CYTOTOXIC ACTIVITY OF BIOACTIVE PEPTIDES DERIVED FROM MALAYSIAN MARINE SPONGE, *XESTOSPONGIA TESTUDINARIA*, AND SOFT CORAL, *SARCOPHYTON GLAUCUM*, ON HELA CELLS

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By

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ABSTRACT

CYTOTOXIC ACTIVITY OF BIOACTIVE PEPTIDES DERIVED FROM MALAYSIAN MARINE SPONGE, *Xestospongia testudinaria*, AND SOFT CORAL, *Sarcophyton glaucum*, ON HELA CELLS

Quah Yixian

Resistance and side effects are common problems for anticancer drugs used in chemotherapy. Thus, continued research to discover novel and specific anticancer drugs is obligatory. Bioactive peptides of marine organisms are valuable resources for the discovery of potent and novel anticancer drugs. The marine biodiversity of Malaysia is a reservoir of bioactive peptides that has not been intensively harnessed for new drug development. Hence, this project aimed to purify and identify cytotoxic peptides from the protein hydrolysates of the giant barrel sponge (*Xestospongia testudinaria*) and soft coral (*Sarcophyton glaucum*) guided by a cytotoxicity assay based on the human cervical cancer cell line (HeLa). Briefly, proteins were isolated from the marine samples followed by enzymatic hydrolysis. The most potent hydrolysates were purified consecutively with ultrafiltration membrane, gel filtration chromatography, solid phase extraction and reversed-phased high performance liquid chromatography. Sequences of potential cytotoxic peptides were determined by liquid chromatography-tandem mass spectrometry. The identified sequences were chemically synthesized and then validated for cytotoxicity. Two peptides were identified from the most cytotoxic RP-HPLC fraction of *X. testudinaria*: KENPVLSLVNGMF and LLATIPKVGVFSIL. Notably, the cytotoxicity of KENPVLSLVNGMF was 3.8-fold more potent

than anticancer drug 5-fluorouracil (5FU). Furthermore, KENPVLSLVNGMF show only marginal 5% cytotoxicity to Hek293, a non-cancerous, human embryonic kidney cell line, when tested at 0.67 mM. Besides, the half-life of KENPVLSLVNGMF peptide was 3.2±0.5 h in human serum *in vitro*. In addition, three peptides AERQ, AGAPGG and RDTQ were identified from the most cytotoxic SPE fraction of *S. glaucum*. Markedly, the cytotoxicity of AERQ, AGAPGG and RDTQ was on average 4.76-fold more potent than 5FU. In conclusion, four novel cytotoxic peptides were successfully isolated, purified and identified from *X. testudinaria* and *S. glaucum*. Results obtained highlight the promising nature of Malaysian marine biodiversity as a source of novel cytotoxic peptides with potential applications in future drug development.

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All glory be to God.

FACULTY OF SCIENCE

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Date: _____

SUBMISSION OF DISSERTATION

It is hereby certified that ***Quah Yixian*** (ID No:***14ADM01185***) has completed this dissertation entitled "Cytotoxic Activity of Bioactive Peptides Derived from Malaysian Marine Sponge, *Xestospongia testudinaria*, and Soft Coral, *Sarcophyton glaucum*, on HeLa Cells" under the supervision of Assoc. Prof. Dr. Chai Tsun Thai (Supervisor) from the Department of Chemical Science, Faculty of Science, and Assist. Prof. Dr. Nor Ismaliza Binti Mohd Ismail (Co-Supervisor) from the Department of Biological Science, Faculty of Science.

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APPROVAL SHEET

This dissertation entitled "**CYTOTOXIC ACTIVITY OF BIOACTIVE PEPTIDES DERIVED FROM MALAYSIAN MARINE SPONGE, *XESTOSPONGIA TESTUDINARIA*, AND SOFT CORAL, *SARCOPHYTON GLAUCUM*, ON HELA CELLS"** was prepared by QUAH YIXIAN and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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LIST OF ABBREVIATIONS

5FU	5-fluorouracil
A549	Human lung adenocarcinoma epithelial
ACE	Angiotensin-converting enzyme
ACN	Acetonitrile
AGS	Human gastric cancer
AO/EB	Acridine orange/ethidium bromide
BSA	Bovine serum albumin
Caco-2	Human colon cancer
Da	Dalton
Daoy	Human medulloblastoma
DDA	Data directed analysis
DH	Degree of hydrolysis
DLD-1	Human colon cancer
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DPP IV	Dipeptidyl peptidase IV
DU-145	Human prostate cancer
EB	Ethidium bromide
EC_{50}	Half maximal effective concentration
ESI	Electrospray ionization
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GF	Gel filtration chromatography

h	Hour(s)
H-1299	Human lung cancer
HCT-116	Human colon carcinoma
Hek293	Human embryonic kidney cell line
HeLa	Human cervical cancer
HepG2	Human liver cancer
HL-60	Human promyelocytic leukemia
HT-29	Human colorectal cancer
IC_{50}	Half maximal inhibitory concentration
IUCN	International Union for Conservation of Nature
kDa	Kilo dalton
L1210	Mouse lymphocytic leukemia
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LH-RH	Luteinising hormone releasing hormone
L-O2	Human normal liver
LSD	Fisher's least significant difference
MALDI	Matrix Assisted Laser Desorption/Ionization
MCF-7	Human breast cancer
MDA-MB-231	Human breast cancer
MGC-803	Human gastric cancer
min	Minute(s)
ML-2	Human acute myelomonocytic leukemia
MOLT-4	Human acute lymphoblastic leukemia
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
MWCO	Molecular weight cut-off
NCI-H446	Human small cell lung cancer
NCI-H510	Human small cell lung cancer
NCI-H69	Human small cell lung cancer
NCI-H82	Human small cell lung cancer
NCL-H1299	Human lung cancer
P388	Mouse leukemia
PC-3	Human prostate cancer
PI	Propidium iodide
ppm	Parts per million
Q-TOF	Quadrupole time-of-flight
RP-HPLC	Reversed-phase high-performance liquid chromatography
RPMI-8226	Human myeloma
SCLC	Small cell lung cancer
SCUBA	Self-contained underwater breathing apparatus
SGC-7901	Human gastric cancer
SPE	Solid phase extraction
SUP-T1	Human T-cell lymphoblastic
TFA	Trifluoroacetic acid
THP-1	Human monocytic
U87	Glioma cells
U-937	Human histiocytic lymphoma

UF	Ultrafiltration
US-FDA	United States Food and Drug Administration
VEGF	Vascular endothelial growth factor
WHO	World health organization

CHAPTER 1

INTRODUCTION

Cancer has been reported as one of the largest single causes of morbidity and mortality worldwide. According to the World Health Organization (2017a), cancer accounted for approximately 17% of all global deaths, which is 8.8 million deaths in the year 2015. A statistical report by the GLOBOCAN 2012 projected that the number of new cancer cases will increase by nearly 70% in the next two decades (Ferlay et al., 2013). Unfortunately, chemotherapy, a frequently used cancer treatment, tends to show non-specific cytotoxicity, damaging not only cancerous cells, but also normal tissues (e.g., bone barrow, gut lining and hair follicles) resulting in side effects (e.g., nausea, vomiting, infection, fatigue and loss of appetite) (Gore and Russell, 2003, Liao et al., 2015). Non-specific cytotoxicity demotes the effectiveness of the treatment (Sutradhar and Amin, 2014). This necessitates the search for more specific cytotoxic drugs.

Peptides are attracting considerable interest in the treatment of cancer due to their specificity as well as other advantages such as good cellular uptake (Xiao et al., 2015) and ease of synthesis and modification (Thundimadathil, 2012). Tumor cells express different proteins on the membrane surface; this may commission these peptides to specifically bind to the target tumor cells (Xiao et al., 2015). Excitingly, bioactive peptides derived from natural sources have been found to show inhibitory effect in

various cancer cells, including human cervical, breast, colon, liver, and lung cancer cells (Xiao et al., 2015, Chai et al., 2017, Daliri et al., 2017, Pangestuti and Kim, 2017).

Bioactive peptides are specific protein fragments that possess various physiological functions, including cytotoxic, antibacterial, antihypertensive and immunomodulatory activities (Harnedy and FitzGerald, 2012). Bioactive peptides usually contain 2 to 20 amino acid residues and are inactive within the sequence of the parent protein (Harnedy and FitzGerald, 2012, Chai et al., 2017). These peptides can be liberated by enzymatic proteolysis (*in vitro* enzymatic hydrolysis and gastrointestinal digestion) as well as heating and fermentation (Daliri et al., 2017).

Enzymatic hydrolysis is the most convenient method to obtain bioactive peptides (Bhat et al., 2015). The most widely used proteases in enzymatic hydrolysis are alcalase, α-chymotrypsin, papain, pepsin and trypsin (Qian et al., 2007, Ngo et al., 2012). Generally, active hydrolysates produced from enzymatic hydrolysis are subjected to bioassay-guided purification procedures which involve membrane ultrafiltration (UF), gel filtration chromatography (GF), solid phase extraction (SPE) and reversed-phase high-performance liquid chromatography (RP-HPLC) to purify and isolate the bioactive peptides (Bhat et al., 2015, Chai et al., 2017). The sufficiently purified bioactive peptides were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) and/or Edman degradation for amino acid sequence identification (Chai et al., 2017).

The marine environment comprises nearly 70% of the earth's surface. This diverse marine environment offers numerous unexploited sources of natural products that could be potential candidates for pharmaceutical drugs in cancer treatments (Ruiz-Torres et al., 2017). Among marine organisms, marine invertebrates contributed almost 65% of the marine natural products reported thus far (Hu et al., 2015). In fact bioactive compounds originated from Porifera (mainly sponge) and Cnidaria (mainly coral) accounted for 56.89% of the total bioactive compounds discovered from marine organisms (Hu et al., 2015). Sponges and corals are sessile marine organisms which lack of physical defence mechanisms; therefore the production of a range of secondary metabolites is essential to protecting themselves from harmful predators (Liang et al., 2014, Mioso et al., 2017). Furthermore, compounds that are released into the seawater are likely to be rapidly diluted, hence the compounds need to be extremely potent to be effective (Haefner, 2003).

Xestospongia testudinaria is a maroon giant barrel sponge in the family of Petrosiidae (El-Gamal et al., 2016). *Sarcophyton glaucum*, also known as the rough leather coral, belongs to the family of Alcyoniidae (van Ofwegen, 2010). *X. testudinaria* and *S. glaucum* are common and sometimes dominant species found in Malaysian reefs (Affendi, 2017). They were chosen because of their abundance, more importantly they are not recognized as endangered species according to the IUCN Red List of Threatened Species™ (International Union for Conservation of Nature and Natural Resources, 2017). Previous bioprospecting studies have been limited to non-peptide bioactive

compounds that were derived from these two species (Hegazy et al., 2011, Al-Lihaibi et al., 2014, Abdel-Lateff et al., 2015, El-Gamal et al., 2016, Chao et al., 2017). In spite of this interest among the scientific community, there have been no reports to date of cytotoxic peptides identified from *X. testudinaria* and *S. glaucum*. Hence, to fill in this gap in knowledge, the objectives of this study were:

1. To prepare protein hydrolysates from *X. testudinaria* and *S. glaucum* by using alcalase, chymotrypsin, papain and trypsin.
2. To evaluate the cytotoxic activity of the protein hydrolysate on human cervical cancer (HeLa) cells.
3. To isolate, purify, and identify cytotoxic peptides from the most active protein hydrolysate.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is a complex disease caused by multiple factors, such as unhealthy dietary habits, aging, smoking, sunlight, radiation, and carcinogenic infections (National Cancer Institute, 2015, Xu et al., 2017). Cancer can be defined as a disease in which abnormal cells divide uncontrollably and invade nearby tissues. The latter process is known as metastasis which is a major cause of death from cancers (Guan, 2015).

Cancers remain to be one of the leading causes of death globally, and accounted for 8.8 million deaths in 2015 (World Health Organization, 2017a). It was predicted that over the next 2 decades the number of new cases will increase by about 70% (World Health Organization, 2017a). In response to that the WHO launched the ‘Global Action Plan for the Prevention and Control of Noncommunicable Diseases 2013-2020’ in 2013. This action plan aims to reduce premature mortality by 25% from noncommunicable diseases, including cancers, by 2025. One of the ways to achieve their aim is through early detection and timely treatment (World Health Organization, 2017b).

In Malaysia, the ten most common cancers among the residents from year 2007 to 2011 were breast, colorectal, lung, lymphoma, nasophynx, leukaemia, cervical, liver, ovary and stomach cancers, based on the report published by the National Cancer Institute (2016). Particularly, cervical cancer was the third most common cancer among the women in Malaysia, almost 60% of such cases were detected at stage I and II (National Cancer Institute, 2016). The estimated annual deaths caused by cervical cancer for 2012 was 621, which makes it the 4th leading cause of cancer deaths among women in the age range from 15 to 44 years old in Malaysia (Bruni et al., 2017).

Cancer treatment options differ depending on the type of cancer, the stage of cancer, and the site of origin. The goals of the treatments are to cure cancer, to extend the survival, and to improve the quality of life of the patient (World Health Organization, 2017b). Cancer treatments usually include surgery, radiotherapy and chemotherapy. Surgery is a local treatment which works best in removing non-metastasized solid tumour. It is not used to treat cancers like lymphoma cancer or cancers that have metastasized. These advanced cancers entail the use of systemic therapies with chemotherapeutic agents (Carvalho et al., 2015).

2.1.1 Drugs Used in Cancer Treatments

In general, chemotherapy drugs act by killing actively dividing cancer cells or by limiting the growth of cancer cells. Different drugs act on different phases of the cell cycle, during which large amount of DNA are accurately

duplicated followed by precise segregation into two genetically identical cells (Alberts et al., 2002). Chemotherapy drugs can be classified into six general categories (American Cancer Society, 2016c) as outlined in Table 2.1.

Table 2.1: Categories and examples of chemotherapy drugs used in cancer treatments (American Cancer Society, 2016c)

Categories	Examples	Types of cancer
Alkylating agents	Busulfan	Chronic myelogenous leukaemia
	Carboplatin	Ovarian cancer
	Carmustine	Brain tumours, Hodgkin lymphoma, multiple myeloma, non-Hodgkin lymphoma
	Chlorambucil	Chronic lymphocytic leukaemia, Hodgkin lymphoma, non-Hodgkin lymphoma
	Cisplatin	Bladder cancer, ovarian cancer, testicular cancer
	Thiotepa	Bladder cancer, breast cancer, malignant pleural effusion, malignant pericardial effusion, and malignant peritoneal effusion, ovarian cancer
Antimetabolites	5-fluorouracil (5FU)	Breast cancer, colorectal cancer, gastric (stomach) cancer, pancreatic cancer
	Capecitabine	Breast cancer, colorectal cancer
	Cytarabine	Acute lymphoblastic leukaemia, acute myeloid leukaemia, chronic myelogenous leukaemia
	Gemcitabine	Breast cancer, non-small cell lung cancer, ovarian cancer, pancreatic cancer
	Hydroxyurea	Chronic myelogenous leukaemia, squamous cell carcinoma of the head and neck

Anthracyclines	Doxorubicin	Acute lymphoblastic leukaemia, acute myeloid leukaemia, breast cancer, gastric cancer, Hodgkin lymphoma, neuroblastoma, non-Hodgkin lymphoma, ovarian cancer, small cell lung cancer (SCLC), soft tissue and bone sarcomas, thyroid cancer, transitional cell bladder cancer
	Epirubicin	Breast cancer
Topoisomerase inhibitors	Topotecan	Cervical cancer, ovarian cancer, SCLC
	Irinotecan	Colorectal cancer
	Etoposide	SCLC, testicular cancer
	Mitoxantrone	Acute myeloid leukaemia, prostate cancer
Mitotic inhibitors	Ixabepilone	Breast cancer
	Paclitaxel	Breast cancer, non-SCLC, ovarian cancer
	Vinblastine	Breast cancer, choriocarcinoma, Hodgkin lymphoma, testicular cancer

The conventional chemotherapy drugs commonly focus on mass cell killing with low specificity and often cause adverse side effects (Huang et al., 2012b). Side effects usually involve damaging healthy cells and tissues such as intestinal cells and stem cells in the bone marrow (American Cancer Society, 2016b). Specifically, Cisplatin, a chemotherapy drug used in bladder, ovarian and testicular cancer treatment (Table 2.1), causes kidney damage, breathlessness and bruising in patients (Cancer Research UK, 2016a). Doxorubicin causes hair loss, diarrhoea, fever and chills (Cancer Research UK, 2017). 5FU causes patients to feel fatigue, loss of appetite and increases risk of infection (Cancer Research UK, 2016b). Besides, the use of chemotherapy drugs in cancer treatment also results in the development of chemical

resistance in cancer cells (Huang et al., 2012b, Wu et al., 2014). For instance, tamoxifen, a chemotherapy drug which works as an estrogen receptor antagonist, was reported to lose its antagonist activity on tumour cells with active growth factor receptor signalling (Housman et al., 2014).

As mentioned in Section 2.1, cervical cancer has been one of the most common cancers among the women in Malaysia. The current drugs that are used for cervical cancer treatment are Cisplatin, Carboplatin, Paclitaxel, Topotecan and Gemcitabine alone, as well as in combination with 5FU (American Cancer Society, 2016a). 5FU is an antimetabolite chemotherapy drug which acts by inhibiting the DNA and RNA synthesis (Thomas et al., 2016). 5FU acts as an analogue of uracil. When 5FU is converted intracellularly into metabolites, namely fluorodeoxyuridine monophosphate, fluorodeoxyuridine triphosphate and fluorouridine triphosphate, it interferes with RNA synthesis and the action of thymidylate synthase (nucleotide synthetic enzyme) (Longley et al., 2003). Besides being used intravenously, 5FU has been used as topical treatment for actinic keratosis, as well as squamous cell carcinoma and basal cell carcinoma (Cohen, 2010). Despite the advancement of 5FU usage in cancer treatments, side effects (Cancer Research UK, 2016b) and drug resistance (Longley et al., 2003) remains a substantial drawback to the clinical use of 5FU. Consequently, there is an urgent need for the development of new anticancer agents (Huang et al., 2012b).

2.1.2 Peptides as Cancer Drugs

Over the past decades, peptides and proteins have gained remarkable interest among the pharmaceutical and biotechnology industries (Craik et al., 2013, Usmani et al., 2017). To date, there are more than 60 therapeutic peptides that were approved by US-FDA for clinical use, over 140 peptide drugs in clinical-phase trials, and more than 500 therapeutic peptides being evaluated in advanced preclinical phases (Fosgerau and Hoffmann, 2015). Some of the approved therapeutic peptides for different non-cancer treatments are presented in Table 2.2.

Table 2.2: Selected examples of FDA-approved therapeutic peptides (Usmani et al., 2017)

Brand names	Generic names	Indications	Number of residue	Origin
Integrilin®	eptifibatide	Acute coronary syndrome, unstable angina undergoing percutaneous coronary intervention	7	Pygmy rattlesnake
Enalapril Maleate, Vasotec®	enalapril maleate (or 2-butanedioate)	Hypertension	3	-
Fuzeon®	enfuvirtide	AIDS/HIV-1 infection	36	-
Acticalcin®, Calcimar®, Caltine®, Miacalcic®	salmon calcitonin	Postmenopausal osteoporosis, Paget's disease, hypercalcaemia	32	Salmon
Byetta®	exenatide	Glycemic control in patients with type 2 diabetes mellitus	39	Gila monster

'-' indicates that the origin of the peptide was not mentioned in the literature.

Currently, the growth rate of the peptide market is substantially faster than that of small molecules (Bruno et al., 2013). This is because therapeutic peptides offer various advantages over small-molecule drugs. Peptides offer higher efficacy, selectivity and specificity than small organic molecules (Vlieghe et al., 2010, Fosgerau and Hoffmann, 2015). Besides, the products of degradation of peptides are amino acids, therefore minimizing the drug-drug interaction, consequently the risk of systemic toxicity can be abated (Vlieghe et al., 2010). Although short half-life of the peptide is often considered as one of their disadvantages, the peptides are less likely to accumulate in the targeted tissues, thus the risks of complications that may be caused by their metabolites can be minimized (Vlieghe et al., 2010).

By studying the nature of the cancer tissue and its microenvironment, researchers have discovered that cancer cells express molecular markers that are not expressed or only expressed at low levels in normal cells (Diaz-Cano, 2012). The discovery of the overexpression of tumour-specific receptors has motivated the use of targeting peptides (Le Joncour and Laakkonen, 2017). The majority of therapeutic peptides are receptor agonists (Vlieghe et al., 2010). These peptides act by targeting molecular markers such as receptors expressed on the cancer cell membrane (Marqus et al., 2017). Peptide agonists function to initiate drug actions by activating the targeted receptors (Vlieghe et al., 2010). An example of the application of peptides in cancer treatment is the use of luteinising hormone releasing hormone (LH-RH) agonists in prostate cancer treatment. These LH-RH agonists, such as buserelin, goserelin, leuprolide and triporelin (Table 2.3), cause down-regulation of LH-RH

receptors in the pituitary gland, resulting in an inhibition of follicle-stimulating hormone and luteinising hormone release, and a simultaneous reduction in testosterone production (Schally et al., 2000). On the other hand, some peptide antagonists, which act by inhibiting receptor-ligand interactions, have also reached the market (Ladner et al., 2004). Cetrorelix is one of the examples of LH-RH antagonist that is used in prostate and breast cancer treatments (Thundimadathil, 2012). A list of peptide-based drugs used for various cancer treatments are depicted in Table 2.3.

Table 2.3: Selected examples of FDA-approved therapeutic peptides used in cancer treatment (Usmani et al., 2017)

Brand names	Generic names	Indications	Number of residue	Origin
Bigonist®, Suprefact®	Buserelin acetate	Advanced prostate cancer	9	Synthetic analogue of GnRH
Zoladex®	Goserelin acetate	Advanced prostate cancer, breast cancer	10	Synthetic antagonist of GnRH
Supprelin®, Supprelin LA®, Vantas®	Histrelin acetate	Advanced prostate cancer, central precocious puberty	9	Synthetic analogue of GnRH
Eligard®, Enantone®, Lucrin Depot®, Lupron®, Lupron Depot®, Prostap®, Viadur®	Leuprolide acetate, or leuprorelin	Advanced prostate cancer, breast cancer, central precocious puberty	9	Synthetic analogue of GnRH
Decapeptyl®, Diphereline®, Gonapeptyl®, Pamorelin®, Trelstar Depot®, Trelstar LA®	Triptorelin pamoate	Advanced prostate cancer, central precocious puberty, endometriosis, uterine fibroids, ovarian stimulation in *in vitro* fecundation	10	Synthetic antagonist of LHRH
Plenaxis™	Abarelix acetate	Advanced prostate cancer	10	Synthetic antagonist of GnRH
Degarelix Acetate, Firmagon®	Degarelix acetate	Advanced prostate cancer	10	Synthetic antagonist of GnRH

Velcade®	Bortezomib	Multiple myeloma, and refractory, mantle cell lymphoma	2	-
Thymogen	Oglufanide disodium	Ovarian cancer – Phase II	2	-

'-' indicates that the origin of the peptide was not mentioned in the literature.

2.2 Cytotoxic Peptides

One of the main disease areas that steers the therapeutic application of peptide drugs is the area of oncology (Fosgerau and Hoffmann, 2015). Hence, research on the use of peptides in cancer treatment has been a fertile ground. This has attracted a great deal of interest among the scientific community to exploit natural resources for potential therapeutic peptides with cytotoxic activity. To date, many researchers have investigated the terrestrial and marine sources for cytotoxic peptides (Daliri et al., 2017).

Cytotoxic peptides derived from terrestrial sources such as wheat (Rivabene et al., 1999), soybean (Rayaprolu, 2015), medicinal mushrooms (Liu et al., 2016), milk (Sah et al., 2015) and egg proteins (Carrillo et al., 2016) have been reported over the last two decades. Table 2.4 shows a list of selected examples of cytotoxic peptides derived from various terrestrial sources. A study of soybean protein hydrolysate prepared by alcalase hydrolysis reported that the fractions of the hydrolysate (800 µg/mL) exhibited cytotoxicity of 73% in colon cancer (HCT-116), 70% in liver cancer (HepG2) and 68% in lung cancer (NCL-H1299) cell lines (Rayaprolu, 2015). Lunasin, a peptide isolated from soybean cotyledon, was reported to possess anticancer activity (González-Montoya M. et al., 2017). When tested on chemical

carcinogens treated fibroblast NIH/3T3 cells, Lunasin showed significant inhibition in cell proliferation (Hsieh et al., 2010). Besides Lunasin, soybean protein hydrolysate also contained many cytotoxic peptides such as SKWQHQQDSC (Fernández-Tomé et al., 2017), GEGSGA, GLTSK, MPACGSS, LSGNK, as well as MTEEY (Luna Vital et al., 2014). These peptides were reported to exhibit significant antiproliferative effect on colorectal cancer (HT-29) cells (Luna Vital et al., 2014, Fernández-Tomé et al., 2017).

Table 2.4: Selected examples of terrestrial cytotoxic peptides

Peptide	Terrestrial source	References
Cn-AMP1 (SVAGRAQGM)	Coconut water (*Cocos nucifera*)	(Silva et al., 2012)
Coccinin (KQTENLADTY)	Large scarlet runner beans (*Phaseolus coccineus*)	(Ngai and Ng, 2004)
Cordymin (AMAPPYGYRTPDAAQ)	Medicinal mushroom (*Cordyceps militaris*)	(Wong et al., 2011, Liu et al., 2016)
Cyclosaplin (RLGDGCTR)	Sandalwood (*Santalum album* L.)	(Mishra et al., 2014)
Cycloviolacin O2 (cyclo-GIPCGESCVWIPCISSAIGCSCKSKVCYRN)	Sweet violet (*Viola odorata*)	(Svangård et al., 2007)
Defensin sesquin (KTCENLADTY)	Ground bean (*Vigna sesquipedalis*)	(Wong and Ng, 2005)
EQRPR	Rice bran	(Kannan et al., 2010)
Limenin (KTCENLADTYKGPCFTTGGC)	Lima bean (*Phaseolus limensis*)	(Wong and Ng, 2006)
Lunasin, SKWQHQQDSC, GLTSK, LSGNK, GEGSGA, MPACGSS and MTEEY	Soybean (*Glycine max*)	(Luna Vital et al., 2014, Rayaprolu, 2015, Fernández-Tomé et al., 2017, González-Montoya M. et al., 2017)

Pyrularia thionin (KSCCRNTWARNCYNVCRLPGTISREI CAKKCRCKIISGTTCPSDYPK)	Mistletoe (*Pyrularia pubera*)	(Evans et al., 1989)
RA-XVII and RA-XVIII (AAYAYY)	Indian madder (*Rubia cordifolia* L.)	(Lee et al., 2008)
RHPFDGPLLPPGD, RCGVNAFLPKSYLVHFGWKLLFHFD and KPEEVGGAGDRWTC	Orchid (*Dendrobium catenatum* Lindley)	(Zheng et al., 2015)
RQSHFANAQP	Chickpea (*Cicer arietinum*)	(Xue et al., 2015)
RQ-8, LQ-10, and YY-11 (RGLHPVPQ, LEEQQQTEDEQ, and YLEELHRLNAGY)	Camel milk	(Homayouni-Tabrizi et al., 2017)

Peptide RQSHFANAQP isolated from chickpea hydrolysate demonstrated dose-dependent antiproliferative activity against human breast cancer (MCF-7 and MDA-MB-231) cells (Xue et al., 2015). On the other hand, rapeseed peptides obtained by using bacterial and enzymatic cooperation have shown antiproliferative activity towards HepG2, HeLa and MCF-7 cell lines (Xie et al., 2015). In another study, three peptides namely RHPFDGPLLPPGD, RCGVNAFLPKSYLVHFGWKLLFHFD and KPEEVGGAGDRWTC were identified from the alcalase hydrolysate of *D. catenatum* Lindley, a medicinal plant. These synthetic peptides showed antiproliferative effects against HepG2, MCF-7 and gastric cancer (SGC-7901) cells but only low inhibitory activity against normal liver (L-O2) cells (Zheng et al., 2015).

Marine organisms have been recognized as reservoirs of structurally diverse bioactive compounds with various biological effects including anticancer activity (Ngo et al., 2012, Pangestuti and Kim, 2017). Particularly, cytotoxic peptides isolated, purified and identified from many marine organisms, such as oysters (Umayaparvathi et al., 2014), clams (Kim et al.,

2013), tuna dark muscle (Hsu et al., 2011), half-fin anchovy (Song et al., 2014), skate (Pan et al., 2016), and algae protein waste (Sheih et al., 2010) have been shown to display cytotoxic activity. Table 2.5 shows a list of marine peptides identified from various sources.

Table 2.5: Selected examples of marine cytotoxic peptides

Peptide	Marine source	References
Aplidine	Tunicate (*Aplidium albicans*)	(Taraboletti et al., 2004)
Arenastatin A	Marine sponge (*Dysidia arenaria*)	(Kobayashi et al., 1994)
BEPT II-1	Marine mollusc (*Bullacta exarata)*	(Ma et al., 2013)
Didemnin B	Tunicate (*Tridemnum solidum*)	(Rinehart et al., 1981)
Dolastatin 10	Marine mollusc (*Dolabella auricularia*)	(Kalemkerian et al., 1999, Aneiros and Garateix, 2004)
Discodermins	Marine sponge (*Discodermia kiiensis*)	(Ryu et al., 1994, Pangestuti and Kim, 2017)
H3	Marine mollusc (*Arca subcrenata*)	(Chen et al., 2013)
Hemiasterlin D, geodiamolides D–F	Marine Sponge (*Pipestela candelabra*)	(Tran et al., 2014)
Jaspamide	Marine sponge (*Jaspis johnstoni*)	(Crews et al., 1986, Takeuchi et al., 1998)
Kahalalide F	Marine mollusc (*Elysia rufescens*)	(Suárez et al., 2003, Suarez-Jimenez et al., 2012)
LPHVLTPEAGAT, PTAEGGVYMVT	Tuna dark muscle (*Thunnus tonggol*)	(Hsu et al., 2011)
Mollamide	Marine ascidian (*Didemnum molle*)	(Carroll et al., 1994)
Phakellistatin 13	Marine sponge (*Phalkellia fusca*)	(Li et al., 2003)
Reniochalistatin E	Marine sponge (*Reniochalina stalagmitis*)	(Zhan et al., 2014)
SCAP1	Oyster (*Saccostrea cucullata*)	(Umayaparvathi et al., 2014)
WPP	Blood clam muscle	(Chi et al., 2015)

	(*Tegillarca granosa*)	
YALPAH	Half-fin anchovy (*Setipinna taty*)	(Song et al., 2014)

One of the lead cytotoxic peptides found from marine organism was didemnin B. When didemnin B was first isolated from Caribbean tunicates *T. solidum* in 1981, it was reported that this cyclic depsipeptide possessed *in vivo* cytotoxic activities against leukemia P388 cells at nanomolar concentration (Rinehart et al., 1981). With noteworthy dose-dependent activity and tolerable toxicity in preclinical model, it was then subjected to phase I and phase II clinical trials, making didemnin B the first natural product from marine source assessed in clinical trials against several human tumours (Cain et al., 1992, Molinski et al., 2009, Suarez-Jimenez et al., 2012). However, clinical trials on didemnin B were suspended due to severe fatigue and anaphylaxis in patient. A simple analogue of didemnin B, aplidine, was found to be more promising in preclinical models (Molinski et al., 2009). Aplidine is also a cyclic depsipeptide which was obtained from the tunicate *A. albicans* (Taraboletti et al., 2004). It is worth noting that aplidine has been evaluated in phase I and phase II clinical trials in the indications including Stage IV melanoma, multiple myeloma, non-Hodgkin's lymphoma, acute lymphoblastic leukemia, prostate cancer and bladder cancer (Molinski et al., 2009, Pangestuti and Kim, 2017). Phase III clinical trials are currently on-going to test for relapsed/refractory myeloma (Cooper and Albert, 2015).

WPP, a tripeptide, derived from blood clam muscle displayed great cytotoxic effect against lung cancer (H-1299), prostate cancer (DU-145 and

PC-3) and HeLa cell lines (Chi et al., 2015). Oyster protein hydrolysates contained cytotoxic peptide SCAP-1 with the sequence of LANAK. This peptide displayed cytotoxic activity on HT-29 cell lines but no cytotoxic effect on Vero cell lines (Umayaparvathi et al., 2014). Apart from shellfish, several cytotoxic peptides have been discovered in molluscs. Dolastatin 10 comprised of several unique amino acid compositions. This cytotoxic pentapeptide was isolated from marine molluscs *D. auricularia*. It has been reported that dolastatin 10 exhibited cytotoxic activity against several cell lines including multiple lymphoma, human promyelocytic leukemia (HL-60), mouse lymphocytic leukemia (L1210), human acute myelomonocytic leukemia (ML-2), SCLC (NCI-H69, NCI-H82, NCI-H446, and NCI-H510), human monocytic (THP-1) and PC-3 cells (Kalemkerian et al., 1999, Aneiros and Garateix, 2004). Another cytotoxic peptide isolated from the Hawaiian marine molluscs *E. rufescens* is a cyclic depsipeptide, Kahalalide F. This peptide has shown selectivity towards prostate-derived cells lines and tumour (Suárez et al., 2003, Suarez-Jimenez et al., 2012). Kahalalide F has displayed promising results in phase I and phase II clinical trials when administered in combination with other cytotoxic agents (Andavan and Lemmens-Gruber, 2010).

Isolation and identification of cytotoxic peptides from fish hydrolysates have been reported for the past decade (Picot et al., 2006, Hsu et al., 2011, Song et al., 2014, Karnjanapratum et al., 2016, Pan et al., 2016). Cytotoxic peptide YALPAH isolated from half-fin anchovy *S. taty* was found to exhibit strong cytotoxicity against PC-3 cells (Song et al., 2014). Furthermore, this peptide was modified into three different analogous peptides

by amino acid modification to reveal the influence of amino acid composition to the antiproliferative effect (Song et al., 2014). In another study, two peptides derived from tuna dark muscle by-product hydrolysate were reported to exhibit cytotoxicity against MCF-7 cell lines. The peptide sequences were identified as LPHVLTPEAGAT and PTAEGGVYMVT (Hsu et al., 2011).

In recent years, marine sponges have been known as a source of novel bioactive peptides with novel structural features and diverse biological activities (Ngo et al., 2012). Discodermins from marine sponge *D. kiiensis* have been shown to be cytotoxic towards human lung adenocarcinoma epithelial (A549) and P388 cells with IC_{50} range from 0.02 to 20 µg/mL (Pangestuti and Kim, 2017). In addition, Jaspamide, a cyclic depsipeptide derived from the marine sponge *J. johnstoni*, has been comprehensively evaluated as a promising cancer therapeutic agent. It has been found to inhibit the growth of several cell lines, such as PC-3, DU-145, and Lewis lung carcinoma (Crews et al., 1986, Takeuchi et al., 1998). A recent study reported that reniochalistatin E, a cyclic octapeptide from a tropical marine sponge *R. stalagmitis* Lendenfeld exhibited cytotoxicity in different cancer cell lines, including RPMI-8226, MGC-803, HL-60, HepG2, and HeLa cell lines (Zhan et al., 2014).

2.3 Enzyme-assisted Production, Purification and Identification of Marine Cytotoxic Peptides

In the discovery of marine bioactive peptides, a number of research groups adopted an enzyme-assisted approach (Ngo et al., 2012, Chai et al., 2017, Daliri et al., 2017). In such an approach, the peptides encrypted within the parent proteins isolated from marine sources were released by enzymatic hydrolysis. The hydrolysates were screened for cytotoxic activities after enzymatic hydrolysis and fractionated according to their sizes by membrane UF (Fan et al., 2017). The most potent fraction was then further purified using size exclusion chromatography and/or reversed phase high performance liquid chromatography. Finally the individual peptide fractions were identified by using the combined techniques of mass spectrometry and protein sequencing (Cheung et al., 2015). The peptide sequences obtained were often chemically synthetized and validated for cytotoxicity. A typical workflow for the enzyme-assisted production, purification and identification of cytotoxic peptides from marine hydrolysates is illustrated in Figure 2.1.

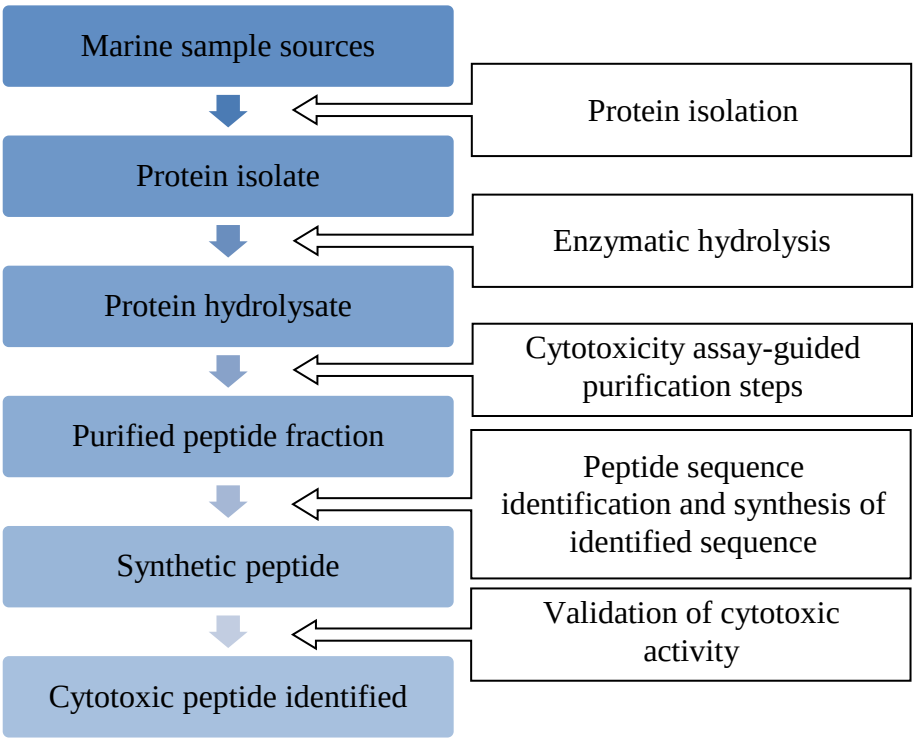


Figure 2.1: A typical workflow describing the process of the purification and identification of cytotoxic peptides from the protein hydrolysates of marine samples modified from Chai et al. (2017)

2.3.1 Production of Cytotoxic Marine Hydrolysates

Several methods were used to isolate proteins from marine organisms prior to enzymatic hydrolysis. One of the methods is the salting-out method using ammonium sulphate precipitation. Lv et al. (2015) used the salting-out method at increasing saturation levels of ammonium sulphate ranging from 70 to 100% to precipitate crude proteins from the homogenate of bivalve mollusc *T. granosa L.*. This method yielded 0.26% of crude protein, based on weight of wet visceral (Lv et al., 2015). Another study reported the use of pH-shift extraction to isolate fish proteins (Picot et al., 2006). On the other hand, frozen specimens of solitary tunicate (Jumeri and Kim, 2011) and oyster (Wang et al.,

2014) were thawed and minced before they were taken for the preparation of hydrolysis. These reports showed that the isolation of proteins together with elimination of non-protein components from marine samples is not always necessary for successful purification and identification of potent antiproliferative peptide fractions from marine samples.

During enzymatic hydrolysis, the physicochemical conditions for instance pH and temperature of the protein solution must be well-regulated to achieve the enzyme's optimum activity (Ngo et al., 2012, Pangestuti and Kim, 2017). Several proteolytic enzymes are available from animal, plant and microbial sources (Umayaparvathi et al., 2014). Digestive enzymes that have been reported to produce cytotoxic hydrolysates are proteases of animal origin (trypsin, α-chymotrypsin and pepsin), plant origin (papain) and microbial origin (Alcalase, Protamex, Esperase and Neutrase) (Picot et al., 2006, Alemán et al., 2011, Hsu et al., 2011, Song et al., 2014, Fan et al., 2017). Table 2.6 shows examples of proteases used by various research groups to generate cytotoxic marine hydrolysates and the optimum ranges of temperatures and pH's used in their studies.

Table 2.6: Examples of proteases and the optimum ranges of temperatures and pH's used in previous studies

Origins	Proteases	Optimum temperature, ^{o}C	Optimum pH	References
Animal	Trypsin	55	8	(Alemán et al., 2011)
		45	8	(Fan et al., 2017)
		37	7	(Kim et al., 2013)
		51	8	(Ding et al., 2011)
		45	8.7	(Ma et al., 2013)
	α-chymotrypsin	37	7	(Kim et al., 2013)

Animal	Pepsin	37	2	(Kim et al., 2013)
		37	3	(Song et al., 2014)
		37	2	(Jumeri and Kim, 2011)
Plant	Papain	37	6	(Kim et al., 2013)
		25	6.2	(Hsu et al., 2011)
Microbial	Alcalase	50	8	(Alemán et al., 2011)
		50	7	(Kim et al., 2013)
		55-57	7.5	(Picot et al., 2006)
		55	8	(Jumeri and Kim, 2011)
	Protamex	60	6.5	(Alemán et al., 2011)
		50	7	(Kim et al., 2013)
		55-57	7.5	(Picot et al., 2006)
	Neutrase	55	8	(Alemán et al., 2011)
		50	7	(Kim et al., 2013)
	Protease XXIII	37	7.5	(Hung et al., 2014)
		37	7.5	(Hsu et al., 2011)
	Esperase	60	8.5	(Alemán et al., 2011)
	Savinase	55	9.5	(Alemán et al., 2011)
	Flavourzyme	50	7	(Kim et al., 2013)
	Thermoase	67	7.5	(Jumeri and Kim, 2011)

Alemán et al. (2011) hydrolysed gelatin from giant squid (*Dosidicus gigas*) using various proteases including Protamex, Neutrase, Alcalase and Esperase. The hydrolysate that showed the highest cytotoxic activity on glioma (U87) and MCF-7 cell lines, was produced by Esperase, followed by the Alcalase hydrolysate (Alemán et al., 2011). Besides, Alcalase was also used to hydrolyse protein of solitary tunicate (*Styela clava*). It was found that the hydrolysate produced by Alcalase had high anticancer activity in stomach (AGS), human colon (DLD-1), and HeLa cancer cells (Jumeri and Kim, 2011).

On the other hand, papain hydrolysate of tuna dark muscle by-product has been reported to possess significant cytotoxic activity against MCF-7 cell line (Hsu et al., 2011). Fractions from loach protein hydrolysates prepared by papain hydrolysis have been reported to have antiproliferative activities against colon (Caco-2) cancer cells (You et al., 2011).

Hydrolysates of marine organisms generated by gastrointestinal digestive enzymes were also found to possess cytotoxic effects. For instance, the protein of *Spirulina platensis* was hydrolysed consecutively using pepsin, trypsin and chymotrypsin. The resulting enzymatic hydrolysate showed strong inhibition in MCF-7 and HepG2 cell lines (Wang and Zhang, 2016b). Fan et al. (2017) hydrolysed seaweed (*Porphyra haitanesis*) protein with trypsin for six hours. Following the tryptic digestion was ultrafiltration to obtain four fractions which showed good inhibitory effects on MCF-7, A549 and HT-29 cell lines. In another study, the oligopeptide prepared by trypsin treatment on cuttlefish ink (*Sepia esculenta*) inhibited the growth of human prostate carcinoma DU-145 cell line (Ding et al., 2011). Lastly, pepsin was used to hydrolyse half-fin anchovy (*S. taty*) to obtain an antiproliferative peptide which possessed cytotoxicity on PC-3 cells (Song et al., 2012, Song et al., 2014).

One of the strategies used by some studies to determine the optimum hydrolysis duration was evaluating the degree of hydrolysis (DH) of several hydrolysates generated by using different enzymes under their optimum physicochemical conditions (Chai et al., 2017). The hydrolysis duration that

generates the highest DH and/or strongest cytotoxicity is usually selected as the optimum hydrolysis duration (Chai et al., 2017). DH is defined as a percentage of cleaved peptide bonds. It is used to describe the hydrolysis of proteins and to monitor the hydrolysis reaction (Guérard et al., 2010). Many studies employed the measurement of DH to evaluate the effectiveness of proteolysis of marine derived proteins. For instance, DH analysis was used in the production of hydrolysates from tuna dark muscle by-product (Hsu, 2010, Hsu et al., 2011), Flathead fish by-product (Nurdiani et al., 2017), and shortclub cuttlefish (Sudhakar and Nazeer, 2015). Depending on the samples, the DH values may range between 20.4% (tuna dark muscle by-product) (Hsu, 2010, Hsu et al., 2011) and 48.2% (Flathead fish by-product) (Nurdiani et al., 2017).

The hydrolytic processing might be one of the most convenient approaches to convert underutilized marine proteins into anticancer peptides (Song et al., 2014). On top of that, enzymatic hydrolysis is more preferred in the nutraceutical and pharmaceutical industries compared to other methods such as organic solvent extraction and fermentation, to avoid toxic chemical and microbial residues in the products (Cheung et al., 2015, Pangestuti and Kim, 2017).

2.3.2 Purification of Cytotoxic Marine Peptides

2.3.2.1 Membrane Ultrafiltration

UF is often used as the initial step of assay-guided purification (Chai et al., 2017). Membrane UF usually uses permeable cellulose membranes with defined molecular weight cut-off (MWCO) specifications to separate the hydrolysate into different fractions based on their sizes. Combined use of different MWCO UF membranes is often employed in the fractionation of cytotoxic marine peptides. For example, UF membranes with 5 and 10 kDa MWCO were used in the fractionation of hydrolysates from roe protein hydrolysates of giant grouper (Yang et al., 2016). According to Pangestuti and Kim (2017), the main advantage of using this separation method is that the molecular weight (MW) range of the desired peptide can be easily manipulated by choosing the UF membrane with the right MWCO specifications.

2.3.2.2 Gel Filtration Chromatography

GF is also known as size exclusion chromatography. This purification technique, which serves to separate the peptides on the basis of differences in size, is the simplest and mildest mean among the chromatography techniques (Wang et al., 2017). The most commonly used GF stationary phases are Sephadex G-15 and Sephadex G-25. The partially purified peptide fraction obtained using membrane UF is usually further fractionated by GF. For example, Fan et al. (2017) used Sephadex G-15 to purify cytotoxic peptides

from the < 3 kDa UF fraction from seaweed. Remarkably, some studies directly separated protein hydrolysates using GF without using membrane UF. Protein hydrolysates from tuna dark muscle (Hsu et al., 2011) and oyster (Umayaparvathi et al., 2014) were directly subjected to GF using the same stationary phase, Sephadex G-25. In another study, a sequential GF purification step was carried out using both Bio-Gel P4 and Sephadex G-25 to purify hydrolysate of half-fin anchovy (Song et al., 2014).

One of the limitations of GF is lower loading volume when compared to UF, and fraction collection can be tedious and time-consuming. However, when parameters such as flow rate, bed height, particle size of stationary phase, sample concentration and volume are carefully controlled, GF is considered to be competent to achieve high selectivity and high resolution purification (Wang et al., 2017).

2.3.2.3 Reversed-phase High Performance Liquid Chromatography

Reversed-phase high performance liquid chromatography (RP-HPLC) has become a widely used, well-established technique for the identification, purification and analysis of bioactive peptides (Singh et al., 2014, Chai et al., 2017). In the procedures of marine peptide isolation, RP-HPLC is a common final purification step after GF and/or ion exchange chromatography (Cheung et al., 2015). In recent years, there are many studies that have employed RP-HPLC to obtain cytotoxic peptides from marine organisms, such as tuna dark muscle (Hsu et al., 2011), *A. subcrenata* (Chen et al., 2013), Flathead by-

products (Nurdiani et al., 2017), half-fin anchovy (Song et al., 2014) and oyster (Umayaparvathi et al., 2014).

Kim et al. (2013) used a semi-preparative RP-HPLC column (20 × 250 mm) to purify the strongest anticancer fraction isolated from hydrolysate of marine bivalve molluscs *Ruditapes philippinarum* using anion exchange chromatography. Further purification of the semi-preparative HPLC fraction with the highest anticancer activity was carried out by using an analytical RP-HPLC column (4 × 250 mm). Other studies that reported the use of analytical column (4.6 × 250 mm) in the purification step of marine cytotoxic peptides were Nurdiani et al. (2017), Song et al. (2014), Chen et al. (2013) and Hsu et al. (2011).

One of the reasons for RP-HPLC to play a central role in identifying and purifying peptides is its high resolution. In another words, RP-HPLC is capable of separating peptides of nearly identical amino acid sequences (Carr, 2002). Other advantages of this automated tool include high sensitivity, reproducibility, recovery and the ease of operation, and it uses shorter time to obtain the elution chromatogram as compared to the manual ion exchange and GF chromatography (Chai et al., 2017).

RP-HPLC separates peptides based on the mechanism of interaction between peptides and the reversed-phase surface. This includes continuous segregating of the peptide between the mobile phase and the hydrophobic stationary phase, which is the reversed phase column (Coskun, 2016).

Generally, the peptides adsorb to the hydrophobic stationary phase and remain adsorbed until the organic mobile phase achieves the critical concentration necessary to initiate desorption (Carr, 2002). Variances in amino acid composition and structure of a peptide will determine the peptide's retention in the column (Carr, 2002).

It is noteworthy that, in most studies, acetonitrile (ACN) with 0.1% trifluoroacetic acid (TFA) was used as the mobile phase in RP-HPLC purification step (Hsu et al., 2011, Chen et al., 2013, Song et al., 2014, Nurdiani et al., 2017). TFA is used as the anionic ion-pairing reagent which serves to set the pH of the eluent to enhance the separation (Chakraborty and Berger, 2005). ACN and TFA are volatile and can be easily removed from collection fractions and have low UV adsorption at low wavelengths. Besides, ACN has low viscosity and thus minimizing column back-pressure (Dunn, 2015).

2.3.2.4 Solid-phase Extraction

Solid-phase extraction (SPE) is a short chromatography separation used for concentration and impurities removal from synthetic, biological, and environmental samples (Herraiz and Casal, 1995, Kamysz et al., 2004). SPE has the advantage over the HPLC for its relatively cheaper cost and lower buffer consumption (Kamysz et al., 2004). There are four common extraction mechanisms used in SPE, namely non-polar (also known as reversed-phase), polar, ion-exchange, and covalent interactions (Kamysz et al., 2004).

Generally, there is very few reports of the use of SPE in the isolation and purification of cytotoxic peptide from marine sources. However, SPE has been used to purify antimicrobial peptides from various marine samples (Sperstad et al., 2011), such as mussel hemocytes (Charlet et al., 1996), sea hare body wall (Iijima et al., 2003), and spider crab hemocytes (Sperstad et al., 2009). For instance, during the isolation of antimicrobial peptides from the mussel hemocytes, Sep-Pak Vac C_{18} column was eluted with stepwise elution of 5, 50 and 80% ACN in 0.05% TFA. Their results showed that antibacterial and antifungal activities were only found in the 50% ACN fraction (Charlet et al., 1996).

Reversed-phase (C_{18}) SPE was also used as one of the purification methods to obtain bioactive peptides with angiotensin-I-converting enzyme (ACE) inhibitory activity from water and methanol extract of mushroom *Pleurotus cornucopiae* (Jang et al., 2011). Besides, C_{18} SPE was also employed by Chernysh et al. (2002) to isolate two peptides with antiviral and antitumor activities from blow fly *Calliphora vicina*.

Notwithstanding, this purification method was also employed in other more sophisticated bioanalyses. Stokvis et al. (2002) employed SPE as sample pre-treatment prior to LC-MS/MS analysis to study the stability of Kahalalide F, a cyclic depsipeptide from the marine mollusc, in human plasma. SPE was used in the isolation of the nanopeptides arginine vasotocin and isotocin which

are the brain neurohormones from fish (*Poecilia sphenops)* in the study of endocrine control of sexual behaviour in fish (Kulczykowska et al., 2015).

2.3.3 Identification of Cytotoxic Marine Peptides

The identification of amino acid sequence of the cytotoxic peptides was normally performed after the RP-HPLC step. Table 2.7 shows some of the examples of the commonly used methods employed by some researchers in the identification of cytotoxic marine peptide sequences. Tandem mass spectrometry is known to be a well-established methodology in peptide sequencing (Chen et al., 2007). According to Chai et al. (2017), a standard LC-MS/MS method combined the quadrupole time-of-flight (Q-TOF) tandem mass spectrometer with an electrospray ionization (ESI) source and analysed in the positive ionization mode. The identification of the peptide sequences was performed by analysing the fragmentation data obtained from a mass spectrometer with *de novo* sequencing algorithms. This method was used by Song et al. (2014) and Umayaparvathi et al. (2014) to successfully identify cytotoxic peptide YALPAH from half-fin anchovy.

On the other hand, the identification of amino acid sequences of cytotoxic peptides derived from algae (Sheih et al., 2010), blood clam (Chi et al., 2015), mollusc (Kim et al., 2013) and oyster (Umayaparvathi et al., 2014) was carried out by using Edman degradation method (Table 2.7). Subsequently, mass spectrometry was employed in some studies to analyse the molecular masses of the peptides. For instance, Chi et al. (2015) determined

the molecular mass of WPP using a Q-TOF MS coupled with ESI source. The molecular mass of LANAK was determined by using ESI-MS (Umayaparvathi et al., 2014).

Table 2.7: Examples of techniques adopted in amino acid sequence identification of cytotoxic marine peptides

Source species	Peptide identified	Techniques adopted	References
Algae (*Chlorella vulgaris*)	VECYGPNRPQF	Edman degradation	(Sheih et al., 2010)
Blood clam (*T. granosa*)	WPP	Edman degradation and ESI-MS	(Chi et al., 2015)
Flathead fish (*Platycephalus fuscus*)	MGPPGLAGAPGEAGR	LC-MS/MS-TOF	(Nurdiani et al., 2017)
Half-fin anchovy (*S. taty*)	YALPAH	ESI-MS/MS	(Song et al., 2014)
Marine mollusc (*R. philippinarum*)	AVLVDKQCPD	Edman degradation	(Kim et al., 2013)
Marine mollusc (*A. subcrenata*)	ISMEDVEESRKNGMHSID-VNHDGKHRAYWADNTY-LMKCMDLPYDVLDTGGK-DRSSDKNTDLVDLFELD-MVPDRKNNECMNMIMD-VIDTNTAARPYYCSLDV-NHDGAGLSMEDVEEDK	MALDI-TOF/TOF-MS	(Chen et al., 2013)
Oyster (*S. cucullata*)	LANAK	Edman degradation	(Umayaparvathi et al., 2014)
Seaweed (*P. haitanesis*)	VPGTPKNLDSPR and MPAPSCALPRSVVPPR	MALDI-TOF-MS	(Fan et al., 2017)
	KPEGMDPPLSEPEDRRD-GAAGPK and KLPPLLLA-KLLMSGKLLAEPCTGR	MALDI-TOF/TOF MS/MS	(Hung et al., 2014)
Tuna fish (*T. tonggol*)	LPHVLTPEAGAT and PTAEGGVYMVT	Q-TOF MS-ESI and Edman degradation	(Hsu et al., 2011)

2.4 Evaluation of the Cytotoxicity of Marine Peptides

Typically, a compound is considered to be cytotoxic if it interferes with the cellular attachment, adversely affects replication rate, or causes morphological changes and cell death (Niles et al., 2009). The choice of assay conditions should take into account the sample under study, nature of the expected response, and the specific target cell (Freshney, 2015). There are several assays that have been utilized for the measurement of cell viability or cytotoxicity *in vitro*.

The traditional cell counting method such as trypan blue exclusion assay was used to detect and measure cell viability based on the selective permeability of living cell membrane towards trypan blue dye (Anghel et al., 2013). This method is simple and inexpensive but very time consuming and sometimes inaccurate (Kanemura et al., 2002). Therefore, many researchers have opted for other means to evaluate the cytotoxic activities of a compound.

One of the most widely applied *in vitro* cytotoxicity measurements is the measurement of mitochondrial metabolic rate which involves the use of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). This cell-based assay has been developed to indirectly reflect the number of viable cells. Briefly, MTT will be reduced by mitochondrial dehydrogenase in viable cells into insoluble purple soluble formazan crystals which can be dissolved in organic solvent. The optical density of the resulting solution can be measured under a multi-well spectrophotometer. This colorimetric assay was originally

described by Mosmann (1983) and then was used extensively in many cytotoxicity experiments with various modifications introduced to match the needs of the studies.

An increasing number of studies used the MTT assay to guide the purification of cytotoxic peptides from marine cyanobacteria (Tripathi et al., 2009), fish proteins (Picot et al., 2006, Naqash and Nazeer, 2010, Hsu et al., 2011, Song et al., 2014, Pan et al., 2016), oyster (Umayaparvathi et al., 2014), giant squid gelatin (Alemán et al., 2011), mollusc (Chen et al., 2013), solitary tunicate (Jumeri and Kim, 2011) and seaweed (Fan et al., 2017). The most frequently used cell lines in MTT assay are MCF-7 (Picot et al., 2006, Tripathi et al., 2009, Alemán et al., 2011, Hsu et al., 2011, Fan et al., 2017), HepG2 (Naqash and Nazeer, 2010, Chen et al., 2013, Fan et al., 2017) and HeLa (Jumeri and Kim, 2011, Chen et al., 2013, Pan et al., 2016) cell lines as shown in Appendix A.

On the other hand, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was used as an alternative to MTT to evaluate the cytotoxicity of marine derived peptides. The formazan formed from reducing MTS is water-soluble, which is comparably less toxic than that of MTT (O'Toole et al., 2003). The water soluble formazan can be dissolved easily in cell culture media, without the need to perform the intermittent steps to remove culture media and add DMSO, which are required in the typical MTT assay. Unlike MTT assay, the formazan dye generated by the cells using MTS is detected with the absorbance at 490

nm (Wang et al., 2010). A number of studies successfully determined the cytotoxic activities of peptides derived from marine sources such as Flathead by-product (Nurdiani et al., 2017), shrimp shell (Kannan et al., 2011), and loach (You et al., 2011) by using MTS assay.

Another more sophisticated method used by the researchers to determine the cytotoxicity of marine peptide is the flow cytometry analysis. This powerful tool is able to investigate cell components, cell proliferation and cell cycle (Adan et al., 2017). The dyes that are commonly used in flow cytometry including Annexin V, fluorescein isothiocyanate (FITC), ethidium bromide (EB) and propidium iodide (PI). Song et al. (2014) utilized Annexin V-FITC/PI double staining to assess the initiation of apoptosis in PC-3 cells treated with synthetic peptide derived from half-fin anchovy. Working on HeLa cells, Pan et al. (2016) used the same staining method in flow cytometry analysis to evaluate the apoptosis rate of the cells with the presence of the peptide FIMGPY from skate. Another study by Fan et al. (2017) determined the phases of cell cycle in MCF-7 cells treated with synthetic peptide VPGTPKNLDSPR derived from seaweed *P. haitanesis* using Annexin V-FITC staining method. On the other hand, fluorescence microscopy and acridine orange/ethidium bromide (AO/EB) staining methods were used to observe the changes of cell morphologic features including chromatin condensation, blebbing, cell shrinkage, and nuclear fragmentation in apoptotic cells (Huang et al., 2012a, Pan et al., 2016).

2.5 Structural Characteristics of Cytotoxic Marine Peptides

The novel structural features of cytotoxic marine peptides have generated considerable interest. The understanding of the structure-activity relationship of cytotoxic peptides may allow researchers to predict and *de novo* design cytotoxic peptides with therapeutic potential (Camilio, 2013). Besides, it also provides useful insights for the methodology development using the appropriate proteases and purification strategies to release and isolate the peptides with the possible desired bioactivities (Li et al., 2017).

In general, a number of structural characteristics of a peptide believed to be essential for cytotoxic activity have been identified, including MW, net charge, hydrophobicity, amino acid composition and sequences (Huang et al., 2011). In the context of MW, a majority of the marine cytotoxic peptides have MW range from 200 – 1700 Da (Picot et al., 2006, Hsu et al., 2011, Umayaparvathi et al., 2014, Chi et al., 2015, Pan et al., 2016, Fan et al., 2017). Peptides with lower MW are commonly believed to exhibit higher cytotoxic activity than those with higher MW (Jumeri and Kim, 2011, Song et al., 2014). Jumeri and Kim (2011) proposed that smaller peptide may have higher mobility and diffusivity, which may contribute to the enhanced cytotoxicity of the peptide. In agreement with this were the reports of UF fractions with the lowest MW range showed the highest cytotoxic activity. These peptidic fractions were derived from marine organisms, including flathead by-product (Nurdiani et al., 2017) and loach (You et al., 2011). However, there is inconsistency in this argument, given the studies by Picot et al. (2006) and

Hsu et al. (2011) suggested the lack of correlation between antiproliferative activity and MW of peptides from other fish species. On top of that, Alemán et al. (2011) concluded that MW cannot be deemed as the most important factor affecting the anticancer activity of a peptide.

Besides, net charge is another important determining factor of cytotoxicity of peptides. In order to understand the contribution of net charge to the antiproliferative activity of peptide derived from half-fin anchovy, YALPAH, Song et al. (2014) replaced the Proline (P) residue with an Arginine (R) residue to increase the net charge of the peptide from +1 to +2. The modified peptide, YALRAH, showed improved antiproliferative activity compared to YALPAH. The authors proposed that the enhanced activity of YALRAH may be due to the increased positive charges and hydrogen bonding formed with the cancer cell membrane resulting from the R residue (Song et al., 2014). In another study conducted by Yang et al. (2004) used modified synthetic peptide to successfully demonstrate that a peptide with a net charge close to +7 has great antitumor activity. This study thereby revealed a strong correlation between net positive charge and antitumor activity.

Yang et al. (2004) suggested that the amino acid sequence and thus the conformation of a peptide contribute to its antitumor activity. The differences in amino acid composition and sequences largely depend on the specificity of the enzyme used in hydrolysis (Jumeri and Kim, 2011) and the protein source. Interestingly, Jumeri and Kim (2011) highlighted that the peptide fractions with higher hydrophobic amino acid content exerted greater anticancer activity.

In accordance with this are the examples of marine cytotoxic peptides containing 16-100% hydrophobic amino acid residues in their sequences (Table 2.8).

Table 2.8: Percentages of hydrophobic residues in cytotoxic marine peptides

Cytotoxic peptides	Hydrophobic amino acid residue (%)*	References
RDGDSCRGGGPV	16.67	(Ma et al., 2013)
VECYGPNRPQF	36.36	(Sheih et al., 2010)
VPGTPKNLDSPR	41.67	(Fan et al., 2017)
PTAEGGVTMVT	45.45	(Hsu et al., 2011)
QP	50	(Chi et al., 2015)
YALRAH	50	(Song et al., 2014)
LPHVTPEAGAT	54.54	(Hsu et al., 2011)
RAALAVVLGRGGPR	57.14	(Ma et al., 2013)
LANAK	60	(Umayaparvathi et al., 2014)
FIMGPY	66.67	(Pan et al., 2016)
YALPAH	66.67	(Song et al., 2014)
YALPAR	66.67	
YALPAG	66.67	
WPP	100	(Chi et al., 2015)

*The percentages of hydrophobic amino acid residues were calculated manually, based on the classification of A, I, L, M, F, P, W, and V as hydrophobic residues (IARC TP53 Database) .

It has been found that the presence of tyrosine and other hydrophobic amino acids is essential to the free radical scavenging ability of the peptide (Jumeri and Kim, 2011). By lowering oxidative stress in the microenvironment, genetic alteration including mutation as well as chromosomal rearrangements which contributes to the initiation step of carcinogenesis may be reduced (Jumeri and Kim, 2011).

Pan et al. (2016) pointed out that the hydrophobic F, I, M and P residues in the peptide sequence of FIMGPY could be accountable for its good anticancer activities. On top of that, Chi et al. (2015) also reported that the presence of hydrophobic residues W and P in WPP peptide might contribute to its antiproliferative activity in PC-3 cells. Although another study by Huang et al. (2011) disclosed that modulation of hydrophobicity of peptides is accountable for its cytotoxicity against cancer cell, mechanism of action of cytotoxic marine peptides on cancer cells needed to be further studied.

2.6 Mechanisms of Cytotoxic Marine Peptides

The understanding of the mechanisms of action of cytotoxic peptides isolated from marine sources plays a crucial role to the development of new chemotherapeutic drugs (Zheng et al., 2013). In general, cytotoxic marine peptides induce cell death via different pathways, for instance apoptosis, angiogenesis inhibition and tubulin-microtubule equilibrium (Zheng et al., 2011).

Briefly, apoptosis is a process of programmed cell death which occurs naturally as homeostatic mechanism to uphold cell populations as well as defence mechanism against formation of cancer (Elmore, 2007, Beesoo et al., 2014). There are two main pathways of apoptosis: the intrinsic (or mitochondrial) pathway, and the extrinsic (death receptor) pathway (Elmore, 2007). In the intrinsic pathway, the pro- and anti-apoptotic proteins such as Bax of the Bcl-2 family play an influential role as the pivotal regulators of

apoptosis (Zheng et al., 2011, Beesoo et al., 2014). A recent study by Pan et al. (2016) reported that FIMGPY peptide from skate might stimulate apoptosis in HeLa cells by upregulating the Bax/Bcl-2 ratio. Besides, in the same study, the activation of caspase-3, an effector caspases which plays a central role in triggering apoptosis, was also determined. Western blot analysis was used to measure the levels of Bax and Bcl-2 protein as well as caspase-3 in their study (Pan et al., 2016).

Another method used to determine the mode of action of cytotoxic peptide is the Annexin V assay with help of flow cytometer for detection. This assay detects the apoptotic changes in the plasma membrane of the cancer cells (Wlodkowic et al., 2009). Under physiological conditions, choline phospholipids are exposed on the outer leaflet of the cell membrane while aminophospholipids are displayed on the inner surface of the membrane (Wlodkowic et al., 2009). When apoptosis happens, one of the aminophospholipids, namely phophatidylserine, is exposed on the outer leaflet of the membrane (Wlodkowic et al., 2011). Annexin V is used as a probe which binds to phophatidylserine residues so that it can be detected by the flow cytometer. PI is a membrane impermeant dye, which can only penetrate through the damaged and disrupted membrane of the dead cells and bind tightly to the nucleic acid of the cell, which is used to indicate cell viability (Stiefel et al., 2015).

Several studies on cell apoptotic rate of cancer cells treated by cytotoxic marine peptides, for example FIMGPY (Pan et al., 2016), VPGTPKNLDSPR

(Fan et al., 2017), YALPAH (Song et al., 2014) BEPT II-1 (Ma et al., 2013) and BCP-A (Chi et al., 2015) have been performed. Particularly, by using Annexin V and PI staining, the cell apoptotic rate of FIMGPY peptide-treated HeLa cells was determined (Pan et al., 2016). The authors reported that the apoptotic effect on the HeLa cells which was treated with FIMGPY peptide significantly increased with peptide concentration as compared to the control (Pan et al., 2016). Moreover, by adopting Annexin V-FITC/PI fluorescence staining, apoptosis induction in MCF-7 cells treated by VPGTPKNLDSPR peptide derived from *P. haitanesis* was reported by Fan et al. (2017).

A number of studies have reported the characteristic pattern of the morphological changes by using AO/EB fluorescence staining (Ma et al., 2013, Umayaparvathi et al., 2014, Chi et al., 2015, Pan et al., 2016). The typical apoptotic changes, including nuclear chromatin condensation, nuclei fragmentation and cytoplasmic blebs formation as well as cell shrinkage were observed in the study of BCP-A (Chi et al., 2015) and BEPT II-1(Ma et al., 2013) against PC-3 cells. On top of the aforementioned observations, features such as orange necrotic cell apoptotic bodies were also observed in HeLa cells after treated with FIMGPY for 24 h (Pan et al., 2016).

Other methods such as DNA fragmentation and cell cycle analysis were also used to study the mechanisms of actions of cytotoxic marine peptide. The induction of DNA damage in HT-29 cells after treated with peptide fraction SCAP1 derived from oyster hydrolysate was reported (Umayaparvathi et al., 2014). On the other hand, the distribution of cell cycle phases of MCF-7 cells

treated with VPGTPKNLDSPR peptide was measured by flow cytometry showing cell cycle arrest in G_0/G_1 phase (Fan et al., 2017).

Another mechanism of action of cytotoxic marine peptide is via angiogenesis inhibition. In short, angiogenesis is the formation of new blood vessels which plays a key role in the progression, invasion and metastasis of most tumours (Wong et al., 2009). The key factors that are accountable for tumour angiogenesis are vascular endothelial growth factor (VEGF) and its receptor, VEGFR-2 (Flk-1/KDR) (Zheng et al., 2011). The disruption of VEGF-VEGFR-2 pathway and downstream intracellular signalling is one of the mechanisms to inhibit cancer growth (Wong et al., 2009, Zheng et al., 2011). A novel linear polypeptide, PG155, with potent anti-angiogenic activity was previously reported by Zheng et al. (2007). This peptide was derived from the cartilage of the shark *Prionace glauca* and was tested using *in vivo* zebrafish embryos model to evaluate its anti-angiogenic effect (Zheng et al., 2007).

According to Hadfield et al. (2003), drugs that cause the disruption of tubulin and microtubule equilibrium are effective cancer drugs. Microtubules play a crucial role in essential cellular functions such as chromosome segregation during cell mitosis, the maintenance of cell shape, motility and organelle distribution (Hadfield et al., 2003). The compounds that affect the tubulin-microtubule equilibrium act by binding to the protein tubulin in the mitotic spindle and subsequently blocking the polymerization of microtubules (Zheng et al., 2011). Marine peptides, for example Dolastatin 10 derived from

marine mollusc *D. auricularia* (Bai et al., 1990), Hemiasterlin from marine sponges *Auletta* and *Siphonochalina* sp.(Anderson et al., 1997, Gamble et al., 1999, Yamashita et al., 2004), and Diazonamide A from marine ascidian *Diazona angulata* (Cruz-Monserrate et al., 2003), were reported to display the ability to disrupt the formation of microtubules.

2.7 *Xestospongia testudinaria*

Marine sponges (phylum of Porifera) are among the phylogenetically oldest phylum still in existence today (Mioso et al., 2017). They are known as filter feeders; they feed by filtering seawater through the small pores or oscules on their bodies (Qaralleh et al., 2011). Marine sponges have great capacity to withstand harsh conditions such as extreme changes in salinity, temperatures and pressures (Thakur et al., 2005, El-Gamal et al., 2016). Mainly due to the lack of natural physical defence mechanisms, these sessile marine invertebrates produce a range of secondary metabolites to protect themselves against harmful pathogens and predators (Liang et al., 2014, Mioso et al., 2017).

Marine sponges from the genus *Xestospongia* are a rich source of secondary metabolites (Longeon et al., 2010, Liang et al., 2014). In recent years, non-peptide bioactive compounds have been identified from *X. testudinaria* (Zhou et al., 2011, El-Gamal et al., 2016). For instance, Zhou et al. (2011) reported that mutafuran H, a brominated ene-tetrahydrofuran, isolated from the alcohol extract of *X. testudinaria* possessed significant anti-acetylcholinesterase activity with the IC_{50} value of 0.64 μM. Another group of

researchers have successfully isolated brominated polyunsaturated lipids from *X. testudinaria*, one of which named methyl xestospongic ester possessed significant pancreatic lipase inhibitory activity with the IC_{50} of 3.11 μM (Liang et al., 2014).

In the literature, there is still no report of any cytotoxic peptide derived from *X. testudinaria*. However, several non-peptide compounds have been identified from *X. testudinaria*, which exhibited cytotoxic activity on several cell lines, including HeLa, HepG2 and human medulloblastoma (Daoy) cell lines (El-Gamal et al., 2016) (Table 2.9).

Table 2.9: Selected examples of non-peptide cytotoxic compounds derived from *X. testudinaria* (El-Gamal et al., 2016)

Compounds	Cell lines	Inhibition at 50 μg/mL, %
Xestosterol	HeLa	35.78
	HepG2	46.25
	Daoy	34.07
Brominated acetylenic fatty acid derivatives: 18,18-dibromo-(9E)-octadeca-9,17-diene-5,7-diynoic acid	HeLa	87.98
	HepG2	89.33
	Daoy	87.02
18-bromooctadeca-(9E,17E)-diene-7,15-diynoic acid	HeLa	67.00
	HepG2	18.40
	Daoy	77.56
l6-bromo (7E,11E,l5E)hexadeca-7,11,l5-triene-5,13-diynoic acid	HeLa	58.61
	HepG2	45.23
	Daoy	24.57

2.8 *Sarcophyton glaucum*

Soft corals are marine invertebrates that are generally bright in color and rich in nutritional substances (Rocha et al., 2011). However, the occurrence of predation in the soft corals is unexpectedly low owing to their effective defence mechanisms (Hooper and Davies-Coleman, 1995). They produce toxic compounds in order to protect themselves from their predators (Rocha et al., 2011). *S. glaucum* (family Alcyoniidae, under Phylum Cnidaria, Class Anthozoa) is a marine soft coral (van Ofwegen, 2010).

In recent years there has been growing interest in the discovery of bioactive secondary metabolites of *S. glaucum*, including cembranoids (Hegazy et al., 2011, Hegazy et al., 2012, Abou El-Ezz et al., 2013), bicembranoids (Huang et al., 2015), and steroids (Chao et al., 2017).Since 2011, much more information on non-peptide compounds derived from *S. glaucum* that exhibited cytotoxic activity has become available. These compounds were found to show cytotoxicity towards various cancer cell lines, including HeLa (Hegazy et al., 2011), HepG2 (Hegazy et al., 2011, Al-Lihaibi et al., 2014, Abdel-Lateff et al., 2015), MCF-7 (Al-Lihaibi et al., 2014, Abdel-Lateff et al., 2015), HCT-116 (Hegazy et al., 2011, Abdel-Lateff et al., 2015), MDA-MB-231, human T-cell lymphoblastic (SUP-T1), and human histiocytic lymphoma (U-937) (Chao et al., 2017) cell lines (Table 2.11). However, there is no previous study has investigated cytotoxic peptide from *S. glaucum.*

Table 2.10: Cytotoxicity of non-peptide cytotoxic compounds derived from *S. glaucum*

Compounds	Cell lines	IC_{50}	References
7beta-acetoxy-8alpha-hydroxydeepoxysarcophine	HepG2 HCT-116 HeLa	3.6 µg/mL 2.3 µg/mL 6.7 µg/mL	(Hegazy et al., 2011)
Sarcophytolol	HepG2	20 µM	(Al-Lihaibi et al., 2014)
Sarcophytolide B	MCF-7	25 ± 0.0164 µM	
Sarcophytolide C	HepG2 MCF-7	20 µM 29 ± 0.030 µM	
10(14)aromadendrene	HepG2	20 µM	
Sarcophinediol	HepG2 HCT116	18.8 ± 0.07 µM 19.4 ± 0.02	(Abdel-Lateff et al., 2015)
Sarcotrocheliol acetate	HepG2 MCF-7	19.9 ± 0.02 µM 2.4 ± 0.04 µM	
Deoxosarcophine	MCF-7 HCT116	9.9 ± 0.03 µM 25.8 ± 0.03 µM	
Sarcotrocheliol	MCF-7	3.2 ± 0.02 µM	
6-oxogermacra-4	HCT116	29.4 ± 0.03 µM	
Sarcomilasterol	MDA-MB-231 MOLT-4 SUP-T U-937	13.8 µg/mL 6.7 µg/mL 10.5 µg/mL 17.7 µg/mL	(Chao et al., 2017)
Sarcoaldesterol B	HepG2 MDA-MB-231 A-549	9.7 µg/mL 14.0 µg/mL 15.8 µg/mL	

CHAPTER 3

MATERIAL AND METHODS

3.1 Reagents and Materials

Ammonium sulfate, phthaldialdehyde, ACN, sodium bicarbonate and ultrafiltration centrifugal units (MWCO 3 kDa and 10 kDa) were purchased from Merck. Dialysis tubing (MWCO 6000-8000 Da) was obtained from Fisher Scientific. Trypsin, α-chymotrypsin and phthalaldehyde were purchased from Nacalai Tesque; alcalase and papain from Calbiochem. Di-sodium tetraborate and TFA were purchased from Fisher Chemical. RPMI 1640 medium, fetal bovine serum (FBS) and Penicillin-Streptomycin were from Gibco, Life Technologies. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Himedia. Phosphate-buffered saline (PBS) was obtained from Takara; MTT from Amresco; Sephadex G25 resin from GE Healthcare, and 5FU from Biobasic. Strata® C18-E SPE cartridges (55 µm, 70Å, 1000 mg/6 mL) were purchased from Phenomenex, Inc. ACN and TFA used were of HPLC-grade, whereas other reagents were of analytical grade.

3.2 Protein Isolation and Fractionation

3.2.1 Preparation of Protein Isolates

Specimens of *X. testudinaria* were collected in September 2013 on the offshore of Mentigi Island in Johor, Malaysia by research collaborator Mr. Affendi Yang Amri from University of Malaya. The specimens of *S. glaucum* were collected in July 2013, from Nanga Kecil Island in Johor, Malaysia by Dr. Jillian Ooi Lean Sim and Mr. Affendi Yang Amri. The samples were collected at 3 - 6 m depth using SCUBA. The identification of the sponge and soft coral species was carried out by Dr. Jillian Ooi Lean Sim and Mr. Affendi Yang Amri, referring to Hooper and Soest (2002) and Fabricius et al. (2001).

The specimens were kept on ice while they were transported back to the laboratory from the site of collection and immediately stored in a -20^{o}C freezer. The specimens were cut into smaller pieces and subjected to freeze-drying before use. The freeze dried samples were then pulverized into fine powder with a Waring blender. Proteins from *X. testudinaria* and *S. glaucum* were isolated according to the procedure used by Balti et al. (2010) with slight modification. Briefly, sample powder was suspended in cold deionized water at the ratio of 1 g: 5 mL. The mixture was stirred for 30 min at 4^{o}C followed by 20 min of heating at 90^{o}C to inactivate endogenous enzyme. To separate water insoluble substances, the heated mixture was then centrifuged (8603 × g for 20 min) for 20 min. The supernatant was collected and was brought up to 80% saturation by adding ammonium sulfate and then stirred at 4^{o}C for 1 h. After centrifugation at 20,000 × g at 4^{o}C for 1 h, the supernatant was carefully

discarded, while the pellet (corresponded to the proteins precipitate) was dialyzed overnight at 4^{o}C against deionized water. Next, the dialyzed protein isolate was freeze dried and stored at -20^{o}C for later use. The quantification of the protein content of the isolate was done by means of the Bradford's assay (Bradford, 1976), based on a bovine serum albumin standard curve.

3.2.2 Preparation of Hydrolysates

The freeze dried protein isolate was dissolved in 50 mM sodium phosphate buffer at a ratio of 1 g protein isolate to 200 mL buffer. The pH of the buffer and the optimum temperature for each protease used were according to Byun et al. (2009), Jung et al. (2007), Forghani et al. (2012) and Tanzadehpanah et al. (2012), as listed in Table 3.1.

Table 3.1: The optimum pH and temperatures for alcalase, chymotrypsin, papain and trypsin

Proteases	Optimum pH	Optimum temperature, ^{o}C
Alcalase	7	50
Chymotrypsin	7	37
Papain	6	37
Trypsin	8	37

Each protease was then added separately into a chilled protein-buffer mixture and 1 mL of aliquot was immediately removed from the mixture which corresponded to the 0-hour aliquot. The hydrolysis was initiated when the mixture was incubated in a water bath maintained at the optimum temperature of the proteases (Table 3.1). Protein hydrolysate sample (1 mL) was taken from each protein-buffer mixture at different intervals up to 8 hours. Each aliquot was heated at 100^{o}C for 10 min to inactivate the protease and

then freeze-dried to be used in the cytotoxicity test as described later. DH was determined as previously described (Chen et al., 2009b) for each protease treatment to pinpoint the optimum proteolysis duration.

3.2.3 Fractionation of Papain Hydrolysate

3.2.3.1 Membrane Ultrafiltration

Fifteen mL of *X. testudinaria* papain hydrolysate (10 mg/mL in deionized water) was added into a 10 kDa MWCO ultrafiltration centrifugal unit. It was then centrifuged at 5000 × g and 25^{o}C for 20 min. The retentate was designated as "> 10 kDa UF", whereas the permeate fraction was transferred into a 3 kDa MWCO ultrafiltration centrifugal unit and centrifuged as illustrated above. The resulting retentate was designated as "3-10 kDa UF", while the permeate fraction was designated as "< 3 kDa UF". The three UFs were freeze-dried and tested for cytotoxicity using the MTT assay as described later. The results from *X. testudinaria* implied that fractionation using 10 kDa was not necessary. Therefore for *S. glaucum*, membrane ultrafiltration was performed by using only 3 kDa MWCO ultrafiltration centrifugal unit. The resulting permeate fraction was designated as "< 3 kDa UF"; the retentate, "> 3 kDa UF". The quantification of peptide contents of the UF samples was performed by using the OPA method (Nielsen et al., 2001).

3.2.3.2 Gel Filtration Chromatography

For *X. testudinaria* and *S. glaucum*, the freeze dried < 3 kDa UF was dissolved in deionized water at the concentration of 25 mg/mL and then filtered through 0.22 µm filters. Two mL of the solution was loaded onto a Sephadex G-25 gel filtration column (1.6 × 70 cm), pre-equilibrated and eluted with deionized water at a flow rate of 1.55 mL/min. Eluate was collected at 2-min intervals and elution profile was established by monitoring the absorbance of each fraction at 280 nm with a UV-Vis spectrophotometer. Pooled fractions (GF1, GF2, and GF3) were collected, freeze dried, and tested for cytotoxicity using the MTT assay described later. Peptide content of the samples was determined as described in Section 3.2.3.1.

3.2.3.3 Semi-preparative Reversed-phase High Performance Liquid Chromatography

For *X. testudinaria*, GF3 was dissolved in deionized water and filtered through 0.22 µm filter membrane and injected into the RP-HPLC column. Generally, the column was eluted with a linear gradient of acetonitrile (40-50% in 60 min) containing 0.1% TFA. The RP-HPLC parameters used for purification of GF3 are summarized in Table 3.2.

Table 3.2: The parameters used in semi-preparative RP-HPLC

Parameters	Descriptions
RP-HPLC system	PerkinElmer Flexar FX-20 UHPLC
RP-HPLC column	Eclipse XDB-C_{18} column (5µm, 9.4 × 250 mm)
Mobile phase	Solvent A: Deionized water containing 0.1% TFA Solvent B: ACN containing 0.1% TFA
Flow rate	0.8 mL/min
Wavelength	214 nm

Pooled fractions namely F3P1, F3P2, F3P3, and F3P4 were collected and vacuum-concentrated at 45^{o}C until fully dried. Their cytotoxicity was determined by using MTT assay described later. Peptide content of the samples was determined as described above.

3.2.3.4 Solid Phase Extraction

For *S. glaucum*, GF3 was further purified by using SPE cartridges. This method was used as an alternative due to the breakdown of RP-HPLC machine at that point in time. The freeze dried pooled fraction GF3 was dissolved in deionized water (50 mg/mL) and filtered through 0.22 µm filter membrane. Then, 2 mL of sample was applied to the Strata® C18-E cartridges which were preconditioned with methanol (6 mL), washed with 100% ACN containing 0.1% TFA (6 mL), and equilibrated with deionized water containing 0.1% TFA (12 mL). GF3 was fractionated by using a stepwise elution (6 mL per step) with increasing ACN concentrations in 0.1% TFA and the fractions were labeled accordingly as summarized in Table 3.3.

Table 3.3: Solid phase extraction stepwise elution

Concentration of ACN containing 0.1% TFA, %	Fractions
0	SPE-F1
10	SPE-F2
20	SPE-F3
30	SPE-F4
40	SPE-F5
50	SPE-F6
80	SPE-F7
100	SPE-F8

Absorbance of each eluted fraction was monitored at 214 nm with a UV-Vis spectrophotometer. The fractions were then vacuum-concentrated at 45^{o}C until fully dried and were tested for cytotoxicity. Peptide content of the samples was determined as described above.

3.2.3.5 Analytical Reversed-phase High Performance Liquid Chromatography

The dried SPE-F7 derived from *S. glaucum*, was dissolved in deionized water, sonicated and filtered through 0.22 µm filter membrane. Ten micro liters of SPE-F7 was injected into the analytical RP-HPLC column. Briefly, the column was eluted with a gradient elution as follows: 0-10 min, 5-35% of solvent B; 10-35 min, 35-95% solvent B; 35-41 min, 95% solvent B; 41-41.01 min 95-5% solvent B; 40.01-50 min, 5% solvent B. The RP-HPLC parameters used to analyze SPE-F7 are shown in Table 3.4.

Table 3.4: The parameters used in analytical RP-HPLC

Parameters	Descriptions
RP-HPLC system	Shimadzu LC-20D dual binary pumps and Shimadzu Prominence SPD-M20A PDA detector
RP-HPLC column	Kinetex C_{18} column (100 Å, 5µm, 4.6 × 250 mm)
Mobile phase	Solvent A: Deionized water containing 0.1% TFA Solvent B: ACN containing 0.1% TFA
Flow rate	0.5 mL/min
Wavelength	214 nm

3.3 Cytotoxicity Assay

3.3.1 Preparation of Culture Medium

The culture medium was prepared by dissolving RPMI-1640 (for HeLa cell line) or DMEM (for Hek293 cell line) powder and 2 g of sodium bicarbonate in 1 L of deionized water according to the manufacturer's instruction. The medium was filter-sterilized through a Corning® 0.2 µm cellulose acetate membrane filtration unit into an autoclaved 1 L Schott bottle and stored at 4^{o}C. The sterile medium was supplemented with 10% (v/v) FBS and 1% Penicillin-Streptomycin.

3.3.2 Cell Culture Preparation

The human cervical cancer cell line (HeLa, cell line number ATCC CCL-2) and human embryonic kidney cell line (Hek293, cell line number ATCC CRL-1573) were grown at 37^{o}C in a humidified incubator in 5% CO_2, in their respective culture medium prepared by the method described above.

The cells were sub-cultured every 3 days or when the cells reached approximately 80% confluency as observed under the inverted microscope. The culture was checked regularly for contamination.

3.3.3 MTT Assay

The cell confluency was checked under inverted microscope and ensured to be more than 80% before they were seeded into a 96-well plate at a density of 1 x 10^4 cells per well and incubated at 37^{o}C in a humidified incubator in 5% CO_2 for 24 h. Then, 100 µL of sample of different concentrations in sterile deionized water were added to each well and were incubated for another 24 h. Sterile water was used as negative control in place of sample. After 24 h of treatment, 20 µL of MTT solution (5 mg/mL) was added to each well and the plate was incubated further for 4 h. Next, the 96-well plate was centrifuged at 1000 × g for 5 min and 70% of supernatant in each well was carefully removed. Next, 200 µL of 100% DMSO was added into each well to solubilize the purple formazan crystals, The absorbance for each well was determined at 570 nm with a 96-well plate reader. 5FU was used as the positive control (Jumeri and Kim, 2011).

3.4 Peptide Sequence Identification

For *X. testudinaria*, the peptide sequence in F3P4 was determined by means of online LC-MS/MS analysis at Fitgene Bio Pte Ltd, China. Briefly, the purified peptide was analyzed by an Acclaim PepMapRSLCC18 column

and then introduced to a Thermo Scientific Q Exactive Hybrid-Quadrupole-Orbitrap mass spectrometer coupled with an electrospray ionization source in the positive ion mode. Mass spectra were searched against a *Xestospongia* sp. database using MASCOT software (version 2.3; Matrix Science) for F3P4 peptide sequence identification. Database search parameters were set as follows: fixed modifications: carbamidomethyl (cysteine); variable modifications: oxidation (methionine); enzyme: no; peptide mass tolerance: 20 ppm; fragment mass tolerance: 0.6 Da; peptide/fragment ion mass values: monoisotopic; and significance threshold: 0.05.

For *S. glaucum*, the identification of the peptide sequences in SPE-F7 was performed with online LC-MS/MS analysis at the Proteomics Core facility, Malaysia Genome Institute, National Institutes of Biotechnology Malaysia. Briefly, the purified peptide was resolved by a Waters nanoACQUITYUPLC, coupled to the Waters SynaptG2HDMS-Q-TOF mass spectrometer. *De novo* peptide sequencing was performed using Data Directed Analysis (DDA) with the positive electrospray ionization mode. ProteinLynx Global Server Software (Version 2.4) was employed for data analysis.

After sequence determination, peptides were chemically synthesized by Bio Basic Inc., Canada. The purity of synthetic peptides was over 95% and the cytotoxicity of the peptides on HeLa and Hek293 cell lines was tested using MTT assay as described in Section 3.3.3.

3.5 Peptide Stability in Human Serum

Peptide stability was assayed in diluted human serum as described in Cudic et al. (2002) and Nguyen et al. (2010). Briefly, synthetic peptide KENPVLSLVNGMF was added to 25% human serum at a final peptide concentration of 1 mg/mL (690 µM) and incubated at 37^{o}C in a shaking incubator. Aliquots of 250 µL of the mixture were taken out at the following time points: 0, 2, 4 and 6 h. The aliquots were mixed with 50 µL of 15% trichloroacetic acid and incubated at 4^{o}C for 15 min to precipitate serum proteins. The mixture was centrifuged at 13000 × g for 10 min, and the supernatant was carefully collected and stored at -20^{o}C for peptide analysis by RP-HPLC with the parameters as summarized in Table 3.5. The column was eluted with a gradient elution designed as follows: 0-10 min, 5-35% of solvent B; 10-35 min, 35-95% solvent B; 35-41 min, 95% solvent B; 41-41.01 min 95-5% solvent B; 40.01-50 min, 5% solvent B.

Table 3.5: The parameters used in analytical RP-HPLC to analyze the peptides presence in human serum

Parameters	Descriptions
RP-HPLC system	Shimadzu LC-20D dual binary pumps and Shimadzu Prominence SPD-M20A PDA detector
RP-HPLC column	Kinetex C_{18} column (100 Å, 5µm, 4.6 × 250 mm)
Mobile phase	Solvent A: Deionized water containing 0.1% TFA Solvent B: ACN containing 0.1% TFA
Flow rate	1.0 mL/min
Wavelength	214 nm

The relative concentrations of the peptides (expressed as a percentage) was calculated from the peak area obtained from the chromatogram at each time point versus the peak area at 0 h. Half-life of the peptide, defined as the time point where peptide concentration is 50% of the initial concentration, was calculated by using linear regression analysis.

3.6 Data Analysis

Data are expressed as mean ± standard errors (n = 3). SAS (Version 9.4) was used for statistical analysis. Data were subjected to analysis by one-way ANOVA, followed by the Fisher's Least Significant Difference (LSD) test to separate means of significant differences where appropriate. Student's T-test was used for comparison of two mean values. A probability (p) value < 0.05 was considered statistically significant.

CHAPTER 4

RESULTS

4.1 *Xestospongia testudinaria*

After ammonium sulfate precipitation and dialysis, proteins of *X. testudinaria* were harvested for enzymatic hydrolysis. The total yield of the crude protein isolate was 1.6% of the weight of freeze-dried *X. testudinaria*. Furthermore, the soluble protein content of the crude protein isolate was determined as 0.13 g soluble protein/g protein isolate, based on a bovine serum albumin (BSA) standard curve.

4.1.1 Hydrolysis of *X. testudinaria* Proteins

The isolated protein was subjected to proteolysis and the changes in the DH of sponge proteins were monitored over a duration of 8 h; the degrees of change varied depending on the proteases used (Figure 4.1). In general, the DH values generated by alcalase hydrolysis were higher when compared to other proteases. The trend lines demonstrated an elevation in the DH values of alcalase and trypsin hydrolysis during the first 6 h, and then declined thereafter. Conversely, DH values of papain and chymotrypsin hydrolysis rose up to 4-5 h, and then dropped from then on.

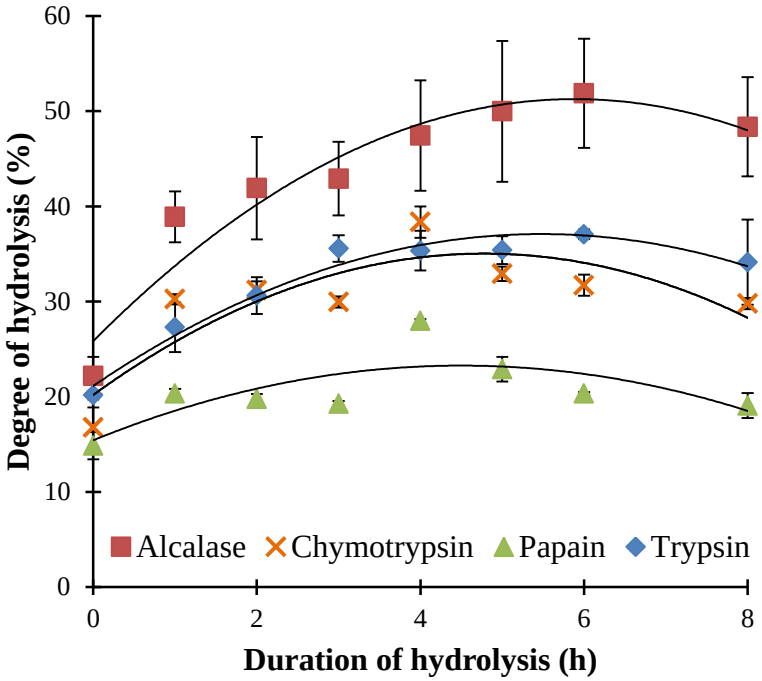


Figure 4.1: Degree of hydrolysis of *X. testudinaria* proteins during hydrolysis with alcalase, chymotrypsin, papain and trypsin. Data are means ± standard errors (n=3)

Both chymotrypsin and trypsin hydrolysis produced very similar DH values over the 8-hour duration. In this study, the duration required for each hydrolysis to achieve maximum DH was taken as the optimal hydrolysis duration. Therefore, the optimum duration for alcalase and trypsin hydrolysis was determined as 6 h, and chymotrypsin and papain was 4 h, which were selected based on the analysis of trend lines (Figure 4.1). The DH values obtained at the optimum duration for alcalase, chymotrypsin, papain and trypsin hydrolysis were 51.9 ± 5.7%, 38.3 ± 1.7%, 28.0 ± 0.2% and 37.1 ± 0.5%, respectively. Hydrolysates that were obtained at their respective optimum durations were collected for cytotoxicity test against the HeLa cell line.

4.1.2 Cytotoxic Activity of *X. testudinaria* Hydrolysates

Following enzymatic hydrolysis, the cytotoxic activities of the sponge protein hydrolysates were assessed using MTT assay. As shown in Figure 4.2, the cytotoxic activity of the alcalase, trypsin and chymotrypsin hydrolysate on showed no dose-dependent trends over the range of concentration tested. On the other hand, when tested at the concentration of 1, 2 and 3 mg/mL, papain hydrolysate displayed dose-dependent increase in cytotoxicity. As a positive control, 5FU exhibited cytotoxicity against HeLa cells in a dose-dependent trend. Particularly, when compared at the concentration of 1-4 mg/mL, the cytotoxicity of papain hydrolysate was either stronger ($p < 0.05$) or similar to that of 5FU. Besides, papain hydrolysate exhibited the highest cytotoxicity among the four hydrolysates at 5 mg/mL. Therefore, papain hydrolysate was selected for further bioassay-guided purification by using ultrafiltration membrane.

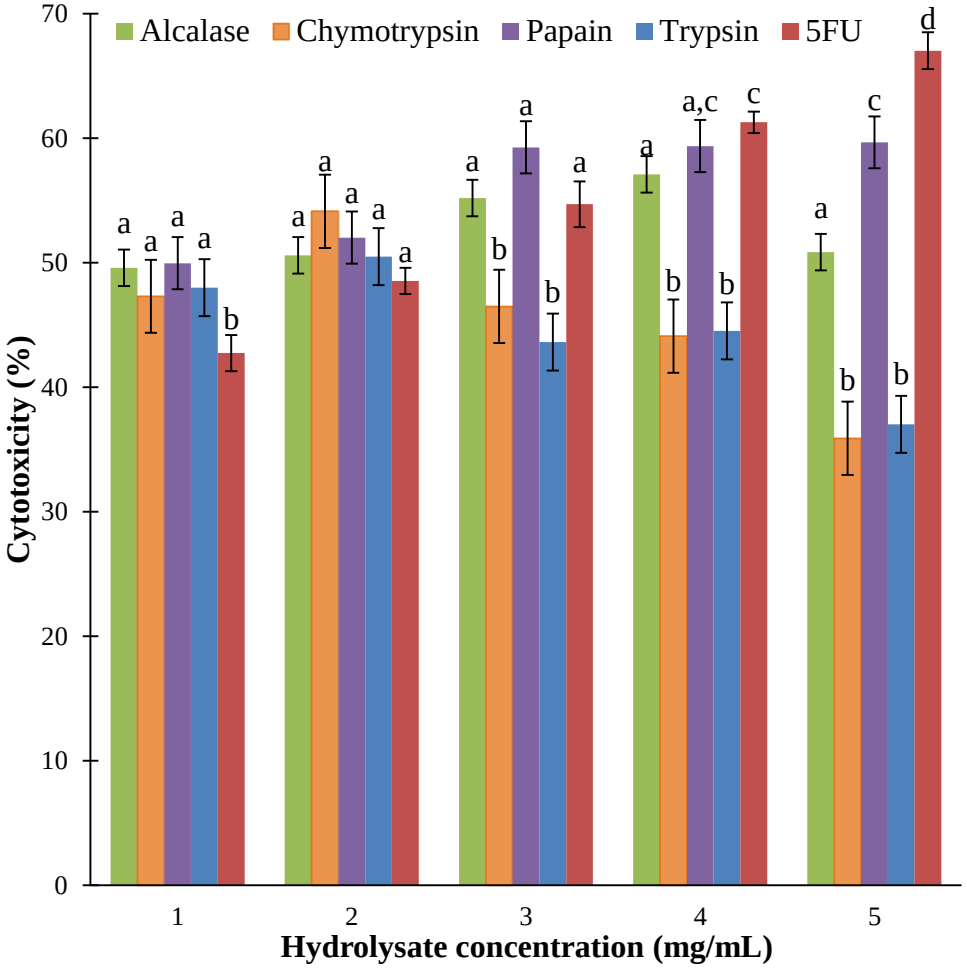


Figure 4.2: Cytotoxicity of sponge hydrolysates produced by the four proteases. Data are means ± standard errors (n=3). Data for the same hydrolysate concentration that are labeled by different letters are significantly different ($p < 0.05$), as determined using the Fisher's LSD test

4.1.3 Purification of Cytotoxic Peptides

4.1.3.1 Membrane Ultrafiltration

Membrane ultrafiltration separated papain hydrolysate into three UF: < 3 kDa, 3–10 kDa and > 10 kDa. The MTT assay results showed that the EC_{50} value of the < 3 kDa UF (0.17mg/mL) was about 7 times lower compared to that of the 3–10 kDa UF (1.18 mg/mL). Furthermore, the EC_{50} values of < 3

kDa UF and 3–10 kDa UF were significantly lower compared to that of 5FU (Figure 4.3). The EC_{50} was not determined for the > 10 kDa UF owing to lack of a dose-dependent increase pattern in cytotoxicity. The result suggests that the < 3 kDa UF was more cytotoxic than 3–10 kDa UF. Therefore, the < 3 kDa UF was taken for further fractionation using GF chromatography.

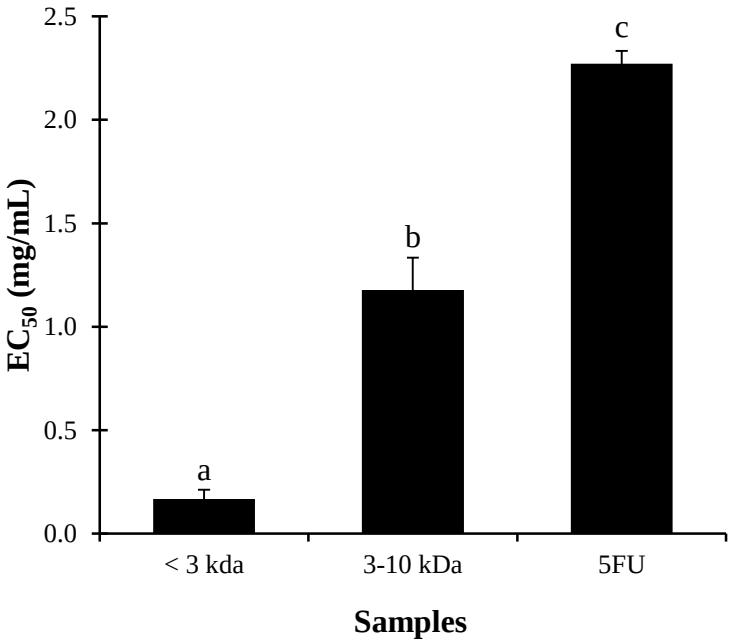


Figure 4.3: Cytotoxicity of the UF fractions and 5FU, expressed as EC_{50} values. Data are means ± standard errors (n=3). Data labeled by different letters are significantly different ($p < 0.05$), as determined using the Fisher's LSD test

4.1.3.2 Gel Filtration Chromatography

Figure 4.4 shows a representative GF elution profile of < 3 kDa UF separated by gel filtration chromatography. Pooled fractions of GF1, GF2 and GF3 were tested against HeLa cell line to evaluate their cytotoxic activity. GF1 (EC_{50} 0.08 mg/mL) and GF3 (EC_{50} 0.03 mg/mL) showed higher

cytotoxicity compared to < 3 kDa UF. Furthermore, the result shows GF3 was more cytotoxic than GF1 as the EC_{50} of GF3 was about 2.7-fold lower than that of GF1. Due to a lack of concentration-dependent increase in cytotoxicity, the EC_{50} of GF2 was not determined. GF3 was then further purified by using semi-preparative RP-HPLC.

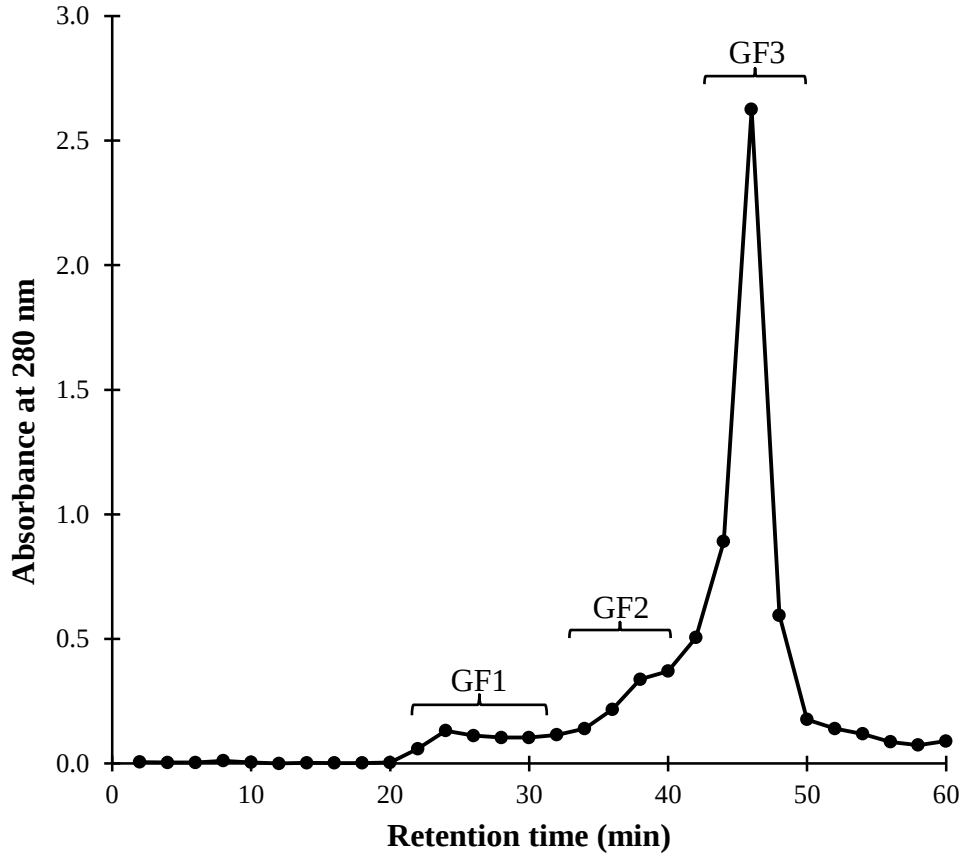


Figure 4.4: A gel filtration chromatography elution profile of the < 3 kDa UF fraction. The peaks eluted were separated into three fractions, namely GF1, GF2 and GF3

4.1.3.3 Semi-preparative RP-HPLC

Figure 4.5 shows a semi-preparative RP-HPLC chromatogram of GF3. The chromatogram revealed four major peaks in GF3 and the pooled fractions, namely F3P1, F3P2, F3P3 and F3P4, were collected and were tested for cytotoxicity at the concentration of 0.03 mg peptide/mL. This concentration was chosen based on the EC_{50} of GF3 determined in the previous step.

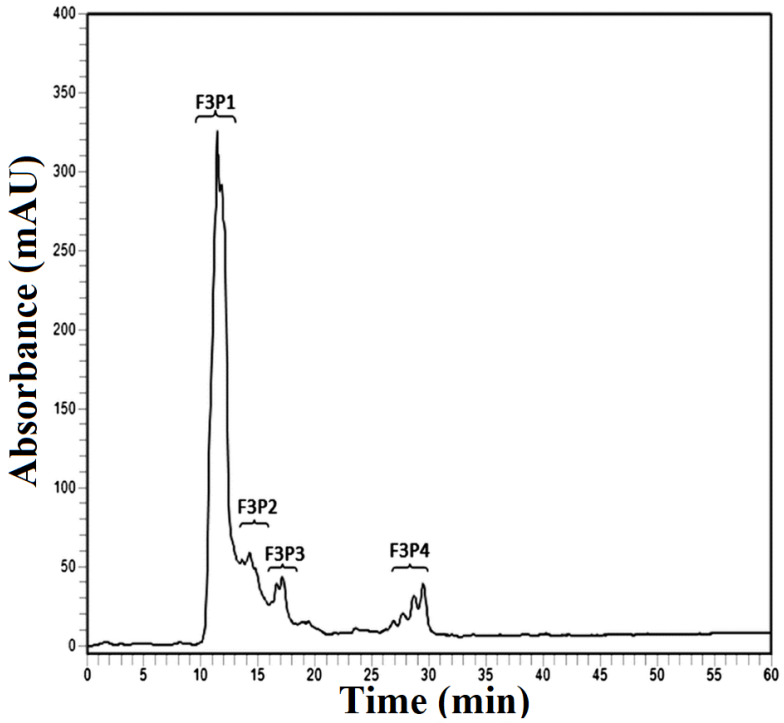


Figure 4.5: RP-HPLC profile of GF3 fraction obtained from gel filtration chromatography. The peaks eluted were pooled into four fractions, designated F3P1, F3P2, F3P3 and F3P4

Of the four fractions collected, only two fractions (F3P1 and F3P4) showed cytotoxicity against HeLa cells (Figure 4.6). Significantly, F3P1 exhibited the strongest cytotoxic activity among the four fractions. Hence, this fraction was subjected to peptide identification by LC-MS/MS analysis.

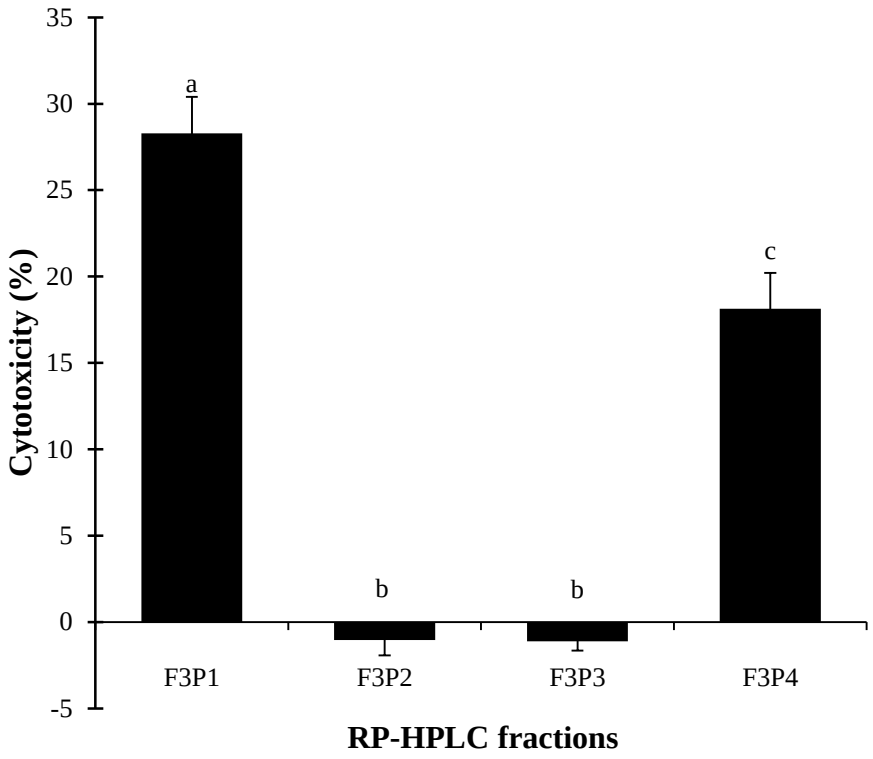


Figure 4.6: Cytotoxicity of semi-preparative RP-HPLC fractions tested at 0.03 mg/mL. Data are means ± standard errors (n=3). Data labeled by different letters are significantly different ($p < 0.05$), as determined using the Fisher's LSD test

4.1.3.4 Peptide Identification

LC-MS/MS analysis and database searching were employed to identify the sequence of peptides present in F3P1. Consequently, the sequences of two potential cytotoxic peptides were identified, namely KENPVLSLVNGMF and LLATIPKVGVFSILV with the MW of 1447.70 Da and 1570.04 Da, respectively.

4.1.3.5 Validation of Cytotoxicity of Synthetic Peptides

The peptides KENPVLSLVNGMF and LLATIPKVGVFSILV were chemically synthesized and their cytotoxic activities were validated. Generally, pentadecapeptide LLATIPKVGVFSILV showed no dose-dependent cytotoxicity on HeLa cells when tested up to 500 µg/mL. Conversely, tridecapeptide KENPVLSLVNGMF exhibited dose-dependent cytotoxicity on HeLa cells (Figure 4.7). Notably, the cytotoxicity of KENPVLSLVNGMF was considerably greater than that of 5FU. KENPVLSLVNGMF exerted 90% cytotoxicity in HeLa cells when tested at 1.24 mM (1.8 mg/mL). On the contrary, 5FU showed only 66% cytotoxicity at 38.4 mM (5 mg/mL). The EC_{50} of KENPVLSLVNGMF and 5FU, expressed on a millimolar basis, were 0.67 mM and 2.56 mM, respectively.

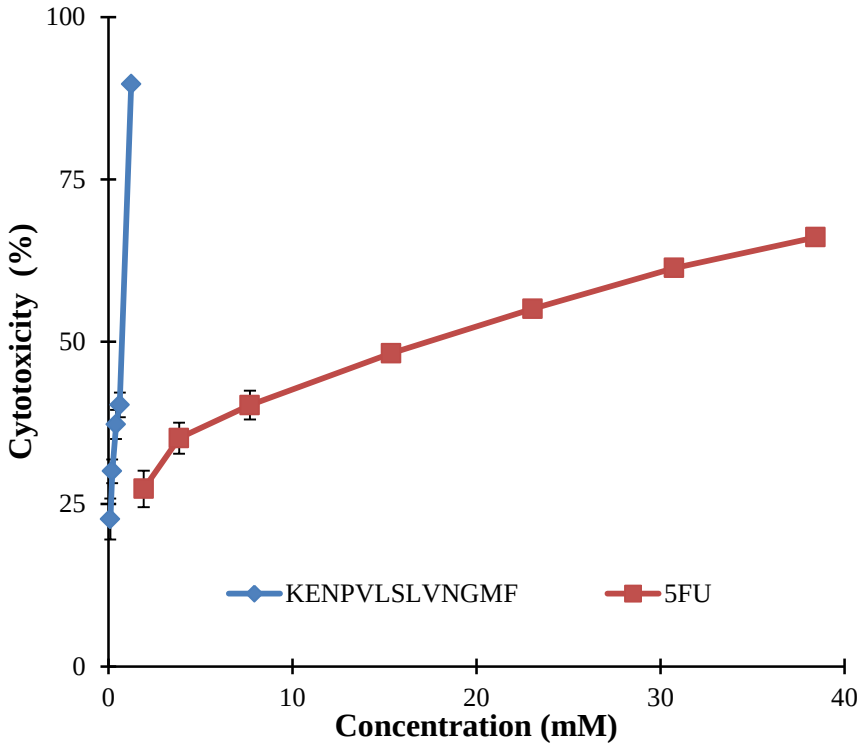


Figure 4.7: Cytotoxicity of KENPVLSLVNGMF and 5FU compared on a millimolar basis. Data are means ± standard errors (n=3)

Besides, the selectivity of tridecapeptide KENPVLSLVNGMF was also evaluated. The peptide was tested against Hek293 (non-cancerous) and HeLa cell lines at the concentration 0.67 mM, which is the EC_{50} of the peptide. The results showed that KENPVLSLVNGMF exhibited less than 5% cytotoxicity in Hek293 cells, but 44% cytotoxicity in HeLa cells (Figure 4.8).

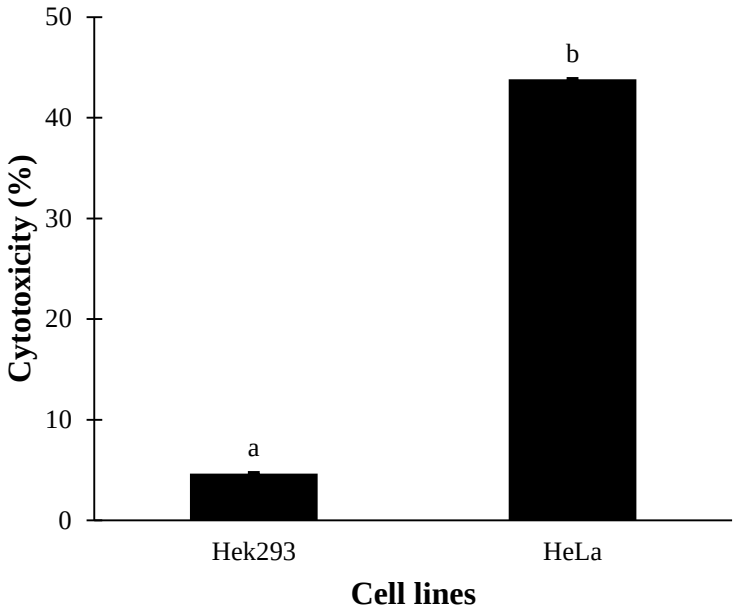


Figure 4.8: Cytotoxicity of KENPVLSLVNGMF, tested at 0.67 mM, on Hek293 and HeLa cell lines. Data are means ± standard errors (n=3). Data labeled by different letters are significantly different ($p < 0.05$), as determined by Student's T-test

As shown in Figure 4.9, potency of peptide fractions (< 3 kDa UF and GF3) purified from hydrolysate of *X. testudinaria* was compared with the potency of synthetic peptide (KENPVLSLVNGMF). By comparison, KENPVLSLVNGMF shows about 140-fold and 25-fold stronger cytotoxicity than < 3 kDa UF and GF3, respectively.

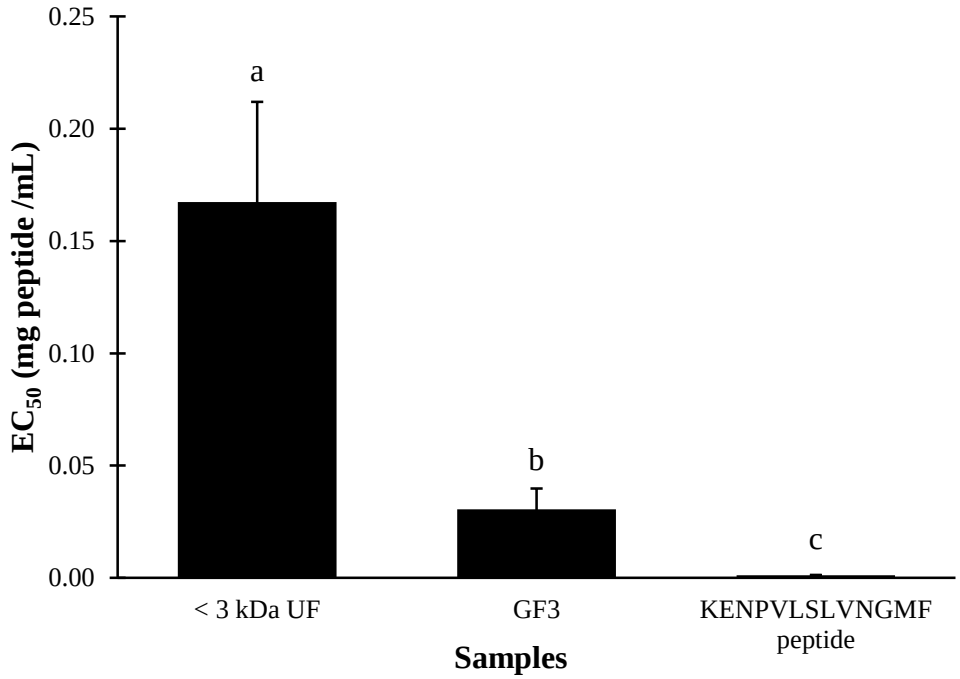


Figure 4.9: Comparison of EC_{50} values of purified *X. testudinaria* peptide fractions and synthetic peptide. Data are means ± standard errors (n=3). Data labeled by different letters are significantly different ($p < 0.05$), as determined using the Fisher's LSD test

4.1.4 Serum Stability Test

By using the analytical RP-HPLC conditions described in the previous chapter, KENPVLSLVNGMF peptide was detected at the average retention time of 17.36 min (Figure 4.10A). Based on the RP-HPLC chromatograms, the peak areas of KENPVLSLVNGMF peptide reduced steadily over the 6 h of incubation time (Figure 4.10A-D). In the first 2 h of incubation, a rapid fall in peptide concentration to 55.5 ± 8.2% was observed (Figure 4.11). Nevertheless, 19% of KENPVLSLVNGMF peptide was detected in the human serum after 6 h of incubation and KENPVLSLVNGMF peptide have half-life of 3.2 ± 0.5 h.

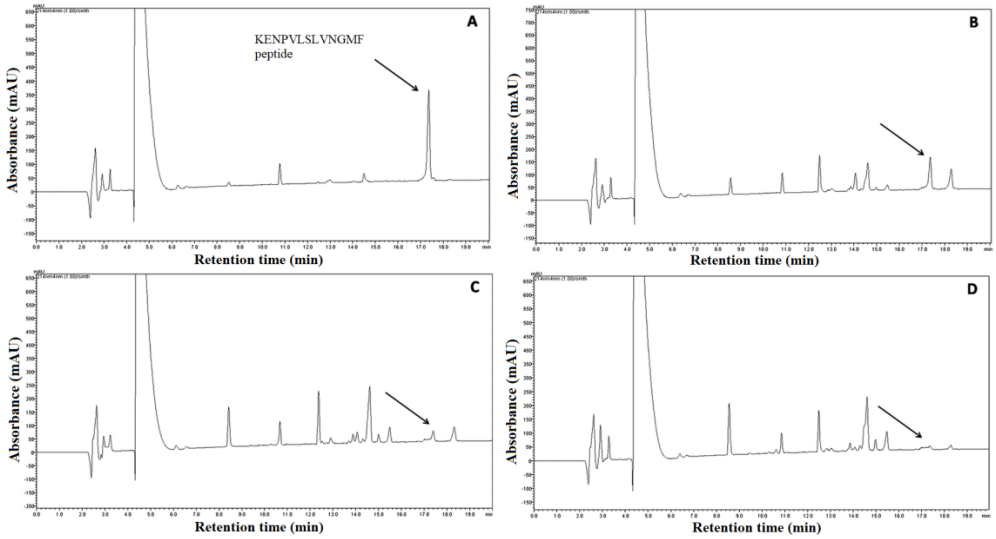


Figure 4.10: Representative RP-HPLC profiles of KENPVLSLVNGMF following incubation in human serum for (A) 0 h, (B) 2 h, (C) 4 h, and (D) 6 h. Arrow indicates the KENPVLSLVNGMF peak, eluted at retention time 17.37 min

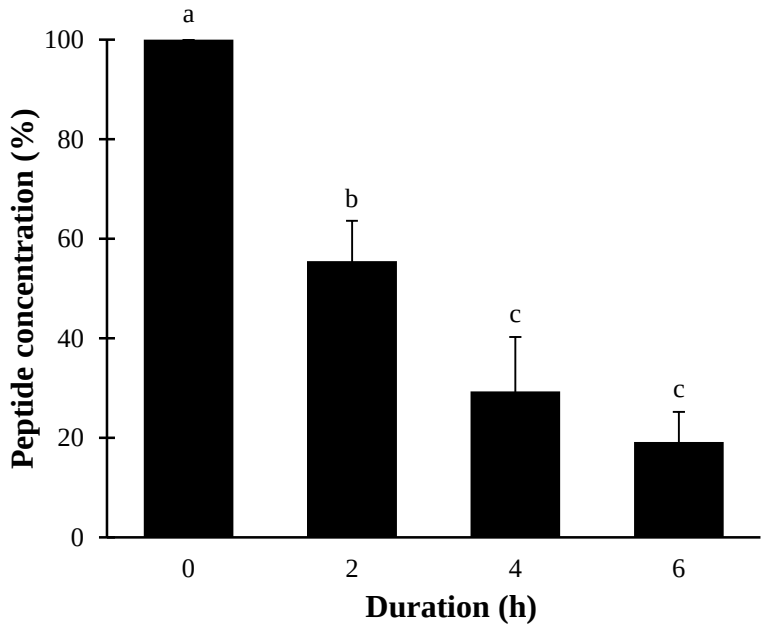


Figure 4.11: KENPVLSLVNGMF concentration in human serum over 6 h of incubation. . Data are means ± standard errors (n=3). Data labeled by different letters are significantly different ($p < 0.05$), as determined by the Fisher's LSD test

4.2 *Sarcophyton glaucum*

The same protein isolation method described previously was used to isolate protein from freeze-dried *S. glaucum* sample. The total yield of the crude protein isolate was 2.1% of the weight of freeze-dried *S. glaucum*. The soluble protein content of the isolate was 443.1 mg proteins/g dry weight.

4.2.1 Hydrolysis of *S. glaucum* Proteins

The isolated protein was subjected to hydrolysis with alcalase, chymotrypsin, papain and trypsin under controlled conditions and monitored up to 8 h (Figure 4.12). Generally, with the same enzyme:substrate ratio, alcalase hydrolysis stands out by generating the highest DH value among all four proteases. After 8 h of hydrolysis, the highest DH value produced by alcalase was 27.5 ± 0.44%. Conversely, there was no obvious trend in DH values produced by hydrolysis using chymotrypsin, papain and trypsin. Throughout the 8-h hydrolysis, chymotrypsin and papain hydrolysates showed very similar DH values, and their highest DH values were 20.65 ± 0.16% and 20.8 ± 0.82% respectively, obtained at the 8^{th} h time point. Whereas trypsin hydrolysis produced a maximum DH value of 20.08 ± 1.14% at the 4^{th} h time point. Hence, the optimum duration for alcalase, chymotrypsin and papain hydrolysis was determined as 8 h, and trypsin was 4 h.

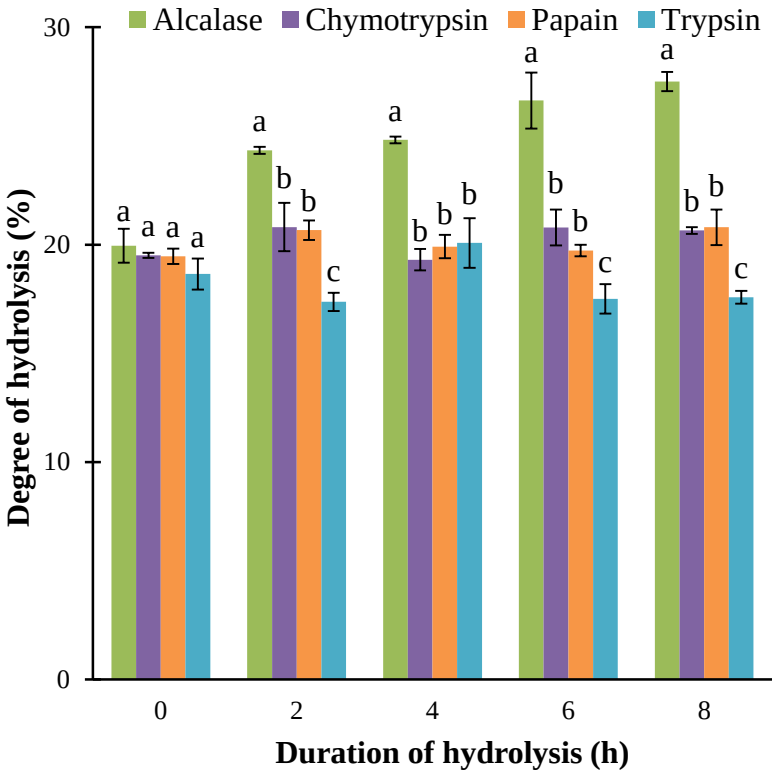


Figure 4.12: DH of soft coral proteins hydrolysed by alcalase, chymotrypsin, papain and trypsin over 8-h duration. Data are means ± standard errors (n=3). Data for the same hydrolysis duration that are labelled with different letters are significantly different ($p < 0.05$) according to the Fisher's LSD test

4.2.2 Cytotoxic Activity of *S. glaucum* Hydrolysates

Cytotoxic activity of hydrolysates harvested at their respective optimum hydrolysis durations were tested on HeLa cell line. Figure 4.13 shows that all four hydrolysates possessed cytotoxicity towards HeLa cells at concentration 1 – 5 mg/mL. Remarkably, only papain hydrolysate exhibited dose-dependent cytotoxicity among the four hydrolysates. On the contrary, alcalase, chymotrypsin and trypsin hydrolysates showed minor fluctuations in cytotoxicity over the concentration range tested. 5FU showed dose-dependent cytotoxicity against HeLa cells and it was more cytotoxic than all four hydrolysates. At 5 mg/mL, papain hydrolysate showed the highest cytotoxicity (50.4%) compared to hydrolysates prepared by alcalase (29.9%), chymotrypsin (31.1%) and trypsin (34%). As a result, papain hydrolysate was chosen for further fractionation by UF membrane.

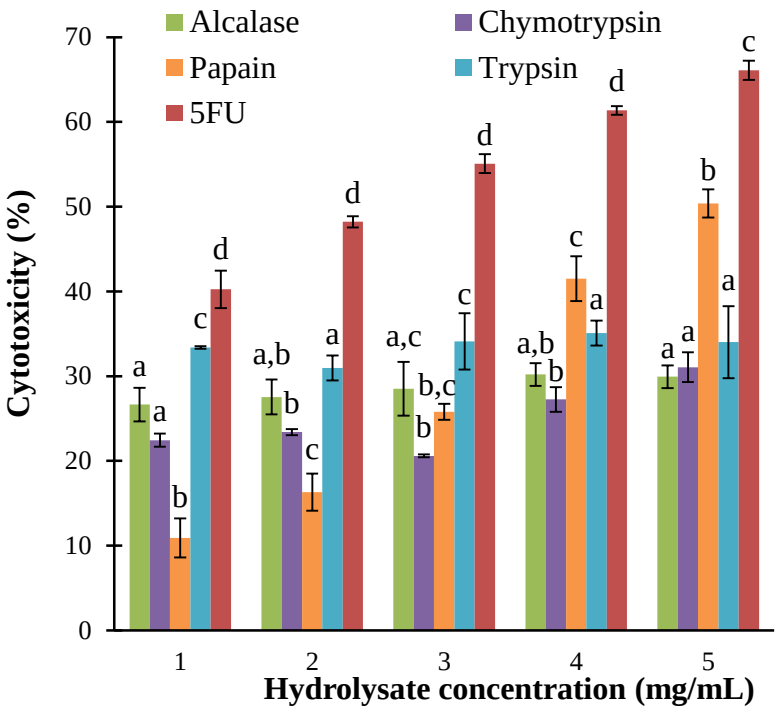


Figure 4.13: Cytotoxicity of *S. glaucum* hydrolysates prepared by using alcalase, chymotrypsin, papain and trypsin against the HeLa cell line. Data are means ± standard errors (n=3). Data for the same hydrolysate concentration that are labelled with different letters are significantly different ($p < 0.05$) according to the Fisher's LSD test

4.2.3 Purification of Cytotoxic Peptides

4.2.3.1 Membrane Ultrafiltration

Papain hydrolysate was separated into two fractions: < 3 kDa UF and > 3 kDa UF. These two fractions were tested against HeLa cell line for their cytotoxicity (Figure 4.14). Notably, the EC_{50} of < 3 kDa UF (0.16 ± 0.01 mg/mL) and > 3 kDa UF (0.14 ± 0.01 mg/mL) were considerably lower than that of 5FU. Contrary to expectations, the observed difference between the two fractions in this study was not statistically significant ($p > 0.05$, Student's T-test). Much of the available literature (Hsu et al., 2011, Hung et al., 2014, Song et al., 2014) often found marine peptides which possess cytotoxic

activity to be less than 3 kDa in size. Therefore, < 3 kDa UF was chosen for purification.

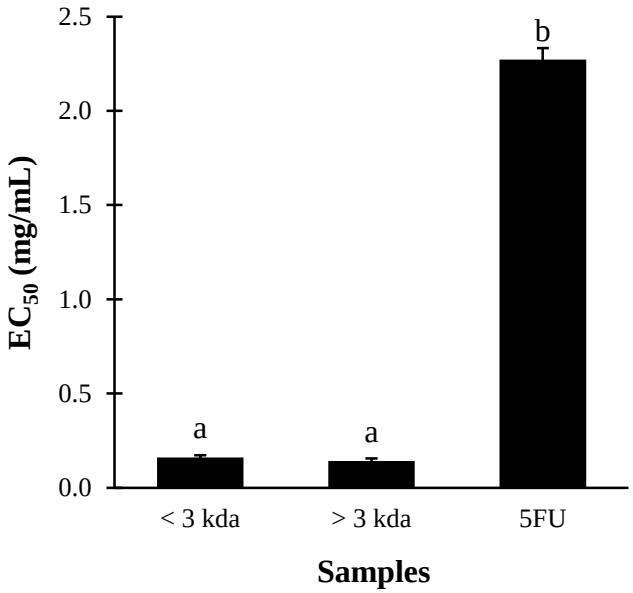


Figure 4.14: Cytotoxicity of the UF fractions and 5FU, expressed as EC_{50} values. Data are means ± standard errors (n=3). Data labeled by different letters are significantly different ($p < 0.05$), as determined using the Fisher's LSD test

4.2.3.2 Gel Filtration Chromatography

Figure 4.15 shows a representative elution profile of < 3 kDa UF fractionated by using Sephadex G-25 column. Pooled fractions of GF1, GF2 and GF3 were tested against HeLa cell. In general, the EC_{50} values of all three fractions were significantly lower compared to that of 5FU (Figure 4.16). The EC_{50} of GF2 was the highest among the three fractions. According to Student's T-test, the EC_{50} value of GF2 was not significantly different from that of < 3 kDa UF ($p > 0.05$). Whereas the EC_{50} of GF1 (0.06 mg/mL) and GF3 (0.04 mg/mL) were almost 2.7- and 4-fold lower than that of < 3 kDa UF, respectively ($p < 0.05$, Student's T-test). Furthermore, the EC_{50} values of GF1

and GF3 were about 37.8- and 56.8-fold lower than that of 5FU, respectively. Therefore, GF3 was subjected to reversed phase SPE for further purification.

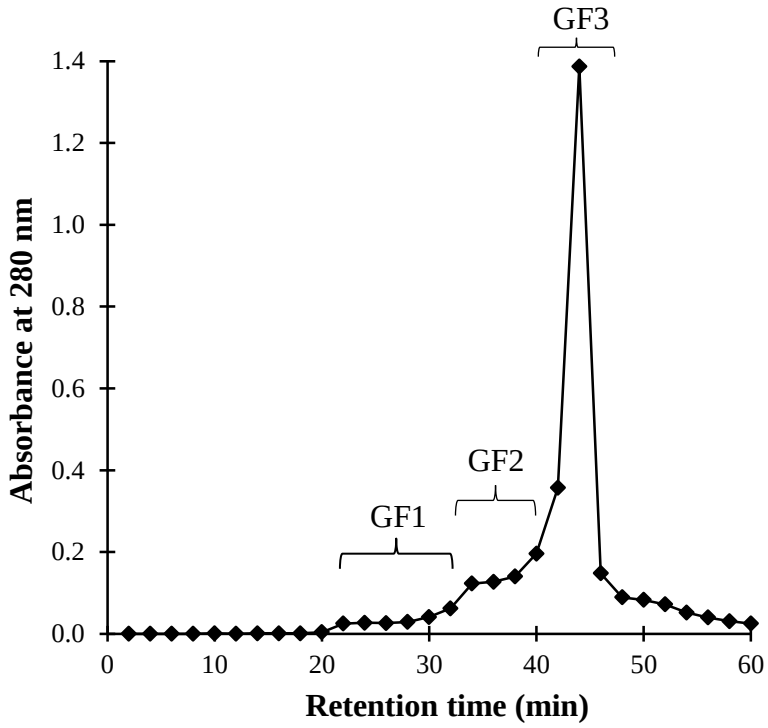


Figure 4.15: A representative gel filtration chromatography elution profile of < 3 kDa UF. The peaks eluted were separated into three pooled fractions, namely GF1, GF2 and GF3

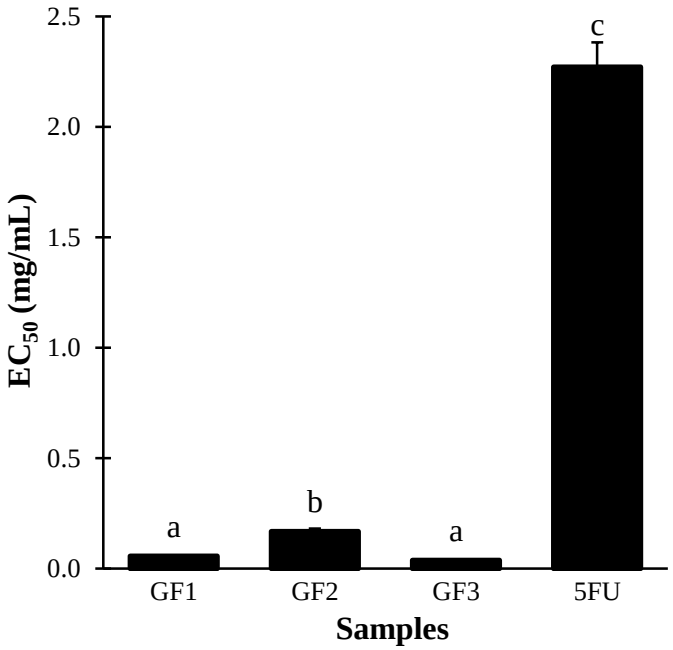


Figure 4.16: Cytotoxicity of the GF fractions and 5FU, expressed as EC_{50} values. Data are means ± standard errors (n=3). Data labelled by different letters are significantly different ($p < 0.05$) according to the Fisher's LSD test

4.2.3.3 SPE

Figure 4.17 shows the peptide content of the SPE fractions. Of all the eight fractions, the peptide content of SPE-F8 which was eluted at 100% ACN appeared to be undetectable. Hence, the cytotoxicity of only seven SPE fractions was tested on HeLa cells at the standardized concentration of 0.04 mg peptide/mL (the EC_{50} of GF3). Result shows that all the SPE fractions possessed cytotoxic activity on HeLa cells (Figure 4.18). Interestingly, SPE-F7 stood out as the most cytotoxic (41.2 ± 0.7%).

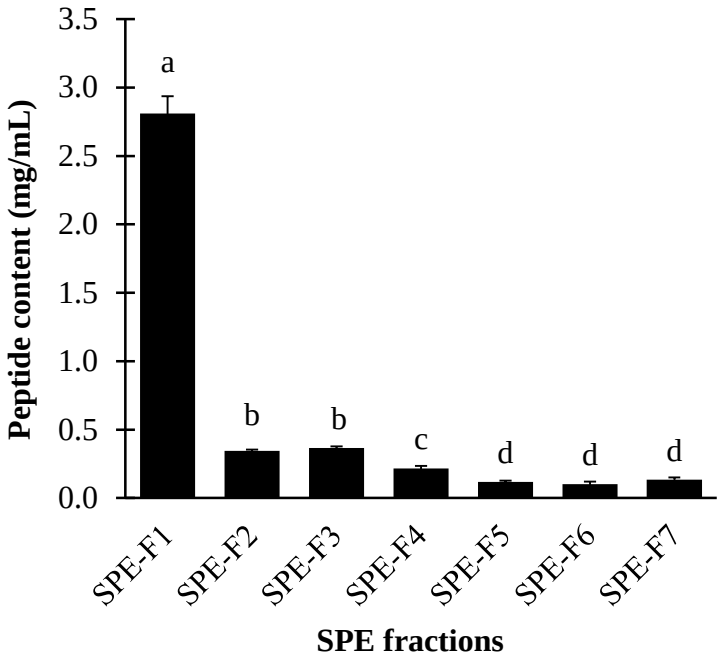


Figure 4.17: Peptide content of SPE fractions. Data are means ± standard errors (n=3). Data labeled by different letters are significantly different ($p < 0.05$) according to the Fisher's LSD test

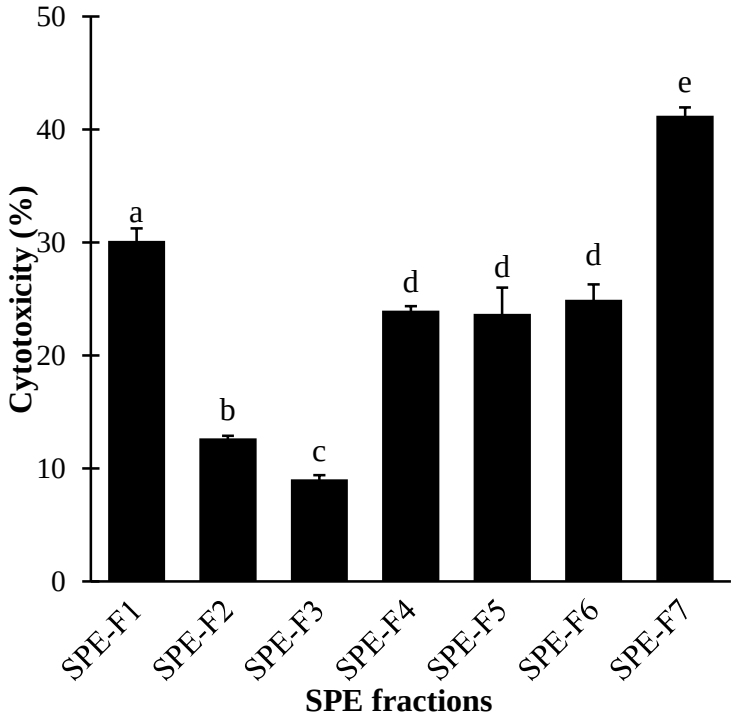


Figure 4.18: Cytotoxicity of SPE fractions tested at 0.04 mg peptide/mL on HeLa cells. Data are means ± standard errors (n=3). Data labeled by different letters are significantly different ($p < 0.05$) according to the Fisher's LSD test

4.2.3.4 RP-HPLC analysis

When SPE-F7 was analysed using an analytical RP-HPLC, three major peaks were observed (Figure 4.19), which comprised of 8.2, 12.6 and 31.8% relative peak area corresponding to the total area of all peaks within the chromatogram. These peaks were eluted with the retention time at 4.677, 5.147 and 5.434 min at 12, 12.7, and 13.1% ACN concentration, respectively. SPE-F7 fraction was then taken for LC-MS/MS analysis and *de novo* sequencing.

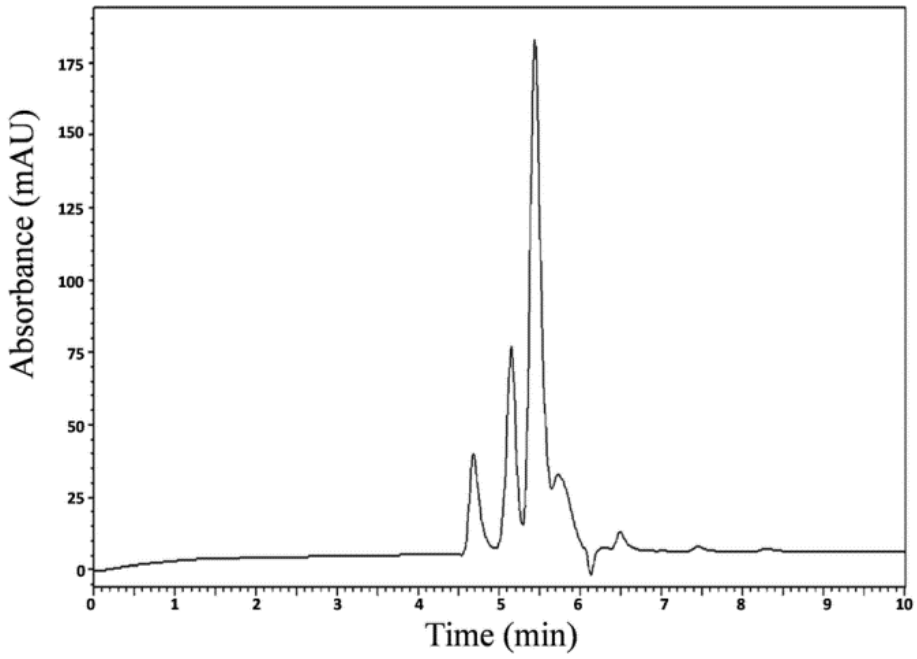


Figure 4.19: A representative RP-HPLC chromatogram of SPE-F7 monitored at 214 nm

4.2.3.5 Peptide Identification

A total of three peptides were identified from SPE-F7: One hexapeptide, AGAPGG, and two tetrapeptides, AERQ and RDTQ. Figure 4.20 shows the MS/MS spectra of the three peptides with their m/z values ranged between 429 and 519, as single-charged ions. The detected molecular masses of AGAPGG (428.2019 Da), AERQ (502.2500 Da) and RDTQ (518.2449 Da) were in agreement with the theoretical molecular masses of the three peptides (428.2013, 502.2492, and 518.2441 Da), as calculated by PepDraw (http://www.tulane.edu/~biochem/WW/PepDraw/). These peptide sequences obtained were chemically synthesized and their cytotoxicity was validated.

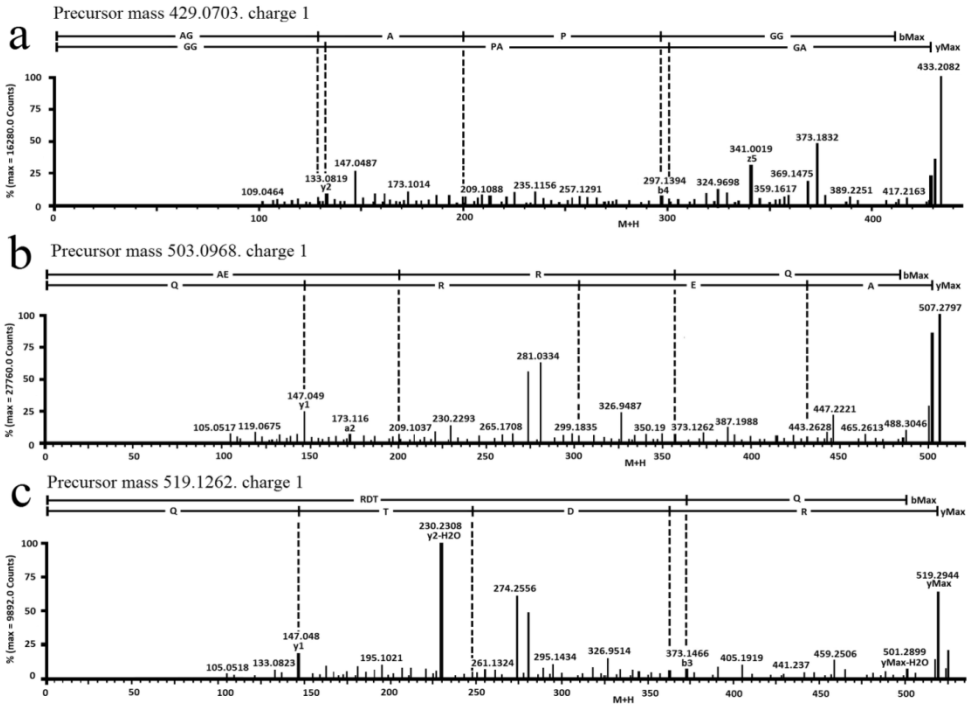


Figure 4.20: MS/MS spectra of (a) AGAPGG, (b) AERQ and (c) RDTQ

4.2.3.6 Validation of Cytotoxicity of Synthetic Peptides

It can be seen from Figure 4.21, AGAPGG, AERQ and RDTQ generally exhibited dose-dependent cytotoxicity in HeLa cells. AERQ exhibited the strongest cytotoxic effect on HeLa cells, closely followed by RDTQ. Remarkably, when tested at 5 mg/mL, both AERQ and RDTQ exhibited cytotoxicity of approximately 90%, exceeding that of 5FU. The cytotoxicity of AGAPGG was almost 1.2-fold higher than that of 5FU at 5 mg/mL. As shown in Figure 4.22, the EC_{50} values of AGAPGG, AERQ and RDTQ were 8.6, 4.9, and 5.6 mM, respectively. By comparison, the EC_{50} of AGAPGG, AERQ and RDTQ were 3.3-, 5.8- and 5.1-fold lower than that of 5FU. Based on the Fisher's LSD test, the EC_{50} values of AERQ and RDTQ are not significantly different.

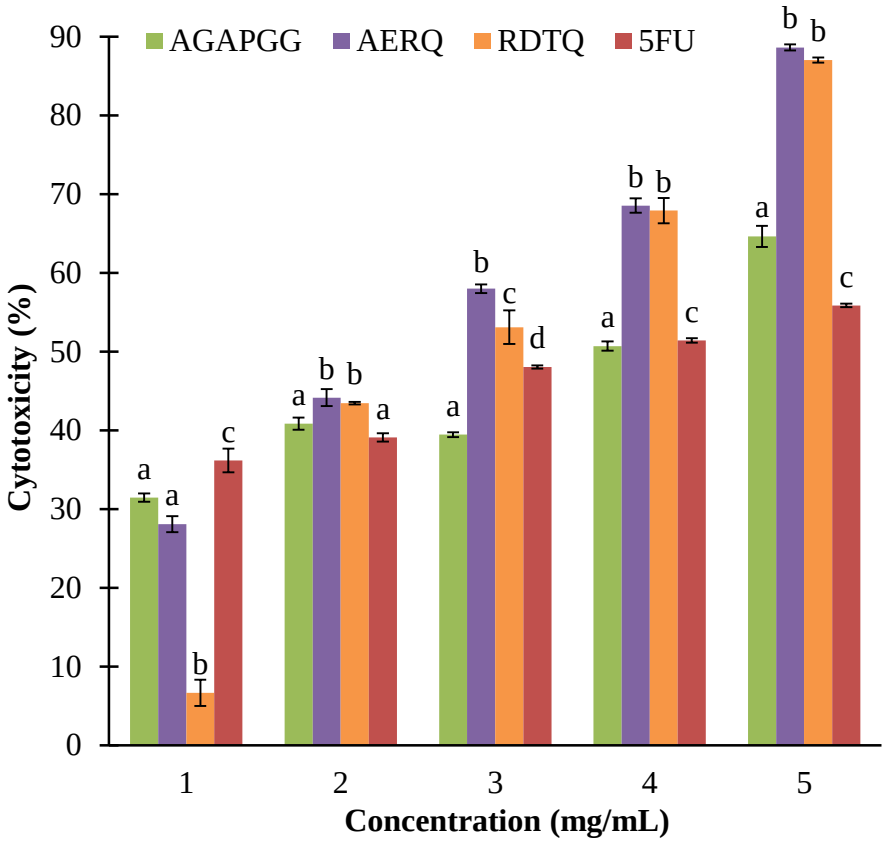


Figure 4.21: Cytotoxicity of synthetic peptides and 5FU against the HeLa cell line. Data are means ± standard errors (n=3). Data labeled by different letters are significantly different ($p < 0.05$) according to the Fisher's LSD test

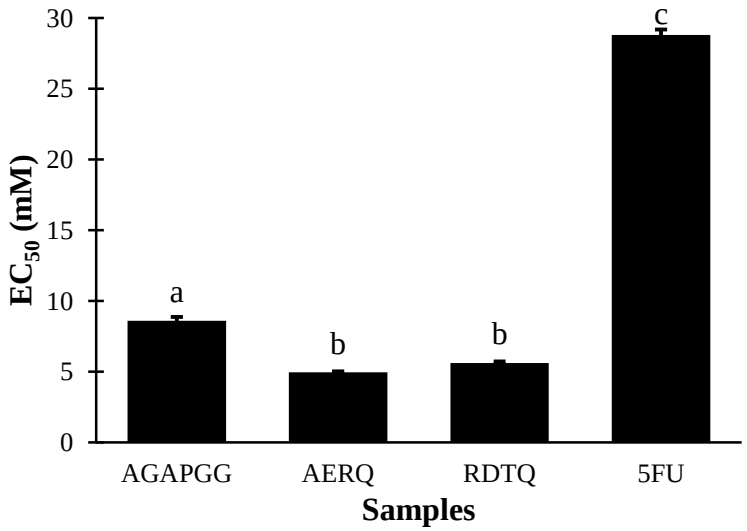


Figure 4.22: EC_{50} of the synthetic peptides and 5FU compared on a millimolar basis. Data are means ± standard errors (n=3). Data labeled by different letters are significantly different ($p < 0.05$) according to the Fisher's LSD test

AGAPGG, AERQ and RDTQ were further tested at their respective EC_{50} for their cytotoxicity against Hek293 cell line to evaluate their selectivity. The relative cytotoxicity of the three peptides against Hek293 cells, in ascending manner, was as follows: RDTQ<AGAPGG<AERQ (Figure 4.23). Notably, at the same concentration, the cytotoxicity of the three peptides against Hek293 cells ranged between 11- 25% was noticeably lower than the 50% cytotoxicity they exerted on HeLa cells.

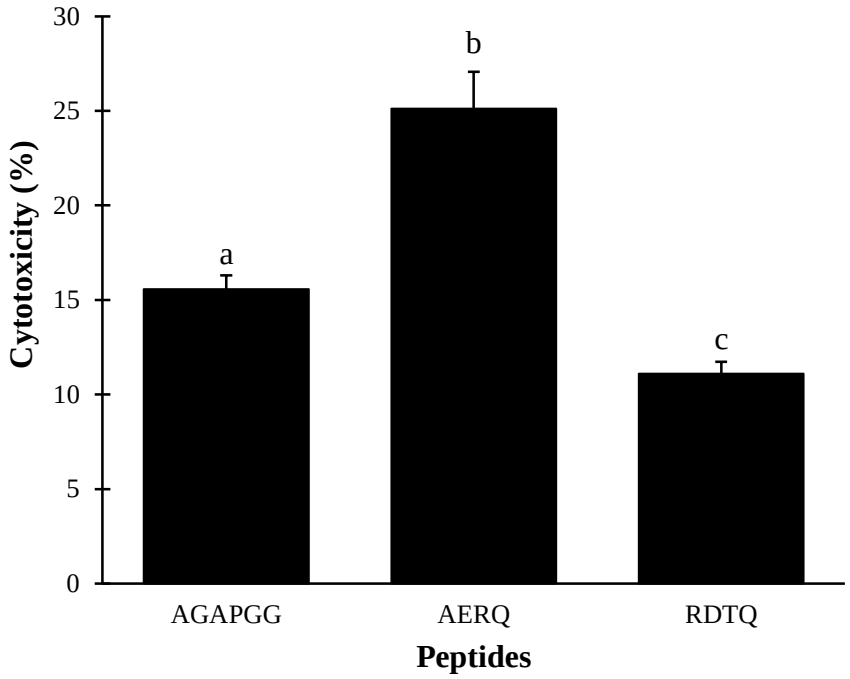


Figure 4.23: Cytotoxicity of AGAPGG, AERQ and RDTQ tested at the respective EC_{50}, on Hek293 cell lines. Data are means ± standard errors (n=3). Data labeled by different letters are significantly different ($p < 0.05$) according to the Fisher's LSD test

Figure 4.24 shows that the synthetic peptides (AGAPGG, AERQ and RDTQ) have higher EC_{50} values compared to < 3 kDa UF and GF3 fractions, when expressed as mg peptide/mL. On average, the synthetic peptides are 19- and 79-fold less potent that < 3 kDa UF and GF3 fractions, respectively.

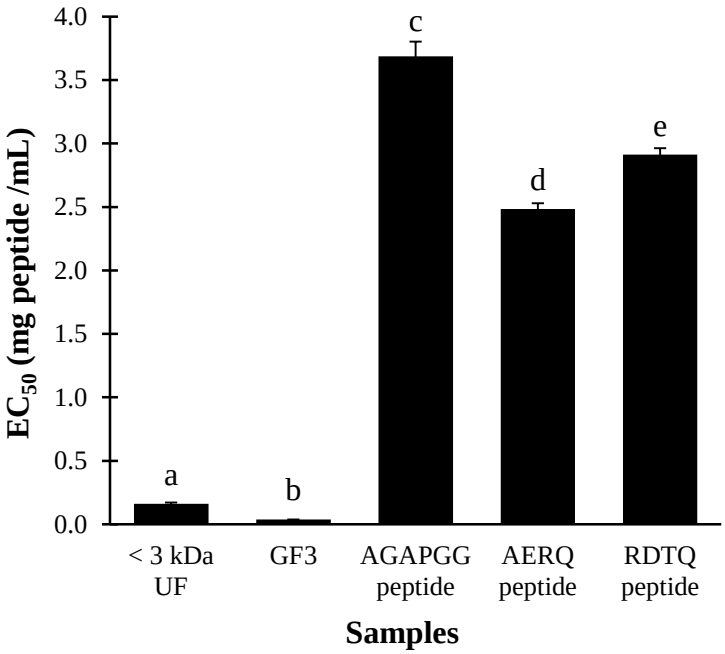


Figure 4.24: Comparison of EC_{50} values of purified *S. glaucum* peptide fractions and synthetic peptides. Data are means ± standard errors (n=3). Data labelled by different letters are significantly different ($p < 0.05$) according to the Fisher's LSD test

CHAPTER 5

DISCUSSION

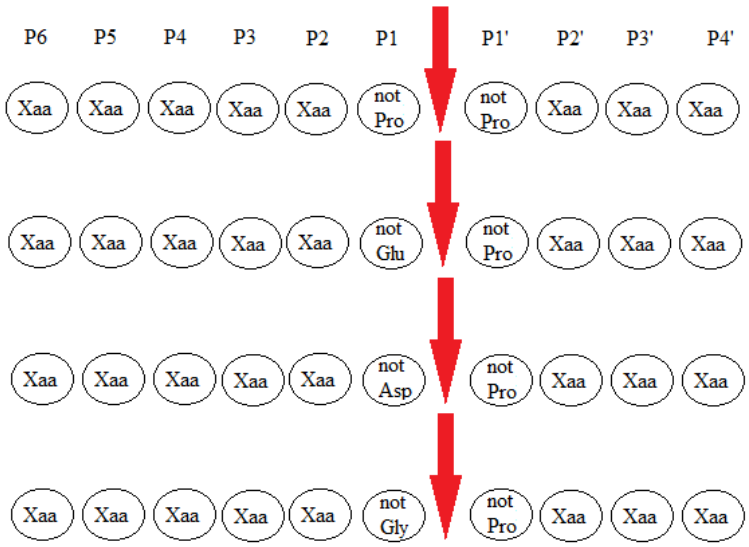
5.1 *Xestospongia testudinaria*

5.1.1 Production of *X. testudinaria* Protein Hydrolysates

Several studies have performed enzymatic hydrolysis of marine proteins to liberate and identify bioactive peptides from mostly edible marine organisms (Cheung et al., 2015, Chai et al., 2017). This is the first time that enzyme-assisted hydrolysis has been used to release cytotoxic peptide from a marine sponge. In this study, alcalase, chymotrypsin, papain and trypsin were used to hydrolyze protein isolates of *X. testudinaria* to different extents (Figure 4.1). The reason these proteases were chosen was that they have been commonly used to produce protein hydrolysates with bioactivities and various bioactive peptides were successfully isolated from the hydrolysates (Suarez-Jimenez et al., 2012, Park and Nam, 2015).

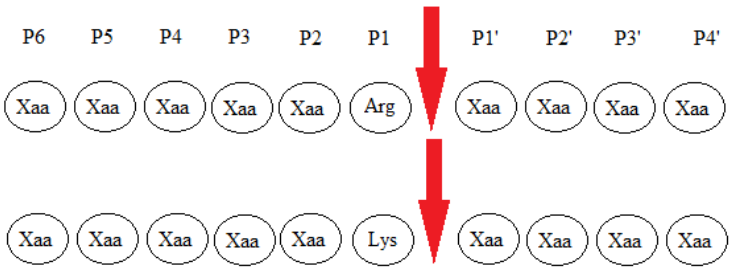
The highest DH for the hydrolysis of sponge protein was produced by alcalase treatment (Figure 4.1). It was therefore the most effective protease treatment for the sponge proteins. This is consistent with the previous studies that showed alcalase to be the most effective among other proteases used (Jin et al., 2016, Sbroggio et al., 2016). Alcalase is an enzyme that has a broad specificity (Adamson and Reynolds, 1996). In this study, it is possible that the

sponge protein may have more cleavage sites for alcalase compared to the other three proteases. Trypsin and chymotrypsin treatments produced very similar DH values. Similar observations were reported in other studies which used these two proteases to hydrolyze lecithin-free egg yolk protein (Aleksandra et al., 2012) and egg white protein (Aleksandra et al., 2010). The similar trend may be due to trypsin and chymotrypsin are both gastrointestinal enzymes with rather restricted cleavage sites as indicated in Figure 5.1 and Figure 5.2 respectively.



Xaa=any amino acid residue;↓ = cleavage site

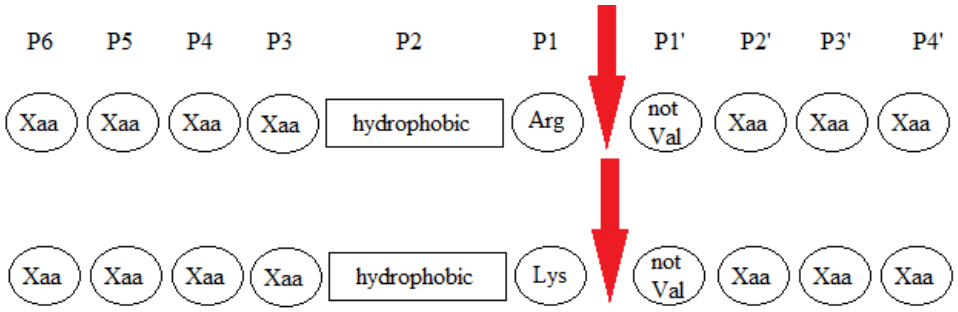
Figure 5.1: Preferential cleavage of chymotrypsin modified from Sigma-Aldrich (Sigma-Aldrich)



Xaa=any amino acid residue;↓ = cleavage site

Figure 5.2: Preferential cleavage of trypsin modified from Sigma-Aldrich (Sigma-Aldrich)

Papain, an endolytic cysteine protease, has a relatively narrow specificity for peptide bonds. Papain cleaves at peptide bonds of basic amino acids, leucine, or glycine, but shows preferential cleavage for amino acid with a large hydrophobic side chain at the P2 position (Figure 5.3)(De Jersey, 1970, Chen et al., 2009a, Yao et al., 2012, Ma et al., 2015). The low DH values and minimal DH changes generated by papain hydrolysis could be the result of restrained cleavage sites for papain in sponge protein isolate.



Xaa=any amino acid residue; hydrophobic=Ala, Val, Leu, Ile, Phe, Trp, Tyr; ↓ = cleavage site

Figure 5.3: Preferential cleavage of papain modified from Sigma Aldrich (Sigma-Aldrich)

There is no clear relationship between DH values and cytotoxicity of the four protein hydrolysates. In this study, the DH value of alcalase hydrolysate was markedly superior to that of papain hydrolysate, yet papain hydrolysate displayed greater cytotoxicity against HeLa cells. This result is in line with the studies of correlation between DH and antiproliferative activity of protein hydrolysates prepared from tuna dark muscle (Hsu et al., 2011), tuna cooking juice (Hung et al., 2014), and proteins from the by-products of other fishes, for instance, Atlantic salmon, Atlantic cod, Atlantic emperor, blue whiting, plaice, pollack and Portuguese dogfish (Picot et al., 2006). Hence, this lack of correlation suggests that DH values may not be a reliable indicator of cytotoxicity of protein hydrolysates.

In this study, papain hydrolysate showed promising dose-dependent trend up to 3 mg/mL, and achieved the highest cytotoxicity among all hydrolysates at 5 mg/mL (Figure 4.2), whereas other three hydrolysates decreased in activity when the concentration increased to 5 mg/mL. These results suggest that there are more potent cytotoxic peptides present in papain hydrolysate than the other three hydrolysates. Particularly, papain's preferential cleavage sites, indicated in Figure 5.3, may result in releasing peptides containing amino acid sequences that could interact with the cancer cell membrane and cause cell death. To date, no report of the cytotoxicity of *X. testudinaria* protein hydrolysates evaluated *in vitro* against any cancer cell lines were found in the literature.

5.1.2 Purification of Cytotoxic Peptides

Ultrafiltration membrane was used as the first purification step in this study. The obtained fraction with the lowest MW range (< 3 kDa UF) showed the strongest cytotoxicity against HeLa cells with the EC_{50} of 0.17 mg/mL. Remarkably, when < 3 kDa UF was further fractionated by GF chromatography, the resulting fraction with the lowest MW (GF3) was also the most potent with the EC_{50} of 0.03 mg/mL. These results are consistent with those of the previous studies during the purification of cytotoxic peptides from *Mercenaria* (Leng et al., 2005) and loach (You et al., 2011). These results suggest that the MW of the potential cytotoxic peptides present, at least in some marine hydrolysates, are likely less than 3000 Da. In support of this,

cytotoxic peptides with MW of < 3 kDa were identified from marine bivalve mollusks (Kim et al., 2013), half-fin anchovy (Song et al., 2014) and skate (Pan et al., 2016). Usually, smaller peptides have greater molecular mobility and diffusivity that may improve their contacts and interactions cancer cell components and therefore enhancing its anticancer activity (Suarez-Jimenez et al., 2012, Kim et al., 2013).

Results showed that F3P1 and F3P4 obtained from RP-HPLC separation to be the two peptide fractions with cytotoxic activity (Figure 4.6). It is possible that F3P1 was more polar than F3P4 since it was the first fraction to be eluted during RP-HPLC. Presumably, the cytotoxic effect of GF3 in HeLa cells may be due to the overall effects of at least two peptides with relatively different polarities. As a result, F3P1 was taken for peptide sequence determination by LC-MS/MS analysis.

5.1.3 Cytotoxicity of Synthetic Peptides

The resulting peptide sequences were KENPVLSLVNGMF and LLATIPKVGVFSILV. When the cytotoxicity of these two peptides assessed which were chemically synthesized, only KENPVLSLVNGMF peptide showed promising cytotoxicity. To the best of my knowledge, KENPVLSLVNGMF is a novel peptide with cytotoxic activity that had not been reported. Furthermore, this is also the first report of cytotoxic peptide identified from *X. testudinaria*. Particularly, KENPVLSLVNGMF was

noticeably more potent than 5FU against HeLa cells. Thus, these results signify that *X. testudinaria* is a promising source of potent cytotoxic peptides.

KENPVLSLVNGMF is an amphiphilic peptide, as indicated by the presence of hydrophobic (L, M, F, P, V) and hydrophilic (N, E, G, K) amino acid residues, with a calculated hydrophobic ratio of 53.8%. The hydrophobic ratio was calculated manually using this equation:

$$\text{Hydrophobic ratio} = \frac{no.\ hydrophobic\ amino\ acid\ residues}{total\ no.of\ amino\ acid\ residues} \times 100\%$$

The cytotoxic effect of this peptide may be associated with its amphiphilic properties. Likewise, anticancer peptides CTLEW, derived from walnut protein and LANAK, from oyster, are also amphiphilic peptides (Umayaparvathi et al., 2014, Ma et al., 2015). Amphiphilicity is thought to play a key role in facilitating the binding and penetration of anticancer peptides to cancer cell membranes (Dennison et al., 2006, Li and Yu, 2015).

On the other hand, KENPVLSLVNGMF was only marginally toxic to non-cancerous, Hek293 cells but exhibiting cytotoxic effect against HeLa cells. Normal cell membranes exhibit an outer leaflet that comprised of mostly zwitterionic phospholipids, whereas cancer cell membranes exhibit more anionic phospholipids (Papo and Shai, 2005).

5.1.4 Stability of Synthetic Peptides in Human Serum

With a half-life of 3.2 ± 0.5 h, the stability of KENPVLSLVNGMF in the human serum was distinctly higher than the two antimicrobial peptides, RRWQWR and RRWWRF, which degraded rapidly with half-life of less than 0.5 h (Nguyen et al., 2010). Besides, KENPVLSLVNGMF was also more stable when compared to RWQ and WQ, short antihypertensive peptides, which have half-lives of 1.9 min and 2.3 h, respectively (Fernández-Musoles et al., 2013). In reviewing the literature, the half-lives of unmodified bioactive peptides in the human serum are usually less than 6 h. Remarkably, cyclization of RRWQWR and RRWWRF results in significant stabilization compared to their unmodified counterparts, displaying half-life of almost 24 h (Nguyen et al., 2010). Besides cyclization, end capping and N- and C- terminus modifications (Werle and Bernkop-Schnurch, 2006) as well as substitution of key amino acid residues and modification using D- or L-beta-amino acids (Nguyen et al., 2010, Arenas et al., 2016) may be used to improve peptide stability.

5.2 *Sarcophyton glaucum*

5.2.1 Production of *S. glaucum* Protein Hydrolysates

This is the first study reporting that protease-assisted hydrolysis can be used to release potent cytotoxic peptides from the proteins of *S. glaucum*. As mentioned in the previous chapter, papain hydrolysate showed the most obvious dose-dependent cytotoxicity in HeLa cells among the four hydrolysates (Figure 4.13). In accordance with the present results, previous studies have demonstrated that papain hydrolysates also showed higher cytotoxicity compared to other enzyme-treated hydrolysates prepared from tuna dark muscle (Hsu et al., 2011) and tuna cooking juice (Hung et al., 2014). These results imply that papain hydrolysis may be the most effective treatment to release potent cytotoxic peptides from the proteins of *S. glaucum* compared to the other three proteases

There is no correlation found between the DH and the cytotoxicity of hydrolysates derived from *S. glaucum*. In the same way, as mentioned in the Section 5.1.1, hydrolysates prepared from *X. testudinaria*, tuna dark muscle (Hsu et al., 2011) and tuna cooking juice also showed lack of correlation between these two values. Therefore, the findings of present and previous studies (Hsu et al., 2011, Hung et al., 2014) suggest that although DH may be useful to gauge the progress of protein hydrolysis, it seems to be insufficient for its value to be used an indicator of cytotoxicity of protein hydrolysates.

5.2.2 Purification of Cytotoxicity Peptides

The papain hydrolysate of *S. glaucum* was separated by UF membrane into < 3 kDa and > 3 kDa UFs. Results show that the two UF fractions displayed similar cytotoxic effect on HeLa cells. This result mirrors those of the previous studies that tested the < 3 kDa and > 3 kDa UF fractions of a *P. haitanesis* hydrolysate against the A549 lung cancer and SGC-7901 gastric cancer cell lines (de Lumen, 2005, Fan et al., 2017). However, the < 3 kDa UF fraction of papain hydrolysate from *X. testudinaria* (Figure 4.9) showed almost 7 times greater cytotoxic effect than that of the 3-10 kDa UF fraction. Furthermore, the < 3 kDa UF fraction derived from flathead by-product protein hydrolysates exhibited the strongest cytotoxicity against the HT-29 colon cancer cell line compared to the other UF fractions (Nurdiani et al., 2017). You et al. (2011) also reported that the < 3 kDa UF prepared from loach papain hydrolysate also showed greater antiproliferative activity against the HepG2 , MCF-7, and Caco-2 colon cancer cell lines, when compared to fractions with larger MW ranges. Moreover, according to Fan et al. (2017), short peptides can be identified by mass spectrometry easily and are easier to synthesize. The cost of synthetizing the smaller peptides are also cheaper than that of the larger ones (Chai et al., 2017). Thus, in this study, < 3 kDa UF was selected for further purification.

The next fractionation step of < 3 kDa UF using Sephadex G-25 gel filtration column resulted in three active peaks as shown in the elution profile (Figure 4.15). This implies that there were several peptides with different MW

found in < 3 kDa UF. Remarkably, the EC_{50} values of three GF fractions were significantly lower than that of 5FU, signifying that the GF fractions were substantially more potent compared to 5FU. This result is noteworthy considering previous reports of GF fractions which showed weaker cytotoxic effect when compared with 5FU. For instance, the GF fractions derived from *Spirulina (Arthrospira) platensis* (Wang and Zhang, 2016a) and *P. haitanesis* (Fan et al., 2017) exhibited poorer cytotoxicity against the MCF-7, HepG2, SGC-7901, A549 and HT-29 cell lines when compared with 5FU. GF3, fraction of the lowest MW range, exhibited the highest cytotoxicity against HeLa cells. This result is consistent with those of GF fractions derived from *X. testudinaria* hydrolysate (Section 4.1.3.2) and half-fin anchovy hydrolysate (Song et al., 2014).

Following fractionation using SPE, the most cytotoxic peptide fraction SPE-F7, was eluted with 80% ACN (Figure 4.18). The strong cytotoxic effect of SPE-F7 against HeLa cells may be attributed to the relatively high hydrophobicity of the peptide. This suggestion was supported by the previous reports of peptides which had higher hydrophobicity exhibited stronger anticancer activity against HeLa, MCF-7 and other cancer cell lines (Huang et al., 2011, Shan et al., 2012). RP-HPLC analysis of SPE-F7 revealed three major peaks (Figure 4.19), implying that there were at least three peptides with different polarities found in the fraction. Furthermore, the RP-HPLC chromatogram also suggests that SPE-F7 was adequately purified to be subjected to peptide sequence determination by LC-MS/MS analysis.

5.2.3 Cytotoxicity of Synthetic Peptides

De novo peptide sequencing led to the identification of three potential cytotoxic peptides: AGAPGG, AERQ and RDTQ. When these peptides were chemically synthesized and tested on HeLa cells, all of them showed cytotoxicity (Figure 4.21). To the best of my knowledge, this study reports for the first time the identification of cytotoxic peptides from *S. glaucum.* Moreover, AGAPGG, AERQ and RDTQ are novel cytotoxic peptides that have not been previously reported. A search of the BIOPEP database (Minkiewicz et al., 2008) (accessed on 19 September 2017) also found these three peptides not documented for any bioactivities previously. Importantly, AGAPGG, AERQ and RDTQ were more powerful cytotoxic agents than 5FU. Hence, *S. glaucum* should be exploited more intensively in future as a source of novel cytotoxic peptides.

The structure-activity relationship of anticancer peptides is still not fully understood (Gabernet et al., 2016). Nevertheless, amphiphilicity is believed to be important to the ability of anticancer peptides to bind to and penetrate cancer cell membranes (Dennison et al., 2006, Li and Yu, 2015). Interestingly, two of the three peptides identified in this study were amphiphilic. The amphiphilicity of AGAPGG is indicated by hydrophobic (A and P) and hydrophilic (G) amino acid residues, with a calculated hydrophobic ratio of 50%. On the other hand, the amphiphilicity of AERQ is indicated by the presence of hydrophobic (A) and hydrophilic (E, R, Q) residues, with a calculated hydrophobic ratio of 25%. The hydrophobic ratio was calculated

manually using equation mentioned in Section 5.1.3. Although AGAPGG and AERQ are both amphiphilic, AERQ was about 1.5-fold more cytotoxic than AGAPGG (Figure 4.22). Song et al. (2014) reported that upon the replacement of a H residue with a G residue in the peptide YALPAH, the modified peptide YALPAG showed weaker inhibitory activity on the PC-3 prostate cancer cells. Thus, the presence of three G residues in AGAPGG could have lowered its cytotoxic effect.

On a related note, the strong cytotoxicity of AERQ may also be associated with the presence of an R residue within the peptide (Schmidt et al., 2010). Tada et al. (2011) demonstrated that replacement of a H residue to an R residue in an EGFR-lytic hybrid peptide enhanced the ability of the peptide to bind to cancer cells, hence increasing its anticancer activity. Among the three peptides identified in this study, RDTQ is not amphiphilic. When tested on HeLa cells, RDTQ was more cytotoxic than AGAPGG and similarly cytotoxic as AERQ. Hence, our results suggest that in contrast to peptide amphiphilicity, the presence of specific amino acid residues and/or their arrangement in a peptide sequence may be a more important determinant of cytotoxicity.

In this study, although AERQ and RDTQ were similarly cytotoxic to HeLa cells, RDTQ was less toxic than AERQ to the non-cancerous Hek293 cells. In other words, our results suggest that RDTQ was more selectively toxic to HeLa cells in comparison to AERQ. Based on this finding, RDTQ seems to be a more promising candidate for future development of selective anticancer therapeutics.

In light of the uses of different assay protocols between studies, comparing potencies of anticancer peptides between studies is challenging. To enhance the reliability of such a comparison, we compared the cytotoxicity of the peptides we identified with those reported in the literature by considering their relative potencies after standardization against the cytotoxicity of the same anticancer drug 5FU (Table 5.1).

Table 5.1: Cytotoxicity of selected reported peptides in comparison with peptides identified in this study

Peptide	EC_{50} (mM)	Positive control	EC_{50} (mM)	Relative potency*	Cell line tested	Reference
A12L/A20L	0.002	5FU	0.353	176.5	HeLa	(Huang et al., 2011)
P	0.010			35.3		
L6A	0.059			6.0		
L21A	0.065			5.4		
L17A/ L21A	> 0.084			< 4.2		
AERQ	4.9	5FU	28.8	5.8	HeLa	
RDTQ	5.6			5.1		
AGAPGG	8.6			3.3		

*Relative potency is defined as EC_{50} of 5FU/EC_{50} of peptide.

The cytotoxicities of peptide P, a 26-residue α-helical peptide, and their analogues were compared with that of 5FU against HeLa cells by Huang et al. (2011). By comparison, the potencies of the three peptides we identified in this study were markedly lower than those of peptide P and peptide A12L/A20L. On the other hand, the relative potencies of AERQ and RDTQ were comparable to those of peptides L6A and L21A, and higher than that of L17A/ L21A. This suggests that despite the apparent lack of α-helical structures in AERQ and RDTQ, these peptides could still exert levels of

cytotoxicity comparable to those of some α-helical anticancer peptides. The effects of some anticancer peptides have been attributed to their helicity and hydrophobicity (Huang et al., 2011, Huang et al., 2012b). Our discovery of RDTQ is thus interesting as this peptide lacks both helicity and hydrophobicity, yet it was apparently more potent than the α-helical, hydrophobic anticancer peptide L17A/ L21A. Furthermore, in comparison with peptides L6A, L21A, and L17A/ L21A, AERQ and RDTQ may also have the advantages of being more economical to manufacture and less prone to protease degradation.

5.3 Limitations of Current Study and Recommendations for Future Studies

The present study has examined the cytotoxicity of KENPVLSLVNGMF, AGAPGG, AERQ and RDTQ peptides against cancerous HeLa cells and non-cancerous Hek293 cells. One limitation in this study is that only one cancer cell line was tested, hence it is unclear whether the peptides are also cytotoxic against other cancer cell types. Thus, it is recommended that in future the evaluation of the cytotoxic effect of these synthetic peptides against other cancer and non-cancer cell lines should be undertaken.

This study demonstrated that the cytotoxicity of the synthetic peptides were generally more potent than that of the anticancer drug, 5FU. The current study was limited by comparing the cytotoxicity of the synthetic peptides with

only one anticancer drug. Therefore, research is also needed to compare the potency of the peptides with other anticancer drugs, such as Cisplatin and Paclitaxel. This may provide more insights into the potential application of the peptide as an anticancer agent.

The results of this study showed that the synthetic peptides were selectively toxic to HeLa cells. To better understand the observed selectivity, further work needs to be carried out to establish whether the selectivity of the peptides for cancer cells is attributable to its differential affinities for cell membranes with different components. Nevertheless, it would be desirable that anticancer agents display great selectivity towards cancer cell and only exert low toxicity to normal cells as they may instigate just minimal side effects (Markman et al., 2013). Besides, future research to unravel the cellular and molecular mechanisms underlying the cytotoxicity of the synthetic peptides is of great interest. Additionally, using *in vivo* approaches to evaluate the toxicity of the identified peptides is also necessary in the future.

The investigation of the half-life of KENPVLSLVNGMF in human serum in this study also serves as a continuous incentive for future research in enhancing the stability of KENPVLSLVNGMF in human blood. Strategies such as cyclization, N- and C- terminus modifications, end capping, replacement of key amino acid residues and modification using D- or L-beta-amino acids may be attempted in future. Considering that there are over 20 peptidases and proteases in the human blood (Werle and Bernkop-Schnurch,

2006), future works of modification in KENPVLSLVNGMF should be guided by careful study on potential peptidase cleavage sites occur in the peptide.

Based on the BIOPEP database (Minkiewicz et al., 2008), AGAPGG shares part of its sequence with 14 previously identified peptides, including those possessing antiamnestic (PG), ACE inhibitory (AP, GA, AG, GG, and PG), and dipeptidyl peptidase IV (DPP IV) inhibitory (AP, APG, GA, AG, GG, and PG) activities. On the other hand, AERQ shares partial homology with a previously identified DPP IV inhibitory dipeptide (AE), whereas RDTQ shares partial homology with an ACE- and DPP IV inhibitory dipeptide (TQ). In the light of this information, future investigations on the potential multifunctionality of AGAPGG, AERQ and RDTQ are warranted. If confirmed to have multiple bioactivities, this finding could be a means of discovery of wider range of the peptides' applications in future therapeutics development.

CHAPTER 6

CONCLUSION

The research presented in this dissertation has advanced our current knowledge of marine bioactive peptides through the findings of four novel cytotoxic peptides, for the first time, from marine sponge *X. testudinaria* and soft coral *S. glaucum*. In this study, papain hydrolysates were successfully prepared from the protein isolates of *X. testudinaria* and *S. glaucum*. Four novel cytotoxic peptides were purified and identified: KENPVLSLVNGMF from the papain hydrolysate of *X. testudinaria*; AGAPGG, AERQ and RDTQ from the papain hydrolysate of *S. glaucum*. These peptides were cytotoxic to the cancerous HeLa cells but displayed low cytotoxicity towards the non-cancerous Hek293 cells. Remarkably, the cytotoxicity of KENPVLSLVNGMF was 3.8-fold more potent than anticancer drug 5FU, whereas the cytotoxicity of AGAPGG, AERQ and RDTQ were 3.3-, 5.8-, 5.1-fold stronger than 5FU. The current study also demonstrated that the half-life of KENPVLSLVNGMF in human serum was 3.2 ± 0.5 h. In short, the findings of this study indicate that these peptides are potential leads for future development of peptide-based anticancer therapeutics.

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APPENDICES

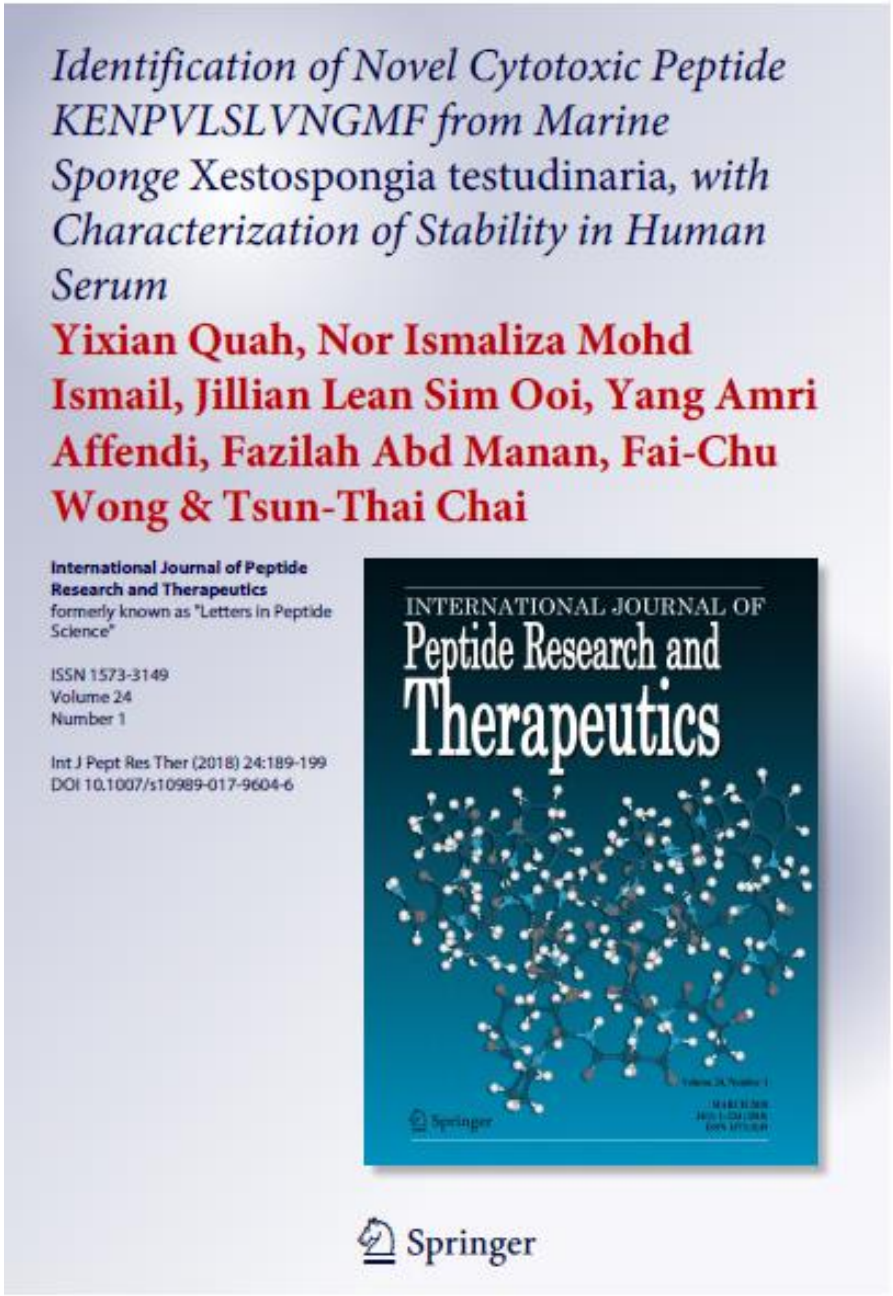
Appendix A

List of commonly used parameters in MTT assay

Sources	Cell lines	No. of cells/ well	Treatment duration, hours	References
Skate fish (*Raja porosa*)	HeLa cells	1×10^4	24	(Pan et al., 2016)
Marine cyanobacteria (*Lyngbya majuscula*)	MCF-7 and human acute lymphoblastic leukemia (MOLT-4) cells	4×10^4	24	(Tripathi et al., 2009)
Seaweed (*P. haitanesis*)	MCF-7, HepG2, SGC-7901, A549, HT-29 and human embryo liver (L-O2) cells	5×10^4	48	(Fan et al., 2017)
Half-fin anchovy (*S. taty*)	PC-3 cells	$1\text{- }2 \times 10^5$	48	(Song et al., 2014)
Solitary tunicate (*S. clava*)	HeLa, stomach cancer (AGS) and colon cancer (DLD-1) cells	1×10^4	72	(Jumeri and Kim, 2011)
Fish proteins (*Salmo salar, Gadus morhua, Pleuronectes platessa, Micromesistius poutassou, Lethrinus atlanticus, Pollachius pollachius, Centroscymnus coelolepis*)	MCF-7 and MDA-MB-231 cells	5×10^3	72	(Picot et al., 2006)
Giant squid gelatin (*D. gigas*)	MCF-7 and glioma (U87) cells	1×10^4	24, 48 and 72	(Alemán et al., 2011)
Oyster (*S. cucullata*)	HT-29 and Vero cells	5×10^3	24, 48 and 72	(Umayaparvathi et al., 2014)

Appendix B

Published Article Entitled Identification of Novel Cytotoxic Peptide KENPVLSLVNGMF from Marine Sponge *Xestospongia testudinaria*, with Characterization of Stability in Human Serum



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Identification of Novel Cytotoxic Peptide KENPVLSLVNGMF from Marine Sponge *Xestospongia testudinaria*, with Characterization of Stability in Human Serum

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Abstract Resistance and side effects are common problems for anticancer drugs used in chemotherapy. Thus, continued research to discover novel and specific anticancer drugs is obligatory. Marine sponges hold great promise as a source of potent cytotoxic peptides with future applications in cancer treatments. This study aimed to purify and identify cytotoxic peptides from the protein hydrolysates of the giant barrel sponge *Xestospongia testudinaria*, guided by a cytotoxicity assay based on the human cervical cancer cell line (HeLa). Comparison among trypsin, chymotrypsin, papain and alcalase hydrolysates of *X. testudinaria* revealed papain hydrolysate (PH) to be the most active. PH was purified consecutively by membrane ultrafiltration, gel filtration chromatography, and reversed-phase high performance liquid chromatography (RP-HPLC). Following liquid chromatography-tandem mass spectrometric analysis, two peptides were identified from the most cytotoxic RP-HPLC fraction: KENPVLSLVNGMF and LLATIPKVGVFSILV. Between the two, only the synthetic peptide KENPVLSLVNGMF showed cytotoxicity toward HeLa cells in a dose-dependent manner. KENPVLSLVNGMF (EC_{50} 0.67 mM) was 3.8-fold more cytotoxic compared with anticancer drug 5-fluorouracil (EC_{50} 2.56 mM). Furthermore, KENPVLSLVNGMF show only marginal 5% cytotoxicity to Hek293, a non-cancerous, human embryonic kidney cell line, when tested at 0.67 mM. The half-life of the peptide was 3.2 ± 0.5 h in human serum in vitro, as revealed by RP-HPLC analyses. These results suggest that KENPVLSLVNGMF identified from *X. testudinaria* papain hydrolysate has potential applications as peptide lead in future development of potent and specific anticancer drugs.

Keywords Cytotoxic peptide · Cancer · Marine sponge · Protein hydrolysate · Peptide stability · *Xestospongia testudinaria*

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Introduction

Cancer is one of the largest single causes of death globally. It accounted for 8.8 million deaths in 2015, nearly one in six of all global deaths (World Health Organization 2017). Chemotherapy, one of the conventional cancer treatments, often leads to side effects such as nausea, vomiting, loss of appetite, loss of hair, mouth sores, fatigue and diarrhea. Chemotherapeutic drugs also tend to show non-specific cytotoxicity, killing not only cancerous cells, but also normal tissues (Liao et al. 2015). Therefore, research and development of more specific cytotoxic drugs is obligatory (Alemán et al. 2011; Song et al. 2014).

Bioactive peptides of marine organisms are valuable resources for the discovery of potent and specific anticancer or antitumor drugs (Zheng et al. 2013). In support of this is the approval of some marine peptides or their derivatives for cancer treatment, in addition to some marine peptides undergoing different phases of clinical trials for cancer treatment (Beena et al. 2016; Cheung et al. 2015). Bioactive peptides can be obtained by enzymatic hydrolysis of proteins isolated from marine organisms (Beena et al. 2016; Chai et al. 2017; Kim et al. 2013; Wiriyaphan et al. 2013). The most commonly used proteases are pepsin, trypsin, α-chymotrypsin, papain and alcalase (Ngo et al. 2012; Qian et al. 2007). Following further purification by chromatographic and/or other techniques, bioactive peptides can be isolated and identified by tandem mass spectrometry or Edman degradation. Peptides can then be chemically synthesized based on the identified sequences and be used for validation of their bioactivities (Chai et al. 2017). Previous studies which purified and identified anticancer peptides from marine protein hydrolysates have not given adequate attention to investigating the stability of the peptides in the human circulatory system. Determination of peptide stability in human serum represents an important assay for characterizing the therapeutic potential of a bioactive peptide (Jenssen and Aspmo 2008). Peptide stability in the human serum can be monitored by using reversed-phase high performance liquid chromatography (RP-HPLC) and mass spectroscopy (Cudic et al. 2002; Nguyen et al. 2010).

Marine sponges have been considered a very promising group of marine organisms for the discovery of bioactive natural products (Perdicaris et al. 2013). Novel natural products isolated from marine sponges include sterols, terpenes, terpenoids, macrolides, polyketones, glucosides, quinines, alkaloids, indole, fatty acids and peptides (Mehbub et al. 2014, 2016; Ye et al. 2015). To date, quite a number of cytotoxic peptides have been discovered from various marine sponge species (Beena et al. 2016). For example, Microcionamides A and B are two linear peptides identified from marine sponge *Clathria (Thalysias) abietina* which showed strong cytotoxicity against human breast tumor cell lines MCF-7and SKBR-3 (Davis et al. 2004). Recently, Zhan et al. (2014) identified five new cytotoxic cyclic peptides from the marine sponge *Reniochalina stalagmitis*.

To date, non-peptide bioactive compounds have been identified from the giant barrel sponge *Xestospongia testudinaria* (El-Gamal et al. 2016; Zhou et al. 2011). However, there are still no reports of identification of bioactive peptides from *X. testudinaria*. Thus, to fill in the gap in knowledge, the objective of this work was to purify and identify cytotoxic peptides from the protein hydrolysates of *X. testudinaria*, guided by a cytotoxicity assay based on the human cervical cancer cell line (HeLa). Effectiveness of trypsin, chymotrypsin, papain and alcalase in producing cytotoxic hydrolysates from the protein isolates of *X. testudinaria* was compared. The most potent hydrolysate was purified consecutively with ultrafiltration membrane, gel filtration chromatography and RP-HPLC. Sequences of potential cytotoxic peptides were determined by liquid chromatography-tandem mass spectrometry (LC–MS/MS). By using chemical synthesized peptides, cytotoxicity of the sequences was verified on HeLa cells, and subsequently compared with effect on Hek293, a non-cancerous human embryonic kidney cell line. The stability of the cytotoxic synthetic peptide in in vitro human serum was also assessed.

Materials and Methods

Reagents and Materials

Ammonium sulfate, phthaldialdehyde and ultrafiltration centrifugal units (MWCO 10 and 3 kDa) were purchased from Merck. Trypsin and α-chymotrypsin were purchased from Nacalai Tesque. Alcalase and papain were purchased from Calbiochem. Dialysis tubing was purchased from Fisher Scientific, whereas di-sodium tetraborate and trifluoroacetic acid (TFA) were from Fisher Chemical. RPMI 1640 medium was purchased from Gibco, Life Technologies; phosphate-buffered saline (PBS) from Takara; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Amresco; and 5-fluorouracil (5FU) from Biobasic. TFA used was HPLC-grade, whereas other reagents were at least AR-grade.

Preparation of Sponge Protein Isolate and Hydrolysates

Specimens of *X. testudinaria* were collected offshore of Mentigi Island, Johor, Malaysia, in September, 2013. The samples were collected at a depth of 3–6 m by using SCUBA. The sponge species identification was referenced to Hooper and Soest (2002). The sponge samples were transported back to laboratory on ice and immediately kept in a −20 °C freezer. Frozen sponge samples were freeze-dried and pulverized with a Waring blender. Sponge powder was suspended in chilled deionized water at the mass (g): volume (mL) ratio of 1:5 and then stirred for 30 min at 4 °C. The mixture was heated at 90 °C for 20 min and then centrifuged at 8603×*g* for 20 min to remove water-insoluble substances. The supernatant was brought up to 80% saturation with ammonium sulfate and stirred at 4 °C for 1 h. Precipitated proteins were pelleted after centrifugation at 20,000×*g* at 4 °C for 1 h. The proteins isolated were dialyzed overnight at 4 °C using deionized water and dialysis tubing (molecular weight

cut-off: 6000–8000 Da). The dialyzed protein isolate was freeze-dried and stored at −20 °C until use. Protein content of the isolate was quantified by using the Bradford's assay (Bradford 1976), based on a bovine serum albumin standard curve.

For protein hydrolysate preparation, the freeze-dried protein isolate (1 g) was dissolved in 200 mL of 50 mM sodium phosphate buffer. For hydrolysis with alcalase, trypsin, chymotrypsin, and papain, the buffer was adjusted to optimum pH, i.e., pH 7, pH 8, pH 7, and pH 6, respectively. Alcalase, trypsin, papain and chymotrypsin were added separately to the corresponding buffer at a ratio of 1 g of enzyme: 10 g of protein. Initially, each enzyme was added into a cold sponge protein-buffer mixture and a 0-hour aliquot (1 mL) was taken. Next, each mixture was placed into a water bath maintained at the optimum temperature of the enzyme (50 °C for alcalase; 37 °C for trypsin, chymotrypsin, and papain). Protein hydrolysate sample (1 mL) was then removed from each sponge protein-buffer mixture after 1, 2, 3, 4, 5, 6, and 8 h. Each aliquot of hydrolysate sample was heated at 100 °C for 10 min to inactivate the protease, cooled on ice, and then freeze-dried to be used in the cytotoxicity test described below. Degree of hydrolysis (DH) was determined as previously described (Chen et al. 2009b) for each protease treatment to identify the optimum proteolysis durations.

MTT Assay

The human cervical cancer cell line (HeLa, cell line number ATCC CCL-2) were cultured using the RPMI 1640 medium supplemented with 2 g sodium bicarbonate, 10% fetal bovine serum and 1% Penicillin–Streptomycin. The cell confluency was checked under inverted microscope and ensured to be more than 80% before seeding. The cells were seeded into a 96-well plate (1×10^4 cells per well) and were cultured for 24 h. Next, 100 µL of sample of different concentrations, dissolved in sterile, autoclaved water, were added to each well. The cells were then cultured for 24 h at 37 °C in a humidified incubator in 5% CO_2. For untreated cells, 100 µL of deionized water was added in place of the sample. Twenty microlitres of MTT (5 mg/mL) was then added to each well and the culture was incubated for another 4 h at 37 °C. The 96-well plate was centrifuged at 1000×*g* for 5 min and 70% of supernatant in each well was removed. Dimethyl sulfoxide (100%, 200 µL) was used to dissolve the purple formazan. The absorbance at 570 nm was determined for each well with a 96-well plate reader. 5FU was used as the positive control.

Fractionation of Papain Hydrolysate by Membrane Ultrafiltration

Fifteen mL of papain hydrolysate (10 mg/mL in deionized water) was added into a 10 kDa MWCO ultrafiltration centrifugal unit. It was then centrifuged at 5000×*g* and 25 °C for 20 min. The retentate was designated ">10 kDa ultrafiltration fraction (UF)". The permeate fraction was transferred into a 3 kDa MWCO ultrafiltration centrifugal unit and centrifuged as described above. The resulting retentate was designated "3–10 kDa UF", whereas the permeate fraction was designated the "<3 kDa UF". The three UFs were freeze-dried and tested for cytotoxicity using the MTT assay described above. EC_{50}, defined as the peptide concentration required to exert 50% cytotoxicity, was determined by using linear regression analysis. EC_{50} values were expressed on the basis of peptide content. Peptide contents of the UF samples were determined by using the OPA method (Nielsen et al. 2001).

Fractionation of UF by Size Exclusion Chromatography

Two milliliter of <3 kDa UF (50 mg/mL in deionized water) was loaded onto a Sephadex G-25 column (1.6×70 cm). Elution was performed at the flow rate of 1.55 mL/min using deionized water as the mobile phase. Eluate was collected at 2-min intervals and absorbance of each fraction was monitored at 280 nm with a UV–Vis spectrophotometer. Pooled fractions designated GF1, GF2, and GF3 were collected, freeze-dried, and tested for cytotoxicity as described above. Peptide content of the samples was determined as described above.

Isolation of Peptides from GF3 by RP-HPLC

The freeze dried GF3 collected from the previous step was dissolved in deionized water and filtered with 0.22 µm filter membrane before it was further purified by using semi-preparative RP-HPLC. GF3 was separated by RP-HPLC (PerkinElmer Flexar FX-20 UHPLC) using an Eclipse CDB-C_{18} column (75 µm, 9.4×250 mm). The column was eluted with a linear gradient of acetonitrile (40–50% in 60 min) containing 0.1% TFA at a flow rate of 0.8 mL/min. Absorbance readings of the eluted fractions were determined at 214 nm. Pooled fractions designated F3P1, F3P2, F3P3, and F3P4, corresponding to four selected peaks in the elution profile, were collected and vacuum-concentrated at 45 °C until fully dried. The dried fractions were tested for their cytotoxicity. Peptide content of the samples was determined as described above.

LC–MS/MS Analysis and Peptide Sequence Identification

The identification of the peptide sequences in F3P4 was carried out by means of online LC–MS/MS analysis at Fitgene Bio Pte Ltd, China. In brief, the peptide was resolved by using an Acclaim PepMapRSLCC18 column prior to being introduced to a Thermo Scientific Q Exactive Hybrid-Quadrupole-Orbitrap mass spectrometer. The spectrometer analysis was conducted in the positive ion mode with an electrospray ionization source. Mass spectral data were searched against a *Xestospongia* sp. database using MASCOT (version 2.3; Matrix Science) to identify peptides in the F3P4 fraction. Database retrieval parameters were as follows: fixed modifications: carbamidomethyl (cysteine); variable modifications: oxidation (methionine); enzyme: no; peptide mass tolerance: 20 ppm; fragment mass tolerance: 0.6 Da; peptide/fragment ion mass values: monoisotopic; and significance threshold: 0.05.

Validation of Cytotoxicity of Synthetic Peptides

Following the LC–MS/MS analysis described above, two peptide sequences identified (KENPVLSLVNGMF and LLATIPKVGVFSILV) were synthesized and used in further validation of their cytotoxicity. The synthetic peptides were manufactured by Bio Basic Inc., Canada. MTT assay was carried out as described above to evaluate the cytotoxicity of the synthetic peptides in HeLa and Hek293 cell lines.

Peptide Stability in Human Serum

Peptide stability in human serum was assayed in diluted serum as described in Cudic et al. (2002) and Nguyen et al. (2010). Synthetic peptide KENPVLSLVNGMF was dissolved in 25% human serum at a final concentration of 1 mg/mL (690 µM) and incubated in a 37 °C shaking incubator. Aliquots of 250 µL of the incubation mixture were taken out at the following time points: 0, 2, 4 and 6 h. Each aliquot was mixed with 50 µL of 15% trichloroacetic acid and incubated at 4 °C for 15 min to precipitate serum proteins. The supernatant was collected after centrifugation at 13,000×*g* for 10 min and stored at −20 °C until used for peptide analysis by RP-HPLC. Peptide concentration, expressed as a percentage, was calculated from the chromatographic peak area at each time point versus the peak area at 0 h. Half-life of the peptide, defined as the time point where peptide concentration is 50% of the initial concentration, was calculated by using linear regression analysis.

Data Analysis

Data are presented as mean ± standard errors (n = 3). Statistical analysis was performed using SAS (Version 9.4). Data were analyzed by the ANOVA test and means of significant differences were separated using Fisher's Least Significant Difference (LSD) test at the 0.05 level of probability. Comparison of only two means was performed by using Student's T-test at the 0.05 level of probability.

Results

Preparation of Sponge Protein Hydrolysates

In this study, ammonium sulfate-precipitated and dialyzed proteins of *X. testudinaria* were used for hydrolysate preparation. The yield of protein isolate was 1.6% of the weight of freeze-dried *X. testudinaria*. Soluble protein content of the protein isolate, determined based on a bovine serum albumin standard curve, was 0.13 g soluble protein/g protein isolate.

When subjected to proteolysis, changes in the DH of sponge proteins were observed over 8 h; the magnitudes of change differed depending on the type of protease used (Fig. 1). Overall, the alcalase treatment produced higher DH values than other protease treatments. When treated with chymotrypsin and trypsin, very similar DH values were observed in sponge proteins over the 8-h duration. Analysis of trend lines showed a rise in the DH of sponge

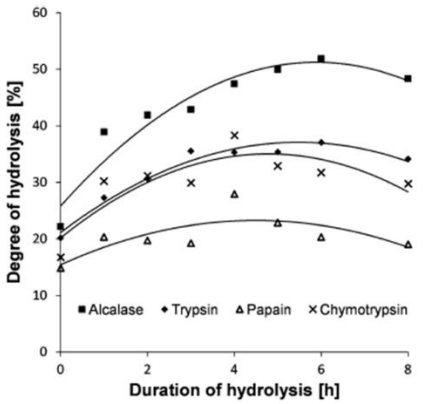

Fig. 1 Degree of hydrolysis of sponge proteins during hydrolysis with alcalase, trypsin, papain and chymotrypsin

proteins treated with trypsin and alcalase during the initial 6 h, with a declining trend thereafter. On the other hand, DH of sponge proteins treated with papain and chymotrypsin exhibited a rising trend up to 4–5 h, declining thereafter. Based on analysis of trend lines, we took optimum hydrolysis duration for trypsin and alcalase to be 6 h and for papain and chymotrypsin, 4 h. The DH values at the optimum duration were 37.1 ± 0.5, 51.9 ± 5.7, 28.0 ± 0.2 and 38.3 ± 1.7% for treatments with trypsin, alcalase, papain and chymotrypsin, respectively. Alcalase and trypsin hydrolysates obtained at the 6th h as well as papain and chymotrypsin hydrolysates obtained at the 4th h were collected for analysis of cytotoxicity against the HeLa cell line.

Cytotoxic Activity of Sponge Protein Hydrolysates

MTT assay was carried out to assess the cytotoxicity of the sponge protein hydrolysates. The cytotoxicity of the alcalase hydrolysate on the HeLa cell line fluctuated, whereas that of trypsin and chymotrypsin hydrolysates showed no concentration-dependent trends over the range of concentration tested (Fig. 2). Anticancer drug 5FU, which was used as positive control in our assay, exhibited cytotoxicity against HeLa cells in a concentration-dependent manner. Papain hydrolysate showed concentration-dependent increase in cytotoxicity when tested at 1, 2 and 3 mg/mL. Notably, when compared at the concentration of 1–4 mg/mL, the cytotoxicity of papain hydrolysate was either stronger ($p < 0.05$) or similar to that of 5FU. At 5 mg/mL, papain hydrolysate had the strongest cytotoxicity among the four hydrolysates evaluated. Thus, papain hydrolysate was chosen for further purification by using ultrafiltration membrane.

Purification of Cytotoxic Peptides

Membrane ultrafiltration yielded three ultrafiltrated fractions (UF): <3 kDa, 3–10 kDa and >10 kDa. When tested on HeLa cells for cytotoxicity, the EC_{50} value of the <3 kDa UF (0.17 mg/mL) was almost 7 times lower compared to that of the 3–10 kDa UF (1.18 mg/mL). Due to a lack of concentration-dependent increase in cytotoxicity (data not shown), the EC_{50} for the >10 kDa UF was not determined. Our result indicates that <3 kDa UF was more cytotoxic than the 3–10 kDa UF. Hence the peptide in <3 kDa UF was subjected to gel filtration (GF) chromatography for further fractionation. A representative GF elution profile is shown in Fig. 3. Three pooled GF fractions, namely, GF1, GF2 and GF3, were tested on HeLa cells to determine their cytotoxicity. GF1 and GF3, with EC_{50} of 0.08 and 0.03 mg/mL respectively, showed enhanced cytotoxicity compared to <3 kDa UF. EC_{50} of GF1 and GF3 were about 2 times and 5.7 times lower than that of <3 kDa UF. GF3 was more cytotoxic than GF1. EC_{50} of GF3 was about 2.7-fold lower than that of GF1. Due to a lack of concentration-dependent increase in cytotoxicity (data not shown), the EC_{50} of GF2 was not determined. GF3 was further purified by using semi-preparative RP-HPLC.

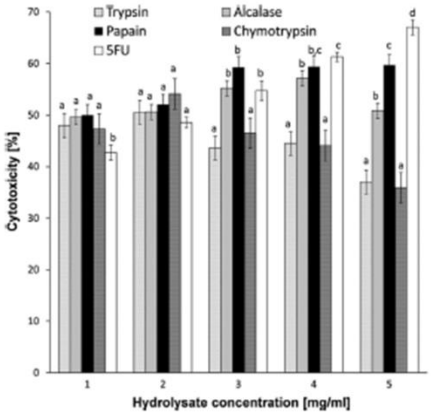

Fig. 2 Cytotoxicity of sponge hydrolysates produced by the four proteases. Data for the same hydrolysate concentration that are labeled by *different letters* are significantly different ($p < 0.05$), as determined using the Fisher's LSD test

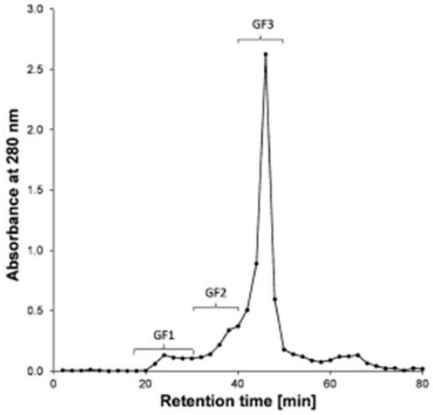

Fig. 3 A gel filtration chromatography elution profile of the <3 kDa UF fraction. The peaks eluted were separated into three fractions, namely GF1, GF2 and GF3

Figure 4a shows a semi-preparative RP-HPLC chromatogram of GF3. Four pooled fractions, namely F3P1-F3P4, were collected. These fractions were tested for cytotoxicity on HeLa cells at the standardized concentration of 0.03 mg peptide/mL. This concentration was chosen based on the EC_{50} of GF3. Among the four purified peptide fractions, two fractions F3P1 and F3P4 showed cytotoxicity against HeLa cells (Fig. 4b). F3P1 exhibited the strongest cytotoxicity among the four semi-preparative RP-HPLC fractions.

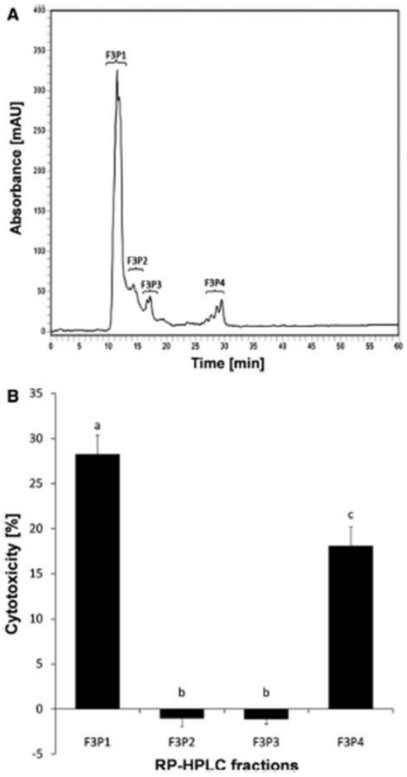

Fig. 4 **a** RP-HPLC profile of GF3 fraction obtained from gel filtration chromatography. The peaks eluted were pooled into four fractions, designated F3P1, F3P2, F3P3 and F3P4. **b** Cytotoxicity of RP-HPLC fractions tested at 0.03 mg/mL. Data labeled by *different letters* are significantly different ($p<0.05$), as determined using the Fisher's LSD test

Consequently, F3P1 was taken to peptide identification by LC–MS/MS analysis.

Identification of Cytotoxic Peptides

Based on LC–MS/MS analysis and database searching, two potential cytotoxic peptide sequences were identified from F3P1, namely KENPVLSLVNGMF (1447.70 Da) and LLATIPKVGVFSILV (1570.04 Da). We have synthesized these two peptide sequences to validate their cytotoxicity. Pentadecapeptide LLATIPKVGVFSILV, tested at up to 500 µg/mL (0.1592 mM), showed no concentration-dependent cytotoxicity on HeLa cells (data not shown). By contrast, tridecapeptide KENPVLSLVNGMF demonstrated concentration-dependent toxicity against HeLa cells (Fig. 5a). Importantly, the cytotoxicity of KENPVLSLVNGMF was drastically superior to that of 5FU. At 1.24 mM (1.8 mg/mL), KENPVLSLVNGMF exerted about 90% cytotoxicity in HeLa cells. By contrast, at 38.4 mM (5 mg/mL), 5FU demonstrated only 66% cytotoxicity. When expressed on a millimolar basis, the EC_{50} of KENPVLSLVNGMF was 0.67 mM, whereas that of 5FU was 2.56 mM.

To evaluate the selectivity of tridecapeptide KENPVLSLVNGMF, we also determined the cytotoxicity of the peptide on Hek293, a non-cancerous, human embryonic kidney cell line. Comparison was made between HeLa and Hek293 cells exposed to KENPVLSLVNGMF at 0.67 mM, the EC_{50} of the peptide. Our results showed that KENPVLSLVNGMF exhibited less than 5% cytotoxicity in Hek293 cells, but 44% cytotoxicity in HeLa cells (Fig. 5b).

Stability of Cytotoxic Peptide in Human Serum

Under the RP-HPLC conditions we used, KENPVLSLVNGMF was detected at the average retention time of 17.36 min. RP-HPLC analysis revealed gradual disappearance of the KENPVLSLVNGMF peptide from the human serum over 6 h, as indicated by the reducing peak areas of the peptide (Fig. 6a–d). There was a rapid drop in peptide concentration within the first 2 h of incubation to $55.5 \pm 8.2\%$ (Fig. 7). After 6 h of incubation, only 19% of KENPVLSLVNGMF was detected in the human serum. Half-life of the peptide was 3.2 ± 0.5 h.

Discussion

In the literature, numerous studies performed proteolytic hydrolysis of marine proteins to release and subsequently to identify bioactive peptides from mainly edible marine organisms (Chai et al. 2017; Cheung et al. 2015). Our study demonstrated for the first time that protease-assisted

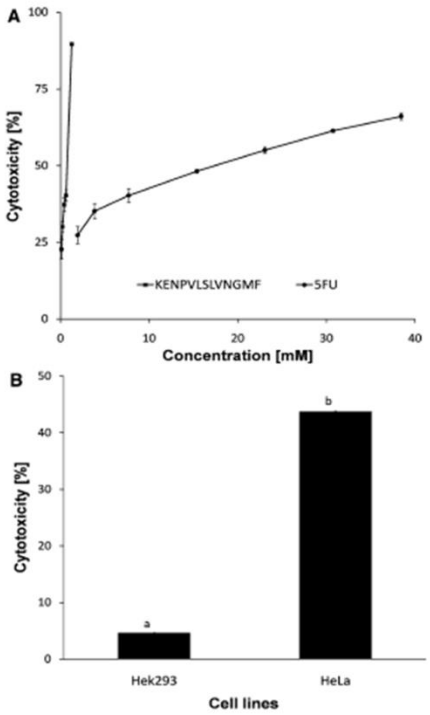

Fig. 5 **a** Cytotoxicity of KENPVLSLVNGMF and 5FU compared on a millimolar basis. **b** Cytotoxicity of KENPVLSLVNGMF, tested at 0.67 mM, on Hek293 and HeLa cell lines. Data labeled by *different letters* are significantly different ($p<0.05$), as determined by Student's T-test

hydrolysis can also be used to liberate a cytotoxic peptide from a marine sponge. In our study, *X. testudinaria* protein isolates were hydrolyzed to different extents by alcalase, trypsin, chymotrypsin and papain (Fig. 1). The four proteases were chosen because they have been widely used to produce bioactive protein hydrolysates from which various bioactive peptides were isolated (Park and Nam 2015; Suarez-Jimenez et al. 2012). Alcalase hydrolysis produced the highest DH values (Fig. 1) and was therefore the most effective protease treatment for the sponge proteins. Previous studies comparing the DH values of protein hydrolysates generated by different proteases treatments also showed alcalase to be the most effective (Jin et al. 2016; Sbroggio et al. 2016). In our study, likely because of the broad specificity of alcalase (Adamson and Reynolds 1996), the sponge protein samples potentially had more cleavage sites for alcalase, relative to the other three proteases. DH values produced by trypsin and chymotrypsin treatments were similar in our study. These two proteases also produced similar DH values when used to hydrolyze lecithin-free egg yolk protein (Aleksandra et al. 2012) and egg white protein (Aleksandra et al. 2010). Papain is an endolytic cysteine protease that does not exhibit a broad specificity for peptide bonds. Papain can cleave peptide bonds of basic amino acids, leucine, or glycine, but exhibits a preference for an amino acid displaying a large hydrophobic side chain at the P2 position (Chen et al. 2009a; De Jersey 1970; Ma et al. 2015; Yao et al. 2012). Limited occurrence of such cleavage sites may have accounted for the relatively low DH values and small DH changes during papain hydrolysis of the sponge protein isolate.

Our results show no clear relationship between DH values and cytotoxicity of the four sponge protein hydrolysates. Although the alcalase hydrolysate had markedly higher DH values than the papain hydrolysate, the latter showed stronger cytotoxicity against HeLa cells. A similar lack of correlation between DH and antiproliferative activity of protein hydrolysates prepared from tuna cooking juice (Hung et al. 2014), tuna dark muscle (Hsu et al. 2011) and proteins from the by-products of Atlantic salmon, Atlantic cod, plaice, blue whiting, Atlantic emperor, pollack and Portuguese dogfish (Picot et al. 2006) was also previously reported. Thus, DH values of protein hydrolysates may not be a reliable indicator of cytotoxicity. In this study, papain hydrolysate showed a concentration-dependent trend in cytotoxicity against HeLa cells when tested at the concentrations of 1–3 mg/mL, achieving the strongest activity among all hydrolysates at 5 mg/mL (Fig. 2). The other three hydrolysates showed reduced cytotoxicity when the concentration tested was increased to 5 mg/mL. Our observations suggest that the papain hydrolysate likely contained more potent cytotoxic peptides compared with the other three hydrolysates. The cytotoxicity of *X. testudinaria* protein hydrolysates against any cancer cell lines has never been reported in the literature.

When the papain hydrolysate was fractionated by membrane ultrafiltration, the peptide fraction with the lowest molecular weight range (<3 kDa UF) showed the highest cytotoxicity against HeLa cells at EC_{50} 0.17 mg/mL. Interestingly, following the fractionation of the <3 kDa UF by gel filtration chromatography, the fraction having the lowest molecular weight (GF3) was also the most cytotoxic (EC_{50} 0.03 mg/mL). Similar observations have been reported by other groups during the purification of cytotoxic peptides from the loach (You et al. 2011) and *Mercenaria* (Leng et al. 2005). Generally, when compared with larger peptides, smaller peptides have greater molecular mobility and diffusivity, which may enhance their interactions with

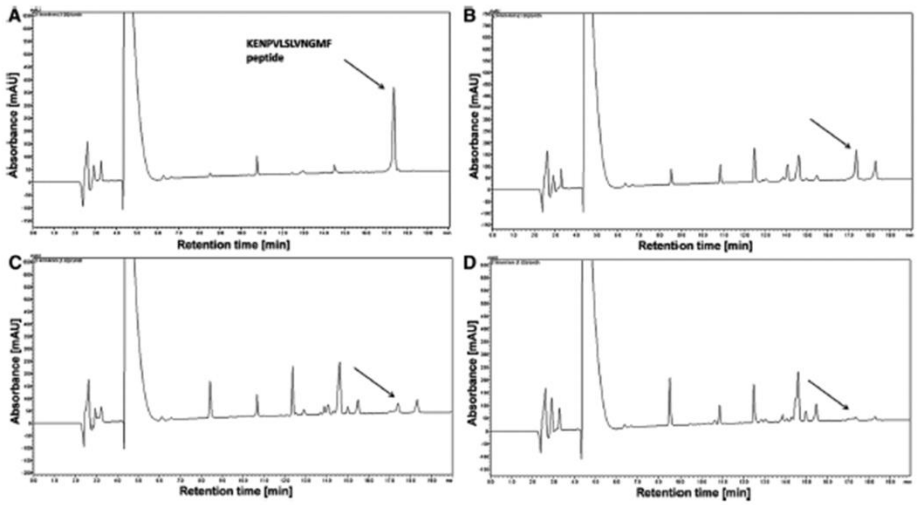

Fig. 6 Representative RP-HPLC profiles of KENPVLSLVNGMF following incubation in human serum for **a** 0 h, **b** 2 h, **c** 4 h, and **d** 6 h. *Arrow* indicates the KENPVLSLVNGMF peak, eluted at retention time 17.37 min

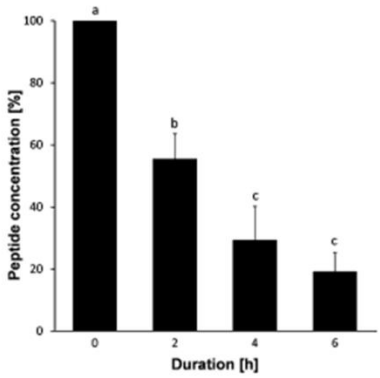

Fig. 7 KENPVLSLVNGMF concentration in human serum over 6 h of incubation. Data labeled by *different letters* are significantly different ($p < 0.05$), as determined by the Fisher's LSD test

cancer cell components, thus improving anticancer activity (Kim et al. 2013; Suarez-Jimenez et al. 2012). Ours and others' findings also suggest that at least in some marine protein hydrolysates, the potential cytotoxic peptides present are likely less than 3000 Da in size. In agreement with this are the reports of <3 kDa cytotoxic peptides identified from marine bivalve mollusks (Kim et al. 2013), half-fin anchovy (Song et al. 2014) and skate (Pan et al. 2016).

Further fractionation of GF3 on RP-HPLC revealed F3P1 and F3P4 to be the two cytotoxic peptide fractions (Fig. 4b). F3P1, being the first eluted fraction during RP-HPLC, was likely more polar in nature than F3P4. Our results thus suggest that the cytotoxicity of GF3 against HeLa cells could be attributed to the overall effects of at least two peptides of rather different polarities. Because F3P1 showed stronger cytotoxicity than F3P4, the former was taken to peptide sequence determination by LC–MS/MS analysis. This resulted in the identification of two potential peptide sequences: KENPVLSLVNGMF and LLATIPKVGVFSILV. When we assessed the cytotoxicity of the two sequences by using synthetic peptides, KENPVLSLVNGMF was the only one showing potent cytotoxicity. To our knowledge, KENPVLSLVNGMF is a novel cytotoxic peptide which has not been previously reported. This is also the first report of identification of a cytotoxic peptide from *X. testudinaria*. Notably, when compared on a millimolar basis, KENPVLSLVNGMF was considerably stronger than 5FU as a cytotoxic agent against HeLa cells. Hence, our results indicate that *X. testudinaria* is a promising source of potent cytotoxic peptides, which deserves more attention in future research.

The cytotoxic effect of KENPVLSLVNGMF may be associated with its amphiphilicity, as indicated by the

presence of hydrophobic (L, M, F, P, V) and hydrophilic (N, E, G, K) residues. CTLEW and LANAK, anticancer peptides isolated from walnut protein and oyster, respectively, are also amphiphilic peptides (Ma et al. 2015; Umayaparvathi et al. 2014). Amphiphilicity is believed to play an important role in the ability of anticancer peptides to bind to and penetrate cancer cell membranes (Dennison et al. 2006; Li and Yu 2015). The molecular mechanisms of KENPVLSLVNGMF as a cytotoxic agent, whether involving cancer cell membrane penetration or by other modes of action, remain to be elucidated.

On the other hand, KENPVLSLVNGMF was selectively toxic to HeLa cells but was only marginally toxic to normal, non-cancerous cells. One of the hallmarks of cancer cell membrane is having more anionic phospholipids in their outer leaflet, whereas normal cell membranes comprised of largely zwitterionic phospholipids (Papo and Shai 2005). Whether the selectivity of KENPVLSLVNGMF for cancer cells is attributable to its differential affinities towards the two types of cell membranes should be addressed in future research. In any case, anticancer agents which effect high cancer cell selectivity and low toxicity to normal cells are highly desirable as they may induce only minimal side effects (Markman et al. 2013). Hence, future investigations of the effects of KENPVLSLVNGMF on other cancer and non-cancer cells are warranted and may provide more insights into the potential application of the peptide as an anticancer agent.

KENPVLSLVNGMF (half-life 3.2 ± 0.5 h) showed higher stability in the human serum when compared to RRWQWR and RRWWRF, two antimicrobial peptides that degraded quickly, with half-life of less than 0.5 h (Nguyen et al. 2010). Half-life of KENPVLSLVNGMF in the human serum was also superior to that of short antihypertensive peptides RWQ and WQ, which was 1.9 min and 2.3 h, respectively (Fernández-Musoles et al. 2013). In the literature, reports of unmodified bioactive peptides having half-life exceeding 6 h in the human serum are scarce. Notably, the cyclised forms of RRWQWR and RRWWRF showed drastically improved stability compared to their unmodified counterparts, with half-life of approximately 24 h (Nguyen et al. 2010). Besides cyclization, other modification strategies may also be used to enhance peptide stability. These include N- and C- terminus modifications, end capping (Werle and Bernkop-Schnurch 2006) as well as replacement of key amino acid residues and modification using D- or L-beta-amino acids (Arenas et al. 2016; Nguyen et al. 2010). Such strategies may be attempted in future to enhance the stability of KENPVLSLVNGMF in human blood. Considering that at least 20 peptidases and proteases occur in the human blood (Werle and Bernkop-Schnurch 2006), future efforts to modify KENPVLSLVNGMF for improved stability should be preceded by an investigation on potential peptidase cleavage sites in the peptide. Future research to discover modified forms of KENPVLSLVNGMF which are more stable in human blood and which also maintain a selective cytotoxicity against HeLa cells is of great interest.

In conclusion, a novel cytotoxic peptide KENPVLSLVNGMF was purified and identified from the papain hydrolysate of marine sponge *X. testudinaria*. KENPVLSLVNGMF was cytotoxic to the cancerous HeLa cells but only marginally cytotoxic to the non-cancerous Hek293 cells. Notably, the cytotoxicity of KENPVLSLVNGMF was 3.8-fold more potent when compared with anticancer drug 5FU. Our study also found the peptide to have a half-life of 3.2 ± 0.5 h in human serum. Taken together, KENPVLSLVNGMF is a potential lead for future development of peptide-based anticancer drugs.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval Ethical approval was obtained from UTAR Scientific and Ethical Review Committee (U/SERC/40/2017). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or research committee and with Helsinki Declaration of 1975.

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Appendix C

Published Article Entitled Purification and Identification of Novel Cytotoxic Oligopeptides from Soft Coral *Sarcophyton glaucum*

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Purification and identification of novel cytotoxic oligopeptides from soft coral *Sarcophyton glaucum*[*‡]

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Abstract: Globally, peptide-based anticancer therapies have drawn much attention. Marine organisms are a reservoir of anticancer peptides that awaits discovery. In this study, we aimed to identify cytotoxic oligopeptides from *Sarcophyton glaucum*. Peptides were purified from among the *S. glaucum* hydrolysates produced by alcalase, chymotrypsin, papain and trypsin, guided by a methylthiazolyldiphenyl-tertrazolium bromide (MTT) assay on the human cervical cancer (HeLa) cell line for cytotoxicity evaluation. Purification techniques adopted were membrane ultrafiltration, gel filtration chromatography, solid phase extraction (SPE), and RP-HPLC. Purified peptides were identified by *de novo* peptide sequencing. From papain hydrolysate, three peptide sequences were identified: AGAPGG, AERQ and RDTQ (428.45, 502.53 and 518.53 Da, respectively). Peptides synthesized from these sequences exhibited cytotoxicity on HeLa cells with EC_{50} values of 8.6, 4.9, and 5.6 mM, respectively, up to 5.8-fold stronger than the anticancer drug 5-fluorouracil. When tested at their respective EC_{50}, AGAPGG, AERQ, and RDTQ showed only 16, 25 and 11% cytotoxicity to non-cancerous Hek293 cells. In conclusion, AERQ, AGAPGG and RDTQ are promising candidates for future development as peptide-based anticancer drugs.

Key words: Anticancer therapy; Bioactive peptides; Cytotoxicity; HeLa cells; *Sarcophyton glaucum*; Soft coral
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1 Introduction

Cancer is one of the leading causes of morbidity and mortality worldwide. There were about 14 million new cancer cases and 8.2 million cancer deaths in 2012 globally. It was projected that in the next 20 years, new cases will increase by about 70% (Ferlay et al., 2013). Unfortunately, chemotherapy, a common cancer treatment, often incurs side effects, in-

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cluding nausea, hair loss, fatigue, and loss of appetite. Many of these side effects take place due to nonspecific actions of the anticancer compounds on non-cancerous tissues, thus reducing the effectiveness of the treatment (Sutradhar and Amin, 2014). Therefore, there is an urgent need to develop more cancer-specific cytotoxic drugs.

More than 60% of cancer therapeutic agents currently used in chemotherapy originate from natural sources (Pangestuti and Kim, 2017). This has driven further interest among the scientific community to bioprospect natural resources for new anticancer agents. Marine-derived bioactive peptides are an invaluable resource for anticancer drug development (Zheng et al., 2013). Some marine bioactive peptides, such as kahalalide F, HTI-286, dehydrodidemnin B, dolastatin 10 and synthadotin, have already made it to preclinical or clinical trials for cancer treatment (Simmons et al., 2005). Bioactive peptides, usually consisting of 2–20 amino acids residues, are inactive within the sequence of the parent proteins and can be liberated by enzymatic hydrolysis (Kim et al., 2013; Chai et al., 2017). Proteases often used for such a purpose include alcalase, α-chymotrypsin, papain, trypsin and pepsin (He et al., 2005), Qian et al. (2007), Ngo et al., 2012). Separation techniques such as membrane ultrafiltration (UF), gel filtration chromatography (GF), fast protein liquid chromatography, and reversed-phase high-performance liquid chromatography (RP-HPLC) are often employed to purify the bioactive peptides before they can be identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) or Edman degradation (Chai et al., 2017). Anticancer peptides have been identified from various marine organisms, including sponges (Quah et al., 2017), tunicates (Jumeri and Kim, 2011), fish (Picot et al., 2006; Hsu et al., 2011, Hung et al., 2014), and oysters (Wang et al., 2010). However, only a few studies have evaluated the cytotoxicity of those peptides on normal or non-cancerous cells to characterize selectivity of the peptides (Chen et al., 2013; Pan et al., 2016; Daliri et al., 2017).

Sarcophyton glaucum is a marine soft coral that belongs to the family Alcyoniidae, phylum Cnidaria, and class Anthozoa (van Ofwegen, 2010). *S. glaucum* has received much attention in the last decade for its diverse bioactive secondary metabolites, including cembranoids (Fahmy et al., 2006; Hegazy et al., 2011), bicembranoids (Huang et al., 2015), and steroids (Chao et al., 2017). A number of non-peptide compounds from *S. glaucum* showed cytotoxicity towards some cancer cell lines, e.g. human breast cancer (MCF-7) (Al-Lihaibi et al., 2014; Abdel-Lateff et al., 2015), lymphoma human liver cancer (HepG2) (Hegazy et al., 2011; Al-Lihaibi et al., 2014; Abdel-Lateff et al., 2015), human colon cancer (HCT-116) (Hegazy et al., 2011; Abdel-Lateff et al., 2015) and human cervical cancer (HeLa) (Hegazy et al., 2011) cell lines. However, the discovery of cytotoxic peptides from *S. glaucum* has not been documented in the literature. Therefore, this study aimed to purify and identify cytotoxic peptides from *S. glaucum*. Guided by a cytotoxicity assay on HeLa cells and using UF, GF, solid phase extraction (SPE), RP-HPLC, and *de novo* peptide sequencing, three peptides AGAPGG, AERQ and RDTQ were identified from a papain hydrolysate of *S. glaucum*.

2 Materials and methods

2.1 Reagents and materials

Ultrafiltration centrifugal units molecular weight cut-off (MWCO) 3 kDa and HPLC-grade acetonitrile (ACN) were purchased from Merck. RPMI 1640 medium was bought from Gibco, Life Technologies, and HPLC-grade trifluoroacetic acid (TFA) was from Fisher Chemical. Methylthiazolyldiphenyl-tertrazolium bromide (MTT) was purchased from Amresco, 5-fluorouracil (5FU) from Biobasic, and phthalaldehyde (OPA) from Nacalai Tesque. Strata® C18-E SPE cartridges (55 μm, 70 Å, 1000 mg/6 ml) were purchased from Phenomenex, Inc., and Acrodisc® syringe filters with Supor® membranes (0.2 μm pore size) from Medigene. Other reagents used were of analytical grade.

2.2 Preparation of *S. glaucum* protein isolate and hydrolysates

S. glaucum was sampled at Nanga Kecil Island, Malaysia, in July 2013. The specimens were obtained using self-contained underwater breathing apparatus (SCUBA) at 3 m depth. Species were identified by reference to Fabricius et al. (2001). The samples were brought back to the laboratory on ice, stored in a freezer at -20 °C, and lyophilized before use. Proteins

from *S. glaucum* were isolated as described by Quah et al. (2017). The protein content of the isolate was determined with Bradford's assay (Bradford, 1976), using a standard curve constructed with bovine serum albumin.

Hydrolysis was performed as previously described (Quah et al., 2017). Briefly, the lyophilized protein isolate was dissolved in 50 mmol/L sodium phosphate buffer at a mass (g): volume (ml) ratio of 1:200. The pH of the buffer was adjusted to 7, 7, 6, or 8, for the hydrolysis with alcalase, chymotrypsin, papain or trypsin, respectively. The proteases were applied according to an enzyme (g): protein (g) ratio of 1:10. Hydrolysis was carried out in water baths at the optimum temperature for each protease (50 °C for alcalase; 37 °C for trypsin, chymotrypsin, and papain). Aliquoted hydrolysate samples were lyophilized before use in an MTT cytotoxicity test as described below. For each protease treatment, the degree of hydrolysis (DH) was measured as reported previously (Chen et al., 2009).

2.3 MTT assay

The MTT assay was performed as reported previously (Quah et al., 2017). Briefly, the HeLa human cervical cancer cell line (ATCC CCL-2) was grown at 37 °C in a humidified incubator in 5% CO_2, in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Filtered samples (100 µl) of various concentrations were added to each microplate well, followed by 24 h of incubation. For a negative control, the sample was substituted with sterile, deionized water. Upon addition of MTT (5 mg/ml) and after 4-h incubation, the microplate was centrifuged at 1000×g. To solubilize the purple formazan, dimethyl sulfoxide (200 µl) was added to each well and the contents of each well were mixed thoroughly prior to measurement of absorbance at 570 nm. 5FU was used as a positive control.

2.4 Fractionation by membrane ultrafiltration

Papain hydrolysate was fractionated by membrane ultrafiltration as reported previously (Quah et al., 2017), with modifications. Briefly, two fractions designated as "<3 kDa UF" and ">3 kDa UF" were obtained using a 3 kDa MWCO ultrafiltration centrifugal unit. The two UFs were lyophilized before being tested for cytotoxicity. Peptide contents were quantified using the OPA method (Nielsen et al., 2001).

2.5 Fractionation by gel filtration chromatography

The <3 kDa UF was fractionated using a Sephadex G-25 column (1.6×70 cm) as described previously (Quah et al., 2017). Briefly, sample fractions were eluted with deionized water and the elution profile was monitored at 280 nm. The pooled fractions obtained, designated GF1, GF2, and GF3, were lyophilized and tested for cytotoxicity. Peptide contents were determined as described above.

2.6 Fractionation by SPE

Pooled fraction GF3 was further purified using SPE cartridges. Two ml of GF3 (50 mg/ml) was applied to Strata® C18-E cartridges which were preconditioned with methanol (6 ml), washed with 100% ACN containing 0.1% TFA (6 ml), and equilibrated with deionized water containing 0.1% TFA (12 ml). GF3 was fractionated using a stepwise elution (6 ml per step) with increasing ACN concentrations (0, 10, 20, 30, 40, 50, 80, and 100%) in 0.1% TFA. This produced a series of fractions designated SPE-F1, SPE-F2, SPE-F3, SPE-F4, SPE-F5, SPE-F6, SPE-F7, and SPE-F8, respectively. Absorbance of each fraction was monitored at 214 nm. The peptide content and cytotoxicity of the SPE fractions were determined as described above.

2.7 Analysis of SPE-F7 by RP-HPLC

Ten µl of SPE-F7 were separated by RP-HPLC (Shimadzu LC-20D dual binary pumps and Shimadzu Prominence SPD-M20A PDA detector) using a Kinetex C_{18} column (100 Å, 5 µm, 4.6×250 mm). The mobile phase consisted of deionized water with 0.1% TFA as solvent A and ACN containing 0.1% TFA as solvent B at a flow rate of 0.5 ml/min. The column was eluted with a gradient elution as follows: 0–10 min 5–35% of solvent B; 10–35 min, 35–95% solvent B; 35–41 min, 95% solvent B; 41–41.01 min 95–5% solvent B; 40.01–50 min, 5% solvent B. The elution profile was monitored at 214 nm.

2.8 *De novo* peptide sequencing

Online LC-MS/MS analysis was performed at the Proteomics Core facility, Malaysia Genome In-

stitute, National Institutes of Biotechnology, Malaysia to identify the peptide sequences in SPE-F7. Briefly, the peptide was analyzed using a Waters nanoACQUITY UPLC system, coupled to a Waters SynaptG2HDMS-Q-TOF mass spectrometer. *De novo* peptide sequencing was carried out using data directed analysis (DDA) with a positive electrospray ionization mode. ProteinLynx Global Server Software (Version 2.4) was used for data analysis.

2.9 Validation of cytotoxicity of synthetic peptides

Synthetic peptides (>95% purity) were manufactured by and purchased from Bio Basic Inc., Canada, based on the sequences identified (AGAPGG, AERQ and RDTQ). The cytotoxicity of the synthetic peptides was tested on HeLa and Hek293 cell lines. Hek293 is a non-cancer, human embryonic kidney cell line.

2.10 Data analysis

Data are expressed as mean±standard errors (n=3). SAS (Version 9.4) was used for statistical analysis. Data were analyzed by one-way ANOVA, and where appropriate, followed by Fisher's Least Significant Difference (LSD) test to separate means of significant differences. Student's T-test was used to compare two mean values. P values less than 0.05 were considered to be statistically significant.

3 Results

3.1 Protein isolation and hydrolysis

Protein isolate prepared from *S. glaucum* yielded 443.1 mg proteins/g dry weight. Hydrolysis of the protein isolate by alcalase, chymotrypsin, papain and trypsin was monitored for up to 8 h (Fig. 1). Yield percentages of the four hydrolysates were 26.4, 22.9, 22.2 and 28.1%, respectively. Overall, hydrolysis using alcalase produced the highest DH value (27.5±0.4% after 8 h of hydrolysis) among all protease treatments. A clear-cut rising trend in DH was not observed in the protein hydrolysates produced using chymotrypsin, papain or trypsin. Chymotrypsin and papain treatments produced very similar DH values over the 8 h period. After 8 h of hydrolysis, the highest DH values were 20.7±0.2% for chymotrypsin

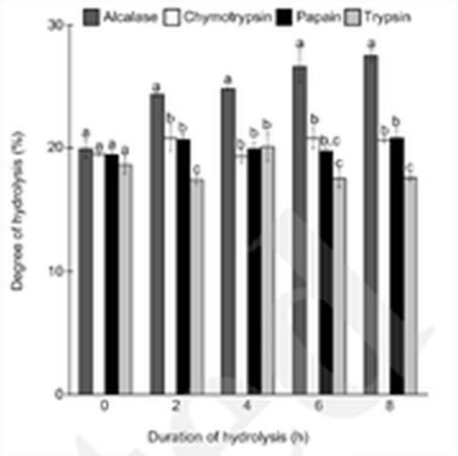

Fig. 1 Degree of hydrolysis of *S. glaucum* protein hydrolysates
Data are mean±standard errors (n=3). Data for the same hydrolysis duration that are labelled with different letters are significantly different (P<0.05) according to the Fisher's LSD test

hydrolysate and 20.8±0.8% for papain hydrolysate. For hydrolysis using trypsin, the maximum DH value of 20.1±1.1% was obtained after 4 h. In this study, the optimal hydrolysis duration was defined as the duration required to achieve the maximum DH value. Therefore, the optimum hydrolysis duration for alcalase, chymotrypsin and papain was 8 h, whereas for trypsin, it was 4 h.

3.2 Cytotoxic activity of *S. glaucum* protein hydrolysates

Hydrolysates obtained after their respective optimum hydrolysis durations were tested for cytotoxicity against the HeLa cell line. The four hydrolysates were cytotoxic at doses of 1–5 mg/ml (Fig. 2). Notably, papain hydrolysate was the only hydrolysate that showed a dose-dependent increase in cytotoxicity. By contrast, the cytotoxicity of the alcalase, chymotrypsin and trypsin hydrolysates fluctuated slightly over the range of concentrations tested. 5FU, an anticancer drug, was used as the positive control in our assay. 5FU was more cytotoxic than all four hydrolysates tested. When the sample concentration tested was 5 mg/ml, the cytotoxicity of alcalase, chymotrypsin, papain and trypsin hydrolysates was 30, 31, 50, and

34%, respectively. Owing to its high cytotoxicity, papain hydrolysate was further fractionated using membrane ultrafiltration.

3.3 Purification of cytotoxic peptides

Membrane ultrafiltration separated papain hydrolysate into two fractions: <3 kDa UF and >3 kDa UF. Yield percentages of <3 kDa UF and >3 kDa UF were 11.5 and 3.2%, respectively. When evaluated for cytotoxic activity against HeLa cells, the EC_{50} values of <3 kDa UF and >3 kDa UF were 0.16±0.01 and 0.14±0.01 mg/ml, respectively. There were no significant differences between the EC_{50} of the two fractions (P>0.05, Student's T-test). Considering that previous reports (Hsu et al., 2011, Hung et al., 2014, Song et al., 2014) often found cytotoxic marine peptides to be smaller than 3 kDa, <3 kDa UF was selected to be further fractionated. A representative elution profile of <3 kDa UF following separation on a Sephadex G-25 column is presented in Fig. 3a. Yield percentages of GF1, GF2 and GF3 were 3.2, 9.3 and 0.8%, respectively. Pooled fractions GF1, GF2 and GF3 were evaluated for cytotoxicity on HeLa cells. Among the three GF fractions, GF2 had the highest EC_{50} (0.17 mg/ml) (Fig. 3b), which was similar to that of <3 kDa UF (P>0.05, Student's T-test). The EC_{50} of GF1 (0.06 mg/ml) and GF3 (0.04 mg/ml) were about 2.7- and 4-fold lower, respectively, than that of <3 kDa UF (P<0.05, Student's T-test). Overall, all three GF fractions had much lower EC_{50} values than 5FU. In comparison to 5FU, the EC_{50} values of GF1 and GF3 were about 37.8- and 56.8-fold lower, respectively. GF3 was, therefore, further purified by reversed phase SPE.

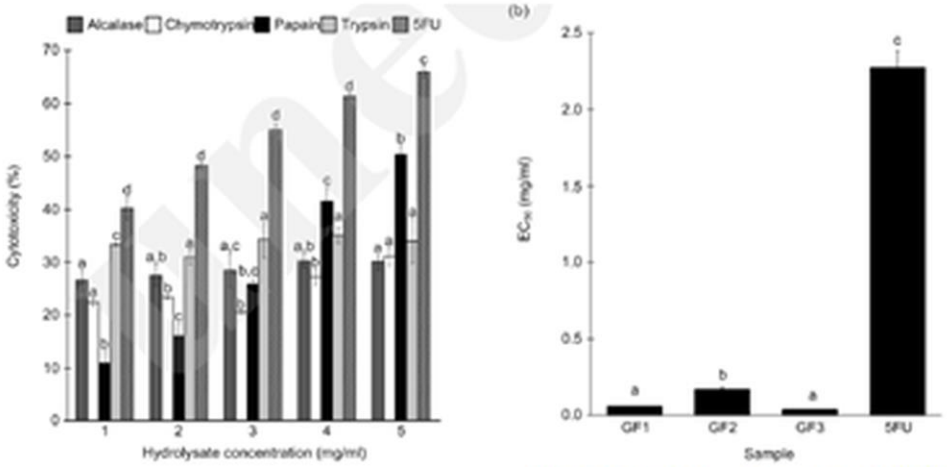

Fig. 2 Cytotoxicity of *S. glaucum* hydrolysates against HeLa cells
Data are mean±standard errors (n=3). Data for the same hydrolysate concentration that are labelled with different letters are significantly different (P<0.05) according to the Fisher's LSD test

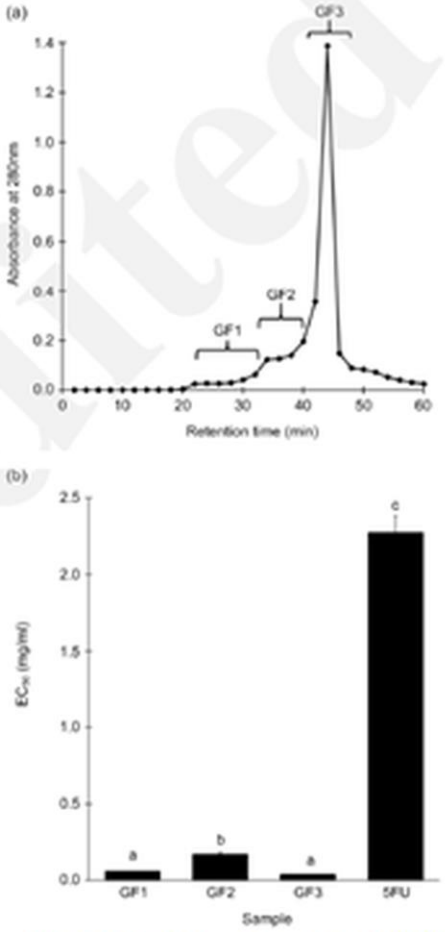

Fig. 3 Gel filtration chromatography of <3 kDa UF
(a) A representative elution profile. Eluates were divided into three pooled fractions, GF1, GF2 and GF3. (b) Cytotoxicity of the GF fractions and 5FU, expressed as EC_{50} values. For (b), data are mean ± standard errors (n=3). Data labelled by different letters are significantly different (P<0.05) according to the Fisher's LSD test

A representative SPE elution profile of GF3, monitored at 214 nm, is presented in Fig. S1. Among the eight fractions, an absorbance reading was undetectable for SPE-F8. Thus, the peptide contents of only seven fractions SPE-F1 to SPE-F7 were quantified. Except for SPE-F1, the peptide contents of most SPE fractions ranged between 0.01 and 0.37 mg/ml (Fig. S2). The cytotoxicity of the seven SPE fractions was then tested at 0.04 mg peptide/ml. This standardized concentration was selected according to the EC_{50} of GF3. Although all the SPE fractions showed cytotoxic activity against HeLa cells, SPE-F7 stood out as the most active (41.2±0.7%) (Fig. 4a). When analyzed using RP-HPLC, the chromatogram for SPE-F7 revealed three major peaks (Fig. 4b), namely P1, P2 and P3, which constituted 8.2, 12.6 and 31.8% of the relative peak area in relation to total area of all peaks in the chromatogram. These peaks were eluted at retention times of 4.677, 5.147 and 5.434 min, respectively. The SPE-F7 fraction was taken for peptide sequencing by *de novo* sequencing.

3.4 Identification of cytotoxic peptides

One 6-residue peptide AGAPGG and two 4-residue peptides AERQ and RDTQ were identified from SPE-F7. Fig. 5 shows the MS/MS spectra of the three peptides. The m/z values of these peptides, as single-charged ions, ranged between 429 and 519. The detected molecular masses of AGAPGG (428.2019 Da), AERQ (502.2500 Da) and RDTQ (518.2449 Da) agreed well with the theoretical molecular masses of the three peptides (428.2013, 502.2492, and 518.2441 Da), as calculated by PepDraw (http://www.tulane.edu/~biochem/WW/PepDraw/). These peptide sequences were synthesized and used for validation of their cytotoxicity.

Among the three synthetic peptides, AERQ exhibited the highest cytotoxic activity on HeLa cells, closely followed by RDTQ. Both these peptides exhibited about 90% cytotoxic activity at 5 mg/ml, surpassing that of 5FU (data not shown). EC_{50} values of the three peptides, when tested on HeLa cells, were 8.6, 4.9, and 5.6 mmol/L, respectively (Fig. 6a). Based on EC_{50} comparisons, AGAPGG, AERQ and RDTQ were 3.3-, 5.8- and 5.1-fold more potent than 5FU. Statistically, the EC_{50} values of AERQ and RDTQ were not significantly different ($P>0.05$, Fisher's LSD).

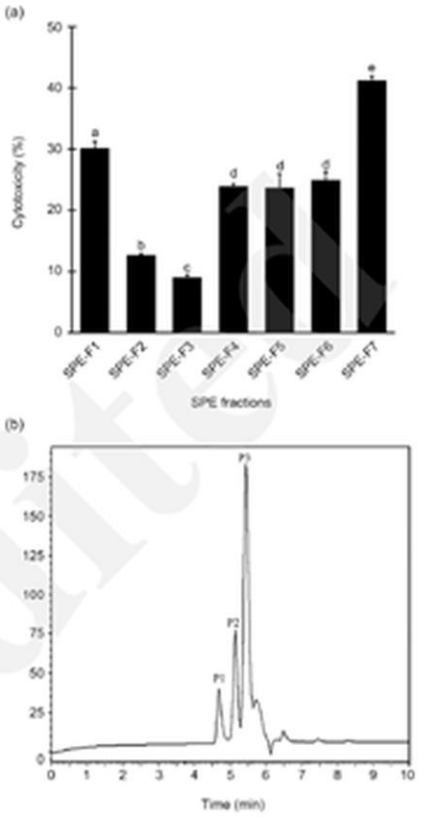

Fig. 4 (a) Cytotoxicity of SPE fractions tested at 0.04 mg peptide/ml on HeLa cells (b) A representative RP-HPLC profile of SPE-F7 monitored at 214 nm
For (a), data are mean±standard errors (n=3). Data labelled by different letters are significantly different ($P<0.05$) according to the Fisher's LSD test

To evaluate their selectivity, AGAPGG, AERQ and RDTQ were further assessed for their cytotoxicity on the Hek293 cell line based on their respective EC_{50} against HeLa cells. The relative cytotoxicity of the three peptides against Hek293 was RDTQ< AGAPGG<AERQ (Fig. 6b). The cytotoxicity of the peptides against Hek293 cells, tested at their respective EC_{50} against HeLa cells, ranged from 11- 25% (Fig. 6b). Notably, whereas 5.6 mmol/L RDTQ

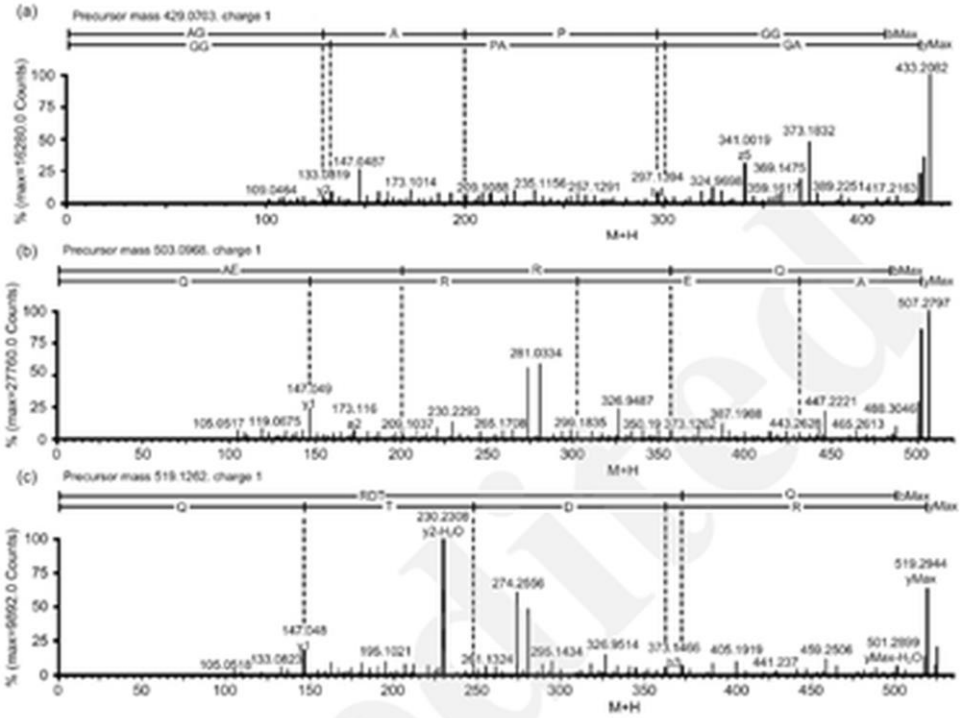

Fig. 5 MS/MS spectra of (a) AGAPGG, (b) AERQ and (c) RDTQ

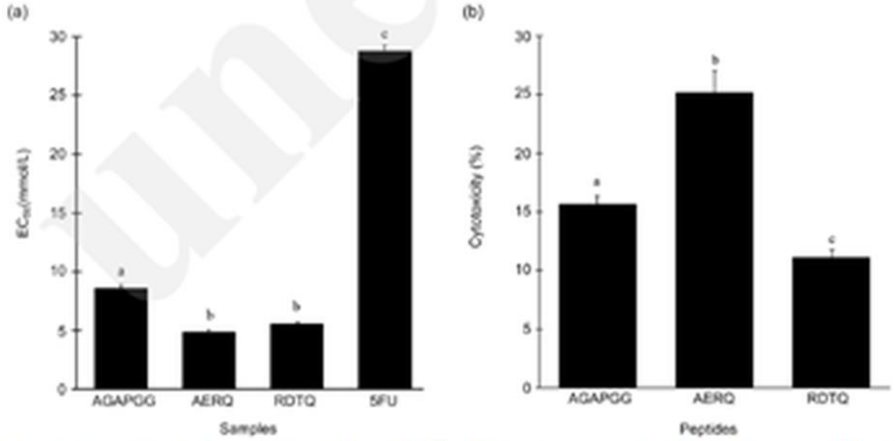

Fig. 6 (a) Cytotoxicity of the synthetic peptides and 5FU on HeLa cells, expressed as EC_{50} values on a mM basis. (b) Cytotoxic activity of synthetic peptides tested at their respective EC_{50} on Hek293 cells

Data are mean±standard errors (n=3). Data labelled by different letters are significantly different (P<0.05) according to the Fisher's LSD test

induced 50% cytotoxicity on HeLa cells (Fig. 6a), the same peptide concentration induced only 11% cytotoxicity on Hek293 cells (Fig. 6b).

4 Discussion

The bioprospecting of edible marine fishes, other seafoods, and their by-products for bioactive peptides is frequently accomplished via bioassay-guided purification and identification of such peptides from protein hydrolysates (Wang et al., 2013; Chai et al., 2017; Nurdiani et al., 2017). Adopting the same strategy, we demonstrated for the first time that protease-mediated hydrolysis can also be used to release potent cytotoxic peptides from *S. glaucum*, a non-food marine animal. In this study, papain hydrolysate exhibited the most prominent dose-dependent cytotoxic effect against HeLa cells (Fig. 2). This result is consistent with those of other studies which reported papain hydrolysates having stronger cytotoxicity than other enzyme hydrolysates prepared from tuna dark muscle (Hsu et al., 2011), tuna cooking juice (Hung et al., 2014) and the marine sponge *Xestospongia testudinaria* (Quah et al., 2017). Our findings suggest that papain hydrolysis likely liberated more potent cytotoxic peptides encrypted in the proteins of *S. glaucum* than did the other three proteases.

In this study, no correlation was found between DH and cytotoxicity. Hsu et al. (2011) reported no correlation between the DH of hydrolysates prepared from tuna dark muscle and their antiproliferative activities against the MCF-7 cell line. Similarly, Picot et al. (2006) observed no correlation between the DH of several fish hydrolysates and their antiproliferative activities. Thus, our findings and those of others (Picot et al., 2006; Hsu et al., 2011) imply that although DH may be useful as an indicator of the progress of protein hydrolysis, its value as an indicator of cytotoxicity ap pears limited.

Our results revealed that the <3 kDa and >3 kDa UFs exhibited similar levels of cytotoxicity against the HeLa cells. Similar levels of cytotoxicity were also observed when <3 kDa and >3 kDa UF fractions of a *Porphyra haitanesis* hydrolysate were tested on the A549 lung cancer and SGC-7901 gastric cancer cell lines (de Lumen, 2005; Fan et al., 2017). Nevertheless, the <3 kDa UF fraction of *X. testudinaria* papain hydrolysate exhibited about 7 times stronger cytotoxicity than the 3-10 kDa UF fraction (Quah et al., 2017). When compared with fractions of larger molecular weight (MW) ranges, the <3 kDa UF prepared from loach papain hydrolysate also exhibited stronger antiproliferative activity against the HepG2 , MCF-7, and Caco-2 colon cancer cell lines (You et al., 2011). Furthermore, according to Fan et al. (2017), short peptides can be identified easily by mass spectrometry and are easier to synthesize. Small peptides are also less costly to synthesize (Chai et al., 2017). Therefore, in this study, <3 kDa UF was chosen for further purification.

Following separation on a Sephadex G-25 gel filtration column, three active peaks were detected in the elution profile (Fig. 3a). This implies that multiple peptides of variable MWs were found in the <3 kDa UF. Notably, the three GF fractions had EC_{50} values that were drastically lower than that of 5FU, indicating that they were considerably more potent than 5FU. This result is interesting in the light of some other studies in which GF fractions were found to have poorer cytotoxicity than 5FU. For example, the GF fractions derived from *Spirulina (Arthrospira) platensis* (Wang and Zhang, 2016) and *P. haitanesis* (Fan et al., 2017) exhibited weaker cytotoxicity on the MCF-7, HepG2, SGC-7901, A549 and HT-29 cell lines than 5FU.

Among the three GF fractions in this study, GF3, which corresponded to the lowest MW range, displayed the strongest cytotoxicity against HeLa cells. Similar trends were also reported for GF fractions prepared from *X. testudinaria* hydrolysate (Quah et al., 2017) and half-fin anchovy hydrolysate (Song et al., 2014). Working on the alcalase hydrolysate of a solitary tunicate, Jumeri and Kim (2011) reported that F2, the GF fraction of a lower MW range, was more cytotoxic to HeLa cells than F1, the GF fraction of a higher MW range. The authors proposed that the enhanced anticancer activity of F2 may be due to its higher molecular mobility and diffusivity compared with F1, which may have enabled better interaction between F2 and cancer cells (Jumeri and Kim, 2011). Thus, in this study, favorable mobility and diffusivity characteristics may have underlain the potency of GF3 as a cytotoxic agent against HeLa cells.

Further separation of GF3 using SPE revealed

that SPE-F7, the most cytotoxic peptide fraction, was eluted with 80% ACN (Fig. 4a). Thus, peptides with relatively high hydrophobicity may have contributed to the strong cytotoxic effect of SPE-F7 against HeLa cells. Supporting this suggestion are previous observations that peptides with greater hydrophobicity showed stronger anticancer activity against HeLa, MCF-7 and other cancer cell lines (Huang et al., 2011, Shan et al., 2012). RP-HPLC analysis of SPE-F7 revealed three major peaks (Fig. 4b), implying the presence of at least three peptides with different polarities in the fraction. Furthermore, the chromatogram suggests that SPE-F7 was sufficiently purified to be subjected to the determination of peptide sequences using LC-MS/MS analysis.

De novo peptide sequencing led to the identification of three potential cytotoxic peptides: AGAPGG, AERQ and RDTQ (Fig. 5). When these peptides were chemically synthesized and tested on HeLa cells, all showed cytotoxicity (Fig. 6a). To our knowledge, this study reports for the first time the identification of cytotoxic peptides from *S. glaucum*. Moreover, AGAPGG, AERQ and RDTQ are cytotoxic peptides that have not been previously reported. A search of the BIOPEP database (Minkiewicz et al., 2008) (accessed on 19 September 2017) also found these three peptides not documented for any bioactivities. Importantly, AGAPGG, AERQ and RDTQ were more powerful cytotoxic agents than 5FU. Hence, *S. glaucum* should be exploited more intensively in future as a source of novel cytotoxic peptides.

The structure-activity relationship of anticancer peptides is still not fully understood (Gabernet et al., 2016). Nevertheless, amphiphilicity is believed to be important to the ability of anticancer peptides to not only bind to but also penetrate cancer cell membranes (Dennison et al., 2006; Li and Yu, 2015). Interestingly, two of the three peptides identified in this study were amphiphilic. The amphiphilicity of AGAPGG is indicated by hydrophobic (A and P) and hydrophilic (G) amino acid residues, with a calculated hydrophobic ratio of 50%. The amphiphilicity of AERQ is indicated by the presence of hydrophobic (A) and hydrophilic (E, R, Q) residues, with a calculated hydrophobic ratio of 25%. Although AGAPGG and AERQ are both amphiphilic, AERQ was about 1.5-fold more cytotoxic than AGAPGG (Fig. 6a). Song et al. (2014) reported that upon the replacement of an H residue with a G residue in the peptide YALPAH, the modified peptide YALPAG showed weaker inhibitory activity on PC-3 prostate cancer cells. Thus, the presence of three G residues in AGAPGG could have lowered its cytotoxic effect. The strong cytotoxicity of AERQ may also be associated with the presence of an R residue within the peptide (Schmidt et al., 2010). Tada et al. (2011) demonstrated that replacement of an H residue by an R residue in an EGFR-lytic hybrid peptide enhanced the ability of the peptide to bind to cancer cells, hence increasing its anticancer activity. Among the three peptides identified in this study, RDTQ is not amphiphilic. When tested on HeLa cells, RDTQ was more cytotoxic than AGAPGG and similarly cytotoxic as AERQ. Hence, our results suggest that, in contrast to peptide amphiphilicity, the presence of specific amino acid residues and/or their arrangement in a peptide sequence may be a more important determinant of cytotoxicity.

In this study, although AERQ and RDTQ were similarly cytotoxic to HeLa cells, RDTQ was less toxic than AERQ to the non-cancerous Hek293 cells. In other words, our results suggest that RDTQ was more selectively toxic to HeLa cells than AERQ. Based on this finding, RDTQ seems to be a more promising candidate for future development of selective anticancer therapeutics. Future research to unravel the cellular and molecular mechanisms that underlie the cytotoxicity of RDTQ and whether it also exerts toxicity against other cancer and non-cancer cell lines is of great interest.

According to the BIOPEP database (Minkiewicz et al., 2008), AGAPGG shares part of its sequence with 14 previously identified peptides, including those possessing antiamnestic (PG), angiotensin-converting enzyme (ACE) inhibitory (AP, GA, AG, GG, and PG), and dipeptidyl peptidase IV (DPP IV) inhibitory (AP, APG, GA, AG, GG, and PG) activities. AERQ shares partial homology with a previously identified DPP IV inhibitory dipeptide (AE), whereas RDTQ shares partial homology with an ACE- and DPP IV inhibitory dipeptide (TQ). In the light of this information, future investigations on the potential multifunctionality of AGAPGG, AERQ and RDTQ are warranted. If confirmed to have multiple bioactivities, the three *S. glaucum*-derived peptides

would have a wider range of applications in the future development of therapeutics.

5 Conclusions

In this study, three novel cytotoxic peptides, AGAPGG, AERQ and RDTQ, were successfully purified and identified from the papain hydrolysate of *S. glaucum*. The three peptides displayed relatively high cytotoxicity on HeLa cells, but low cytotoxicity on non-cancerous Hek293 cells. Significantly, the cytotoxic activities of AGAPGG, AERQ and RDTQ were 3.3-, 5.8- and 5.1-fold stronger than that of the anticancer drug 5FU. The findings of this study demonstrated that these peptides have remarkable potential for development as anticancer agents in future.

Acknowledgements

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Compliance with ethics guidelines

Yixian QUAH, Nor Ismaliza MOHD ISMAIL, Jillian Lean Sim OOI, Yang Amri AFFENDI, Fazilah ABD MANAN, Lai-Kuan TEH, Fai-Chu WONG and Tsun-Thai CHAI declare that they have no conflict of interest.

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List of electronic supplementary materials

Fig. S1
Fig. S2

中文概要

题　目：从软珊瑚 (*Sarcophyton glaucum*) 中纯化与鉴定新型细胞毒性肽

目　的：纯化和鉴定软珊瑚（*Sarcophyton glaucum*）蛋白水解物中的细胞毒性肽。

创新点：从软珊瑚的木瓜蛋白酶水解物中鉴定出三种新型肽序列：AGAPGG, AERQ 和 RDTQ（分别为 428.45,502.53 和 518.53Da）。此三种短肽含抗癌细胞的功能，EC_{50} 值各为 8.6, 4.9 和 5.6 mM，比抗癌药物 5-氟尿嘧啶功效高达 5.8 倍。当以各自的 EC_{50} 测试时，AGAPGG，AERQ 和 RDTQ 对非癌细胞 Hek293 显示仅 16, 25 和 11%的细胞毒性。

方　法：通过碱性蛋白酶，胰凝乳蛋白酶，木瓜蛋白酶和胰蛋白酶产生软珊瑚蛋白质水解物。采用 MTT 方法，研究水解物和分离的肽组分对 HeLa 癌细胞的毒性。跟据所鉴定的序列，以合成肽来确定对 HeLa 癌细胞的毒性。

结　论：综上所述，AERQ，AGAPGG 和 RDTQ 是有潜能成为肽类抗癌药物的。

关键词：抗癌治疗，活性肽，细胞毒性，HeLa细胞，*Sarcophyton glaucum*, 软珊瑚

Appendix D

Ethical Approval for Human Serum Stability Test Obtained from UTAR Scientific and Ethical Review Committee (U/SERC/40/2017)

UNIVERSITI TUNKU ABDUL RAHMAN
Wholly Owned by UTAR Education Foundation (Company No. 578227-M)

Re: U/SERC/40/2017

23 May 2017

Dr Chai Tsun Thai
Department of Chemical Science
Faculty of Science
Universiti Tunku Abdul Rahman
Jalan Universiti
Bandar Baru Barat
31900 Kampar
Perak

Dear Dr Chai,

Ethical Approval For Research Project/Protocol

We refer to your application dated 18 April 2017 which was circulated for the consideration of the UTAR Scientific and Ethical Review Committee (SERC). We are pleased to inform that your application for ethical approval for your research project involving human subjects has been approved by SERC.

The details of your research project are as follows:

Research Title	Reactions of Peptide in Human Blood
Investigator(s)	Dr Chai Tsun Thai Dr Teh Lai Kuan
Research Area	Science
Research Location	UTAR Kampar
No of Participants	15 participants
Research Costs	Self-funded
Approval Validity	23 May 2017 - 22 May 2018

The conduct of this research is subject to the following:

(1) The participants' informed consent be obtained prior to the commencement of the research.

(2) Confidentiality of participants' personal data must be maintained; and

(3) Compliance with procedures set out in related policies of UTAR such as the UTAR Research Ethics and Code of Conduct, Code of Practice for Research Involving Humans and other related policies/guidelines.

Address: Jalan Sg. Long, Bandar Sg. Long, Cheras, 43000 Kajang, Selangor D.E. **Postal Address:** P O Box 11384, 50744 Kuala Lumpur, Malaysia
Tel: (603) 9086 0288 **Fax:** (603) 9019 8868 **Homepage:** http://www.utar.edu.my

Should you collect personal data of participants in your study, please have the participants in the research signed the attached Personal Data Protection Statement for your records.

The University wishes you all the best in your research.

Thank you.

Yours sincerely,

Professor Ir Dr Lee Sze Wei
Chairman
UTAR Scientific and Ethical Review Committee

c.c Dean, Faculty of Science
Director, Institute of Postgraduate Studies and Research