IDENTIFICATION OF LIVER CANCER (HEP 3B) INHIBITING FRACTION FROM SOFT CORAL *SINULARIA* SPECIES

LIM LEONG RUI

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Lim Leong Rui

MZ1521001T

CERTIFICATION

NAME : **LIM LEONG RUI**

MATRICS NO. : **MZ1521001T**

TITLE : **IDENTIFICATION OF LIVER CANCER (HEP 3B) INHIBITING FRACTION FROM SOFT CORAL *SINULARIA* SPECIES**

DEGREE : **MASTER OF SCIENCE (BIOTECHNOLOGY)**

VIVA DATE : **07 NOVEMBER 2018**

CERTIFIED BY;

1. **MAIN SUPERVISOR**
   Dr. Ling Yee Soon Signature

2. **CO-SUPERVISOR**
   Assoc. Prof. Dr. Lee Ping Chin

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ABSTRACT

Soft corals are sessile marine invertebrates having vast number of promising anti-cancer compounds. Various studies have proven the effectiveness of soft coral extract on various cancer cell lines including liver cancer cell lines. Currently, high-throughput metabolomics is applied to replace conventional technique in drug discovery which is time-consuming and laborious. The current study aimed to determine the efficiency of a selected soft coral's fraction, *SC11-F5* against hepatocellular carcinoma Hep 3B and its cell death triggering activities using mass spectrometry based metabolomics. Sixteen soft corals were collected and extracted using modified Folch extraction protocol. Soft corals' crude extracts were screened against the hepatocellular carcinoma (Hep 3B) cells. The most effective crude extracts of *SC08* and *SC11* were further isolated using chromatography technique, producing eight fractions for each crude extract. These fractions were acquired and tested at different concentrations (25, 50 and 100 µg/mL) for 48 h. *SC11-F5* was found to be the most effective in inducing cell death in Hep 3B. The fraction was screened with MTT assay to evaluate cell proliferation. From the assay, the lowest inhibitory concentration at 50% of total cell survival (IC_{50}) was determined at 45.31 µg/mL at 72 h post-treatment. Following, cell metabolomics was applied at IC_{50} against *SC11-F5* and then underwent the extraction process. The cell crude extracts were subjected to high-throughput profiling using ultra-high performance liquid chromatography coupled with quadrupole time-of-flight. Chemometric analysis revealed over 50 metabolites were significantly (p-value < 0.05) perturbed and these metabolites were found involving in the programmed cell death via tandem mass spectral matching. Up-regulation of vitamin D and its derivatives increased the concentration of pro-apoptotic factor, calcium ions (Ca^{2+}) which caused direct programmed cell death. For triacylglycerols, their concentration was high in the treated Hep 3B cells that might cause distortion in plasma membrane. For diaclyglycerols, they act as second lipid messenger to activate proteins in cellular activities. On the other hand, perturbation of phospholipids such as phosphatidylcholine and phosphatidylethanolamine affected the rigidity of plasma membrane and regulation of signaling molecules synthesis and cell death. Up-regulation of ceramides caused programmed cell death by disrupting mitochondria while down-regulated ceramides were involved in synthesis of sphingomyelins in which sphingomyelins helped to recover plasma membrane. To conclude, *SC11-F5* has cytotoxicity effect towards Hep 3B cell line. In the future, a structural identification of the *SC11-F5* is needed.

ABSTRAK

IDENTIFICATION OF LIVER CANCER (HEP 3B) INHIBITING FRACTION FROM SOFT CORAL SINULARIA SPECIES

Karang lembut merupakan invertebrat laut sesil yang menunjukkan banyak sebatian antikanser yang berpotensi. Terdapat banyak kajian membuktikan keberkesanan sebatian karang lembut dalam pelbagai sel kanser termasuk sel kanser hati. Kini, penabiran celusan tinggi (HTS) digunakan untuk menggantikan teknik konvensional yang ambil masa lama dan banyak kerja. Kajian penyelidikan ini bertujuan untuk menentukan keberkesanan SC11-F5 terhadap titisan sel kanser hati, Hep 3B dan aktiviti kematian sel melalui jisim spektrometri berasaskan metabolomik. Enam belas karang lembut dikutip dan disari dengan menggunakan protokol penyarian Folch terubah. Sari karang lembut terkutip disaringkan terhadap titisan sel kanser Hep 3B. Sari yang paling berkesan (SC08 dan SC11) ditulenkan melalui teknik kromatografi. Lapan pecahan bagi setiap sari karang lembut dihasilkan dan pecahan-pecahan ini diuji terhadap sel kanser tersebut pada kepekatan 25, 50 dan 100 µg/mL dalam 48 jam. SC11-F5 ditentukan sebagai pecahan yang paling berkesan untuk mencetuskan kematian dalam sel kanser Hep 3B. Pecahan ini disaringkan terhadap titisan kanser sel Hep 3B melalui ujian MTT. 50% kepekatan rencatan (IC_{50}) yang terendah bagi SC11-F5 ditentukan pada 45.31 µg/mL. Selepas itu, metabolomik sel diaplikasikan selepas sel kanser tersebut diubat dengan IC_{50} SC11-F5 dan seterusnya disarikan. Analisis sari sel dijalankan melalui kromatografi cecair ultraprestasi terkupel dengan spectrometer jisim masa terbang (LC-QToF). Analisis kemometri menunjukkan lebih lima puluh metabolit penting (p-value < 0.05) dan sebahagian metabolit ditemui terlibat dalam kematian sel berprogram melalui pemadanan spektrum jisim tandem. Pengawalaturan tinggi vitamin D dan bahan terbitannya meningkatkan kepekatan ion Kalsium (Ca^{2+}) yang menyebabkan kematian sel terprogram. Bagi trigliserol, kepekatan lipid ini adalah tinggi dalam sel kanser Hep 3B yang dirawat. Kepekatan lipid yang tinggi ini menyebabkan herotan dalam membran plasma. Selain itu, usikan fosfolipid seperti fosfatidilkolina dan fosfatidiletanolamina menganggukan kerigidan membran plasma dan pengawalaturan molekul isyarat dan kematian sel. Pengawalaturan tinggi seramida menyebabkan kematian sel terprogram dengan menganggukan struktur mitokondria manakala pengawalaturan rendah seramida terlibat dalam sintesis sfingomielin di mana sfingomielin dapat membantu dalam pemulihan membran plasma. Kesimpulannya, SC11-F5 mempunyai kesan kesitotoksikan terhadap titisan sel kanser Hep 3B. Pada masa depan, identiti SC11F5 perlu dijelaskan melalui pengecaman struktur kompaun.

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LIST OF ABBREVIATIONS

µg/mL	Microgram per mililitre
EtoAC	Ethyl acetate
g	Gram
mg	miligram
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
UHPLC	Ultra High Performance Liquid Chromatography

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CHAPTER 1

INTRODUCTION

1.1 Natural Product

Livings organisms synthesize biologically complex organic compounds for self-defence and environmental adaptation. These biologically complex organic compounds are commonly known as natural products. Natural products are distinctive because of their various kinds of chemical structures and biological functions [Ji *et al.*, 2009]. This special property allows them being the main source of novel drug productions. In 2008, Newman's article mentioned that there were 70% of marketed drugs were originated from either natural products or their derivatives, while the remaining 30% were synthetic drugs [Newman, 2008]. This fact has showed the importance of natural products in pharmaceutics applications. Natural products are crucial as they are proven to be effective treatments for chronic diseases such as cancer.

Discovery of natural products can be sourced from both terrestrial and marine resources. Terrestrial-based natural products are always the dominant in the development of natural products discovery, but discovery of marine-based natural products starts on half a decade ago and it grows rapidly. Rapid growth of marine natural products discovery is not only due to the technology advancement or high throughput screening, but also due to the comparable effectiveness of the promising novel marine compounds. Marine natural products (MNPs) have a better biological performance compared to terrestrial natural products as they showed ten-fold effectiveness in anti-cancer potential [Munro *et al.*, 1999].

To-date, there are more than 30,000 marine bioactive compounds discovered [Blunt and Munro, 2007]. These marine compounds are mostly originated from marine invertebrates such as soft corals [Mehbub *et al.*, 2014]. Soft corals are parts of coelenterates and they are physically weak. Because of having weak physical appearance, they have to release toxic marine compounds for self-defense and harsh environment adaptation [Mander and Liu, 2010]. Ability of these chemical compounds has ignited the research study on the possibility of MNPs in anti-cancer treatment.

1.2 Liver Cancer

Liver cancer is the fifth most common cancer globally but it contributes to the second highest mortality rate in the world [Ferlay *et al.*, 2015]. This cancer mostly occurs in less developed countries and the most dominant type of the cancer is hepatocellular carcinoma (HCC). Factors of the liver cancer remain unknown but certain risk factors such as liver cirrhosis, hepatitis virus infection, alcohol abuse and non-alcoholic fatty liver disease can develop liver cancer.

Liver cancer can be treated through curative therapies, palliative therapies and best supportive care. These three main therapies are briefly explained as followings:

a) Curative therapies

Curative therapies are mostly applied in the first or second stage of the liver cancer. Liver resection, liver transplantation and local ablative therapy are kinds of curative therapies. Liver resection is recommended when a patient's liver functions normally and the tumor is less than 3 cm [Barone *et al.*, 2013]. Other than resection, liver transplantation is a common approach that could restore the cancerous injury and the coexisting liver diseases. However, liver donation source is the main obstacle in liver transplantation [Lin *et al.*, 2012]. Additionally, local ablative therapy such as radiofrequency ablation (RFA) increases electrode temperature to remove cancer tumours around the electrode [Parikh *et al.*, 2002]. However, HCC curative therapies can highly cause postoperative side effects such as obesity, blood loss and postoperative bile leak [Wrighton *et al.*, 2012].

b) Palliative therapies: Transarterial therapy and chemotherapy

Transarterial therapy is applied in the intermediate stage of HCC to extend the overall patients' survival. Throughout the palliative therapies, patients could extend their survival up to 20 months [Llovet and Bruix, 2003]. Chemotherapy is applied to treat the advanced stage of HCC. Currently, sorafenib tosylate is the only drug approved by the Food, Drug and Administration (FDA) and it is prescribed to the patients with the advanced HCC and extending patients' survival up to 10.7 months [Llovet *et al.*, 2008]. However, prolonged prescription of chemothrerapy drug can suppress cancer patients' bone marrow and cause gastrointestinal issues such as nausea, vomiting and diarrhoea [MacDonald, 2009].

c) Best supportive care

Best supportive care is applied in the advanced and the last stage of HCC. In these stages, the median overall survival is less than 4 months [Kumar and Panda, 2014]. Therefore, patients in these stages are given psychological support, pain and nutrition management to improve life quality of the patients [Kumar and Panda, 2014].

1.3 Metabolomics

Metabolomics is a comprehensive study of metabolome which consists of a complete set of small metabolites (< 1500 Da) within a biological sample [Kuehnbaum and Britz-McKibbin, 2013]. Although it is the end stage of the –omics sequel, it strongly interconnects with genomics, transcriptomics and proteomics. It is getting important in the current –omics research because it can bridges the gap in between genotype and phenotype of a living organism [Fiehn, 2002].

Metabolomics can be rapid and cost-effective by quantifying large sample size in a short time [Gomez-Casati *et al.*, 2013]. However, it is challenging because of the complexity and abundance difference of metabolites [Goodacre, 2005]. Furthermore, it is also facing the challenges on time effectiveness as it requires more time to extract and interpret raw metabolic data [Roesnner and Bowne, 2009].

Metabolomics is categorized into targeted and untargeted metabolomics. Targeted metabolomics is a technique whereby a set of experimental metabolites

data is quantified, compared and matched to a set of pre-calibrated standard metabolites data [Menni *et al.*, 2017]. On the other hand, untargeted metabolomics studies all the known and unknown metabolites in a biological sample without the need of pre-defined list of metabolites [Menni *et al.*, 2017]. There are common technologies applied in metabolomics, which are nuclear magnetic resonance (NMR), gas chromatography coupled with mass spectrometry (GC-MS) and liquid chromatography coupled with mass spectrometry (LC-MS). NMR can give better accuracy in determination of metabolites and their structures, while for GC-MS and LC-MS, they can determine molecular formula of potential metabolites of a biological sample.

Metabolomics is widely applied in plant, animal, food and medical research areas. In plant research field, metabolomics is applied to discover the plant biological system by predicting novel gene function, relating novel gene function with known biochemical process and elucidating food metabolites synthesis mechanism [Hirai *et al.*, 2007; Putri *et al.*, 2013a; Sawada *et al.*, 2009]. In animal study, metabolites involved in animal growth are well discovered through metabolomics [Hayashi *et al.*, 2011]. In food quality study, metabolomics is an alternative to replace human sensory evaluation by determining and analysing the quantity of taste-active compounds in a food sample [Putri *et al.*, 2013a].

Additionally, medical metabolomics is useful in toxicology, organ transplantation.. Toxicity of a drug can be determined by measuring and comparing metabolites changes between treated and untreated group of an interested biological sample [Jones and Cheung, 2007]. Meanwhile, after organ transplantation, organ rejection and dysfunction can be lessen as metabolomics could quantify biomarkers involved in impaired organ [Feng *et al.*, 2002].

1.4 Significance of Study

Palliative drugs are prescribed to the patients with the advanced stage (stage 3) of liver cancer to prolong their life span. However, side effects of the drugs are unfavourable such as hair loss, nausea, vomiting and diarrhoea [MacDonald, 2009]. Different patient from ethnics or races may response differently to the current

available chemotherapy drugs. Therefore, it is urgent to search for an effective drug which could exhibit lesser side effects to the patients.

Marine natural products are good sources for biomedical applications. They are mostly biologically active and have high relevant biological chemical space [Montaser and Luesch, 2011]. These properties make them be the alternative source for lead compound(s) synthesis. Furthermore, their effectiveness on cancer treatment have been studied and they are found to be useful in anticancer [Simmons *et al.*, 2005]. Therefore, lead compound(s) synthesized from marine resources could overcome the drawbacks of the chemotherapy drugs potentially.

1.5 Problem statement

Liver cancer is the second cancer causing death in the world. Various marine natural product studies had reported the treatment of soft corals' extract on the most commonly liver cancer cell lines used (Hep G2). However, treatment of soft corals' extract on another liver cancer cell lines (Hep 3B) was little studied. Hep 3B cells consisted of hepatitis B virus genome and in Malaysia, hepatitis B virus infection is the main risk factor developing liver cancer. Therefore, this research study aimed to identify potential soft coral's fraction on the liver cancer (Hep 3B) cell lines.

1.6 Objectives

1. To screen liver cancer (Hep 3B) inhibiting fraction from the potential soft coral.
2. To evaluate the effectiveness of the potential soft coral's fraction on inhibiting liver cancer (Hep 3B).
3. To elucidate the liver cancer cell inhibiting activities treated with the potential soft coral's fraction.

CHAPTER 2

LITERATURE REVIEW

2.1 Natural Products

Natural products are the biological active compounds synthesized by living organisms for the purpose of self-defence and environmental adaptation. Researches on natural products are getting more focused due to their proven biological activities such as anti-cancer, anti-inflammation, anti-oxidant and anti-bacterial. Generally, most of the natural products are originated from terrestrial resources but number of natural products discovered from marine resources is increasing over years. There were more than 30,000 compounds discovered from marine resources over the past two decades [Blunt and Munro, 2007].

Discovery of marine natural products (MNPs) begun since 20th century [Rangel and Falkenberg, 2015]. Number of marine natural products discovered is increasing because of advancement of high throughput screening technologies and improved laboratory techniques such as sample preparation, extraction and compounds isolation. Other than that, marine natural products study is getting popular because not only MNPs are found to inherit potential pharmaceutics properties such as anti-cancer, anti-viral and pain relief, but they were also found to have better bioactivity performance compared to terrestrial natural products [Munro *et al.*, 1999]. Table 2.1 showed the current approved or under clinical trials of the marine compounds.

Table 2.1: Discovery of marine-based compounds leads to the drug development in pharmaceutical industry.

Compound(s)	Marine resources	Species	Function	Citation
Cytosine arabinoside	Sponge	*Cryptotheca crypta*	Antitumor Antiviral	[Sneader, 2005]
Eribulin mesylate	Sponge	*Halichondria okadai*	Antitumor	[Towle *et al.*, 2001]
Ecteinascidin 743	Tunicate	*Ecteinascidia turbinate*	Antitumor	[Rinehart, 2000]
Ziconotide	Cone snail	*Conus magus*	Pain relieve	[McGivern, 2007]

2.2 Soft Corals

Marine natural products are mainly obtained from marine invertebrates such as sponges, coelenterates and tunicates. Coelenterates such as soft corals are the secondary sources after the sponges [Leal *et al.*, 2012] and they are focused in this review.

Soft corals are marine invertebrates under family Alcyoniidae [Rocha *et al.*, 2011]. They are coelenterates as they have tube-shaped body with single opening ringed with nematocysts [Stevenson, 2010]. They are also known as octocorals because they have polyps with eight tentacles and they mostly survive as colonies [Snyderman and Wiseman, 1996]. Although soft corals are physically weak, they have strong chemical defence. They have rich sources of terpenoids such as sesquiterpenes and diterpenes which help some of them for self-protection by inhibiting the growth of hard corals and other marine organisms [Atta-ur-Rahman, 2000].

The development of soft corals compounds discovery increases over the years. In 2012, the number of new marine natural products in family Alcyoniidae increased about 66.9% from 1990s to 2000s [Leal *et al.*, 2012]. Compounds exerted from soft corals had the ability to prevent invasive growth of other competitive marine organisms nearing them [Wang *et al.*, 2008]. This ability has

therefore greatly leading to the discovery of the pharmaceutical potential of soft corals in various researches applications such as biomedical and drug discovery. To date, various studies had discovered the effectiveness of soft corals against the cancer cells [Huang *et al.*, 2017; Lei *et al.*, 2014; Lin *et al.*, 2009; Tung *et al.*, 2010].

Researches on discovery of soft coral compounds and their cytotoxicity against the various kinds of cancer cells were covered from 2007 to 2017. In 2009, flexilarin D, a cembranoid-type compound was extracted from *Sinularia flexibilis* in Taiwan [Lin *et al.*, 2009]. The compound was cytotoxic against the hepatocellular carcinoma cell, Hep2 cell at IC_{50} 0.07 µg/mL and the concentration was comparable to the concentration of the control, mitomycin C (IC_{50} 0.06 µg/mL).

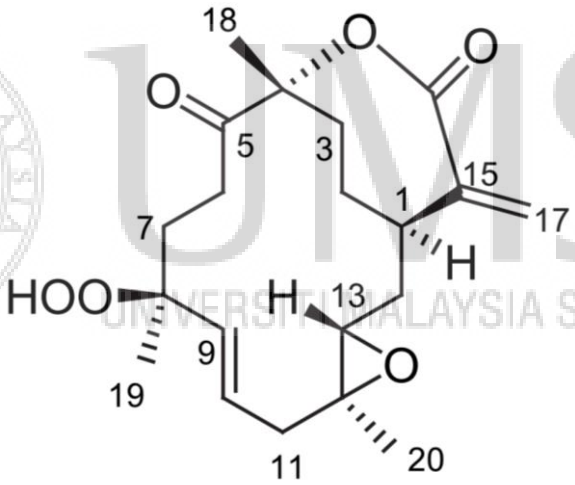

Figure 2.1 : Flexilarin D.
Source : [Lin *et al.*, 2009].

In 2010, six compounds were extracted from *Lobophytum* sp. in Vietnam [Tung *et al.*, 2010]. Among six compounds, the (1S,2S,3E,7E,11E)-3,7,11,15-cembratetraen-17,2-olide was significantly inhibiting the proliferation of the lung cancer cell (A-549) and the colorectal cancer cell (HT-29) at 5.1 µM and IC_{50} =1.8 µM respectively. Both concentrations were lower than the concentration of the control, mitoxantrone which is IC_{50} = 6.1 µM for A-549 and IC_{50} = 6.5 µM for HT-29.

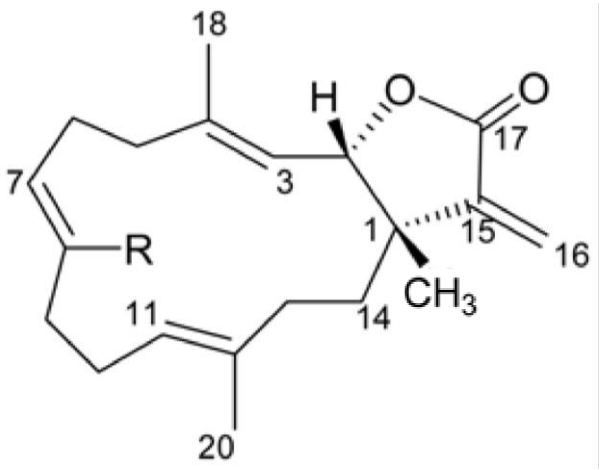

Figure 2.2 : (1S,2S,3E,7E,11E)-3,7,11,15-cembratetraen-17,2-olide.
Source : [Tung *et al.*, 2010].

In 2014, eight cembranoid-type compounds were extracted from the soft coral *Sinularia* sp [Lei *et al.*, 2014]. Sinularoid A was shown to have potent cytotoxicity against hepatocellular carcinoma multidrug-resistance cell line, HepG2/ADM at IC_{50} = 9.70±1.77 μM. The IC_{50} of the compound was almost four fold lower than the concentration of the doxorubicin which is the positive control used to treat the cell line.

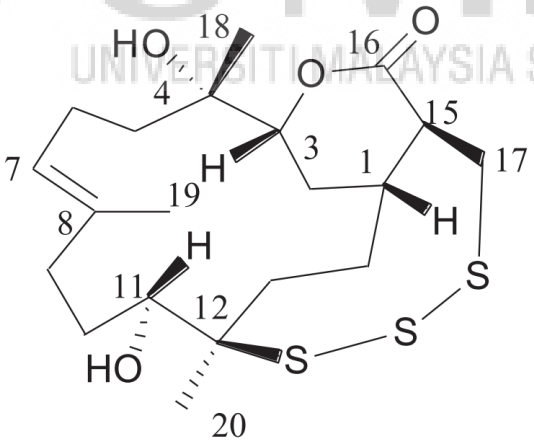

Figure 2.3 : Sinularoid A.
Source : [Lei *et al.*, 2014].