, non-tumourigenic 184B5 breast cells. The column chromatographic separation of the crude extracts of the seaweeds and sponge therefore yielded purer compounds which resulted in more potent antiproliferative activity.

4.8   Liquid Chromatography-Mass Spectrometry (LC-MS) Profiling

Following determination of antiproliferative activity, the pooled fractions P2, P1, P1 and P2 for *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda*, respectively, were subjected to bioassay guided fractionation through liquid chromatography-mass spectrometry (LC-MS). Analysis of the mass spectral data for peaks at m/z 371 revealed the presence of camptothecin eluted at 26.5 min. Pseudopelletierine and lycodine eluting at 12.7 and 20.5 min, respectively show fragments at m/z 171 and 277, respectively (Figures 4.10 – 4.16). The compounds detected are summarised in (Tables 4.27 and 4.28).
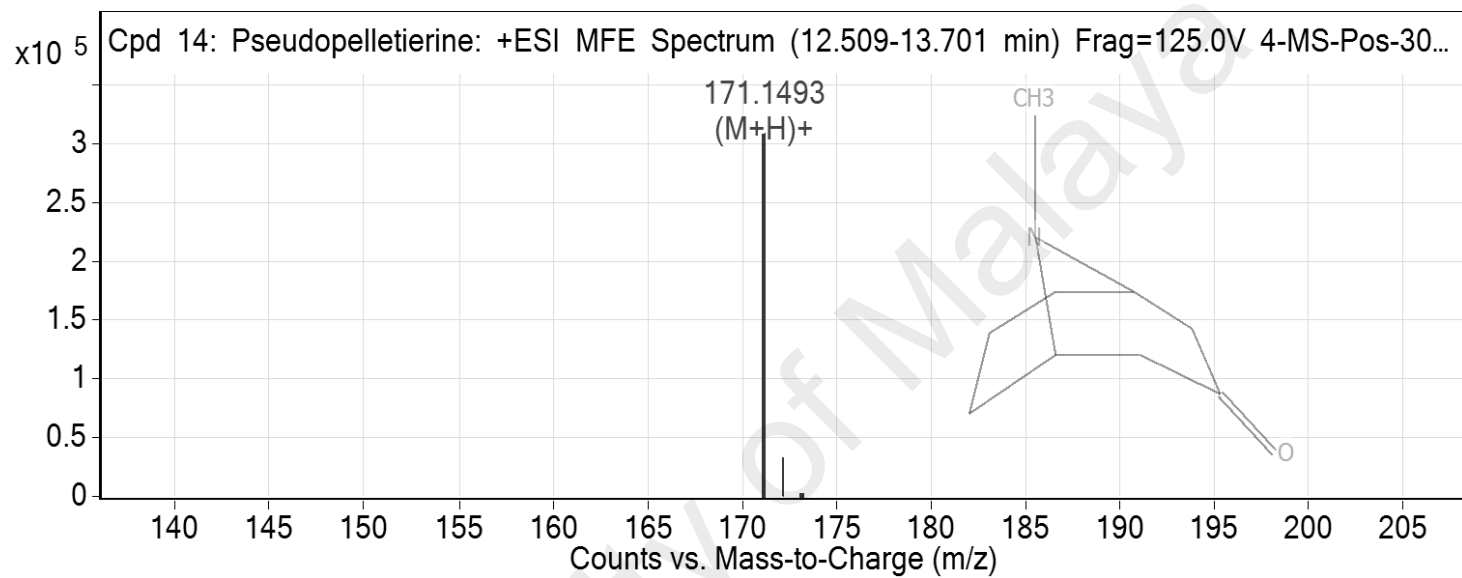
Figure 4.10: MS spectra of protonated molecule (Pseudopelletierine)isolated from *P. tetrastromatica*. Pseudopelletierine at [M+H]+ of m/z 171.
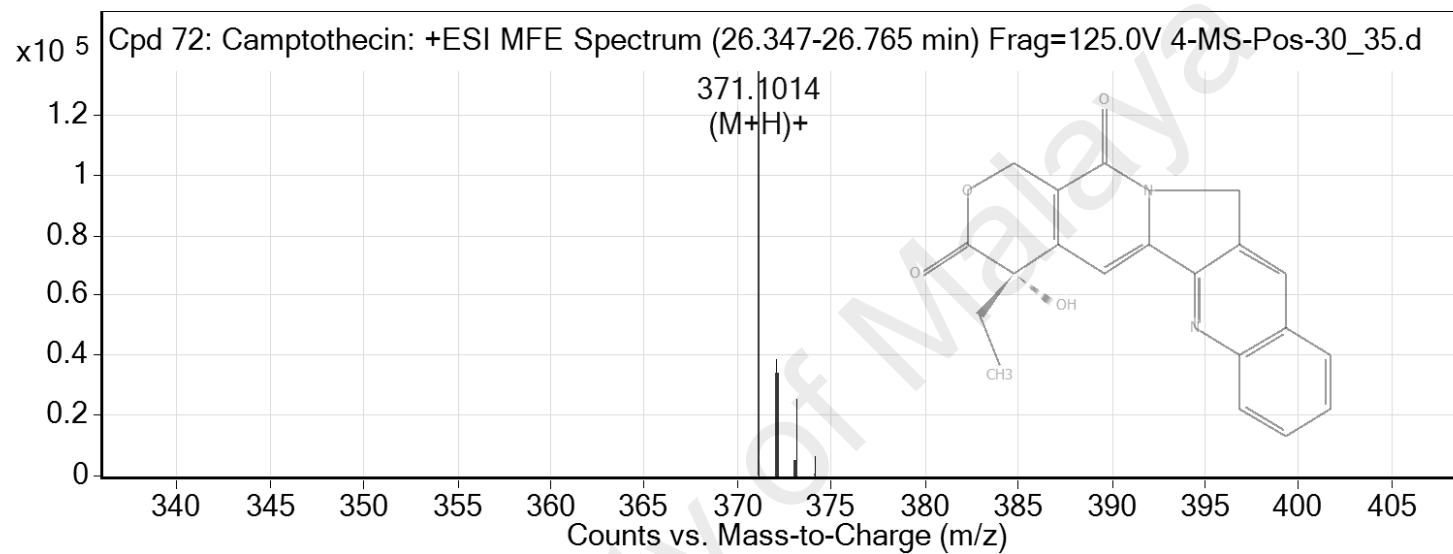
Figure 4.11: MS spectra of protonated molecule (Camptothecin) isolated from *P. tetrastromatica*. Camptothecin at [M+H]+ of m/z 371.
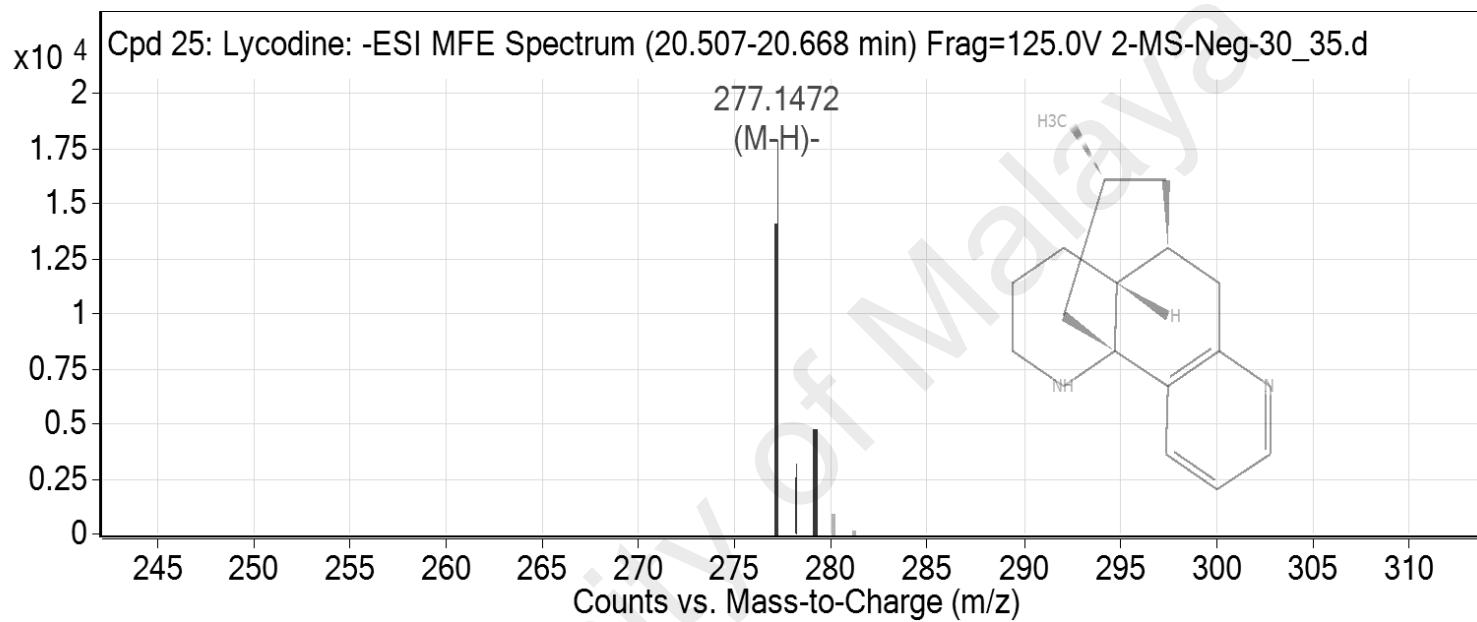
Figure 4.12: MS spectra of deprotonated molecule (Lycodine) isolated from *C. racemosa*. Lycodine at [M-H]- of m/z 277.
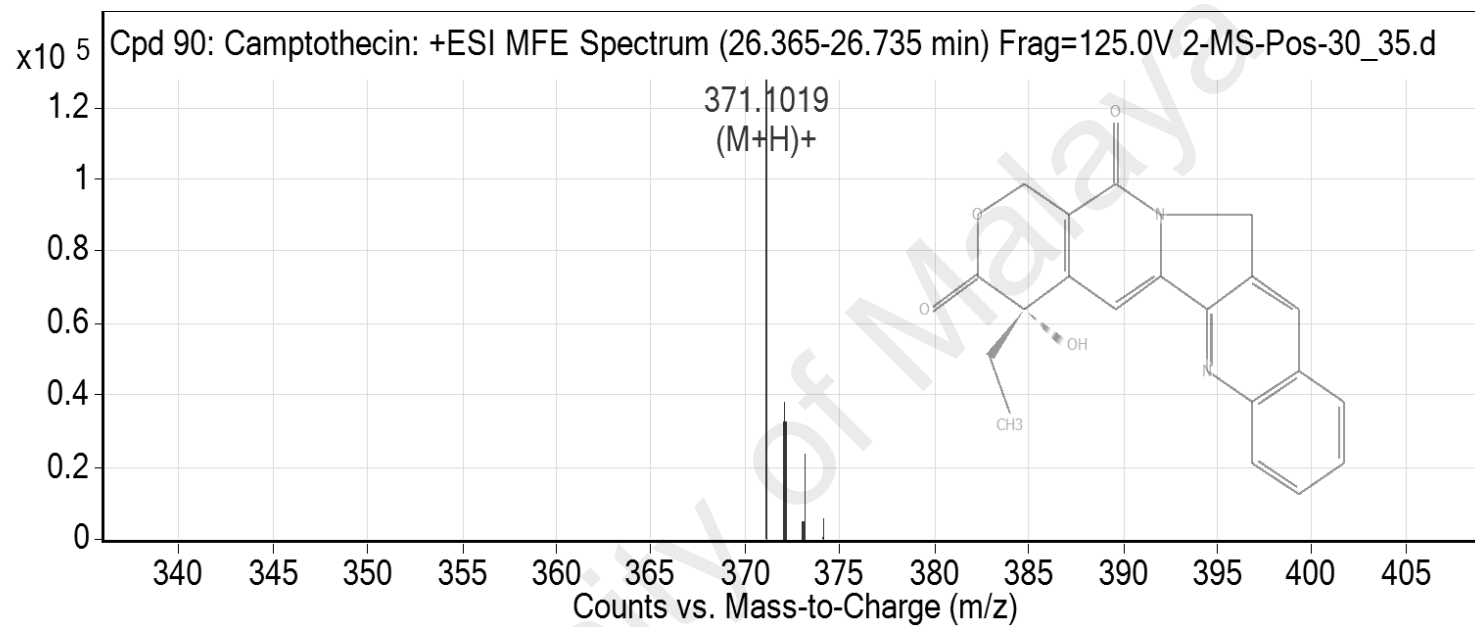
Figure 4.13: MS spectra of protonated molecule (Camptothecin) isolated from *C. racemosa*. Camptothecin at [M+H]+ of m/z 371.
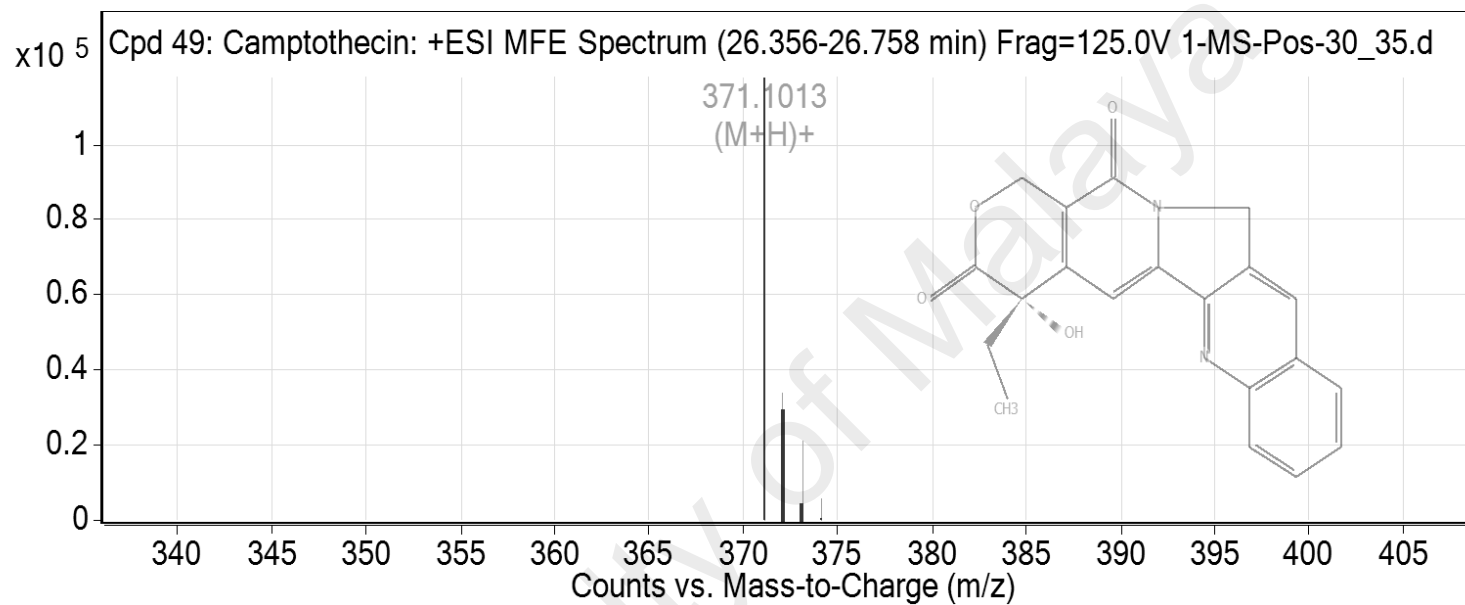
Figure 4.14: MS spectra of protonated molecule (Camptothecin) isolated from *T. ornata*. Camptothecin at [M+H]+ of m/z 371.
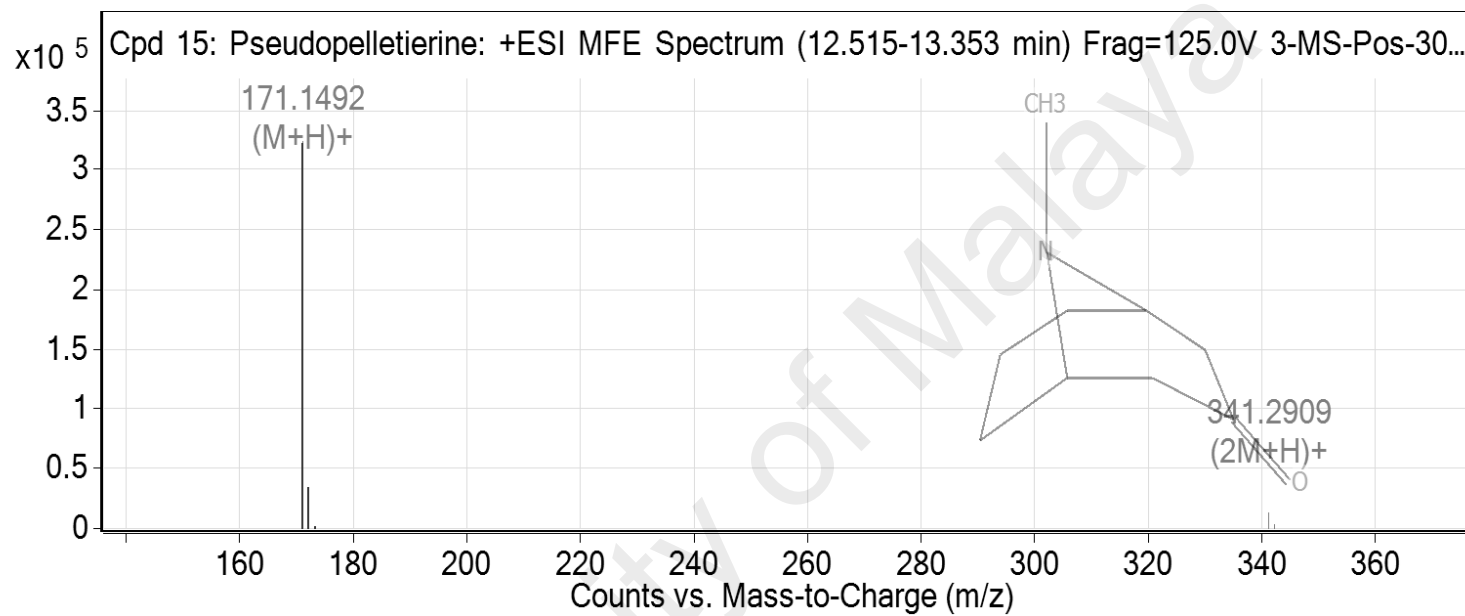
Figure 4.15: MS spectra of protonated molecule (Pseudopelletierine)isolated from *S. vagabunda*. Pseudopelletierine at [M+H]+ of m/z 171.
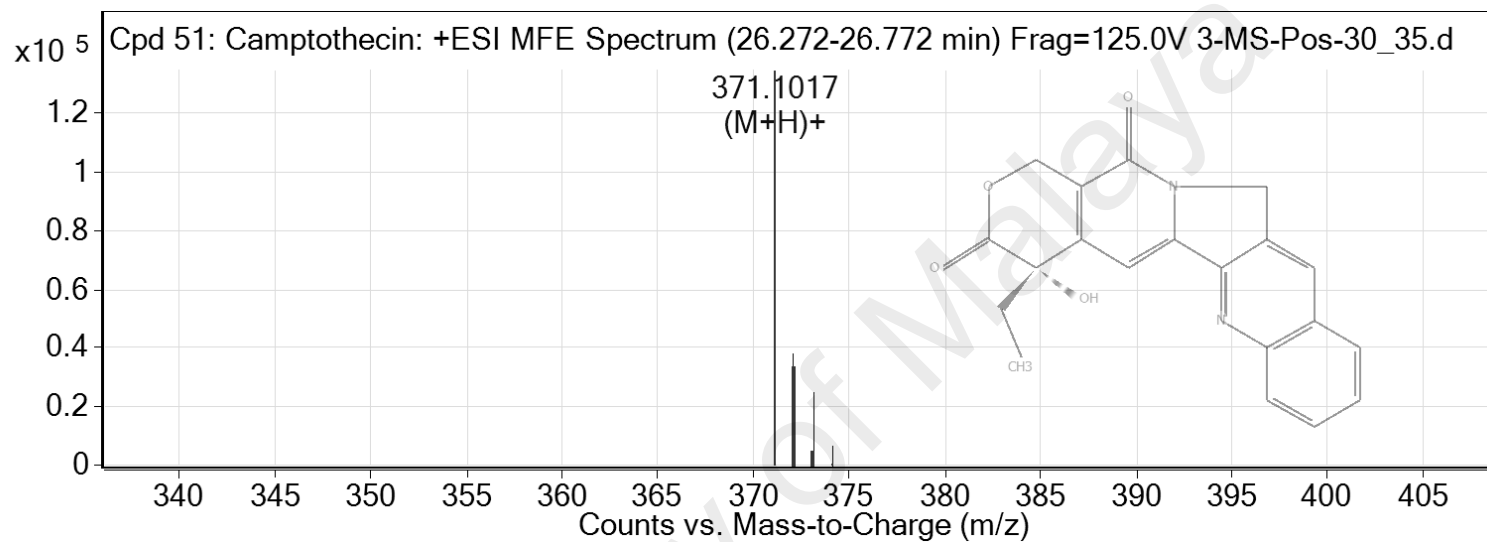
Figure 4.16: MS spectra of protonated molecule (Camptothecin)isolated from *S. vagabunda*. Camptothecin at [M+H]+ of m/z 371.

Table 4.27: Compounds identified by LC-MS in the seaweeds, *P.tetrastromatica*, *C. racemosa* and *T. ornata*

Samples	Compounds	Mass-to-charge ratio (m/z)	Retention time (min)	Volume percentage (%)
P. tetrastromatica	Camptothecin	371.1019	26.51	0.32
	Pseudopelletierine	171.1493	12.71	0.99
C. racemosa	Camptothecin	371.1019	26.51	0.42
	Lycodine	277.1472	20.57	0.61
T. ornata	Camptothecin	371.1013	26.51	0.48

Table 4.28: Compounds identified by LC-MS in the sponge, *S. vagabunda*

Samples	Compounds	Mass-to-charge ratio (m/z)	Retention time (min)	Volume percentage (%)
S. vagabunda	Camptothecin	371.1017	26.50	0.48
	Pseudopelletierine	171.1492	12.71	1.55

4.9 Evaluation of Antiproliferative Activity of Pure Compounds

Based on bioassay guided fractionation of the partially purified column chromatographic fractions, three pure compounds responsible for the cytotoxic activity were identified namely camptothecin, pseudopelletierine and lycodine. The MTT assay was conducted on these compounds to evaluate their antiproliferative effects in the tumorigenic MCF-7 and the non-tumourigenic 184B5 cells. No cytotoxicity was observed in the compounds-treated 184B5 cells. Figure 4.17 shows the antiproliferative effects of camptothecin, pseudopelletierine and lycodine in MCF-7 cells.
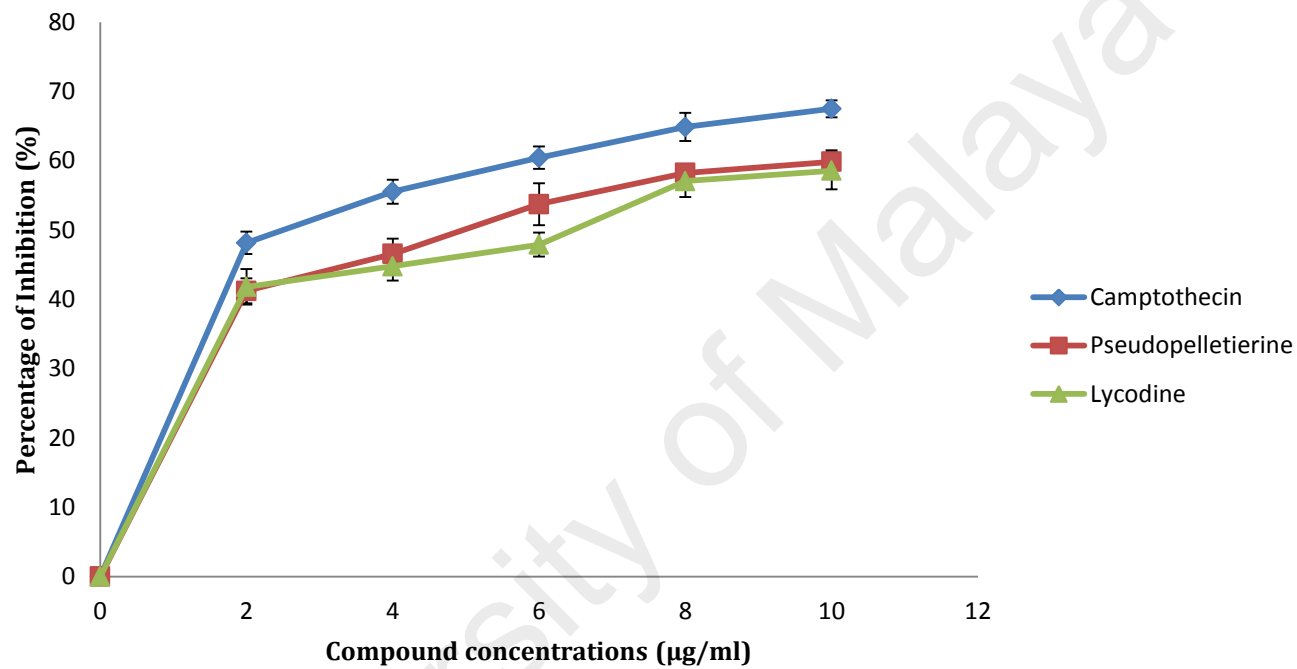
Figure 4.17: Antiproliferative activities of camptothecin, pseudopelletierine and lycodine on MCF-7 cells. Antiproliferative activity is expressed as IC_{50} (in μg/ml) $\pm$ SD (n = 3), which is the concentration of extract at which 50% of cell growth was inhibited relative to cells incubated in the presence of 0.1% DMSO vehicle control.Significance levels were determined using an unpaired, two-tailed Student's t-test, whereby ($p < 0.05$).

4.10   Cellular Mechanisms Related to Decreased Cancer Cell Viability

Following compound identification, the following studies were performed on the partially purified column chromatographic fractions and pure compounds: analysis of mitochondrial membrane potential (MMP), determination of caspase activities and DNA fragmentation analysis.

4.10.1   Partially Purified Fractions and Compounds-Induced Alterations in MMP

JC-1 can selectively enter the mitochondria and reversibly change colour from green to red as the MMP increases. As shown in Figure 4.18(a), untreated MCF-7 cells (negative control) showed only red fluorescence, indicating healthy cells with high MMP in which JC-1 exists in aggregates. Treatment with the partially purified fractions of the three seaweeds decreased the MMP, thus, enabling JC-1 to remain in the monomeric form which showed only green fluorescence [Figures 4.18(b) – (d)].
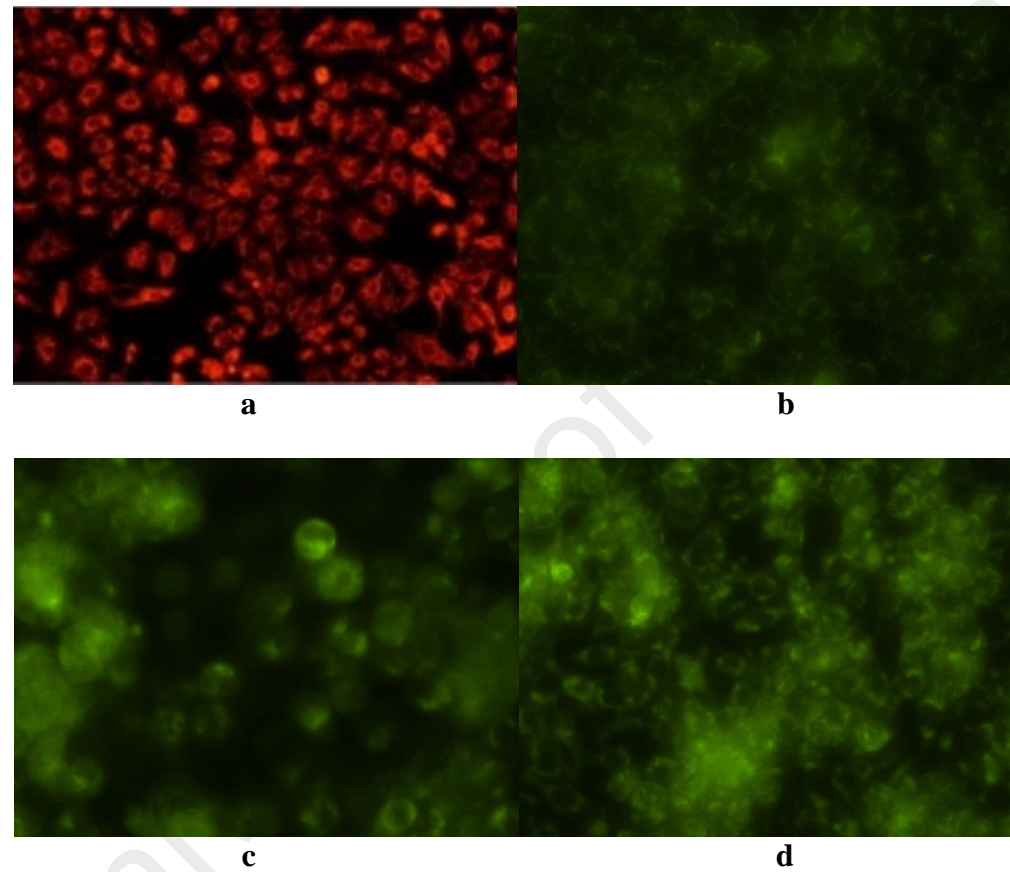
Figure 4.18: Representative image of MCF-7 cells treated with column chromatographic fractions of the seaweeds, _P. tetrastromatica_, _C. racemosa_ and _T. ornata_(a) 0.1% DMSO vehicle control, (b) 12 µg/ml of partially purified _P. tetrastromatica_ column chromatographic fraction, (c) 18 µg/ml of partially purified _C. racemosa_ column chromatographic fraction and (d) 12 µg/ml of partially purified _T. ornata_ column chromatographic fraction. Cells were stained with JC-1 stain as described in section 3.9.1 of Chapter 3.

Figures 4.19 – 4.21 show that the number of apoptotic cells increased by at least 1.04, 1.10 and 1.10 folds compared to untreated control cells upon treatment with 10 µg/ml of *P. tetrastromatica*, *C. racemosa* and *T. ornata* fractions, respectively while treatment with 50 µg/ml of the fractions increased the number of apoptotic cells by 1.56, 1.30 and 1.74 folds, respectively. Hence, it is postulated that the partially purified fraction of *T. ornata* is the most potent in inducing apoptosis in MCF-7 cells among the three seaweeds tested (Figure 4.21).
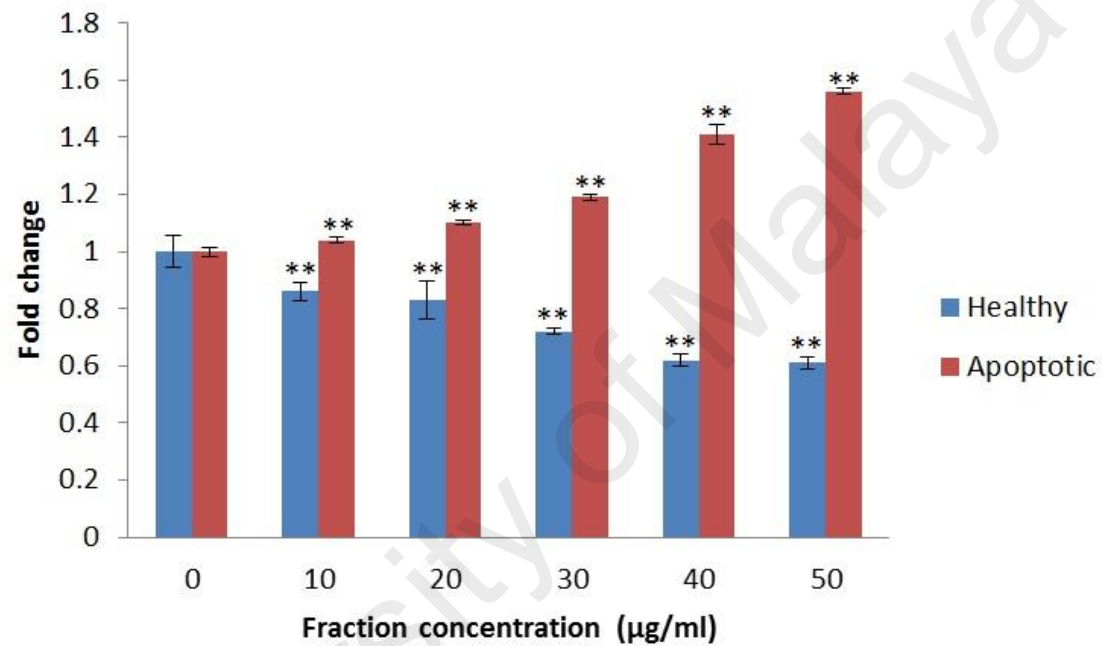
Figure 4.19: Analysis of MMP of MCF-7 cells treated with the column chromatographic fraction of *P. tetrastromatica*. Histogram showing the average fluorescent intensities for MMP expressed in fold change relative to untreated MCF-7 cells. The histogram represents MMP of MCF-7 cells treated with $10-50$ μg/ml of *P. tetrastromatica*. Data are shown as mean $\pm$ SD (*$p < 0.05$; **$p < 0.01$).
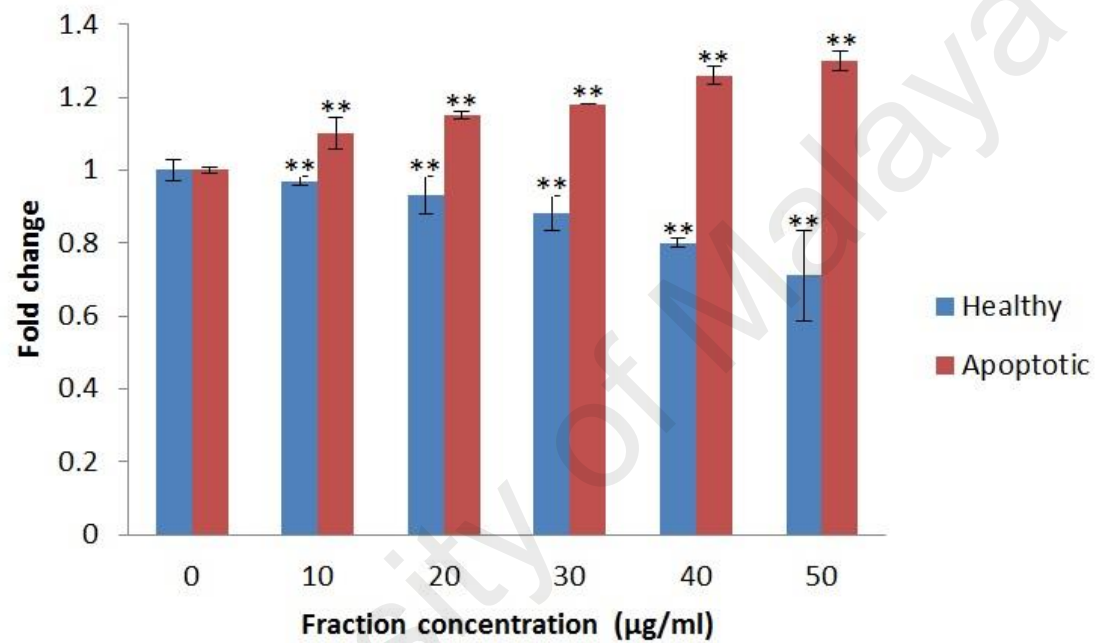
Figure 4.20: Analysis of MMP of MCF-7 cells treated with the column chromatographic fraction of *C. racemosa*. Histogram showing the average fluorescent intensities for MMP expressed in fold change relative to untreated MCF-7 cells. The histogram represents MMP of MCF-7 cells treated with 10 – 50 µg/ml of *C. racemosa*. Data are shown as mean ± SD (*$p < 0.05$; **$p < 0.01$).
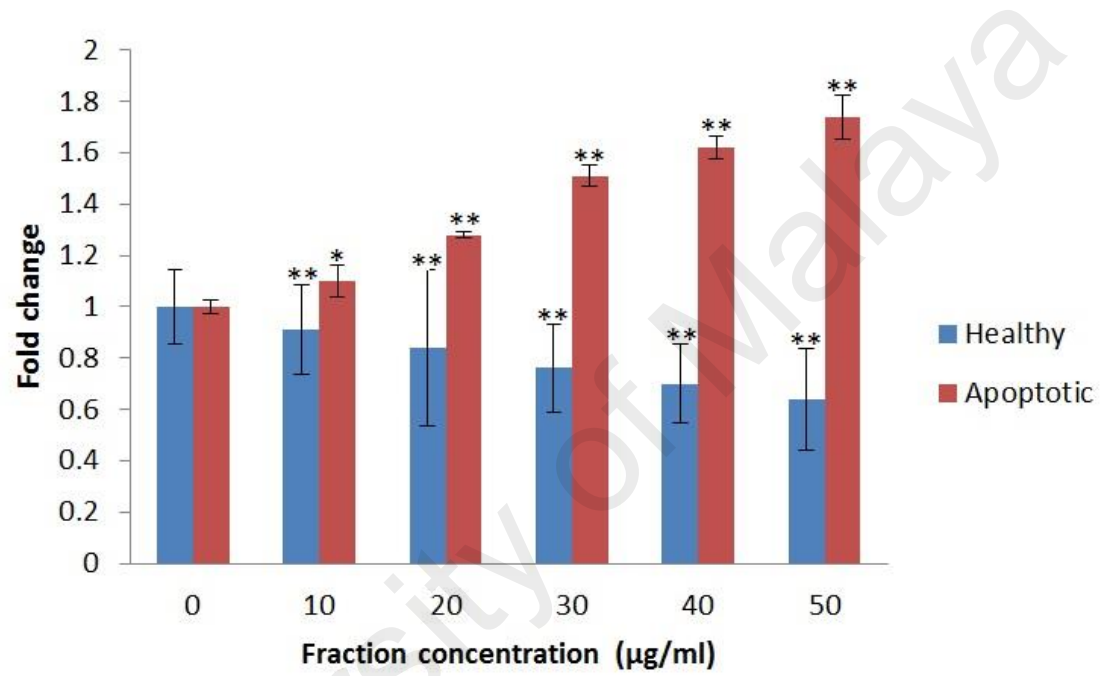
Figure 4.21: Analysis of MMP of MCF-7 cells treated with the column chromatographic fraction of *T. ornata*. Histogram showing the average fluorescent intensities for MMP expressed in fold change relative to untreated MCF-7 cells. The histogram represents MMP of MCF-7 cells treated with 10 – 50 μg/ml of *T. ornata*. Data are shown as mean ± SD (*$p < 0.05$; **$p < 0.01$).

Figures 4.22(b) shows the existence of green fluorescence, indicating a collapse in MMP upon treatment of MCF-7 cells with the partially purified fraction of *S. vagabunda*.

a   b
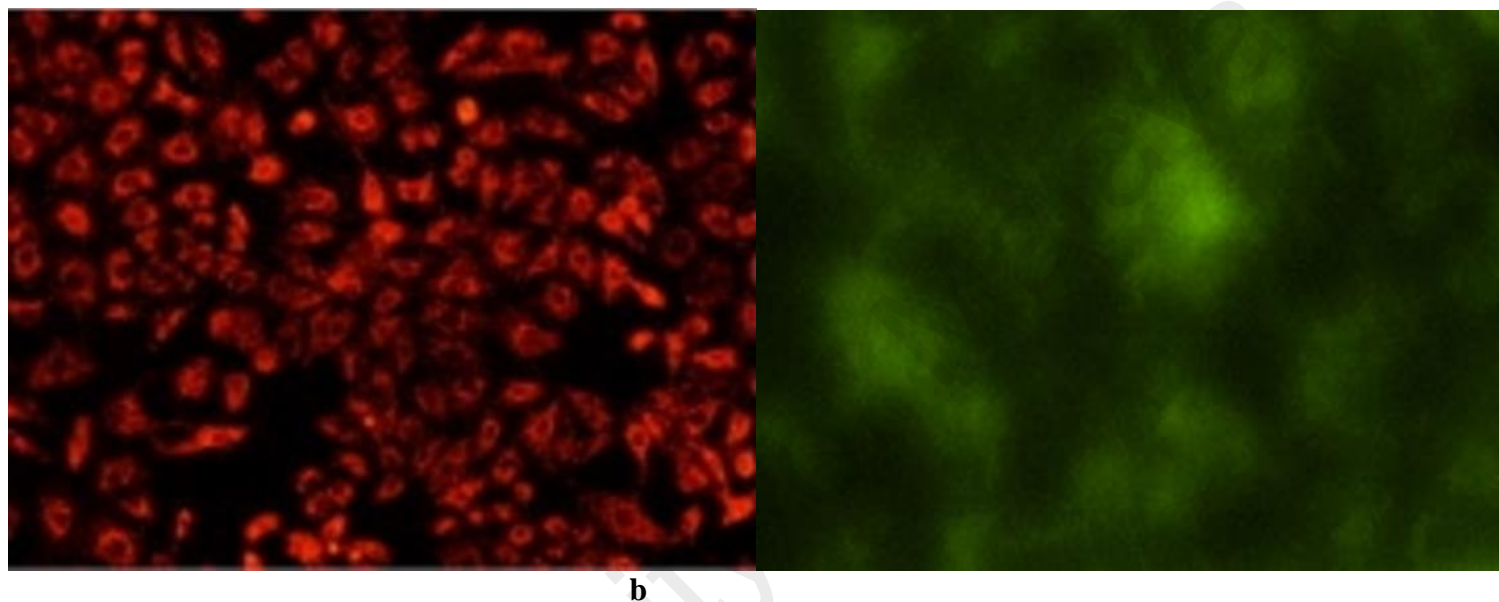
Figure 4.22: Representative image of MCF-7 cells treated with column chromatographic fraction of the sponge, *S. vagabunda* (a) 0.1% DMSO vehicle control and (b) 12 µg/ml of partially purified *S. vagabunda* column chromatographic fraction. Cells were stained with JC-1 stain as described in section 3.9.1 of Chapter 3.

The number of apoptotic cells increased by 1.08 folds compared to untreated control cells upon treatment with 10 µg/ml of the *S. vagabunda* fraction, while treatment with 50 µg/ml of the fractions increased the number of apoptotic cells by 1.56 folds (Figure 4.23). After treatment with 50 µg/ml of fractions, *S. vagabunda* had a higher potency (comparable to the brown seaweeds, *P. tetrastromatica* and *T. ornata*) in inducing apoptosis in MCF-7 cells, as compared to the green seaweed, *C. racemosa*.
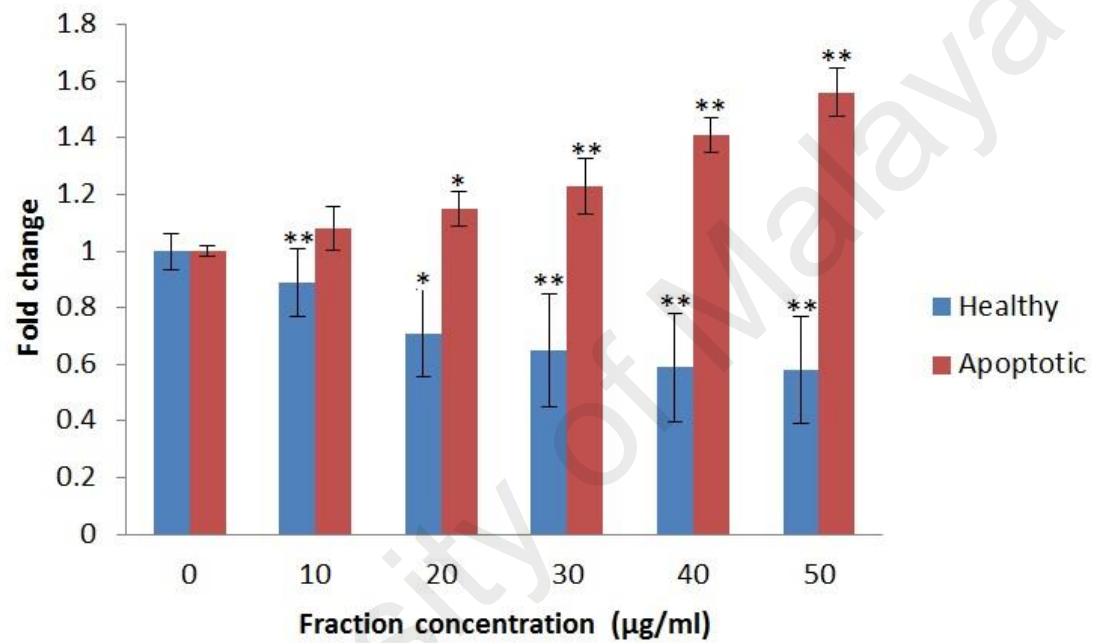
Figure 4.23: Analysis of MMP of MCF-7 cells treated with the column chromatographic fraction of the sponge, *S. vagabunda*. Histogram showing the average fluorescent intensities for MMP expressed in fold change relative to untreated MCF-7 cells and represents MMP of MCF-7 cells treated with 10 – 50 µg/ml of *S. vagabunda*. Data are shown as mean ± SD (*$p < 0.05$; **$p < 0.01$).

On the other hand, figures 4.24 (b) – (d) show a collapse in MMP (indicated by the appearance of green fluorescence) after treatment with the pure compounds, camptothecin, pseudopelletierine and lycodine.
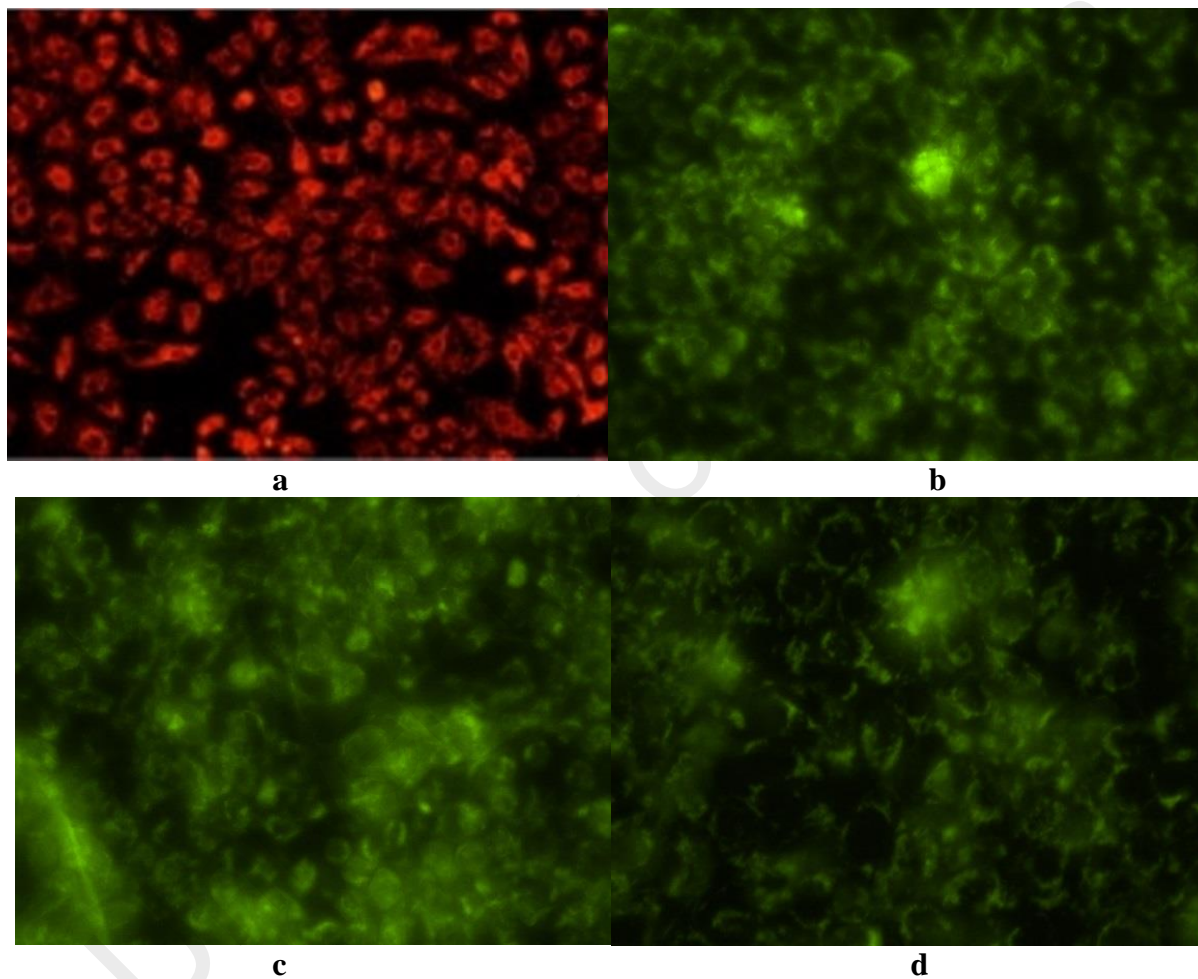
Figure 4.24: Representative image of MCF-7 cells treated with the pure compounds (a) 0.1% DMSO vehicle control, (b) 1 µM of camptothecin, (c) 1 µM of pseudopelletierine and (d) 1 µM of lycodine. Cells were stained with JC-1 stain as described in section 3.9.1 of Chapter 3.

Treatment with 10 µg/ml of camptothecin, pseudopelletierine and lycodine increased the number of apoptotic cells by at least 1.34, 1.08 and 1.17 folds, respectively, compared to untreated negative control. Treatment with 50 µg/ml of the compounds increased the number of apoptotic cells by 1.94, 1.57 and 1.46 folds, respectively (Figures 4.25 – 4.27).
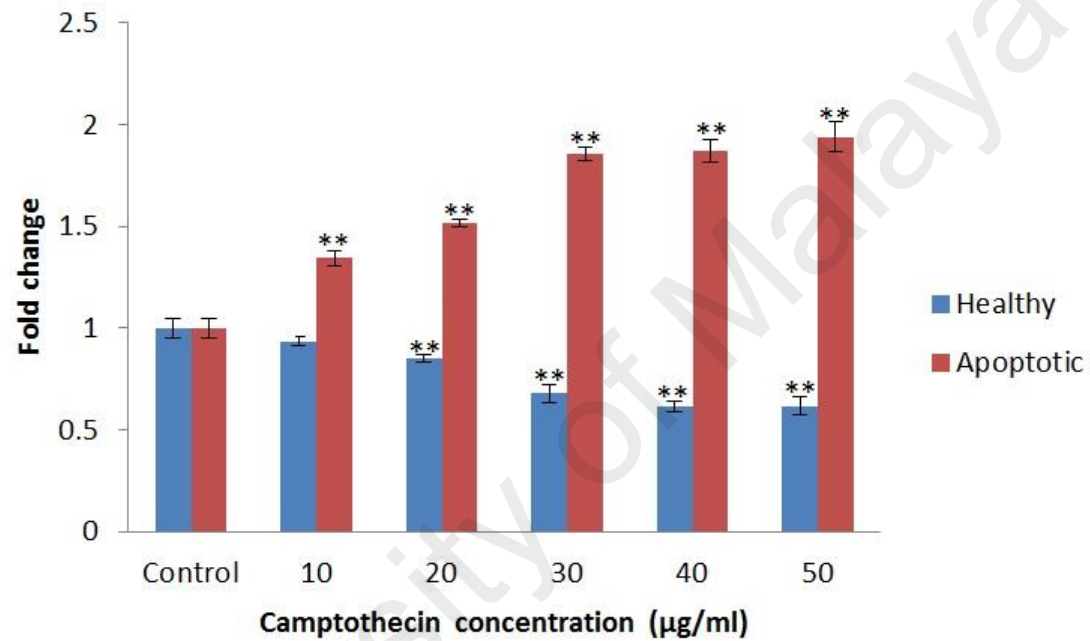
Figure 4.25: Analysis of MMP of MCF-7 cells treated with camptothecin. Histogram showing the average fluorescent intensities for MMP expressed in fold change relative to untreated MCF-7 cells. The histogram represents MMP of MCF-7 cells treated with $10 - 50$ µg/ml of camptothecin. Data are shown as mean ± SD (*$p < 0.05$; **$p < 0.01$).
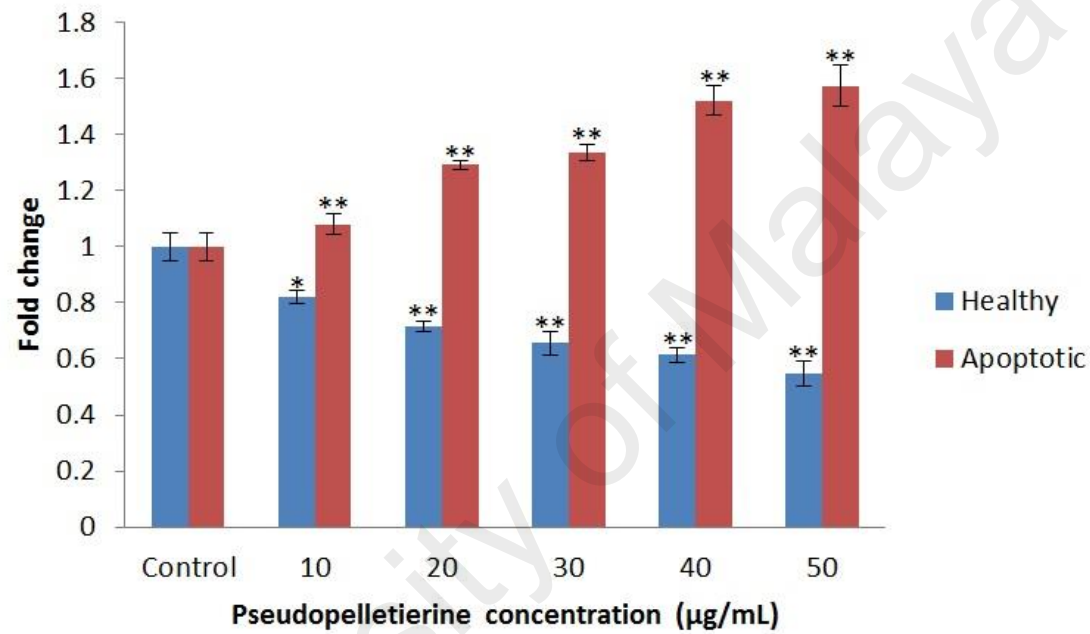
Figure 4.26: Analysis of MMP of MCF-7 cells treated with pseudopelletierine. Histogram showing the average fluorescent intensities for MMP expressed in fold change relative to untreated MCF-7 cells. The histogram represents MMP of MCF-7 cells treated with 10 – 50 µg/ml of pseudopelletierine. Data are shown as mean ± SD (*$p < 0.05$; **$p < 0.01$).
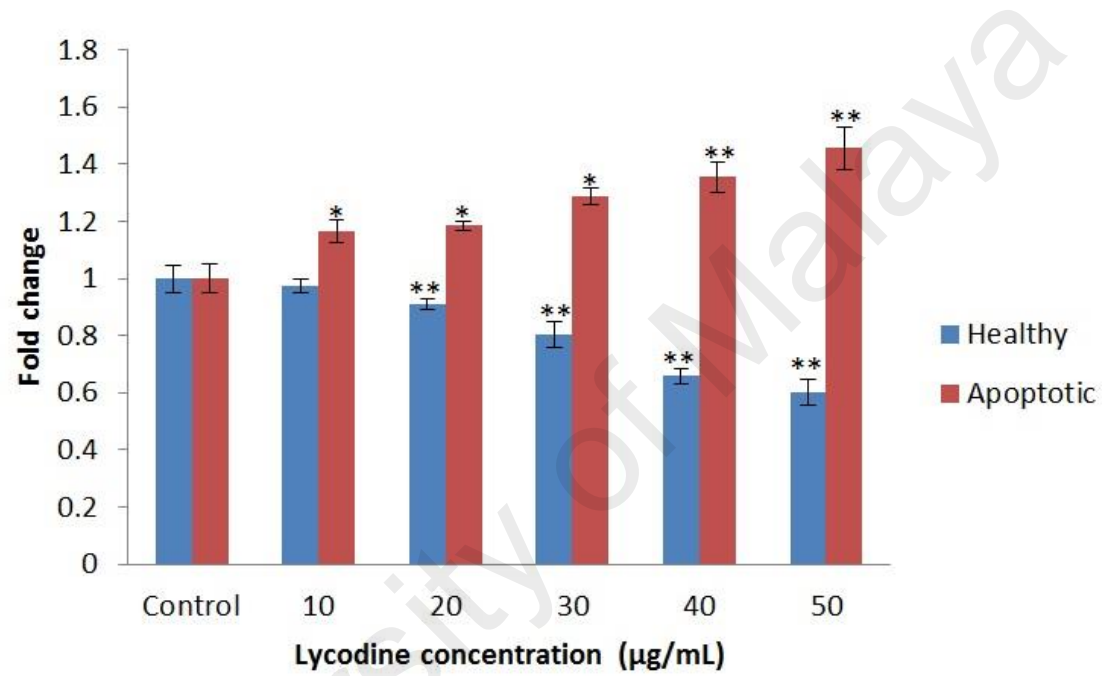
Figure 4.27: Analysis of MMP of MCF-7 cells treated with lycodine. Histogram showing the average fluorescent intensities for MMP expressed in fold change relative to untreated MCF-7 cells. The histogram represents MMP of MCF-7 cells treated with 10 – 50 µg/ml of lycodine. Data are shown as mean ± SD (*$p< 0.05$; **$p< 0.01$).

4.10.2 Determination of Caspase Activities

The caspase-8, -9 and -3 activity of the partially purified fractions of the seaweed and sponge samples as well as the pure compounds were measured at time points of 8, 16, 24 and 45 h, and expressed as percentages relative to the negative control (untreated cells). The caspase-8 activity of *P. tetrastromatica* increased and peaked at 16 h treatment (by 1.4 fold) and then decreased while caspase-8 activity of *C. racemosa* and *T. ornata* peaked at 8 h treatment (by 1.2 and 1.5 folds, respectively) and then decreased (Figure 4.28). For *S. vagabunda*, the caspase-8 peaked at 8 h treatment by 1.5 folds and then decreased (Figure 4.29). As shown in Figure 4.30, the caspase-8 activity of camptothecin, pseudopelletierine and lycodine-treated MCF-7 cells peaked at 24 h (by 5.1, 3.9 and 4.6 folds, respectively) relative to the negative control (untreated MCF-7).
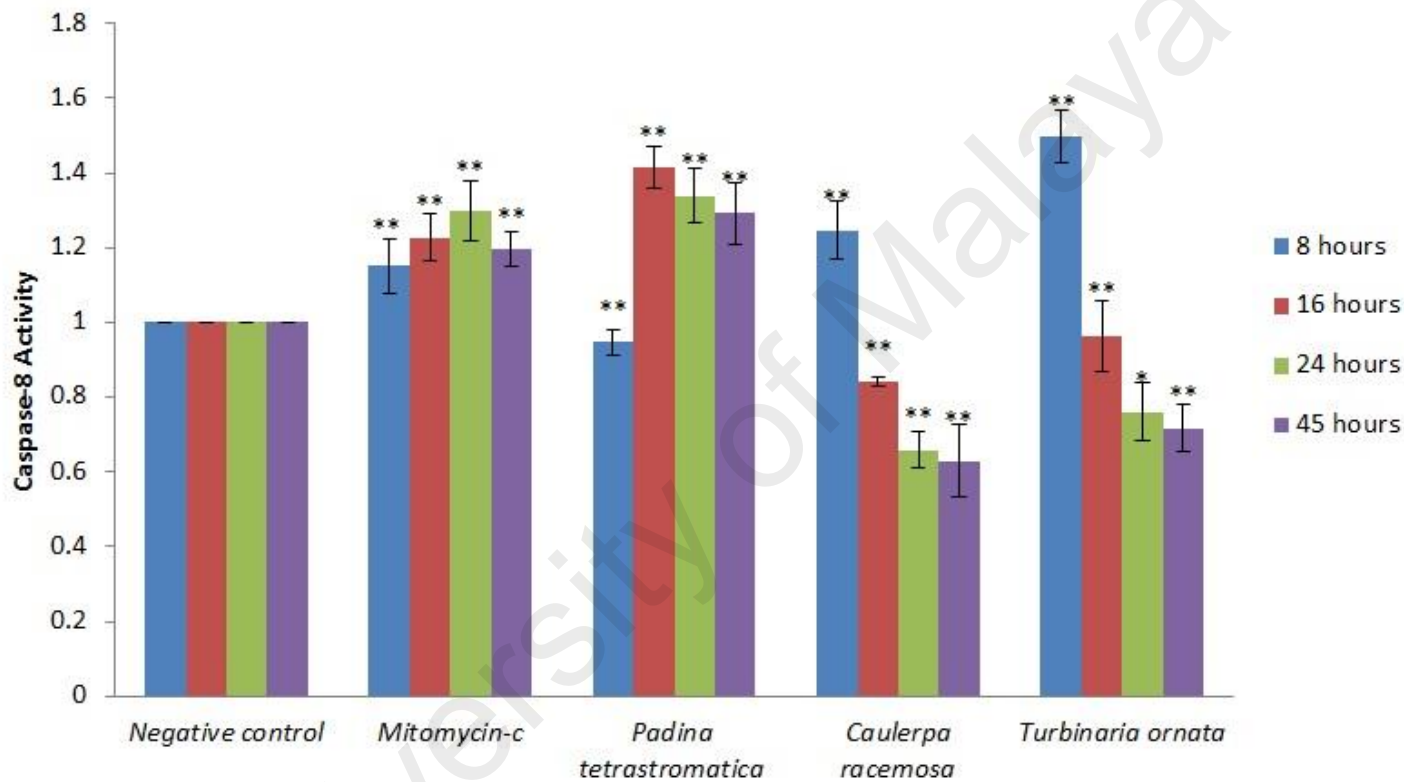
Figure 4.28: Caspase-8 activity of MCF-7 cells treated with the partially purified fractions of the seaweeds. Caspase-8 activity of MCF-7 cells treated with 12 μg/ml of partially purified *P. tetrastromatica* column chromatographic fraction, 18 μg/ml of partially purified *C. racemosa* column chromatographic fraction and 12 μg/ml of partially purified *T. ornata* column chromatographic fraction for 8, 16, 24 and 45 h (*$p < 0.05$; **$p < 0.01$). Mitomycin-c was used as a positive control for caspase-8 activity.
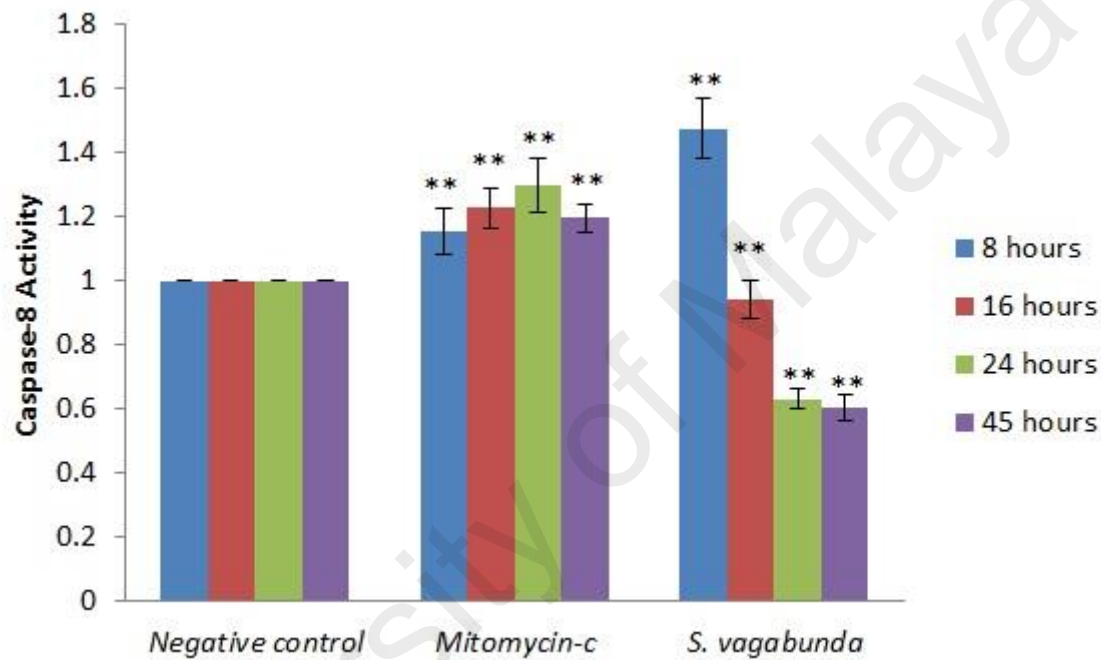
Figure 4.29: Caspase-8 activity of MCF-7 cells treated with the partially purified fractions of the sponge. Caspase-8 activity of MCF-7 cells treated with 12 μg/ml of partially purified *S. vagabunda* column chromatographic fraction for 8, 16, 24 and 45 h (*$p < 0.05$; **$p < 0.01$). Mitomycin-c was used as a positive control for caspase-3 activity.
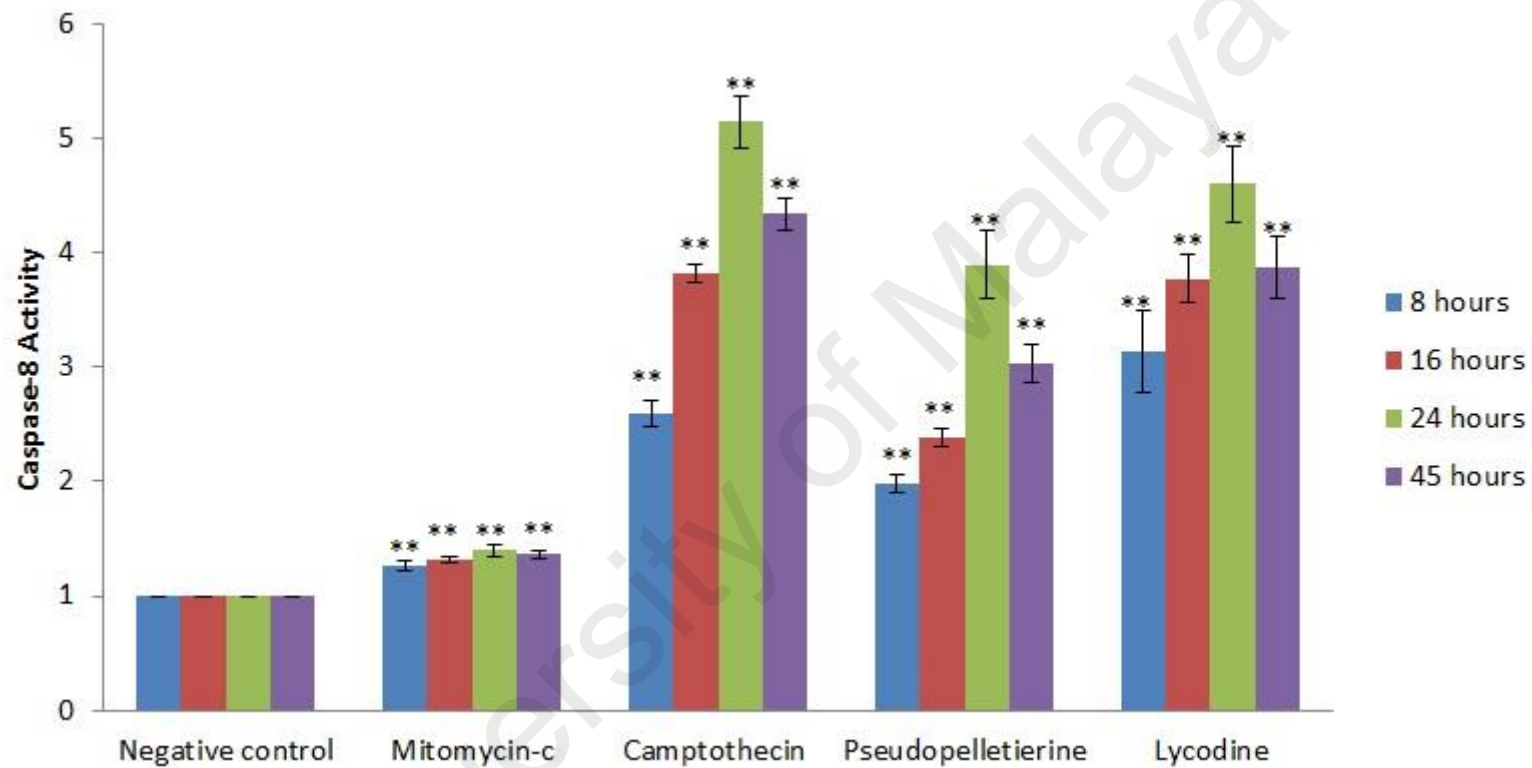
Figure 4.30: Caspase-8 activity of MCF-7 cells treated with the isolated pure compounds. Caspase-8 activity of MCF-7 cells treated with 1 µM of camptothecin, pseudopelletierine and lycodine for 8, 16, 24 and 45 h ($*p < 0.05$; $**p< 0.01$). Mitomycin-c was used as a positive control for caspase-8 activity.

Caspase-9 activity peaked at 8 h treatment (by 1.3, 1.2 and 1.3 folds, respectively) and then decreased in a time-dependent manner for *P. tetrastromatica*, *C. racemosa* and *T. ornata* as presented in Figure 4.31. The caspase-9 activity of *S. vagabunda* peaked at 8 h treatment by 2.0 folds and then decreased in a time-dependent manner as shown in Figure 4.32. The caspase-9 activity of camptothecin, pseudopelletierine and lycodine-treated MCF-7 cells peaked at 24 h by 6.5, 4.9 and 5.5 folds, respectively (Figure 4.33).
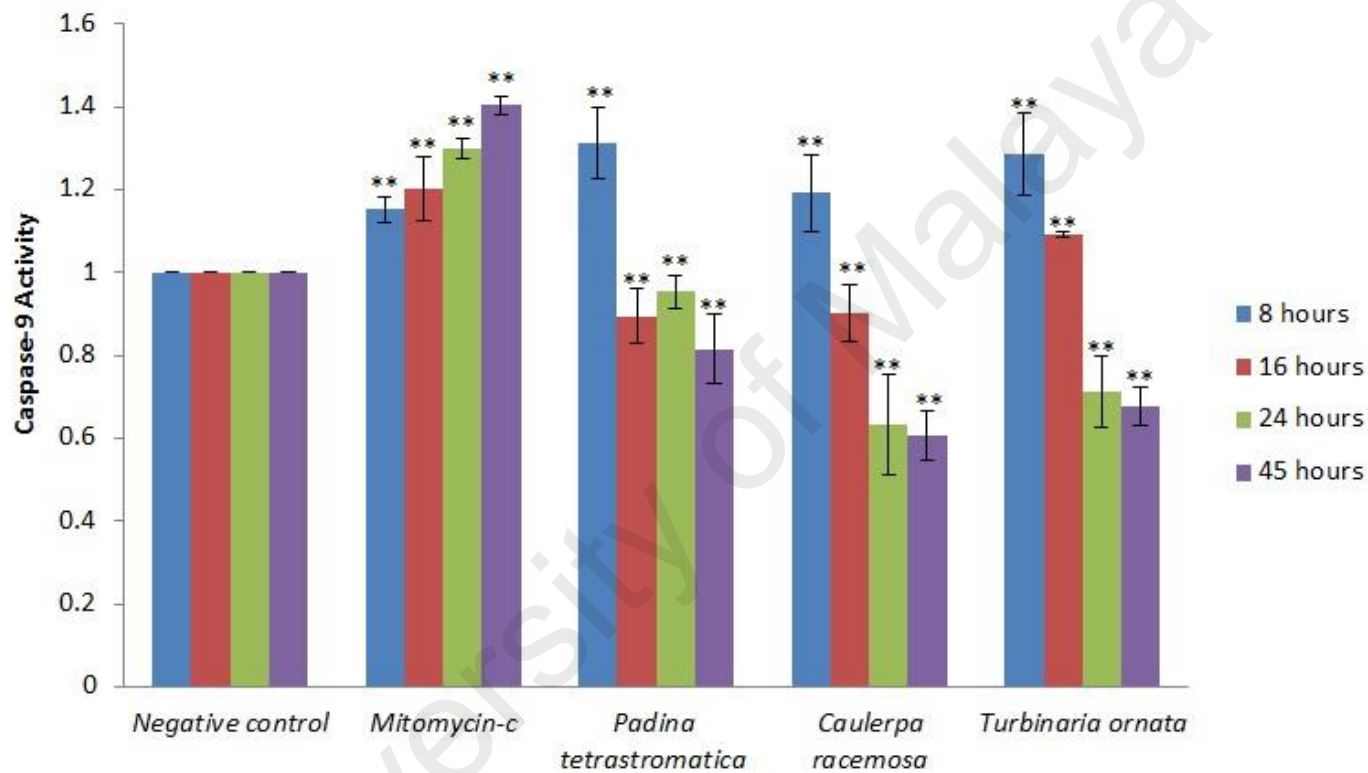
Figure 4.31: Caspase-9 activity of MCF-7 cells treated with the partially purified fractions of the seaweeds. Caspase-9 activity of MCF-7 cells treated with 12 μg/ml of partially purified *P. tetrastromatica* column chromatographic fraction, 18 μg/ml of partially purified *C. racemosa* column chromatographic fraction and 12 μg/ml of partially purified *T. ornata* column chromatographic fraction for 8, 16, 24 and 45 h (*$p < 0.05$; **$p < 0.01$). Mitomycin-c was used as a positive control for caspase-9 activity.
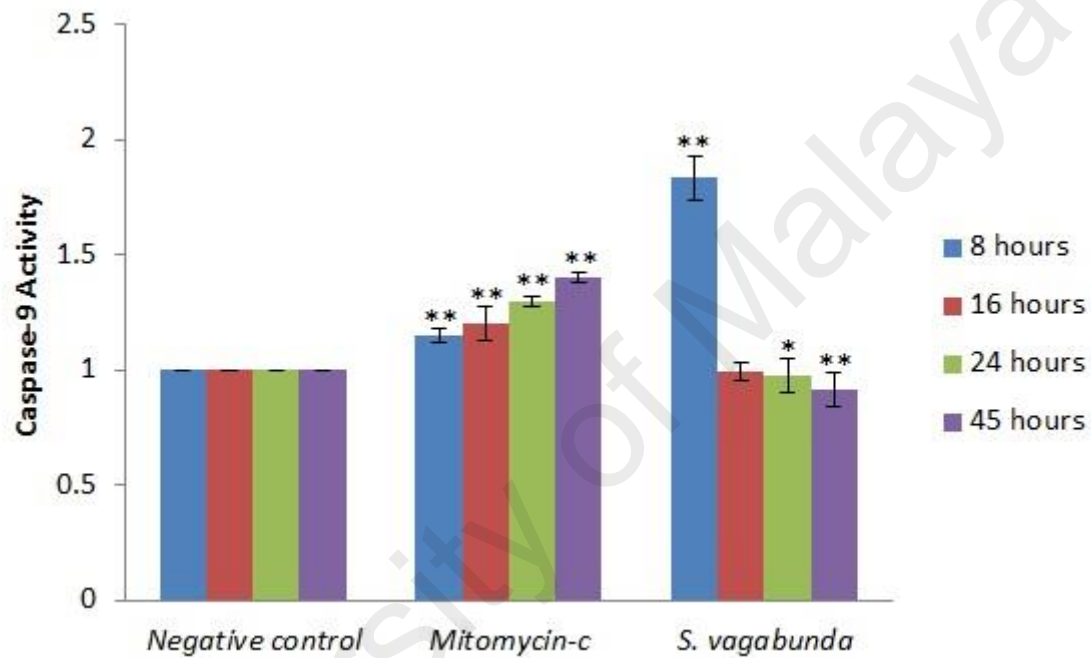
Figure 4.32: Caspase-9 activity of MCF-7 cells treated with the partially purified fractions of the sponge. Caspase-9 activity of MCF-7 cells treated with 12 μg/ml of partially purified *S. vagabunda* column chromatographic fraction for 8, 16, 24 and 45 h (*$p < 0.05$; **$p < 0.01$). Mitomycin-c was used as a positive control for caspase-9 activity.
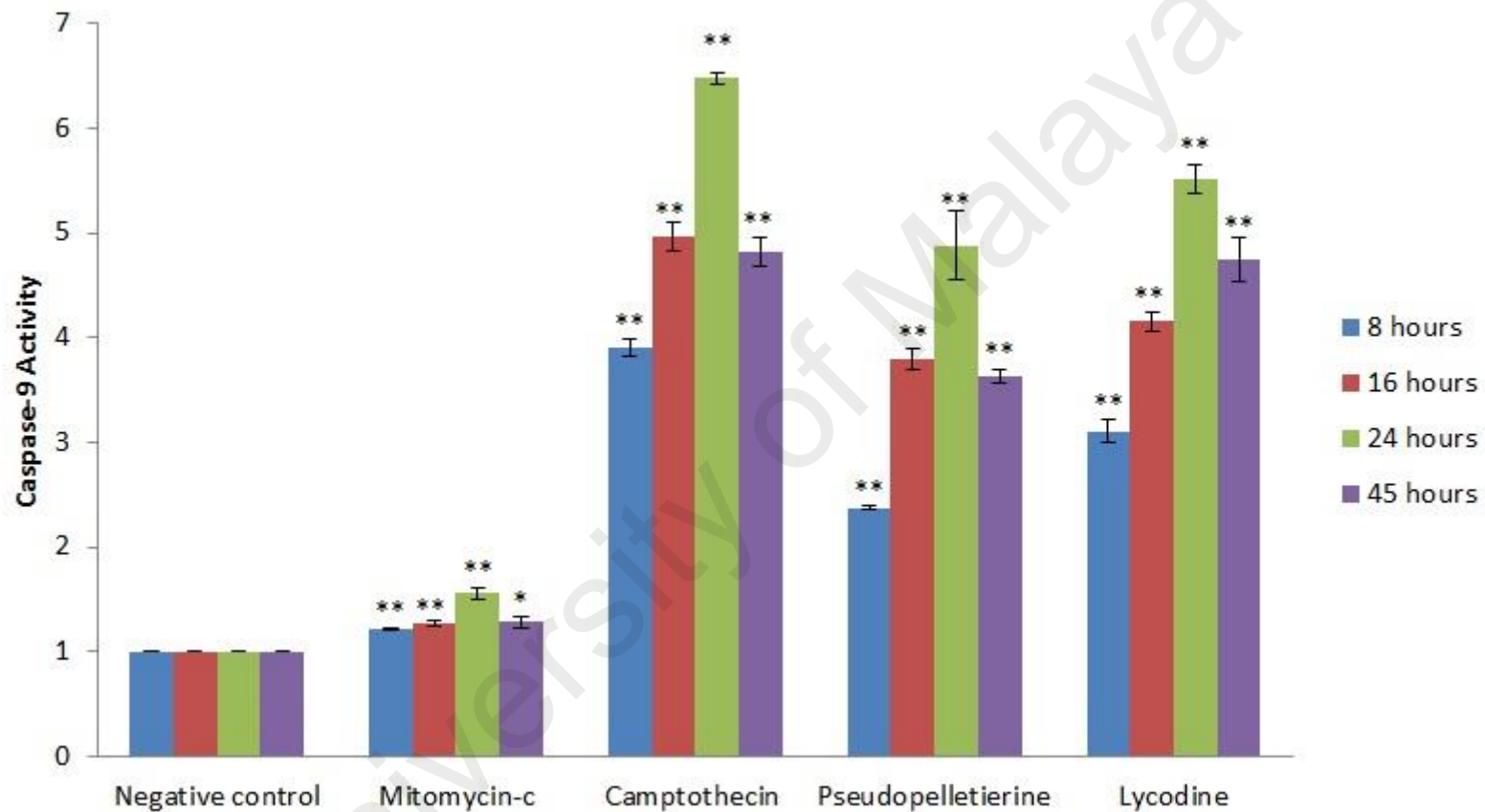
Figure 4.33: Caspase-9 activity of MCF-7 cells treated with the isolated pure compounds. Caspase-9 activity of MCF-7 cells treated with 1 µM of camptothecin, pseudopelletierine and lycodine for 8, 16, 24 and 45 h (*$p < 0.05$; **$p < 0.01$). Mitomycin-c was used as a positive control for caspase-9 activity.

Caspase-3 activity of *P. tetrastromatica* and *C. racemosa* increased and peaked at 24 h treatment (by 2.0 and 2.4 folds, respectively) and then decreased at 45 h treatment. Caspase-3 activity of *T. ornata*, on the other hand, peaked at 8 h treatment by 2.6 folds and then decreased in a time-dependent manner as shown in Figure 4.34. Caspase-3 activity of *S. vagabunda* peaked at 8 h treatment by 2.9 folds and then decreased in a time-dependent manner as shown in Figure 4.35. Activation of caspase-8 and 9 activity eventually lead to the activation of caspase-3. Caspase-3 activity of camptothecin, pseudopelletierine and lycodine-treated MCF-7 cells also peaked at 24 h by 6.9, 5.7 and 5.7 folds, respectively, relative to untreated cells (Figure 4.36).

In a nutshell, the caspase-8, -9 and -3 activities increased significantly ($p < 0.01$) upon treatment with the partially purified fractions and pure compounds, as compared to the untreated MCF-7 cells.
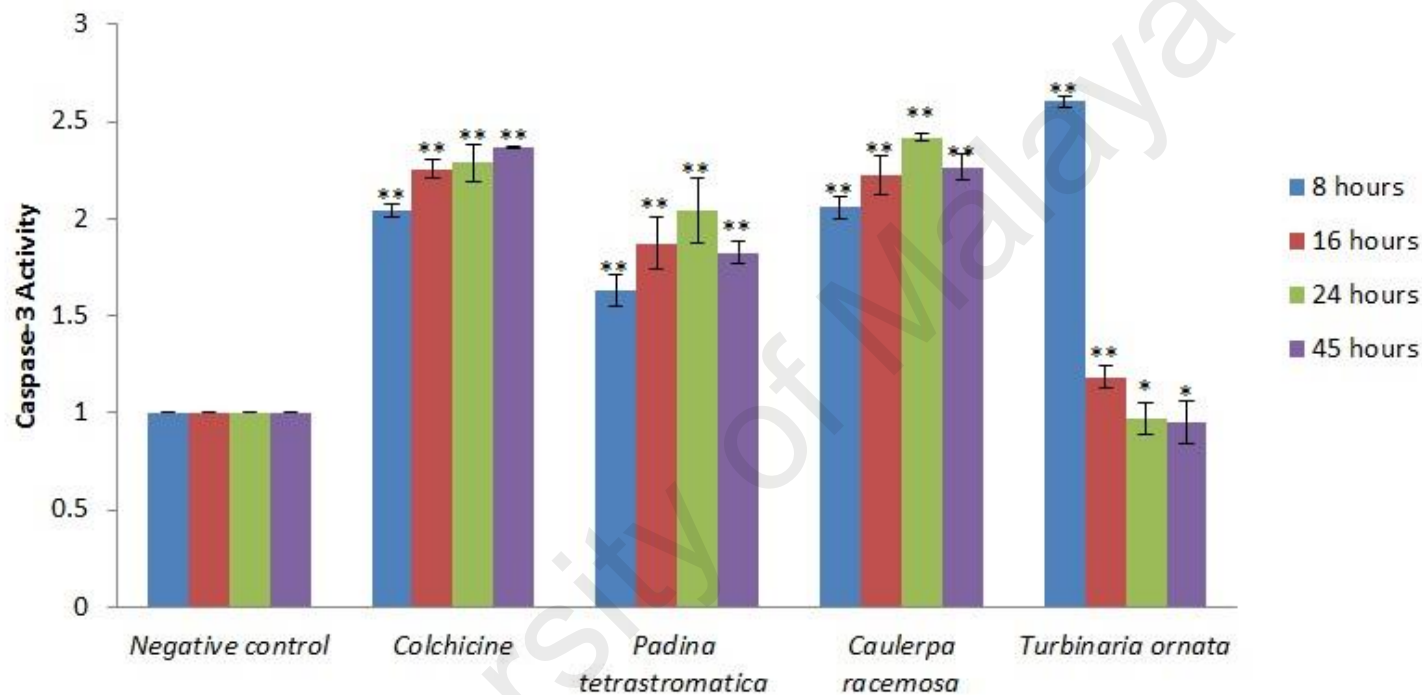
Figure 4.34: Caspase-3 activity of MCF-7 cells treated with the partially purified fractions of the seaweeds. Caspase-3 activity of MCF-7 cells treated with 12 μg/ml of partially purified *P. tetrastromatica* column chromatographic fraction, 18 μg/ml of partially purified *C. racemosa* column chromatographic fraction and 12 μg/ml of partially purified *T. ornata* column chromatographic fraction for 8, 16, 24 and 45 h (*$p < 0.05$; **$p < 0.01$). Colchicine was used as a positive control for caspase-3 activity.
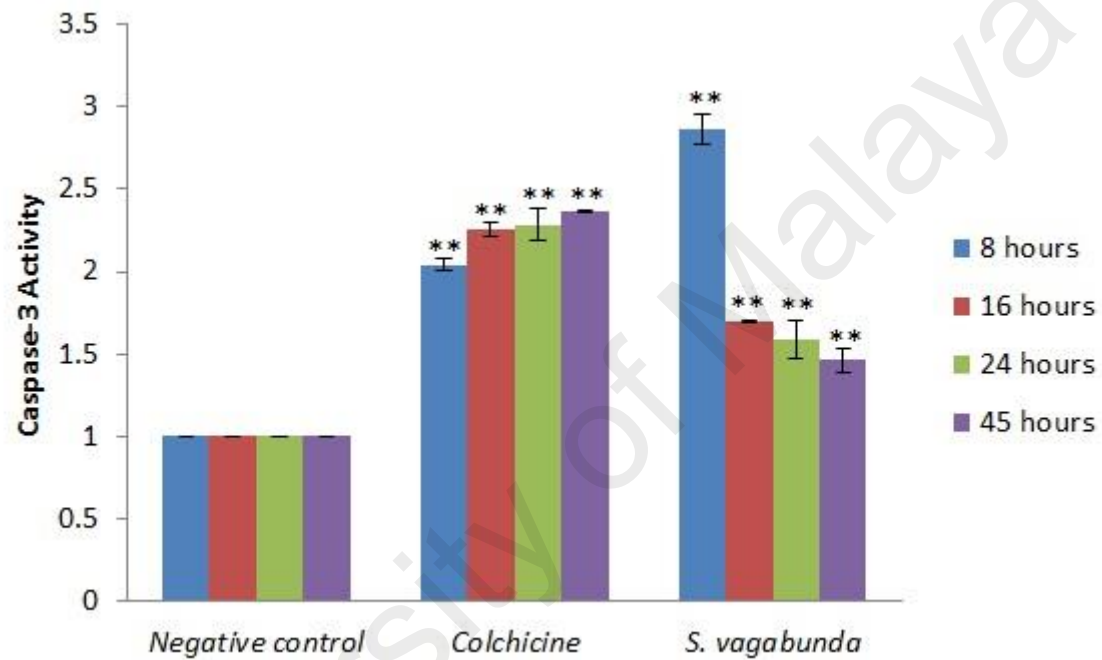
Figure 4.35: Caspase-3 activity of MCF-7 cells treated with the partially purified fractions of the sponge. Caspase-3 activity of MCF-7 cells treated with 12 µg/ml of partially purified *S. vagabunda* column chromatographic fraction for 8, 16, 24 and 45 h (*$p < 0.05$; **$p < 0.01$). Colchicine was used as a positive control for caspase-3 activity.
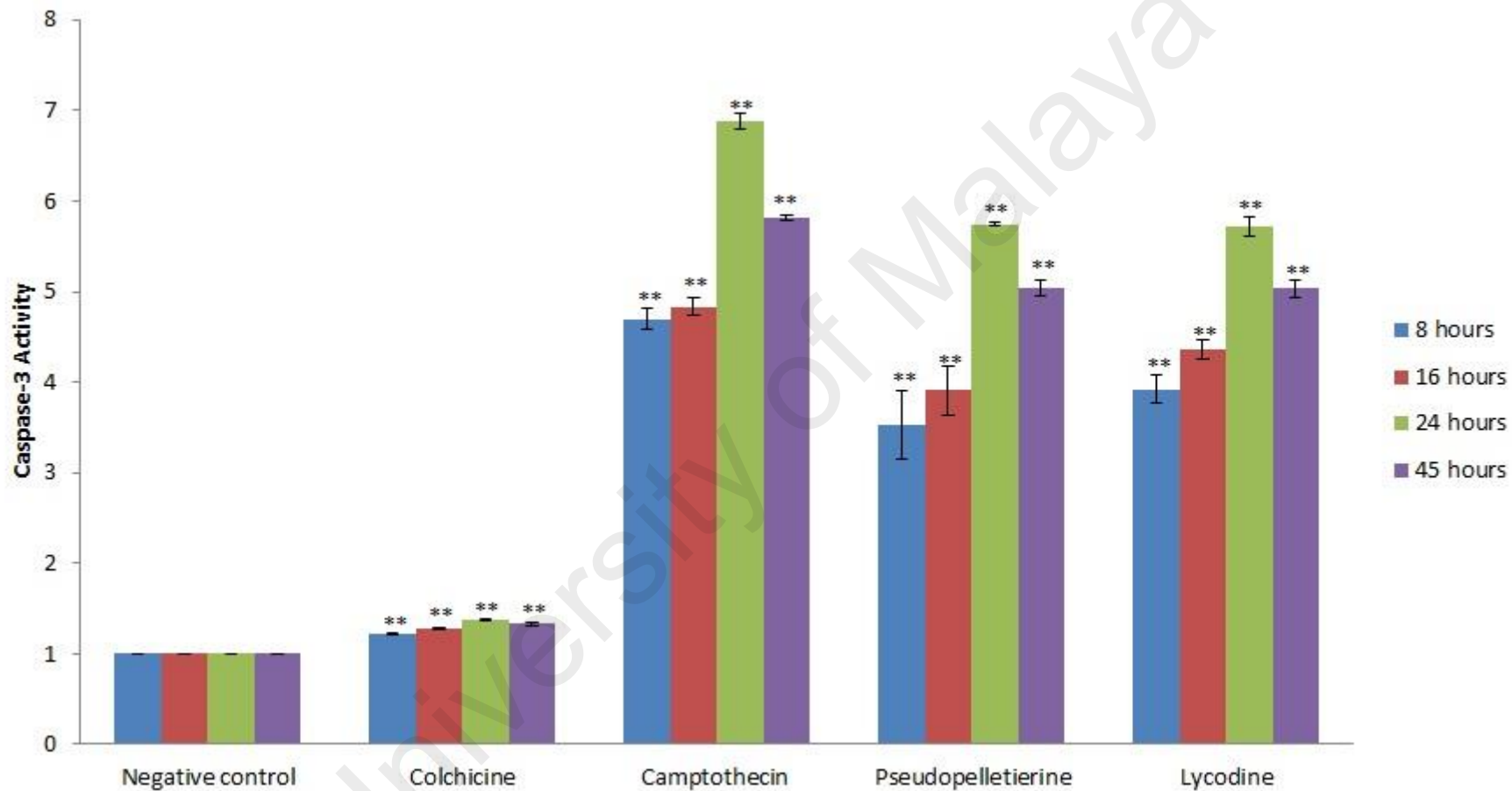
Figure 4.36: Caspase-3 activity of MCF-7 cells treated with the isolated pure compounds. Caspase-3 activity of MCF-7 cells treated with 1 μM of camptothecin, pseudopelletierine and lycodine for 8, 16, 24 and 45 h (*$p < 0.05$; **$p < 0.01$). Colchicine was used as a positive control for caspase-3 activity.

4.10.3 Analysis of DNA Fragmentation

The appearance of a DNA laddering pattern in MCF-7 cells was examined following treatment with the partially purified fractions and pure compounds for 24 and 48 h. Figure 4.37 shows DNA degradation in the seaweed fractions-treated MCF-7 cells whereas the untreated control cells showed intact genomic DNA when observed with a UV transilluminator. A smearing pattern was observed in lanes 2, 4 and 6 upon treatment with *P. tetrastromatica*, *C. racemosa* and *T. ornata* for 24 h, respectively, whereas a typical laddering pattern was observed in lanes 3, 5 and 7 after treatment with the same fractions for 48 h.

As for *S. vagabunda*, treatment with the partially purified fraction in MCF-7 cells resulted in a smearing pattern at a time point of 48 h, as observed in Figure 4.38. Hence, the seaweeds showed higher internucleosomal DNA fragmentation than the sponge, *S. vagabunda*.
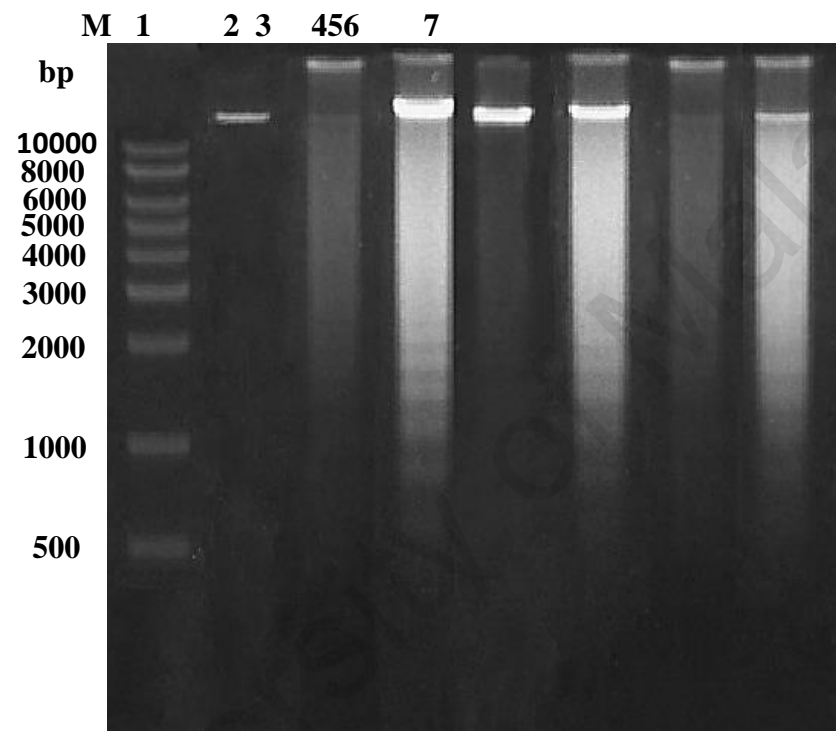
Figure 4.37:DNA fragmentation of seaweed fractions-treated MCF-7 cells. The gel pattern of 1kbDNA ladder (lane M) and DNA sample isolated from untreated control MCF-7 cells (lane 1); MCF-7 cells treated with 12 µg/ml of *P. tetrastromatica* for 24 and 48 h (lanes 2 and 3, respectively), 18 µg/ml of *C. racemosa* for 24 and 48 h (lanes 4 and 5, respectively); 12 µg/ml of *T. ornata* for 24 and 48 h (lanes 6 and 7, respectively).
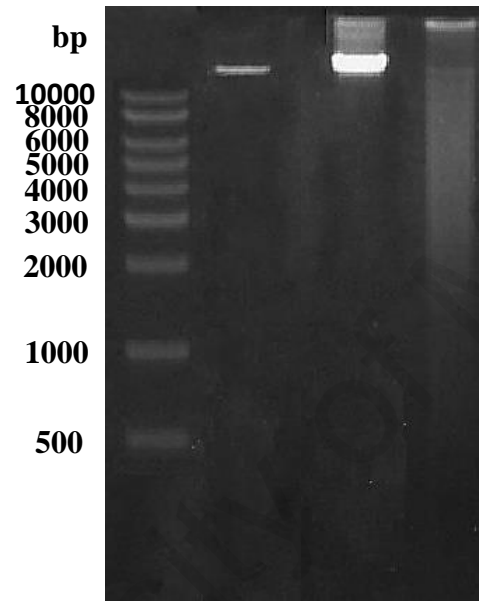
Figure 4.38:DNA fragmentation of sponge fraction-treated MCF-7 cells. The gel pattern of 1kbDNA ladder (lane M) and DNA sample isolated from untreated control MCF-7 cells (lane 1); MCF-7 cells treated with 12 µg/ml of *S. vagabunda* for 24 and 48 h (lanes 2 and 3), respectively.

MCF-7 cells were treated with 1 µM of camptothecin, pseudopelletierine and lycodine for 24 and 48 h before extraction of genomic DNA for the analysis of DNA fragmentation. Gel electrophoresis of chromosomal DNA of MCF-7 was conducted to examine the compounds-induced DNA damage. The laddering pattern was only observed in treated cells but not in untreated cells (negative control) (Figure 4.39). Cleavage of DNA by endogenous endonucleases into fragments is a hallmark of apoptosis while a smeared pattern is observed in cells which undergo cell death by necrosis. All treated samples showed significant DNA fragmentation after 24 and 48 h treatment (lanes 2 - 7 in Figure 4.39) as compared to the untreated cells (lane 1 in Figure 4.39).
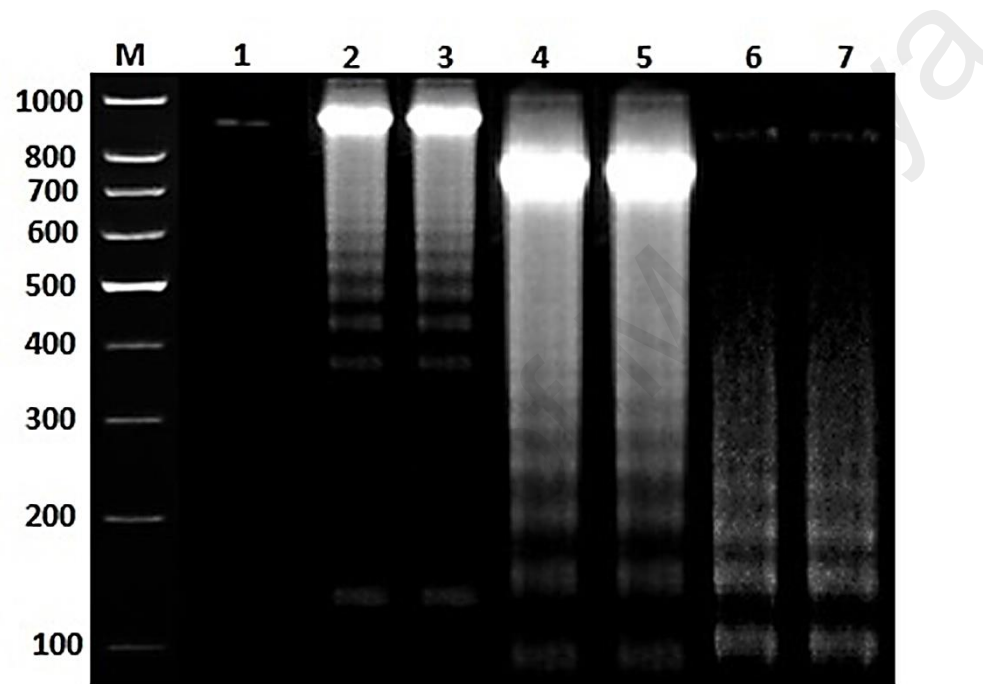
Figure 4.39: DNA fragmentation of compounds-treated MCF-7 cells (M) 1000 bp DNA marker, Lane 1 : Untreated MCF-7 cells (negative control), Lanes 2 and 3 : MCF-7 cells treated with 1 μM of camptothecin for 24 and 48 h, respectively, Lanes 4 and 5 : MCF-7 cells treated with 1 μM of pseudopelletierine for 24 and 48 h, respectively, Lanes 6 and 7 : MCF-7 cells treated with 1 μM of lycodine for 24 and 48 h, respectively.

4.11 Proteomics Profiling of the Differentially Expressed Proteins in Untreated and Treated MCF-7 Cells

A two dimensional (2D)-gel based proteomics approach was used to analyse the untreated and partially purified fractions-treated MCF-7 cells for the investigation of the molecular mechanisms of the anticancer activity of the partially purified fractions. Protein expression profiles in MCF-7 cells with or without treatment were compared by 2D-PAGE. Approximately 500 protein spots in untreated and treated cells were detected in the silver stained 2D-PAGE gels. All protein spots were aligned and matched by gel-to-gel comparison using the Progenesis SameSpots Software Q1 v2.0. The difference in the relative abundance of each protein spot was analysed. A total of 42 protein spots were differentially expressed in the untreated and treated MCF-7 cells, whereby 15 were up-regulated and 27 were down-regulated. Those highly repeatable proteins were picked, excised, subjected to in-gel tryptic digestion and subsequently subjected to peptide fragment analysis by the Agilent 1200 HPLC-Chip/MS Interface coupled with Agilent 6520 Accurate-Mass Q-TOF LC-MS and the proteins were identified against a database search of NCBInr, species of *Homosapiens* (human) (Agilent Technologies Inc., California, USA).

Differentially expressed proteins were statistically defined based on two criteria: (a) degree of intensity > 1.0 fold (protein score > 60 are considered as significant, $p< 0.05$) and (b) reoccurrence of the same proteins in the triplicates. Protein maps obtained for untreated MCF-7 cells (Figure 4.40) and MCF-7 cells treated with the partially purified fractions of *P. tetrastromatica*, *C. racemosa* and *T. ornata* are presented in Figures 4.41 – 4.43. Figure 4.44 shows the representative protein map for the MCF-7 cells treated with the partially purified fraction of *S. vagabunda*. The fold change in the expression levels of the proteins were analysed by the ImageMaster 2D Platinum 7.0 Software (GE

Healthcare, Buckingham, UK). A total of 42 differentially expressed proteins were identified and grouped into 10 biological processes based on their biological functions described in the UniProtKB/Swiss-Prot protein database (Table 4.29).
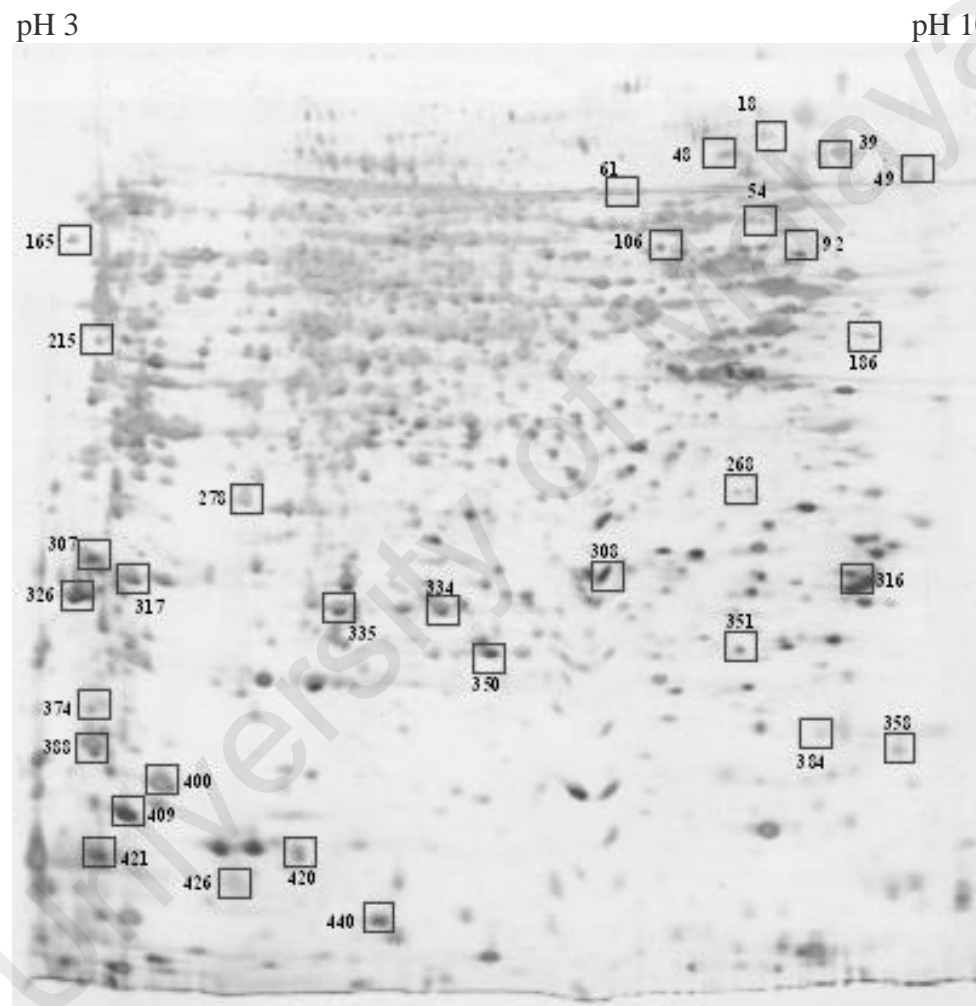
Figure 4.40: Protein map for untreated MCF-7 cells. The differentially expressed protein spots excised for mass spectrometry analyses are indicated by the labelled numerals.
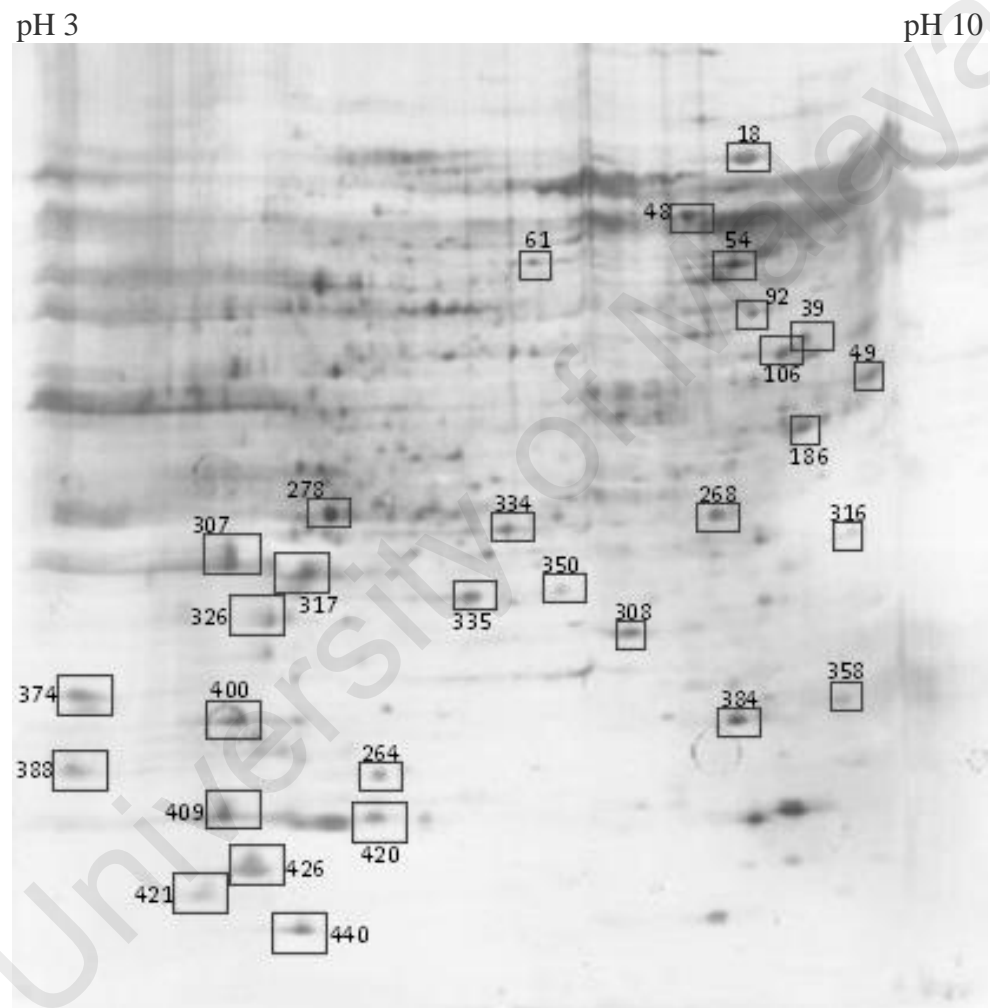
Figure 4.41: Protein map for MCF-7 cells treated with 12 μg/ml of *P. tetrastromatica* fraction. The differentially expressed protein spots were excised for mass spectrometry analyses are indicated by the labelled numerals.
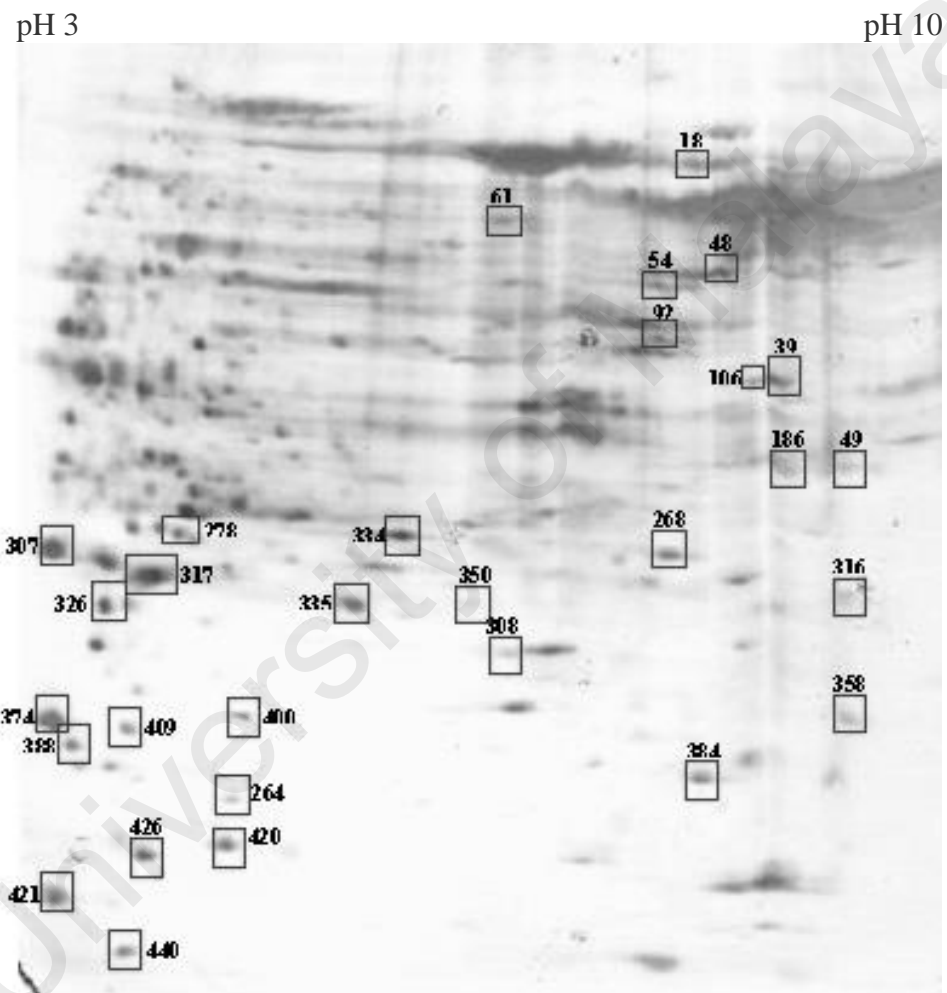
Figure 4.42: Protein map for MCF-7 cells treated with 18 μg/ml of *C. racemosa* fraction. The differentially expressed protein spots were excised for mass spectrometry analyses are indicated by the labelled numerals.
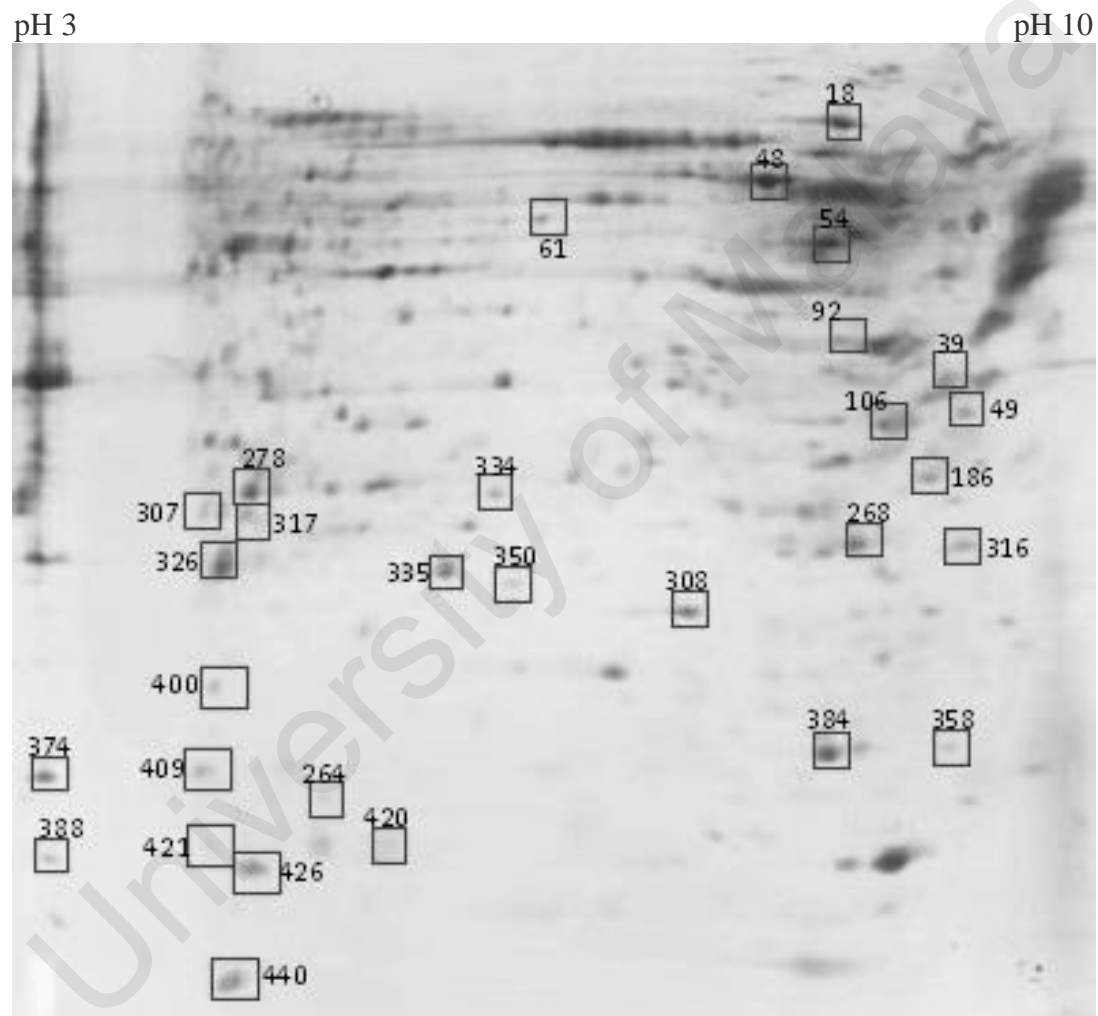
Figure 4.43: Protein map for MCF-7 cellstreated with 12 μg/ml of *T. ornata* fraction.The differentially expressed protein spots were excised for mass spectrometry analyses are indicated by the labelled numerals.
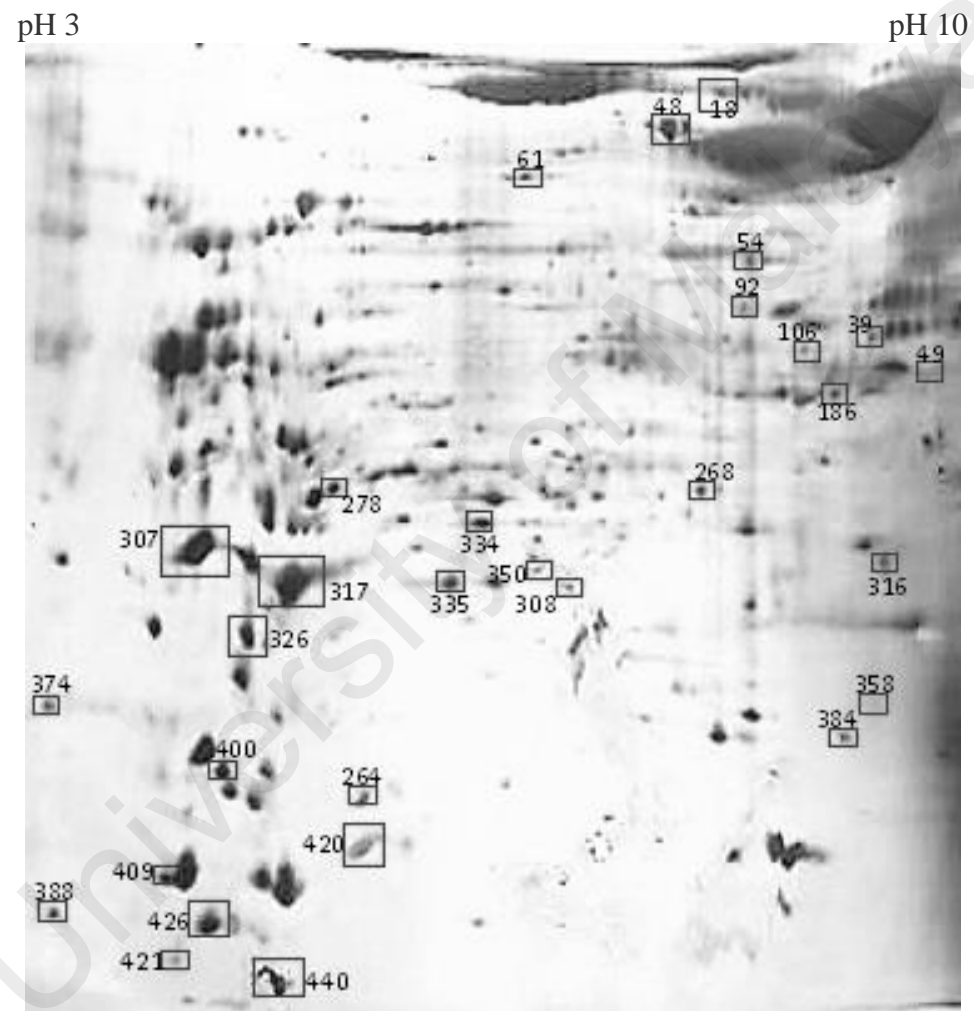
Figure 4.44: Protein map for MCF-7 cells treated with 12 μg/ml of the partially purified fraction of *S. vagabunda*. The differentially expressed protein spots were excised for mass spectrometry analyses are indicated by the labelled numerals.

In Group I (regulation of cell adhesion, cell proliferation, cell cycle, transcription, cell differentiation, signal transduction and apoptosis), 12 proteins were differentially expressed in treated MCF-7 cells. Of these, the cellular tumour antigen p53, pro-apoptosis regulator Bax and the poly [ADP-ribose] polymerase 1 (PARP-1) were found to be up-regulated ($p < 0.05$). The expressions of p53 were up-regulated by 2.91, 2.52 and 2.78 folds for *P. tetrastromatica*, *C. racemosa* and *T. ornata*, respectively. Upon treatment of MCF-7 cells by *P. tetrastromatica*, *C. racemosa* and *T. ornata*, the Bax levels of expression were up-regulated by 2.85, 2.73 and 2.93 folds, respectively while the expression levels of PARP-1 were up-regulated by 2.32, 2.81 and 2.44 folds, respectively. In contrast, the expression levels of Bcl-2 were down-regulated after treatment with *P. tetrastromatica*, *C. racemosa* and *T. ornata* by 2.36, 2.58 and 2.79 folds, respectively (Table 4.29).

As for *S. vagabunda*, the expressions of p53, Bax and PARP-1 were up-regulated by 2.84, 2.66 and 2.59 folds, respectively. Treatment of MCF-7 cells by the fraction of *S. vagabunda* also caused a down-regulation of the anti-apoptotic Bcl-2 protein by 2.52 folds (Table 4.29).

Table 4.29: Differentially expressed proteins identified by Q-TOF LC-MS analysis

Spot no.	UniProtKB accession no.	Protein name	Gene symbol	Protein scores	Protein molecular weight (Da)	Protein pI	Fold change			
							P. tetrastromatica	*C. racemosa*	*T. ornata*	*S. vagabunda*
I Regulation of cell adhesion, cell proliferation, cell cycle, transcription, cell differentiation, signal transduction and apoptosis										
018	P07900	Heat shock protein HSP 90-alpha	HSP90AA1	73	85057.6	4.94	1.17	1.03	1.32	1.52
264	P08238	Heat shock protein HSP 90-beta	HSP90AB1	85	83605.0	4.97	1.28	1.05	1.57	1.35
317	O14818	Proteasome subunit alpha type-7	PSMA7	88	28057.5	8.84	1.88	1.42	1.49	1.57
278	4R3O_D	Chain D, Human Constitutive 20s Proteasome	PSMA7	104	27511.8	7.79	1.52	1.31	1.27	1.04
278	4R3O_R	Chain R, Human Constitutive 20s Proteasome	PSMA7	79	27511.8	7.79	1.55	1.47	1.06	1.08
054	Q8TAA3	Proteasome subunit alpha type-7-like	PSMA8	95	28700.4	9.26	1.73	1.72	1.30	1.15
350	P10415	Apoptosis regulator Bcl-2	BCL2	158	26266.0	6.75	-2.36	-2.58	-2.79	-2.52
268	Q07812	Apoptosis regulator Bax	BAX	177	21814.0	5.08	2.85	2.73	2.93	2.66
278	P04637	Cellular tumour antigen p53	TP53	185	43653.2	6.33	2.91	2.52	2.78	2.84
384	P09874	Poly[ADP-ribose] polymerase 1	PARP-1	196	113084.1	8.99	2.32	2.81	2.44	2.59
048	P11021	78 kDa glucose-regulated protein	HSPA5	80	72445.7	5.07	-1.95	-1.52	-1.56	-1.89
186	P08865	40S ribosomal protein	RPSA	70	32967.6	4.79	-1.38	-1.74	-1.42	-1.45
II p53/TP53 stabilization and increased transcription activity										
326	Q13268	Dehydrogenase/reductase SDR family member 2, mitochondrial	DHRS2	87	30325.4	9.75	2.01	1.48	1.63	1.85
III Chaperone-mediated protein assembly and folding										
374	P23284	Peptidyl-prolyl cis-trans isomerase B	PPIase B	65	23799.1	9.48	1.36	1.43	1.65	1.31
061	P07900	Heat shock protein HSP 90-alpha	HSP90AA1	62	85057.6	4.94	1.54	1.76	1.65	1.34
061	P08238	Heat shock protein HSP 90-beta	HSP90AB1	69	83605.0	4.97	1.32	1.04	1.43	1.23
186	NP_001291217	SA 40S ribosomal protein SA isoform 2	RPSaB	74	33484.2	4.79	-1.27	-1.14	-1.46	-1.24
186	NP_002286	40S ribosomal protein SA isoform 1	RPSaA	77	32967.6	4.79	-1.51	-1.24	-1.42	-1.36

Table 4.29, continued. Differentially expressed proteins identified by Q-TOF LC-MS analysis

Spot no.	UniProtKB accession no.	Protein name	Gene symbol	Protein scores	Protein molecular weight (Da)	Protein pI	Fold change			
							P. tetrastromatica	*C. racemosa*	*T. ornata*	*S. vagabunda*
048	P17066	Heat shock 70 kDa protein 6	HSPA6	95	71483.3	5.81	-1.80	-1.46	-1.47	-1.37
215	P11142	Heat shock cognate 71 kDa protein	HSPA8	86	71125.0	5.37	-1.35	-1.84	-1.58	-1.33
335	P34931	Heat shock 70 kDa protein 1-like	HSPA1L	82	70773.2	5.76	-1.57	-1.68	-1.42	-1.73
334	P08107	Heat shock 70 kDa protein 1A/1B	HSPA1A	74	70336.2	5.48	-1.43	-1.36	-1.54	-1.54
165	P54652	Heat shock-related 70 kDa protein 2	HSPA2	68	70305.0	5.56	-1.26	-1.76	-1.32	-1.59
409	4H5N_A	Chain A, Hsc70 Nbd With Po4, Na, Cl	HSC70	61	42222.0	6.62	-1.04	-1.42	-1.69	-1.25
409	4H5N_B	Chain B, Hsc70 Nbd With Po4, Na, Cl	HSC70	83	42222.0	6.62	-1.13	-1.08	-1.47	-1.04
316	4H5R_A	Chain A, Hsc70 Nbd With Na, Cl And Glycerol	HSC70	76	42222.0	6.62	-1.40	-1.23	-1.56	-1.85
307	4H5R_B	Chain B, Hsc70 Nbd With Na, Cl And Glycerol	HSC70	82	42222.0	6.62	-1.42	-1.74	-1.88	-1.54
308	4H5T_A	Chain A, Hsc70 Nbd With Adp And Mg	HSC70	69	42222.0	6.62	-1.27	-1.04	-1.43	-1.87
388	4H5V_A	Chain A, Hsc70 Nbd With Mg	HSC70	61	42222.0	6.62	-1.16	-1.32	-1.76	-1.44
092	4H5W_A	Chain A, Hsc70 Nbd With Betaine	HSC70	89	42222.0	6.62	-1.38	-1.65	-1.82	-1.28
039	4H5W_B	Chain B, Hsc70 Nbd With Betaine	HSC70	96	42222.0	6.62	-1.32	-1.72	-1.39	-1.15
358	P48741	Putative heat shock 70 kDa protein 7	HSPA7	103	40471.9	7.90	-1.25	-1.65	-1.54	-1.63

IV Regulation of cell shape

Spot no.	UniProtKB accession no.	Protein name	Gene symbol	Protein scores	Protein molecular weight (Da)	Protein pI	*P. tetrastromatica*	*C. racemosa*	*T. ornata*	*S. vagabunda*
215	Q8TF72	Protein Shroom3	SHROOM3	68	21845.1	8.24	-1.53	-1.04	-1.17	-1.46

V Protein synthesis

Spot no.	UniProtKB accession no.	Protein name	Gene symbol	Protein scores	Protein molecular weight (Da)	Protein pI	*P. tetrastromatica*	*C. racemosa*	*T. ornata*	*S. vagabunda*
440	P25398	40S ribosomal protein S12	RPS12	75	14914.1	6.84	-1.49	-1.57	-1.39	-1.52

VI Maintaining homeostasis of cellular nucleotides

Spot no.	UniProtKB accession no.	Protein name	Gene symbol	Protein scores	Protein molecular weight (Da)	Protein pI	*P. tetrastromatica*	*C. racemosa*	*T. ornata*	*S. vagabunda*
317	P27144	Adenylate kinase 4, mitochondrial	AK4	84	25381.7	8.71	1.55	1.94	1.52	1.41

VII Redox regulation of cell

Spot no.	UniProtKB accession no.	Protein name	Gene symbol	Protein scores	Protein molecular weight (Da)	Protein pI	*P. tetrastromatica*	*C. racemosa*	*T. ornata*	*S. vagabunda*
308	P32119	Peroxiredoxin-2	PRDX2	107	22062.7	5.66	1.96	2.01	1.93	1.99
421	Q06830	Peroxiredoxin-1	PRDX1	97	22338.2	8.65	1.75	1.94	1.64	1.95

Table 4.29, continued. Differentially expressed proteins identified by Q-TOF LC-MS analysis

Spot no.	UniProtKB accession no.	Protein name	Gene symbol	Protein scores	Protein molecular weight (Da)	Protein pI	Fold change			
							P. tetrastromatica	*C. racemosa*	*T. ornata*	*S. vagabunda*
400	Q06830	Peroxiredoxin-1	PRDX1	106	22338.2	8.65	1.84	1.89	2.05	1.93
		VIII Gluconeogenesis and glycolysis								
335	P60174	Triosephosphate isomerase	TPI1	96	31075.7	5.65	-2.45	-2.76	-2.17	-2.62
316	4POC_A	Chain A, Structure Of Triosephosphate Isomerase Wild Type Human Enzyme	TPI	83	27477.8	6.26	-1.83	-1.53	-1.62	-1.68
316	4POC_B	Chain B, Structure Of Triosephosphate Isomerase Wild Type Human Enzyme	TPI	76	27477.8	6.26	-1.31	-1.65	-1.57	-1.74
307	4POD_A	Chain A, Structure Of Triosephosphate Isomerase I170v Mutant Human Enzyme	TPI	82	27463.8	6.26	-1.62	-1.40	-1.25	-1.25
307	4POD_B	Chain B, Structure Of Triosephosphate Isomerase I170v Mutant Human Enzyme	TPI	89	27463.8	6.26	-1.57	-1.28	-1.74	-1.59
		VIIII Regulation of the colloidal osmotic pressure of blood								
048	P07268	Serum albumin	ALB	149	71362.3	8.36	1.34	1.86	1.42	1.45
421	P07268	Serum albumin	ALB	164	71362.3	5.92	1.60	1.62	1.32	1.40
		X Transferase activity								
421	Q96MS3	Glycosyltransferase 1 domain-containing protein 1	GLT1D1	96	38734.6	6.02	-1.68	-1.57	-1.76	-1.34

A positive fold change indicates up-regulation while a negative fold change indicates down-regulation of protein expression relative to the untreated negative control. The differentially expressed proteins were statistically defined based on (a) degree of intensity > 1.0 fold (protein score > 60 are considered as significant, $p < 0.05$) and (b) reoccurrence of the same proteins in the triplicates.Data was obtained from triplicate analyses (n = 3).

4.12   Validation of Differentially Expressed Proteins by Western Blotting

The expression levels of some of the identified proteins were determined by Western blot analysis in order to validate the proteomics findings in section 4.11 of this chapter. Proteins were selected for Western blot analysis based on their high match score and their probable role in cell proliferation and apoptosis. p53, Bax, PARP-1 and Bcl-2 are crucial proteins which play an important role in apoptosis. In agreement with our earlier observation in Table 4.29, the expressions of Bax, p53 and PARP-1 were up-regulated while the expression of Bcl-2 was down-regulated in the partially purified fractions-treated MCF-7 cells for the seaweed fractions (*P. tetrastromatica*, *T. ornata* and *C. racemosa*) (Figure 4.45). Similar result was observed for *S. vagabunda* fraction as shown in Figure 4.46.
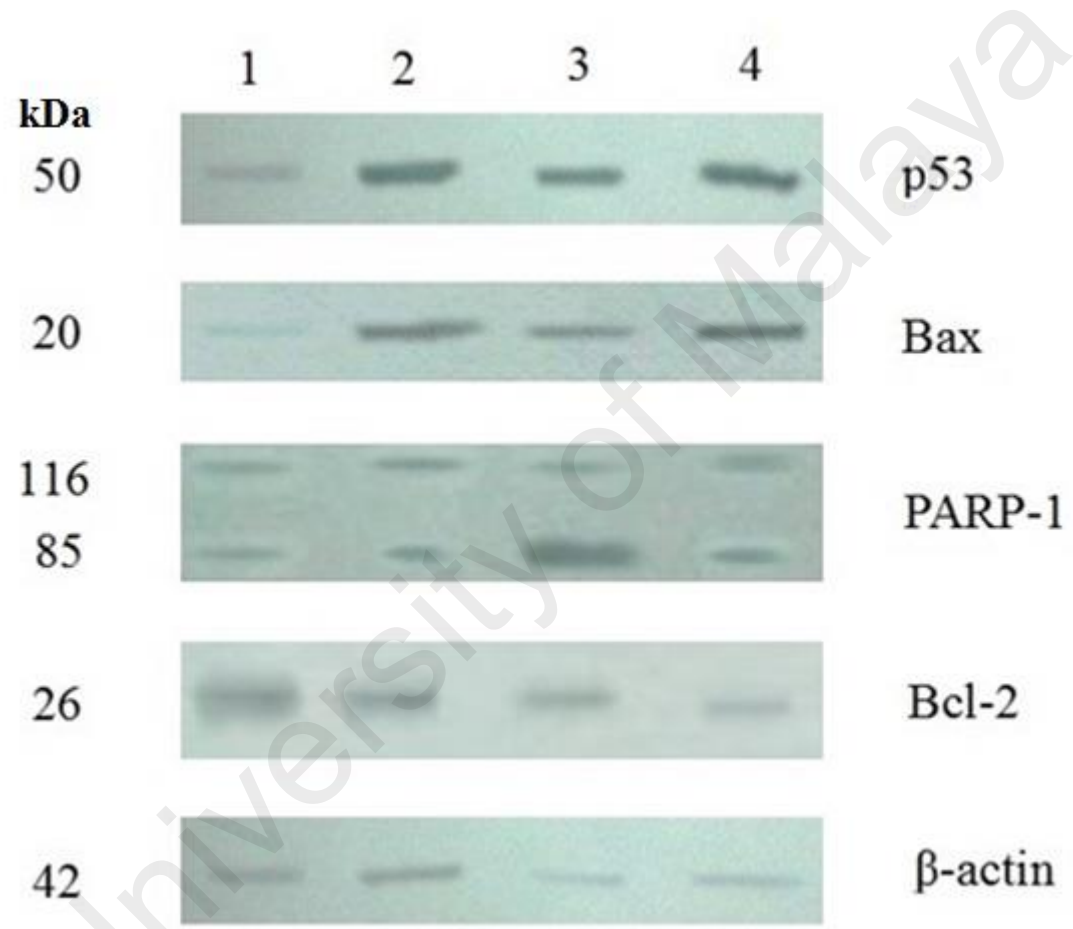
Figure 4.45: The effects of the partially purified fractions of the seaweed samples on the expression of different apoptotic proteins. Western blot analysis of p53, Bax, PARP-1 and Bcl-2 in MCF-7 cells treated for 48 h with the partially purified fractions of *P. tetrastromatica*, *C. racemosa* and *T. ornata* (12, 18 and 12 μg/ml, respectively). β-actin was used as internal control. Lane 1 indicates untreated control MCF-7 cells, lanes 2 to lane 4 denote MCF-7 cells treated with 12 μg/ml of *P. tetrastromatica*, 18 μg/ml of *C. racemosa* and 12 μg/ml of *T. ornata*, respectively.
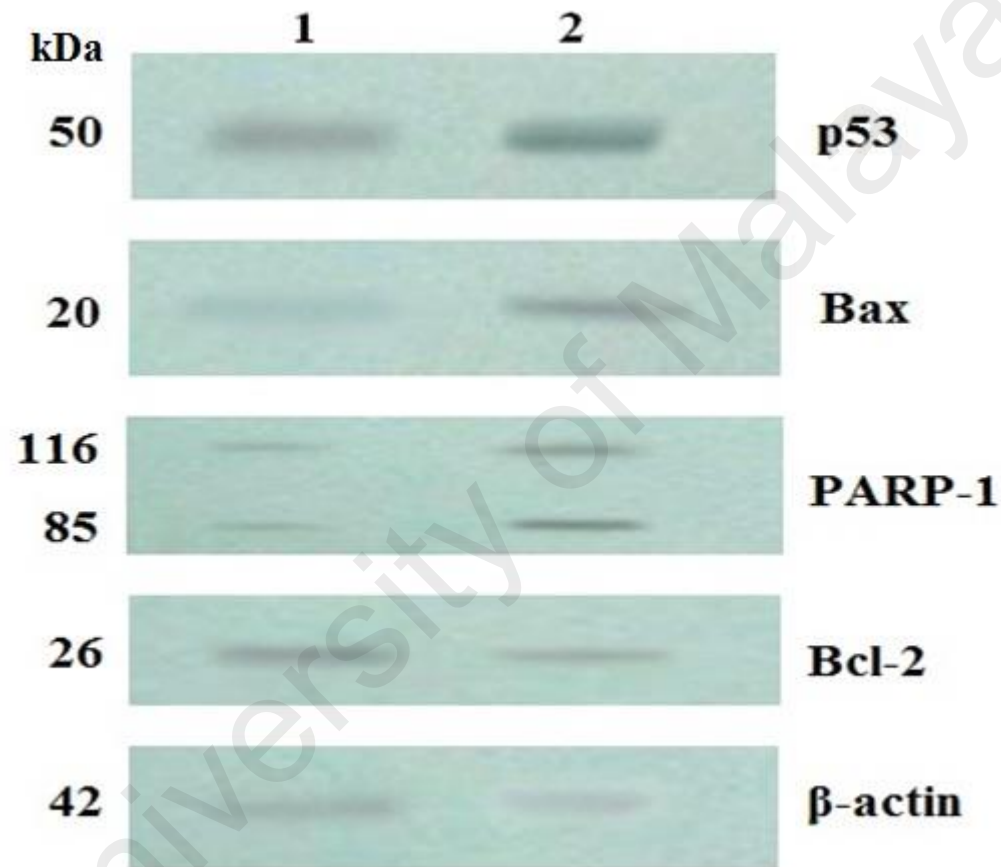
Figure 4.46: The effects of the partially purified fractions of the sponge sample on the expression of different apoptotic proteins. Western blot analysis of p53, Bax, PARP-1 and Bcl-2 in MCF-7 cells treated for 48 h with 12 μg/ml of *S. vagabunda* fraction. β-actin was used as internal control. Lane 1 indicates untreated control MCF-7 cells, lane 2 denotes MCF-7 cells treated with 12 μg/ml of *S.vagabunda*.

The expression levels of p53, Bax, Bcl-2 and PARP-1 were analysed by ImageJ analyser and software. Figure 4.47 shows that treatment with the partially purified fractions of *P. tetrastromatica*, *C. racemosa* and *T. ornata* for 48 h increased the expression level of p53 to 2.46, 1.73 and 1.98 folds, respectively, as compared to the negative control (untreated MCF-7). The expression level of Bax increased to 2.74, 2.51 and 2.94 folds with treatment of *P. tetrastromatica*, *C. racemosa* and *T. ornata*, respectively, for 48 h. The expression of PARP-1 increased to 2.17, 2.84 and 2.41 after treatment with *P. tetrastromatica*, *C. racemosa* and *T. ornata*, respectively, for 48 h. The Bcl-2 level of expression decreased to 2.27, 2.49 and 2.71 folds upon treatment with *P. tetrastromatica*, *C. racemosa* and *T. ornata*, respectively, for 48 h.
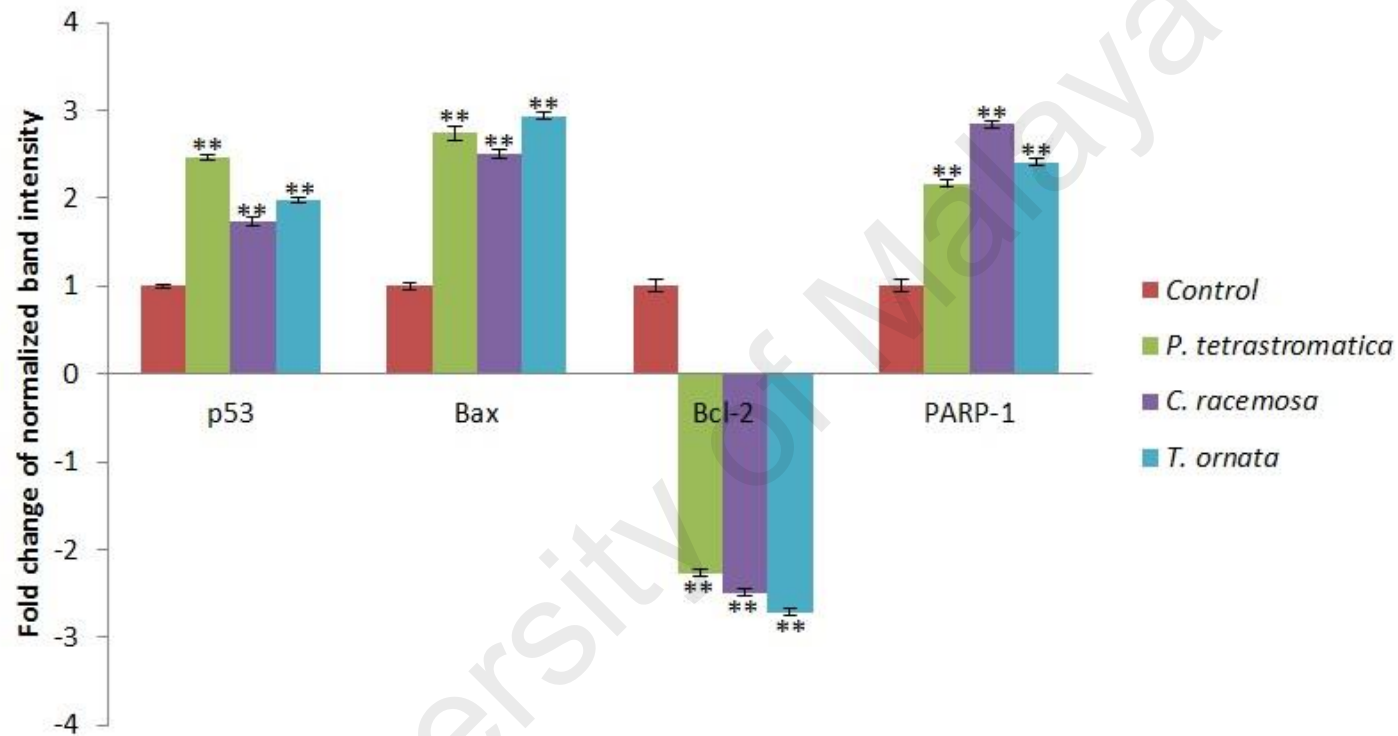
Figure 4.47: The effects of the partially purified fractions of the seaweed samples on the fold change of different apoptotic proteins. Fold change in the expressions of apoptotic proteins in MCF-7 cells treated with the partially fractions of the seaweeds, *P. tetrastromatica*, *C. racemosa* and *T. ornata*. Significance levels were determined using an unpaired, two-tailed Student's t-test, whereby (*$p < 0.05$; **$p < 0.01$).

Treatment with the partially purified *S. vagabunda* for 48 h increased the expression levels of p53, Bax and PARP-1 to 2.17, 2.18 and 2.63 folds, respectively, as compared to control (untreated MCF-7). The Bcl-2 level of expression, on the contrary, was down-regulated to 2.43 folds upon treatment with *S. vagabunda* for 48 h (Figure 4.48). The results are consistent with the observations in the 2D gel proteomics analysis.
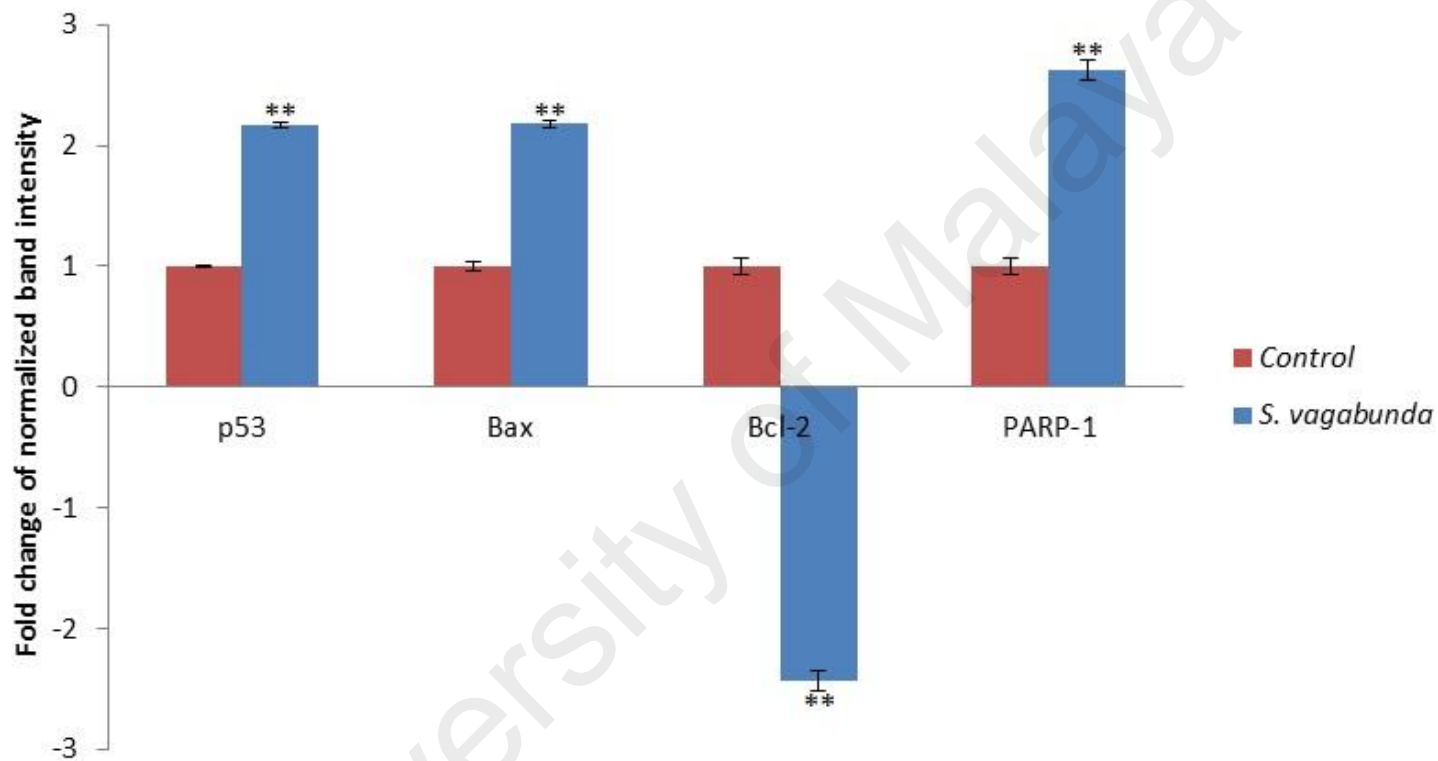
Figure 4.48: The effects of the partially purified fraction of the sponge sample on the fold change of different apoptotic proteins. Fold change in the expressions of p53, Bax, PARP-1 and Bcl-2 proteins in MCF-7 cells treated with the partially fraction of the sponge, *S. vagabunda*.Significance levels were determined using an unpaired, two-tailed Student's t-test, whereby (*$p < 0.05$; **$p < 0.01$).

CHAPTER 5

DISCUSSION

5.1   Discussion

Marine organisms have been used as a natural source of healthcare dating back to prehistoric times. It is perceived that natural products are generally non-toxic with less side effects and complications.However, this statement does not hold true albeit being a common general perception as there are natural products which are extremely toxic, including snake venoms and plant toxins such as ricin and cotinine. It is necessary to systematically and scientifically explore ethnobotanically important natural products in order to evaluate their medicinal benefits and properties, safety and efficacy. Many studies have proven that extracts from natural products and their components which are pharmacologically active can be used as therapeutic agents (Souza-Fagundes *et al*., 2002; Chang *et al*., 2013; Gurnani *et al*., 2014; Ramana *et al*., 2014; Harvey *et al*., 2015). Screening of natural products is mostly carried out to validate their ethnobotanical properties. Reliable bioassays and tests are required for screening of natural products (Hussain *et al*., 2007). Researchers worldwide are competing in exploring and seeking natural products with a broad spectrum of bioactivities from antibiotic to anticancer. Most anticancer drugs which are currently used in treatment expose patients to considerable health risks and complications as they bring about detrimental effects to normal cells in addition to causing multidrug resistance (MDR) among cancer cells (Bai *et al*., 1992; Mascarenhas, 1994; Shanmugaraju *et al*., 2011). Therefore, much attention has been diverted to natural products in terms of controlling and preventing cancer (Wet *et al*., 2009).

5.2   Assessment of Cell Proliferation

Despite tremendous effort and considerable progress made over the past few decades in oncology research and treatment, cancer remains as one of the foremost causes of morbidity and mortality worldwide, with 12.7 million new cases being diagnosed and 7.6 million deaths in 2008 (Msyamboza *et al*., 2012). The most widely used cancer treatments are restricted to chemotherapy, immunotherapy, surgery and radiation (Topcul & Cetin, 2013). These are severely fraught with challenges concerning the adverse side effects of chemotherapeutic drugs (Jiang *et al*., 2010) due to their non-specific systemic distribution (Wang *et al*., 2009c; Drabu *et al*., 2010), development of multidrug resistance (MDR) acquired upon repeated chemotherapeutic cycles (Shahbazi *et al*., 2012) and intolerable toxicity (Jeyaraj *et al*., 2013). Thus, there is clearly a need for novel chemotherapeutic agents with enhanced specificity, selectivity and potency. For this purpose, marine organisms seem promising as they contain novel bioactive compounds with distinctive chemical and structural features which are not discovered in compounds isolated from terrestrial plants (Güven *et al*., 2010). Moreover, the marine environment remains largely unexplored.

The human breast adenocarcinoma cell line, MCF-7, was used for the evaluation of the antiproliferative effect of the extracts in this study by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. MCF-7 is an oestrogen receptor positive cell line which is able to show rapid responses such as inhibitory effects and serves as a model for studying the response of therapeutic agents against breast cancer cells (Nor Aini *et al*., 2008). Cytotoxic assays serve as a guide for the preliminary screening of potent natural products against cancer (Al-Fatimi *et al*., 2007). The MTT assay was employed in this study to evaluate the antiproliferative effects of the extracts. The principle of the MTT assay is based on evaluation of the capability of an extract to

impart the reducing ability of mitochondrial dehydrogenase in cancer cells. This is because mitochondrial dehydrogenase is able to reduce MTT to a violet formazan product only in viable cells (Mosmann, 1983). Hence, this assay is used to evaluate the reduction of viability of MCF-7 cells in the presence and absence of extracts (Son *et al.*, 2003).

The human tumourigenic (MCF-7) and non-tumourigenic (184B5) cell lines were treated with the standard anticancer chemotherapeutic drug, doxorubicin, as a positive control. The drug showed IC_{50} values at 0.21 ± 1.25 µg/ml and 0.02 ± 1.97 µg/ml in MCF-7 and 184B5 cells, respectively (Figures $4.2 - 4.3$). From the results obtained in Table 4.1, the extracts of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* are more appropriate to be used as a chemotherapeutic agent as compared to the standard drug, doxorubicin. This is because treatment with doxorubicin in normal breast cells (184B5) resulted in a high toxicity in the cells (IC_{50} of 0.02 ± 1.97 µg/ml).

As presented in Tables 4.1 and 4.2, the hexane extracts of *P. tetrastromatica* and *S. vagabunda* exhibited the strongest antiproliferative activity (as represented by the lowest IC_{50} of 130.0 ± 1.72 and 80.0 ± 2.18 µg/ml, respectively) against MCF-7 cells, while the methanolic extracts of *C. racemosa* and *T. ornata* were more effective as compared to the other organic solvent extracts. These data suggest that the nature of the cytotoxic compound(s) tend to be non-polar in the hexane extracts of *P. tetrastromatica* and *S. vagabunda* and tend to be polar in the methanolic extracts of *C. racemosa* and *T. ornata*. The hexane extracts of *P. tetrastromatica* and *S. vagabunda* were the only extracts that were able to achieve IC_{50} among all the five solvents tested. Hence, it was postulated that MCF-7 cells were more susceptible to the effects of the hexane extracts of *P. tetrastromatica* and *S. vagabunda* than the other solvents. However, this is not the

case for *C. racemosa* and *T. ornata*. The methanolic extracts of both seaweeds exhibited greater potency among the five tested solvents. Thus, it is suggested that MCF-7 cells were more susceptible to the effects of polar compound(s) from the methanolic extracts of *C. racemosa* and *T. ornata* as methanol is a polar solvent. The susceptibility and resistance of MCF-7 cells to different extracts vary as the compound(s) present in all the marine organisms have variable polarity and extractability (Wissam *et al*., 2012). The crude extracts of the seaweeds and sponge showed no cytotoxicity in the non-tumourigenic 184B5 cells, thus further implying their suitability to be used as chemotherapeutic agents.

The antiproliferative activity of the extracts might be attributed to the presence of active phytochemicals such as quinine and alkaloids (Milugo *et al*., 2013). Quinine derivatives confer antiproliferative activity via interference in DNA and RNA replication and mitochondrial oxidative pathways; as well as through the formation of peroxide, superoxide and hydroxyl radicals in the cell (Mohamed, 2012). Antiproliferative activity of alkaloids, on the other hand, is due to the presence of microtubule interfering agents that can bind to beta tubulin, thus inhibiting the formation of the mitotic spindle fibre required for cell division (Solanki *et al*., 2008).

Furthermore, chemical and biological investigations indicated that the main substances biosynthesised, particularly by brown algae, with antiproliferative potential include phlorotannins, fucoidans, terpenes and sulphated polysaccharides (Mhadhebi *et al*., 2014). Besides eliciting *in vitro* antiproliferative activity as well as inhibiting tumour growth in mice, the sulphated polysaccharides and fucoidans found in brown seaweeds were also found to have antimetastatic activity by blocking the interactions between cancer cell lines and the basement membrane (Rocha *et al*., 2005). It has also been

reported that the induction of autophagy and apoptosis by the sulphated polysaccharides resulted in the inhibition of the growth of human gastric adenocarcinoma cells (Cantrell *et al*., 2003). In addition to the polar components such as phlorotannins and fucoidans, brown algae also contain non-polar components such as terpenes with antiproliferative activity, meroterpene with antitumour property and hydroquinone diterpene mediterraneol with mitotic cell division inhibitory activity (Mhadhebi *et al*., 2014).

Namvar *et al*. (2013) evaluated the effect of the brown seaweed, *Sargassum muticum*, methanolic extract against MCF-7 breast cancer cells dose- and time-dependently with an IC_{50} of 22 µg/ml. In the report of Harada and Noro (1997), 47 species of algae exhibited strong cytotoxic activity against the mouse lymphocytic leukemia cell line, L1210 cells, with low cytotoxicity in normal cells. Previous studies reported that hot water extracts of several brown algae were effective against some mouse cancer cells and the active principle was found to be the polysaccharide fraction (Holdt & Kraan, 2011). The crude extracts of the marine sponge, *Dendrilla nigra*, exhibited cytotoxicity in liver hepatocellular carcinoma (HepG2 cells) and the human breast adenocarcinoma cell line (MCF-7) (Valentin *et al*., 2011). One of the well-studied cytotoxic sponges, *Axinella donnani*, was reported to have vast potential of antitumour activity (Quershi *et al*., 1998).

5.3   Determination of Percentage Yield of Extraction

Extraction, defined pharmaceutically, is the method of separating medicinally active portions of plant tissues or other natural products (such as marine organisms) from the inert or inactive components using selective solvents (Das *et al*., 2010) which will diffuse into the solid plant material (or natural products), thereby solubilising compounds with similar polarity (Tiwari *et al*., 2011). The potency of an extract is

dependent on several parameters such as the choice of solvent and the extraction method (Ncube *et al.*, 2008). Extraction of natural products with solvents of different polarities could provide a comprehensive study for a variety of bioactive compounds (Das *et al*., 2010).

Solvent extractions are the most widely used procedure in the preparation of extracts from plant materials or other natural products such as marine organisms. The percentage yield of extraction is affected by numerous factors, including chemical composition and the physical characteristics of the sample, particle size of the sample, extraction time and temperature, type of solvents with varying polarities, and the solute to solvent ratio (Herode *et al*., 2003). The solubility of the compounds is governed by the chemical nature of the sample and the polarity of the solvent used. The samples may contain phenolics varying from simple (such as phenolic acids) to highly polymerised substances (tannins) in different quantities. Hence, there is no universal extraction procedure for the extraction of all plant phenolics (Dai & Mumper, 2010). A good extraction method involves procedures which are efficient in dissolving cellular components, soluble fibres and probably some high molecular weight polysaccharides.

The hexane extract of *P. tetrastromatica* showed considerably high antiproliferative activity (Table 4.1) despite having the lowest percentage yield of 0.04%. This is in agreement with research by Dellavalle *et al*. (2011) which reported that the chemical characteristics of the solvent, method employed during the extraction process, as well as the distinguishing structural and compositional aspects of the natural products or compound(s) may show distinct behaviour. Higher efficiency in the extraction of solutes is not automatically directly related to the biological activity of the sample.

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Differences in the solubility of the active compound(s) present are accountable for the variations in the biological activities (Dellavalle *et al*., 2011).

S. vagabunda showed relatively lower percentage of yield compared to the three seaweeds in this study (Tables 4.3 – 4.4). One plausible explanation for this is that sequential extraction might not be the best extraction method for the extraction of phytochemicals from the sponge, *S. vagabunda*. Several studies have reported the use of single extraction by polar solvents such as ethyl acetate, methanol and water as a means of extraction of phytochemicals from sponge samples (Rivera & Uy, 2011; Chairman *et al*., 2012; Mhya & Mankilik, 2014).

Methanol is generally the most suitable solvent for the extraction of polyphenolic compounds due to its ability to inhibit the action of polyphenol oxidase that leads to the oxidation of polyphenols (Yermilo *et al*., 1995). It was also reported that extraction with methanol usually results in a higher recovery of total extractable compounds (Manzoor *et al*., 2012). Other studies have also reported methanolic extracts as having the highest TPC in edible Japanese brown seaweeds, *Hibiscus sabdariffa* calyx and *Tecoma stans*, also known as yellow elder or small tree (Airanthi *et al*., 2010; Anokwuru *et al*., 2011b; Govindappa *et al*., 2011). Methanolic extracts contain significantly high TPC as phenolic compounds are typically more polar compounds. In this study, the solute to solvent ratio of 1:20 was chosen for maceration. The percentage of yield is directly proportional to solvent to solute ratio. The driving force during mass transfer is the concentration gradient between solute and solvent which is greater when a higher solvent to solute ratio is used as in the principle of mass transfer (Cacace & Mazza, 2003).

5.4   Estimation of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Generally, the two brown seaweeds (*P. tetrastromatica* and *T. ornata*) had higher TPC compared to the green seaweed, *C. racemosa* (as shown in Table 4.5). This might be attributed to the presence of phlorotannins, bi-polar polyphenols commonly found in brown seaweeds. Phlorotannins show antioxidative properties due to the presence of multiple phenolic groups that assist the algae to overcome oxidative stress arising from their environment (Airanthi *et al*., 2010).

The different TPC values of the different extracts might be due to the dilution of phenolic concentration per g extract by extracted matter other than phenolics with methanol being an effective solvent for the extraction of phenolics and also other compounds (Airanthi *et al*., 2010). All four samples showed significantly higher TPC (ranging from 6.4 to 71.3 mg GAE/g) than the methanolic-chloroform, petroleum ether, ethyl acetate, butanol and aqueous extracts (ranging from 2.8 to 33.4 mg GAE/g) of five brown seaweeds (Luo *et al*., 2010). Some higher plants have higher TPC values as compared to seaweeds. For instance, Anokwuru *et al*. (a) (2011) reported that the acetone extract of *Solanum scabrum* leaf extract showed the highest TPC of 34.2 ± 0.26 g GAE/100g dried weight. However, seaweeds might exhibit higher TPC than other higher plants, such as the methanolic extract of *Hibiscus sabdariffa* calyx (TPC value of 29.2 mg GAE/g) (Anokwuru *et al*., 2011b).

Flavonoids are one of the most widespread and diverse group of natural compounds and are probably the most important natural phenolics. They are the major subclass of

polyphenols and antioxidants in plants. Generally, flavonoids occur as glycosides, containing several phenolic hydroxyl groups on their ring structures. The role of flavonoids as effective ROS scavengers is due in part to the presence of phenolic hydroxyl groups (Anokwuru *et al*., 2011). It was postulated that flavonoids may have direct contribution to antioxidative actions. Antioxidant activity depends on the number and position of hydroxyl groups, other substituents and glycosylation of flavonoid molecules (Bouaziz *et al*., 2005).

It was found that the acetone extract of *T. ornata* in this study showed relatively low TFC despite having a high TPC value (Table 4.5). This suggests that flavonoids are not the most abundant polyphenols present in *T. ornata*, and the high TPC values might be due to the presence of other polyphenols such as phenolic acids, stilbenes, lignans, alkaloids, essential oils, ascorbic acid, tocopherols, carotenoids and steroids (Arfan *et al*., 2009).

The TFC values of the four marine organisms in the present study were higher than that reported for two seaweeds (*Ulva lactuca* and *Sargassum wightii*) with TFC values of 1.35 ± 0.04 and 2.02 ± 0.07 mg GAE/g, respectively (Meenakshi *et al*., 2009). As reported by Cox *et al*. (2010), the TFC values for six species of edible Irish seaweeds are in the range of $6.83 - 52.50$ mg/g, which is comparable to the TFC values of the samples in this study. Hence, the higher flavonoid content in the seaweed and sponge might contribute to more significant antioxidant activities.

5.5   Evaluation of Antioxidant Activities

The term "antioxidant' was coined in 1920 and is defined as a substance that fights against any sort of oxidation process (Mazumdar, 2011). It has been recognised for

many years that naturally occurring substances in natural products, be it terrestrial plants or marine organisms, contain antioxidant activities. It was proposed that antioxidant activity involves several mechanisms such as termination of free radical chain reactions, hydrogen donation, elimination of peroxides, chelation of catalytic ions and prevention of hydrogen abstraction (Gordon, 1990).

Phenolic compounds act as antioxidants via several potential pathways. The most significant pathway is the scavenging of free radicals during which free radical chain reactions can be interrupted by the phenolic compounds (Prakash *et al*., 2007).In addition, polyphenols demonstrate antioxidant activity by the inactivation of lipid radicals and by the prevention of decomposition of hydroperoxides into free radicals. Antioxidant activities are predominantly attributed to their redox properties, allowing them to act as reducing agents, singlet oxygen quenchers and hydrogen donors.

Owing to the complex reactive nature of phytochemicals, the antioxidant activities of the natural products cannot be evaluated by a single, universal method. In fact, at least two test systems have to be conducted for the determination of antioxidant activity to establish authenticity (Schlesier *et al*., 2002; Chanda & Dave, 2009). Hence, in this study, the hexane, DCM, acetone, EA and methanol extracts of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* were analysed by the DPPH radical scavenging activity, superoxide anion scavenging activity, nitric oxide scavenging activity and hydroxyl radical scavenging activity, all of which demonstrate antioxidant activity based on the scavenging of different free radicals. These radical scavenging assays measure the mixture of compounds in the extracts which are likely to show a range of biological potencies. However, they do not predict biological activity in the whole animal.

The free radical scavenging assay using the DPPH radical is a preliminary test for the analysis of the antioxidant potential of extracts. The assay has been used extensively as it allows high throughput screening and has high sensitivity for the detection of active ingredients even at low concentrations. One of the reasons that this method is widely used is due to its simplicity and high sensitivity. The DPPH radical is one of the several stable and commercially available organic nitrogen radicals (MacDonald *et al*., 2006). Antioxidant activity is predominantly related to the presence of bioactive compounds such as phenolics, flavonols and flavonoids. The antioxidant effect is directly proportional to the disappearance of DPPH radicals in the test samples. Polyphenols and anthocyanins scavenge DPPH via the donation of hydrogen, thus reducing DPPH (DPPH-H). Since the DPPH radical scavenging activity of the seaweeds in our study is considerable, they could be used as substitutes to replace harmful synthetic antioxidants such as butylated hydrotoluene (BHT) (DPPH radical scavenging of 42.3%) and butylated hydroxyanisole (BHA) (DPPH radical scavenging of 86.7%), which have been reported to be carcinogenic and tumorigenic at high doses (Aruoma, 2003).

The results obtained in this study, as shown in Tables 4.7 and 4.8, revealed that the hexane extract of the seaweed, *C. racemosa*, and the ethyl acetate extract of the sponge, *S. vagabunda*, showed the highest DPPH radical scavenging activity among the four marine organisms tested, despite having low TFC values (Tables 4.5 and 4.6). This indicates that only part of the flavonoids of *C. racemosa* and *S. vagabunda* participate in the DPPH radical scavenging activity (Siatka & Kašparová, 2010).

In this study, all the four samples demonstrated higher DPPH radical scavenging activity (ranging from $10.96 \pm 0.81\%$ to $75.17 \pm 2.04\%$) compared to the methanolic-

chloroform, petroleum ether, butanol and aqueous extracts of five brown seaweeds from China (ranging from $6.08 \pm 0.32\%$ to $58.25 \pm 1.36\%$) (Luo *et al.*, 2010). This might be attributed to the different extraction methods used. As reported by Luo *et al.* (2010), the mixture was ultrasonicated for 2 h whereas the mixture in our study was macerated for 24 h. The antioxidant activity of the extracts on DPPH radical may be attributable to a direct role of the extracts in terms of trapping free radicals by donating hydrogen atoms. Reduction of DPPH radical by phenolics and flavonoids also occur due to their hydrogen donating abilities (Zhao *et al.*, 2006).

Superoxide anion is the reduced form of molecular oxygen produced from the mitochondrial electron transport system upon the acceptance of a single electron. Energy is generated from mitochondria using electron transport chain reactions. Any loose electrons from the electron transport chain reactions will react directly with molecular oxygen, forming superoxide anion, which is the precursor for the formation of other ROS, including hydrogen peroxide, hydroxyl radicals and singlet oxygen (Lee *et al.*, 2004). These ROS will in turn induce oxidative damage in DNA, proteins and lipids (Wickens, 2001). In the phenazine methosulphate-nicotinamide adenine dinucleotide-nitroblue tetrazolium (PMS-NADH-NBT) system, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. Antioxidants have the capability of inhibiting the formation of blue NBT. The consumption of superoxide anion in the reaction mixture is implied by a decrement in absorbance at 560 nm with antioxidants (Elmastas *et al.*, 2006).

Among the seaweeds and sponge tested, the EA, acetone and methanol extracts of *T. ornata* showed superoxide anion scavenging activity at an IC_{50} of 20.0 µg/ml with a percentage of superoxide anion scavenging ranging from $4.8 - 75.3\%$. This implies that

P. tetrastromatica, *C. racemosa* and *S. vagabunda* might not use superoxide scavenging abilities as the main method to scavenge free radicals. *T. ornata* demonstrated stronger superoxide anion scavenging activity compared to the positive controls. This might be attributed to the presence of sulphated polysaccharides from *T. ornata* which have been reported to be excellent antioxidants for the management of oxidative stress (Kelman *et al*., 2011).

Nitric oxide (NO) is an important cellular messenger molecule involved in numerous physiological functions of the body. Nitric oxide is generated from L-arginine by vascular endothelial cells, certain brain cells and phagocytes. The damage brought about by nitric oxide and oxygen free radicals is further exacerbated as they react to produce peroxynitrites, which in turn lead to severe toxic reactions in nucleic acids, proteins and lipids (Yermilo *et al*., 1995).

The NO generated from sodium nitroprusside (SNP) reacts directly with oxygen to produce nitrite. The results shown in Table 4.3 indicate that the dichloromethane (DCM) extract of *C. racemosa* and the DCM and acetone extracts of *T. ornata* in SNP solution are capable of decreasing the levels of nitrite. The reduction in the nitrite level might be due to the direct competition between the extracts and oxygen in the reaction with NO. The DCM extract of *C. racemosa* and the DCM and acetone extracts of *T. ornata* demonstrated lower IC_{50} (in the range of 25-38 µg/ml) than the IC_{50} of ethyl acetate extract of *Cassia auriculata* leaves (51.3 µg/ml) in NO scavenging activity (Jain *et al*., 2011).

The hydroxyl radical is the most reactive radical and has the shortest half-life among other ROS due to its ability to induce severe damage to adjacent molecules. It can cause cell damage by reacting with lipids, saccaharides, polypeptides and nucleotides (Arfan

et al., 2009). In this study, EDTA, which acts as an iron chelator, was not used. Hence, hydroxyl radicals are generated site-specifically whereby unchelated iron ions are weakly associated with deoxyribose. These iron ions then react with hydrogen peroxide through the Fenton reaction; forming hydroxyl radicals that launch an immediate attack on the deoxyribose. When extracts are added to the reaction mixture, they remove the hydroxyl radicals from deoxyribose, thus directing the damage towards them and preventing the reaction (Halliwell *et al.*, 1987). The IC_{50} values indicate that these extracts are better hydroxyl radical scavengers than rutin (Tables 4.7 and 4.8). The four samples under study showed a higher percentage of inhibition of hydroxyl radicals (ranging from 37.22 to 74.66%) than the methanolic-chloroform, petroleum ether, EA, butanol and aqueous extracts of five brown seaweeds (ranging from 13.65 to 67.65%) (Luo *et al.*, 2010).

5.6   Evaluation of Antioxidant Enzyme Activities

Among the various causes of mutations in genetic materials, DNA damage due to increased oxidative stress is apparently the main contributing factor in raising the number of significant changes in DNA. The types of mutations arising from oxidative stress include alterations in DNA methylation, recurrent mutations in the Ras pathway and the occurrence of single nucleotide polymorphisms (SNPs) in oxidative DNA repair genes and antioxidant genes (Klaunig *et al.*, 2010; Schott *et al.*, 2015; Venza *et al.*, 2015). A large number of mutations can aggravate the severity of tumour cells as well as alter the behaviour of the tumour cells, thus enabling them to invade nearby tissue or spread to organs distant from the site of origin (Gorrini *et al.*, 2013). Tumour cells often have impaired antioxidant system. Moreover, system failure can occur repeatedly due to mutations in crucial genes involved in the repair of damaged molecules in addition to deficiency of enzymes and compounds to combat oxidative stress (Khan *et al.*, 2010;

Gorrini *et al*., 2013). Endogenous antioxidants such as SOD, CAT and GR are regarded as the first line of the antioxidant defence system and they are responsible for the elimination of ROS which are largely generated from mitochondrial energy metabolism through oxidative phosphorylation (Sohn *et al*., 2013).

Antioxidant enzymes are involved in the direct elimination of ROS in cells. SOD constitutes the first line of defence against ROS in living cells. SOD catalyses the dismutation of the superoxide radical, which is the precursor of hydroxyl radicals and other ROS, to hydrogen peroxide (H_2O_2) and water. CAT, on the other hand catalyses the conversion of H_2O_2 to oxygen and water. GR facilitates the removal of H_2O_2 and organic peroxides by reducing glutathione disulphide (GSSG) to the sulfhydryl form (GSH) with the help of NADPH (Nazifi *et al*., 2010). The reduction in CAT activity is considered a general response to stress. It has been suggested that the reduction in CAT activity is due to the inhibition of enzyme synthesis or alterations in the assembly of enzyme subunits under stress conditions.A decrease in CAT and GR activities in treated MCF-7 cells might be due to increasing ROS. CAT can be downregulated by ROS while peroxides and hydroxyl radicals inactivate GR (Min *et al*., 2010).

Upon exposure to H_2O_2, the burden of oxidative stress increases in cells which results in fast oxygenation of DNA nucleotides, thereby giving rise to DNA damage through the disintegration of DNA (Collins *et al*., 1993; Dusinska & Collins, 1996). The suppression of the antioxidant enzyme activities (SOD, CAT and GR) in this study causes the accumulation of H_2O_2, thus leading to cancer cell death.

5.7 Correlation between TPC and (i) TFC, (ii) DPPH Radical, (iii) Superoxide Anion, (iv) Nitric Oxide and (v) Hydroxyl Radical Scavenging Activities; and (vi) Antioxidant Enzyme Activities

The TPC of all four samples were highly correlated with their respective TFC. The highest Pearson correlation coefficient was shown by *C. racemosa* (0.99), implying that flavonoids constitute a major proportion of the total phenolic content of *C. racemosa*. Relatively strong linear positive relationships were also observed between the TPC and TFC values of *P. tetrastromatica*, *T. ornata* and *S. vagabunda* (Pearson correlation coefficient of 0.86, 0.71 and 0.74, respectively). Wan *et al*. (2011) and Akhtar *et al*. (2015) also reported similar results for 61 medicinal plants and *Gynura divaricata* leaf extracts, whereby the TPC is highly correlated to their TFC values.

The Pearson correlation coefficients for *P. tetrastromatica* and *T. ornata* were 0.96 and 0.93, respectively, and that of *C. racemosa* was 0.77 (as shown in Table 4.9) indicating that their DPPH radical scavenging activity might be attributed to their similar antioxidant content as both *P. tetrastromatica* and *T. ornata* are brown seaweeds (Javanmardia *et al*., 2003). The DPPH scavenging activity of the brown seaweeds (*P. tetrastromatica* and *T. ornata*) might be due to the presence of phlorotannins, the major antioxidants in brown seaweeds (Airanthi *et al*., 2010). The scavenging activity of the green seaweed, *C. racemosa*, on the other hand, might be due to the presence of polyphenols such as ascorbic acid, folic acid, Vitamin A and B1 (Chang *et al*., 2002). *S. vagabunda* has a low correlation between TPC and DPPH radical scavenging activity with a Pearson correlation coefficient of 0.37. A plausible explanation for this is because the antioxidant activity of the extracts is not limited to phenolic compounds, but also due to the presence of other secondary metabolites such as carotenoids, vitamins and volatile oils.

The TPC of all four marine organisms under study was positively correlated to their respective DPPH radical scavenging activity (Table 4.11). This is in agreement with a

number of studies by different researchers. As previously reported by Gorinstein and co-workers (2007), a positive correlation was shown between TPC and DPPH radical scavenging activity. Since these solvent extracts have the highest phenolic content, it was postulated that they would represent the major source of antioxidant capacity. Several other studies also reported a positive correlation between TPC and DPPH radical scavenging activity and suggested that the correlation is due to the major contribution of phenolic compounds to antioxidant activity (Cai *et al*., 2004; Shan *et al*., 2005; Wong *et al*., 2006;Maizura *et al*., 2011). The positive correlation may also be due to the fact that the phenolic compounds present in the extracts are potent radical chain breakers, radical scavengers and peroxide decomposers (Lai & Lim, 2011).

The TPC of *S. vagabunda* is weakly correlated with its DPPH scavenging activity (Table 4.12). This is in agreement with an earlier report by Lai and Lim (2011), who postulated that the compounds in the *S. vagabunda* extract may be weak ion chelators. However, in another study conducted by Azlim *et al*. (2010), weak negative correlations were observed for the methanolic and ethanolic extracts of the leaves of four aromatic plants. This could be due to several reasons. According to Prior *et al*. (2005), the FC assay provides a crude estimate of the TPC present in an extract, whereas the free radical scavenging assay is not only specific to polyphenols but measures the overall antioxidant capacity due to interaction between polyphenols and non-phenolic compounds. Various phenolic compounds have different responses in the DPPH assay, depending on the number of phenolic groups they contain (Singleton & Rossi, 1965). Hence, TPC is not always positively correlated with DPPH assay as the TPC might be high (contains a high amount of phenolic compounds) but the DPPH radical scavenging activity might be low (due to a low number of phenolic groups). More to the point, Tawaha *et al*. (2007) further suggested that the negative correlation may be due to the

TPC that does not necessarily incorporate all the antioxidants that may be present in the extract, but also the non-phenolic compounds.

C. racemosa, *T. ornata* and *S. vagabunda* showed weak correlation between the TPC and superoxide anion scavenging activity (Tables 4.13 and 4.14). Hence, it is suggested that the phenolic compounds in the *C. racemosa*, *T. ornata* and *S. vagabunda* extracts might be weak scavengers of superoxide anion radicals. These extracts exert less inhibitory effects on the superoxide anion radicals (Li *et al*., 2009). In agreement with the results obtained in this study, Zhao *et al*. (2008) also reported a low correlation between the TPC and superoxide anion scavenging activity in malting barley. Parejo *et al*. (2002) also reported a low correlation between the TPC and superoxide anion scavenging activity of six herbs and aromatic plants. In contrast to the results obtained in these studies, Li *et al.* (2009) reported a high correlation between the TPC and superoxide anion scavenging activity in ferulic acid and caffeic acid, indicating the significant contribution of phenolics to the superoxide anion scavenging potential.

The high correlation between the TPC and nitric oxide scavenging activity of *P. tetrastromatica* in this study implies that the compounds in this extract might be scavengers of nitric oxide radicals. This result is in agreement with the results obtained in a study by Akiri *et al*. (2010) using the methanolic extracts from Njavara rice bran.

The TPC of *P. tetrastromatica* and *S. vagabunda* were highly correlated (Pearson correlation coefficients of 0.87 and 0.94, respectively) with their respective hydroxyl radical scavenging activities (Tables 4.17 and 4.18). These results indicated that strong hydroxyl radical scavenging activities of the extracts were closely associated with their high levels of phenolic compounds which have the potential to scavenge hydroxyl

radicals through the donation of hydrogen. These results are in agreement with the findings by Zhang *et al*. (2011) who reported high correlation between the phenolic content of grape cane extracts with the hydroxyl radical scavenging ability. On the other hand, the low Pearson correlation coefficients of *C. racemosa* and *T. ornata* imply that the compounds in these extracts are weak chelators of Fe^{2+} ions, therefore enabling the production of hydroxyl radicals which eventually render damage to deoxyribose (Tawaha *et al*., 2007). Among the four antioxidant assays examined (DPPH radical, superoxide anion, nitric oxide and hydroxyl radical scavenging assays), *S. vagabunda* only showed a strong correlation between the TPC and the hydroxyl radical scavenging activity. This correlation was even stronger than that of the seaweeds. Therefore, it is suggested that the free radical scavenging ability of *S. vagabunda* is mainly attributed by its hydroxyl radical scavenging potential.

The difference in the correlations between TPC and antioxidant activities indicates the diversity of the phenolic compounds in the extracts and their different responses to different methods for the determination of antioxidant activity (Lai & Lim, 2011). Numerous previous studies reported that the antioxidant activities of natural products are well correlated with the phenolic compounds. One of the many mechanisms of the overall antioxidant activities is governed by the contribution of phenolic compounds, due to their redox properties. These usually involve neutralisation of lipid free radicals and preventing the decomposition of hydroperoxides into free radicals (Li *et al*. 2006). However, the TPC is not necessarily always correlated to the antioxidant activities. This might be due to the difference between the mechanisms of action of the antioxidant assays and the nature of the marine organisms under study as observed by Yu *et al*. (2002) and Akhtar *et al*. (2015). In addition, the low correlation indicates that the antioxidant activities are not limited to phenolic compounds but instead, other

secondary metabolites may also contribute to the antioxidant potentials, thus pointing to the difference in the level of potency of various compounds (Javanmardi *et al*., 2003; Akhtar *et al*., 2015). Therefore, sometimes there is still a vague correlation between TPC and antioxidant activity (Tawaha *et al*., 2007).

The reduction in activities of the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR), were determined after treatment of MCF-7 cells with the extracts of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda*. All four marine organisms showed a strong correlation between the TPC and the reduction of the antioxidant enzyme activities (Tables 4.19 – 4.24). The antioxidant enzyme activities may be directly related to the content of phenols, flavonoid and tannins; and consequently to their free radical scavenging activities (Sharma & Ramawat, 2013). Moreover, the response of the MCF-7 cells to oxidative stress is associated with the antioxidant capacity of the extracts. Stress damage is preventable by increased levels of antioxidant enzymes and vice versa (Bettaieb *et al*., 2011). Thus, the high correlation between the TPC and the reduction in antioxidant enzyme activities of the four marine organisms in this study would result in oxidative stress in the treated tumourigenic MCF-7 cells, which eventually leads to cell death.

5.8 Antiproliferative Activity of the Partially Purified Fractions Obtained by Bioassay Guided Fractionation of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda*

Sequential extraction was chosen as a method of extraction in this study as it is conducted based on different solvent polarities. This is due in part to reports pertaining to the nature of cytotoxic compounds which can be non-polar or polar. Most of the studies reported have shown that the majority of the cytotoxic and antiproliferative

compounds are polar in nature (Uddin *et al*., 2011; Vijayarathna & Sasidharan 2012; Durga *et al*., 2013; Kuete *et al*., 2013). Nonetheless, there are also reports showing that the cytotoxic compounds are phenolics of low polarity (Umthong *et al*., 2011; Jemia *et al*., 2013; Swiatek *et al*., 2013). Therefore, a series of solvents with increasing polarities were chosen for extraction. As reported in Tables 4.1 and 4.2, the cytotoxic and antiproliferative compounds present in *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* are either non-polar or polar, as reflected by the IC_{50} values, whereby the cytotoxic compounds in *P. tetrastromatica* and *S.vagabunda* are of low polarity (hexane extracts) while those in *C. racemosa* and *T. ornata* are polar compounds (methanol extracts).

According to the American National Cancer Institute (NCI), an extract can only be considered as active if it has an IC_{50} value of 20 µg/ml or less following incubation between 48 to 72 h (Lee & Houghton, 2005). As shown in Tables 4.25 and 4.26, there was significant enhancement of antiproliferative and cytotoxic activity in the partially purified fractions of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* as compared to the crude extracts. A plausible explanation of this is that most unwanted components which are not bioactive, such as colour pigments, resin or wax, were removed during sequential extraction, leaving only cytotoxic compounds or other bioactive phenolic compounds (Umthong *et al*., 2013). In addition, the partially purified fractions were apparently more active compared to the crude extracts due to the presence of certain compounds which might inhibit the activities of other compounds in the crude extracts. This phenomenon is due to the existence of antagonistic interactions in the crude extracts, thus rendering the weak activities (Okokon & Nwafor, 2010). Moreover, crude extracts may be inactive due to the fact that the active fractions or components may be present in minute quantity. Hence, their effects are suppressed by

other compounds. However, upon fractionation, the aforementioned compounds became concentrated and therefore exhibited higher activities (Babayi *et al*., 2004).

In agreement with the results obtained in this study, Umthong *et al*. (2011) also reported a significantly higher antiproliferative activity after performing bioassay guided fractionation on the hexane crude extract against five cancer and two normal cell lines using the MTT assay. In a recent study reported by Zainal *et al*. (2014), cocoa leaf extract demonstrated the highest antiproliferative activity against MCF-7 cells among all the non-consumed parts of the cocoa plant tested. This extract was then selected for further purification using bioassay guided fractionation. The bioassay guided fractionation yielded the most potent hexane partitioned fraction with significant antiproliferative activity. Prabhu *et al*. (2014) also reported similar outcomes whereby the bioassay guided fractionation of *Aspergillus japonica* extract showed more potent antiproliferative effect in the cervical cancer cell line, HeLa.

5.9 Liquid Chromatography-Mass Spectrometry (LC-MS) Profiling

Marine organisms such as fungi, bacteria, seaweeds and sponges are taxonomically diverse and biologically active, offering a wide array for the discovery of new anticancer drugs derived from bioactive compounds with medicinal properties such as terpenoid derivatives, flavonoids, flavones, alkaloids, glycosides, polyphenolics and steroids (Boopathy & Kathiresan, 2010). Alkaloids are widely present in natural products particularly in ethnobotanically important plants and marine organisms. Furthermore, alkaloids have specifically been of interest due to their remarkable physiological activities including anticancer, antiviral and antimalarial activities (Silva *et al*., 2008). Camptothecin (quinoline alkaloid) is one of the most well-known examples of anticancer alkaloids and accounts for approximately one-third of the global anticancer market (Oberlies & Kroll, 2004). Pseudopelletierine, isolated from the bark

of the stem and the root of pomegranate was reported to have antimicrobial, anthelmintic and molluscicidal as well as antiuraemic activities (Neuhofer *et al*., 1993). Orhan *et al*. (2007) reported that lycodine (tetracyclic alkaloid) isolated from the clubmoss, *Lycopodium clavatum* L., contributed to its antioxidant, antimicrobial and antiviral activities.

5.10 Evaluation of Antiproliferative Activity of Camptothecin, Pseudopelletierine and Lycodine

As shown in Figure 4.17, the IC_{50} of camptothecin, pseudopalletierine and lycodine were 2.4 µg/ml, 4.8 µg/ml and 6.4 µg/ml, respectively. These data suggested that the pure compounds accountable for the cytotoxic activity are significantly more active individually as compared to when they exist together as a whole (as in the crude extracts and partially purified fractions) despite their low volume percentages as shown in Tables 4.27 and 4.28. (It should be noted here that concentrations between $0 - 5$ µg/ml were not done, and therefore the IC_{50} values mentioned above are approximations).

Camptothecin is one of the most promising anticancer drugs. Human DNA topoisomerase I is firmly established to be the molecular target of camptothecin (Liu *et al*., 2000). The inhibition of topoisomerase I by camptothecin eventually results in the accumulation of the cleavable complex. The primary mechanism of cell killing by camptothecin is attributable to the lethal collision between the advancing replication forks and topoisomerase I cleavable complexes during the S-phase of the cell cycle. This would ultimately lead to the induction of double strand breaks and cell death (Liu *et al*., 2000; Legarza & Yang 2006; Lv *et al*., 2013). Pseudopelletierine is an alkaloid isolated from *Punica granatum* or pomegranate with anticancer and antiangiogenic activities (Rahimi *et al*., 2012). Pseudopelletierine exhibited antiproliferative activity on

prostate cancer cell lines *in vitro* through the inhibition of NF-κB (Hong *et al*., 2008). Lycodine, on the other hand, has not been as intensively studied. Therefore, it is postulated that camptothecin, pseudopelletierine and lycodine are the main contributors to the antiproliferative activity of the seaweeds and sponge in this study. It is also implied that the compounds exhibit antagonistic effects wherein their activities were suppressed in crude extracts and partially purified fractions.

5.11   Cellular Mechanisms Related to Decreased Cancer Cell Viability

There are two main distinct modes of cell death namely the apoptotic and necrotic pathways. Researchers have been giving much attention to apoptosis, particularly pertaining to the mechanism of action. Apoptosis can be further divided into two distinct pathways: the extrinsic or receptor-linked pathway and the intrinsic or mitochondria-mediated pathway (Li *et al*., 2012).

Mitochondria are the key regulators of mechanisms controlling cell death and survival and play a vital role in the intrinsic pathway of apoptosis (Gogvadze *et al*., 2008; Indran *et al*., 2011). Mitochondria are semiautonomous subcellular organelles which play vital roles in cellular metabolism energy generation and apoptotic pathways (Talari *et al*., 2014). Genomic, structural and functional mitochondrial alterations have been linked with cancer. Mitochondria may serve as promising targets for cancer therapy due to the specific alterations occurring in cancer cell mitochondria (Barbosa *et al*., 2012). Mitochondrial membrane potential (MMP) is defined as the voltage difference across the mitochondrial inner membrane, whereby the exterior is positively charged whilst the interior is negatively charged. This subsequently generates the proton-motive force, hence driving the synthesis of ATP by the process of oxidative phosphorylation. The MMP status is an important parameter of mitochondrial function (Salido *et al*., 2007).

Non-receptor-mediated stimuli such as toxins, viral infections, hypoxia, hyperthermia as well as the absence of certain growth factors and hormones, govern the opening of the mitochondrial permeability transition (MPT) pore and loss of the MMP, thus resulting in the release of two main groups, the normally sequestered apoptotic proteins (the Bcl-2 family of proteins and the pro-apoptotic proteins) from the intermembrane space into the cytosol, thereby triggering the intrinsic signalling pathways of apoptosis (Lin *et al*., 2015). Mitochondrial outer membrane permeabilisation (MOMP), accompanied by the collapse of MMP, is a key commitment step in the induction of mitochondrion-dependent apoptosis, as it serves as a point of convergence for various intracellular apoptotic signalling pathways (Korper *et al*., 2003). The release of apoptogenic factors such as cytochrome c from mitochondria into cytosol is facilitated by alterations in the MMP. The released cytochrome cwill then form an apoptosome which leads to the activation of downstream apoptogenic signals such as caspase-3 (Kumar, 1999).

A collapse of MMP indicates mitochondrial toxicity (Ferraresi *et al*., 2004). Tumour mitochondria are more resistant to mitochondrial or intrinsic pathway of apoptosis as they are less susceptible to mitochondrial membrane permeabilisation (Gogvadze *et al*., 2008). However, in this study, we showed that attenuation of MMP in MCF-7 cells was induced by treatment with fractions and pure compounds (Figures 4.18 – 4.27). Depolarisation of the MMP due to chemical compounds will thus imply mitochondrial involvement in the cell death pathway. The collapse of MMP was postulated to be caused by the production of reactive oxygen species (ROS) which promotes mitochondrial membrane permeabilisation and gradual induction of apoptotic cell death (Looi *et al*., 2013). Both dissipation of MMP and the release of cytochrome c from mitochondria are crucial indicators of cellular apoptosis and important endpoints for the determination of mitochondrial dysfunction (Talari *et al*., 2014).

Various fluorescent probes are commonly used for assessing the physiological and biochemical functions of mitochondria and for monitoring MMP. There is accumulation of lipophilic cations in the mitochondrial membranes due to their delocalised positive charge and solubility in both the inner mitochondrial membrane and matrix space (Atamna *et al*., 2012). The lipophilic cation dye, JC-1 (5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolyl-carbocyanine iodide) is able to detect alterations in MMP at single cell level (Troiano *et al*., 2007). JC-1 shows green fluorescence when it exists as a monomer in the cytosol and shows red fluorescence when it aggregates and accumulates in the mitochondria. The mitochondria are stained red when JC-1 accumulates in intact mitochondria of healthy non-apoptotic cells. However, a collapse in MMP in apoptotic cells disables the JC-1 stain from entering the mitochondria, thus forming primarily green fluorescence in apoptotic cells with depolarised MMPs (Smiley *et al*., 1991).

 As shown in the results (Figures 4.18-4.27) obtained in this study, a collapse of MMP was observed as green fluorescence upon treatment of MCF-7 with the fractions and pure compounds. This implies that the fractions and pure compounds induced apoptosis through MOMP, leading to the subsequent collapse of MMP.

In a recent study by Wang *et al*. (2015), treatment of a coumarin derivative, esculetin, induced both *in vitro* and *in vivo* antiproliferative activity against hepatocellular carcinoma via the collapse of the MMP. Zheng *et al*. (2011) concluded that the inhibition of the growth of HT-29 cells by the ethanolic extract of *Spica prunellae* was due to apoptosis as the treatment resulted in a collapse of MMP, accompanied by activation of caspase-9 and -3 and an increase in the Bax/Bcl-2 ratio. Talari *et al*. (2014)

noted a point in their findings which stated that 250 μg/ml of the *Dracocephalum kotschyi* extract significantly caused H_2O_2 production, mitochondrial swelling, collapse of MMP and the release of cytochrome c in treated liver cancer cells. Similar to the results obtained in our study, Thamizhiniyan *et al*. (2015) reported that the hexane fraction of the *Acanthopanax sessiliflorus* stem bark extract dose-dependently increased the ratio of green fluorescence in the MCF-7 and MD-MB-231 human breast cancer cell lines, implying the involvement of mitochondrial membrane depolarisation during the induction of cell death by the extract.

Cells can undergo apoptosis by at least two fundamental pathways, both of which involve the activation of caspases. Distinct cell types differ in the mechanisms by which the death receptor Fas induces apoptosis. In type I cells such as lymphocytes, activation of effector caspases such as caspase-3 or -7 following the Fas-induced activation of caspase-8 suffices for cell killing. On the other hand, in type II cells which include hepatocytes and pancreatic β-cells, caspase cascade amplification via caspase-8-mediated activation of the pro-apoptotic Bcl-2 family member Bid (BH3 interacting domain death agonist) is essential (Kaufmann *et al*., 2007; McKenzie *et al*., 2008). A regulatory cascade eventually leads to the activation of effector caspases which are responsible for the downstream caspase activities which include cleavage of cellular proteins such as cytoskeletal components, resulting in typical morphological changes observed in cells undergoing apoptosis (Kothakota *et al*., 1997).

MCF-7 cells are not known to express full length caspase-3 due to a 47-base pair deletion within the exon of the caspase-3 gene. Since then, there had been continuous debates regarding the ability of MCF-7 to undergo apoptosis (Jänicke *et al*., 1998; Blanc *et al*., 2000). The MCF-7 cell line serves as an important tool for the study of caspase-3

dependent and independent effects. Many studies have reported that MCF-7 cells lack caspase-3 (Devarajan *et al*., 2002; Mooney *et al*., 2002; Jänicke, 2009; Zhong *et al*., 2009). Since the expression of caspase-3 was reported to be deficient in MCF-7 cells, caspase-3 cleavage products will not be observed through Western blotting. However, caspases-3 and -7 have essentially the same function as they have similar substrate specificity. Thus, the caspase-3 substrate used in this study, DEVD, serves as the same substrate for caspase-7. Therefore, the activation of caspase-3 as observed in this study might indicate the activation of caspase-7 instead, as caspase-7 is expressed in MCF-7 cells and it exhibits a certain degree of functional redundancy with caspase-3 (Choi *et al*., 2009). Nonetheless, arguments pertaining to the deficiency of caspase-3 in MCF-7 cells are no longer valid in recent years as shown in numerous studies (Gooch & Yee, 1999; Yang *et al*., 2006; Zhang e*t al*., 2006;Alshatwi *et al*., 2010). The activation of caspase-3 in this study is in accordance with the study by Karimian *et al*. (2014) who reported that the hexane extract of *Ferulago angulata* leaves induced mitochondrial-dependent apoptosis in MCF-7 cells via the activation of caspase-3. Moreover, the caspase-3 activation observed in this study might also be due to the different variants or strains of MCF-7 used (Gooch & Yee, 1999). Hence, caspase-3 is referred to as caspase-3/7 thereafter in this thesis.

The caspase activity shown in this study suggests that the fractions and pure compounds inhibited the proliferation of MCF-7 cells by inducing apoptosis via both extrinsic (ligand-receptor mediated) and intrinsic (mitochondrial mediated) pathways, since caspase-8, -9 and -3/7 were all activated (Figures 4.28 – 4.36). The mechanism by which caspase-8 is activated is unclear. However, since autoactivation induced by oligomerization can lead to activation of caspase-8, it could be possible that the samples induced oligomerization of caspase-8 (Muzio *et al*., 1998). In the extrinsic pathway,

interaction of ligands (such as TNF or Fas) with their death receptors, Fas-associated death domain (FADD), leads to activation of initiator caspase, i.e., caspase-8. The executioner caspase, caspase-3/7, appears as a zymogen in cells and has no activity until it is cleaved by an initiator caspase (caspase-8 or 9) after being stimulated by apoptotic stimuli. Activated caspase-8 cleaves and activates caspase-3/7, which subsequently cleaves various substrates, leading to apoptosis (Walters *et al*., 2009).

In the intrinsic pathway, caspase-8 cleaves BID (pro-apoptotic member of Bcl-2 family) and causes the release of cytochrome c from the mitochondria. Cytochrome c together with apoptotic protease activating factor-1 (APAF-1), ATP and procaspase-9 form an apoptosome, which then activates caspase-9. Caspase-9 in turn activates caspase-3/7 (Li *et al*., 2004). Caspase-9 and caspase-3/7, both of which are the members of the cysteine protease family, have been identified as the major regulators of apoptosis. Caspase-9 and caspase-3/7 are not only involved in the initiation but also in the execution phase of apoptosis by cleaving more than 400 substrates, including various protein kinases, intracellular proteins, molecules involved in DNA repair and many nuclear and cytoplasmic components (Baharara *et al*., 2015). This cleavage mediates most of the morphological and biological alterations in apoptotic cells such as cell shrinkage, chromatin condensation and DNA fragmentation, and subsequently interferes with the survival pathway which then leads to apoptotic cell death (Earnshaw *et al*., 1999).

In agreement with the results obtained in this study, Wong *et al*. (2013) reported that the exposure of MCF-7 cells to *Vernonia amygdalina* extract for 48 h resulted in activation of caspases-7, -8 and -9, suggesting that both extrinsic and intrinsic apoptotic pathways were activated. The results obtained in a study by Li *et al*. (2012) indicated that the methanolic extract of *Momordica charantia* increased caspase-3 activity in Hone-1,

AGS, HCT-116, and CL1-0 cells in a time-dependent manner. An increase in the caspase-3/7 and -6 activities was noted in the human malignant carcinoid of the small intestine, KRJ-1 cells, treated with the subfractions of *Trailliaedoxa gracilis* (Svejda *et al*., 2010). Similar results were obtained in a study by Chandrappa *et al*. (2014) whereby the treatment of the human hepatoma cell line, HepG2, by quercetin isolated from *Carmona retusa* increased caspase-3 activity after 24 and 48 h of incubation. In recent research, the human non-small-cell lung cancer A549 cells, were treated with dioscoreanone for 24, 48 and 72 h and showed a time-dependent activation of caspase-3, peaking at 24 h of treatment and then decreasing over time (Hansakul *et al*., 2014).

In contrast to the results obtained in this study, Chao *et al*. (2012) reported that the α-tomatine induced cell death was independent of caspase activation as caspases-8, -9 and -3 were not activated even after exposure to a high concentration of α-tomatine in HL60 and K562 cells. This may be a unique characteristic of α-tomatine as a prior study by Yang *et al*. (2002) also showed that α-tomatine-induced EL4 thymoma cell death was independent of caspase activation. Moreover, many other previous studies have shown that there are natural products that do not require caspase activation for the induction of leukaemic cell death (Elangovan & Hsieh, 2008; Iwasaki *et al*., 2009). However, in this study, the fractions and pure compounds isolated from the seaweeds and sponge induced apoptotic cell death via the activation of both extrinsic and intrinsic pathways (as indicated by the increase in caspase-8 and -9 activities). Caspase-3/7 was then activated and this ultimately leads to apoptotic cell death.

DNA fragmentation is one of the hallmarks of apoptosis. The activity of caspase-3/7 is closely related to the initiation of DNA fragmentation. Under normal physiological conditions, the DNA fragmentation factor-40, DFF-40, also known as the caspase

activated DNase, CAD, exists as an inactive zymogen with its inhibitor, DNA fragmentation factor-45 (DFF-45) also known as the inhibitor of caspase activated DNase, ICAD (Li *et al*., 2012). The proteolytic inactivation of the DFF-45 by caspase-3/7 upon treatment of MCF-7 cells with the extracts releases the active endonuclease, DFF-40, followed by a rapid fragmentation of the nuclear DNA (Zhivotovsky *et al*., 1997). It is thus suggested that caspase-3/7 is essential for DNA fragmentation during apoptosis.

Apoptosis involves the activation of caspases and the subsequent cleavage of several substrates. One of which is poly (ADP-ribose) polymerase (PARP) (Slee *et al*., 2001). PARP is involved in DNA repair by catalysing the synthesis of poly (ADP-ribose), binding to DNA strand breaks and modifying nuclear proteins (Shu *et al*., 2009). However, the ability of PARP to repair DNA damage is attenuated by cleavage of PARP via the activation of caspase-3/7 (Soldani & Scovassi, 2002). Hence, the observed DNA fragmentation as shown in Figures 4.37 - 4.39 indicates the occurrence of fragmentation of the nucleosomal DNA.

The DNA laddering pattern observed upon treatment of MCF-7 with the partially purified fractions and pure compounds in this study, as shown in Figures 4.9(a) – (c), is a characteristic of DNA damage and apoptosis. This is in accordance with the results obtained in numerous studies using various plant extracts. Lawal *et al.* (2012) accounted that the ethanol extract of *Securidaca longepedunculata* induced DNA fragmentation in Ehrlich ascites carcinoma, comparable to the effect of a commercially available chemotherapy drug, 5-fluorouracil. In a study by Li *et al*. (2012), after the caspase-3 activity was activated by the methanolic extract of *Momordica charantia*, DFF-45 was cleaved and PARP was activated, leading to DNA fragmentation. Solowey *et al*. (2014)

reported that treatment of Colo205 cells with an *Urtica membranacea* extract for 24, 48 and 72 h exhibited DNA laddering patterns, indicating that the extract induced apoptotic DNA fragmentation. Similar outcomes were obtained in a number of studies in which treatment of cancer cell lines with extracts of plants or natural products resulted in DNA fragmentation and eventually apoptosis (Gandhiappan & Rengasamy, 2012;Dwivedi *et al*., 2013; Maliyakkal *et al*., 2013; Samarghandian & Shabestari, 2013; Sofi *et al*., 2014).

5.12 Proteomics Profiling of the Differentially Expressed Proteins in Untreated and Treated MCF-7 Cells

Comprehending cell signalling in cancer is of paramount importance as it may provide insights concerning the potential targets which play a role in the induction of apoptosis for the development of anticancer agents. For the past decade, modern cancer therapies which include chemotherapy, surgery and immunotherapy are deemed relatively unsuccessful when factors such as safety issues, effectiveness and cost were taken into consideration (Shoeb, 2006). Although chemotherapy was advocated at a point of time, recent studies have proven that these chemotherapeutic agents were no longer effective as they used to be. This is due in part to the capability of cancer cells to evoke other survival pathways, rendering the chemotherapeutic agents less cytotoxic (Tang *et al*., 2013).

Determination of the target proteins is the ideal way to elucidate the mechanism of action of an effective compound. This study used the 2D gel electrophoresis proteomics approach to investigate the alterations of the protein expression profile in MCF-7 cells as a result of treatment with the partially purified fractions of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda*. Instead of pure compounds, the partially purified fractions were selected for this proteomics study as it was hypothesised that

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there is no single or universal compound that can possibly elicit a particular effect, for instance, the antiproliferative effect. The antiproliferative effect is attributed to the crosstalk and interactions between various signalling pathways which include the involvement of a tremendous amount of signalling molecules (or compounds) (Garcia-Becerra *et al*., 2013). Hence, the compounds either work synergistically or antagonistically to elicit the antiproliferative effect. Since no single or pure compound is responsible for the antiproliferative activity, partially purified fractions of the seaweeds and sponge were chosen instead of the pure compounds (camptothecin, pseudopeletierine and lycodine). A total of 42 proteins were differentially expressed as a result of the treatment. The differentially expressed proteins were grouped into 10 biological processes based on their biological functions (Table 4.29). Only those proteins that are related to cell death were considered in greater detail as the main purpose of the study is to investigate the molecular events occurring during the treatment of MCF-7 cancer cells with the partially purified fractions.

The key factor to the survival of cancer cells is the inhibition of apoptosis, which is accomplished by the suppression of the expression of pro-apoptotic proteins such as p53, Bax and PARP-1, as well as elevating the expression of anti-apoptotic proteins such as Bcl-2 (Reed, 1999). The results obtained in this study showed that the expressions of p53, Bax and PARP-1 in the MCF-7 cells treated with the partially purified fractions were significantly up-regulated, accompanied by the suppression of Bcl-2 expression (Table 4.29).

p53, Bax, PARP-1 and Bcl-2 are important regulators of apoptosis. p53 is the most extensively studied tumour suppressor which acts in response to a variety of cellular stresses to mediate diverse forms of antiproliferative processes (Fridman & Lowe,

2003). Since the discovery of p53, many studies have focused on its functions as well as its role in cancer. Besides being involved in apoptosis, p53 is also a key player in cell cycle regulation, differentiation, development, cellular senescence and gene amplification (Oren & Rotter, 1999). In fact, defects in the p53 tumour suppressor gene have been associated to more than 50% of human cancers (Bai & Zhu, 2006).

The pro-apoptotic effector protein (Bax) and anti-apoptotic Bcl-2 protein (Bcl-2) belong to the Bcl-2 protein family. Activation of Bax by direct interaction with a member of the BH3-only protein family is required for the permeabilisation of the mitochondrial outer membrane. This activation causes conformational changes in Bax, leading to their oligomerisation within the mitochondrial outer membrane. These Bax oligomers will then permeabilise the mitochondrial outer membrane either indirectly by inducing lipid pores or by directly forming pores themselves (Lopez & Tait, 2015). This would in turn lead to MOMP, the release of mitochondrial cytochrome c, activation of caspases and finally apoptosis. On the other hand, the anti-apoptotic Bcl-2 protein prevents MOMP and apoptosis by binding to activated Bax or to BH3-only proteins, thereby preventing the interaction between Bax and the BH3-only proteins (Lopez & Tait, 2015).

PARP-1 is a chromatin-associated protein which is the most abundant isoform of the PARP superfamily (Amé *et al*., 2004). The normal function of PARP-1 is the routine repair of DNA damage in response to various cellular stresses. Interestingly, the repair of double strand breaks is decreased by the over-expression of PARP-1, indicating that its enzymatic activity is not entirely essential to all repair processes (Susse *et al*., 2004). PARP-1 is also a preferred substrate for several proteolytic actions of suicidal proteases such as caspases, calpains and matrix metalloproteinases (MMPs). Studies have shown that PARP-1 is also involved in many biochemical and molecular signalling processes

which include apoptosis, cell proliferation, DNA repair and transcriptional regulation (Phulwani & Kielian, 2008; Hernandez *et al*., 2009; von Lukowicz *et al*., 2009).

In this study, the up-regulation of the pro-apoptotic protein p53 could induce its transcriptional activity to produce other pro-apoptotic proteins such as Bax, Bid NOVA and PUMA, which subsequently lead to the release of cytochrome c and activation of caspases (Schuler *et al*., 2000; Fridman & Lowe, 2003). The highly expressed Bax protein in MCF-7 cells after treatment could induce changes in mitochondrial outer membrane permeabilisation (MOMP) thus resulting in the release of cytochrome c from mitochondria which in turn can induce proteolytic activation of procaspase-9 (Yip & Reed, 2008). This subsequently activates caspase-3/7 as shown in the results of this study in Figure 4.8(a) and Figure 4.8(b), inhibits the inhibitors of apoptosis (IAPs) and ultimately lead to the induction of apoptosis in treated MCF-7 cells (Chang & Yang, 2000; Yip & Reed, 2008). The up-regulation of PARP-1 in the treated MCF-7 cells triggers apoptotic cell death (Chaitanya *et al*., 2010).

Heat shock proteins (HSPs) are inhibitors of apoptosis and high levels of HSPs are usually detected in tumours (Samali & Orrenius, 1998; Jaattela, 1999; Vayssier & Polla, 1999). Inducible Hsp70 was suggested to have multiple roles in cytoprotection against apoptosis. Consistent with this proposal, high levels of Hsp70 prevent stress-induced apoptosis (Jolly & Morimoto, 2000). Hsp70 protects cells against apoptosis by binding to pro-apoptotic proteins such as p53 and c-myc (Koskinen *et al*., 1991). In addition, Hsp70 also interacts with and is repressed by Bag11, an anti-apoptotic protein that enhances the activities of Bcl-2 and Raf-1 (Takayama *et al*., 1997; Jaattela *et al*., 1998).

In prior studies, heat shock protein 70 (Hsp70) was shown to be crucial for the survival of cancer cells (Beere & Green, 2001; Dudeja *et al*., 2010). It was observed that most proteins critical for modulating protein assembly and folding or HSPs, were down-regulated (Table 4.29). The down-regulation of these proteins attenuated the expression of chaperone proteins such as the 78 kDa glucose-regulated protein. In a study by Dudeja *et al*. (2010), Hsp70 inhibits apoptosis in cancer cells via two mechanisms: the attenuation of cytosolic calcium and the stabilisation of lysosomes. In this study, both the heat shock 70 kDa protein 6 and heat shock cognate 71 kDa protein were down-regulated in the fractions-treated MCF-7 cells, implying that there might be alteration in the intracellular calcium level in MCF-7 cells, thus leading to the induction of apoptotic cell death (Chen *et al*., 2013; Tang *et al*., 2013).

Being the central energy resource for cells, glucose metabolism is rather complicated. Increased aerobic glycolysis, glutamine metabolism and gluconeogenesis have been closely associated to therapeutic resistance in cancer. It was speculated that cell proliferation was promoted by deregulated cancer metabolism due to increased energy production and metabolite synthesis, which decreased drug-induced apoptosis, thus conferring therapeutic resistance (Zhao *et al*., 2013). As shown in the results in Table 4.29, the partially purified fractions were noted to inhibit the glycolytic pathway and energy production in MCF-7 cells by down-regulating the glycolytic protein, triose phosphate isomerase. The down-regulation of the glycolytic protein reduces the production of vascular endothelial growth factor (VEGF) and therefore inhibits tumour angiogenesis and cancer progression (Tang *et al*., 2013).

Scientific literature concerning the use of 2D-PAGE for proteome profiling in extract-treated cancer cells is still scarce to date. In a study conducted by Tang *et al.* (2015), the

apoptotic protein, p53, was up-regulated in treated human melanoma (MeWo) cells. This could induce apoptosis through its transcriptional activity to produce pro-apoptotic proteins, which subsequently leads to the release of cytochrome c and activation of caspases. In agreement with the results obtained in this study, Tang *et al*. (2013) reported that apoptosis was induced in the *Phyllanthus*-treated prostate cancer (PC-3) cells through the up-regulation of the pro-apoptotic Bax protein as well as the down-regulation of the anti-apoptotic protein, Bcl-2. In the same study, *Phyllanthus* extracts inhibited the glycolytic pathway and energy production in prostate cancer cells by down-regulation of HIF-1α protein. Similar results were also reported by Dihal *et al*. (2008) who observed that glycolytic proteins were significantly down-regulated.

5.13   Validation of Differentially Expressed Proteins by Western Blotting

The differentially expressed apoptotic proteins (p53, Bax, PARP-1 and Bcl-2 proteins) obtained from the 2D-PAGE analysis were selected for further validation by Western blotting following 2D-PAGE. The fold change from 2D-PAGE was higher than those from Western blots due to variations between different techniques but the direction of change (up- or down-regulation) was consistent.

The regulation of p53 is of utmost importance to enable normal cell division as p53 is an efficient inhibitor of cell growth, inducing cell cycle arrest and/or apoptotic cell death (Mahfudh & Pihie, 2008). There are numerous mechanisms through which p53 is regulated. The major mechanisms include regulation of p53 protein levels, control of the localisation of p53 protein and the modulation of the p53 activity, particularly as a sequence-specific transcription factor (Vousden, 2002).

Both biochemical and genetic evidence imply that the Bcl-2 family of proteins, including the pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins, can regulate apoptotic cell death induced by caspases (Ola *et al*., 2011). The Bax/Bcl-2 ratio is recognised as a key factor in apoptosis. An increase in the Bax/Bcl-2 ratio favours apoptosis by releasing cytochrome c from the mitochondria into the cytosol and promoting activation of caspase-9 and subsequently binding to the apoptotic protease activating factor-1 (APAF-1), which eventually results in the activation of the executioner caspase-3/7 and cleavage of PARP (Bossy-Wetzel & Green, 1999; Pandey *et al*., 2000).

Poly (ADP-ribose) polymerase-1 (PARP-1) is one of the major targets for caspases and the cleavage of PARP-1 into the 116 and 85 kDa fragments triggered by activation of caspases is considered as a hallmark of apoptosis (Srivastava *et al*., 2014).

In this study, the expression level of p53 was up-regulated in the fractions-treated MCF-7 cells possibly due to the increasing of p53 stability. A previous report stated that the activation of p53 could be invoked by as little as one DNA double strand break which could dramatically increase the stability of p53 as the signal can be amplified in the cells (Lakin & Jackson, 1999). Treatment of MCF-7 cells with the partially purified fractions caused an increase in the expression level of Bax and a decrease in the level of Bcl-2. Therefore, the Bax/Bcl-2 ratio was augmented, indicating that mitochondria-mediated apoptosis is involved in the partially purified fractions-induced cell death in MCF-7 cells (Figures 4.45 – 4.48). As shown in the Western blot analysis (Figures 4.45 – 4.48), treatment of MCF-7 cells with the partially purified fractions led to significant increase in PARP-1 cleavage. The activation of caspases-8 and -9, which subsequently activated caspase-3/7 (Figures 4.28 – 4.33), triggered the cleavage of PARP-1,

suggesting the activation of both the extrinsic and intrinsic pathway of apoptosis to induce cytotoxicity in MCF-7 cells.

Karimi *et al*. (2010) reported that an extract from the terrestrial plant *Brachylaena ramiflora* (Asteraceae) induced p53-dependent apoptosis in human tumour cells (human Saos-2 osteosarcoma, H1299 lung adenocarcinoma cells and human HCT116 colon carcinoma cells). Ravi *et al*. (2012) reported that the induction of apoptosis by the ethanolic extract of the aerial parts of *Pupalia lappacea* (L) Juss (Amaranthaceae) on chronic myeloid leukemia K562 cells was dose dependent with the activation of p53. An increase in the Bax/Bcl-2 ratio as well as the cleavage of PARP was also observed. In a study conducted by Abdullah *et al*. (2010), treatment of a natural compound from *Hydnophytum formicarium* induced apoptosis in MCF-7 cells by the up-regulation of the pro-apoptotic protein, Bax, without affecting the expression of the anti-apoptotic Bcl-2. Yeh *et al*. (2011) investigated the role of Bcl-2 family of proteins in gallic acid-induced apoptosis and observed an increase in the expression of Bax protein and a decrease in the expression of Bcl-2 protein in HL-60 cells. The results of this study agree with the results obtained by Li *et al*. (2012) who reported that the extract of *Momordica charantia* increased the Bax/Bcl-2 ratio in treated human nasopharyngeal carcinoma cells (Hone-1), gastric adenocarcinoma cells (AGS), colorectal carcinoma cells (HCT-116), and lung adenocarcinoma cells (CL1-0). Wang *et al*. 2009a reported that curcumin significantly induced growth inhibition and apoptosis of the human colon cancer cell line, HT-29, by down-regulation of Bcl-2 and up-regulation of Bax and PARP-1. Gridling *et al*. (2009) reported that the apoptotic cell death triggered by the treatment of the dichloromethane extract of *Pluchea odorata* in promyelocytic leukemia (HL-60) cells was brought about by the activation of caspase-3 and the subsequent cleavage of PARP-1 by the activated caspase-3. In a report by Srivastava *et al*. (2014),

treatment of the human B cell leukemia (NALM6) cells with the methanolic extract of Sapota fruits activated the mitochondrial pathway of apoptosis through the cleavage of PARP-1 and activation of caspase-9.
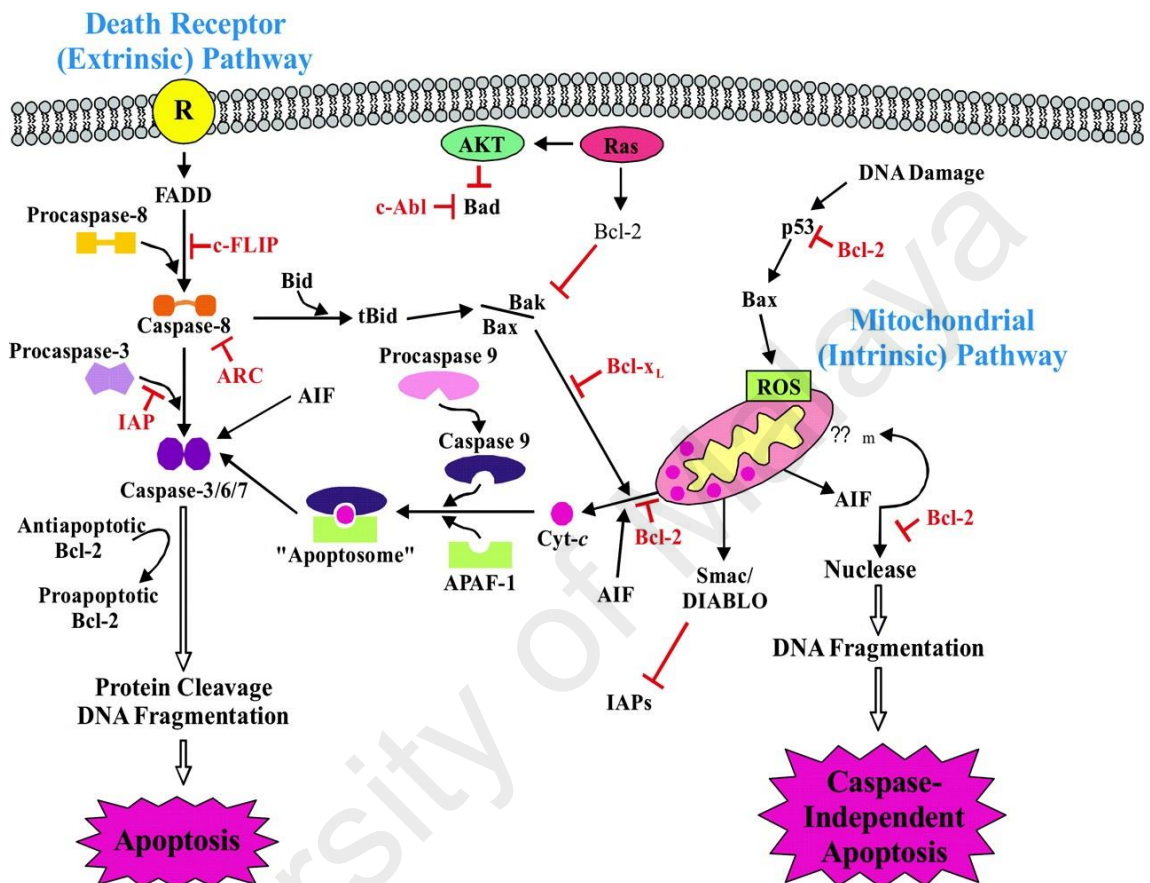
Figure 5.1: Apoptosis as the mode of cell death through the extrinsic and intrinsic pathways. The up-regulation of the pro-apoptotic proteins, p53, Bax and PARP-1 and the down-regulation of the anti-apoptotic Bcl-2 protein eventually result in DNA fragmentation and apoptosis.
[Figure from Activation of K$^+$ channels: an essential pathway in programmed cell death by Remillard & Yuan, 2004. *American Journal of Physiology – Lung Cellular and Molecular Physiology*, *286*(1), L49-L67].

5.14   General Summary

The seaweeds, *P. tetrastomatica*, *C. racemosa* and *T. ornata*, and the sponge, *S. vagabunda*, extracts showed higher superoxide and hydroxyl radical scavenging activity than the reference compounds, quercetin and rutin. In this study, the cytotoxic compounds responsible for the antiproliferative activity in the seaweeds and sponge

samples were identified via LC-MS profiling. The cellular mechanisms associated with the decreased viability of the MCF-7 cells upon treatment with the partially purified fractions and pure compounds were also studied. Upon treatment of the MCF-7 cells with the fractions and pure compounds, a collapse in MMP, activation of caspase-3, 8 and 9 activity and DNA fragmentation were observed. These cellular events indicated the induction of apoptosis as a mode of cell death. To further verify this hypothesis, a proteomics approach using the 2D gel electrophoresis and Western blotting were used to study the protein expression profiles of the MCF-7 cells treated with the partially purified fractions. Treatment of the MCF-7 cells with the fractions caused up-regulation of the pro-apoptotic p53, Bax and PARP-1 proteins and down-regulation of the anti-apoptotic Bcl-2 protein. In summary, the results obtained in this study indicate for the first time that the partially purified fractions of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* induced apoptosis, thus making them a promising chemotherapeutic agent for further evaluation in the treatment of cancer-related pathologies.

This study is the first report on the antioxidant and antiproliferative activities of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* in MCF-7 cells. The cellular and biochemical mechanisms through which MCF-7 cells undergo apoptosis upon treatment with the extracts were also the first reported. In addition, this is also the first study that reported the protein expression profiles through a proteomics approach.

CHAPTER 6

CONCLUSION

6.1   Conclusion

The crude extracts of the seaweeds (*P. tetrastromatica*, *C. racemosa* and *T. ornata*) and sponge (*S. vagabunda*) have high levels of antioxidants with free radical scavenging ability. These extracts showed higher superoxide and hydroxyl radical scavenging activities than the reference compounds (quercetin and rutin). Hence, these extracts can be used as substitutes to replace harmful synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated toluene (BHT) which arecarcinogenic and tumourigenic at higher doses.

Bioassay guided fractionation was performed on the most potent crude extract with the highest antiproliferative activity. The partially purified fractions also demonstrated significant antiproliferative effects against MCF-7 cells compared to the crude extracts. From the results obtained in this study, the crude extracts and the partially purified fractions are more appropriate to be used as chemotherapeutic agents as the standard chemotherapeutic drug, doxorubicin, was cytotoxic to normal breast cells even at low doses.

LC-MS profiling was conducted on the most potent fraction with the highest antiproliferative activity to identify the compounds accountable for the antiproliferative activity. LC-MS analysis indicated the presence of cytotoxic compounds responsible for the antiproliferative activity, which include camptothecin, pseudopelletierine and lycodine.

The antiproliferative activity of the partially purified fractions and the pure compounds was elicited through the induction of apoptosis. This is proven through a number of cellular mechanisms involved in this study which include a collapse of MMP, activation of caspase-3, 8 and 9 activities and the formation of DNA fragmentation in the fractions- and compounds-treated MCF-7 cells. The induction of apoptosis was via the activation of both the extrinsic and intrinsic pathways, whereby there was an increase in caspase-8 and -9 activities, respectively. Furthermore, the collapse in MMP explains that the occurrence of apoptosis was mitochondrial-dependent.

The proteomics profiling of the fractions-treated MCF-7 cells suggested that the primary events leading to apoptosis and eventually cell death were due in part to the up-regulation of the pro-apoptotic proteins (p53, Bax and PARP-1) and the down-regulation of the anti-apoptotic Bcl-2 protein. In addition, the down-regulation of the heat shock proteins (HSPs), which are the inhibitors of apoptosis involved in protein assembly and folding, certainly plays a crucial role in the induction of apoptosis.

The efficacy of the crude extracts and the partially purified fractions of the four marine organisms against MCF-7 cells without causing significant cytotoxicity in normal breast cells support their potentials as sources of natural chemotherapeutic agents.

6.2 Limitations of the study

The seaweeds and sponge in this study are distributed in many other areas around the world. Therefore, the area and also the time of year that the samples were collected might influence the content of the seaweeds and the sponge samples. This could introduce a small difference in the extent or intensity of the activities observed in this study.

The MTT assay used in this study for the determination of antiproliferative activity does not measure the bioavailability or the pharmacokinetics of the compounds despite their important characteristics pertaining to the actions of the compounds in the body.

6.3 Future Studies

Exploration of the *in vitro* pharmacological properties of the partially purified fractions of *P. tetrastromatica*, *C. racemosa*, *T. ornata* and *S. vagabunda* revealed that the compounds present in these fractions may represent a new generation of potential drug candidates for the treatment of cancer. However, further *in vivo* studies (such as animal studies) and clinical trials to assess the bioavailability, pharmacokinetic and pharmacodynamics profiles in humans are required to ascertain the efficacy, safety and mechanisms of action of the cytotoxic compounds (either individually or synergistically) prior to application in the pharmaceutical industry as natural therapeutic agents.

Limitations are inevitable in consideration of therapeutic application of the partially purified fractions as anticancer agents due to the relatively high production cost, either through purification from the crude extracts or via cloning. Hence, the design of new chemotherapeutic drugs involving the knowledge of bioinformatics and molecular

docking studies which mimic the mode of antiproliferative action of the partially purified fractions could be a promising alternative.

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APPENDIX A

List of Conferences Attended

Conferences	Date	Title of Poster
36th Annual Conference of Malaysian Society of Biochemistry and Molecular Biology (MSBMB)	27th-28th July 2011	Anticancer and antioxidant activities of several marine organisms.
38th Annual Conference of Malaysian Society of Biochemistry and Molecular Biology (MSBMB)	28th-29th August 2013	Identification of phytochemicals from several marine organisms that lead to the induction of apoptosis and antiproliferative effects.
39th Annual Conference of Malaysian Society of Biochemistry and Molecular Biology (MSBMB)	25th-26th June 2014	Induction of apoptosis in human breast cancer cells by camptothecin, pseudopelletierine and lycodine.
International Conference on Antioxidants and Degenerative Diseases (ICADD 2015)	3rd – 4th June 2015	Antioxidant and antiproliferative effects of several marine algae on MCF-7 cells.
CENAR Colloquium on Drug Development from Natural Products 2015	12th August 2015	Marine algae in the fight against breast cancer.
7th Biennial Meeting of Society for Free Radical Research-Asia (SFRR-Asia 2015)	29th November 2015 – 2nd December 2015	Proteomics of apoptosis induced in breast cancer cells by marine organisms.

APPENDIX B

List of Publications in ISI-Indexed Journals

1. Chia, Y.Y., Kanthimathi, M.S., Jayakumar, R., Khoo, K.S., & Cheng, H.M. (2015). Antioxidant, antiproliferative, antigenotoxic and cytoprotective effects of the methanolic extract of *Padina tetrastromatica* on human breast adenocarcinoma and embryonic fibroblast cell lines. *Frontiers in Life Sciences*, doi: 10.1080/21553769.2015.1051245.

2. Chia, Y.Y., Kanthimathi, M.S., Khoo, K.S., Jayakumar, R., Cheng, H.M., & Yap, W.S. (2015).Antioxidant and cytotoxic activities of threespecies of tropical seaweeds. *BMC Complementary and Alternative Medicine*, *15*(339), 1-14.

APPENDIX C

List of Manuscripts in Preparation

1. Caspase-dependent induction of apoptosis in human breast cancer cells by camptothecin, pseudopelletierine and lycodine isolated from three tropical seaweeds.

2. Evaluation of the antiproliferative and antioxidant activities of the sponge, *Spheciospongia vagabunda*.

3. Suppression of breast cancer cells, MCF-7 and induction of apoptosis by *Padina tetrastromatica*, *Caulerpa racemosa*, *Turbinaria ornata* and *Spheciospongia vagabunda* through the Bax/Bcl-2, p53 and PARP-1 pathway.