Identification of Novel Compounds in *Undaria pinnatifida* with Anti-Cancer Potential

by

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Abbreviations

Ara-A: Vidarabine

Ara-C: Cytrarbine

AUT: Auckland University of Technology

Bax: Bcl-2-associated X protein

Bcl-2: B-cell lymphoma 2

Carbon dioxide: CO_2

CV: Coefficient Variation

DoC: Department of Conservation

FBS: Fetal bovine serum

HCl: Hydrochloric acid

HPLC: High performance liquid chromatograph

hr: Hour

HTS: High-Throughput Screening

IC_{50}: the concentration (μM) of the experimental compounds generating a 50% inhibition in cell growth

IR: Inhibition rate

Log: Logarithm

MAE: Microwave-Assisted Extraction

MAF: Ministry of Agriculture and Forestry

MFish: Ministry of Fisheries

MPI: Ministry for Primary Industries

mg/mL: milligram/millilitre

mM: millimoles per litre

MTT: [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide]

NaOH: Sodium hydroxide

PARP: Poly-ADP-ribose polymerase

PBS: Preparation of Phosphate Buffered Saline

PI3K: Phosphatidylinositol 3-kinase

PLE: Pressurized Liquid Extraction

SFE: Supercritical Fluid Extraction

µM: micromoles per litre

***U. pinnatifida*:** *Undaria pinnatifida*

US: The Unite State of America

UV: Ultraviolet

VIS: Visible

v/v: Volume/ volume

WHO: World Health Organization

Abstract

In New Zealand, the rate of registered cancer cases increased significantly in recent years especially for breast, melanoma, colorectal cancer and lung cancer (Ministry of Health, 2008b). The success rate of cancer chemotherapy is generally low mainly due to cytotoxic side effects and multi-drug resistance of current anti-cancer drugs (Szakács, Paterson, Ludwig, Booth-Genthe, & Gottesman, 2006). Two major components of *U. pinnatifida*, fucoxanthin and fucoidan, have recently been found to induce apoptosis in various cancer cell lines (Nishibori, Itoh, Kashiwagi, Arimochi, & Morita, 2012). The primary objective of this study was to investigate whether fucoxanthin could inhibit proliferation of different types of cancer cells in a time- and concentration-dependant pattern. The secondary objective of this study was to determine other potential anti-cancer compounds from both New Zealand and Japanese *U. pinnatifida*. In addition, a novel extraction method has been developed by Japanese scientists to isolate fucoxanthin and fucoidan by supercritical carbon dioxide and subcritical water in a single process. The third purpose was to evaluate biological activities of these two major components isolated by using the novel extraction method. Based on the particular statistical reported several types of human cancer cell lines were used in this study including lung carcinoma A549, NCI-H522, colon adenocarcinoma WiDr, Lovo, hepatocellular carcinoma Hep G2, breast adenocarcinoma MCF-7, malignant melanoma Malme-3M, cervix squamous carcinoma SiHa, and neuroblastoma SK-N-SH. Anti-proliferative effects were determined by 24 hr-72 hr MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assays.

Pure fucoxanthin showed anti-proliferative effects in all types of cancer cell lines in dose- and time- dependent manners. The ranking of anti-proliferative sensitivity to fucoxanthin is in the order of SiHa > MCF-7, Lovo, NCI-H522 > Hep G2, A549, WiDr, SK-N-SH, and Malme-3M.

Similarly, anti-proliferative effects of fucoxanthin extracted from New Zealand seeweeds were found in all types of cancer cell lines in dose- and time- dependent manners. These three fucoxanthin extracts (purity of fucoxanthin: 0.2%, 43.5% and 60.77%) were achieved through a series of fucoxanthin isolation and purification procedures. Compared with anti-proliferative IC_{50} of pure fucoxanthin standards, the

significant anti-proliferative effects of fucoxanthin extracts were found in some cancer cell lines including WiDr, Lovo, and NCI-H522.

Similar results were also found from the determination of anti-proliferative effects fucoxanthin extracts by the novel extraction method. However, SiHa and Malme-3M were special cases, suggesting some of compounds might effectively inhibit growth of those cancer cells. In comparison to other published studies, similar anti-proliferative effects from Japanese fucoidan fraction were found in dose- and time dependence, but its anti-proliferative effects were much lower.

In conclusion, fucoxanthin as a type of marine carotenoid possessed effectively anti-proliferative effects to multiple types of cancer cell lines. Thus, fucoxanthin can be the important phytochemical with chemopreventive effects. Furthermore, some of novel compounds with potential anti-cancer effects might be contained in New Zealand and Japanese *U. pinnatifida*. This suggested *U. pinnatifida* as part of a human diet could possibly decrease the risk of cancer especially for human cervix, colon and lung cancer. Finally, crude fucoxanthin was successfully isolated by supercritical carbon dioxide from novel extraction method. However, fucoidan fraction which possessed biological anti-cancer activity might not be obtained from extract using subcritical water.

Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no materials previously published or written by another person (except where explicitly in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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Signature: 

Date: **24/10/2012**

Chapter 1 Introduction & Literature Review

1. Introduction & Literature Review

1.1 Overview

Cancer is one of major health problem in the world. Before 2005, cancer was ranked as the second serious disease behind the cardiovascular problem to be the most common cause of death in the world (Sener & Grey, 2005). However, the global cancer statistics in 2011 reported cancer cases in the world have replaced ischemic heart disease as the overall leading cause of death in the world especially in economically developed countries (Jemal et al., 2011). According to data from the World Health Organization (WHO) in April 2003, the global cancer rates could significantly increase by 50% to 15 million new cases in 2020. In recent report from WHO in 2008, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred. However, WHO cancer statistical reported in 2011 only 30% of these diagnosed with cancer could be cured or prevented after clinical treatment.

In NZ, the rate (per 100,000 people) of registered cancer cases (including all types of cancer diseases) included both male and female and both adult and children recorded from 1956 to 2016 is not significantly changed. The NZ Ministry of Health in 2010 statistically reported the total number of registered cancer cases in all NZ adult was increased especially in adult age of over 75, and their registered cancer rate was increased as twice as before from 1956 to 2012. For NZ childhood, the registered cancer cases in both male and female are unchanged from 1956 to 2012 (Ministry of Health, 2010). According to data report from Ministry of Health in 2008, breast cancer, melanoma, colorectal cancer and lung cancer are the most common causes of death in NZ population (Ministry of Health, 2008b). In order to solve these serious health problems, cytotoxic chemotherapy is one of the most common methods. However, current cytotoxic chemotherapies often cause serious adverse side effects to patients after treatment. Therefore, it is vitally important to discover and develop new anti-cancer agents.

The marine natural environment occupies approximate 70% of the earth's surface, and it represents 95% of the biosphere (William, 2004). Thus, the marine natural products can possibly contains a rich source of novel chemical compounds, and all these new chemical compounds can be potentially used in many areas of human life, such as pharmaceutical, nutritional supplements, cosmetics, agrichemicals and enzymes respectively. Since the 1960s, some chemicals have been successfully isolated that have anti-cancer potentials in cancer pharmacology. These chemicals were purified as new anti-cancer agents to be involved in clinical trials or cancer treatment. For example, the chemical components ara-A (vidarabine) and ara-C (cytrarbine) isolated from marine sponges were developed for chemical synthesis in the 1960s, and these synthetic chemical compounds were then developed to be used in clinical cancer treatment (Glaser & Mayer, 2009). In addition, other examples include Ecteinascidin 743 (isolated from *Ecteinascidia turbinate*) in clinical phase II (Taamma et al., 2001), Bryostatin 1 (isolated from *Bugula neritina*) in clinical phase II (Varterasian et al., 2001), KRN-7000 (Agelasphin derivative) (isolated from *Agelas mauritianus*) in clinical phase I (Chaulagain, Postema, Valeriote, & Pietraszkewicz, 2004), and Vitilevuamide (isolated from *Didemnin cucliferum / Polysyncration lithostrotum*) in pre-clinical phase (Edler, Fernandez, Lassota, Ireland, & Barrows, 2002).

U. pinnatifida is a type of brown seaweed that was recently allowed to be harvested in NZ. This type of brown seaweed contains a large amount of nutritive components. As food, *U. pinnatifida* forms an important part of diet in some Asian countries such as Japan, Korea, and China. As a medicinal treatment, *U. pinnatifida* is source of biologically active phytochemicals. There are two important bioactive compounds which were already isolated from *U. pinnatifida*. Fucoxanthin is a pigment compound responsible for the brown colour in brown seaweeds. Several research studies have already defined its anti-proliferative effects to several types of cancer cell lines including leukemia HL-60 (Peng, Yuan, Wu, & Wang, 2011), colon cancer (Caco-2, WiDr, HT-29 and DLD-1) (S. K. Das et al., 2005; Hosokawa et al., 2004; Sugawara, Baskaran, Tsuzuki, & Nagao, 2002), lung cancer (A549, NSCLC-N6 and SRA 01/04) (Hazra et al., 2007; D. Moreau et al., 2006), prostate cancer (PC-3, DU 145, and LNCaP) (Eiichi Kotake-Nara, Asai, & Nagao, 2005; Satomi, 2012; Yoshiko & Hoyoko, 2007), liver cancer (Hep G2) (Swadesh K. Das, Takashi Hashimoto, & Kazuki Kanazawa, 2008), gastric adenocarcinoma (MGC-803) (R.-x. Yu, X.-m. Hu, S.-q. Xu, Z.-j. Jiang, & W. Yang, 2011), and neuroblastoma cancer (GOTO) (Okuzumi et al., 1990). Another

isolated bioactive compound from *U. pinnatifida* was fucoidan. In fact, the structure of fucoidan is different between species (B. Li, Lu, Wei, & Zhao, 2008). Fucoidan isolated from *U. pinnatifida*, was determined to have anti-cancer potential in breast carcinoma (MCF-7) (Yamasaki-Miyamoto, Yamasaki, Tachibana, & Yamada, 2009), human epithelial carcinoma (A549) (H.-J. Boo et al., 2011), and human hepatocellular carcinoma (Hep G2) (Nagamine et al., 2009). Besides these major components that were already found in *U. pinnatifida*, the possibility that other minor components in *U. pinnatifida* may also be involved in anti-proliferative effects cannot be completely ruled out.

Extraction methods are the most important to identify the desirable bioactive compounds from marine natural products. The most common method in many pharmaceutical industries is the high-throughput screening or a pilot-scale screening. This type of extraction method largely relies on analytic chemistry to obtain desirable bioactive extraction with high-performance liquid chromatography purification. Another concern of extraction method was developed to rely on microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE).

In general, the main objective of this study was to undertake a pilot-scale screen of anti-proliferative effects of fucoxanthin by using several cancer cell lines. These cancer cell lines were chosen based on the incidence of cancer in NZ including lung cancer, breast cancer, colon cancer and melanoma cancer. The second objective was to identify potentially novel anti-cancer compounds from whole *U. pinnatifida* extract. In addition, it is necessary to compare the two extraction methods which are mostly used in pharmaceutical industries.

1.2 Cancer and Marine Products

1.2.1 What is Cancer?

Cancer is a collective name that describes a devastating human disease of which there are many types and varying degrees of severity. Cancer is generated by a series of cumulative changes, both genetic and epigenetic occurring in a normal cell (Prendergast & Jaffee, 2007). Over time, these abnormal cells continually grow without control and finally generate a cancerous growth known as a tumour. These tumours could continuously grow into bigger size and can interfere with the digestive, nervous, and circulatory systems. In some cases, they also release hormones that could change the

normal bodily functions. Tumours can either be benign or malignant. Benign tumours do not spread to other parts of the body, so that they are rarely life-threatening. On the other hand, malignant neoplasm is cancer cells where cell survival, proliferation and differentiation are uncontrollable. Malignant neoplasm can invade adjacent tissue and destroy its function through blood circulation or lymph (Illemann et al., 2006). There are a wide range of potential causes that encourage the development of cancer diseases such as aging, exposure to carcinogens, genetic predisposition, infection with certain viruses or bacteria, poor diet, and weakened immune system.

1.2.2 A Serious Health Problem of Cancer in the World & in New Zealand

Cancer is one of the major growing health problems around the world, particularly with the steady rise in life expectancy, increasing urbanisation and the subsequent changes in environmental conditions, including lifestyle (Surh, 2003). It accounts for approximately 20% of current fatality statistics in the world, making it one of the leading causes of death. According to the statistic report by the World Health Organization (WHO) in 2011, more than 10 million cancer cases per year were reported worldwide with increasing rate at 3% per annum over the last 20 years. There were approximately 20 million people alive with cancer in 2002, also the number of cancer patients will be expected to increase to 30 million by 2020. In 2003, about 1,300,000 new cancer cases were diagnosed, and approximately 550,000 people died from cancer in USA (Beyer & Rushton, 2009). In 2009, it was estimated that more than 1,500,00 new cancer cases were diagnosed, and approximately 600,000 people died from cancer in USA (American Cancer Society, 2012). Therefore, based on these reports, cancer was globally identified as one of the most common health problem to cause of death in the world.

1.2.3 Statistical Data about Cancer in New Zealand

In NZ, there were approximately 7,600 people who died from cancer in 1999 which accounted for 27.2% of the total cause of deaths that year (Ranta et al., 2010). Just after a year, 16,000 cancer cases were registered for the year 2000 (Ministry of Health, 2003). The number of people who suffered in cancer has gradually increased throughout the years. In 2002, about 9,500 new cases were diagnosed with different types of cancer in NZ. There were a more than 20,317 cancer cases reported in NZ in 2008. These data

show that cancer is the leading cause of death in NZ, with nearly 1 in every 3 deaths due to cancer (Ministry of Health, 2008a). In a recent study, the Ministry of Health in NZ reported 37.5% of cancer cases (22,000 people registered in cancer) increased in the NZ population in 2011 (Cox & Sneyd, 2011), but also the increased growth rate was expected about 4% per annum on average over the next year (Ministry of Health, 2005).

The NZ Ministry of Health reported in 2008 that breast cancer, melanoma and lung cancer were the serious types of cancer cases, and they were the most common causes of death in registered cancer cases for Maori or non-Maori population in NZ. Colorectal cancer was a new type of cancer registered in NZ, and this was another serious health reason to cause of death in NZ. In the record from Ministry of Health, 2,716 of colorectal cancer cases were reported with 1,222 death cases, and there were 15.3% of all deaths from all cancer cases in 2005 (Ministry of Health, 2006).

Table 1: Rankings of major cancers statistic count in 2004/2008 and projected 2014/18 in NZ. (Source: Ministry of health, 2010).

Rankings of Major Types of Cancer from 2004/2008 and projected 2014/18		
	Male	**Female**
Rank	**Registrations**	**Registrations**
1	Prostate	Endometrium
2	Liver	Kidney
3	Oesophageal	Lung
4	Non-Hodgkin Lymphoma (NHL)	Melanoma
5	Melanoma	Breast
6	Leukaemia	Oesophageal
7	Brain	Pancreatic
8	Colorectal	Colorectal
9	Kidney	Bladder
10	Lung	Cervix

According to the statistical data from Ministry of Health in 2011 reported (Table 1) in these ten years lung cancer, colorectal cancer, melanoma cancer were the top ten types of cancer which NZ people suffered from, and breast cancer and cervix cancer are particularly important cancer diseases in NZ female. In addition, these types of cancer case will be expected to be the major percentage of registered cancer cases in NZ in the following years.

1.2.4 Chemotherapy in Clinical Cancer Treatment

Due to the high mortality rate of cancer, continuous cancer researches have been performed in several countries such as the USA, the United Kingdom, Japan, Germany, and China to contribute to this serious worldwide medical disease problem (Cea-Soriano, Wallander, & García Rodríguez, 2012; Ohsumi & Shimozuma, 2012; Reade & Elit, 2012; Retz et al., 2008; Wu & Zhou, 2012). Chemotherapy primarily refers to the treatment of cancer, it started in the treatment of cancer by chemicals since 1940s (Papac, 2001), and the chemical treatment can destroy tumour cells, so that this can be called as anti-cancer or anti-neoplastic drugs. The most common chemotherapy is to destroy cells due to uncontrolled tumour cells with rapid dividing property, so that tumour cell can be a higher possibility to be harmed by chemotherapy drugs than normal cell or tissue, this can be thought as conventional cytotoxic chemotherapy.

The conventional cytotoxic chemotherapy agents include alkylating agents, platinum drugs, anti-metabolites, topoiosmerase inhibitors, and anti-microtubule drugs. Based on analysis of the cell cycle, mechanism for all cytotoxic drugs except anti-microtubule drugs are directly involved in inhibition of DNA synthesis and arrest synthesis (S) phase of cell cycle to promote cell apoptosis. The anti-microtubule drugs can promote its cytotoxic effect in mitotic (M) phase of the cell cycle. Therefore, the conventional cytotoxic anti-cancer agents can mainly focus on the stage of dividing cells, and DNA synthesis during replication stage. In previous years, cancer research efforts had been concentrated in discovering and the development of an effective anti-cancer drug (or anti-neoplastic drugs) such as vinca alkaloids, bleomycin, anthracyclines, and mitomycin respectively. However, this type of chemotherapy is toxic to the cancerous cells but also to normal cells. Furthermore, the chemotherapy can generate a myriad of side-effects and even cause death (Vander Els & Stover, 2004). Due to the seriously unpleasant side effects, acceptability of this treatment is quite low and treatment usually needs to be discontinued on many occasions. In addition, tumour resistance such as multidrug resistance (MDR), P-glycoprotein can be developed on tumour cells, and it can render the anti-cancer drug ineffective against the tumours that were initially effective (Szakács et al., 2006). Therefore, cancer is a deadful human disease, and its treatments do not have potent medicine as the currently available drugs are causing side effects in some instances. Due to these several reasons above, there is a constant demand for the invention of more potent therapeutic agents with novel mechanisms of action.

1.3 Marine Products for Treatment of Cancer

1.3.1 Marine Natural Products as the Drug Leads

There is an increasing demand for new pharmaceuticals to be developed in order to treat various cancers which are on the rise. The main reason to develop novel drugs for contemporary diseases is due to the increasing levels of drug resistance in current therapies, and the most important in novel drugs development is to define some new mechanisms of action used in current therapies. Nature has always been our main source of food, protection, transportation and remedies (Cragg, Newman, & Snader, 1997). Hence, nature can be an attractive source of new therapeutic candidate compounds as a tremendous chemical diversity is found in millions of species of plants, animals, marine organisms and microorganisms (Da Rocha, Lopes, & Schwartsmann, 2001). In fact, natural products or their derivatives have played an important role in the chemotherapy of a variety of illness throughout history (B. Das & Satyalakshmi, 2012). This is because natural products usually contain a wide range of source of medicines, and in current research natural products also contribute to an important role in the discovery of novel drug leads. Drugs derived from natural resources have been obtained from a variety of source including terrestrial plants and microorganisms, and marine organisms (Chin, Balunas, Chai, & Kinghorn, 2006). These novel compounds from natural plants and marine invertebrate contain a wide range of biological and ecological reasons to contribute to new drugs development.

The marine ecosystem occupies approximate 70% of the earth's surface, and it represents 95% of the biosphere (William, 2004). Thus, the marine organisms can be a rich source of novel chemical compounds, and this is known as the marine natural products. The interest in understanding the function of marine ecosystems has been accelerated in recent years with growing recognition of their importance for human life. The ocean represents an abundant resource of novel compounds with great potential for pharmaceutical, nutritional supplements, cosmetics, agrichemicals and enzymes which have a strong potential market value (Carté, 1996). There are so many structurally and pharmacologically important substances contained in marine environment, and in recent years these have been isolated with novel anti-microbial, anti-cancer, anti-tumour and anti-inflammatory properties.

1.3.2 Historical Perspective and Current Status

The earliest record in history showed the Chinese pharmacopoeia pointed out seaweed-based recipes was already used in clinical treatment for a number of disorders such as pain, abscesses, menstrual difficulties and cancer (Bessey, 1976). The medicinal effects from some marine plants was also found, so that seaweed remedies were also used to treat patients by the San Blas Indians in Panama, and Romans attributed medicinal effects to some marine animals (Jimeno, Faircloth, Sousa-Faro, Scheuer, & Rinehart, 2004). However, the marine seaweed species was still not taken to focus on as the potential drugs in clinical treatment in Western countries.

The first marine drug to be discoved dates back to the 1950s. The Werner Bergmann published reports about unusual *arabino*- and *ribo*-pentosyl nucleosides obtained from marine sponges collected in Florida, USA (Molinski, Dalisay, Lievens, & Saludes, 2009). The chemical compounds, ara-A (vidarabine) and ara-C (cytrarbine) were eventually developed for chemical synthesis in 1960s. Ara-C is a basic component in the clinical treatment of acute myeloid leukaemia. In the following years, the research for novel deoxycytidine analogues led to the identification and development of gemcitabine, and the results from clinical trials indicated that it play an important role in palliative therapy for pancreatic and non-small cell lung cancers (Jimeno et al., 2004). This clinical information suggested that marine ecosystem can potentially contribute to cancer therapy. At the beginning of the 1970s, the pharmacology of marine natural products was combined to the basic scientific research in chemistry for a large of effects in marine drug development. A second drug isolated was from a tropical sea-squirt and was known to be an anti-tumour agent. This agent is trabectedin (Yondelis/ecteinascidin-743/ET-743), and was approved by the European Union in October 2007 for the treatment of soft-tissue sarcoma (Schmitt et al., 2010). Based on these drug discoveries from marine environment, scientists began to systematically focus on the oceans for discovery of new novel drugs such as anti-cancer agents in the middle of the 20th century. In recent years, research into the pharmacological properties of marine natural products has contributed to the discovery of many active agents considered worthy of clinical application. The marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural/chemical features not found in terrestrial natural products (Carté, 1996). It has been reported in 2004 there were approximately 7,000 marine natural products which have been isolated from marine sources; 25 percent of which were from algae, 33 percent were from

sponges, 18 percent were from coelenterates, and 24 percent were from representatives of other invertebrate phyla (Carté, 1996). In 2012, there were more than 10,000 bioactive molecules discovered and isolated from marine systems, and hundreds of new compounds are still being discovered every year (Hussain Md, Fareed, Ansari, & Khan, 2012).

In anti-cancer drug discovery, the type of chemicals in marine environment can be a defense mechanism against predators, competitors and fouling organisms (Paul & Ritson-Williams, 2008). These compounds could be potentially useful candidates used as chemotherapeutic agents (Table 2). Commonly these compounds have been developed as derivatives or synthetic mimics in pharmaceutical industries.

Table 2: Anti-cancer chemotherapy agent candidates from marine natural products, and their current status in pre-clinical or clinical trials

Compounds	**Source Organism**	**Chemical Class**	**Molecular Target**	**Trial**	**Reference**
Ecteinascidin 743	*Ecteinascidia turbinate* (tunicate; bacterial source)	Tetrahydroisoquinolone alkaloid	Tubulin	Phase II	(Taamma et al., 2001)
Dolastatin 10	*Dolabellaauricularia / Symploca* sp. (Mollusc / cyanobacterium)	Linear peptide	Tubulin	Phase II	(Von Mehren et al., 2004)
Bryostatin 1	*Bugulaneritina* (bryozoan)	Macrocyclic lactone	PKC	Phase II	(Varterasian et al., 2001)
Synthadotin	*Dolabellaauricularia / Symploca* sp. (analogue)	Linear peptide	Tubulin	Phase II	(Attard, Greystoke, Kaye, & De Bono, 2006)
Kahalalide F	*Elysiarefescens / Bryopsis* sp. (mollusc / green alga)	Cyclic depsipeptide	Lysosomes / erbB pathway	Phase II	(Suárez et al., 2003)
HTI-286 (Heminasterlin derivative)	*Cymbastella* sp. (synthetic analogue of sponge metabolite)	Linear peptide	Tubulin	Phase I	(Ireland et al., 2003)
LAF-389 (Bengamide B derivative)	*Jaspisdigonoxea* (sponge, synthetic)	ε-Lactam peptide derivative	Methionine aminopeptidase	Phase I	(Sashidhara, White, & Crews, 2009)
KRN-7000 (Agelasphin derivative)	*Agelasmauritianus*(sponge, synthetic)	α-Galacosylceramide	Vα24 + NKT cell activation	Phase I	(Chaulagain et al., 2004)
Curacin A	*Lyngbyamajuscula* (cyanobacterium)	Thiazole lipid	Tubulin	Preclinical	(Muir, Pattenden, & Ye, 2002)
Lamellarin D	*Lamellaria* sp. (mollusc and various soft corals)	Pyrrole alkaloid	Topoisomerase I / Mitochondria	Preclinical	(Ballot et al., 2009)
Dictyodendrins	*Dictyodedrillaverongiformis* (sponge)	Pyrrolocarbazole derivatives	Telomerase	Preclinical	(Buchgraber et al., 2009)
ES-285 (Spisulosine)	*Mactromerispolynyma* (mollusc)	Alkylamino alcohol	Pho (GTP - bp)	Preclinical	(Massard et al., 2012)

1.3.3 Current Technique to Screen Potential Bioactive Compounds from Marine Natural Products

The most common method to screen potential bioactive compounds is high-throughput screening or a pilot-scale screening. This is about a type of mechanism-based *in vitro* assays. These assays are amenable to large scales of operation and the concept of high-throughput screening rapidly became the paradigm for discovery (Bugni, Harper, McCulloch, Reppart, & Ireland, 2008). High-throughput screening or a pilot-scale screening can be a common method for scientific experimentation; especially this method can be used to determine biologically or chemically active compounds in drug discovery from marine natural products. The biological or pharmacological determination from high-throughput screening can be scientifically achieved by combination of biomedical and chemical fields. The results from screening can be the good evidence for drug design or understanding the interaction and biomedical process. In high-throughput screening or pilot-scale screening, the critical process is to distinguish between undesirable or desirable compounds in marine natural product extracts. This process allows the identification and focuses on the most bioactive compounds from marine natural products, and this identification for bioactive compounds also contributes to drug design in further. At the beginning of screening process, the bioactive marine natural compounds in crude natural product extracts requires some form of feedback from the bioassay, and the result can be essential to detect the biological activities (K. Duarte, Rocha-Santos, Freitas, & Duarte, 2012). Then, a series of extraction techniques were applied to identify or focus on the most bioactive compounds from marine natural products. Sometimes, this identification for bioactive compounds also contributes to drug design in further.

Extraction techniques contribute to the removal impurities from marine natural products, and eventually identify and obtain the most desirable bioactive extracts or fraction from marine natural products. There are two common extraction techniques which are mostly used in pharmaceutical industries. The first and the most common methods are based on analytical chemistry. This type of analytical chemistry methods contains a series of extraction step to identify some potential novel compounds from marine natural products. Depending on specific characteristics of the extracts, a common strategy has been to collect fractions from the high-performance liquid chromatography (HPLC) separation in deep-dish microtiter plates or tubes and then

resubmit the individual fractions to the original assay to determine and compare the biological activities to crude marine natural products (Van Elswijk & Irth, 2002). Finally, this purified extraction could be re-submitted to the original bioassay to determine and compare the biological activities to crude marine natural products. The extraction methods are the most important process to contribute to eventual process for the novel bioactive compound isolation and purification. The most common extraction method is achieved by analytic chemistry methods with a large amount of organic solvent used.

In order to reduce or minimize the use of organic solvents and improve the extraction process, another type of extraction method concern for marine plants was already developed such as microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE). Commonly, these extraction methods have already been used in many chemical & pharmaceutical industries to extract bioactive compounds from marine plants. The MAE extraction method depends on microwave energy, and the energy contribute to solution heating in order to significantly reduce the extraction time. Usually the time for extraction is less than 30 minutes (Liu et al., 2008). Other extraction methods are about the use of carbon dioxide and some form of modifiers instead of organic solvents to extract bioactive compounds from marine products, and this can remarkably reduce the consumption of organic solvents. By application of these methods, the number of compounds that were isolated from diversity of marine life dramatically increased, and they were already entered into preclinical or clinical trials in the past decade (Guodong, Urooj, & Birendra, 2009). It is useful to consider the evolution of the field of marine natural products drug discovery in this context as it may help to identify future directions which will be even more successful (Simmons, Andrianasolo, McPhail, Flatt, & Gerwick, 2005).

1.4 Seaweed and *U. pinnatifida*

1.4.1 Algae and Seaweeds

Algae are a large and diverse group of simple marine organisms. This group of organisms is widely distributed in the ocean and can be found in unicellular, colonial, filamentous or multicellular forms. Marine algae grow in seawater, thus they have different chemical composition than plants that grow on land. Marine algae are the origin of the ocean’s food chain and mainly contribute to marine taxonomy and ecology

in sea. Seaweed is the collective name for marine macroalgae which are the plants of the sea (Venugopal, 2008).

This group of algae "seaweed" can be found in the sea and brackish water. In addition, the seaweed can be found along the edge of the ocean. Seaweed is a diverse group of photosynthetic protists, and they use sunlight as a source of energy to convert water and carbon dioxide into carbohydrates. There are approximately 20,000 species of seaweeds in the world, in recent years the cultivation techniques of seaweed are standardised, perfected and made economically favourable. There are five most cultivated seaweed in the world including *Laminaria, Porphyra, Undaria, Eucheuma and Gracilaria* (Phang, 2010). China, Korea, Japan, Philippines, Indonesia, Chile, Vietnam, Russia and Italy are the nine countries where seaweed is mainly produced to the world (Marine Products for Healthcare, 2008).

1.4.2 Classification of Seaweed

There are approximately 36,000 species of algae described to date. Each species of algae is classified based on size, colour, morphology, polysaccharides, type of chlorophyll, and cell wall composition. There are three types of seaweeds (Table 3), brown algae (Phaeophyceae), red algae (Rhodophyceae), and green algae (Clorophyceae) (Jiménez-Escrig & Sánchez-Muniz, 2000).

Table 3: The classification of the marine algae (Source: Helen Fitton, 2003)

Classes of Marine Algae	Examples of Edible Seaweed	Latin Binomials
Brown	Wakame; mekabu	*U. pinnatifida*
	Kelp or kombu	Laminaria species
Red	Nori (Japan) or Laver bread (Wales)	Porphyra species
Green	Sea lettuce	Ulva and Enteromorpha

Brown seaweeds are a large group of mostly marine multicellular algae that belong to heterokontophyta, a eukaryotic group of organisms distinguished most prominently by having chloroplasts surrounded by four membranes, suggesting an origin from a symbiotic relationship between a basal eukaryote and another eukaryotic organism (Kordiš & Turk, 2009). Brown seaweeds are usually quite large in size and its giant kelp can often be as long as 2 to 4 meters. The size of red seaweed can generally be from a

few centimetres to about a meter in length. Brown seaweed is larger than red seaweed. The major pigments of algae can play the main role to contribute to colour of the algae such as chlorophyll a, b and c; β-carotene; phycocyanin; xanthophylls; and phycoerythrin respectively. All these pigments compounds usually can be potentially applied in food, cosmetics, and pharmaceuticals. Brown seaweed contains the pigment fucoxanthin, and it overrides the green pigment chlorophyll and takes the major responsibility for distinctive greenish-brown colour (de Quirós, Frecha-Ferreiro, Vidal-Pérez, & López-Hernández, 2010). Both pigments are responsible for the photosynthesis of light.

1.4.3 *U. pinnatifida*

The brown algal genus *Undaria* (Phaeophyceae, Order Laminariales, Family Alariaceae), a laminarian kelp, has three species: *U. pinnatifida* (William, 2004) Suringar, *U. undarioides* (Yendo) Okamura, and *U. peterseniana* (Kjellm.) (M.J. Parsons, 1995). *U. pinnatifida* is native to the Japan Sea, particular on the coasts of Japan, and west of Hokkaido (Floc'h, Pajot, & Wallentinus, 1991b). It can also be found in the coasts of Korea and Chenshan Island of the Zhoushan Archipelago near Shanghai in China (S. Y. Zhang, Wang, & Wang, 2009). According to the Global Invasive Species Database 2009, *U. pinnatifida* is an edible plant in marine environment and one of the traditional foodstuffs in Japan. It has been cultured for hundreds of years. It is one of the most commercially important species and consequently extensively cultivated. *U. pinnatifida* is a healthy low-fat food. In dried *U. pinnatifida*, it was described as having low lipid content (1g/100g dry weight), low protein content (11-24%) with the relatively high level of polyunsaturated fatty acids (Plaza, Cifuentes, & Ibáñez, 2008; Plaza, Herrero, Alejandro Cifuentes, & Ibáñez, 2009). In addition, *U. pinnatifida* contains a wide variety of essential elements including calcium, iron, protein, iodine, magnesium, and zinc respectively (MacArtain, Gill, Brooks, Campbell, & Rowland, 2007).

1.4.4 Historic Trace of *U. pinnatifida* in the world

1.4.4.1 Commercial History of *U. pinnatifida*

Due to the high nutritional value in *U. pinnatifida*, it was harvested for different purpose and utilised differently in the world. In history, cultivation of *U. pinnatifida* was first studied at Dalian, northeast China, by Youshiro Ohtsuki who patented cultivation techniques of *U. pinnatifida* and for kombu (*Laminaria japonica* Areschoug) in 1943

(Yamanaka & Akiyama, 1993). After 1955 when several enterprising fishermen began cultivating it on ropes, *U. pinnatifida* cultivation spread to various places in Japan, especially in the Sanriku and Naruto areas (Yamanaka & Akiyama, 1993). Since that time, *U. pinnatifida* had been employed as food and medicines in many Eastern Asian countries for a long time. In recent years, commercial activity of seaweed including *U. pinnatifida* was reported in 42 countries around the world, and most countries are from Asia with the ranking as China, North Korea, South Korea, Japan and Philippines respectively. These countries commercially contribute to most of the *U. pinnatifida* products in the world. Due to high demand, the cultivation of *U. pinnatifida* was largely developed, promoted and industrialized since 1970s (Ko, A. Jones, Heo, Kang, & Kang, 2010).

1.4.4.2 History of *U. pinnatifida* in Spread

The spread of *U. pinnatifida* around the world can be attributed to accidental transfer associated with aquaculture and transfer via boat fouling is implicated in many of these invasions (Barrie M. Forrest, Brown, Taylor, Hurd, & Hay, 2000; Silva, Woodfield, Cohen, Harris, & Goddard, 2002). In 1971 this type of brown seaweed was first discovered and reported on the Mediterranean coast of France where it was probably introduced accidentally with oyster spat (Rueness, 1989). Then, because of commercial reasoning, *U. pinnatifida* was transplanted to the French Atlantic coast off Brittany by the French Institute for Exploitation of the Sea (Cameron H. Hay, 1990). Since 1981 *U. pinnatifida* has extended its range to Australia and NZ it was first discovered in Wellington Harbour in 1987, and then it was found spread to Argentina in early 1990s by accidental introduction of shipping from Asia (Barrie M. Forrest et al., 2000). The algae was in the first time discovered and reported on the west coast of the United States in 2000 from Los Angeles Harbour (Silva et al., 2002). Within a year, it had been found 500 km to the north of Los Angeles, in Monterey Harbour by 2002, it was reported to have a range from Ensenada, Mexico to Monterey (Aguilar-Rosas, Aguilar-Rosas, Ávila-Serrano, & Marcos-Ramírez, 2004; Silva et al., 2002). In May 2009 a numerous large sporophytes has been discovered in San Francisco Bay (Zabin, Ashton, Brown, & Ruiz, 2009).

1.5 Biology of *U. pinnatifida*

1.5.1 Description of *U. pinnatifida*

U. pinnatifida (Figure 1) is an annual, heteromorphic life-cycle plant, and its colour can beyellowish-brown to brown depends on its life cycle.

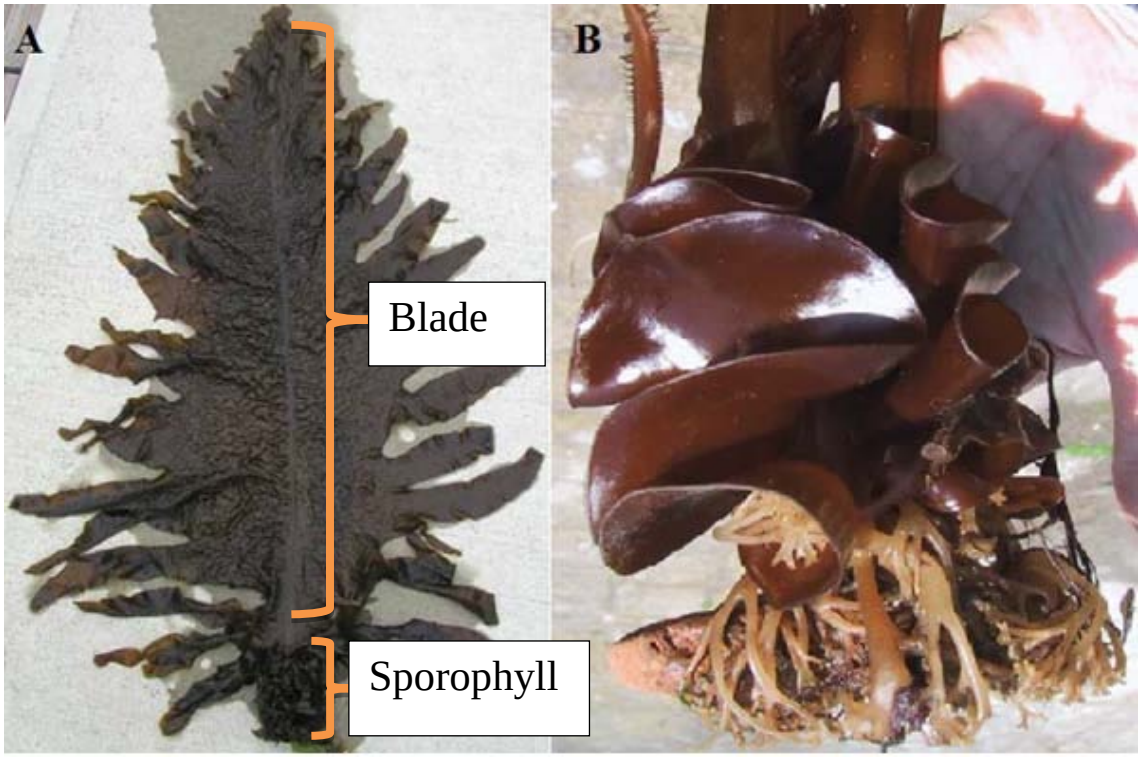

Figure 1: *U. pinnatifida*. (A) Mature algae containing divided blade and sporophyll; (B) Details of sporophylls on the base of the stipes (Redrawn from Wallentinus & Sea, 2007).

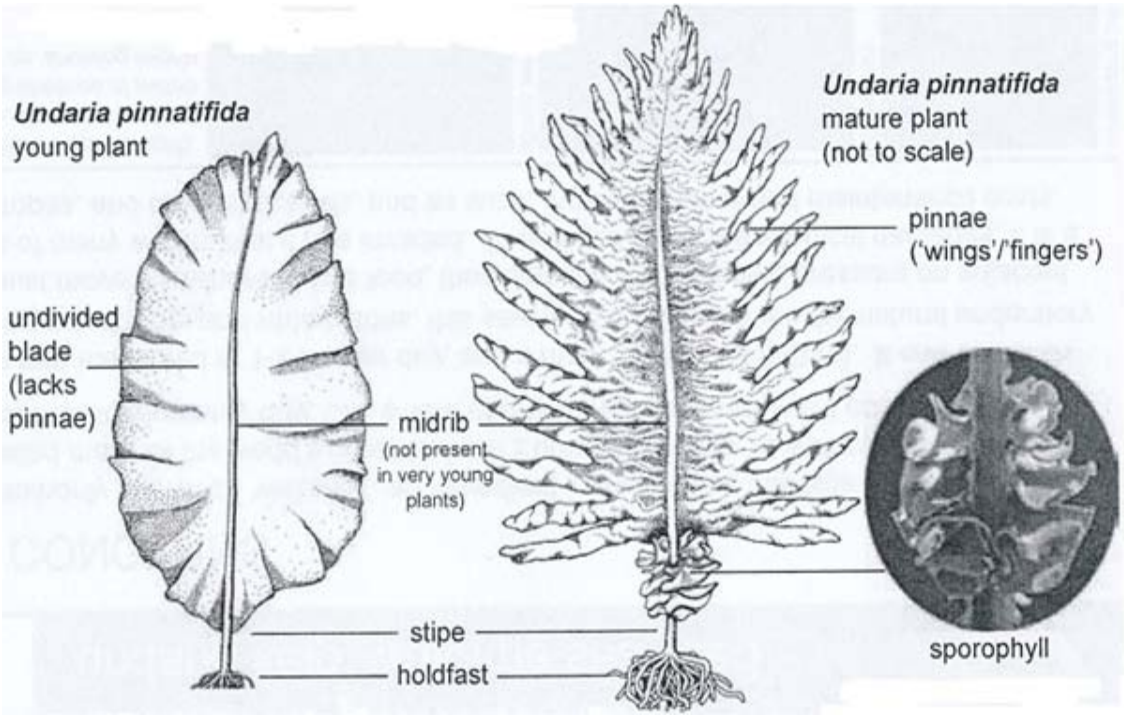

Figure 2: Physical description of mature *U. pinnatifida*. Adapted from"Guid to marine invaders in the gulf of maine" by S. Lonhart (2011, July 22). Retrieved from http://www.mass.gov/czm/invasives/docs/potentialinvaders/u_pinnatifida.pdf

The structure of *U. pinnatifida* sporophyte (Figure 2) includes blade (undivided blade lacks pinnae in young plant; pinnae or wings/fingers in mature plants), sporophyll (only present in mature plants), stipe and holdfast. The stipe extents away from the holdfast, it gradually becomes the midrib in the front of large and translucent blade of the sporophytes grow the margins of the midrib. The holdfast of *U. pinnatifida* is slender branches of haptera which resembles the roots of plants. The common length of mature *U. pinnatifida* is about 1-2 metres in its native habitat, and the maximum length was 3 metres which was reported cultivated only in Japan (Silva et al., 2002). The size of blade is quite large, usually it is about 50-80 cm broad (Wallentinus, 2007). The midrib is present in the middle of blade, and only can be found in mature *U. pinnatifida* plants, and not present in the young plants. This characteristic midrib of *U. pinnatifida* can be used to distinguish it from other seaweed species such as the NZ kelp *Ecklonia radiata*. The sporophyll of the sporophyte is found around the base of the plant stem, and it is often more yellowish than blade. The size of the mature sporophyll is about 6-20 cm length and 1-4 cm of width (Abbott & Hollenberg, 1976). A young sporophyte consists only of a holdfast, stipe and undivided blade, and its morphology is much simpler than mature plants.

1.5.2 General Biology of *U. pinnatifida*

U. pinnatifida has an annual life cycle, characterized by sporophyte stage which is macroscopic and the gametophyte stage of being microscopic in size (Stuart, 2004).

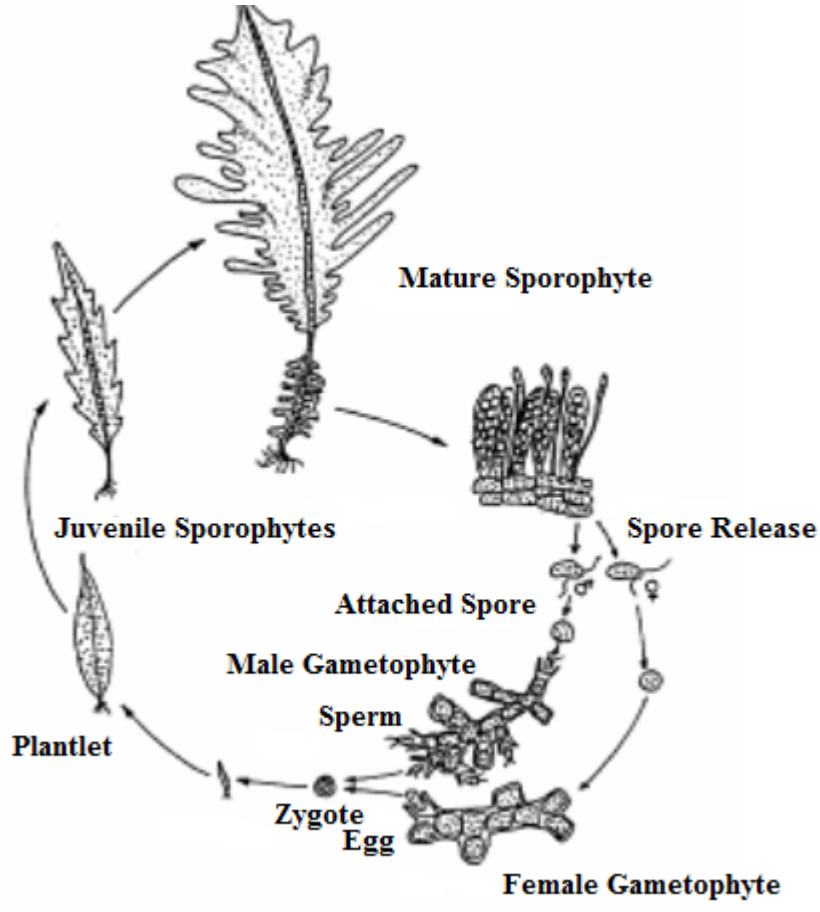

Figure 3: The life cycle of *U. pinnatifida* showing the visible sporophyte (seaweed) stage and the microphyte stage. (Redrawn from Stuart, 2004)

The longevity of the sporophyte stage is about 6 months, and the gametophyte stage can remain for more than 24 months (Stuart, 2004). The reproduction of *U. pinnatifida* is by the release of asexual zoospores annually. In the life cycle of *U. pinnatifida* (Figure 3), the sporophyll of the mature *U. pinnatifida* can generate millions of zoospores, which are released to the ocean where they find a surface for attachment. Then, the million of haploid zoospores germinate into male and female gametophytes. These gametophytes can be able to remain viable for up to three years in their dormant state before they begin germination (M.J Parsons, 1994). Male gametophytes release mobile sperms into the surrounding water while female gametophytes produced eggs which remain on the gametophyte themselves (M.J Parsons, 1994). Mobile sperm fertilises the egg, this

fertilised egg begins to form a germling which can develop into a sporophytes (M.J Parsons, 1994). After a new sporophyte is reproduced through sexual reproduction, the life cycle will repeat again.

In Asia countries such as Japan, Korea and China, spring is the main growth period for *U. pinnatifida.* Usually new sporophytes develop and grow more rapidly from end of winter through to spring, and mature *U. pinnatifida* degenerate in late summer and early autumn. Therefore, growth rate of sporophyte depends on temperature of water. *U. pinnatifida* favours cold water with a preference for around 12 °*C* (Ministry of Fisheries (MFish), 2001). In Asian countries sporophytes can grow fast between 5 to 13 °C from winter to spring. The optimal temperature for growth of *U. pinnatifida* is about 10 °C (Henkel & Hofmann, 2008). The sporophyte starts to degrade after the temperature increases above 20 ℃. If the temperature keeps increasing to 23 ℃, the sporophyte begins to completely die off or degrade (Ministry of Fisheries (MFish), 2001). This contrasts with NZ, where sporophytes are present throughout the year (Kennelly & Larkum, 1983). This may due to the narrower range of annual sea temperature range and much cooler summer and spring temperatures (C.H. Hay & Villouta, 1993).

1.5.3 Distribution of *U. pinnatifida* in New Zealand

U. pinnatifida is native to Japan, Korea, and China, and has spread to France, Australia, Spain, North and South American, and NZ (Ministry of Fisheries (MFish), 2001). *Undaria* is not an aggressive species, but can be described as the third most invasive seaweed in Europe and was ranked as the top 100 invasive species in the world (Wallentinus, 2007). The first discovery of *U. pinnatifida* in NZ was in the Wellington Harbour in 1987. The kelp was accidentally introduced into NZ waters from Japan sometime before 1987 (Williams & West, 2000). Its further spread within NZ can be partially attributed to natural dispersal as each fertile plant can generate millions of zoospores, and these can be transplanted by the wave and currents to other parts of the oceans (Ministry of Fisheries (MFish), 2001). The spore of *U. pinnatifida* can be translocated over hundreds of metres, while the whole sporophytes can be spread up to a few kilometres (Russell, Hepburn, Hurd, & Stuart, 2008). Furthermore, spores of *U. pinnatifida* can attach itself on to hulls of ships or boats to be transferred to other location of the coast. Therefore, *U. pinnatifida was* distributed through vessel fouling, and this distribution (Figure 4) was already certainly occurred. However, in recent years

the *Undaria* gametophytes were transported through vessel fouling in the introduction of *Undaria* to ports and harbours at Gisborne, Wellington, Porirua, Marlborough Sounds, Nelson, Lyttelton, Akaroa, Timaru, Oamaru, Bluff and Halfmoon Bay (Stuart, 2004)

U. sporophytes can be often found on rock and immersed artificial substrate, such as boats and mooring ropes. Its sporophytes can live in some extreme environmental conditions such as its gametophytes can survive being out of water for about a month. In addition, they have very high capability to compete with other seaweed for resource (Floc'h, Pajot, & Wallentinus, 1991a). In fact, Biosecurity NZ generally restricts to ports and harbours frequented by coastal vessels, and area utilise for marine farming (Stuart, 2004). *Undaria* remained as unwanted organism in NZ under section 164c of the Biosecurity Act 1993.

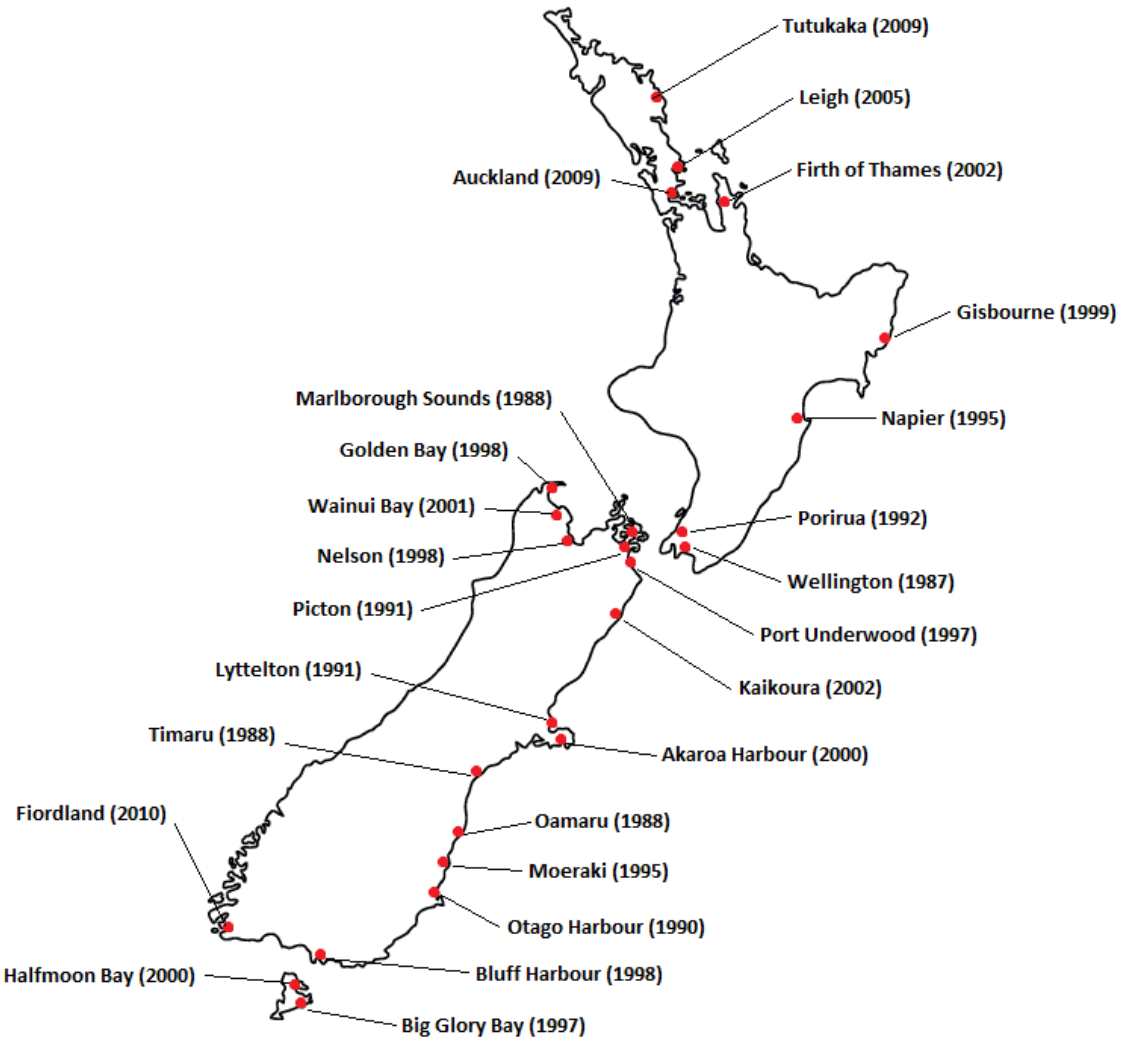

Figure 4: Distribution of *U. pinnatifida* in NZ, and its year of discovery (Redrawn from Stuart, 2004 and Carter, 2010)

1.5.4 Impact of *U. pinnatifida* in New Zealand

Introduction of a non-indigenous species in new environment is usually referred to as invasive, and this can possibly generate huge economic or ecological concerns. It may be as detrimental to native species and ecosystems worldwide as loss and degradation of habitats (Vitousek, D'Antonio, Loope, & Westbrooks, 1996). There are several features of biology of new species which can be generated by the successful invasion including rapid growth, reproductive rate, ability to reproduce vegetatively or asexually, quick maturation, and high dispersal efficiency (Colautti, Grigorovich, & MacIsaac, 2006). In the case of *U. pinnatifida*, the growth rate is quite high, but also the suitable temperature leads to ability of *U. pinnatifida to* reproduce through the whole year in NZ. This invasive species in NZ could possibly change benthic and biological assemblages and reduce biodiversity and could have serious impacts on an ecosystem-level over a broad geographic range (Piazzi & Cinelli, 2003; Scheibling & Gagnon, 2006), but also this possibly change with the availability of light, change of the nutritive food cycling to ecosystem and food availability to herbivore, and finally this can displace native species by the development of monocultures (B. M. Forrest & Taylor, 2002).

1.5.5 Legislation Management of *U. pinnatifida* in New Zealand

Since it arrived in 1987, *U. pinnatifida* has been classified as an invasive species. The Ministry for Primary Industries biosecurity (MPI) as the lead agency manages the marine biosecurity and takes the major responsibility for implementing Central Government's *Undaria* management policies. All decision made by The Ministry of Fisheries (MFish) depends on the advice about biosecurity impacts on native species and high conservation value areas from the Department of Conservation (DOC). In 2000 *U. pinnatifida* was classified as an unwanted species according to the Biosecurity Act 1993 under section 164c (Williams & West, 2000). In the next year, the MFish made an action plan about decisions not to allow harvest of *Undaria* commercially, and decrease rate of *U. pinnatifida* spread in NZ. This plan was about high restriction to spread of *U. pinnatifida*. In 2004 a new policy was developed to allow the commercial harvest of the seaweed in two situations such clearing of mussel farming lines or as part of a control program (Ministry of Agriculture and Forestry (MAF), 2009). In 2009 the NZ government reviewed the policy from previous year, and put forward a new policy to allow the harvest *of U. pinnatifida* in wider surfaces such as marinas or wharves. Due to the high risk of ecological and economic impacts, the eradication programs were used

to control the spread of this founding population in some areas of NZ such as the Nelson region and in Chatham Islands.

1.6 *U. pinnatifida* in Cancer

1.6.1 *U. pinnatifida* as a Source of Anti-Cancer Agents

Since ancient times, many seaweeds have been used in most part of human life such as the dietary foods, medical and medicinal treatment for human (Ye et al., 2005). *U. pinnatifida* is one of the most important species of brown seaweed. As a food, *U. pinnatifida* is a particularly important dietary compound in Japan and Korea, where the most of the brown seaweed intake in the daily diet is the *U. pinnatifida* (Helen Fitton, 2003). In addition, *U. pinnatifida* has been used as the important dietary components since at least sixth century in China (Ye et al., 2005). In medical and medicinal treatment, *U. pinnatifida* is source of biologically active phytochemicals, and it contains a wide range of components including carotenoids, phycobilins, fatty acids, polysaccharides, vitamins, sterols, tocopherol, and phycocyanins (Plaza & Cifuentes, 2008). The earliest record showed the use of *U. pinnatifida* was used in 2700BC in the compilation of "Chinese Herbs" by Emperor ShenNung (Helen Fitton, 2003). Chinese and Kampo (Japanese) medicine both use dried thallus (stem and spore areas) area of brown seaweeds (*Laminaria, Undaria, or Ecklonia* species) (Helen Fitton, 2003). These are used to "eliminate phlegm and more water" and are also recognized source of iodine (Chen et al., 2006). In Korea, it was believed that new mothers after birth should be provided the diets with the rich seaweed *U. pinnatifida*; this is because people thought seaweed diet contains a wide range of health benefits for mothers and their children (Helen Fitton, 2003). In addition, crude brown seaweed such as *U. pinnatifida was* used as an important detoxifying agent. This is because iodine and other elements that are present in brown seaweed can strongly inhibit absorption of similar radioactive elements by the body. In recent studies, it has been demonstrated that *Undaria* ingestion assists in eliminating dioxins in rats (Morita & Nakano, 2002). In fact, there are several potential benefits from consumption of *U. pinnatifida* including control of hyperliqidemia, thrombosis, tumours, and obesity.

This thesis focuses on the anti-cancer activity of constituents of brown seaweed particularly in *U. pinnatifida.* The anticarcinogenic properties of brown seaweeds are

not completely understood. In traditional Chinese medicine, the brown seaweed such as *Laminaria* or *Undaria* was also used in treatment of cancer and it has also been recommended in ancient Ayurvedic texts. In the 1960s, *U. pinnatifida* was used to treat cancer in the first time in Western Medicine, and this was called Algasol T331 in Italy. Claudio & Standardo in 1966 reported 68% of 162 patients made good recovery following intramuscular injections (Chapman, 1970). Good recovery was suggested from the patients that they received the improved treatment of following chemotherapy including less serious adverse side effects such as increasing appetite and hair regrowth. This possibly suggested some of potential compounds that were present in the *U. pinnatifida* directly contributed to anti-cancer treatment or might be the important chemotherapy supplement. In addition, the epidemiological data evidence suggests that brown seaweed such as *U. pinnatifida* can be an important chemopreventive food. The evidence was that compared to the Western countries the Japanese women has one third the rate of pre-menopausal breast cancer and one ninth the rate of postmenopausal breast cancer (Teas et al., 2007). Furthermore, Japanese women were found to be more likely to survive at least five years longer than other women diagnosed with breast cancer in the USA (Kanemori & Prygrocki, 2005; Pineda, White, Kristal, & Taylor, 2001). In a more recent study, Asian women who were born in the US have 60% higher risk of developing breast cancer than Asian women born in Asia (Ziegler et al., 1993). This data possible leads cancer research to focus on Asian food diet habits.

Following from the studies above, *U. pinnatifida* intake has been assessed against the incidence of cancer in detail. There were several both *in vitro* and *in vivo* studies to determine the relationship between incidence of cancer and brown seaweed *U. pinnatifida* consumption. In a large prospective dietary study 21,852 Japanese nurses in Japan with a 9 year follow-up, the data suggested high intakes of miso-*U. pinnatifida* was highly associated with the lowest breast cancer risk. It was particular indicated that the water extracted dietary seaweed was the most effective against induced tumours, and the *in vivo* study may be valuable to identify potential compounds with anti-cancer potentials present in water extracts of *U. pinnatifida* powder. Therefore, the evidence was approved a significant reductions in cancer risk associated with increasing seaweed consumption. Moreover, compared to conventional chemotherapies, it was suggested some of compounds in *U. pinnatifida* may be present to decrease the risk of cancer in 1980s. In the further studies, Hoshiyama and Takafuni, *et al.* 1992 reported consumption of *U. pinnatifida* two or more times in a week could strongly reduce the

incidence of both single and multiple stomach cancer. In addition, the consumption of *U. pinnatifida* also related to significantly decrease the risk of rectal cancer and colon cancer (Hosokawa et al., 1999). The major components of *U. pinnatifida* including major pigment compound fucoxanthin and sulfate polysaccharide fucoidan have been found to induce apoptotic damage to various tumour cells after analysis of major compounds of *U. pinnatifida.*

1.6.2 Fucoxanthin

Fucoxanthin is an abundant marine xanthophyll with the formula $C_{42}H_{58}O_6$. Fucoxanthin can be found in red or green seaweeds, but its amount is richest in brown seaweed which it can be the most important pigment compound in brown seaweed (D'Orazio et al., 2012), Fucoxanthin has been isolated for its bioactivity studies from the marine brown seaweeds including *Alariacrassi folia* (Airanthi, Hosokawa, & Miyashita, 2011), *Cladosiphon okamuranus* (Mise, Ueda, & Yasumoto, 2011), *Cystoseira hakodatensis* (M. K. W. A. Airanthi et al., 2011), *Eisenia bicyclis* (M. W. Airanthi et al., 2011), *Fucus serratus* (Zaragozá et al., 2008), *Fucus vesiculosus* (Strand, Herstad, & Liaaen-Jensen, 1998), *Hijikia fusiformis* (H. Nishino, 1998), *Ishige okamurae* (K. N. Kim, S. J. Heo, S. M. Kang, G. Ahn, & Y. J. Jeon, 2010), *Kjellmaniella crassifolia* (M. W. Airanthi et al., 2011), *Laminaria japonica* (S. K. Das, T. Hashimoto, & K. Kanazawa, 2008), *Laminariao chotensis* (Miyata, Koyama, Kamitani, Toda, & Yazawa, 2009), *Myagropsis myagroides* (Heo et al., 2010), *Padina tetrastromatica* (Sangeetha, Bhaskar, Divakar, & Baskaran, 2010), *Petalonia binghamiae* (Murakami et al., 2002), *Sargassum fulvellum* (Yan, Chuda, Suzuki, & Nagata, 1999), *Sargassum heterophyllum* (Afolayan, Bolton, Lategan, Smith, & Beukes, 2008), *Sargassumhorneri* (M. K. W. A. Airanthi et al., 2011), *Sargassum siliquastrum* (Heo et al., 2008), and *U. pinnatifida* (Hosokawa et al., 2004; Hosokawa et al., 1999; Ikeda et al., 2003), and the diatoms *Chaetoseros* sp.(Iio, Okada, & Ishikura, 2011; Kumiko, Yumika, & Masaharu, 2011), *Cylindrotheca closterium* (Rijstenbil, 2003), *Odontella aurita* (D. Moreau et al., 2006), and *Phaeodactylum tricornutum* (Nomura, Kikuchi, Kubodera, & Kawakami, 1997) respectively. Fucoxanthin is an accessory carotenoid pigment in the chloroplasts of brown algae. It is responsible for the generation of brown or olive-green colour of brown seaweed (Peng et al., 2011).

The structure of fucoxanthin (Figure 5) contains an unusual allelic bond and some oxygenic functional groups such as epoxy, hydroxyl, carbonyl and carbonyl moieties in its molecule that contribute to its unique structure. The allelic bond was found mainly in carotenoids such as fucoxanthin, which was the first allelic carotenoid found in brown seaweeds (Peng et al., 2011). This contributes to the high anti-oxidant of biological properties of fucoxanthin (Lin, Tsai, & Chiu, 2012). It is one of the most abundant carotenoids accounting for more than 10% of estimated total natural production of carotenoids (Maeda, Tsukui, Sashima, Hosokawa, & Miyashita, 2008).

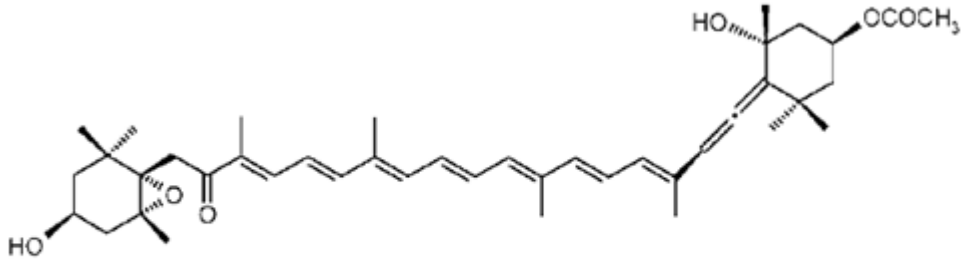

Figure 5: The structure of fucoxanthin (Source: Sachindra et al., 2007).

There is a long polyene chain of a carotenoid as constructed in the structure of fucoxanthin. Based on this structural characteristic, there are two configurations including *trans* and *cis*-formation of fucoxanthin. All-*trans* fucoxanthin is the major isomer present in fresh *Undaria* or other natural marine products with approximate 88% (Holdt & Kraan, 2011). 9'-*cis* and 13'-*cis* can be other types of isomer for fucoxanthin with about 9% when it stores in dark conditions (Nakazawa, Sashima, Hosokawa, & Miyashita, 2009). Based on the principle of steric organic chemistry, the *trans* forms of fucoxanthin are more sterically stable than *cis*-isomers. This is because the dipoles of the substituent at either side in *trans*-formation of fucoxanthin can reduce the steric hindrance. Therefore, compared to its *cis* counterpart, the *trans* forms of fucoxanthin are the thermodynamical products.

1.6.2.1 Bioavailability, Metabolism of Fucoxanthin

In animal study, fucoxanthin has been shown to be totally digested and deacetylated into fucoxanthinol in the intestinal tract by lipase and esterase from the pancreas or intestinal cells (Sugawara et al., 2002). Furthermore, fucoxanthinol was converted into other metabolite and identified as amarouciaxanthin A (Asai, Sugawara, Ono, & Nagao,

2004). This study suggested fucoxanthinol was bio-converted into amarouciaxanthin A through dehydrogenation /isomerisation, and this further bio-conversion was mainly achieved from the contribution of liver microsomes. So, the metabolic processes of dietary fucoxanthin comprise of the conversion to fucoxanthinol in the gastrointestinoal tract, then further biotranformation from fucoxanthinol to amarouciaxanthin A in the liver.

Actually, the absorption of carotenoids in intestinal cells highly depends on the solubility of the carotenoid in mixed micelles. In addition, phospholipids increase the absorption of carotenoid, but also cellular absorption was increased based on its lipophilicity of carotenoids (E. Kotake-Nara & Nagao, 2012). Depending on this characteristic, the absorption of carotenoid can be increased after metabolism of pancreatic phosphlipase A2 and lysophosphatidylcholine. The absorption of carotenoid from the intestinal epithelium was achieved through a simple diffusion mechanism to blood stream. Therefore, there is no fucoxanthin which can be detected in plasma and liver (Sangeetha, Bhaskar, & Baskaran, 2009), while fucoxanthinol and amarouciaxanthin A can be detected in plasma, erythrocytes, liver, lung, kidney, heart, spleen, and adipose tissue (Sugawara et al., 2002). In *in vivo* animal studies, the daily oral administration of fucoxanthin for 1 week showed that a small amount of fucoxanthin was not metabolised and detectable in the liver, lung, kidney, heart, spleen, and adipose tissue of the mice. Compared fucoxanthin to each metabolite, the amarouciaxanthin A can be the highest amount with 55% in adipose tissue, and about 63-76% of fucoxanthinol in other tissue. In human studies, *U. pinnatifida* was orally administered for a week in order to estimate the intestinal absorption of fucoxanthin. The results showed that the plasma concentration of fucoxanthinol was quite low, and no fucoxanthin and amarouciaxanthin A was detected in plasma. The possible explanation for that was that some compounds in algal matrix can inhibit the intestinal absorption of fucoxanthin.

1.6.2.2 Cancer Chemoprevention by Fucoxanthin

Fucoxanthin has remarkable biological properties based on its unique molecular structure including anti-oxidant, anti-inflammatory, anti-obese, anti-diabetic, anti-angiogenic, anti-malarial, and anti-cancer activities (Peng et al., 2011). The anti-cancer property of fucoxanthin will be the main focus in this study. One of the causes of cancer is that free radicals and oxidative stress are clearly involved in the pathogenesis of

cancer disease. The basic principle about anti-cancer activity is that fucoxanthin as a type of carotenoid possesses anti-oxidative properties, so that it should relatively be considered with anti-cancer potentials (D'Orazio et al., 2012).

From *in vitro* studies, fucoxanthin induces apoptosis and enhances anti-proliferative effects on several cancer cell lines.

Leukemia cancer cell lines

Apoptosis induction has been suggested to be the biochemical mechanism by which fucoxanthin exerted an inhibitory effect on tumour cells (Peng et al., 2011). The first apoptosis-inducing effect was found on human promyelocytic leukemia HL-60 cell line in 1999 after treatment with fucoxanthin. The result indicated that fucoxanthin showed strong anti-proliferative effects by cleavages of procaspase-3 and poly-ADP-ribose polymerase.

Colon Cancer Cell Lines

The anti-proliferative effect from fucoxanthin was also found on other types of human colon carcinoma including Caco-2, WiDr, HT-29 and DLD-1 respectively. In the mechanism analysis, Hosokawa, M, et al (2004) reported the possible mechanism for human colon cancer cell lines Caco-2 that the decreased expression level of Bcl-2 protein might contribute to fucoxanthin-induced apoptosis (Hosokawa et al., 2004). The anti-proliferative effects of fucoxanthin to other cancer cells were also focused including human colorectal adenocarcinoma HCT116 and WiDr colon cancer cells. Das *et al*. in 2005 indicated the possible mechanism of growth inhibition to human colon cancer cells was attributed to the generation of a cyclin-dependent kinase (cdk) inhibitory protein $p^{21WAF1/Cip1}$, which could inhibit the phosphorylation of pRb site, resulting in cell cycle arrest in G0/G1 stage.

Lung Cancer Cell Lines

In 1993, the relationship was determined between intake of carotenoids and the incidence of lung cancer (Michaud et al., 2000). This was a statistic analysis including 230 men and 102 women with lung cancer and 597 men and 268 women as controls, frequency-matched to the patients by age and sex. This statistic analysis was validated quantitative diet history assessed the usual intake of foods rich in carotenoids. There was a positive correlation between intake of β-carotene, α-carotene, and lutein and low

risk of lung cancer (Michaud et al., 2000). Among components of seaweeds, fucoxanthin showed dose-dependent anti-proliferative activities on both the bronchopulmonary and epithelial cells NSCLC-N6 and A549 respectively (Dimitri Moreau et al., 2006). Fucoxanthin induced morphological change including rounding up, reduction of cell volume, chromatin condensation, nuclei fragmentation, and formation of apoptotic bodies in NSCLC-N6 and A549 cells (Peng et al., 2011).

Prostate Cancer Cell lines & Liver Cancer Cell Lines

The anti-proliferative effects of fucoxanthin were also found in many prostate cancer cell lines such as PC-3, DU 145, and LNCaP respectively. The inhibition mechanism for PC-3 cancer cells is quite similar to human promyelocytic leukemia HL-60 cellsthrough caspase-3 activation (E. Kotake-Nara, Sugawara, & Nagao, 2005). Satomi and Nishino showed the fucoxanthin induced cell cycle arrest at the G1 phase and Growth arrest and DNA-damage-inducible protein GADD45 alpha protein expression to inhibit the growth of DU145 cells by MAPKs modulation. The similar mechanism of inhibition effect also can be found in human hepatocellular carcinoma Hep G2 cells. In the further study, Das et al. found the growth-inhibitory effect of fucoxanthin on the cancer cells was chiefly due to an arrest in the G0/G1 phase of the cell cycle, and no apoptosis was observed, indicating that fucoxanthin possessed cytostatic rather than cytocidal activity in Hep G2 cells (S. K. Das et al., 2008).

Human gastric adenocarcinoma

Fucoxanthin was found to generate anti-proliferative effect to the human gastric adenocarcinoma MGC-803. In mechanism study, the result indicated fucoxanthin induced cell cycle arrest in G2/M phase and apoptosis of MGC-803, and down-regulated by the expressions of CyclinB1 (Z. Zhang et al., 2008). Therefore, the main molecular mechanism proposed are the fucoxanthin mediated inhibition of cell cycle by inducing cell cycle arrest and enhancing gap junctional intercellular communication.

Human Neuroblastoma Cancer Cell Lines

The human neuroblastoma GOTO cells are the unique human neuroblastoma. It was involved in determination of anti-proliferative effect by culturing with fucoxanthin. The result suggested the fucoxanthin at 10μg/mL reduced the growth rate of GOTO cells to 38% of that of control in a 3-day assay (Okuzumi et al., 1990).

1.6.3 Fucoidan

Fucoidan can be largely found in *U. pinnatifida* as another major bioactive compound. Since 1913, there were a large amount of published research articles related to fucoidan. These research papers found that fucoidan, a bioactive compound from *U. pinnatifida* polysaccharides possess several pharmacological properties including anti-tumour, anti-coagulant, anti-thrombotic, anti-virus, immunomodulatory, anti-oxidant, and anti-inflammatory (Li & Lu, 2008). In this study, the anti-tumour properties of fucoidan will be focused on.

1.6.3.1 Structure of Fucoidan

Since 1913, fucoidan was successfully isolated from the brown seaweed, and in fact it is a natural polysaccharide made essentially of sulfated L-fucose residues. The structure of fucoidan (Figure 6) is mainly composed of fucose and sulfate. Fucoidan extracted from different type of seaweeds is found to be largely different each other (Table 4).

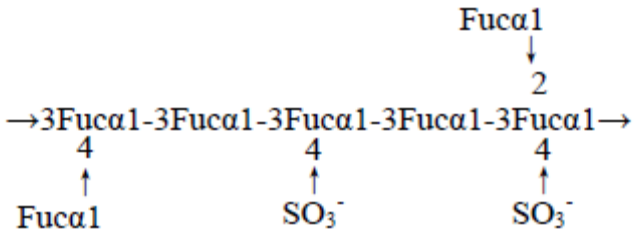

Figure 6: Pankter model of fucoidan structure (Source: Li, Lu et al., 2008).

In its basic structure, fucodian contains sulfated L-fucose as primary sugar in fucoidan, and another important structure is the sulfate group, which is strongly related to biological activities of fucoidan (Wijesinghe & Jeon, 2012). In addition, fucoidan also contains other monosaccharides including mannose, galactose, glucose, xylose, etc. and uronic acids, eve, acetyl groups and protein (Li & Lu, 2008).

Table 4: Chemical composition of fucoidan in different brown seaweed.

Chemical Composition of Fucoidan in Different Brown Seaweed		
Brown Seaweed	**Chemical Composition**	**Reference**
F. Vesiculosus	fucose, sulfate	(T. Nishino, Nishioka, Ura, & Nagumo, 1994)
F. Evanescens C. Ag.	fucose/sulfate/acetate (1/1.23/0.36)	(Bilan et al., 2002)
F. Serratus L.	fucose/sulfate/acetate (1/1/0.1)	(Bilan, Grachev, Shashkov, Nifantiev, & Usov, 2006)
Lessonia vadosa	fucose/sulfate(1/1.12)	(Chandía & Matsuhiro, 2008)
Laminaria angustata	fucose/galactose/sulfate (9/1/9)	(Percival, 1968)
Pelvetia wrightii	fucose/galactose (10/1), sulfate	(Chandía & Matsuhiro, 2008)
U. pinnatifida	fucose/galactose(1/1.1), sulfate	(Lee, Hayashi, Hashimoto, Nakano, & Hayashi, 2004)
Padina pavonia	fucose, xylose, mannose, glucose, galactose, sulfate	(Ponce, Pujol, Damonte, Flores, & Stortz, 2003)
Sargassum stenophyllum	fucose, galactose, mannose, GlcA, glucose, xylose, sulfate	(M. E. R. Duarte, Cardoso, Noseda, & Cerezo, 2001)
Spatoglos sumschroederi	fucose/xylose/galactose/sulfate (1/0.5/2/2)	(Lee et al., 2004)

1.6.3.2 Cancer Chemoprevention by Fucoidan

Anti-cancer activity of many polysaccharides has been reported in recent years (B. Li et al., 2008). Based on the type of saccharide contained, anti-proliferative effect from fucoidan has been investigated in many types of cancer cell lines including breast carcinoma cancer cell lines MCF-7 (Zhongyuan Zhang, Teruya, Eto, & Shirahata, 2011), human epithelial carcinoma cell lines A549 (H. J. Boo et al., 2011), and human hepatocellular carcinoma Hep G2 (Nagamine et al., 2009). Due to structural difference in species, the fucoidan extracted from *U. pinnatifida* was paid more attention in the following.

Yang et al (2008) studied the extraction method for fucoidan of *U. pinnatifida*. The two methods including analytical chemistry method and microwave-heating assistance were compared. The results from this study indicated native fucoidan showed to decrease cell viability to 37.6%, and its inhibition effect was significantly increase to 75.9% after

hydrolysis in boiling water with hydrochloric acid for 5 minutes. However, a little of improvement of anti-cancer activity was found in extraction with microwave oven assistance (C. Yang et al., 2008). In 2010, the structure of fucoidan from *U. pinnatifida* was defined, and its relative anti-cancer activities were also determined. The result showed isolated polysaccharide contained α-fucose (50.9 mol%) and β-galatose (44.6 mol%) as main sugar units. Its anti-proliferative effects were shown fucoidan extract against prostate cancer cell lines, cervical cancer, alveolar epithetical carcinoma, and hepatocellular carcinoma cell line (Synytsya et al., 2010). After one year, a similar study was achieved with different cancer cell lines. Moreover, the most interesting part in that study was the comparison of anti-cancer activity of fucoidan extracted from different brown seaweed between *U. pinnatifida* and *Saccharina japonica*. The cancer cell lines applied were human breast cancer T-47D cell lines and melanoma SK-MEL-28 cell lines. Vishchuk, Ermakova, & Zvyagintseva 2011 suggested fucoidan extracted from both types of seaweed distinctly inhibited proliferation and colony formation in both breast cancer and melanoma cell lines in a dose-dependent manner (Vishchuk, Ermakova, & Zvyagintseva, 2011). Interestingly, the fucoidan extracted from *U. pinnatifida* could possess high anti-proliferative effects on breast cancer cell line than extraction from Saccharina japonica.

Several studies focused on mechanism analysis about the anti-cancer effects of fucoidan from *U. pinnatifida* in human carcinoma cell lines. Hye-Jin Boo, et al. (2011) reported the fucoidan can induce Bcl-2 expression (B-cell lymphoma 2) and the expression of Bax (Bcl-2-associated X protein) in dose dependent manner to promote cell apoptosis (H.-J. Boo et al., 2011). In addition, fucoidan extracted from *U. pinnatifida* could generate cleavage of poly-ADP-ribose polymerase (PARP), and regulate the phosphatidylinositol 3-kniase (PI3K)/Akt pathway (Aisa et al., 2005). In fact, the phosphatidylinositol 3-kniase (PI3K)/Akt pathway take the major responsibility to regulate cell growth survival, and apoptosis (Schade, Powers, Wlodarski, & Maciejewski, 2006). Thus, the effect of fucoidan from this result of study showed to have negatively regulates and promotes cell apoptosis. Therefore, fucoidan as suggested from this study might have therapeutic potential for lung cancer treatment.

1.6.4 Extraction Method Overview for Bioactive Compounds Fucoxanthin & Fucoidan from *U. pinnatifida*

1.6.4.1 Analytical Chemistry Extraction Methods

Fucoxanthin Extraction Methods Review

Fucoxanthin (3'-acetoxy-5,6-epoxy-3,5'-dihydroxy-6',7-didehydro-5,6,7,8,5',6'-hexahydro-β,β- caroten-8-one, Figure 6) cannot dissolve in high polar solvent such as water under neutral pH condition (Mínguez-Mosquera, Jarén-Galán, & Gandul-Rojas, 1992). However, it can easily dissolve in esters and non-polar organic solvent. Therefore, the proposed extraction can be achieved by the use of non-polar solvent with some less polar solvent. There are several non-polar solvent such as chloroform, n-hexane, cyclohexane, petroleum ether, dichloromethane and tetrahydrofuran. There are some choices of less polar solvent such as ethanol, acetone and methanol.

The most common process of fucoxanthin analytic chemistry extraction (Figure 7) was described in diagram above. The basic principle of analytic extraction is that *U. pinnatifida* powder can be soaked in non-polar organic solvent, this allows less-polar or non-polar compounds to dissolve in non-polar solvent; vacuum filter contributes to remove all non-dissolved *U. pinnatifida* powder; combination of organic polar and non-polar solvent with specific ratio allows to remove all polar compounds to remain desirable less polar crude fucoxanthin products. Therefore, it simply says the process of extraction depends on polarity of fucoxanthin, and the extraction can be achieved by the changing of polarity in elution solvent.

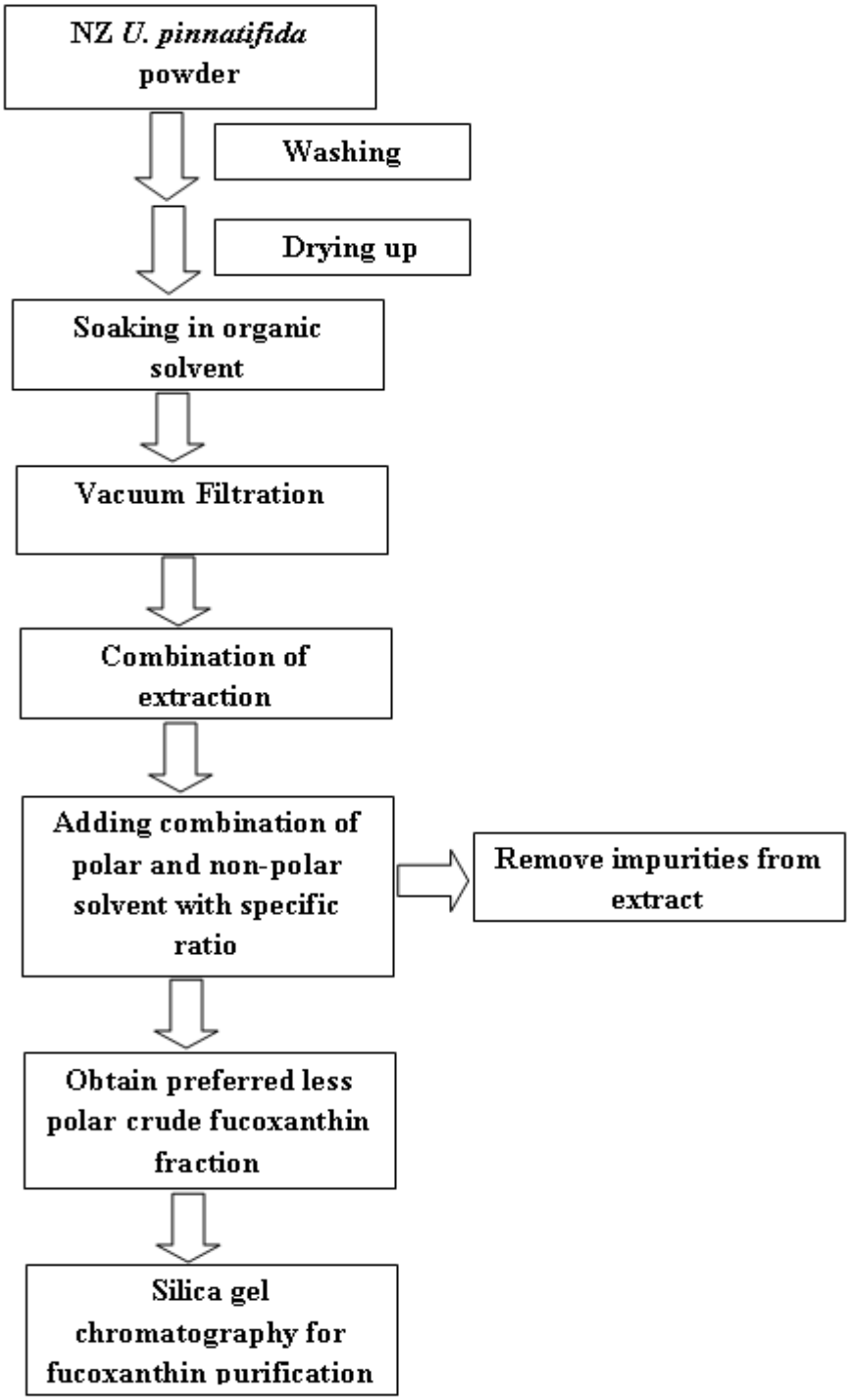

Figure 7: Common process of fucoxanthin extraction & purification

Fucoidan Extraction Methods Review

Based on the chemical composition, the structure of fucoidan contains fucose, sulfate, uronic acid and small quantities of monosaccharides. Thus, compared to fucoxanthin, fucoidan is not a single compound in seaweed, and its structure contains several components. This is the main reason that fucoidan is sometimes called fucose-

containing sulphated polysaccharides. In addition, there are a large numbers of structural studies which were indicated above, these studies concluded the compositions and structures of fucoidan from seaweed are extremely complex, and their structures are different between species (B. Li et al., 2008). Also, the structures of fucoidan are largely different between different season, geographic location and maturity of the plant (Rioux, Turgeon, & Beaulieu, 2007). Therefore, the structure of fucoidan was not accurately defined, and this problem relates the extraction, purification and biosynthesis methods for fucoidan are difficultly defined.

The extensive methods that are currently used contribute to generation of crude fucoidan with multi-components. The methods used to extract fucoidan may result in fucoidan extract that vary in chemical composition and structure. Extraction using dilute acetic acid was the first method used by Kylin in 1913 (Ale, Mikkelsen, & Meyer, 2011). This method was used to isolate “fucoidan”, subsequently referred to as fucoidan (Ale et al., 2011). Usually the crude sulphate extracts was obtained from brown seaweed soaking of in water or the precipitation of the ethanol extraction. However, the high level of ash was found in the ethanol extraction products. In recent study, water was approved to retain the stability if the molecular weight and overall charge of the polysaccharide, and it commonly can be the critical solvent to extract high quality of fucoidan with its bioactivity.

Acids such as diluted sulphate acid, diluted hydrochloric acid were another important solvent to generate the crude fucoidan extracts. For the compositional analysis, acids solvent H_2SO_4 can typically contribute to a substantial amount of sulphate in the step of acidic hydrolysis (Ale et al., 2011). The hydrochloric acid is another consideration, and this acidic solvent can generate a higher yield of crude fucoidan (Michaud et al., 2000). However, the present of sulphate was generated in the form of ester groups in the precipitated products (Dimitri Moreau et al., 2006), but some of undesirable products such as alginic and metals were also found in the fucoidan extracts. In the following, the fucoidan extract could be neutralized with aqueous sodium carbonate and precipitated with ethanol. This crude sulphated polysaccharide structure was proposed to be built of glucuronic acid, mannose, and galactose results with partially sulphate side-chains composed of galactose, xylose and fucose residues (Okuzumi et al., 1990). Prior to extraction, the typical pretreatment for seaweed powder involves using of a mixture of methanol, chloroform and water (Chen Yang et al., 2008). This contributes to remove all colour matter from the seaweed.

1.6.4.2 Novel Extraction Methods to Bioactive Compounds Fucoxanthin & Fucoidan from *U. pinnatifida*

Instead of the use of amount of organic solvent, another extraction methods including microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE). The most common materials which were used in this type of extraction method are carbon dioxide gas and water (Synytsya et al., 2010).

Physical & Chemical Characteristics of Carbon Dioxide & Supercirtical Carbon Dioxide

For the physical characteristics of carbon dioxide, it usually is a gas form in the air in the standard temperature and pressure. After freezing CO_2 it can be changed to solid form as dry ice. When standard of temperature and pressure increases to reach to or over the critical point, the CO_2 gas form can be changed to a form between a gas and a liquid. The critical temperature and critical pressure for carbon dioxide gas are 31.1°C and 7.4MPa (K. Duarte et al., 2012).

In order to achieve these critical points, this depends on the chemistry structural characteristics of carbon dioxide. Basically, carbon dioxide is a linear symmetry molecule, and two oxygen atoms are more electronegative than the central carbon atom. Moreover, this symmetry of the molecule structure contains the two polar carbonyl groups that have dipole moments in opposite direction. Therefore, the net dipole moment is zero, so that carbon dioxide is the non-polar compound. Depends on this characteristics, the high temperature with high pressure can contribute to make carbon dioxide in liquid formation for extraction, and this is the basis of the supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE).

Supercritical carbon dioxide can be the most important industrial solvent with high solvating power, and this possesses high solubility to organic compounds without gas toxicity and seriously environmental pollution. In the industry, supercritical carbon dioxide can be the most important extraction solvent instead of using traditional organic solvent such as hydrocarbons and perchloroethylene. The main advantage of using supercritical carbon dioxide is less cost, less toxic and non-flammable. In addition, supercritical carbon dioxide possesses gas-like viscosity, but also it has higher diffusion coefficient than liquid.

Physical & Chemical Characteristics of Water & Subcritical Water

For the physical characteristics of water, water usually can be liquid form in the standard temperature and pressure. The subcritical water refers to liquid water at temperature between the atmospheric boiling point and the critical temperature (374°C) of water. The pressure also contributes to generate the form of subcritical water, the pressure is about 22.1MPa (Van Elswijk & Irth, 2002).

Water is a polar molecule. This is because the structure of water is not exactly symmetry. The oxygen in the water structure can be more electronegative than the other two hydrogen groups, so that oxygen atom can attract more electrons and generate more electronegative charge than hydrogen. Therefore, the electronegativity is different between hydrogen and oxygen in the water molecular structure. In order to generate subcritical water, microwave heating can be the perfect option. The main reason for this is that the excitation with microwave radiation can generate the polar molecules aligning their dipoles within the external field, and the strong agitation, provided by the reorientation of molecules, causes an intense internal heating. Water can absorb microwave radiation, and the radiation can be converted into heat with high efficiency. This is called superheating, so that the enormous accelerations in the time can be achieved. If this superheating can be generated in closed vessels under high pressure, the reaction, which requires several hours in the normal conventional condition, can be performed over the course of minutes. Therefore, the microwave heating can generate the internal heat flow from inside out, and this is a selective heating to polar compounds to increase rate of reaction.

Water can be subcritical after heating water above 100°C. The dielectric constant of water decreases and its ionic product increases. These contributions can allow water that is suitable for hydrolysis and other organic reactions, and a variety of organic compounds can be dissolved in subcritical water. For example, when water temperature increases to 200°C, the dielectric constant of water is the same as methanol in the room temperature. After water temperature increases at 297°C, it can be completely mixed with benzene. Thus, subcritical water can be better solvent for hydrophobic organics than ambient water. In fact, there is a critical point for both water and carbon dioxide. Therefore, based on description above, supercritical CO_2 can be suitable for extraction of low polar compounds such as oils, terpenoids, flavours and essential oil, and the

subcritical water can be suitable for extraction of highly polar compounds such as sugar, proteins, and pectins etc.

1.7 Objectives of Study

U. pinnatifida is endemic to South East Asia and Japan. This type of brown seaweed has been extensively studied in the past with interest in its pharmaceutical properties. So far, anti-cancer pharmacological research about *U. pinnatifida* extracts fucoxanthin has been carried out in Japan, Korea, and China. Since 1980s, *U. pinnatifida* was discovered in NZ, and treated as an exotic pest. Until now, *Undaria* was recognized to be legally grown, harvested, sold and exported from NZ.

Depending on studies in previous years, fucoxanthin as a single pigment compound can be essential standard in this standard. Its anti-cancer effects were not determined through screening a large number of cancer cell lines in previous studies. Therefore, the first and the most important aim in this study was to determine whether fucoxanthin could generate inhibition effects to different types of cancer cells or generate particularly higher inhibition effect to the specific types of cancer cells investigated. By comparison about anti-proliferative effects of fucoxanthin between cancer cell lines used in this study, the results can indicate fucoxanthin can potentially be used to treatment with type of cancer or used as anti-cancer drug supplement in combination chemotherapies.

Furthermore, in NZ *U. pinnatifida*, the fucoxanthin as the major pigment in brown seaweed and fucoidan as an important type of polysaccharide are the main components in *U. pinnatifida* with apoptotic damage to tumour cells. However, besides these major components, the possibility that other minor components of *U. pinnatifida* may also be involved in anti-proliferative effects has not yet been excluded. Therefore, in this research study, the second aim of this study was to identify novel potential compounds from NZ *U. pinnatifida* with anti-proliferative effects to cancer cell lines. Another purpose in this research study was to identify novel potential compounds from Japanese *U. pinnatifida* with anti-proliferative effects on cancer cell lines. The final purpose in this research study was to determine whether two important components fucoxanthin and fucoidan in *U. pinnatifida* can be effectively extracted by the Japanese novel extraction method.

Chapter 2 Methodology

2.1 Design of Research Study

The diagram (Figure 8) is the design of experiment in this study. There are nine cancer cell lines used in this study including two types of human lung carcinoma cell line, two types of human colon adenocarcinoma cell line, human hepatpcellular carcinoma, human neuroblastoma, human breast adenocarcinoma, human cervix squamous carcinoma and human malignant melanoma. This study contained six highly correlated screening culture experiments with nine cancer cell lines. The anti-proliferative effects were determined after culturing cancer cells with pure standards and seaweed *U. pinnatifida* extracts. The quantitative data for anti-proliferative effects was calculated to describe as the half maximal inhibitory concentration (IC_{50}). This quantitative data for anti-proliferative IC_{50} values can be achieved by the modified Karber method, and the detailed calculation method was described in the Methodology section 2.7.6.

In this study, pure fucoxanthin was an important standard compound. The first and the most important screening experiment was that the quantitative anti-proliferative effects were determined from cancer cell line culturing with pure fucoxanthin. These anti-proliferative quantitative standard values were the essential basis to make contribution to intra-comparison between different types of cancer cells in this study, this comparison was to determine effectiveness of inhibition to cancer cells. In addition, these standard values made contribution to inter-comparison to determine possible novel compounds with anti-cancer potential contained in *U. pinnatifida* extracts. Therefore, these basic standard values were the most important in this study. In order to achieve the most accurate anti-proliferative values, there are two (high & wide and low & narrow) concentration ranges of pure fucoxanthin that was used to culture with cancer cells. The high & wide concentration range is from 0.391μM to 200μM, and the IC_{50} value can be roughly determined. Based on the rough IC_{50} results, the low & narrow concentration range of pure fucoxanthin was considered to use for culturing with nine cancer cell lines, and the low and narrow concentration range was from 1.5625μM to 100μM. In fact, this was the repeating experiment in order that the IC_{50} values obtain from nine cancer cell lines are reliable and correct.

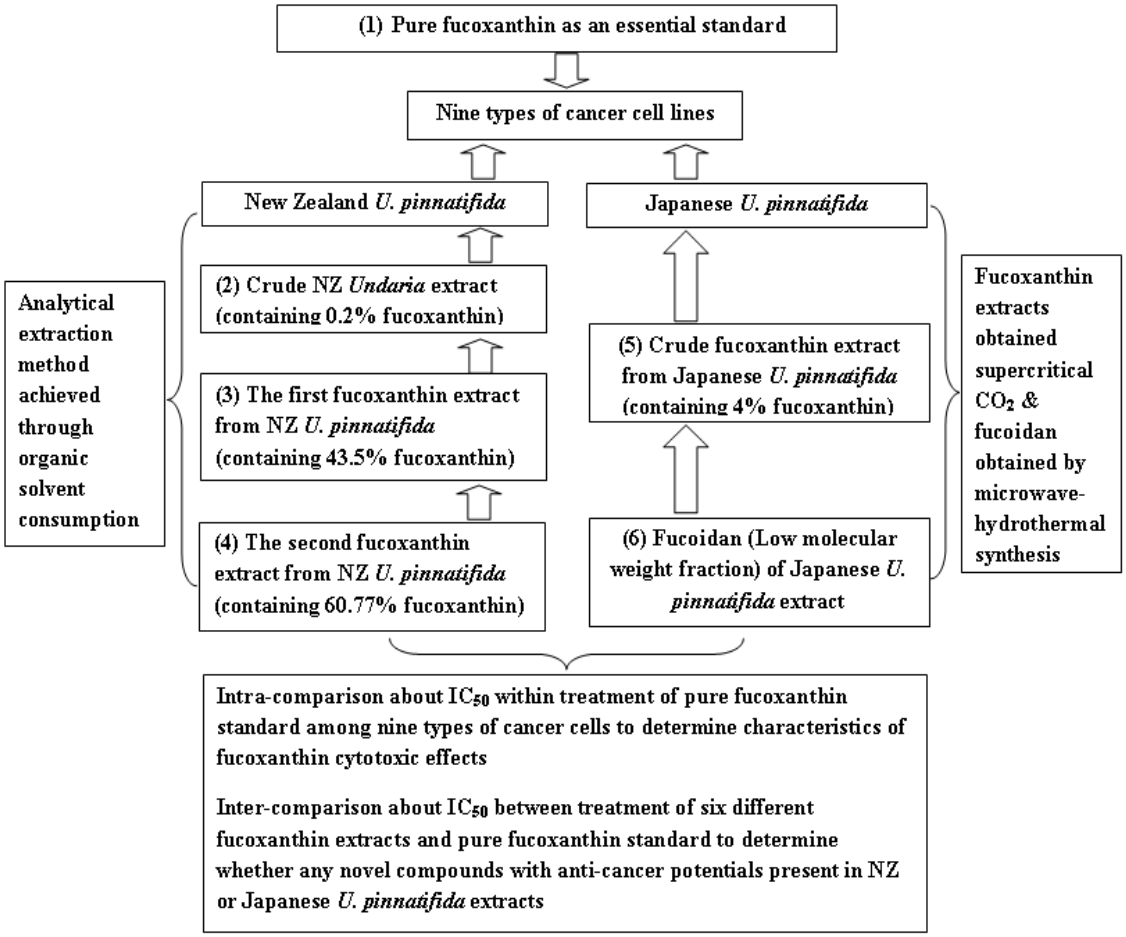

Figure 8: The design of screening experiments in this study: this study contains six screening culture experiments with nine cancer cell lines including (1) pure fucoxanthin standard; (2) crude NZ *Undaria* extracts (containing 0.2% fucoxanthin); (2) crude NZ *Undaria* extracts (containing 0.2% fucoxanthin); (3) the first fucoxanthin NZ *Undaria* extract (containing 43.5% fucoxanthin); (4) the second fucoxanthin NZ *Undaria* extract (containing 60.77% fucoxanthin); (5) crude fucoxanthin Japanese *Undaria* extract (containing 4% fucoxanthin) & (6) fucoidan (low molecular weight fraction) of Japanese *Undaria* extract.

The following experiments (Figure 8: Left Side*)* were that nine cancer cell lines was cultured with three NZ *U. pinnatifida* extracts as the second, third and forth screening culture experiments. The purity of fucoxanthin gradually increased, and these three NZ *U. pinnatifida* extracts contained 0.2%, 43.5%, and 60.77% of fucoxanthin. The quantitative anti-proliferative values were also determined from these three screening experiments. The purity of fucoxanthin at 0.2% can be obtained through soaking in pure methanol for 2 days without further extraction. Other two extracts with fucoxanthin purity at 60.77% and 43.5% were involved in analytical chemistry extraction with silica gel chromatography purification. If significant inhibition could be found from the low

fucoxanthin purity *Undaria* extracts, the inter-comparison to the pure fucoxanthin standard could suggest possible novel compounds contained in NZ *U. pinnatifida*.

Then, the fifth and sixth screening (Figure 8: Right Side) were that nine cancer cell lines were cultured with two Japanese extracts including 4% fucoxanthin extract and crude fucoidan (Low molecular fraction) respectively. This extraction procedure was achieved in a single step through the microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE). Inter-compared to anti-proliferative effects of pure fucoxanthin standard, the inter-comparison determined whether novel compounds with anti-cancer potentials contained in Japanese *U. pinnatifida*. Moreover, the anti-proliferative bioactivity of crude fucoidan (Low molecular fraction) was determined through culturing with nine cancer cell lines. In fact, this was an extra comparison to published anti-proliferative data of fucoidan in literature, this contributed to determine whether bioactive compounds could be effectively extracted from the *U. pinnatifida* by the novel extraction method.

All nine cancer cell lines should be treated with pure fucoxanthin and *U. pinnatifida* extract (NZ & Japanese crude fucoxanthin and Japanese fucoidan in low molecular weight fraction) in a series of diluted extraction concentration for 24, 48 and 72 hours. This is to determine whether the inhibition effect can be the dose-dependent in time manners. The growth inhibition effects for nine cancer cell lines were demonstrated through comparison between treatment group with *U. pinnatifida* extract and control group. Due to low solubility of fucoxanthin in cell completed culture medium, fucoxanthin should be dissolved in biological pure ethanol (100%) first, followed by a dilution by culture medium with a dilution factor of 50. This dilution leads to the maximum final ethanol concentration of 2% in culture medium. It is necessary to examine whether diluted ethanol can generate any positive or negative effects on cancer cell growth. The ethanol effect examination is extremely important. If no effect from pure ethanol can be found, this indicates only fucoxanthin can generate effects to cancer cell growth. The cancer cell viability was then determined by MTT assay.

PART I: Materials and Methods for Fucoxanthin Extraction from New Zealand *U. pinnatifida*

2.2 Materials for Fucoxanthin Extraction

2.2.1 *U. pinnatifida* from South Island New Zealand

2.2.1.1 Sample Collection

U. pinnatifida were collected from Port Underwood (Marine Farms PE327 and 106) and Pelorus Sound of Marlborough Sound (Marine Farms 122 and 353) in NZ, and the samples were collected at monthly intervals from June to December 2011. Seaweed was removed from selected mussel-harvesting lines by hand. Samples were washed by seawater to completely remove debris and epiphytic organisms from the thallus. Samples were then dried with paper towels. The blades and sporophyll of the samples were stored separately in individual labelled plastic bag. Samples were then kept in the freezer overnight prior to be sent to Vitaco Limited, Avondale Auckland to be freeze-dried (John Morris Scientific Ltd). Samples were lyophilised in bulk within 48 hours of frozen storage in Vitaco Limited.

2.2.1.2 Sample Collection Location

U. pinnatifida were collected from two mussel farms from each location (Figure 9), Port Underwood (PE327 and 106) and Pelorus Sound (122 and 353) (Table 5) that were situated around Nelson in NZ.

Table 5: The location of mussel farms: *U. pinnatifida* sampling area

***U. pinnatifida* Sampling Location**			
Sample Collection Location	**Farm Codes**	**Latitude**	**Longitude**
Port Underwood	PE 327	41° 20' 50.05"	174° 07' 20.96"
	106	41° 19' 37.74"	174° 08' 57.54"
Pelorus Sounds	122	41° 06' 30.89"	173° 54' 58.05"
	353	41° 01' 56.95"	173° 56' 12.55"

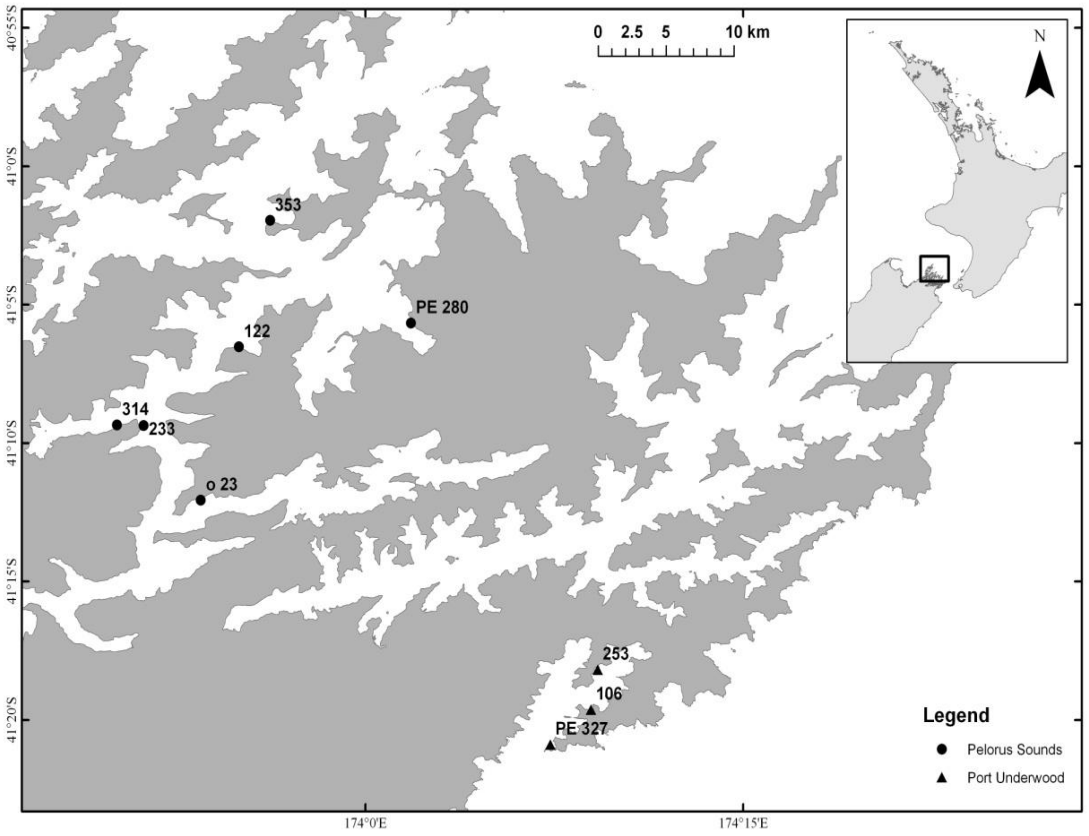

Figure 9: *U. pinnatifida* sample collection farms from the Port Underwood and Pelorus Sound coastal area around Nelson in NZ

2.2.2 Materials for Fucoxanthin Extraction

Methanol (FSBA452-4), hexane (FSBH/0406), and chloroform (FSBC/4966) were certified as HPLC grade and purchased from Thermofisher in Auckland, NZ; Silica gel powder (60-200 MESH) was from J.T. Baker Chemical Co., and Benzene (Analytic Reagent) was from British Drug House B.D.H. LTD.

2.2.3 Materials for Fucoxanthin HPLC Quantification

Pure standard fucoxanthin (F6932) and internal standard canthaxanthin (32993) were purchased from Sigma-Aldrich (St. Louis, MO, USA) for extraction quantification.

2.3 Method for Fucoxanthin Extraction from New Zealand *U. pinnatifida*

2.3.1 Sample Preparation

Freeze dried samples were ground to fine powder using a Breville CG2B Coffee 'n' Spice Grinder and sieved though a 600 μm screen to obtain fine powder. Each sample was stored in 200 mL PET bottle or 1L of solvent glass bottle, and kept at the room temperature in the dark to await analysis.

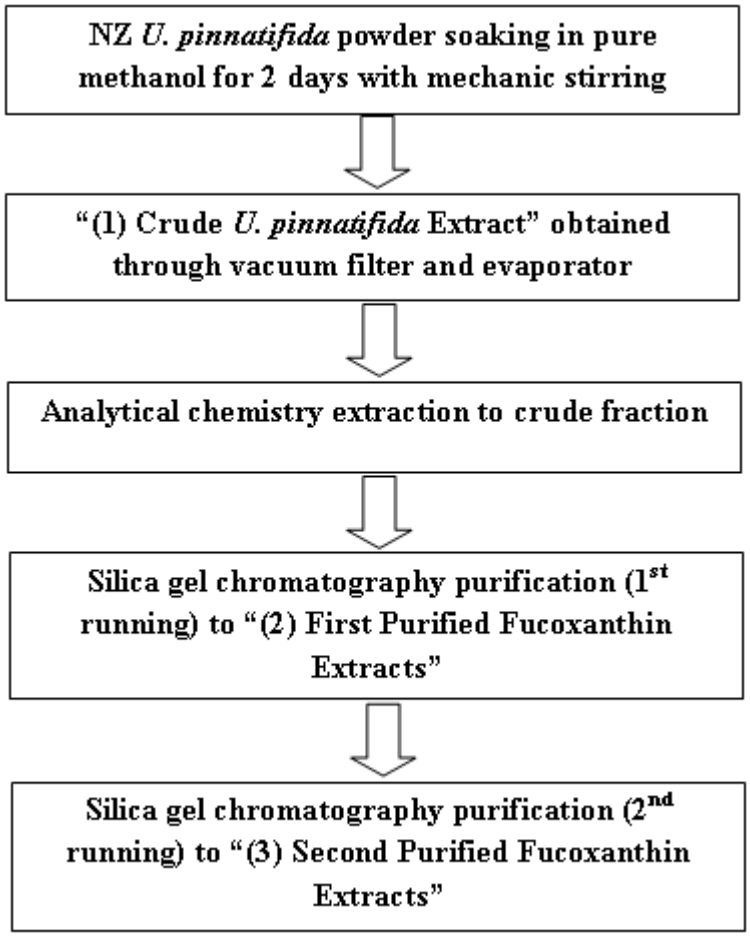

Figure 10: Process of fucoxanthin extraction and purification from NZ *U. pinnatifida* to obtain (1) Crude *U. pinnatifida* Extract; (2) First Purified Fucoxanthin Extract"; (3) Second Purified Fucoxanthin Extract

2.3.2 Analytical Chemical Extraction

NZ *U. pinnatifida* samples prepared from section 2.2.1 were dissolved into pure methanol and stirred mechanically for 48 hours in dark condition at the room

temperature to reduce the possibility of oxidation by sunlight. The ratio for extraction between *U. pinnatifida* sample and pure methanol is 100 g: 1 L. Methanol extract was then filtered using a filter paper (Whatman No.1 filter paper, Thermofisher, NZ) to remove the solids. This procedure was repeated until the seaweed powder was colorless. All methanol extraction was then combined. This will be the "Crude *U. pinnatifida* Extract" (Figure 10 (1)).

Hexane and distilled water were added into the crude fucoxanthin extract and vortex for about 30 seconds. This mixture was loaded into a separating funnel. The lower layer consisted of the methanol/water fraction was collected and the hexane fraction in the upper layer was removed. The chloroform was then added into methanol/water extract and vortexed for 30 seconds. The ratio for pure methanol, pure hexane and pure chloroform was 15 : 15 : 10 (v/v/v). The purpose of this extraction was to remove non-polar substances such as carotene and chlorophyll and water soluble compounds. The mixture was then transferred to centrifuge for 15 minutes at room temperature, 12,000 rpm in a Sorvall RC5C instruments using the Fiberlite F21-8×50y rotor Thermofisher USA. There were two layers were found after centrifuge, the upper layer contains methanol/water fraction where as the lower layer contained fraction of fucoxanthin. The fraction in the lower layer was completely dried by rotary evaporator by using cold water in order to prevent oxidation to reduce amount of fucoxanthin product. Then, the fucoxanthin fraction was freeze dried further to completely remove chloroform (Figure 11). The extracted fucoxanthin was then flushed with argon gas to prevent oxidation and store at -80°C until further purification.

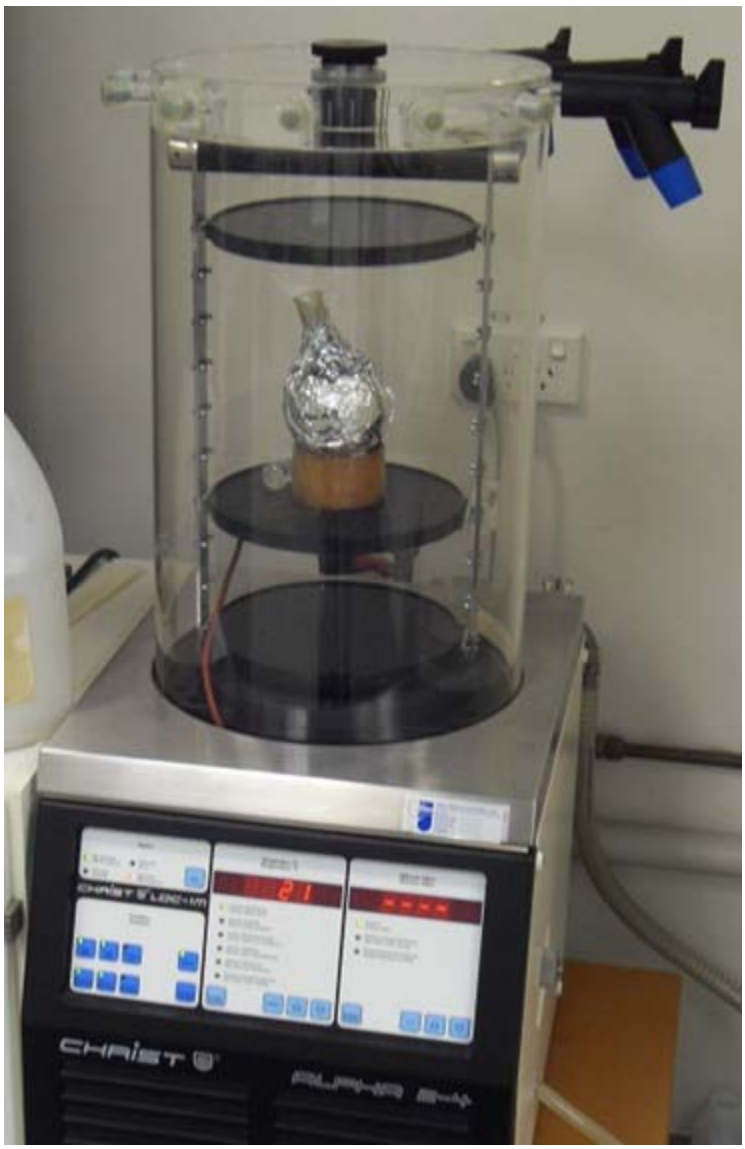

Figure 11: Freeze-drying the NZ crude fucoxanthin

2.3.3 Silica Gel Column Packing Preparation

The silica column needed to be set up for further purification of fucoxanthin extraction in order to increase purity of fucoxanthin for analysis. The 20 cm × 2.5 cm silica gel column was set up for fucoxanthin purification. A glass bulb with B24 quickfit top was used for fucoxanthin purification silica gel column. Approximate 1 cm of laboratory sand was added into the column. This column should be pre-rinsed with pure HPLC graded hexane as the major solvent for purification. Before adding silica into column, 10 cm of pure hexane was added to down side of the column with about 1 cm of laboratory sand.

About 40-50 ml of silica gel powder was measured in a fume hood and was then made into slurry by adding fresh pure hexane solvent. A layer of laboratory sand about 5mm was added to the top of the column in order to protect the top of the column. Another 5 to 10 cm of pure hexane column solvent should be added into the column to protect column for further use.

2.3.4 Silica Gel Column Chromatography Purification

Due to a higher polarity solvent, about 5 to 10 mL of pure benzene was used to dissolve the fucoxanthin sample from Section 2.3.2 Analytic Chemistry Extraction. The column was filled with 5 to 6 cm of hexane to allow crude fucoxanthin to be loaded into the column. There were four types of elution including pure hexane, about 300 mL of n-hexane/acetone (8:2, v/v) (which remove chlorophyll, carotenoids other than fucoxanthin and other impurities), about 400 mL of n-hexane/acetone (6:4, v/v) (which elute crude purified fucoxanthin out of the column) and acetone. Each fraction was collected into test tubes and the absorbance for each test tube was detected using a UV-visible spectrophotometer (UV-visible Amersham pharmacia Biotech, Ultrospec 2100) at 450nm. Fractions that have high absorbance values at 450nm were combined. This will be the First Fucoxanthin Extract (Figure 10 (2)). Half of the extract was dried using a rotary evaporator at 30 °C and freeze-dried in a freeze dryer overnight. The remaining half of the first fucoxanthin extract was run into the silica column repeating the procedure above to obtain the Second Fucoxanthin Extract (Figure 10 (3)). The second fucoxanthin extract was dried using a rotary evaporator and sample was freeze dried in a freeze dryer overnight. Before drying two fucoxanthin extracts above, the purified fucoxanthin was quantified using HPLC. Both first and second fucoxanthin extract were flushed with argon gas and stored in -80^{o}C freezer until further use.

2.3.5 HPLC Quantification for Fucoxanthin Extracted from New Zealand *U. pinnatifida*

HPLC as a quantification system consists of a LC-20AT pump system (Shimadzu), a UV-Vis SPD-20A (Shimadzu) absorbance detector and online analysis software (LC Solution version 1.25). The column for fucoxanthin quantification is a Luna 5µM C18 (4.6mm × 250mm, Phenomenex). The mobile phase was HPLC graded pure methanol at flow rate of 1ml/min. The volume of sample injection was 50µL. The wavelength for

fucoxanthin detection was set at 450nm (Jaswir, Noviendri, Salleh, Taher, & Miyashita, 2011).

2.3.5.1 Calibration Stock Solution

The stock solution of pure fucoxanthin as standard (0.1 mg/mL) was prepared by dissolving 5 mg of pure fucoxanthin in 50 mL pure methanol and stored in -80°C. The fucoxanthin working standards (0.0078µg/mL, 0.0156µg/mL, 0.0312µg/mL, 0.0625µg/mL and 0.125µg/mL) were prepared by a series of dilution, and standard solution was stored in -80°C. The stock solution of pure canthaxanthin as internal standard (0.1 mg/mL) was prepared by dissolving 5 mg of pure canthaxanthin in 50 mL pure acetone and stored in -80°C. The canthaxanthin working standards (0.0625µg/mL) were prepared by acetone dilution standard solution was stored in -80°C. Each concentration of fucoxanthin standard (0.5mL) was combined with internal standard canthaxanthin (0.0625µg/mL, 0.5mL). This mixture was then filtered through a 0.22µm membrane filter (Phenomenex) before each HPLC analysis. The mixed solution (fucoxanthin and canthaxanthin) was injected in HPLC in duplicates and average value was used to construct the quantification calibration curve.

The calibration curve constructed was

$$\frac{Peak\ Area\ of\ Standard}{Peak\ Area\ of\ Internal\ Standard}\ v.s.\ \frac{Concentration\ of\ Standard}{Concentration\ of\ Internal\ Standard}$$

The relationship was determined between ratio of peak area and ratio of concentration as an equation indicated above. Therefore, the concentration of extracted fucoxanthin can be determined from the relative ratio of peak area of analyte and peak area of internal standard.

2.3.5.2 HPLC Quality Control Parameters

Accuracy and Precision

Both accuracy and precision was achieved through the intra-assay and inter-assay. In order to achieve intra-assay accuracy and precision, five replicates of the extracted fucoxanthin from the same *U. pinnatifida* was quantitatively determined by HPLC instrument. The extraction method depended on the Methodology Section 2.3.2., 2.3.3., and 2.3.4 respectively. The HPLC quantitative method for extracted fucoxanthin depended on the Methodology section 2.3.5. After running five replicates, mean and

standard deviation of extracted fucoxanthin concentration was obtained, these two values were used to determine coefficient of variation. The calculate formula was indicated below:

$$\text{Coefficient of Variation} = \frac{\textit{Standard Deviation of Five Replicates}}{\textit{Mean of Five Replicates}} \times 100$$

The value from intra-assay contributed to accuracy and precision of this HPLC quantification method. The acceptable coefficient of variation for intra-assay should be less than 10%, and in this study the coefficient variation of intra-assay around the mean observed sample concentration was 9.21%.

In order to determine inter-assay accuracy and precision, the experiment was then performed for other four days with the same method, the extract was obtained from the same *U. pinnatifida* sample. Constantly, the mean and standard deviation was used to calculate the coefficient of variation. The value from inter-assay contributed to the reproducibility of method between days, and this value indicates the method can be used and repeated between days. The acceptable inter-assay of coefficient variation should be less than 15%, and the coefficient of variation of inter-assay around the mean observed in the samples in different day was 12.96%.

Recovery

A spike and recovery experiment was determined to measure the extraction efficiency of the fucoxanthin method used in this study. The pure fucoxanthin was used to make a fucoxanthin standard solution with concentration in 2mg/mL by HPLC graded pure methanol. This standard solution was added into five fucoxanthin extracts replicates with known concentration. The standard (125μL) was added into 100 mg of freeze dried *Undaria* sample and mixed with 15 mL of methanol, and this mixture was completely sealed with mechanic stirring using a magnetic bar for 2 days at the room temperature in dark condition. The fucoxanthin then was extracted by using the Methodology Section 2.3.2., 2.3.3., and 2.3.4 respectively. The HPLC quantitative method for extracted fucoxanthin depended on the Methodology Section 2.3.5.

The recovery was expressed as a percentage and calculated as equation below

$$\textit{Recovery} = \frac{C_{spike} - C_{sample}}{C_{added}} \times 100$$

In this equation, C_{spike} was the concentration of the analyte determined from the spike and recovery experiment. C_{sample} was about the concentration of the known extracted

fucoxanthin analyte. C_{added} was about the total concentration of the standard added and the concentration the known extracted fucoxanthin analyte.
The recovery of this method was 75%, indicating reproducibility of the procedure.

Instrument Error

The fucoxanthin pure standard concentration at 0.0078µg/mL was used to determination of quantification HPLC instrument error. This standard concentration was run by HPLC for five times, and the coefficient of variation was determined to indicate as the instrument error. In this study, the coefficient of variation in each run was 5%, this indicated the accuracy and precision of the HPLC instrument.

2.3.6 Purity Calculation for New Zealand *U. pinnatifida* Extraction Fucoxanthin

Based on a linear relationship as indicated above between $\frac{Peak\ Area\ of\ Standard}{Peak\ Area\ of\ Internal\ Standard}$ and $\frac{Concentration\ of\ Standard}{Concentration\ of\ Internal\ Standard}$, the concentration of extracted fucoxanthin was calculated by this linear relationship. For fucoxanthin extract from NZ *U. pinnatifida*, the percentage of fucoxanthin (purity) was also obtained in Table 6.

Table 6: Purity of fucoxanthin from NZ *U. pinnatifida*

Percentage (Purity) of Fucoxanthin from NZ *U. Pinnatifida*		
Extract	**HPLC Area Value**	**Purity of Fucoxanthin**
Crude *U. pinnatifida* Extract	3,321,697	0.20%
First Fucoxanthin Extract	226,331	43.50%
Second Fucoxanthin Extract	188, 962	60.77%

PART II: Materials and Methods for Fucoxanthin (Purity: 4%), Fucoidan (Low Molecular Fraction) Extraction from Japanese *U. pinnatifida*

This section was referenced from the publication by Quitain et al in 2012. In this section, the two bioactive compound extracts fucoxanthin (Purity: 4%) and crude fucoidan (Low Molecular Fraction) were provided by Dr. Armando T. Quitain from Kumamoto University in Japan. The *U. pinnatifida* sample preparation and extraction for those two compounds was performed by him at the Graduate School of Science and Technology in Kumamoto University in Japan.

2.4 Materials

2.4.1 Sample Collection and Preparation

U. pinnatifida was provided by ISekonbu Co. Ltd, Mie, Japan. Dry *U. pinnatifida* samples were grounded using a IKA-Werke mill (MF 10B S1, Germany), and then sieved using a mesh size of 60 (Quitain et al., 2012).

2.5 Extraction Methods for 4% of Fucoxanthin & Fucoidan from Japanese *U. pinnatifida*

The Japanese extraction was a single step combining three different methods including supercritical fluid extraction (SFE), pressurized liquid extraction (PLE) and microwave-assisted extraction (MAE) (Figure 12). This single extraction method led to extract two important bioactive compounds fucoxanthin and fucoidan from Japanese *U. pinnatifida*. The first extraction was a process of Japanese crude fucoxanthin extraction, and this extraction process used supercritical liquid carbon dioxide. The supercritical carbon dioxide was obtained by high temperature and high pressure. This extraction method depended on methods including supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) respectively.

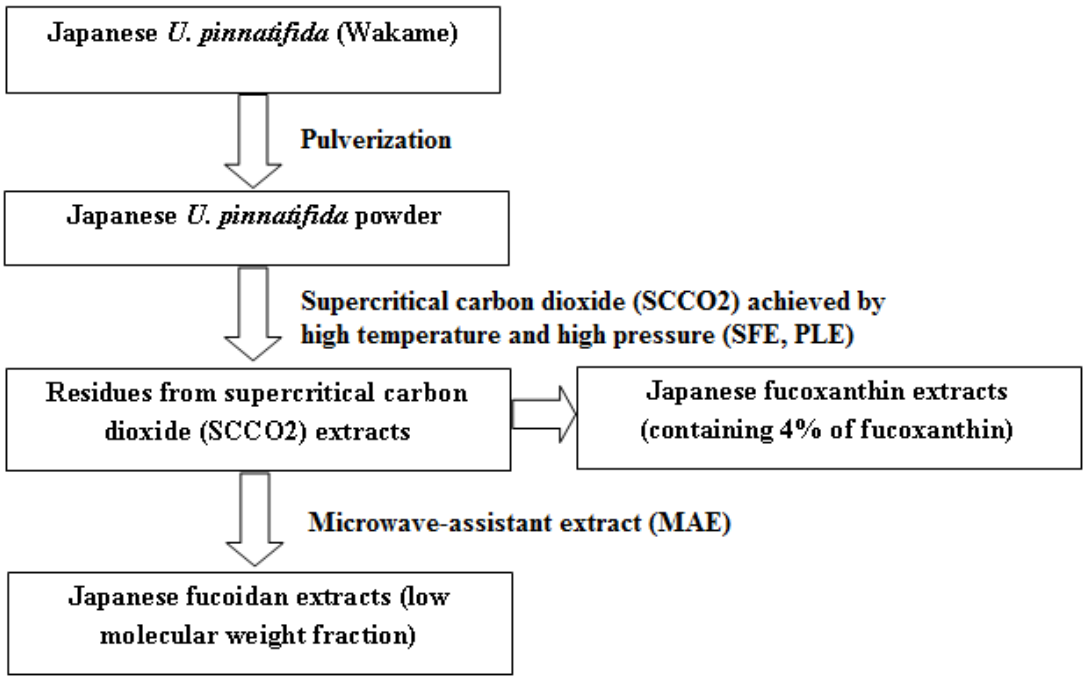

Figure 12: A single process of combination supercritical fluid extract (SFE), pressurized liquid extract (PLE) and microwave-assisted extraction (MAE) for fucoxanthin and fucoidan extract from Japanese *U. pinnatifida* powder (Redrawn: Kai, Quitain, et al 2012, March)

In the following extraction, the Japanese fucoidan in low molecular weight fraction was obtained by the use of water in high temperature by microwave energy. This superheating to water was based on the polarity of water. The subcritical water was formed by microwave radiation under high pressure. Hydrothermal synthesis contributes to a single compound extraction depends on the solubility in hot water. Therefore it was simply said the crude fucoidan extracts in low molecular fraction was obtained by subcritical water, and the extraction was controlled to generate by a gradient of temperature.

2.5.1 Fucoxanthin (Purity: 4%) Extraction Method by Supercritical Carbon Dioxide

The supercritical carbon dioxide extractions were achieved in a semi-continuous flow-type apparatus. Carbon dioxide gas was supplied by CO_2 pump where a chiller was connected to decrease CO_2 temperature down to -5°C. This chiller contributed to cool CO_2 gas in liquid form at -5°C with HPLC high pressure. The CO_2 liquid gradually was injected into oven with flow rate at 4 mL/min. The *U. pinnatifida* powder was loaded into cell in the middle of the extractor where the remaining top and bottom section were

trapped with cotton (Quitain et al., 2012). The extraction was placed in the oven in order to maintain the operation temperature at 40°C with high pressure at 40MPa (Quitain et al., 2012). The high pressure and heating made liquid CO_2 to generate supercritical CO_2 for fucoxanthin extraction. The extraction process was performed in 3 hours and extraction of 180 mins was the best extraction time for the highest fucoxanthin recovery at 80% (Quitain et al., 2012).

2.5.2 Fucoidan (Low Molecular Weight Fraction) Extraction Method by Microwave-Hydrothermal Synthesis from Japanese *U. pinnatifida*

The extraction procedure for the fucoidan in low molecular weight fraction was quite simple. The residue from supercritical carbon dioxide extraction was placed in Teflon vessel reactor. The treatment time and temperature was set up by controller. After treatment time, the crude fucoidan extracts in aqueous mixture were separated from the solid residues by centrifuge (Quitain et al., 2012). A treatment time of 30 minutes was the most appropriate time to obtain the fucoidan fraction between 5-30kDa in the temperature of 140°C (Quitain et al., 2012).

PART III: Cancer Cell Lines and MTT Cell Viability Assay

2.6 Materials

2.6.1 Cancer Cell Lines

Table 7: Nine cancer cell lines

Cancer Cell Line			
Number	**Cancer Cell Line Designation**	**ATCC Catalog No.**	**Cancer Cell Line Description**
1	A-549	CCL-185	A549; Lung Carcinoma; Human
2	WiDr	CCL-218	WiDr; Colon Adenocarcinoma; Human
3	Lovo	CCL-229	Lovo; Colon Adenocarcinoma; Human
4	NCI-H522	CRL-5810	NCIH522; Human Lung
5	Hep G2	HB-8065	Hep G2; Hepatocellular Carcinoma; Human
6	SK-N-SH	HTB-11	SK-N-SH; Neuroblastoma
7	MCF-7	HTB22	MCF; Breast Adenocarcinoma; Human
8	SiHa	HTB-35	SIHA; Cervix Squamous Carcinoma Human
9	Malme-3M	HTB-64	Malme-3M; Malignant Melanoma; Human

Cancer cell lines (Table 7) employed in this study were purchased from ATCC (Cryosite Ltd, NSW, AU). The cell lines were maintained in 25 cm^2 tissue culture flask containing 5 mL of completed growth culture medium consisting of RPMI 1640 culture medium with 1% of Penicillin-Strptomycin, 1% of L-glutamine and 10% of fetal bovine serum except Malme-3M (20% of fetal bovine serum). All cancer cell lines were maintained in tissue culture incubator at 37°C in 5% carbon dioxide humidified air.

2.6.2 Cell Culture Experiment Materials & Reagents

Cell culture medium, L-glutamine-200mM (100×) liquid, Penicillin-Strptomycin (100mL), Trypan Blue Stain, TrypLE™ Express Stable Trypsin Replacement Enzyme without phenol Red and cryotube (1.8 mL) were supplied from Invitrogen NZ Limited.

Sterile filtered fetal bovine serum (FBS) was purchased from Medica Pacifica (Auckland, NZ).

All cell culture flasks, sterile disposable pipette tips, centrifuge tubes, 96-well tissue culture plates were supplied from BD (Becton Dickinson) Bioscience (Auckland, NZ).

2.6.3 Cell Viability Assay Materials

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide], Cat No. M2128-1G, was supplied by Sigma (St Louis, USA).

DMSO (Dimethyl sulfoxide), Cat No. 1029521000 was purchased by Merck-Chemicals.

PBS (Phosphate buffered saline) pH at 7.2, composed of potassium phosphate monobasic (KH_2PH_4, 1, 440mg/L), sodium chloride (NaCl, 90, 000 mg/L), and sodium phosphate dibasic ($Na_2HPO_4.7H_2O$, 7, 950mg/L). The preparation of PBS was described in the following section 2.7.3.

2.7 Methods

2.7.1 Pure Fucoxanthin Standard Preparation

20 mM of pure fucoxanthin ethanolic stock solution for Culture Plan 1 was made by 6.59mg of pure fucoxanthin powder standard dissolving in 500µL of biological pure ethanol.

5 mM of pure fucoxanthin ethanolic stock solution for Culture Plan 2 was made by 6.59mg of pure fucoxanthin powder standard dissolving in 2mL of biological pure ethanol.

After that, the ethanolic solution was thoroughly vortexed to dissolve fucoxanthin completely in ethanol.

2.7.2 Extraction Preparation for Inhibition Effects Determination

2.7.2.1 Fucoxanthin Extraction Sample Preparation

The fucoxanthin ethanolic stock solution concentration was 5mM. Based on these values, the amount of ethanol was added in each extract as indicated in Table 8 below,

Table 8: Amount of NZ & Japanese fucoxanthin extracts, and amount of pure ethanol added to obtain 5mM ethanolic fucoxanthin contained in each extract

Extract	Amount of Fucoxanthin Extracts	Purity of Fucoxanthin	Amount of Ethanol Added
Crude *U. pinnatifida* Extract	329.45mg (Each cell line)	0.20%	200 µL (Each cell line)
First Fucoxanthin Extract	66.60mg	43.50%	8.794 mL
Second Fucoxanthin Extract	39.80mg	60.77%	7.341 mL
Japanese Fucoxanthin Extract	16.4mg (Each cell line)	4%	200 µL (Each cell line)

A series of working solutions for cell viability experiments were prepared by diluting ethanolic stock solution with culture medium.

2.7.2.2 Japanese Fucoidan Crude Fraction (Low Molecule Fraction) Sample Preparation

Due to high solubility, the crude fucoidan fraction 40 mg was directly dissolved in 5 mL of culture medium as a fucoidan stock solution (8mg/mL). The fucoidan stock solution was filtered through a Millex GV 0. 22µm sterile filter to remove any pathogens or undissolved particles.

2.7.3 Preparation of Phosphate Buffered Saline (PBS) pH at 7.2

The phosphate buffered saline (PBS, 10×) was composed of potassium phosphate monobasic (KH_2PH_4, 1, 440mg/L), sodium chloride (NaCl, 90, 000 mg/L), and sodium phosphate dibasic ($Na_2HPO_4.7H_2O$, 7, 950mg/L). The phosphate buffered saline (1×) with pH 7.2 was prepared by making 1 in 10 dilution of phosphate buffered saline (10×) in millipore water. The pH value was adjusted to 7.2 by using 1M sodium hydroxide (NaOH) or 1M hydrochloric acid (HCl).

2.7.4 Preparation of MTT

5mM of MTT solution was prepared in the phosphate buffered saline (PBS). Before use, the 5mM MTT phosphate buffered saline was filter through a sterile Millex GV 0.22µm syringe filter to remove any pathogens, undissolved MTT and any spontaneously formed formazan crystals.

2.7.5 Method for Cell Viability MTT Assay

2.7.5.1 General MTT Assay Protocol

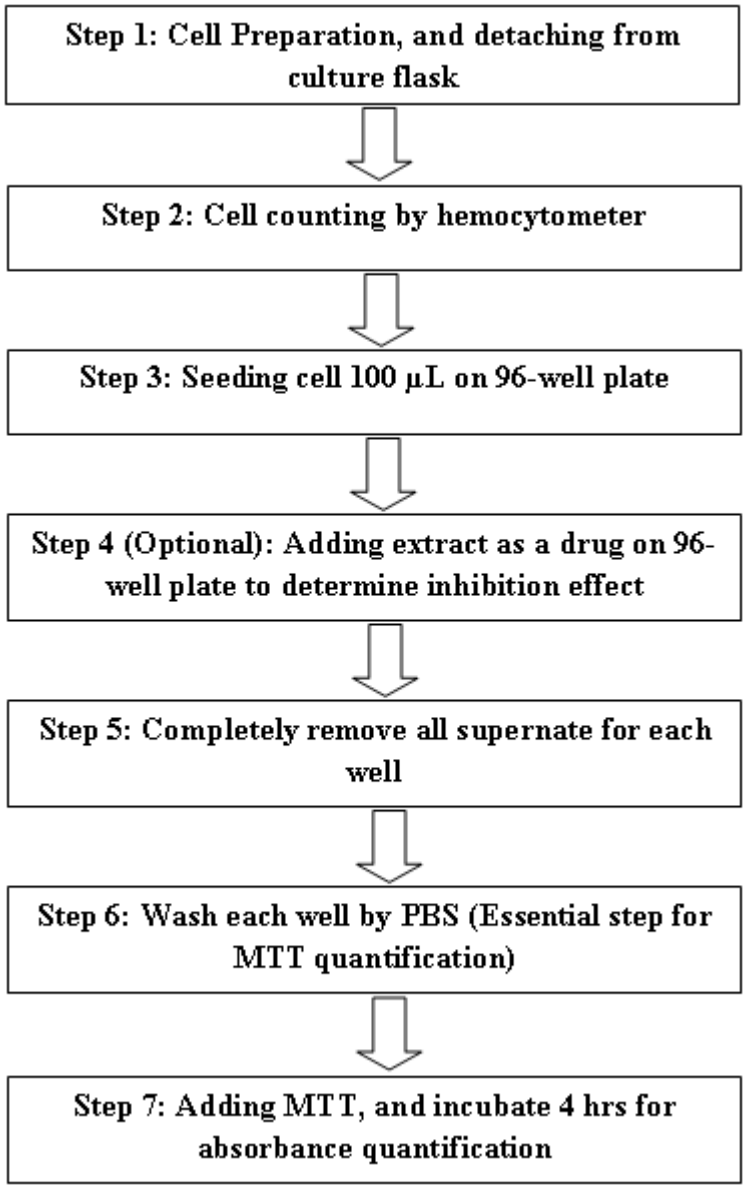

Figure 13: Diagram for general MTT assay protocol

The general MTT assay protocol is described in diagram in above (Figure 13).

Step 1: Cell Preparation

The old culture medium was carefully removed with the aid of pipette. 5mL of sterile pre-warmed PBS was used to wash cancer cells. Approximately 2.5 mL of TrypLE^{TM} Express solution was used to treat cells for no more than 15 minutes in cell incubator to

detach cells. 5-10 mL of completed growth medium was then added into culture flask to stop trypsinization. Solution was transferred into 15 mL centrifuge tube, and centrifuged (HITACHI) at 125*g* x for 5-7 mins. The supernante was then carefully removed and 1 mL of new completed culture medium was added into tube to re-suspend cells gently but thoroughly.

Step 2: Cell Counting

10 µL of the cell suspension was placed onto a piece of parafilm and mixed thoroughly with 10 µL of Trypan Blue. 10 µL of this mixture was then placed to one side of the hemocytometer and the number of cells was determined under the microscope. After counting at least four squares, average number of cells was generated. The total number of cell in 1 mL culture medium was calculated by using the formula below:

$$\text{Average number of cells per square} \times 2 \times 10^4 = \text{number of cells/ml}$$

Step 3: Seeding Cells

Based on cell concentration, the supplement of complete culture medium was applied to make preferred cell concentration for cell seeding on 96-well plates. A detailed description of cell seeding for preparation of cell standard curve and anti-proliferative effect determination will be discussed below (Section 2,7,5,2,; 2.7.5.3.).

Step 4: Adding Extracts as the Treatment

This step was an optional step, and only applied to determine anti-proliferative effect. After cell seeding, 100µL of pure standard or *U. pinnatifida* extracts as treatment with various dilution was added to a 96-well plate to determine the anti-proliferative effects. A detailed description of the process to add pure standard or *U. pinnatifida* extracts will be discussed below (Section 2.7.5.3.).

Step 5: Removing Supernante

After cell seeding onto the 96-well plate, the plate was incubated for at least 4 hours to enable recover and reattach of cells to the plate. All supernante in each well was completely removed including culture medium and extract solution.

Step 6: Washing by PBS

This was an essential step for MTT quantification. An aliquot of 150 µL of sterile PBS was used to wash each well to remove the colour or precipitate of extract from each well.

Another 100 µL of completely culture medium was added to each well after washing with PBS.

Step 7: Quantification

An aliquot of 20 µL of MTT reagent was added into each well. The whole plate was incubated in cell incubator for at least 4 hours at 37°C. When purple precipitate was clearly visible under inverted microscope, 150µL of dimethyl sulfoxide was added into each well to dissolve all purple precipitate. Before adding dimethyl sulfoxide, 100 µL supernant in each well were carefully removed. 96-plate well was then incubated for 10 to 20 minutes further to ensure all precipitate to be completely dissolved. The UV absorbance (OD value) was measured by a plate reader (FLUOstar Omega, Alphatech) at the wavelength of 540nm with reference wavelength at 680nm. The average absorbance value (OD value) was determined from triplicate readings, and then these average values subtracted the average value from the blank for final recording.

For cell linearity standard curve, plot absorbance (OD value) graph was plotted on the y-axis versus cell number per mL on the x-axis. The cell number selected should fall within the linear portion of the curve.

For dose dependent anti-proliferative effect curve, the inhibition rate (IR) was plotted on the y-axis aganist log each extract concentration (µM for fucoxanthin extract concentration; mg/mL for crude fucoidan concentration) on the x-axis. The inhibition rate (IR) calculation was described in the following Section 2.7.6 in detail.

2.7.5.2 Linearity of MTT Assay

The cell number vs absorbance standard curve can be generated to determine cell growth or proliferation under various living conditions. This is to determine the MTT assay linearity as cell number used in cell viability studies should fall within the linear portion of the curve. After a series one in two (1:2) dilution of cells in culture medium, an aliquot of 100µL cells were seeded in each well in triplicates into a 96-well plate.

There were two dilution plans (Table 9 & 10) to generate cancer cell growth curve, depending on cancer cell types. After seeding for 18 hours, the step 7 was undertaken to measure absorbance at 540nm. A linear relationship between absorbance and cell number was generated by plotting absorbance on Y-axis against cancer cell numbers at X-axis.

Table 9: The first dilution plans for construction of cell linearity standard curve

Plan 1: Cancer Cell Line Growth Curve Determination on 96-Well Plate			
Number	**Label Tubes (Cells/mL)**	**Cell Number per Well**	**Cell Culture Concentration**
1	800,000	80,000	200 µL of 8×10^5 cells/mL stock
2	400,000	40,000	200 µL of 4×10^5 cells/mL stock
3	200,000	20,000	200 µL of 2×10^5 cells/mL stock
4	100,000	10,000	200 µL of 1×10^5 cells/mL stock
5	50,000	5,000	200 µL of 5×10^4 cells/mL stock
6	25,000	2,500	200 µL of 2.5×10^4 cells/mL stock
7	12,500	1,250	200 µL of 1.25×10^4 cells/mL stock
8	6,250	625	200 µL of 6.25×10^3 cells/mL stock
9	3,130	313	200 µL of 3.13 × 103 cells/mL stock

Table 10: The second dilution plans for construction of cell linearity standard curve

Plan 2: Cancer Cell Line Growth Curve Determination on 96-Well Plate			
Number	**Label Tubes (Cells/mL)**	**Cell Number per Well**	**Cell Culture Concentration**
1	1.25×10^6	125,000	200 µL of 8×10^5 cells/mL stock
2	6.25×10^5	62,500	200 µL of 4×10^5 cells/mL stock
3	3.13×10^5	31,300	200 µL of 2×10^5 cells/mL stock
4	1.56×10^5	15,600	200 µL of 1×10^5 cells/mL stock
5	7.81×10^4	7,810	200 µL of 5×10^4 cells/mL stock
6	3.91×10^4	3,910	200 µL of 2.5×10^4 cells/mL stock
7	1.95×10^4	1,950	200 µL of 1.25×10^4 cells/mL stock
8	9.77×10^3	977	200 µL of 6.25×10^3 cells/mL stock
9	4.88×10^3	488	200 µL of 3.13 × 103 cells/mL stock

2.7.5.3 Method to Anti-proliferative Effect Determination from Pure Fucoxanthin Standard, New Zealand Extracted Fucoxanthin & Japanese Crude Fucoxanthin

Seeding Cell Procedure for Anti-proliferative Effect Determination

The optimized cell density was 5, 000 cells/well for A549 and WiDr, while for Hep G2, Malme-3M, SiHa, NCI-H522, Lovo, SK-N-SH, and MCF-7, the cell density was 10,000 cells/well. Each well contained 100μL of cancer cells.

Adding Pure Fucoxanthin Standard or Extracts as a Treatment for Anti-proliferative Determination

An aliquot of 100μL culture medium containing pure fucoxanthin or fucoxanthin extracts at different concentrations was added into each well, after cancer cells completely re-attached on the 96-well plate about 4 to 6 hours. There were four 96-well plates that could be used for each types of cell with the same series of concentration treatment. The four 96-well plates indicated treatment in each day including Day 0, Day 1, Day 2, and Day3 respectively (Figure 14). Then, MTT assay was performed at designated time points. This was to determine whether pure fucoxanthin standard or fucoxanthin extracts could generate anti-proliferative effects to nine cancer cell lines in dose- and time- dependent manners.

The UV absorbance (OD) values from MTT assay were used to determine the IC_{50} value. The effective concentration necessary for 50% growth inhibition (IC_{50}) represents the effective concentration of drug which gives 50% inhibition of the maximum achieved. The IC_{50} calculation method was described in Section 2.7.6 in detail.

IC_{50} determination by pure fucoxanthin was the most important value for intra-comparison among nine cancer cell lines and inter-comparison with NZ extracted fucoxanthin and Japanese extracted crude fucoxanthin. For intra-comparison, the statistical comparison about IC_{50} value obtained from pure fucoxanthin treatment was used to rank the anti-proliferative sensitivity of fucoxanthin to nine cancer cell lines. In addition, the statistical comparison between IC_{50} value from treatment of pure fucoxanthin standard and the IC_{50} value from other extract treatment was used to determine whether novel compounds is present in *U. pinnatifida*.

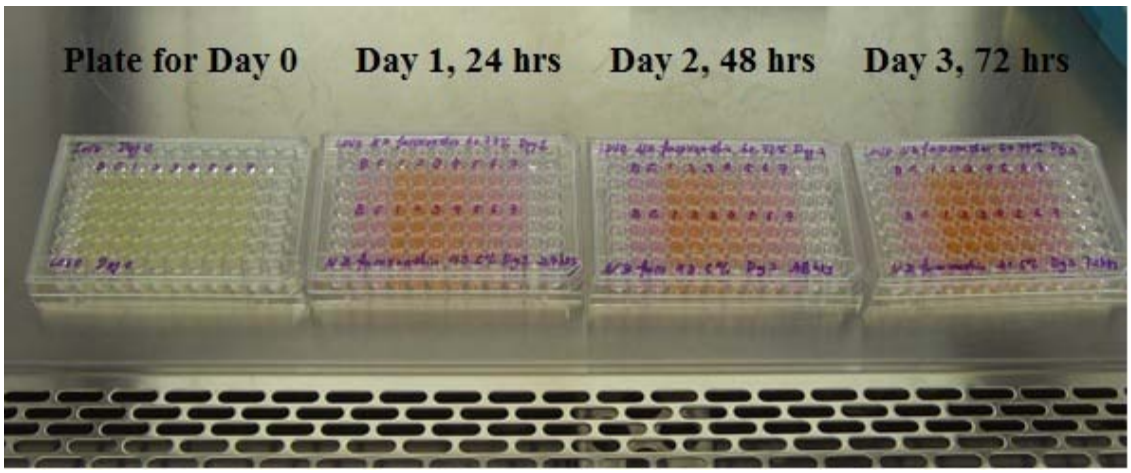

Figure 14: Human colon adenocarcinoma Lovo involved in Dose-Dependence in Time Manner Inhibition Determination

The First Fucoxanthin Concentration Culture Plan (Culture Plan 1)

The fucoxanthin ethanolic stock solution was 20mM, and the fucoxanthin culture plan and relative ethanol testing concentration plan are shown in Table 11 & Table 12.

Table 11: First fucoxanthin culturing plan

The First Plan: Fucoxanthin Inhibition Effect to Cancer Cells Concentration			
Number	**Test Concentration**	**Medium Concentration**	**Stock Solution Concentration**
1	200 μM	400 μM	20 mM
2	150 μM	300 μM	15 mM
3	100 μM	200 μM	10 mM
4	50 μM	100 μM	5 mM
5	25 μM	50 μM	2.5 mM
6	12.5 μM	25 μM	1.25 mM
7	6.25 μM	12.5 μM	0.625 mM
8	1.5625 μM	3.125 μM	0.15625 mM
9	0.391 μM	0.78125 μM	0.0391 mM

1: Test Concentration: Fucoxanthin Inhibition Test Concentration on 96-plate well
2: Medium Concentration: Fucoxanthin Diluted Concentration by Culture Medium
3: Stock Solution Concentration: Fucoxanthin Powder dissolved in Pure Biological Ethanol as the fucoxanthin stock solution

Table 12: Ethanol effect test from the first fucoxanthin culturing plan on cancer cell line

The First Plan: Ethanol Effect Test Concentration			
Number	**Test Concentration**	**Medium Concentration**	**Stock Solution Concentration**
1	1%	2%	100%
2	0.75%	1.5%	75%
3	0.50%	1%	50%
4	0.25%	0.5%	25%
5	0.125%	0.25%	12.5%
6	0.0625%	0.125%	6.25%
7	0.03125%	0.0625%	3.125%
8	0.00781%	0.01562%	0.781%
9	0.001953%	0.003906%	0.019530%

1: Test Concentration: Ethanol Concentration to Dissolve Fucoxanthin on 96-well plate
2: Medium Concentration: Ethanol Concentration Diluted by Culture Medium
3: Stock Solution Concentration: Ethanol Concentration in Fucoxanthin Stock Solution

For the second plan (Culture Plan 2)

The fucoxanthin ethanolic stock solution was 5 mM, and the fucoxanthin culture plan and relative ethanol testing concentration culture plan are shown in Table 13 & Table 14.

Table 13: Second fucoxanthin culturing plan

The Second Plan: Fucoxanthin Inhibition Effect to Cancer Cells Concentration			
Number	**Test Concentration**	**Medium Concentration**	**Stock Solution Concentration**
1	100 μM	200 μM	5 mM
2	80 μM	160 μM	4 mM
3	50 μM	100 μM	2.5 mM
4	25 μM	50 μM	1.25 mM
5	12.5 μM	25 μM	0.625 mM
6	6.25 μM	12.5 μM	0.3125 mM
7	1.5625 μM	3.125 μM	0.078125 mM

1: Test Concentration: Fucoxanthin Inhibition Test Concentration on 96-well plate
2: Medium Concentration: Fucoxanthin Diluted Concentration by Culture Medium
3: Stock Solution Concentration: Fucoxanthin Powder dissolved in Pure Biological Ethanol as the fucoxanthin stock solution

Table 14: Ethanol effect test from the second fucoxanthin culturing plan on cancer cell line

The Second Plan: Ethanol Effect Test Concentration			
Number	**Test Concentration**	**Medium Concentration**	**Stock Solution Concentration**
1	2%	4%	100%
2	1.6%	3.2%	80%
3	1%	2%	50%
4	0.5%	1%	25%
5	0. 25%	0.5%	12.5%
6	0.125%	0.25%	6.25%
7	0.03125%	0.0625%	1.5625%

1: Test Concentration: Ethanol Concentration to Dissolve Fucoxanthin on 96-well plate
2: Medium Concentration: Ethanol Concentration Diluted by Culture Medium
3: Stock Solution Concentration: Ethanol Concentration in Fucoxanthin Stock Solution

2.7.5.4 Method to Anti-proliferative Effect Determination from Japanese Extracted Crude Fucoidan (Low Molecular Fraction)

Step 4: Adding Japanese Fucoidan in Low Molecular Fraction as a Drug for Inhibition Determination

Due to high solubility of the Japanese crude fucoidan, it was directly dissolved in culture medium to make stock solution concentration as 8mg/mL. The anti-proliferative effects from the Japanese crude fucoidan were determined as shown in Table 15.

Table 15: Test concentration: Japanese fucoidan inhibition test concentration on 96-well plate

Fucoidan Inhibition Effect to Cancer Cells Concentration			
Number	**Test Concentration**	**Medium Concentration**	**Stock Solution Concentration**
1	4 mg/mL	8 mg/mL	8 mg/mL
2	3 mg/mL	6 mg/mL	6 mg/mL
3	2 mg/mL	4 mg/mL	4 mg/mL
4	1 mg/mL	2 mg/mL	2 mg/mL
5	0.5 mg/mL	1 mg/mL	1 mg/mL
6	0.25 mg/mL	0.5 mg/mL	0.5 mg/mL
7	0.125 mg/mL	0.25 mg/mL	0.25 mg/mL
8	0.03125 mg/mL	0.0625 mg/mL	0.0625 mg/mL
9	0.0078125mg/mL	0.015625mg/mL	0.015625mg/mL

2.7.6 IC_{50} Calculation Method

This section was to describe calculation method for IC_{50} after culturing cancer cell lines to pure fucoxanthin standard, NZ *Undaria* & fucoxanthin extracts, Japanese fucoxanthin extract and Japanese crude fucoidan (low molecular weight fraction).

After absorbance OD values obtained, the IR of cancer cell proliferation was calculated (N. Li, 2004), and then IC_{50} was calculated by modified Karber method (Mu, 2007). The IR and IC_{50} calculation equation were in the below:

$$IR\ (\%) = \frac{1 - OD\ of\ Treatment\ Group}{OD\ of\ Control\ Group} \times 100\%$$

IR was inhibition rate. The treatment group: OD absorbance values from cancer cell lines involving treatment of pure fucoxanthin standard, NZ *Undaria* extracts, the first & second fucoxanthin extracts and Japanese fucoxanthin extracts. The control group meant OD absorbance values from cancer cell lines without any standard or extracts treatment.

$$IC_{50} = \log_{10}[Xm - I \times (P - \frac{3 - Pm - Pn}{4})]$$

Xm was logarithm of maximum dose. I was difference of the two adjacent logarithmic dose. P was the sum of positive reaction rate. Pm was maximum inhibition rate. Pn was minimum inhibition rate.

2.8 Statistical Analysis

All results were examined by statistic software Minitab® (Version 16). Two-way ANOVA Tukey's multiple comparison test was carried out to examine the quantitative anti-proliferative data for anti-proliferative effects in dose-dependent and time manners for each cancer cell lines. Compared to control group (the group without standard or extract treatment added), the anti-proliferative effects in dose and time-dependence were statistically demonstrated if *p*-values from Minitab output were less than 0.05. To compare about treatments, one way ANOVA with Tukey's comparison test was employed to determine the significant difference between anti-proliferative data from the pure fucoxanthin standard and all extracts including crude *U. pinnatifida* extract,

first fucoxanthin extract, second fucoxanthin extract, the Japanese extracted fucoxanthin, and crude fucoidan extracts (low molecular weight fraction).

All data analysed using ANOVAs met the assumptions of equal variance and homogeneity using Minitab® (Version 16).

Chapter 3 Results

3. Results

3.1 Result of Fucoxanthin Quantification HPLC Calibration & Fucoxanthin Extraction Quantification

3.1.1 Result of Fucoxanthin Quantification HPLC Calibration

The fucoxanthin calibration results are shown (Table 16), and relative calibration curve are shown in (Figure 15). The HPLC chromatography result was shown in (Figure 16), there were three peaks in the HPLC chromatography result, the peak corresponding to all-*trans*-fucoxanthin were found at the retention time of 1.8 min, and the *cis*-isomer of fucoxanthin was found at the retention time of 2.4 min. The peak at around retention time of 4.2 mins was the internal standard, canthaxanthin.

Table 16: HPLC chromatography result for fucoxanthin calibration; pure fucoxanthin as standard, unit: mg/mL, canthaxanthin as internal standard, unit: mg/mL

Fucoxanthin Quantification Calibration Curve	
Fucoxanthin Concentration, mg/mL	**Fucoxanthin Area Value**
0.00125	3089588
0.000625	1563623
0.000313	820280
0.000156	421316
0.000078	213253

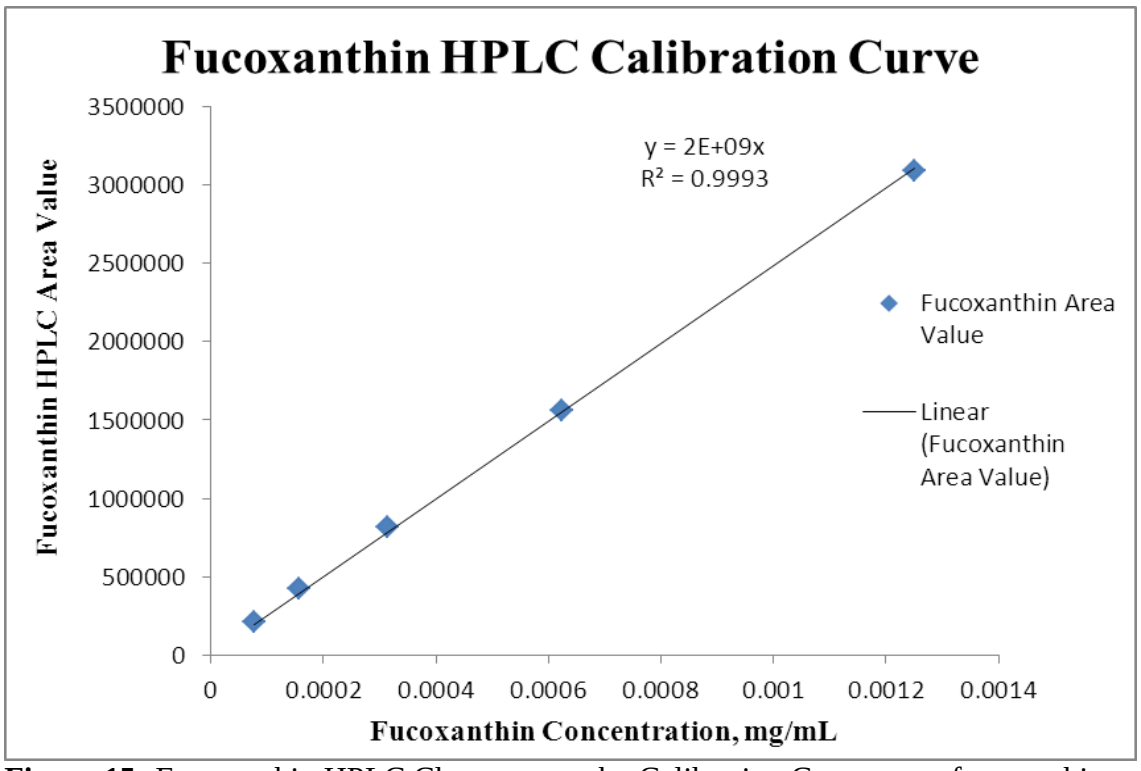

Figure 15: Fucoxanthin HPLC Chromatography Calibration Curve: pure fucoxanthin as standard, unit: mg/mL; canthaxanthin as internal standard, unit: mg/mL

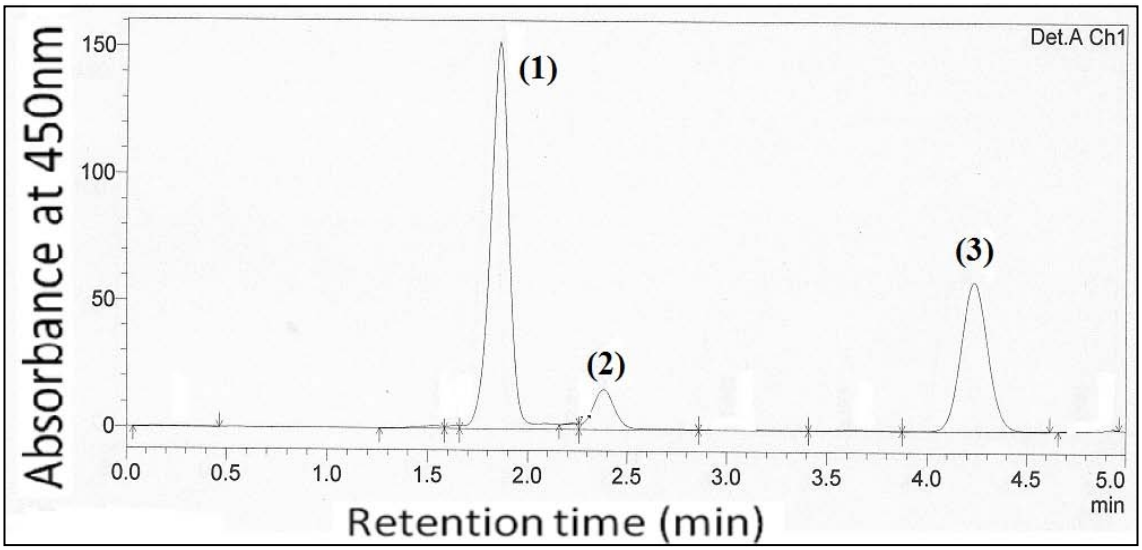

Figure 16: HPLC Chromatography Result for Fucoxanthin Calibration Result: (1) Pure all-*trans*-Fucoxanthin, (2) *cis*-isomer of Fucoxanthin, (3) Canthaxanthin, Internal Standard

3.1.2 Result of Quantification of Fucoxanthin Extraction from New Zealand *U. pinnatifida*

The "NZ fucoxanthin" was extracted from combination blade and sporophyll of NZ *U. pinnatifida*, and it was detected for quantification by using the UV-VIS spectrum at

450nm. After running HPLC for internal standard, canthaxanthin concentration at 0.0625µg/mL was most suitable for HPLC calibration and quantification.

3.1.2.1 Detailed Result for HPLC Quantification Result for "Crude *U. pinnatifida* Extract"

The area value of all *trans*-fucoxanthin was 3,321,697, and the purity of crude fucoxanthin was 0.2%. In order to make 5mM of fucoxanthin (as the NZ fucoxanthin stock concentration), 10.59 mL of pure biological ethanol was added into solution. This was sealed and stored in the -80°C freezer for further experiments.

3.1.2.2 Result for "First Fucoxanthin Extract" after Purification (the First Time) by Silica Gel Chromatography

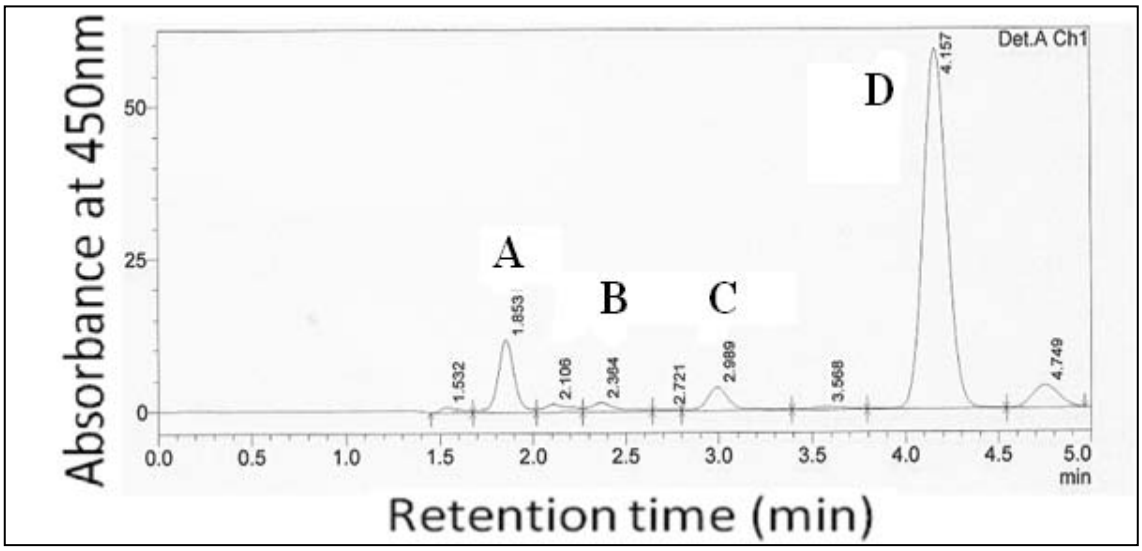

Figure 17: 43.5% of Fucoxanthin Quantification HPLC Chromatography Result. (A) All-*trans*-Fucoxanthin with Dilution Factor in Methanol at 12,800, (B) *cis*-isomer of Fucoxanthin, (C) Unidentified Peak, (D) Canthaxanthin, Internal Standard

The area value of all *trans*-fucoxanthin was 226,331 as showed in HPLC result in Figure 17, and the purity of the first purified fucoxanthin fraction was 43.5%. In order to make 5mM of fucoxanthin ethanolic stock solution (as the "the First NZ Fucoxanthin extracts" stock solution), the freezing dried first NZ fucoxanthin extracts dissolved in 8.794mL of pure biological ethanol. This fucoxanthin ethanolic solution was sealed and stored in the -80°C freezer for further experiments.

3.1.2.3 Result for "Second Fucoxanthin Extract" after Purification (the Second Time) by Silica Gel Chromatography

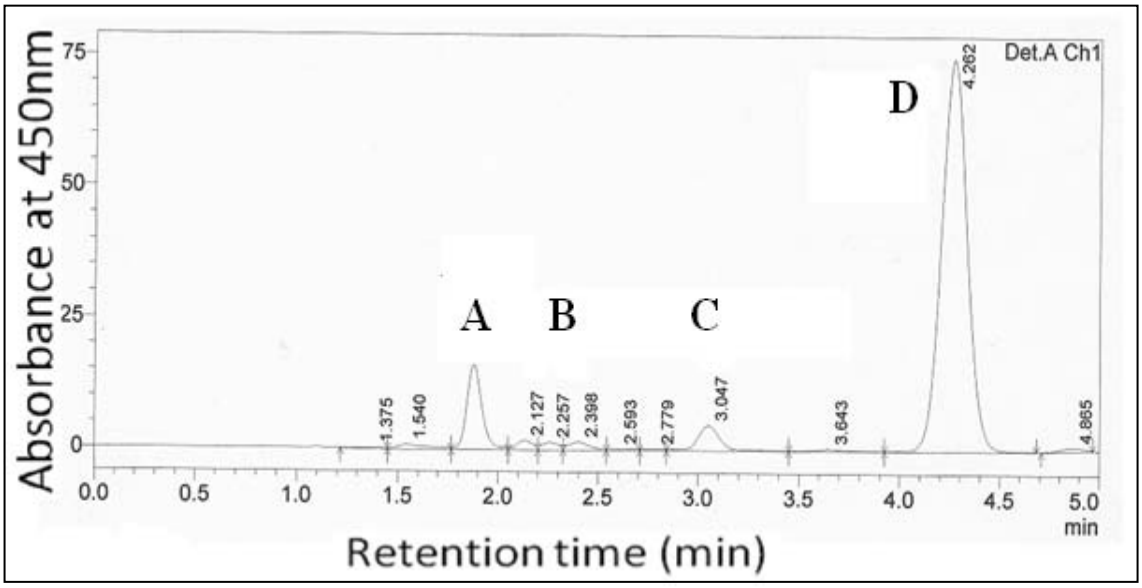

Figure 18: 60.77% of Fucoxanthin Quantification HPLC Chromatography Result. (A) All-*trans*-Fucoxanthin with Dilution Factor in Methanol at 12,800, (B) *cis*-isomer of Fucoxanthin,(C) Unidentified Peak, (D) Canthaxanthin, Internal Standard

The area value of all *trans*-fucoxanthin was 188, 962 as showed in HPLC result in Figure 18, and the purity of purified fucoxanthin was 60.77%. In order to make 5mM of fucoxanthin ethanolic stock solution (as the "the second NZ Fucoxanthin extracts" stock solution), the freezing dried second NZ fucoxanthin extracts dissolved in 7.341mL of pure biological ethanol. This fucoxanthin ethanolic solution was sealed and stored in the -80°C freezer for further experiments.

3.2 MTT Results for Determination of Anti-proliferative Effects from Treatment of Pure Fucoxanthin Standard

3.2.1 Linearity of MTT Assay for Different Cancer Cell Lines

A series of diluted cancer cell lines (one in two dilutions) were seeded on the 96-well-plate. After attachment of the cells to the 96-well-plate for 6, 8 and 18 hours, the linear relationship between absorbance at 540nm and cell number was obtained for each cell line. All detailed results were attached in Appendix 1.

In addition, this linear relationship contributed to determine the period of cell attachment on 96-well-plate. This attachment period value is most important for further

anti-proliferative effect determination to add pure fucoxanthin standard and *U. pinnatifida* extracts. After seeding cells, all cancer cells were completely attached on the 96-well-plate at least 4 hours, and 6 hours was the optimum value. This pre-incubation time was used in this thesis so that the Day 0 value determined in MTT assay can be used as a reliable and valid reference for anti-proliferative effect determination from the pure fucoxanthin standard and *U. pinnatifida* extracts.

3.2.2 Effect of Ethanol on cancer cell growth

In the methodology indicated, two ethanol concentration ranges were used to dissolve fucoxanthin in this study. The first ethanol concentration range for dissolve fucoxanthin are 1%, 0.75%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.03125%, 0.007813%, and 0.001953%. The second first ethanol concentration range for dissolve fucoxanthin are 2%, 1.6%, 1%, 0.5% 0.25%, 0.125% and 0.03125% respectively. The result shows that biological pure ethanol at a reasonably low concentration does not affect cancer cell proliferation. This ruled out any interference from ethanol on fucoxanthin effect to cancer cell proliferation. The detailed MTT results were showed in Appendix 2.

In each cancer cell line, cell viability from completed culture medium with 0% of ethanol compared to cell viability in culture medium with relative concentration of biological ethanol. As MTT result shown in Day 0, 1, 2 and 3, the cell viability without biological ethanol was nearly same as the cell viability in the presence of ethanol in culture medium in all days treatment (p-value > 0.05). These results were found in all nine cancer cell lines.

3.2.3 Dose-Dependent Effects of Pure Fucoxanthin Standard on Cancer Cell Proliferation

The effects of the pure fucoxanthin on cell viability in all nine cancer cell lines were investigated by MTT assay. Since as p-value < 0.05, the dose-dependent anti-proliferative effects of pure fucoxanthin standard in time manner were statistically approved in all cancer cell lines. This indicated the pure fucoxanthin standard generated anti-proliferative effects to all nine cancer cell lines in dose- and time-dependent manners. The illustration for p-value calculation by application of Statistical software Minitab was shown in Appendix 3 in detail.

All nine cancer cell lines were treated with pure fucoxanthin standard for 24 hours, 48 hours, & 72 hours. All results were shown from Figure 19 to 27 below. Values were calculated as the percentage of the values of the cells treated with culture medium only. Points, mean for each time points (n=6 wells); bars, ± standard errors.

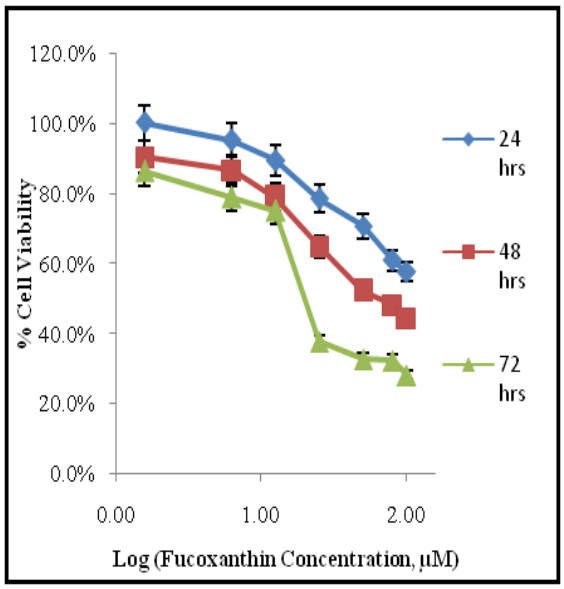

Figure 19: Inhibitory effects of pure fucoxanthin standard on HEPG2 cell proliferation. Human hepatocellular carcinoma Hep G2 cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

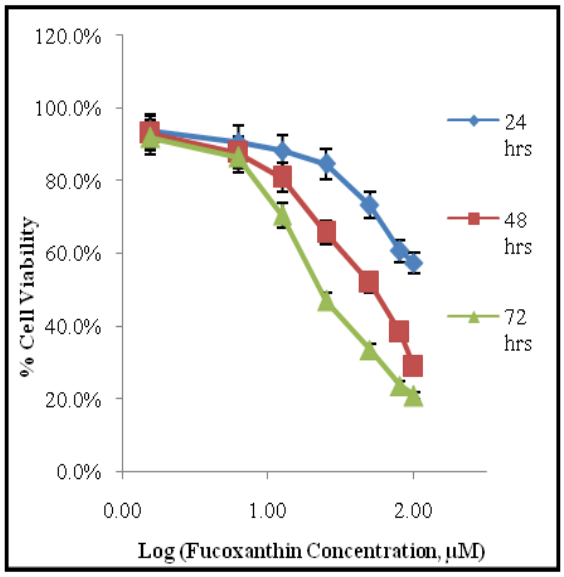

Figure 20: Inhibitory effects of pure fucoxanthin standard on A549 cell proliferation. Human lung carcinoma A549 cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

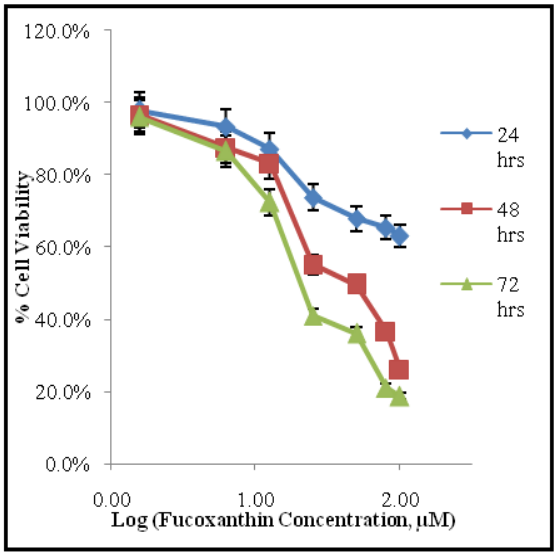

Figure 21: Inhibitory effects of pure fucoxanthin standard on WIDR cell proliferation. Human colon adenocarcinoma WiDr cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

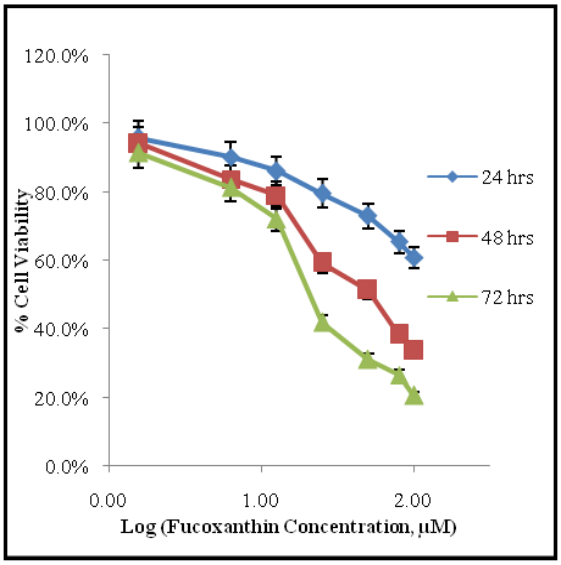

Figure 22: Inhibitory effects of pure fucoxanthin standard on NCI-H522 cell proliferation. Human lung carcinoma NCI-H522 cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

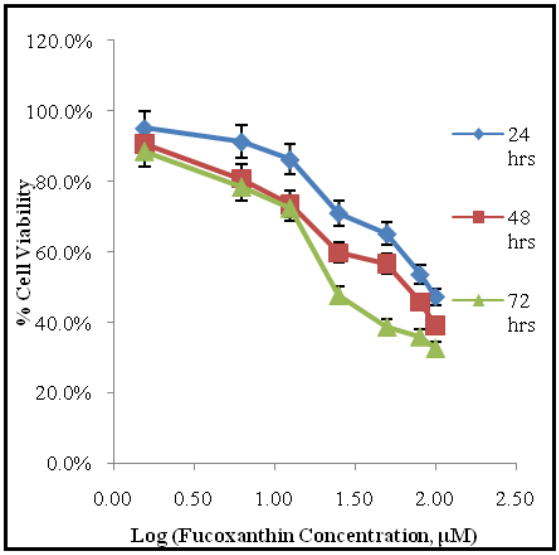

Figure 23: Inhibitory effects of pure fucoxanthin standard on SK-N-SH cell proliferation. Human neuroblastoma SK-N-SH cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

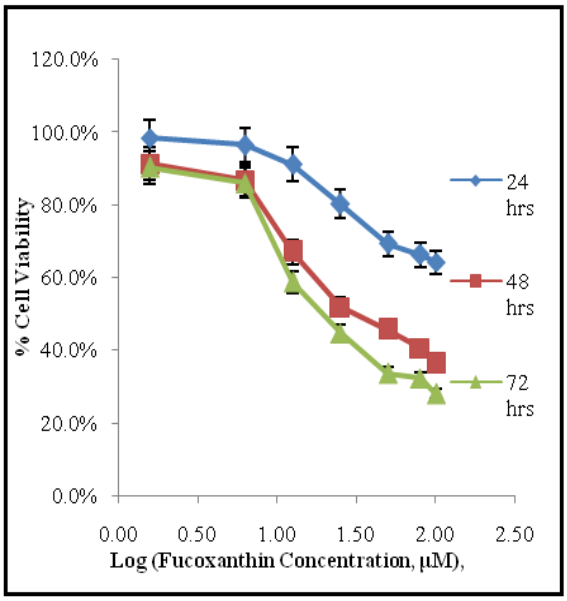

Figure 24: Inhibitory effects of pure fucoxanthin standard on LOVO cell proliferation. Human colon adenocarcinoma Lovo cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

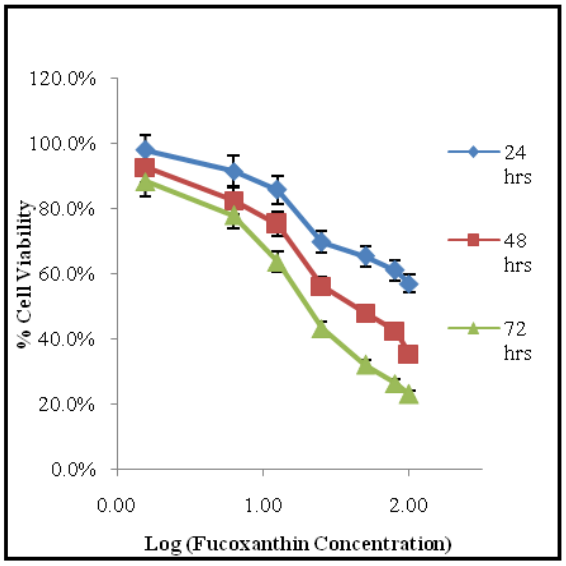

Figure 25: Inhibitory effects of pure fucoxanthin standard on MCF-7 cell proliferation. Human breast adenocarcinoma MCF-7 cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

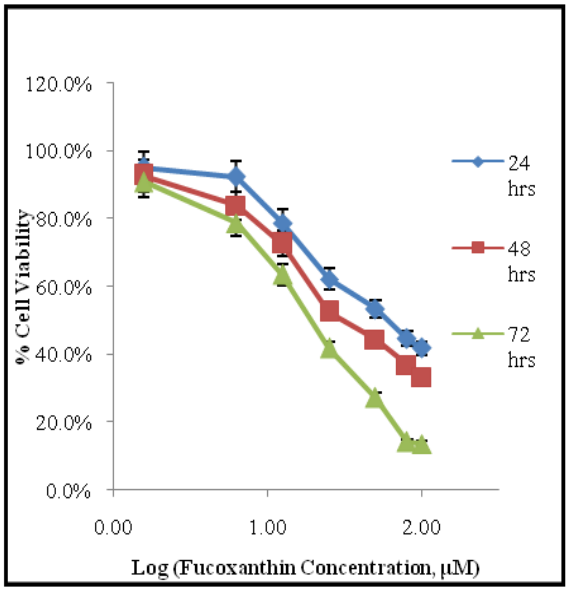

Figure 26: Inhibitory effects of pure fucoxanthin standard on SIHA cell proliferation. Human cervix squamous carcinoma SiHa cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

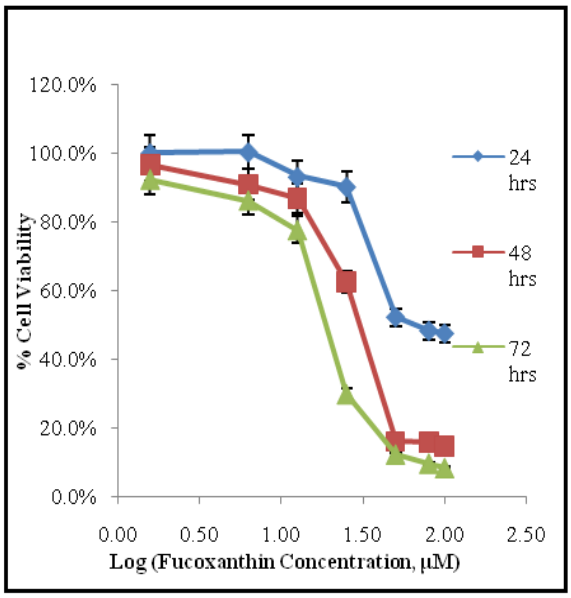

Figure 27: Inhibitory effects of pure fucoxanthin standard on MALME-3M cell proliferation. Human malignant melanoma Malme-3M were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

Overall, there were three distinctive lines in all cancer cell lines after treatment with pure fucoxanthin standard for three days shown from Figure 19 to 27. Each line in figure indicated all fucoxanthin treatment concentration in each day. All these three lines were gradually decreased, indicating cell viability of each cancer cell lines was decreased with increased concentration of fucoxanthin. Therefore, the cell viability decreased with relatively increased exposure of fucoxanthin to each type of cancer cell lines. Thus, all figures clearly showed the pure fucoxanthin standard could generate anti-proliferative effects in dose and time-dependent manners.

In detail, there were 7 concentration of pure fucoxanthin standard used for treatment with all cancer cell lines including 1.5625, 6.25, 12.5, 25, 50, 80, 100µM. All concentration cell viability curves fit well with sigmoid model of negative exponential distribution, showing slightly decreased cell viability in the low concentration range (1.5625 to 6.25µM) followed by relatively steep drop of cell viability and then a plateau. In all three days, the cell viability could be significantly decreased starting concentration of fucoxanthin at 12.5 or 25 µM except Malme-3M in Figure 27. For human malignant melanoma Malme-3M, the cell viability could be significantly decreased starting in pure fucoxanthin standard at 50 µM of treatment within 24 hours. Cell viability could not be decreased to 50% after treatment with pure fucoxanthin standard within 24 hours. This was found in most types of cancer cell lines except SK-N-SH, SiHa, and Malme-3M respectively. For these three cancer cell lines, the high concentration of pure fucoxanthin (from 80 to 100µM) allowed cell viability to decrease to 50%. In treatment within 48 hours, cell viability in most cancer cell lines was largely decreased in treatment of low fucoxanthin concentration. However, Malme-3M was the

exception, and its cell viability was still higher than 80% in fucoxanthin concentration at 25µM. After treatment about 72 hours, cell viability in all types of cancer cell lines was decreased to approximately 20% in the highest concentration of pure fucoxanthin standard as 100 µM. Even in some cancer cell lines including SiHa and Malme-3M, the cell viability was decreased to less than 20% in the highest fucoxanthin concentration after treatment of 72 hours.

3.2.4 An Overview of Anti-proliferative IC_{50} Results of Pure Fucoxanthin Standard

These IC_{50} values for anti-proliferative effects of pure fucoxanthin standard on all nine cancer cell lines were showed in Table 17 and Figure 28.

Table 17: IC_{50} values determination for anti-proliferative effects from pure fucoxanthin standard in three days treatment, Unit: μM; The 1st screening: IC_{50} values from fucoxanthin treatment for three days with the high & wide concentration range of 0.39-200μM; the 2nd screening: the IC_{50} values from fucoxanthin treatment for three days with the low & narrow concentration range of 1.5625-100μM. CV: Coefficient variation.

IC_{50} Value Determination for Anti-proliferative Effects from Pure Fucoxanthin Standard, μM									
	(1) Hep G2			**(2) A549**			**(3) WiDr**		
	24 hours	**48 hours**	**72 hours**	**24 hours**	**48 hours**	**72 hours**	**24 hours**	**48 hours**	**72 hours**
First Screening	53.72	26.00	18.11	64.35	21.78	16.27	69.45	23.38	17.25
Second Screening	57.81	29.74	15.72	69.02	24.98	14.76	65.45	25.38	15.01
Average Value	**55.77**	**27.87**	**16.92**	**66.69**	**23.38**	**15.52**	**67.45**	**24.38**	**16.13**
Standard Deviation	2.89	9.49	1.69	3.30	2.26	1.07	2.83	1.41	1.58
CV	5.19	5.73	9.99	4.95	9.68	6.88	4.19	5.78	9.82
	(4) NCI-H522			**(5) SK-N-SH**			**(6) Lovo**		
	24 hours	**48 hours**	**72 hours**	**24 hours**	**48 hours**	**72 hours**	**24 hours**	**48 hours**	**72 hours**
First Screening	51.57	26	12.81	49.06	24.72	17.76	50.3	22.14	15.01
Second Screening	55.44	24.38	14.36	46.91	25.36	17.68	52.96	19.98	12.19
Average Value	**53.51**	**25.19**	**13.59**	**47.99**	**25.04**	**17.72**	**51.63**	**21.06**	**13.6**
Standard Deviation	2.74	1.15	1.10	1.52	0.45	0.06	1.88	1.53	1.99
CV	5.11	4.55	8.07	3.17	1.81	0.32	3.64	7.25	9.13
	(7) MCF-7			**(8) SiHa**			**(9) Malme-3M**		
	24 hours	**48 hours**	**72 hours**	**24 hours**	**48 hours**	**72 hours**	**24 hours**	**48 hours**	**72 hours**
First Screening	47.99	20.84	12.39	26.64	17.84	9.76	36.08	22.31	17.3
Second Screening	46.77	23.12	13.68	28.84	19.95	10	31.69	19.52	15.47
Average Value	**47.38**	**21.98**	**13.04**	**27.74**	**18.89**	**9.88**	**33.89**	**20.92**	**16.39**
Standard Deviation	0.86	1.61	0.91	1.56	1.49	0.17	3.10	1.97	1.29
CV	1.82	7.33	7.00	5.61	7.90	1.72	9.16	9.43	7.90

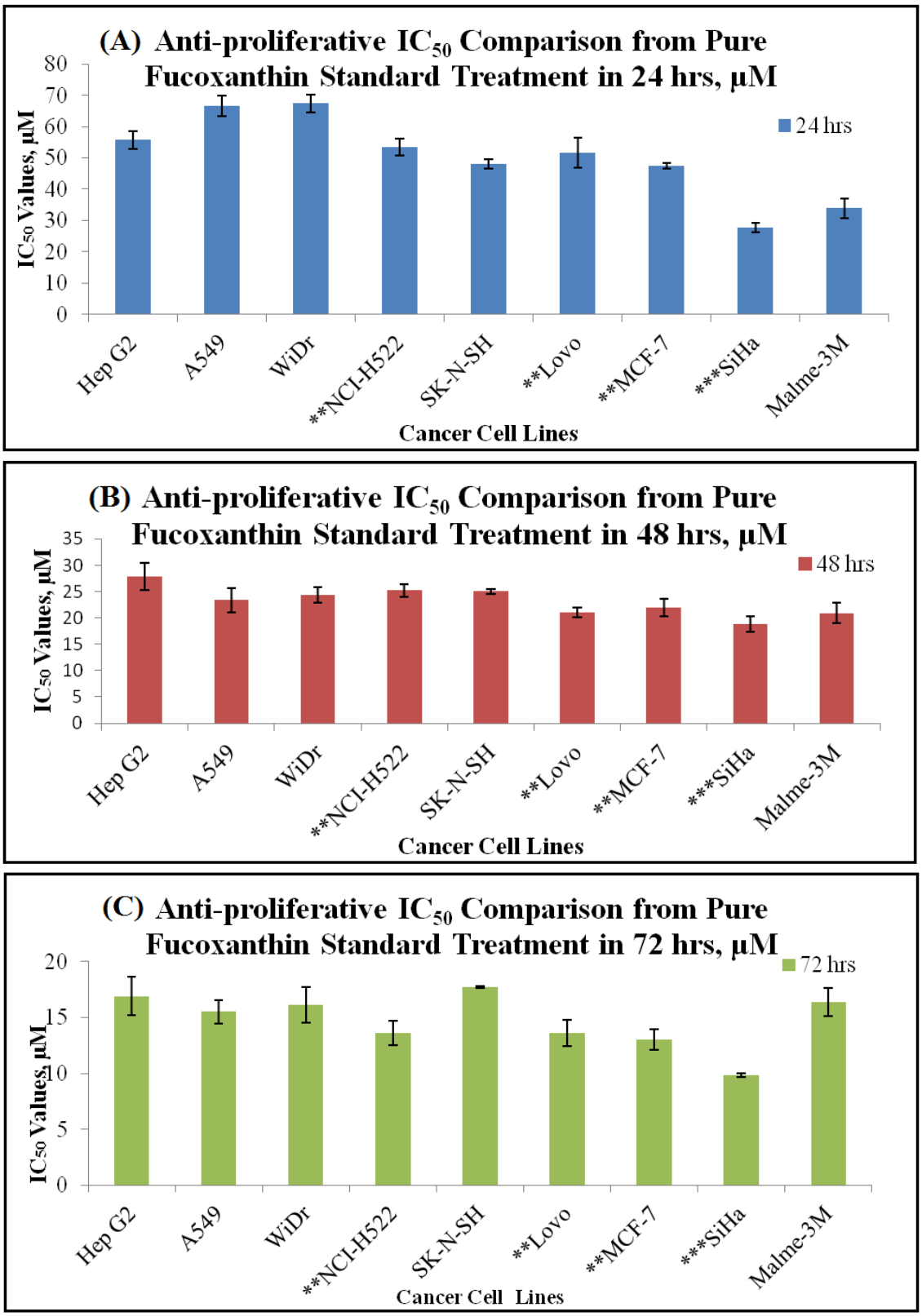

Figure 28: Comparison of anti-proliferative IC_{50} values from pure fucoxanthin standard treatment for three days on nine cancer cell lines, (A) pure fucoxanthin treatment for 24 hrs; (B) pure fucoxanthin treatment for 48 hrs & (C) pure fucoxanthin treatment for 72 hrs. All the values are means ± standard errors of six treatment replicates. The method of statistic data analysis was One-way ANOVA followed by Turkey Test. ***: highly sensitive to pure fucoxanthin standard treatment. **: mildly sensitive to pure fucoxanthin standard treatment.

3.3 Comparison (Intra-Comparison) of Anti-proliferative IC_{50} Values of Pure Fucoxanthin Standard on Nine Cancer Cell lines.

Figure 28 (A, B & C) showed the comparison of anti-proliferative IC_{50} values derived from pure fucoxanthin standard treatment for nine cancer cell lines tested for three days. Overall, there were large differences in the anti-proliferative IC_{50} values determined within 24 hours among all 9 cancer cell lines (Figure 28 A). However, the IC_{50} values for treatments in 48 and 72 (Figure 28 B & C) hours were not significantly different between each other except human cervix squamous carcinoma.

In treatments within 24 hours (Figure 28 A), the IC_{50} values for SiHa and Malme-3M were the lowest values, ranging was from 25 to 35µM. The middle range of IC_{50} values was from 45 to 56µM pure fucoxanthin standard for cancer cell lines including Hep G2, NCI-H522, SK-N-SH, Lovo, and MCF-7 respectively. The high range of IC_{50} values was from 65 to 70µM fucoxanthin standard, for cancer cell lines A549 and WiDr. These three ranges of IC_{50} values were statistically and significantly different with *p*-value < 0.05. IC_{50} values for SiHa determined at 48 and 72 hours after fucoxanthin treatment were the lowest values compared with those for other cancer cell lines (Figure 28 B & C). After treatment for 72 hours (Figure 28 C), the IC_{50} values for cancer cell lines including NCI-H522, Lovo, and MCF-7 were statistically different to other cancer cell lines with *p*-value < 0.05. The range of IC_{50} values for NCI-H522, Lovo, and MCF-7 were from 13 to 14µM, and this range of IC_{50} values was significantly lower than other cancer cell lines after treatment of 72 hours.

3.4 Fucoxanthin Extracts from New Zealand *U. pinnatifida*

3.4.1 Anti-proliferative Effects of Crude *U. pinnatifida* Extract (Purity: 0.2% Fucoxanthin) on Cancer Cell Lines

All nine cancer cell lines were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM of 0.2% of fucoxanthin crude *U. pinnatifida* extract for 24 hours, 48 hours, & 72 hours. All results were shown from Figure 29 to 37 below. Values were calculated as the percentage of the values determined from cells without any treatment at time 0 hours. Points, mean for each time points (n=6 wells).

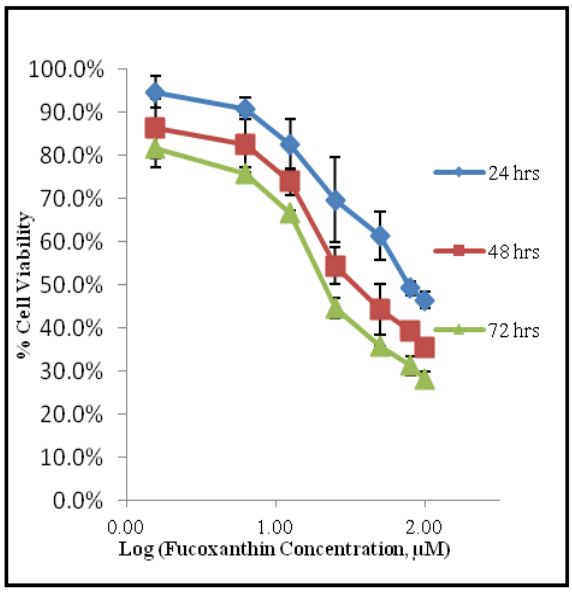

Figure 29: Inhibitory effects of crude NZ *U. pinnatifida* (0.2% fucoxanthin) on cell proliferation in human hepatocellular carcinoma Hep G2. Hep G2 cells were treated with fucoxanthin at 1.5625, 6.25, 12.5, 25, 50, 80, 100µM for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

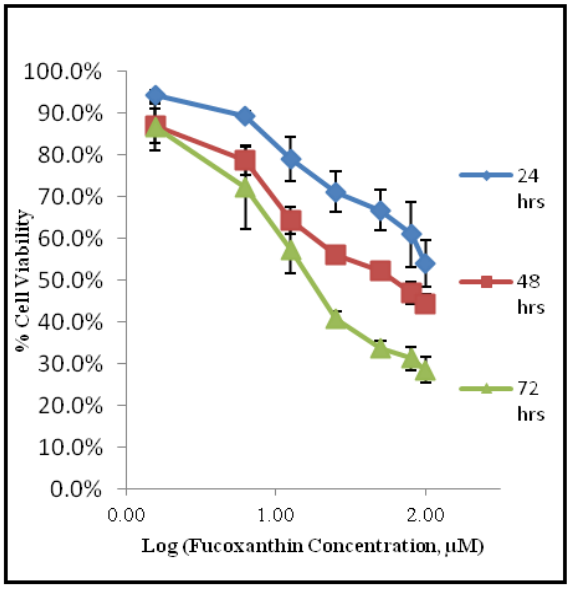

Figure 30: Inhibitory effects of crude NZ *U. pinnatifida* (0.2% fucoxanthin) on cell proliferation in human lung carcinoma A549. A549 cells were treated with fucoxanthin at 1.5625, 6.25, 12.5, 25, 50, 80, 100µM for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

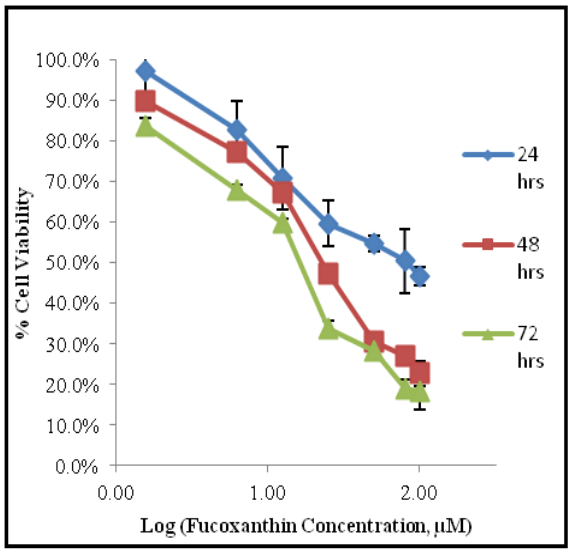

Figure 31: Inhibitory effects of crude NZ *U. pinnatifida* (0.2% fucoxanthin) on cell proliferation in human colon adenocarcinoma WiDr. WiDr cells were treated with fucoxanthin at 1.5625, 6.25, 12.5, 25, 50, 80, 100µM for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

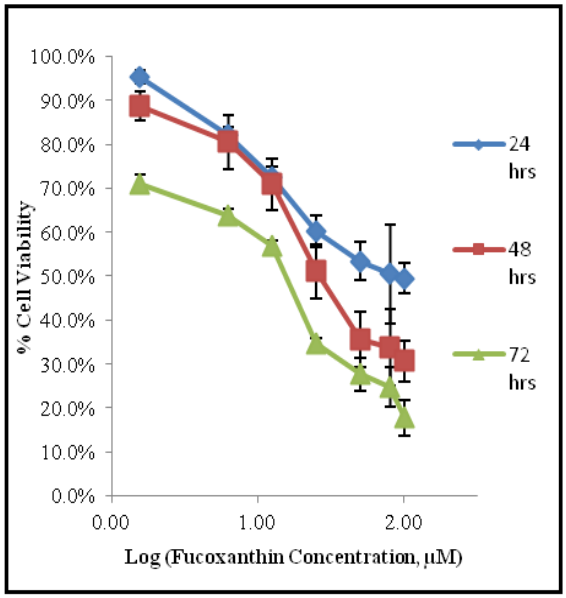

Figure 32: Inhibitory effects of crude NZ *U. pinnatifida* (0.2% fucoxanthin) on cell proliferation in human lung carcinoma NCI-H522. NCI-H522 cells were treated with fucoxanthin at 1.5625, 6.25, 12.5, 25, 50, 80, 100µM for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

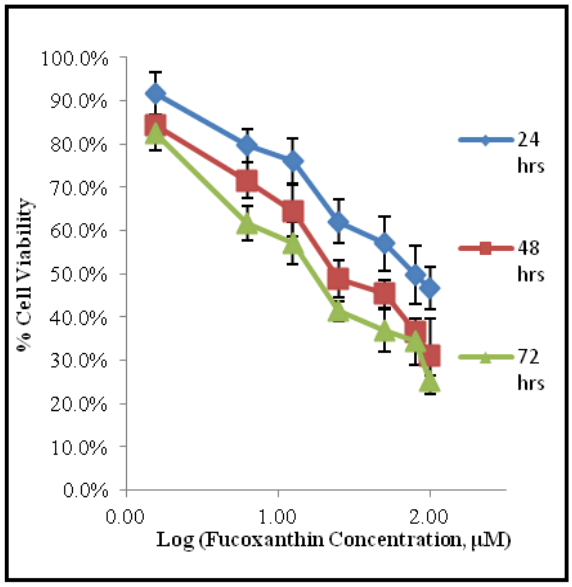

Figure 33: Inhibitory effects of crude NZ *U. pinnatifida* (0.2% fucoxanthin) on cell proliferation in human neuroblastoma SK-N-SH. SK-N-SH cells were treated with fucoxanthin at 1.5625, 6.25, 12.5, 25, 50, 80, 100µM for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

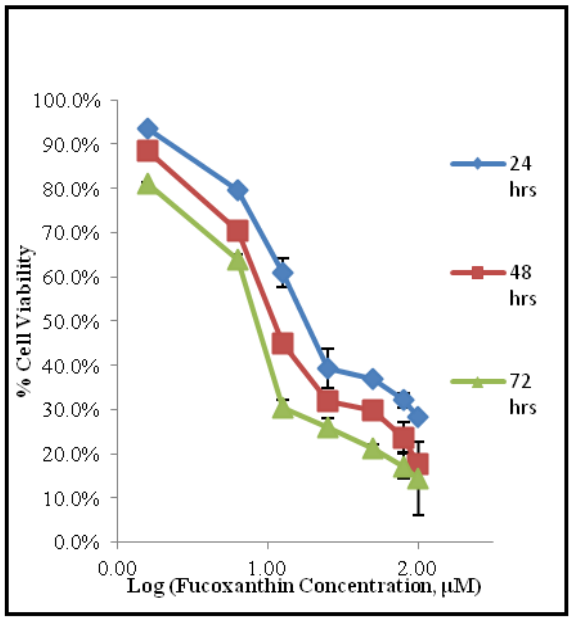

Figure 34: Inhibitory effects of crude NZ *U. pinnatifida* (0.2% fucoxanthin) on cell proliferation in human colon adenocarcinoma Lovo. Lovo cells were treated with fucoxanthin at 1.5625, 6.25, 12.5, 25, 50, 80, 100µM for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

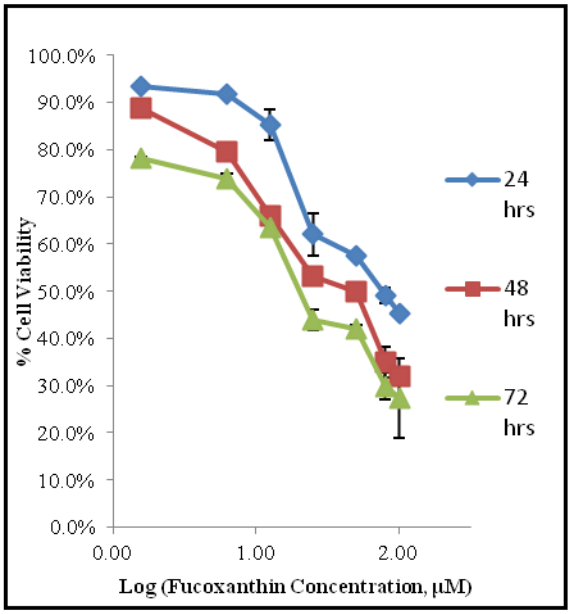

Figure 35: Inhibitory effects of crude NZ *U. pinnatifida* (0.2% fucoxanthin) on cell proliferation in human breast adenocarcinoma MCF-7. MCF-7 cells were treated with fucoxanthin at 1.5625, 6.25, 12.5, 25, 50, 80, 100µM for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

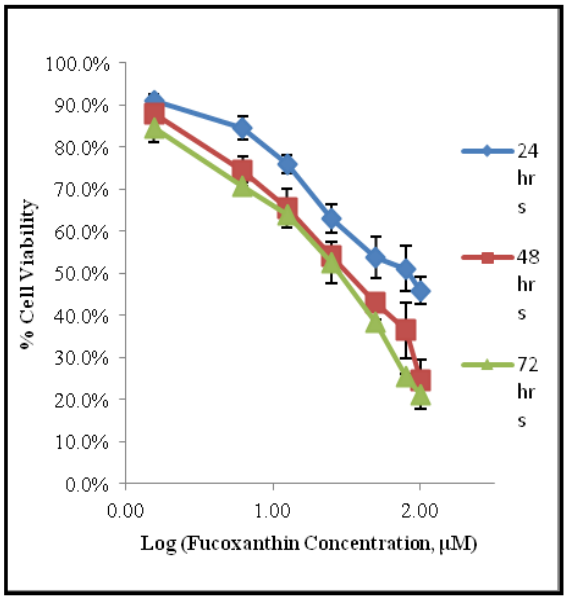

Figure 36: Inhibitory effects of crude NZ *U. pinnatifida* (0.2% fucoxanthin) on cell proliferation in human cervix squamous carcinoma SiHa. SiHa cells were treated with fucoxanthin at 1.5625, 6.25, 12.5, 25, 50, 80, 100µM for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

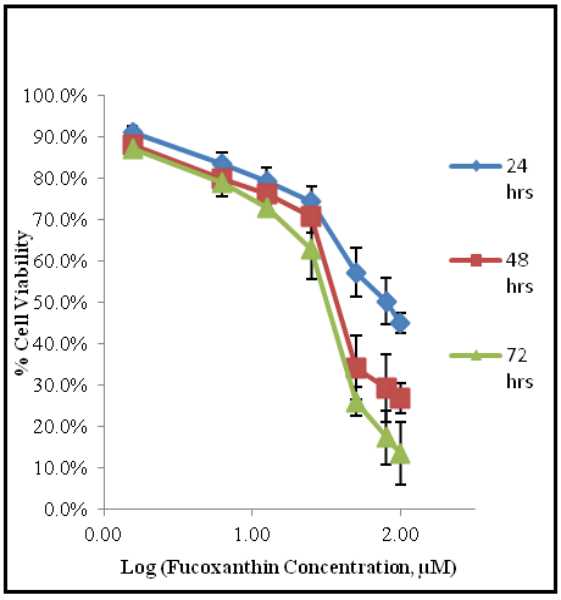

Figure 37: Inhibitory effects of crude NZ *U. pinnatifida* (0.2% fucoxanthin) on cell proliferation in human malignant melanoma Malme-3M. Malme-3M cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

The Figure 29 to 37 showed the results about anti-proliferative effect from crude *U. pinnatifida* extracts, and the crude *U. pinnatifida* contained 0.2% of fucoxanthin.

Overall, the anti-proliferative effects from crude fucoxanthin were statistically found in dose- and time-dependent manners with p-value = 0.000 < 0.05. Most concentration cell viability curves fit well with sigmoid model of negative exponential distribution, showing slightly decreased cell viability in the low concentration range followed by relatively steep drop of cell viability and then a plateau. There were three distinctive lines indicated each day of treatment with a series of fucoxanthin dilution. Cell viability significantly decreased with increasing concentration of fucoxanthin treatment.

In detail, within 24 hours treatment cell viability was decreased to 50% in the high concentration of fucoxanthin treatment showed in dose-response curve. This was found in most cancer cell lines including Hep G2 (Figure 29), WiDr (Figure 31), Lovo (Figure 34), MCF-7 (Figure 35), SiHa (Figure 36), Malme-3M (Figure 37). After treatment for 48 and 72 hours, cell viability was decreased to about 20%. Cell viability was decreased to less than 20% after fucoxanthin treatment for 72 hours in some of cancer cell lines including WiDr (Figure 31), NCI-H522 (Figure 32), Lovo (Figure 34), and Malme-3M (Figure 37).

For Malme-3M, the cell viability was not remarkably reduced by inhibition effects from crude *U. pinnatifida* extract containing 0.2% fucoxanthin from 1.5625 to 25μM in all three days treatment.

3.4.2 Anti-proliferative Effects of First Fucoxanthin Extract (Purity: 43.55% Fucoxanthin) on Cancer Cell Lines

All nine cancer cell lines were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100μM of 43.55% of the first fucoxanthin extract for 24 hours, 48 hours, & 72 hours. All results were shown from Figure 38 to 46 below. Values were calculated as the percentage of the values in 0 hours cells. Points, mean for each time points (n=6 wells).

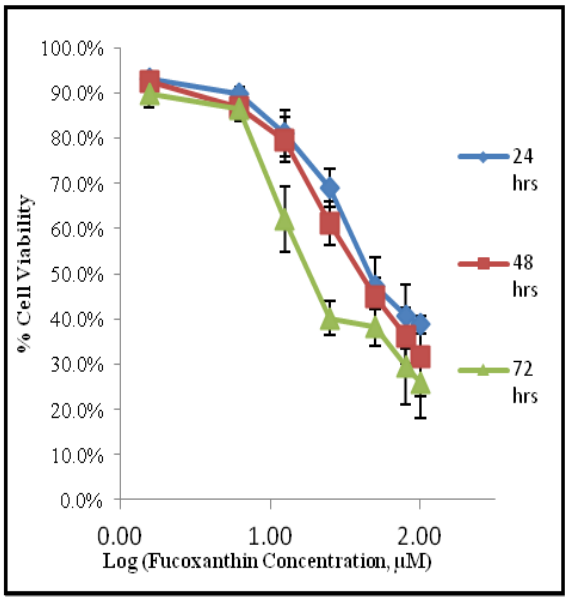

Figure 38: Inhibitory effects of the first fucoxanthin extracts from NZ *U. pinnatifida* (43.5% fucoxanthin) on cell proliferation in human hepatocellular carcinoma Hep G2. Hep G2 cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

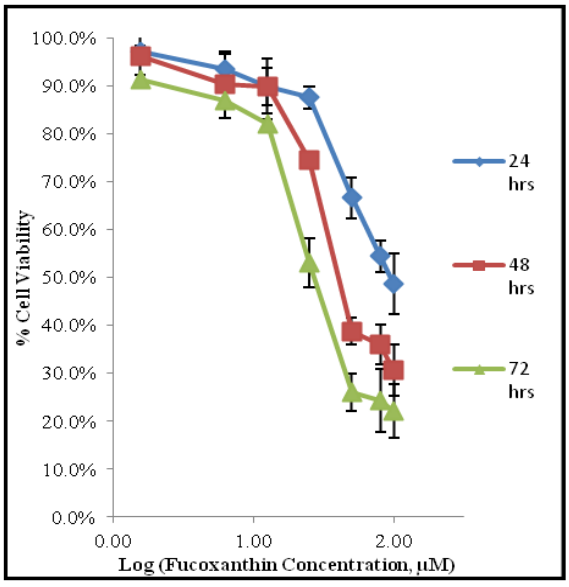

Figure 39: Inhibitory effects of the first fucoxanthin extracts from NZ *U. pinnatifida* (43.5% fucoxanthin) on cell proliferation in human lung carcinoma A549.A549 cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

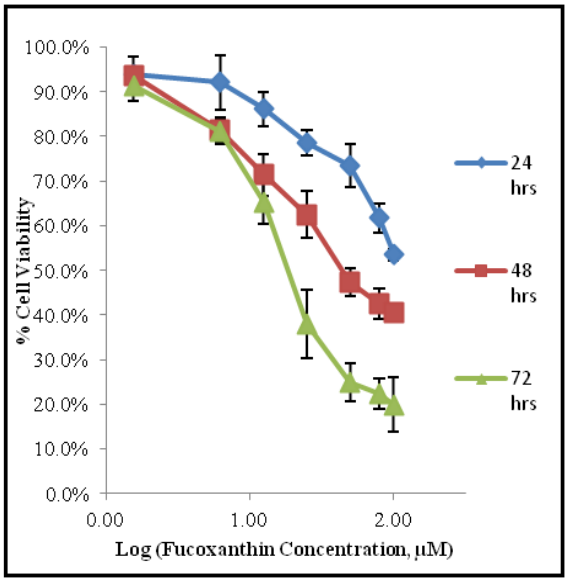

Figure 40: Inhibitory effects of the first fucoxanthin extracts from NZ *U. pinnatifida* (43.5% fucoxanthin) on cell proliferation in human colon adenocarcinoma WiDr. WiDr cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

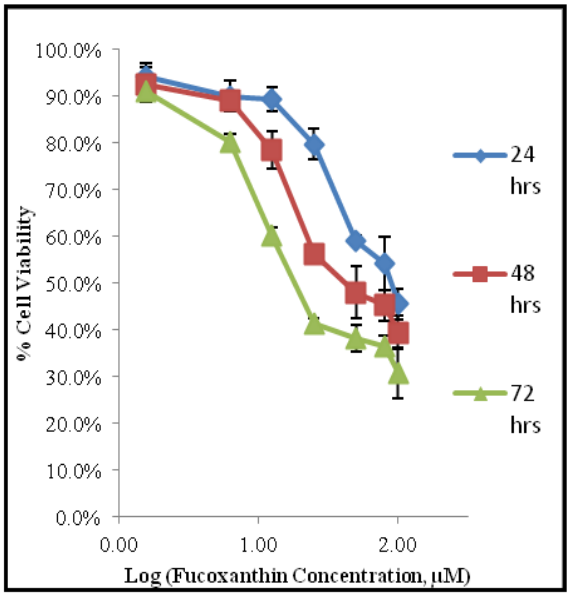

Figure 41: Inhibitory effects of the first fucoxanthin extracts from NZ *U. pinnatifida* (43.5% fucoxanthin) on cell proliferation in human lung carcinoma NCI-H522. NCI-H522 cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48hours, & 72 hours. Data are means ± standard errors (n=6).

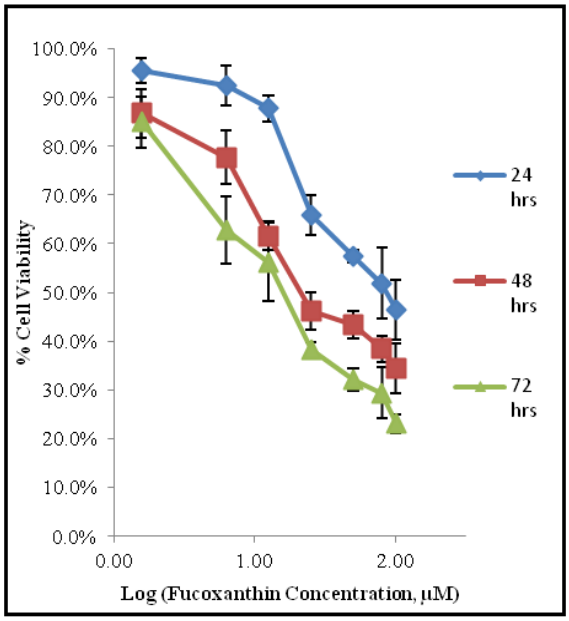

Figure 42: Inhibitory effects of the first fucoxanthin extracts from NZ *U. pinnatifida* (43.5% fucoxanthin) on cell proliferation in human neuroblastoma SK-N-SH. SK-N-SH cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

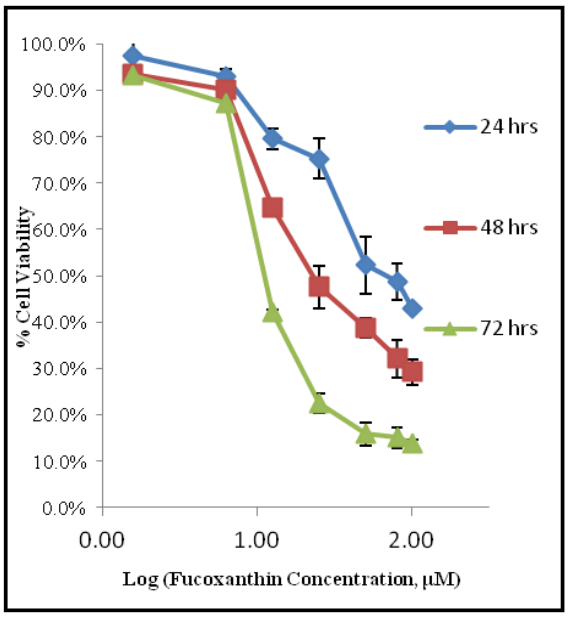

Figure 43: Inhibitory effects of the first fucoxanthin extracts from NZ *U. pinnatifida* (43.5% fucoxanthin) on cell proliferation in human colon adenocarcinoma Lovo. Lovo cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

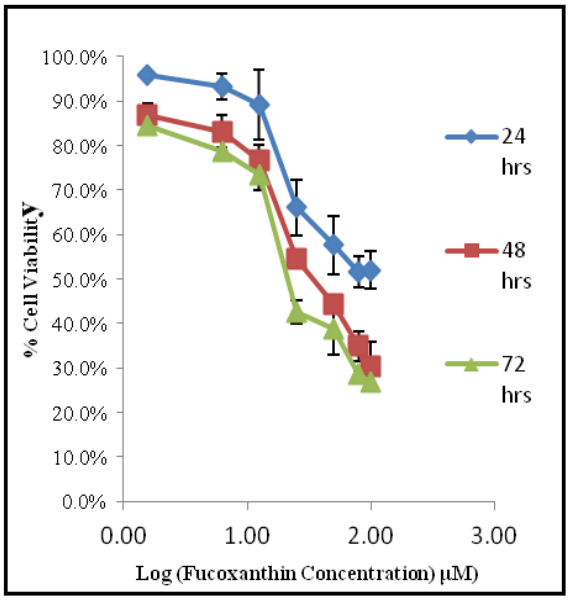

Figure 44: Inhibitory effects of the first fucoxanthin extracts from NZ *U. pinnatifida* (43.5% fucoxanthin) on cell proliferation in human breast adenocarcinoma MCF-7. MCF-7 cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

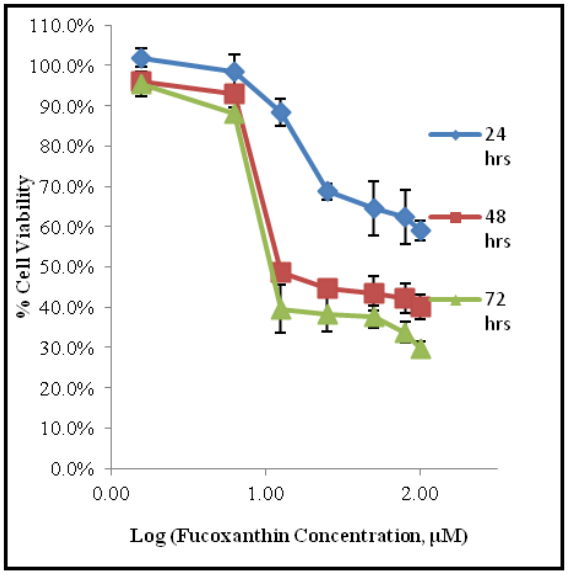

Figure 45: Inhibitory effects of the first fucoxanthin extracts from NZ *U. pinnatifida* (43.5% fucoxanthin) on cell proliferation in human cervix squamous carcinoma SiHa. SiHa cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

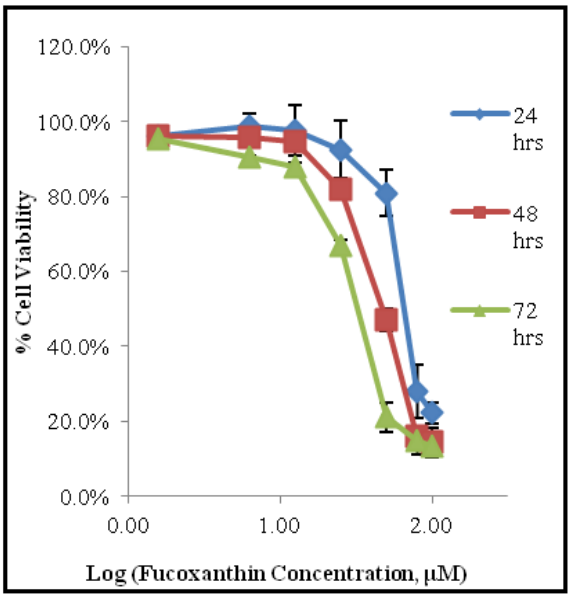

Figure 46: Inhibitory effects of the first fucoxanthin extracts from NZ *U. pinnatifida* (43.5% fucoxanthin) on cell proliferation in human malignant melanoma Malme-3M. Malme-3M cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

The Figure 38 to 46 showed the results about anti-proliferative effect from crude *U. pinnatifida* extracts, and the first fucoxanthin extracts contained 43.5% of fucoxanthin.

Overall, the anti-proliferative effects from crude fucoxanthin were statistically found in dose- and time-dependent manners with p-value = 0.000 < 0.05. Most concentration cell viability curves fit well with sigmoid model of negative exponential distribution, showing slightly decreased cell viability in the low concentration range followed by relatively steep drop of cell viability and then a plateau. There were three distinctive lines indicated each day of treatment with a series of fucoxanthin dilution. Cell viability significantly decreased with increasing concentration of fucoxanthin treatment.

In detail, within 24 hours treatment cell viability was decreased to 50% in the high concentration of fucoxanthin treatment showed in dose-response curve. This was found in most cancer cell lines including Hep G2 (Figure 38), A549 (Figure 39), WiDr (Figure 40), NCI-H522 (Figure 41), SK-N-SH (Figure 42), Lovo (Figure 43), Malme-3M (Figure 46). After treatment for 48 and 72 hours, cell viability was decreased to about 20%. Cell viability was decreased to less than 20% after fucoxanthin treatment for 72 hours in some of cancer cell lines including WiDr (Figure 40), Lovo (Figure 43), and Malme-3M (Figure 46).

For Malme-3M, the cell viability was not remarkably reduced by inhibition effects from crude *U. pinnatifida* extract containing 43.5% fucoxanthin from 1.5625 to 50μM in all three days treatment. In addition, cell proliferation was a little bit of dominant effect than anti-proliferative effects from the first fucoxanthin extract in concentration from 1.5625 to 12.5μM in the 24 and 48 hours treatment (Figure 46).

3.4.3 Anti-proliferative Effects of Second Fucoxanthin Extract (Purity: 60.77% Fucoxanthin) on Cancer Cell Lines

All nine cancer cell lines were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100μM of 60.77% of the first fucoxanthin extract for 24 hours, 48 hours, & 72 hours. All results were shown from Figure 47 to 55 below. Values were calculated as the percentage of the values in 0 hours cells. Points, mean for each time points (n=6 wells).

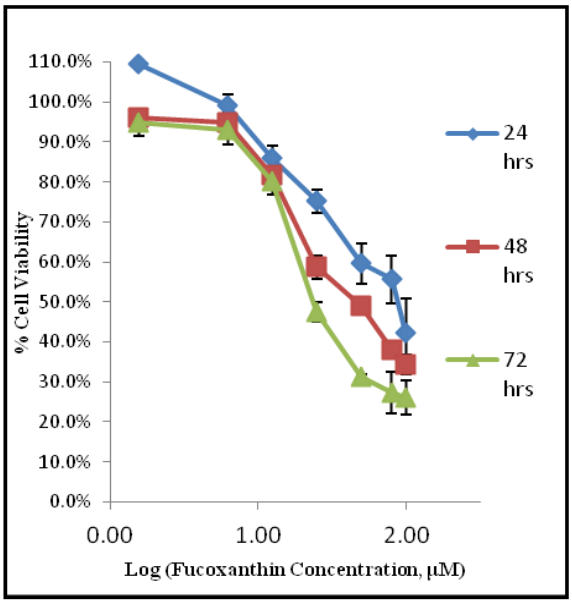

Figure 47: Inhibitory effects of the second fucoxanthin extracts from NZ *U. pinnatifida* (60.77% fucoxanthin) on cell proliferation in human hepatocellular carcinoma Hep G2. Hep G2 cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

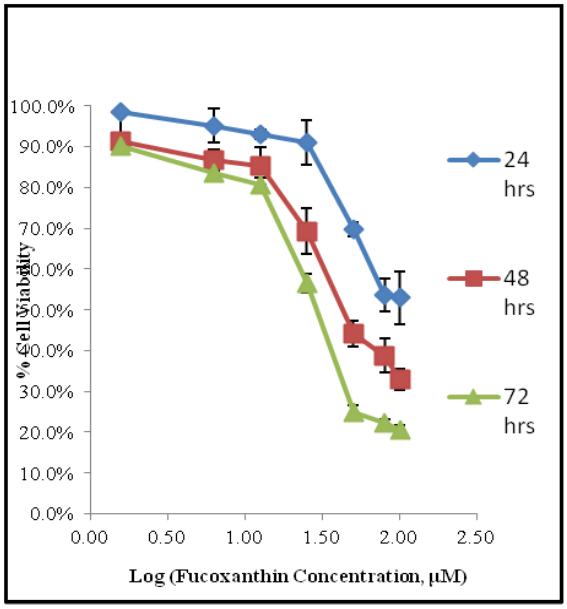

Figure 48: Inhibitory effects of the second fucoxanthin extracts from NZ *U. pinnatifida* (60.77% fucoxanthin) on cell proliferation in human lung carcinoma A549. A549 cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

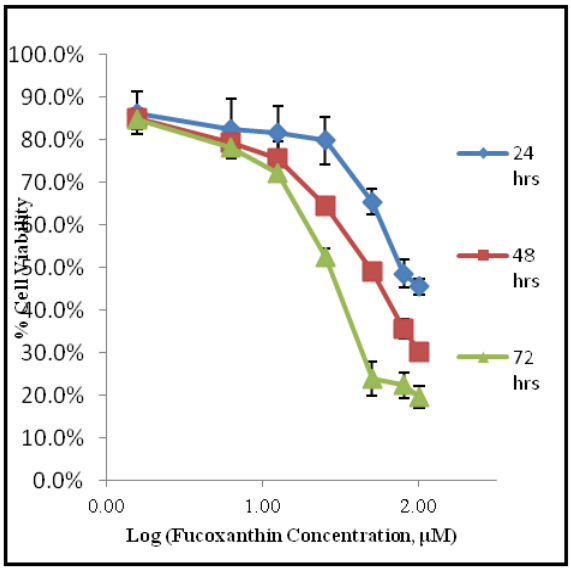

Figure 49: Inhibitory effects of the second fucoxanthin extracts from NZ *U. pinnatifida* (60.77% fucoxanthin) on cell proliferation in human colon adenocarcinoma WiDr. WiDr cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48hours, & 72 hours. Data are means ± standard errors (n=6).

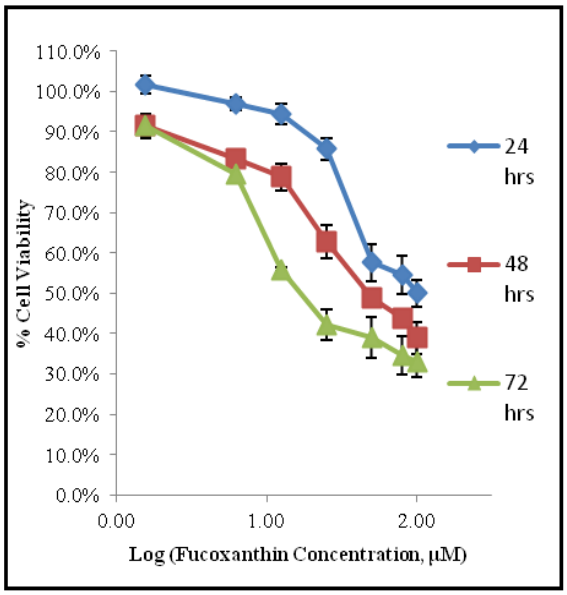

Figure 50: Inhibitory effects of the second fucoxanthin extracts from NZ *U. pinnatifida* (60.77% fucoxanthin) on cell proliferation in human lung carcinoma NCI-H522. NCI-H522 cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

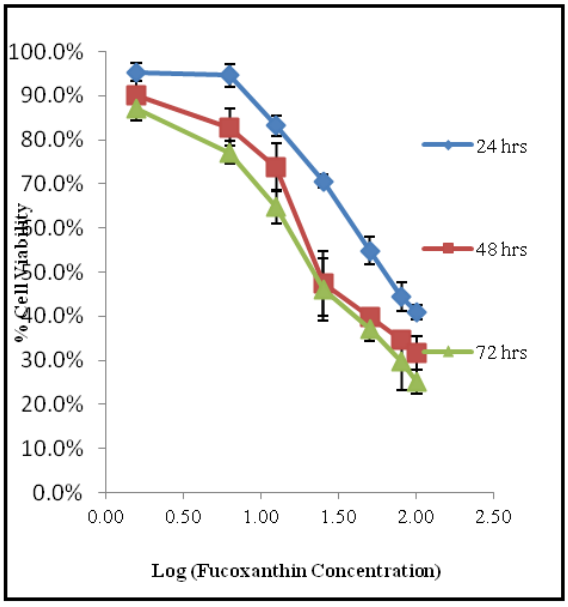

Figure 51: Inhibitory effects of the second fucoxanthin extracts from NZ *U. pinnatifida* (60.77% fucoxanthin) on cell proliferation in human neuroblastoma SK-N-SH. SK-N-SH cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

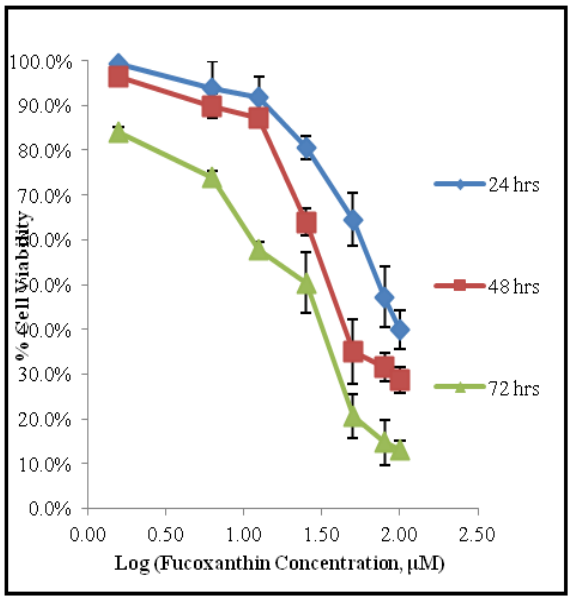

Figure 52: Inhibitory effects of the second fucoxanthin extracts from NZ *U. pinnatifida* (60.77% fucoxanthin) on cell proliferation in human colon adenocarcinoma Lovo. Lovo cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48hours, & 72 hours. Data are means ± standard errors (n=6).

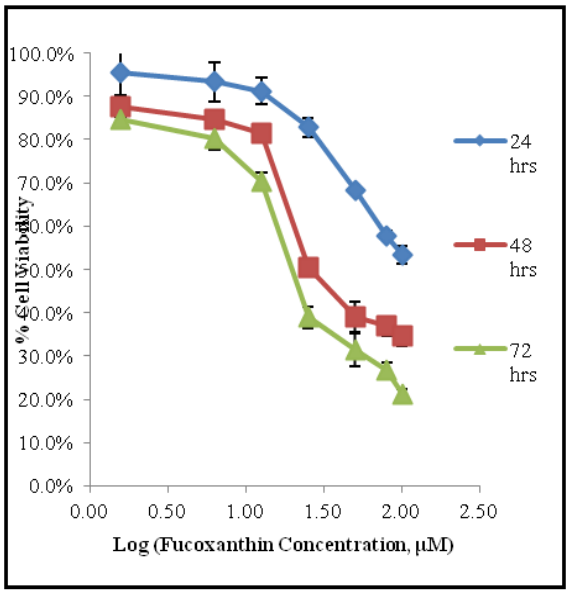

Figure 53: Inhibitory effects of the second fucoxanthin extracts from NZ *U. pinnatifida* (60.77% fucoxanthin) on cell proliferation in human breast adenocarcinoma MCF-7. MCF-7 cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

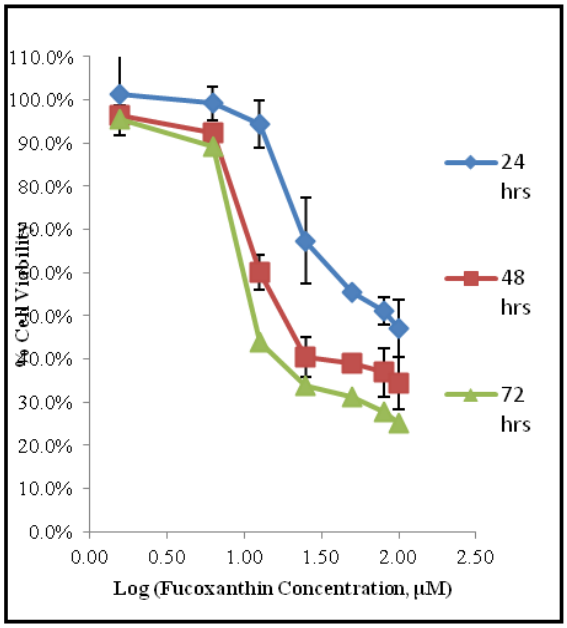

Figure 54: Inhibitory effects of the second fucoxanthin extracts from NZ *U. pinnatifida* (60.77% fucoxanthin) on cell proliferation in human cervix squamous carcinoma SiHa. SiHa cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

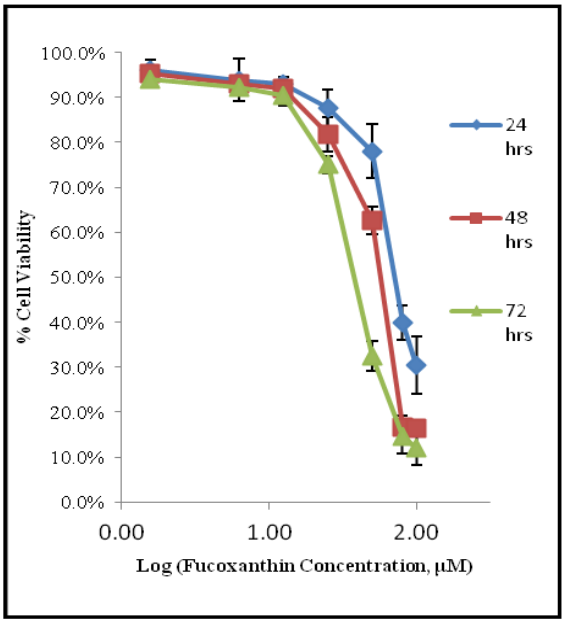

Figure 55: Inhibitory effects of the second fucoxanthin extracts from NZ *U. pinnatifida* (60.77% fucoxanthin) on cell proliferation in human malignant melanoma Malme-3M. Malme-3M cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

The Figure 47 to 55 showed the results about anti-proliferative effect from crude *U. pinnatifida* extracts, and the second fucoxanthin extracts contained 60.77% of fucoxanthin.

Overall, the anti-proliferative effects from crude fucoxanthin were statistically significant in dose- and time-dependent manners with p-value = 0.000 < 0.05. There were three distinctive lines indicated each day of treatment with a series of fucoxanthin dilution. Most concentration cell viability curves fit well with sigmoid model of negative exponential distribution, showing slightly decreased cell viability in the low concentration range followed by relatively steep drop of cell viability and then a plateau. Cell viability significantly decreased with increasing concentration of fucoxanthin.

In detail, within 24 hours treatment cell viability was decreased to 50% in the high concentration of fucoxanthin treatment showed in dose-response curve. This was found in most cancer cell lines including Hep G2 (Figure 47), WiDr (Figure 49), NCI-H522 (Figure 50), SK-N-SH (Figure 51), Lovo (Figure 52), SiHa (Figure 54), Malme-3M (Figure 55). After treatment for 48 and 72 hours, cell viability was decreased to about 20%. Cell viability was decreased to less than 20% after fucoxanthin treatment for 72 hours in some of cancer cell lines including WiDr (Figure 49), Lovo (Figure 52), and Malme-3 M (Figure 55).

For Malme-3M, the cell viability was not remarkably reduced by inhibition effects from crude *U. pinnatifida* extract containing 60.77% fucoxanthin from 1.5625 to 50μM in all three days treatment (Figure 55).

3.5 Anti-proliferative Effects of Japanese Fucoxanthin Extract (Purity: 4% Fucoxanthin) on Cancer Cell Lines

All nine cancer cell lines were treated with 4% of fucoxanthin extract from Japanese *U. pinnatifida* for 24 hours, 48 hours, & 72 hours. All results were shown from Figure 56 to 64 below. Values were calculated as the percentage of the values in 0 hours cells. Points, mean for each time points (n=6 wells).

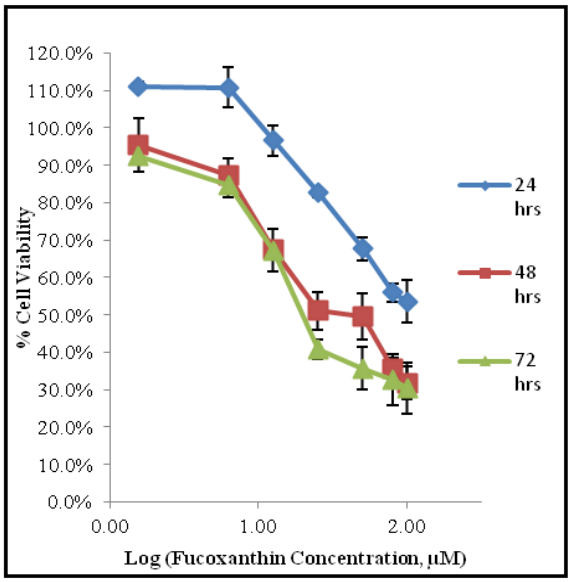

Figure 56: Inhibitory effects of fucoxanthin extracts from Japanese *U. pinnatifida* (4% fucoxanthin) on cell proliferation in human hepatocellular carcinoma Hep G2. Hep G2 cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

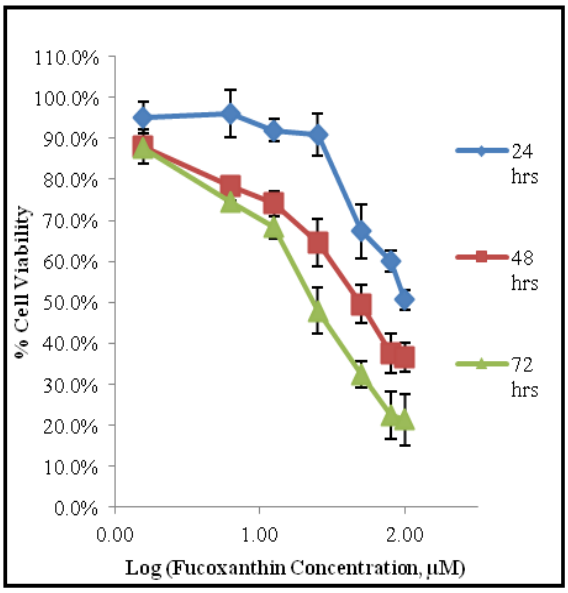

Figure 57: Inhibitory effects of fucoxanthin extracts from Japanese *U. pinnatifida* (4% fucoxanthin) on cell proliferation in human lung carcinoma A549. A549 cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

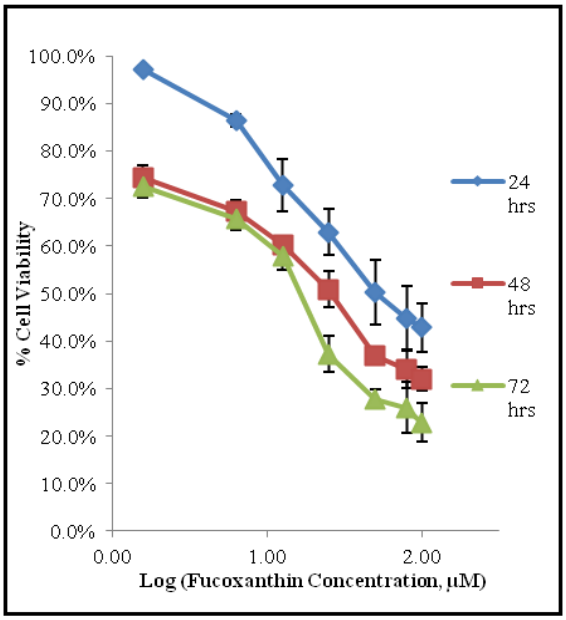

Figure 58: Inhibitory effects of fucoxanthin extracts from Japanese *U. pinnatifida* (4% fucoxanthin) on cell proliferation in human colon adenocarcinoma WiDr. WiDr cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours & 72 hours. Data are means ± standard errors (n=6).

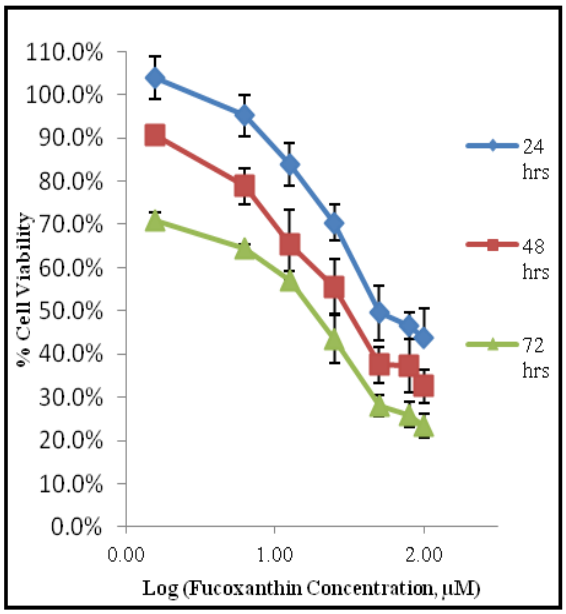

Figure 59: Inhibitory effects of fucoxanthin extracts from Japanese *U. pinnatifida* (4% fucoxanthin) on cell proliferation in human lung carcinoma NCI-H522. NCI-H522 cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

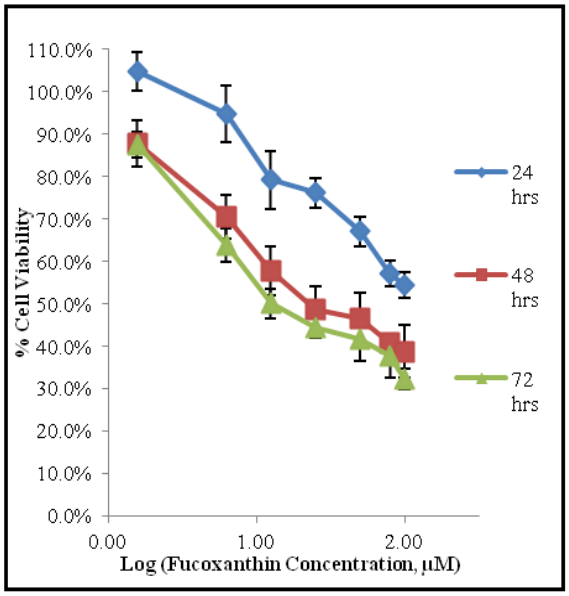

Figure 60: Inhibitory effects of fucoxanthin extracts from Japanese *U. pinnatifida* (4% fucoxanthin) on cell proliferation in human neuroblastoma SK-N-SH. SK-N-SH cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, &72 hours. Data are means ± standard errors (n=6).

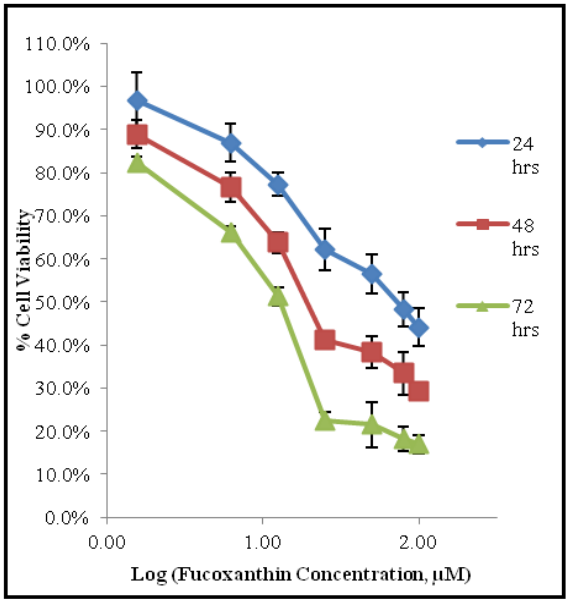

Figure 61: Inhibitory effects of fucoxanthin extracts from Japanese *U. pinnatifida* (4% fucoxanthin) on cell proliferation in human colon adenocarcinoma Lovo. Lovo cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours & 72 hours. Data are means ± standard errors (n=6).

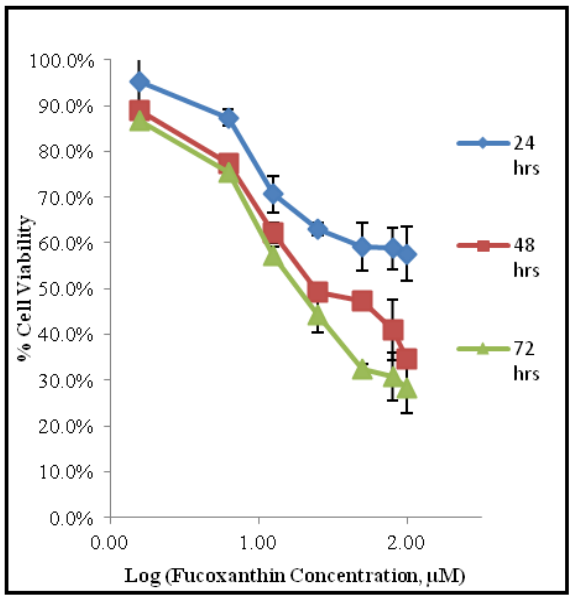

Figure 62: Inhibitory effects of fucoxanthin extracts from Japanese *U. pinnatifida* (4% fucoxanthin) on cell proliferation in human breast adenocarcinoma MCF-7. MCF-7 cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

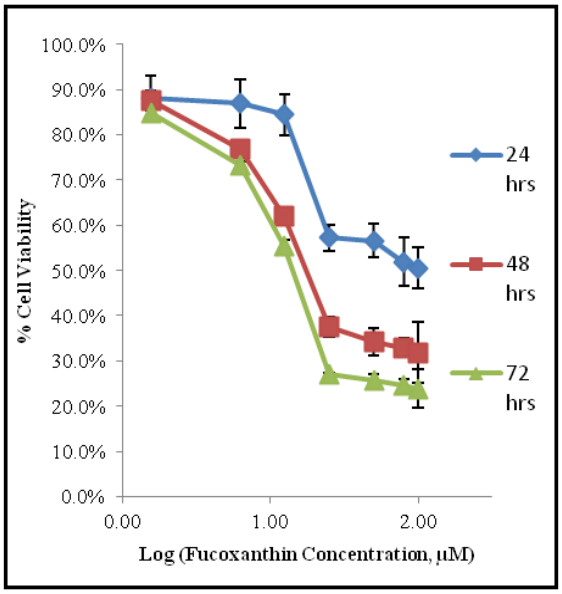

Figure 63: Inhibitory effects of fucoxanthin extracts from Japanese *U. pinnatifida* (4% fucoxanthin) on cell proliferation in human cervix squamous carcinoma SiHa. SiHa cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

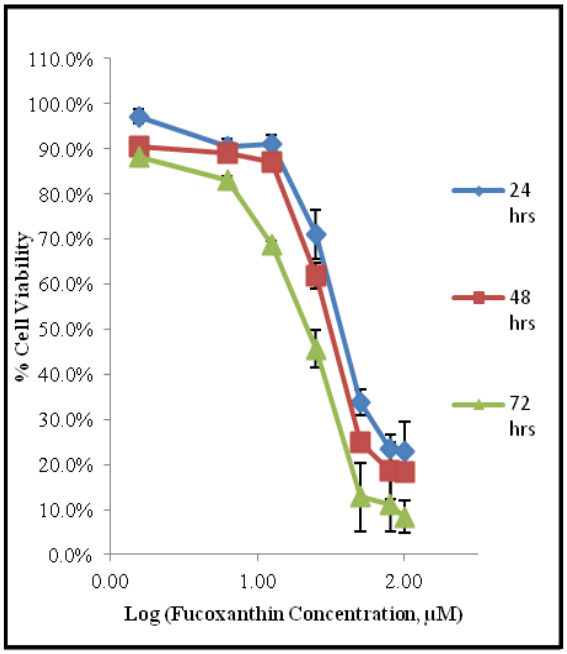

Figure 64: Inhibitory effects of fucoxanthin extracts from Japanese *U. pinnatifida* (4% fucoxanthin) on cell proliferation in human malignant melanoma Malme-3M. Malme-3M cells were treated with 1.5625, 6.25, 12.5, 25, 50, 80, 100µM fucoxanthin for 24 hours, 48 hours, & 72 hours. Data are means ± standard errors (n=6).

The Figure 56 to 64 showed the results about anti-proliferative effect from Japanese fucoxanthin extracts with purity of 4% fucoxanthin.

Overall, the anti-proliferative effects from crude fucoxanthin were statistically found in dose- and time-dependent manners with *p*-value = 0.000 < 0.05. There were three distinctive lines indicated each day of treatment with a series of fucoxanthin dilution. Most concentration cell viability curves fit well with sigmoid model of negative exponential distribution, showing slightly decreased cell viability in the low concentration range followed by relatively steep drop of cell viability and then a plateau. Cell viability significantly decreased with increasing concentration of fucoxanthin treatment.

In detail, within 24 hours treatment cell viability was decreased to 50% in the high concentration of fucoxanthin treatment showed in dose-response curve. This was found in most cancer cell lines including WiDr (Figure 58), NCI-H522 (Figure 59), Lovo (Figure 61), SiHa (Figure 63), and Malme-3 M (Figure 64). After treatment for 48 and 72 hours, cell viability was decreased to about 20%. Cell viability was decreased to less than 20% after fucoxanthin treatment for 72 hours in some of cancer cell lines including WiDr (Figure 58), Lovo (Figure 61), and Malme-3 M (Figure 64).

For Malme-3M, the cell viability was not remarkably reduced by crude *U. pinnatifida* extracts containing 60.77% fucoxanthin (from 1.5625 to 50µM) after 24, 48 and 72-hour treatment (Figure 64).

3.6 Comparison of Anti-proliferative IC_{50} Values of Pure Fucoxanthin Standard and New Zealand Fucoxanthin Extracts & Japanese Fucoxanthin Extracts.

This section is to compare the IC_{50} value between anti-proliferative effect from pure fucoxanthin standard with NZ and Japanese fucoxanthin extracts. There were three NZ *U. pinnatifida* extracts with different purity of fucoxanthin including Crude *U. pinnatifida*: 0.2% of fucoxanthin; First fucoxanthin extract: 43.5% of fucoxanthin; and Second fucoxanthin extract: 60.77% of fucoxanthin. The Japanese fucoxanthin extract contained 4% fucoxanthin. If significant difference of anti-proliferative IC_{50} values between treated with pure fucoxanthin and NZ fucoxanthin extracts or Japanese

fucoxanthin extracts were found, this possibly indicated some of novel compounds with potential anti-cancer effects were present in the those fucoxanthin extracts.

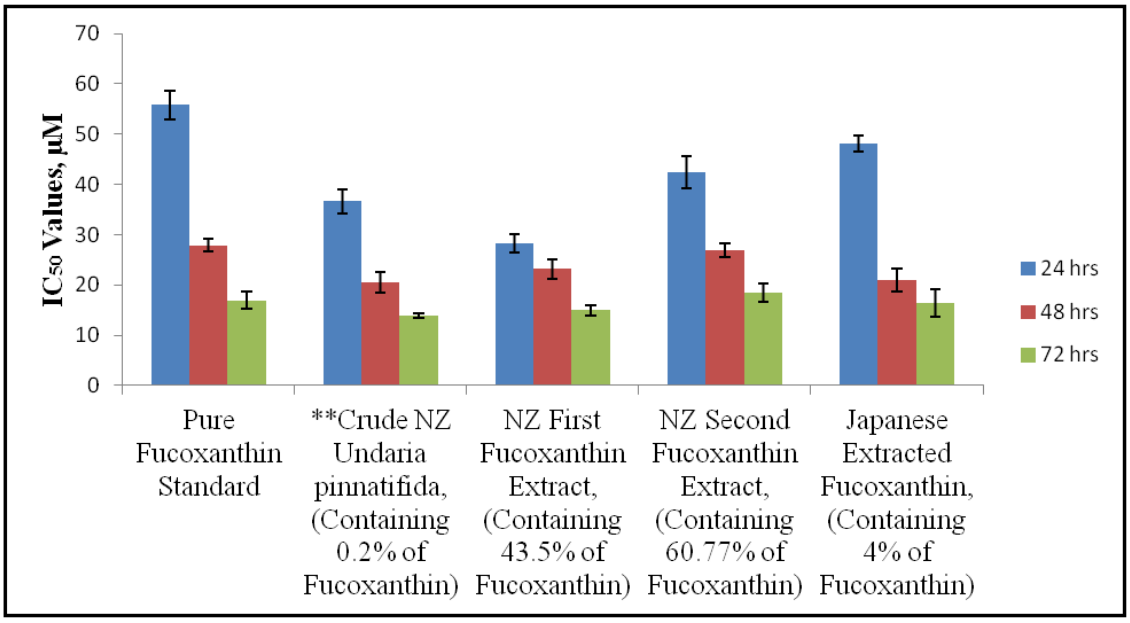

Figure 65: Hep G2: Comparison of anti-proliferative IC_{50} values for human hepatocellular carcinoma Hep G2 cells between pure fucoxanthin and three NZ fucoxanthin extracts (containing 0.2, 43.5 and 60.77% fucoxanthin) & Japanese fucoxanthin extract (containing 4% fucoxanthin). Data are means ± standard errors (n=6).

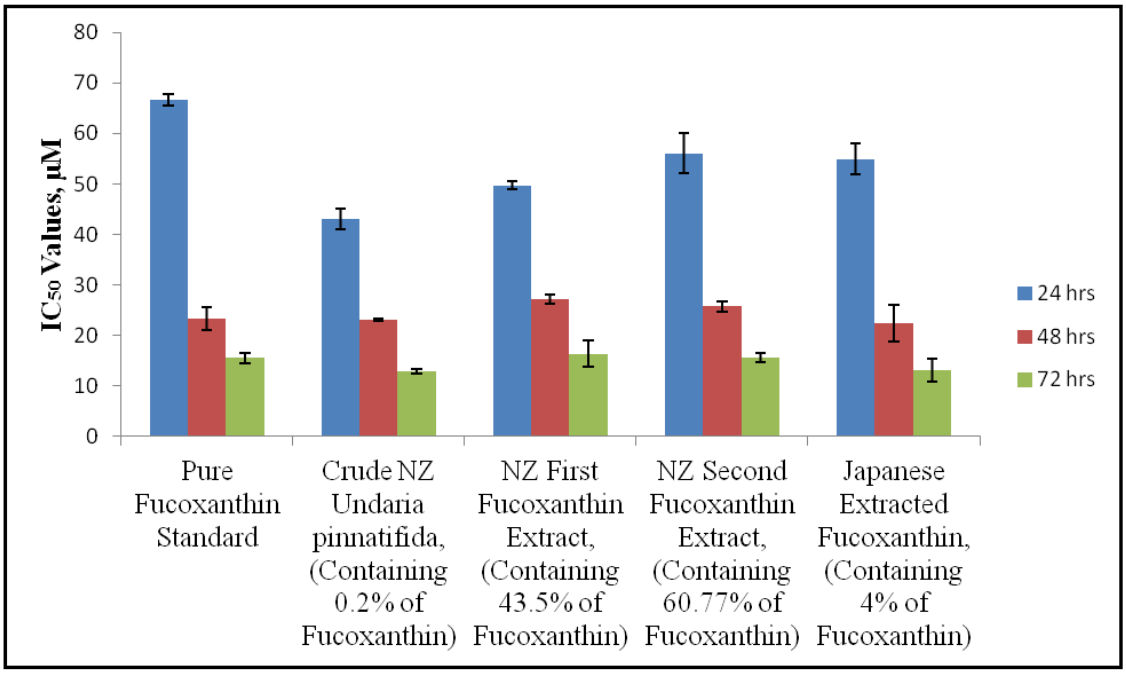

Figure 66: A549: Comparison of anti-proliferative IC_{50} values for human lung carcinoma A549 cells comparison between pure fucoxanthin and three NZ fucoxanthin extracts (containing 0.2, 43.5 and 60.77% fucoxanthin) & Japanese fucoxanthin extract (containing 4% fucoxanthin). Data are means ± standard errors (n=6).

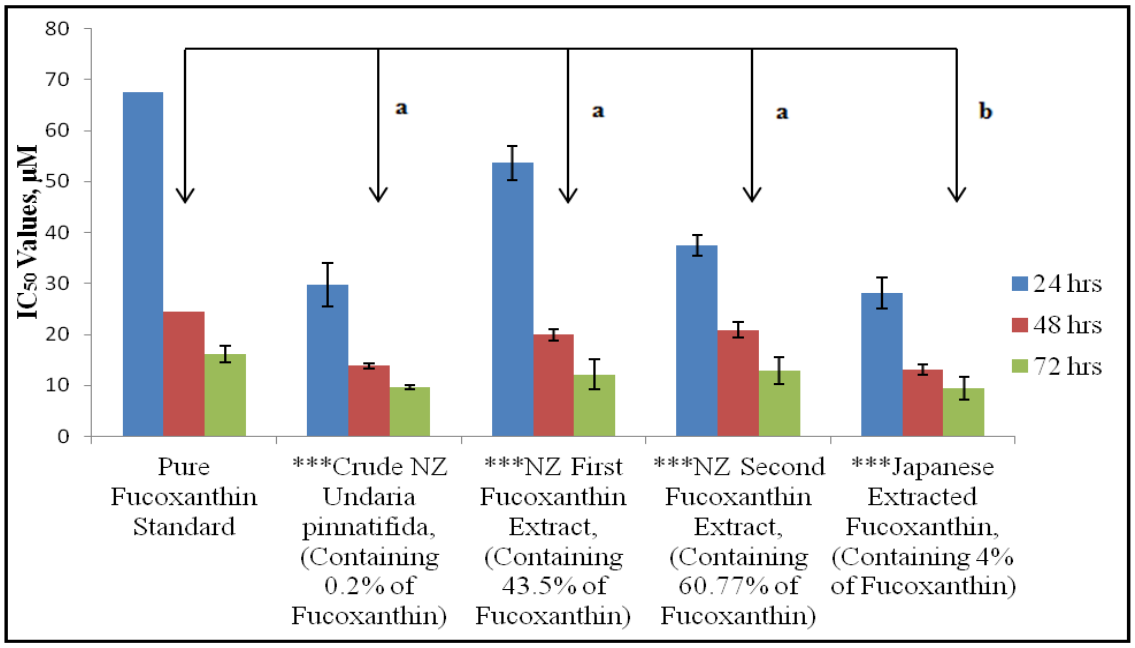

Figure 67: ***WiDr: Comparison of anti-proliferative IC_{50} values for human colon adenocarcinoma WiDr cells between pure fucoxanthin and three NZ fucoxanthin extracts (containing 0.2, 43.5 and 60.77% fucoxanthin) & Japanese fucoxanthin extract (containing 4% fucoxanthin); Data are means ± standard errors (n=6). *** means significant difference about anti-proliferative IC_{50} value compared to treatment with pure fucoxanthin standard; (a) Comparison between pure fucoxanthin and NZ fucoxanthin extracts. (b) Comparison between pure fucoxanthin and the Japanese fucoxanthin extracts.

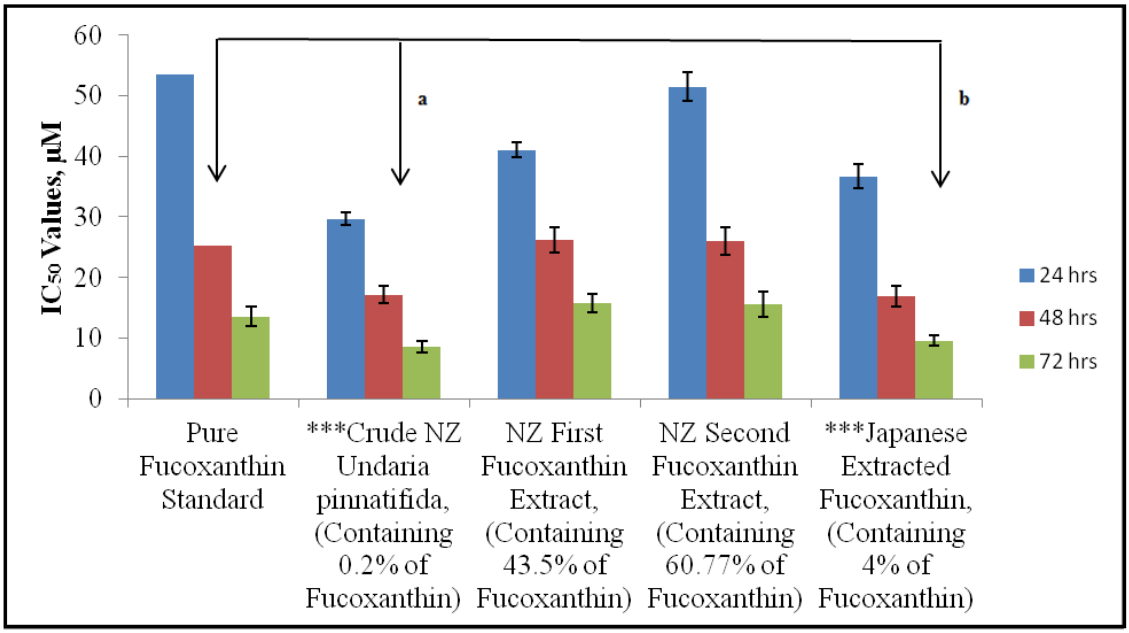

Figure 68: ***NCI-H522: Comparison of anti-proliferative IC_{50} values for human lung carcinoma NCI-H522 cells between pure fucoxanthin and three NZ fucoxanthin extracts (containing 0.2, 43.5 and 60.77% fucoxanthin) & Japanese fucoxanthin extract (containing 4% fucoxanthin); Data are means ± standard errors (n=6). *** means significant difference about anti-proliferative IC_{50} value compared to treatment with pure fucoxanthin standard. (a) Comparison between pure fucoxanthin and NZ fucoxanthin extracts. (b) Comparison between pure fucoxanthin and the Japanese fucoxanthin extracts.

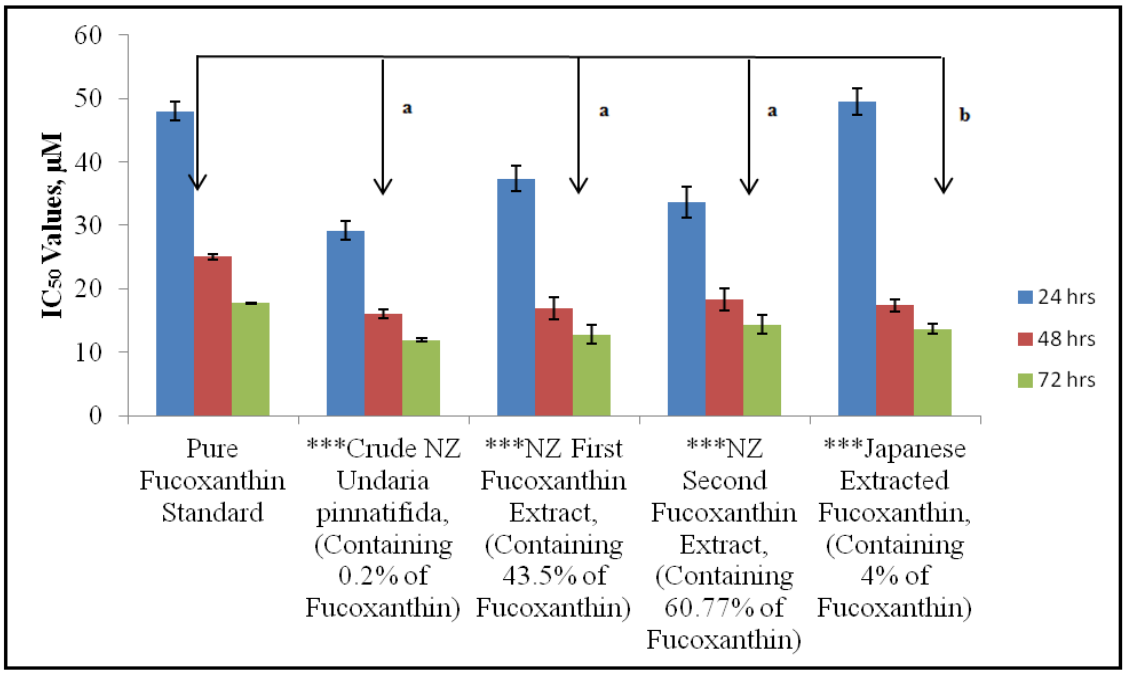

Figure 69: ***SK-N-SH: Comparison of anti-proliferative IC_{50} values for human neuroblastoma SK-N-SH cells between pure fucoxanthin and three NZ fucoxanthin extracts (containing 0.2, 43.5 and 60.77% fucoxanthin) & Japanese fucoxanthin extract (containing4% fucoxanthin); Data are means ± standard errors (n=6). *** means significant difference about anti-proliferative IC_{50} value compared to treatment with pure fucoxanthin standard. (a) Comparison between pure fucoxanthin and NZ fucoxanthin extracts. (b) Comparison between pure fucoxanthin and the Japanese fucoxanthin extracts.

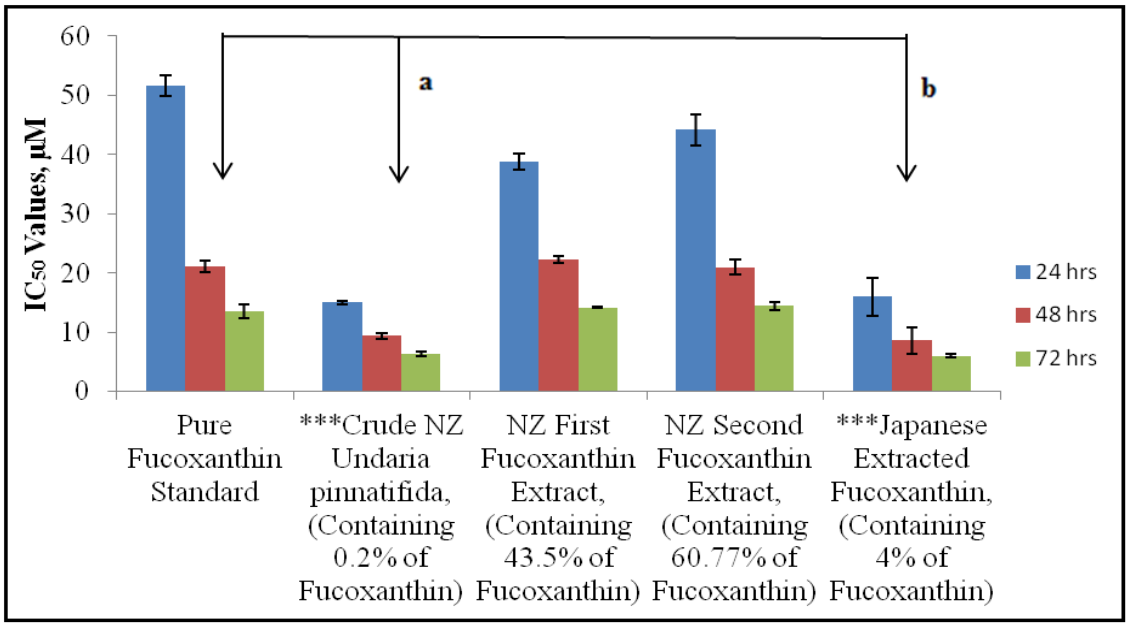

Figure 70: ***Lovo: Comparison of anti-proliferative IC_{50} values for human colon adenocarcinoma Lovo cells between pure fucoxanthin and three NZ fucoxanthin extracts and Japanese fucoxanthin extract. Data are means ± standard errors (n=6). *** means significant difference about anti-proliferative IC_{50} value compared to treatment with pure fucoxanthin standard. (a) Comparison between pure fucoxanthin and NZ fucoxanthin extracts. (b) Comparison between pure fucoxanthin and the Japanese fucoxanthin extracts.

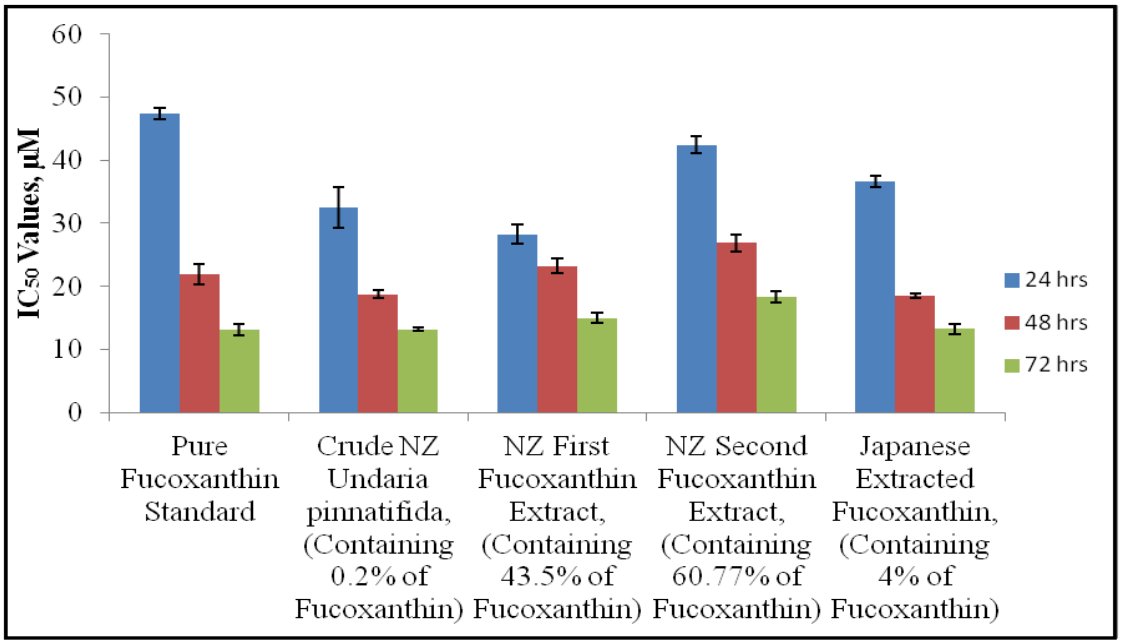

Figure 71: MCF-7: Comparison of anti-proliferation IC_{50} values for human breast adenocarcinoma MCF-7 between pure fucoxanthin and three NZ fucoxanthin extracts (containing 0.2, 43.5 and 60.77% fucoxanthin) & Japanese fucoxanthin extract (containing 4% fucoxanthin). Data are means ± standard errors (n=6).

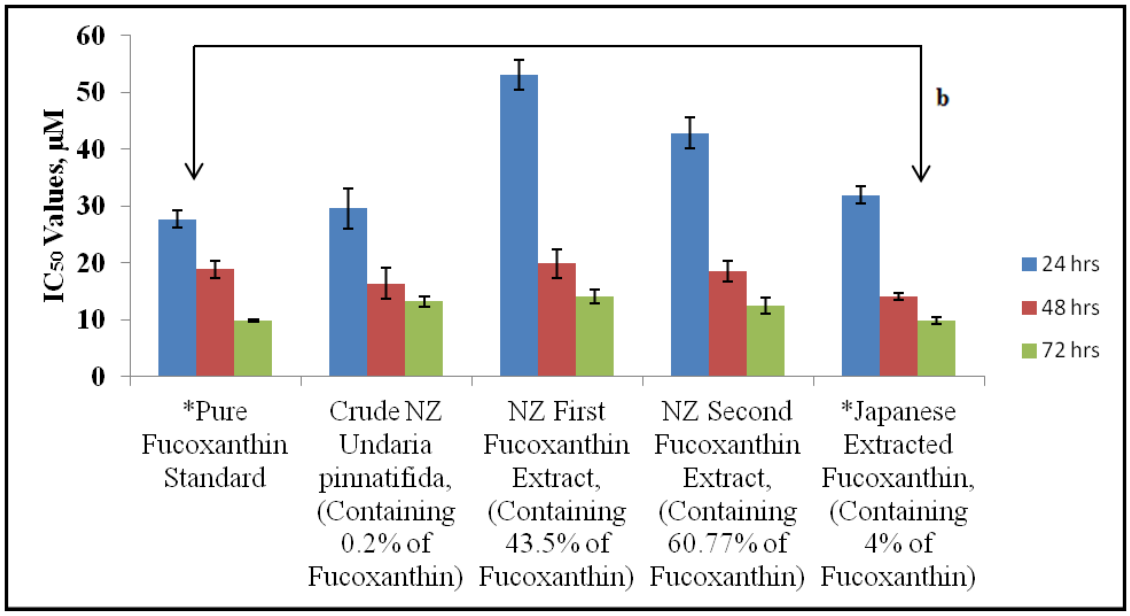

Figure 72: *SiHa: Comparison of anti-proliferative IC_{50} values for human cervix squamous carcinoma SiHa between pure fucoxanthin and three NZ fucoxanthin extracts (containing 0.2, 43.5 and 60.77% fucoxanthin) & Japanese fucoxanthin extract (containing 4% fucoxanthin). Data are means ± standard errors (n=6). * means similar about anti-proliferative IC_{50} value compared to treatment with pure fucoxanthin standard. (b) Comparison between pure fucoxanthin and the Japanese fucoxanthin extracts.

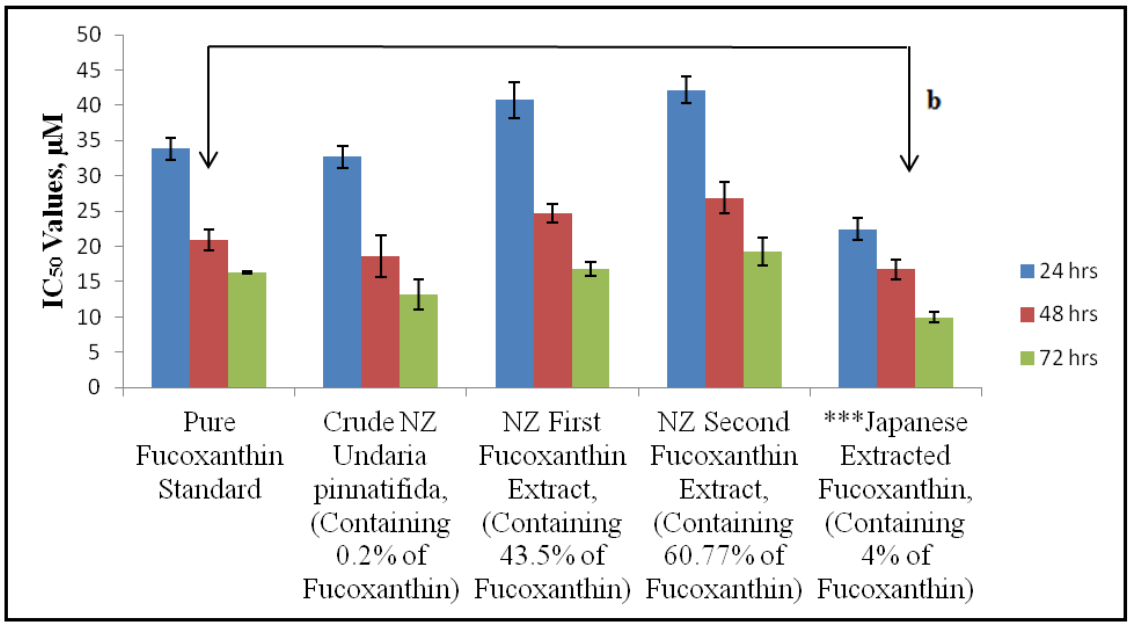

Figure 73: ***Malme-3M: Comparison of anti-proliferative IC_{50} values for human malignant melanoma Malme-3M between pure fucoxanthin and three NZ fucoxanthin extracts (containing 0.2, 43.5 and 60.77% fucoxanthin) & Japanese fucoxanthin extract (containing 4% fucoxanthin) Data are means ± standard errors (n=6). *** means significant difference about anti-proliferative IC_{50} value compared to treatment with pure fucoxanthin standard. (b) Comparison between pure fucoxanthin and the Japanese fucoxanthin extracts.

For comparison about anti-proliferative IC_{50} values between pure fucoxanthin and three NZ fucoxanthin extracts (Purity: 0.2%, 43.5% and 60.77% fucoxanthin) from Figure 65 to 73, some significant difference of anti-proliferative IC_{50} values were found in some of cancer cell lines. These will be described in the following.

For comparison about IC_{50} value between pure fucoxanthin and different NZ *U. pinnatifida* extracts, the anti-proliferative IC_{50} values after treatment for 24 hours were remarkably and statistically different in all types of cancer cells with p-value < 0.05. The example of Minitab output for statistical analysis was shown in Appendix 3. After treatment with NZ *U. pinnatifida* fucoxanthin extracts for 48 and 72 hours, the significant differences about anti-proliferative IC_{50} values between treated with pure fucoxanthin and NZ fucoxanthin extracts were found in some of cancer cell lines including WiDr*** (Figure 67), NCI-H522*** (Figure 68), SK-N-SH*** (Figure 69) and Lovo*** (Figure 70) respectively with p-value < 0.05. The anti-proliferative IC_{50} values after treatment of the crude NZ *U. pinnatifida* (0.2% fucoxanthin) for 72 hours were significantly lower than corresponding IC_{50} values determined with pure fucoxanthin standard treatment. Especially for colon adenocarcinoma cell lines WiDr*** (Figure 67), Lovo*** (Figure 70), the IC_{50} values from treatment of crude *U.*

pinnatifida was almost as twice as lower than IC_{50} value from treatment of pure fucoxanthin standard.

For comparison about IC_{50} value between pure fucoxanthin and different Japanese *U. pinnatifida* extracts from Figure 65 to 73, the anti-proliferative IC_{50} values after treatment for 24 hours were remarkably and statistically different between pure fucoxanthin and Japanese fucoxanthin extract (4% fucoxanthin) in all types of cancer cells with *p*-value = 0.000 < 0.05. After treatment for 48 and 72 hours, the significant differences on anti-proliferative IC_{50} values between treated with pure fucoxanthin and Japanese fucoxanthin extracts were found in some of cancer cell lines including WiDr*** (Figure 67), NCI-H522*** (Figure 68), SK-N-SH*** (Figure 69), Lovo*** (Figure 70), and Malme-3M*** (Figure 73) respectively. The anti-proliferative IC_{50} values after treatment of the Japanese fucoxanthin extract for 72 hours were largely lower than corresponding IC_{50} values determined from pure fucoxanthin standard treatment in several cancer cell lines including WiDr (Figure 67), NCI-H522 (Figure 68), SK-N-SH (Figure 69), Lovo (Figure 70), and Malme-3M (Figure 73). Especially for colon adenocarcinoma cell lines WiDr (Figure 67), Lovo (Figure 70), the IC_{50} values from treatment of Japanese fucoxanthin extract was almost as twice as lower than IC_{50} value from treatment of pure fucoxanthin standard.

For comparison about human cervix squamous carcinoma SiHa* (Figure 72), the anti-proliferative IC_{50} value from treatment of Japanese fucoxanthin extract was statistically significantly lower than any corresponding IC_{50} value determined from all three NZ fucoxanthin extracts treatment for 72 hours. However, the IC_{50} value determined from treatment of Japanese fucoxanthin extract was similar to that from 72 hours pure fucoxanthin standard treatment.

3.7 Anti-proliferative Effects of Fucoidan Fraction (Low Molecular Fraction) Extracted from Japanese *U. pinnatifida* on Cancer Cell Lines

All results on the effects of fucoidan fraction (low molecular weight fraction) on 9 cancer cell proliferation were shown from Figure 74 to 82

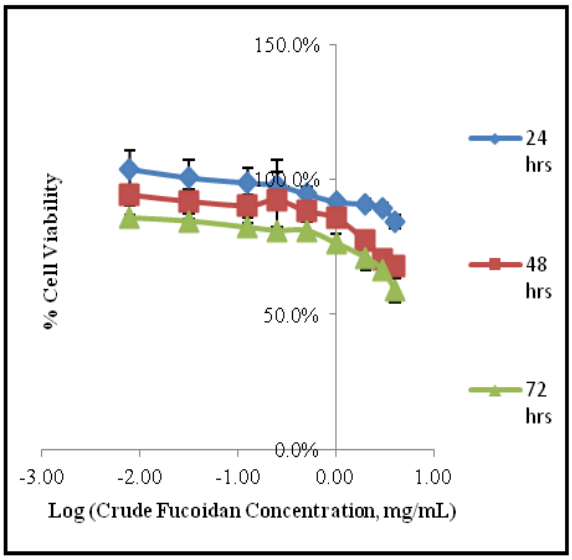

Figure 74: Inhibitory effects of fucoidan (low molecular weight fraction) extracted from Japanese *U. pinnatifida* on proliferation of human heptocellular carcinoma Hep G2. Hep G2 cells were treated with 0.0078125, 0.03125, 0.125, 0.25, 0.5, 1, 2, 3, 4mg/mL for 24, 48 & 72 hours. Data are means ± standard errors (n=3).

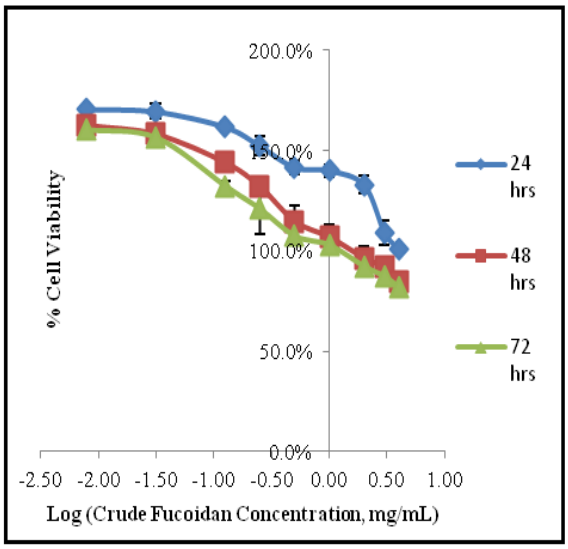

Figure 75: Inhibitory effects of fucoidan (low molecular weight fraction) extracted from Japanese *U. pinnatifida* on proliferation of human lung carcinoma A549. A549 cells were treated with 0.0078125, 0.03125, 0.125, 0.25, 0.5, 1, 2, 3, 4mg/mL for 24, 48 & 72 hours. Data are means ± standard errors (n=3).

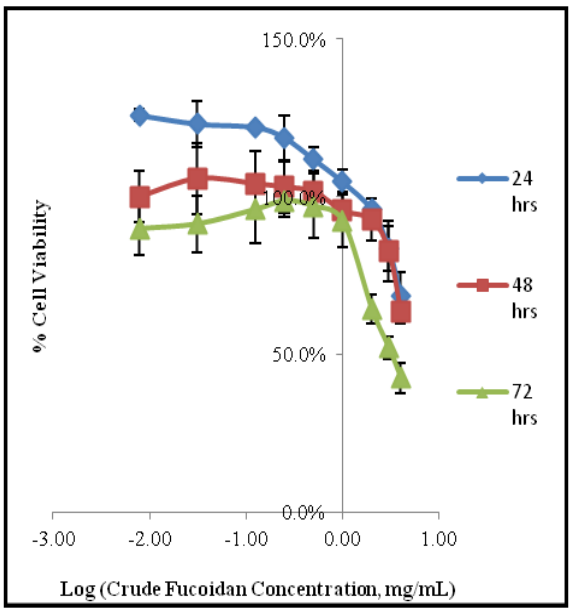

Figure 76: Inhibitory effects of fucoidan (low molecular weight fraction) extracted from Japanese *U. pinnatifida* on proliferation of human colon adenocarcinoma WiDr. WiDr cells were treated with 0.0078125, 0.03125, 0.125, 0.25, 0.5, 1, 2, 3, 4mg/mL for 24, 48 & 72 hours. Data are means ± standard errors (n=3).

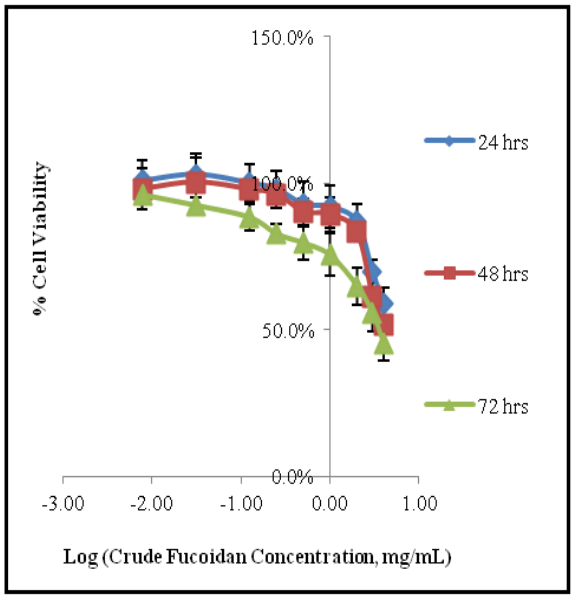

Figure 77: Inhibitory effects of fucoidan (low molecular weight fraction) extracted from Japanese *U. pinnatifida* on proliferation of human lung carcinoma NCI-H522. NCI-H522 cells were treated with 0.0078125, 0.03125, 0.125, 0.25, 0.5, 1, 2, 3, 4mg/mL for 24, 48 & 72 hours. Data are means ± standard errors (n=3).

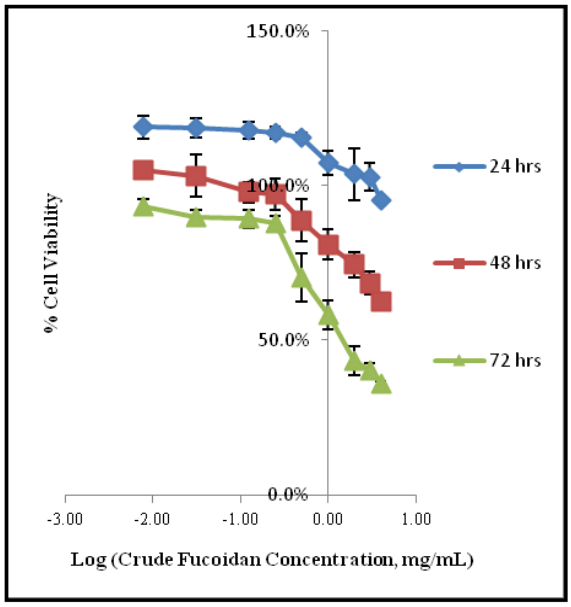

Figure 78: Inhibitory effects of fucoidan (low molecular weight fraction) extracted from Japanese *U. pinnatifida* on proliferation of human neuroblastoma SK-N-SH. SK-N-SH cells were treated with 0.0078125, 0.03125, 0.125, 0.25, 0.5, 1, 2, 3, 4mg/mL for 24, 48 & 72 hours. Data are means ± standard errors (n=3).

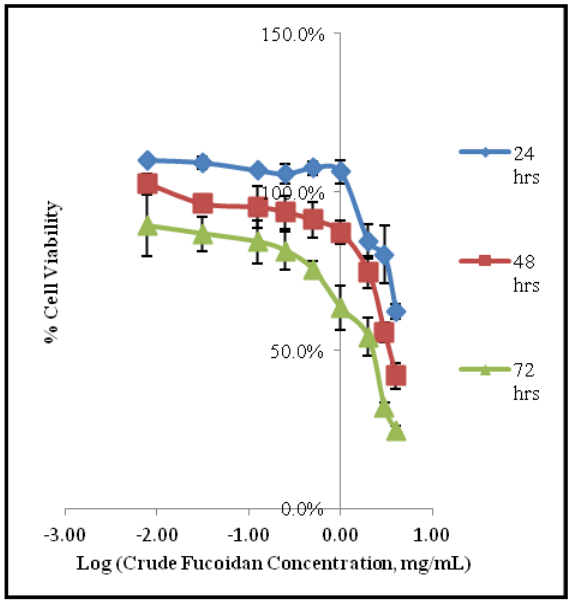

Figure 79: Inhibitory effects of fucoidan (low molecular weight fraction) extracted from Japanese *U. pinnatifida* on proliferation of human colon adenocarcinoma Lovo. Lovo cells were treated with 0.0078125, 0.03125, 0.125, 0.25, 0.5, 1, 2, 3, 4mg/mL for 24, 48 & 72 hours. Data are means ± standard errors (n=3).

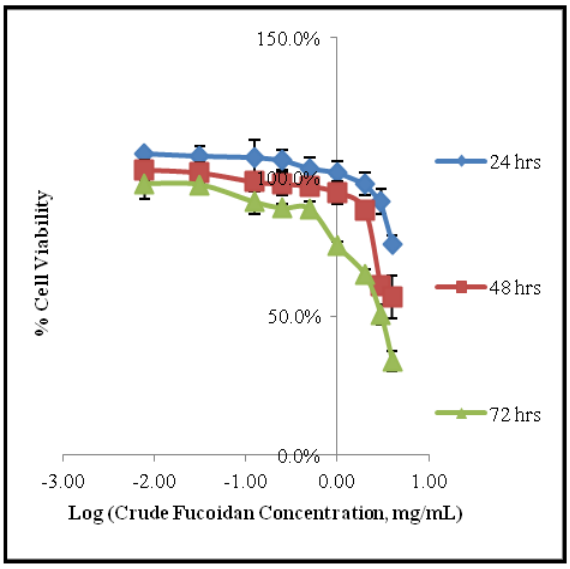

Figure 80: Inhibitory effects of fucoidan (low molecular weight fraction) extracted from Japanese *U. pinnatifida* on proliferation of human breast adenocarcinoma MCF-7. MCF-7 cells were treated with 0.0078125, 0.03125, 0.125, 0.25, 0.5, 1, 2, 3, 4mg/mL for 24, 48 & 72 hours. Data are means ± standard errors (n=3).

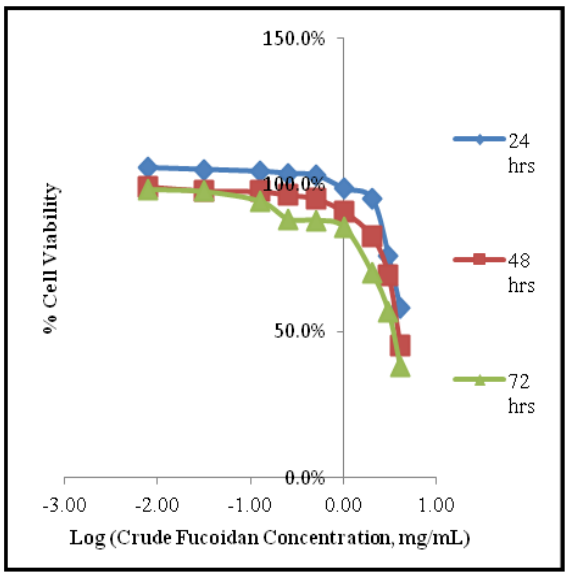

Figure 81: Inhibitory effects of fucoidan (low molecular weight fraction) extracted from Japanese *U. pinnatifida* on proliferation of human cervix squamous carcinoma SiHa. SiHa cells were treated with 0.0078125, 0.03125, 0.125, 0.25, 0.5, 1, 2, 3, 4mg/mL for 24, 48 & 72 hours. Data are means ± standard errors (n=3).

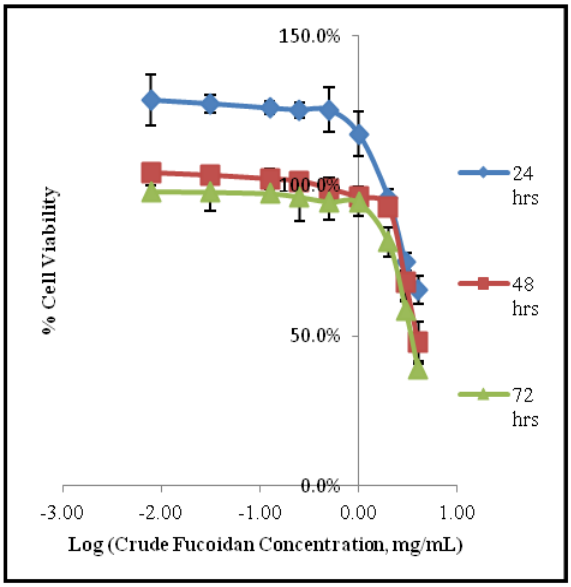

Figure 82: Inhibitory effects of fucoidan (low molecular weight fraction) extracted from Japanese *U. pinnatifida* on proliferation of human malignant melanoma Malme-3M. Malme-3M cells were treated with 0.0078125, 0.03125, 0.125, 0.25, 0.5, 1, 2, 3, 4mg/mL for 24, 48 & 72 hours. Data are means ± standard errors (n=3).

Overall, the crude fucoidan (low molecular fraction) generated anti-proliferative effects to all cancer cell lines in dose and time dependence with relative *p*-value < 0.05, however the anti-proliferative efficacy from the crude fucoidan were quite low, and its anti-proliferative effects usually were found in the concentration starting from 1 mg/mL.

In detail, within 24 hours treatment with the crude Japanese fucoidan fraction, there were minimum anti-proliferative effects on all cancer cell lines tested. Especially for neuroblastoma SK-N-SH (Figure 78), the decreased cell viability only was found in the treatment with crude fucoidan at any concentration above 3 mg/mL in the first treatment day. For cancer cell lines A549 (Figure 75), WiDr (Figure 76), Lovo (Figure 79), MCF-7 (Figure 80) and Malme-3M (Figure 82), the crude fucoidan treatment led to decrease cell viability less than 100% at the concentration above 1 mg/mL. After treatment for 48 hours with the crude fucoidan, the cell number remained original level (100%) in low concentration range from 0.0078125 mg/mL to 1 mg/mL. This was found in cancer cell lines including Hep G2 (Figure 74), WiDr (Figure 76), NCI-H522 (Figure 77), SK-N-SH (Figure 78), MCF-7 (Figure 80), SiHa (Figure 81) and Malme-3M (Figure 82). Within treatment for 72 hours, cell viability was remarkably decreased to less 50% in most types of cancer cell lines including WiDr (Figure 76), NCI-H522 (Figure 77), SK-N-SH (Figure 78), Lovo (Figure 79), MCF-7 (Figure 80), SiHa (Figure 81) and Malme-3M (Figure 82). However, cell viability was reduced to 50% in high concentration of Japanese fucoidan (Low molecular weight fraction) treatment. In some types of cancer cell lines such as SK-N-SH, Lovo & MCF-7, the fucoidan fraction concentration that significantly reduced cell viability was started at 0.5mg/mL. In dose-response curve of Hep G2 (Figure 74) and A549 (Figure 75) shown, the cell viability remained above 50% after treatment for three days with fucoidan at concentration up to 4 mg/mL.

Chapter 4 Discussion

4. Discussion

4.1 Quantification of Fucoxanthin in Extracts from New Zealand *U. pinnatifida* by HPLC

As Figure 16 shows, all-*trans*-fucoxanthin in HPLC result of pure fucoxanthin standard showed in the retention time at the middle of 1.5 to 2.0 mins, and 13'-*cis*-isomer of fucoxanthin showed in the retention time at the middle of 2.5 to 2.5 mins. Based on a previous study, all-*trans* fucoxanthin was the major geometrical isomer (~88%) found in the fresh brown seaweed (*U. pinnatifida*) apart from a small amount of 13-*cis* and 13'-*cis* isomers (~9%) (Nakazawa et al., 2009). In addition, *U. pinnatifida* powder samples were stored in a refrigerator for 2 months at 5 °C in the dark condition in previous study, resulting in significant amount of 9'-*cis* isomer (Nagamine et al., 2009). A similar condition was applied to store *U. pinnatifida* powder sample in this study. Therefore, all-*trans*-fucoxanthin was constantly found in the retention time at the middle of 1.5 to 2.0 mins, and another small pink in both Figure 24 and 25 might be 9- and/or 13-*cis*-isomer of fucoxanthin.

4.2 Interference Factors in MTT Cell Proliferative Viability Assays

4.2.1 Effects from Fucoidan to Anti-proliferative Data Analysis

Since nearly all of the identified components from plants active against microorganisms are aromatic or saturate organic compounds, they are most often obtained initially through ethanol, methanol and water extraction (Mendonça-Filho, 2006). There are two major components including fucoxanthin and fucoidan contained in *U. pinnatifida* (Nishibori et al., 2012). The anti-cancer potentials were already determined in these two compounds extracted from *U. pinnatifida* in previous studies (Aisa et al., 2005; S. K. Das, Ren, Hashimoto, & Kanazawa, 2010; Hosokawa et al., 2004; Kolanjinathan, Ganesh, & Govindarajan, 2009; Yamasaki-Miyamoto et al., 2009). Fucoidan refers to a type of sulfated polysaccharide. The solubility of polysaccharide compound is

reasonably high in water, but insoluble in organic solvents such as methanol and ethanol. In this study, *U. pinnatifida* powder was soaked in the pure methanol for 2 days first, and then undissolved materials in the pure methanol were removed through filtration. Thus, fucoidan contained in NZ *U. pinnatifida* was removed after filtration of pure methanol solution. In this study, there was three extracts from NZ *U. pinnatifida* including crude *Undaria* extracts, first fucoxanthin extract and second fucoxanthin extract respectively. Crude *Undaria* extract was obtained from filtration of pure methanol solution after NZ *U. pinnatifida* was soaked in pure methanol for 2 days. The first and the second fucoxanthin extracts were involved in analytical chemistry extraction and silica gel purification. Therefore, fucoidan compound was not expected to be present in all three fucoxanthin extracts obtained from NZ *U. pinnatifida*. Therefore, fucoidan compounds did not generate any effects in the following MTT cell viability experiments.

4.2.2 Determination of Biological Pure Ethanol as Major Fucoxanthin Solvent

In this study, two ethanolic concentration ranges were applied to dissolve fucoxanthin to make 20mM and 5mM of fucoxanthin ethanolic stock solution first. These two ethanolic stock solutions were diluted by amount of completed culture medium to culture with cancer cell lines for cell viability determination. In this study, pure fucoxanthin ethanolic stock solution (20mM) was diluted with culture medium to determine the standard of anti-proliferative IC_{50} values for further comparison with NZ and Japanese fucoxanthin extracts. 5mM of ethanolic stock solution was used for determination of anti-proliferative IC_{50} values for pure fucoxanthin, NZ and Japanese fucoxanthin extracts. 20mM of pure fucoxanthin ethanolic solution were dilution to 50 fold, and 5mM of pure fucoxanthin ethanolic solution were dilution to 50 fold by completed culture medium. Therefore, 0.391 to 200μM fucoxanthin culture solution contained 0.001953% to 1% (v:v) ethanol, and 1.5625 to 100μM fucoxanthin culture solution contained 0.03125% to 2% (v:v) ethanol.

The results indicated cell viability was not affected by biological ethanol, suggesting biological ethanol at concentration range (0.001953% to 1% (v:v) & 0.03125% to 2% (v:v)) has no positive or negative effect to cancer cell growth compared to the control group (0% of pure ethanol). Therefore, the biological ethanol could be used as the major

solvent used for pure fucoxanthin standard or other fucoxanthin extraction in all anti-proliferative experiments with all nine cancer cell lines tested in this thesis.

4.2.3 Analysis of Systemic Variations in MTT Cell Viability Assays

Cell viability of fucoxanthin was the core of MTT absorbance measurement in this study. However, colour of extracts or drug is the most important interference in the results of MTT cell viability assay. In this study, colour of pure fucoxanthin, first and second NZ fucoxanthin extracts was red-orange. Due to high impurity contained such as chlorophyll, the colour of crude NZ *U. pinnatifida* extract and Japanese fucoxanthin extract was green. As expected, a negative-distribution sigmoid relationship should be obtained which shows cell viability decreases with increased concentration of fucoxanthin applied. However, colours of those extract significantly affected MTT absorbance cell viability results. In MTT theory higher absorbance value indicates higher number of living cells as a large amount of MTT can be reduced to purple formazan by mitochondrial dehydrogenase of living cells. However, red-orange colour or green colour from pure fucoxanthin or NZ and Japanese fucoxanthin extracts could interfere with values generated by formazan, leading to false negative effects of pure fucoxanthin standard or NZ and Japanese fucoxanthin extracts in MTT assay. This problem can be solved by a simple one-step gentle wash of cells with PBS to remove majority of interference. On the other hand, some of cancer cells might be removed as well when the phosphate buffer solution was used to wash each well. However, the washing step was not only applied to all fucoxanthin standard and extracts treatment groups, but also applied to control group (without treatments). Thus, the interference about losing cell was about the systemic variation, so that this interference did not affect the accuracy and reliability of cell viability measurement.

4.2.4 Reliability of Results from MTT Cell Viability Method

In previous year, a large amount of efforts was paid to focus on determination of anti-proliferative effect from pure fucoxanthin. Many types of cancer cell lines were used to examine the anti-proliferative effects from pure fucoxanthin including leukemia cancer cell lines (HL-60) (K.-N. Kim, S.-J. Heo, S.-M. Kang, G. Ahn, & Y.-J. Jeon, 2010), colon cancer cell lines (Caco-2, WiDr, HT-29 and DLD-1) (Swadesh K. Das et al., 2005; Masashi & Kazuo, 2007; Sugawara et al., 2002; Sugawara, Kinoshita, Ohnishi, Nagata, & Saito, 2003), lung cancer cell lines (A549, NSCLC-N6 and SRA 01/04) (D. Moreau

et al., 2006), prostate cancer cell line (PC-3, DU 145, and LNCaP) (E. Kotake-Nara et al., 2001), liver cancer cell lines (Yoshiko & Hoyoko, 2007), human gastric adenocarcinoma (R. X. Yu, X. M. Hu, S. Q. Xu, Z. J. Jiang, & W. Yang, 2011), human neuroblastoma cancer cell lines (Okuzumi et al., 1990), and human breast carcinoma (Ayyad et al., 2011) respectively. In this study, some of cancer cell lines included human bronchopulmonary epithelial cell line A549, human breast carcinoma MCF-7, human heptocellular carcinoma Hep G2 and human colon adenocarcinoma WiDr. The quantitative anti-proliferative effects (IC_{50}) from fucoxanthin were determined for those types of cancer cell lines. Our IC_{50} results were consistent with data published previously, suggesting all cell viability results based on MTT assays were valid and reliable in this study.

4.3 Anti-proliferative Effects of Pure Fucoxanthin Standard

4.3.1 Anti-proliferative Effects of Pure Fucoxanthin Standard

As Figure 19 to 27 shown, anti-proliferative effects of pure fucoxanthin standard were found in all types of cancer cell lines in dose- and time-dependent manners. This indicated cell viability can be largely decreased with significant increased dose and prolonged treatment with pure fucoxanthin standard. As described in results section, in most types of cancer cell lines, the cell viability could be significantly decreased starting concentration of fucoxanthin at 12.5 or 25 μM in all three days treatment except Malme-3M in Figure 27.

For Malme-3M, its IC_{50} value after treatment with pure fucoxanthin standard was similar to other cancer cell lines. In fact, this was the disadvantage to use the modified Karber method for IC_{50} calculation. The modified Karber method used all absorbance OD values from a series of fucoxanthin concentration treatments. Therefore, in order to define or compare anti-proliferative effects of compounds, the IC_{50} value from the modified Karber method calculation was not the unique scientific criteria. The relationship between concentration of treatment and cell viability also needed to be referenced.

In this study, the Malme-3M IC_{50} values after pure fucoxanthin treatment were similar to other types of cancer cell lines (Table 17). However, the cell viability of Malme-3M was significantly decreased in exposure of pure fucoxanthin concentration at 50μM of treatment within 24 hours (Figure 27). In addition, cell viability was not decreased to

80% in the low treatment concentration after treatment for 48 and 72 hours. This possibly suggests anti-proliferative effects of pure fucoxanthin standard was minor on human malignant melanoma Malme-3M.

4.3.2 Comparison (Intra-Comparison) of Anti-proliferative Effects from Pure Fucoxanthin Standard on Nine Cancer Cell Lines

As Figure 19 to 27 shown, anti-proliferative effects from pure fucoxanthin standard were found in all types of cancer cell lines in dose- and time-dependent manners. In addition, after treatment of fucoxanthin for 72 hours, cell viability was decreased to 20% or less 20% in the high concentration of fucoxanthin. This suggested the anti-proliferative effect from pure fucoxanthin was very effective to multiple types of cancer cell lines.

The Figure 28 (A, B & C) showed about the comparison of anti-proliferative IC_{50} value from pure fucoxanthin standard treatment with all cancer cell lines in 24 hours, 48 hours and 72 hours. Overall, the anti-proliferative IC_{50} values on treatment for 24 hours were largely different between each cancer cell line. However, the IC_{50} values in treatment for 48 and 72 hours were not significantly different between each cancer cell line. The sensitivity of anti-proliferative effects can be used to analysis for all cancer cell lines. The sensitivity of fucoxanthin inhibition depends on anti-proliferative IC_{50} data, but cell doubling time and mechanism of fucoxanthin action were also considered to determine sensitivity from fucoxanthin inhibition. The sensitivity of anti-proliferative effects from fucoxanthin was analysed to categorize into Group of High Sensitivity, Mild Sensitivity and Low Sensitivity based on the IC_{50} value in each cancer cell line.

As comparison about IC_{50} values (Table 17, Figure 28 A, B & C) in all nine cancer cell lines, the first and the most obvious feature was that the lowest IC_{50} values was found in all three days in human cervix squamous carcinoma SiHa. Based on data shown, anti-proliferative IC_{50} value for cancer cell lines NCI-H522, Lovo and MCF-7 was higher than anti-proliferative IC_{50} values of SiHa and lower than other types of cancer cell lines in all three treatment days. These three cancer cell lines were mild sensitive to treatment with pure fucoxanthin standard. Due to higher IC_{50} values, other cancer cell lines showed minor anti-proliferative effects after treatment with the pure fucoxanthin standard. The 48-hour anti-proliferative effects for human neuroblastoma SK-N-SH was not significantly different to the mild sensitivity of cancer cell lines. However, 72-hour

IC_{50} value of SK-N-SH was the highest compared with all the other cancer cell lines, suggesting human neuroblastoma SK-N-SH is less sensitive to pure fucoxanthin treatment.

Doubling time for near all types of cancer cell lines were less than 30 hours except human colon adenocarcinoma Lovo (about 37 hours) and malignant melanoma Malme-3M (about 36 hours). Therefore, the doubling time for those types of cancer cell lines was not largely different, so that this is not the important reason to affect inhibition effects from pure fucoxanthin standard.

For mechanism of fucoxanthin action, fucoxanthin can arrest in the G0/G1 phase of the cell cycle for cancer cell lines including Hep G2, WiDr. Therefore, fucoxanthin for these two cancer cell lines can directly inhibit the DNA replication step in cell cycle, and directly reduce proliferation of cancer cell. However, compared between WiDr and Hep G2, the anti-proliferative IC_{50} values in 48 hours and 72 hours were not significantly different (*p*-value > 0). For human breast adenocarcinoma, however, fucoxanthin can be a cell apoptosis inducer to reduce the proliferation of MCF-7. For human lung carcinoma A549, fucoxanthin contributes to morphological change and triggers the terminal differentiation of cancerous cells *in vitro* (D. Moreau et al., 2006). Thus, the differential and anti-proliferative sensitivities of fucoxanthin on various cancer cell lines (Figure 28) may be due to the different mechanism of action of fucoxanthin.

Therefore, human cervix squamous carcinoma SiHa is the most sensitive to anti-proliferative effects from pure fucoxanthin standard. The cancer cell lines NCI-H522, Lovo and MCF-7 can be categorised in the mild sensitivity to the anti-proliferative fucoxanthin effects. For the cancer cell lines Hep G2, A549, and WiDr, these can be grouped in less sensitivity to anti-proliferative fucoxanthin effects. For neuroblastoma SK-N-SH, The 48-hour anti-proliferative effect for SK-N-SH was not significantly different to the mild sensitivity of cancer cell lines. However, 72-hours IC_{50} value of SK-N-SH was the highest compared to all cancer cell lines. This suggested human neuroblastoma SK-N-SH should belong to group of insensitivity to anti-proliferative effects of pure fucoxanthin standard.

Malignant melanoma Malme-3M appeared to be another cancer cell line that was sensitive to anti-proliferative effects of fucoxanthin in this study (Table 1). However, the fucoxanthin at low range of concentration (from 1.5625 to 12.5 μM) showed no

significant effects on malme-3M cell viability within 72 hour treatment. Especially for treatment in day 3, fucoxanthin concentration at 12.5 μM can only decreased cell viability to 70%. The cell viability of Malme-3M decreased dramatically after fucoxanthin treatment at higher concentrations.

4.3.3 Fucoxanthin as a Potential Anti-Cancer Drug and Chemopreventive Agent

From all result and analysis above, fucoxanthin generated anti-proliferative effects to all cancer cell lines in this study. Especially human cervix adenocarcinoma SiHa cells were the most sensitive to anti-proliferative effects from fucoxanthin. In addition, the anti-proliferative effects from fucoxanthin can effectively decrease cell viability to some types of lung, colon and breast cancer cell lines with mild sensitivity. This means fucoxanthin can effectively decrease risk of cancer or can be potentially used for treatment of multiple types of cancer.

Fucoxanthin was reported to have few adverse effects on normal cells both *in vitro* and *in vivo* (Ishikawa et al., 2008; Yamamoto, Ishikawa, Katano, Yasumoto, & Mori, 2011). At a single dose, no mortality and abnormalities were found after dosing of 1000 and 2000 mg/kg in mice *in vivo* (Beppu, Niwano, Tsukui, Hosokawa, & Miyashita, 2009). The 50% lethal dose of fucoxanthin was more than 2000 mg/kg body weight (Peng et al., 2011). At repeated doses, oral administration of fucoxanthin (purity at 95%) at 750 mg/kg was applied to rats for 28 days. The result suggested fucoxanthin did not show obvious toxicity (Kadekaru, Toyama, & Yasumoto, 2008). Furthermore, histological study suggested no abnormality was found in different tissue including liver, kidney, spleen, and gonadal, after repeating dose of 500 and 1000 mg/kg for 30 day in animal mice study (Peng et al., 2011). In addition, the genotoxic/mutagenic effects were also not found on bone marrow cells of mice (Iio et al., 2011; Kumiko et al., 2011). Thus, compared to other existed cancer cytotoxic chemotherapy, fucoxanthin can generate anti-proliferative effects to multiple types of cancer cell with low toxicity. At this stage, fucoxanthin may be considered as chemopreventive agent and can be potentially used in cancer chemotherapy.

However, the dietary fucoxanthin was totally deacetylated into the fucoxanthinol in the intestinal tract by lipase and esterase from the pancreas or intestinal cells, and incorporated as fucoxanthinol from the digestive tract into the blood circulation system

in mammals, but also the bioavailability of fucoxanthinol was higher than its origin fucoxanthin in the body (Sugawara et al., 2002), furthermore, the fucoxanthinol was converted into amarouciaxanthin A through dehydrogenation/isomerization in liver. Therefore, a large amount of fucoxanthin was metabolised and anti-cancer activities only were attributed to its two metabolites fucoxanthinol and amarouciaxanthin A. The low fucoxanthin concentration in drug action site may translate to extremely low anti-cancer activities. Therefore, if fucoxanthin can be considered as a potential anti-cancer drug, the oral administration of fucoxanthin is not an effective way of administration for cancer treatment.

Basically, all cancer cell lines in this study were sensitive to anti-proliferative effects of fucoxanthin. As a chemopreventive phytochemical, the risk of cancer might be reduced if administration of fucoxanthin or foods contained fucoxanthin in rich such as *U. pinnatifida*. In addition, fucoxanthin or its relative metabolite fucoxanthinol generate few adverse effects on normal and uninfected cells in both *in vitro* and *in vivo* study (Ishikawa et al., 2008; Yamamoto et al., 2011). Thus, fucoxanthin might be potentially used in combination with existing anti-cancer drugs as an important sensitizer in the clinical cancer chemotherapy.

4.4 Anti-proliferative Effects of New Zealand Fucoxanthin Extracts

There were three fucoxanthin extracts including crude *U. pinnatifida* extracts (0.2% fucoxanthin), the first fucoxanthin extracts (43.55%), and the second fucoxanthin extracts (60.77%).

4.4.1 Anti-proliferative Effects of Crude *U. pinnatifida* Extracts (Purity: 0.2% Fucoxanthin)

The Figure 29 to 37 showed the results about anti-proliferative effect from crude *U. pinnatifida* extracts, and the crude *U. pinnatifida* contained 0.2% of fucoxanthin. The extract cultured with cancer cell lines was made based on the purity of 0.2% fucoxanthin. As results shown, the anti-proliferative effects of crude fucoxanthin were in dose- and time-dependent manners. There were three distinctive lines indicated each day of treatment with a series of fucoxanthin dilution. Cell viability significantly decreased with increasing concentration of fucoxanthin treatment. After treatment for

72 hours, cell viability was reduced to less 20% in some of cancer cell lines including WiDr (Figure 31), NCI-H522 (Figure 32), Lovo (Figure 34), and Malme-3 M (Figure 37). However, for human malignant melanoma Malme-3M the cell viability was decreased slightly in low concentration range in all three days treatment. This clearly indicated anti-proliferative effects of crude *U. pinnatifida* extracts with 0.2% fucoxanthin were less potent on Malme-3M cell growth with other cell lines tested.

4.4.2 Anti-proliferative Effects of First & Second Fucoxanthin Extracts (Purity: 43.5% & 60.77% Fucoxanthin)

The Figure 38 to 55 showed results about anti-proliferative effect from the first and second *U. pinnatifida* extracts, the first and second fucoxanthin extracts contained 43.5% and 60.77% of fucoxanthin. As results shown, the anti-proliferative effects from the first and second fucoxanthin extracts were statistically found in dose- and time-dependent manners. After treatment with these extracts, cell viability in most types of cancer cell lines was remarkably decreased with increased concentration of fucoxanthin, and these types of cancer cell lines are the same between two treatments in different purity of fucoxanthin extract. In addition, the cell viability was reduced by treatment of these two fucoxanthin extracts within low concentration. The results also suggested the growth of Malem-3M was inefficiently inhibited by fucoxanthin.

4.5 Anti-proliferative Effects of Japanese Fucoxanthin (purity: 4% Fucoxanthin) & Crude Fucoidan Fraction (Low Molecular Weight Fraction) Extracts

4.5.1 Anti-proliferative Effects of Japanese Fucoxanthin Extracts (Purity: 4% Fucoxanthin)

The Figure 56 to 64 showed results about anti-proliferative effect from the Japanese fucoxanthin extracts, the Japanese fucoxanthin extracts contained 4% of fucoxanthin. As results shown, the anti-proliferative effects from the Japanese fucoxanthin extracts were statistically found in dose- and time-dependent manners.

4.5.2 Anti-proliferative Effects of Japanese Fucoidan Extracts (Low Molecular Weight Fraction)

Based on statistical values, the anti-proliferative effects from the Japanese fucoidan in low molecular fraction were found as dose- & time-dependent manners. However, the significant anti-proliferative effects from Japanese fucoidan (low molecular weight fraction) usually were found in the concentration starting from 1 mg/mL in most types of cancer cell lines. In addition, cell proliferative effect of human neuroblastoma SK-N-SH was dominant than anti-proliferative effects from Japanese fucoidan fraction. As Figure 78 shown, it can only change cell viability at a concentration at 3 mg/mL or above in the first day treatment. Therefore, the Japanese fucoidan (low molecular weight fraction) definitely generated anti-proliferative effects to all nine cancer cell lines, but these anti-proliferative effects were not strong enough to be considered as potential compound with anti-cancer effects.

4.6 Comparison (Inter-Comparison) of Anti-proliferative Effects to Nine Caner Cell Lines between Pure Fucoxanthin Standard and New Zealand & Japanese Fucoxanthin Extracts

4.6.1 Comparison of Anti-proliferative Effects on Nine Caner Cell Lines between Pure Fucoxanthin Standard and Crude *U. pinnatifida* Extracts, First & Second Fucoxanthin Extracts

This section is to compare the IC_{50} value between three NZ *U. pinnatifida* extract with different purity of fucoxanthin (Crude *U. pinnatifida*: 0.2% of fucoxanthin; First fucoxanthin extract: 43.5% of fucoxanthin; and Second fucoxanthin extract: 60.77% of fucoxanthin) and pure fucoxanthin. There were three batches of extracts from NZ *U. pinnatifida*, which were used to treat nine cancer cell lines. These three fucoxanthin extracts were highly correlated and were from the sample NZ *U. pinnatifida* powder.

24-hour anti-proliferation results show significant differences in anti-cancer activities between pure fucoxanthin and different NZ fucoxanthin extracts in all types of cancer cells. However, 48- and 72-hour anti-proliferation results only show the significant differences of IC_{50} values between pure fucoxanthin standard and three fucoxanthin

extracts in several cancer cell lines including two colon adenocarcinoma cell lines Lovo and WiDr (Figure 67, 70), and lung carcinoma NCI-H522 (Figure 68). The IC_{50} values from treatment of crude *Undaria* extract were significantly lower than corresponding IC_{50} values after 72 hours treatment with pure fucoxanthin in those three cancer cell lines. In addition, IC_{50} values for hepatocellular carcinoma, lung carcinoma A549 and human neuroblastoma SK-N-SH (Figure 69) after treatment of crude *U. pinnatifida* extract for 72 hours were marginally but statistically significantly lower than the corresponding values derived from treatment with pure fucoxanthin. These results suggested some other compounds with anti-cancer properties might be present in crude NZ *U. pinnatifida*.

Despite of low amount these compounds still significantly inhibited the growth of cancer cell, suggesting they were very potent anti-cancer compounds and may be extremely important for further development as a novel drug for colon and lung cancers. In addition, due to presence of these compounds, this suggested dietary NZ *U. pinnatifida* potentially generate the healthy benefit to cancer prevention especially for colon and lung cancer. These compounds might be a single compound or a group of compounds present in the NZ *U. pinnatifida* fucoxanthin extracts. In addition, the amount of these unknown compounds was tiny, but its effects to dramatically reduce cancer cell viability. Thus, these unknown compounds might be more effective than fucoxanthin to reduce cell viability or might combine with fucoxanthin to generate higher anti-proliferative effects to growth of cancer cell. In addition, other possible compounds could generate potent anti-proliferative effects to human neuroblastoma SK-N-SH in all three fucoxanthin extracts from NZ *U. pinnatifida*. The possible compounds were also present in the first and second fucoxanthin extracts, so these compounds might be different to those compounds with anti-cancer potentials to colon and lung cancer cell lines as mentioned above.

In fact, the crude *U. pinnatifida* extracts were achieved by soaking its powder in pure methanol. In the process of analytical chemistry extraction, the main reason to use of pure hexane was to remove non-polar substance such as some of carotene and chlorophyll compounds. The silica gel chromatography in this study was a purification step of normal phase chromatography, and the main purpose was to remove polar compounds from extraction. Therefore, these purification steps contribute to remove those possible compounds with anti-cancer potentials, so that this was the main reason

that the IC_{50} value from crude *U. pinnatifida* extracts in colon and lung cancer cell lines were lower than corresponding IC_{50} value from the first and second fucoxanthin extracts.

4.6.2 Comparison of Anti-proliferative Effects to Nine Caner Cell Lines between Pure Fucoxanthin Standard and Japanese Fucoxanthin Extracts

This section is to compare the IC_{50} values between the Japanese *U. pinnatifida* extract with 4% of fucoxanthin and pure fucoxanthin. This 4% of Japanese fucoxanthin extract was achieved by separation of supercritical carbon dioxide instead of using analytical chemistry method. As mentioned, the fucoxanthin extraction highly depends on the ratio of polarity. The greatest advantage is that fucoxanthin can be extracted in supercritical carbon dioxide extraction method without using many types of organic solvent with different polarity. Therefore, this novel supercritical carbon dioxide extraction method can preserve most of compounds from *U. pinnatifida* sample, which probably possess anti-cancer potentials. Therefore comparison of IC_{50} values between the Japanese fucoxanthin extract and pure fucoxanthin standard can determine whether there are any novel compounds with anti-cancer potentials in the Japanese fucoxanthin extract.

Overall, 24-hour anti-proliferative results between effects of Japanese fucoxanthin extracts and pure fucoxanthin were largely different. After 48 and 72-hour treatment of the Japanese fucoxanthin extracts, higher anti-proliferative effects were found in treatment with the Japanese fucoxanthin extracts in several cancer cell lines including two human colon adenocarcinoma (Figure 67, 70), human lung carcinoma NCI-H522 (Figure 68), human neuroblastoma SK-N-SH (Figure 69) and human malignant melanoma Malme-3M (Figure 73). Especially for WiDr, Lovo and Malme-3M, their anti-proliferation activities from the Japanese fucoxanthin extract appeared as twice as more potent than the pure fucoxanthin standard. This possibly suggested some of novel compounds that possess anti-cancer potential were present in the Japanese fucoxanthin extracts, and these compounds might be more potent than fucoxanthin.

Interestingly, in human malignant melanoma Malme-3M cells, the anti-proliferative IC_{50} values from the treatment of the Japanese fucoxanthin were significantly lower than pure fucoxanthin standard. This probably indicated some of compounds with anti-cancer potentials were present in the Japanese fucoxanthin extracts. However, this high anti-proliferation was not found after treatment of three NZ fucoxanthin extracts. Thus,

the possible compounds might be removed during fucoxanthin extraction and purification processes using conventional methods.

4.7 Novel Japanese Extraction Methods for Fucoxanthin and Fucoidan

There are two main compositions in *U. pinnatifida,* namely fucoxanthin and fucoidan. Many isolation methods have been developed to extract these two important compositions from *U. pinnatifida*. Commonly, analytical chemistry methods were always used for isolation of the marine compounds such as fucoxanthin and fucoidan with large amount of organic or inorganic solvent consumed. To extract fucoxanthin, the common organic solvents such as HPLC grade pure methanol, hexane, acetone, and analytical graded chloroform were used in this study. For extract of fucoidan, some inorganic solvents such as hydrochloride, calcium chloride (C. Yang et al., 2008), sodium bicarbonate (Vishchuk et al., 2011) were commonly used in isolation of fucoidan from *U. pinnatifida*. However, the Japanese extract was obtained by using a novel method to extract two components from *U. pinnatifida* simutanously. In addition, this method mainly used carbon dioxide gas and water instead of a large amount of organic solvent consumption.

For extraction of fucoxanthin, the anti-proliferative effects from Japanese fucoxanthin extracts were found to be in dose and time-dependent on all cancer cell lines tested, and these effects were generally similar to pure fucoxanthin standard and all three NZ fucoxanthin extracts in terms of dose-response pattern. The interesting difference was found the anti-proliferative IC_{50} values from human cervix squamous carcinoma SiHa in Figure 72. The 72-hour anti-proliferation activity of the Japanese fucoxanthin extracts was similar to that of pure fucoxanthin standard. However, the IC_{50} value was lower than anti-proliferative effects from all three NZ *U. pinnatifida* fucoxanthin extracts. Given the low purity of fucoxanthin (4%) in Japanese extracts, this possibly suggests some novel compounds which could inhibit growth of SiHa were only found in the Japanese fucoxanthin extracts, and were not found in the NZ *U. pinnatifida* fucoxanthin extracts. Similarly, the difference in anti-proliferative effects from NZ *U. pinnatifida* extracts and the Japanese fucoxanthin extracts is that anti-proliferation of Japanese fucoxanthin to Malme-3M (Figure 73) was higher than corresponding anti-proliferative effects from both pure fucoxanthin standard and all three NZ fucoxanthin extracts. This indicated some novel compounds that possess inhibitory effects to human malignant

melanoma Malme-3M may be present in the Japanese fucoxanthin extracts and may not be present in the NZ fucoxanthin extracts. Possibly the composition of NZ and Japanese *U. pinnatifida* powder was different, possibly because some potentially anti-cancer potential compounds to malignant melanoma was removed from organic solvent, but not removed by supercritical carbon dioxide. This also implicates different extraction methods could also contribute to findings of novel bioactive compounds from seaweed *U. pinnatifida*. Thus, fucoxanthin was effectively and successfully isolated from the Japanese *U. pinnatifida* sample by supercritical carbon dioxide.

For extraction of fucoidan (low molecular weight fraction), the anti-proliferative effects from the Japanese fucoidan in low molecular fraction were found in dose- & time-dependent manners. However, as discussed above the anti-proliferative effects from Japanese fucoidan fraction was very low. From literature data shown, human lung carcinoma A549 and hepatocellular Hep G2 were already studied about anti-proliferative effects of fucoidan extract from *U. pinnatifida*. Fucoidan fraction was successfully isolated from sporophyll of *U. pinnatifida* in boiling water with hydrochloride, and the fucoidan fraction showed the significant anti-cancer activity of A549 to 75.9% at concentration of 1 mg/mL (C. Yang et al., 2008). In further study, the fucoidan was isolated with similar extraction method, A549 and Hep G2 was used to determine anti-proliferative effects from fucoidan fraction of *U. pinnatifida*. The treatment period was 24 hours for these two cancer cell lines with concentration range from 0.1 mg/mL to 0.8 mg/mL. Fucoidan showed much more potent anti-proliferation effects for A549 than Hep G2. The cell viability decreased to 60% following the treatment of fucodian at 0.1 mg/mL, and 40% at 0.8 mg/mL. Anti-proliferative effects of fucoidan fraction were quite weak for Hep G2 in the same concentration ranges.

Compared to previous data, the anti-proliferative effects were quite weak from the Japanese fucoidan extract. As shown in Figure 82, the cell viability increased to more than 100% in the concentration range from 0.1 mg/mL to 0.8 mg/mL fucoidan fraction treatment on human lung carcinoma A549 cells, and this could be found in all three-day treatment. This suggested the Japanese extracts stimulated A549 proliferation and other compounds in Japanese extracts may overwhelm the expected anti-proliferation effects by fucoidan fraction in the low concentration range. For Hep G2 in Figure 81, after treatment with fucoidan fraction for 3 days, the cell viability was still above 80% in low concentration range. However, its quantitative anti-proliferative effects on Hep G2 from fucoidan fraction of Japanese *U. pinnatifida* were not consistent with IC_{50} values

reported in the literature data as discussed above. Although effects were quite weak, the fucoidan fraction (low molecular fraction) from the Japanese *U. pinnatifida* appears to generate the anti-proliferative effects to all types of cancer cell lines. Therefore, these results definitely suggested the fractions that possess anti-cancer potential were largely removed from the single step of fucoxanthin and fucoidan extraction.

In fact, the Japanese extraction method depends on the physical and chemical characteristics of carbon dioxide and water. Increasing temperature and pressure can change the polarity of carbon dioxide gas and water in liquid form. In form of supercritical carbon dioxide, the carbon dioxide has both gas-like and liquid-like qualities, which is suitable for extraction of low-polar compounds such as oils, terpenoid, flavours and essential oil. Polarity of water can be decreased in high temperature and pressure to be in form of subcritical water, and the subcritical water is suitable for extraction of highly polar compounds such as sugar, proteins, and pectin. Thus, high temperature and pressure contribute to change carbon dioxide and water in the polar range of terpenoid, oils, essential oil, carotenoids, polyphenols, amino acids, and sugar proteins. Therefore, the supercritical carbon dioxide and subcritical water after changing of polarity were similar to several organic solvents used in the extraction fucoxanthin and fucoidan in the Japanese extraction method.

In Japanese method, the Japanese *U. pinnatifida* was milled to powder using a mesh size of 60 first, then powder was directly loaded into extraction apparatus to obtain fucoxanthin extracts from supercritical carbon dioxide. Then, the residues from fucoxanthin extract was dissolved in water only and loaded into apparatus for extraction of fucoidan (low molecular weight fraction) by microwave-assistance. As the result, the low-molecular weight fucoidan 13kDa could be obtained at 140°C, and fucoidan fraction was obtained at 110°C has higher molecular weight of around 240 kDa. In addition, there was no fucoidan fraction which could be detected above extraction temperature of 160°C. In fact, a similar methods was use in 2008, this method still used organic and inorganic solvent to obtain the crude fucoidan fraction first. The organic solvent 85% ethanol as organic solvent contributed to remove pigments, proteins and low molecular weight compounds (Chen Yang et al., 2008), and then inorganic solvent calcium chloride was used to remove insoluble components from *U. pinnatifida* which could affect the purity of fucoidan. The last step was to use nylon membranes to remain all hydrophilic compounds including fucoidan as the crude fucoidan fraction. Microwave-assistance extraction only contributed to purify the extraction of fucoidan

with adjusting pH value by hydrochloride and sodium hydroxide. After determination of anti-cancer activity, Chen Yang, et al in 2008 reported cell viability of A549 decreased to more than 75.9% by treatment with purified fucoidan fraction by extraction of microwave-assistance (Chen Yang et al., 2008). Therefore, this comparison indicated the fucoidan fraction was not completely isolated by using the Japanese extraction method, this is because fucoidan from *U. pinnatifida* is a mixture containing monosaccharide fucose and galactose. Also, the compounds in mixture of monosaccharide in fucoidan that possess anti-cancer effects might not be completely isolated from *U. pinnatifida*. Compared to previous method, the fucoidan fraction (low molecular weight fraction) was isolated from the residue of fucoxanthin extraction. Therefore the structures of fucoidan might be altered during extraction of fucoxanthin. In addition, some organic or inorganic solvent was applied to remove impurities from *U. pinnatifida* to achieve crude fucoidan fraction, conversely this step was not applied in the Japanese extraction method. Thus, some of impurities possibly were in the Japanese fucoidan fraction, and these might be affect the anti-proliferative effects from the Japanese fucoidan extracts. Moreover, in this study there are two fractions obtained from the Japanese extraction methods including fucoidan fraction 13kDa and 240kDa respectively. However, anti-proliferative effects on nine cancer cell lines only were determined with fucoidan fraction in 13kDa (low molecular weight fraction). The anti-proliferative effects of the fucoidan fraction 240kDa remain unknown and cannot be completely rule out.

Chapter 5 Conclusion

5. Conclusion & Future Study Direction

5.1 Fucoxanthin as an Important Chemopreventive Agent with Future Direction of Study

Pure fucoxanthin possesses effectively and efficiently anti-proliferative effects to multiple types of cancer cells. Especially, the significant anti-proliferative effects from fucoxanthin contribute to treatment with human squamous carcinoma SiHa with high sensitivity. Also, fucoxanthin generated anti-proliferative effects to human colon carcinoma, lung carcinoma and breast adenocarcinoma with mild sensitivity. Other types of cancer cell lines were the less sensitivity to anti-proliferative fucoxanthin effects. Thus, anti-proliferative effects of pure fucoxanthin standard to cancer cell lines used in this studies can be ranked as SiHa > MCF-7, Lovo, NCI-H522 > Hep G2, A549, WiDr, SK-N-SH, and Malme-3M.Therefore, fucoxanthin as a type of marine carotenoid is the most important chemopreventive phytochemical, which may be also considered as a potential candidate used in cancer chemoprevention and clinical cancer chemotherapy.

Due to previous *in vitro* and animal *in vivo* studies shown, adverse side effects from fucoxanthin can be quite low. However, plasma concentration of fucoxanthin was quite low through oral administration in human study, and therapeutic plasma concentration cannot be achieved through oral administration. In future study, concentration of fucoxanthin in plasma should be examined through intravenous administration. Due to multiple anti-cancer effects, fucoxanthin should be combined with existed cancer chemotherapy to treat with cancer cell lines. If high anti-proliferative effects were found as treatment with combination fucoxanthin and existed chemotherapy, amount of existed chemotherapy can be reduced in clinical cancer treatment in order to reduce adverse side effects of current chemotherapy.

5.2 *U. pinnatifida* as A Type of Brown Seaweed with Anti-Cancer Potential with Direction of Study in Future

This *in vitro* study indicated anti-proliferative effects from both NZ and Japanese *U. pinnatifida* extracts were more potent than corresponding anti-proliferative effects from

pure fucoxanthin standard. The significantly potent anti-proliferative effects from both NZ and Japanese fucoxanthin extracts was found in some cancer cell lines including human colon adenocarcinoma, lung carcinoma, neuroblastoma, cervix squamous carcinoma and malignant melanoma. This possibly indicated some novel compounds with anti-cancer potentials were present in those NZ and Japanese fucoxanthin extracts. Due to low amount in extracts, these novel compounds may possess very potential anti-cancer properties. In most cancer cell lines tested, higher anti-proliferative effects were mostly found from the crude *U. pinnatifida* fucoxanthin extracts. Therefore, in order to determine new compounds, other extraction methods should be considered in the further studies such as gel permeation chromatography-based separation of ethanolic extracts of *U. pinnatifida* powder based on molecular weights. By comparison of cell viability, the possible compounds can be found in a fraction with a specific range of molecular weight. Then, mass spectrometry can be used to determine masses of particles, and this can combined with infrared spectroscopy to determine the functional groups contained in the novel compounds. Finally, ^{13}C NMR and ^{1}H NMR can be used to define the position of carbon and proton in the structure of novel compounds.

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Appendix 1

A.1. MTT Results about Linearity of Proliferation for Cancer Cell Lines

The linearity of proliferative standard curve for nine cancer cell lines was conducted for anti-proliferative effect determination of pure fucoxanthin standard, NZ seaweed fucoxanthin extracts and Japanese seaweed fucoxanthin & fucoidan extracts. The Figure 83 to 91 showed nine cancer cell lines below. There was a nearly linear relationship between cell concentration (cells/mL) and MTT absorbance at 540 nm. The correlation factor R^2 in the linear regression is higher than 0.97 with a line formula shown in each figure.

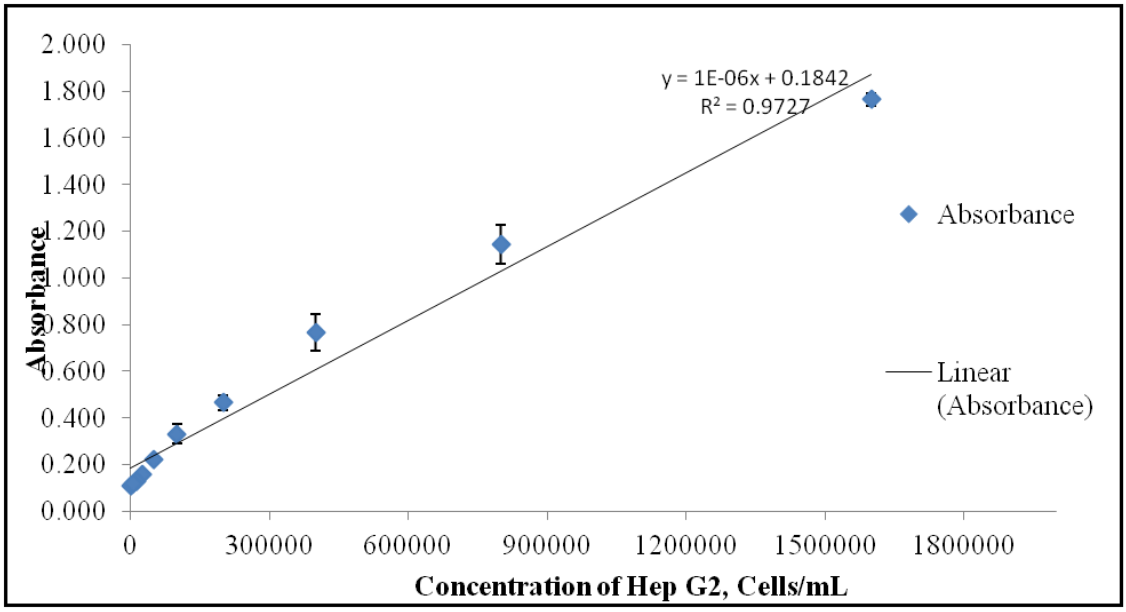

Figure 83: Linearity of proliferative standard curve for human hepatocellular carcinoma Hep G2 cells. Data are means ± standard errors (n=3).

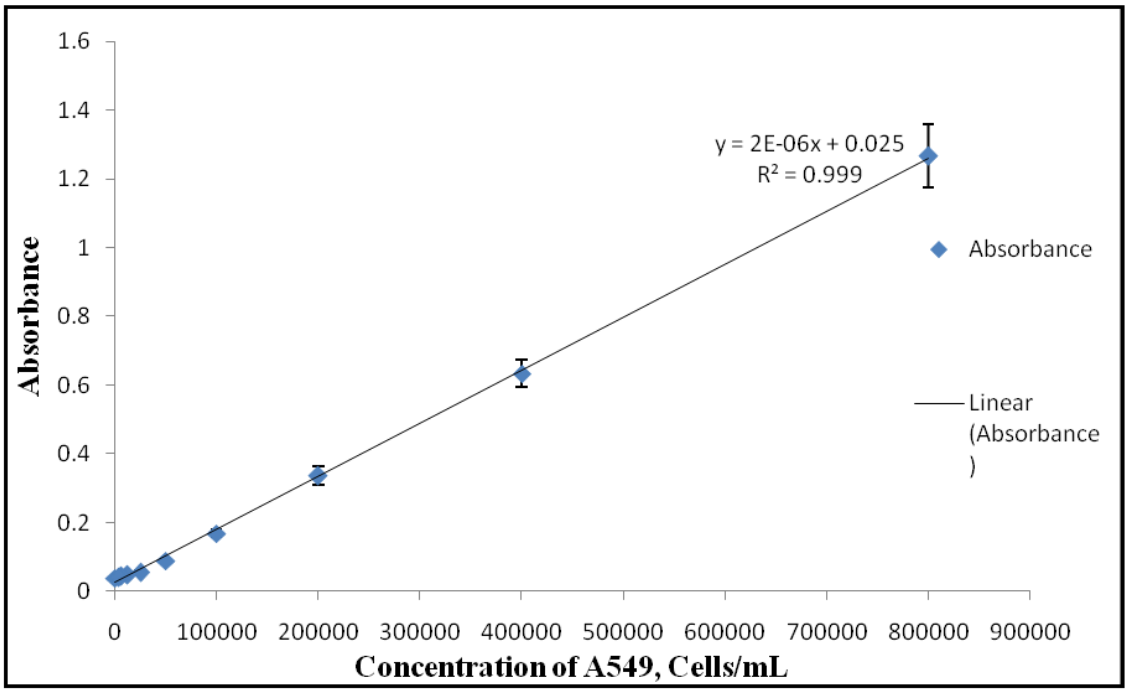

Figure 84: Linearity of proliferative standard curve for human lung carcinoma A549 cells. Data are means ± standard errors (n=3).

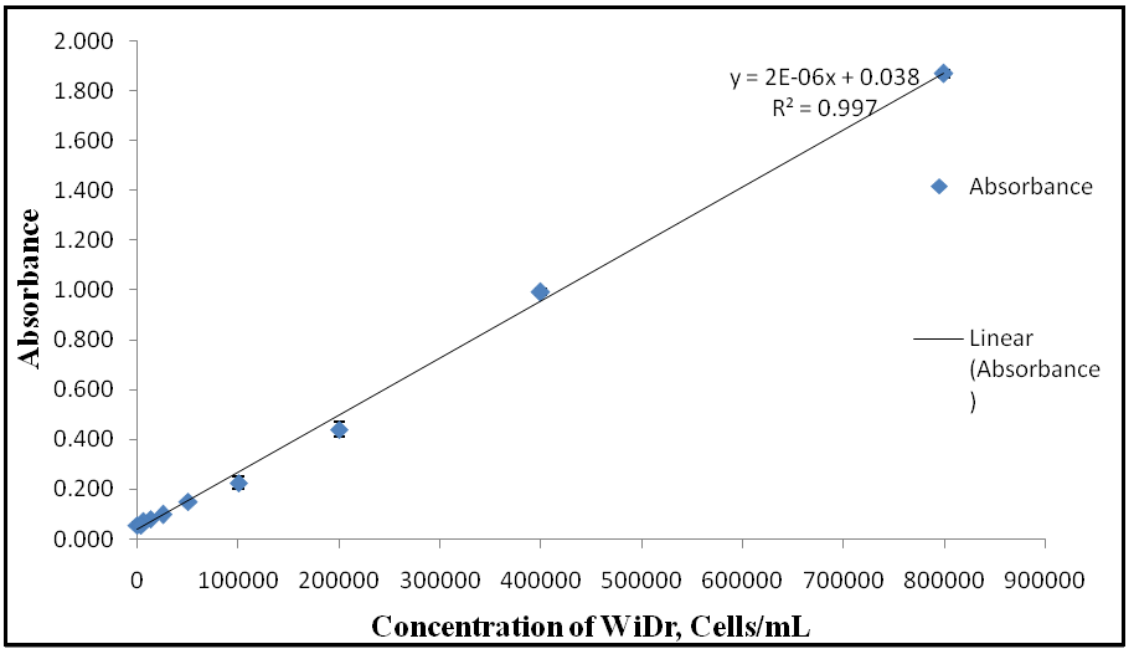

Figure 85: Linearity of proliferative standard curve for human colon adenocarcinoma WiDr cells. Data are means ± standard errors (n=3).

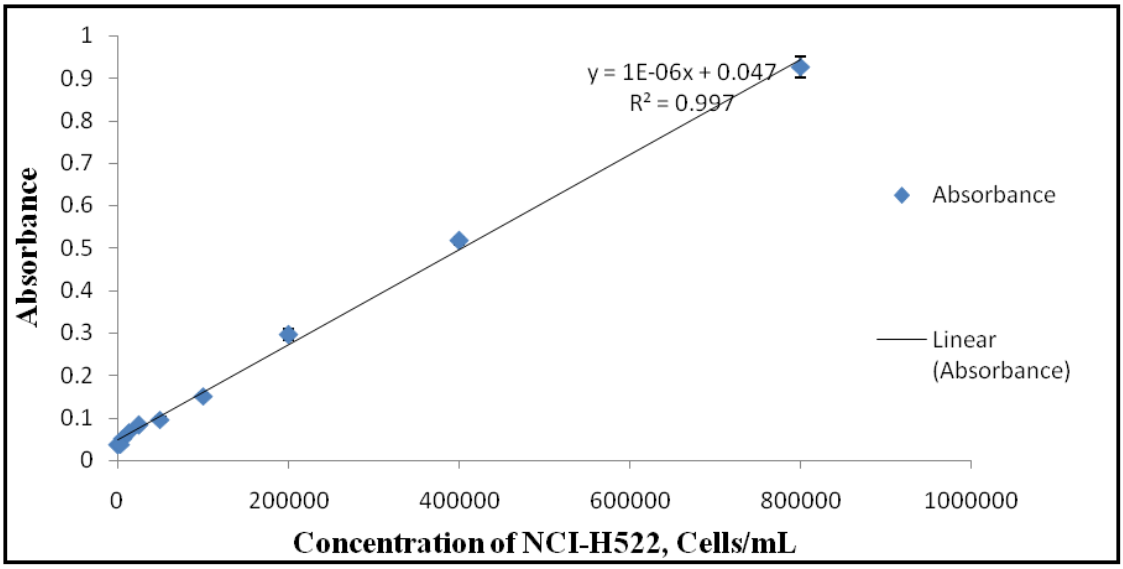

Figure 86: Linearity of proliferative standard curve for human lung carcinoma NCI-H522 cells. Data are means ± standard errors (n=3).

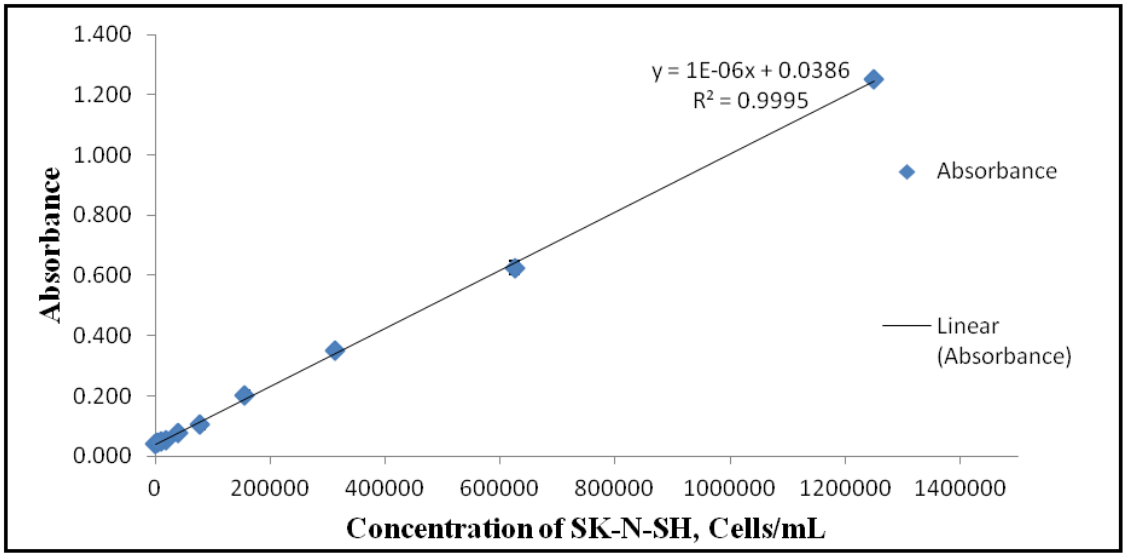

Figure 87: Linearity of proliferative standard curve for human neuroblastoma SK-N-SH cells. Data are means ± standard errors (n=3).

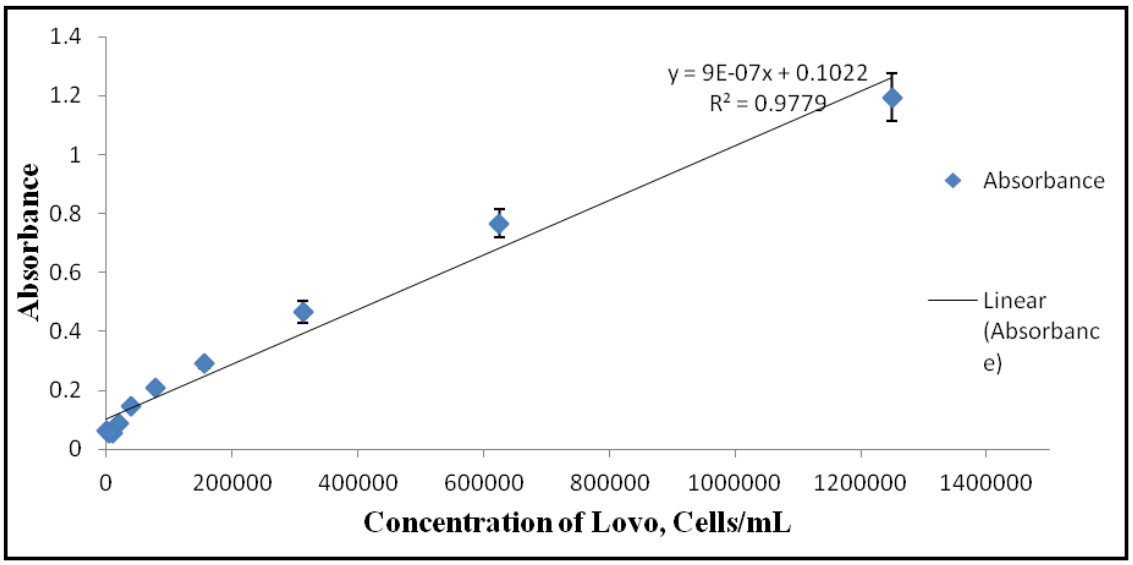

Figure 88: Linearity of proliferative standard curve for human colon adenocarcinoma Lovo cells. Data are means ± standard errors (n=3).

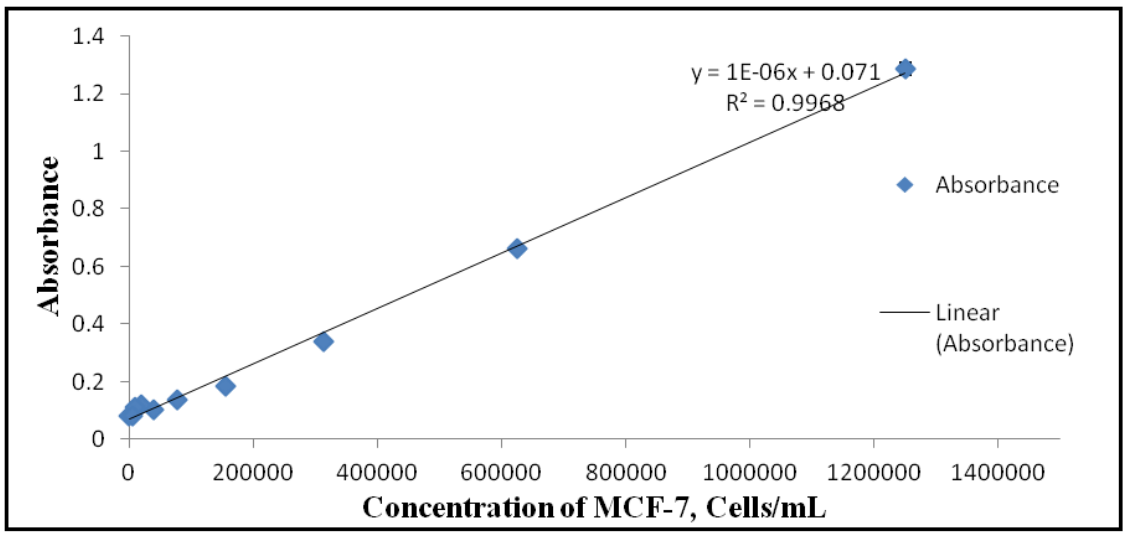

Figure 89: Linearity of proliferative standard curve for human breast adenocarcinoma MCF-7 cells. Data are means ± standard errors (n=3).

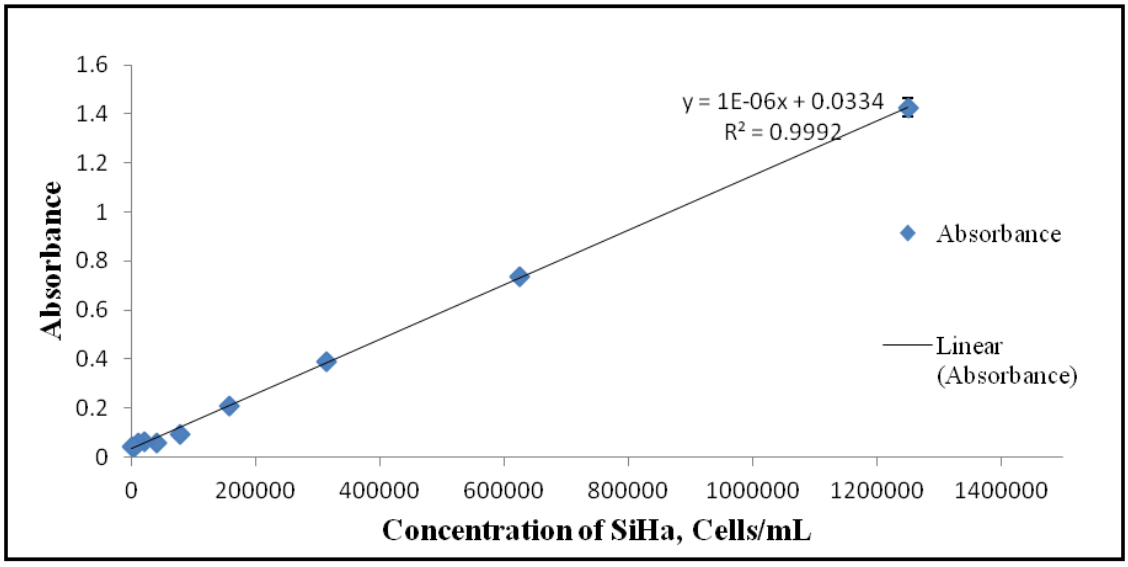

Figure 90: Linearity of proliferative standard curve for human cervix squamous carcinoma SiHa cells. Data are means ± standard errors (n=3).

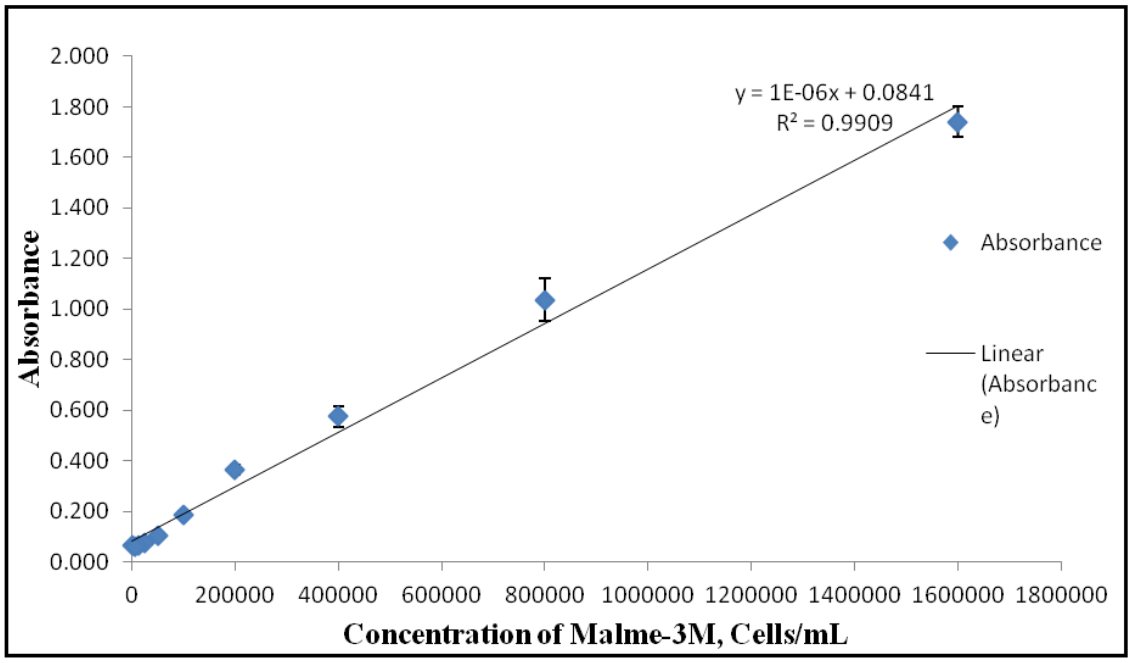

Figure 91: Linearity of proliferative standard curve for human malignant melanoma Malme-3M cells. Data are means ± standard errors (n=3).

Appendix 2

A.2. MTT Results about Proliferation Determination of Each Type of Cancer Cell Line Affected by Biological Ethanol

Biological ethanol was an important solvent for pure fucoxanthin standard and NZ & Japanese seaweed fucoxanthin extracts. It was necessary to determine whether ethanol as the solvent affected viability of cancer cell or not. The Figure 92 to 100 showed the change of cancer cell viability within completed culture medium containing 1%, 0.75%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.03125%, 0.007813% and 0.001953% of biological ethanol. The Figure 101 to 109 showed change of cancer cell viability within completed culture medium containing 2%, 1.6%, 1%, 0.5%, 0.25%, 0.125% and 0.03125% ethanol. In nine cancer cell line, the cell viability in completed culture medium without ethanol was not statistically different in completed culture medium containing ethanol as indicated concentration above (p-value > 0.05).

A.2.1. The First Ethanolic Concentration Range (1%, 0.75%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.03125%, 0.007813%, 0.001953%, and 0%)

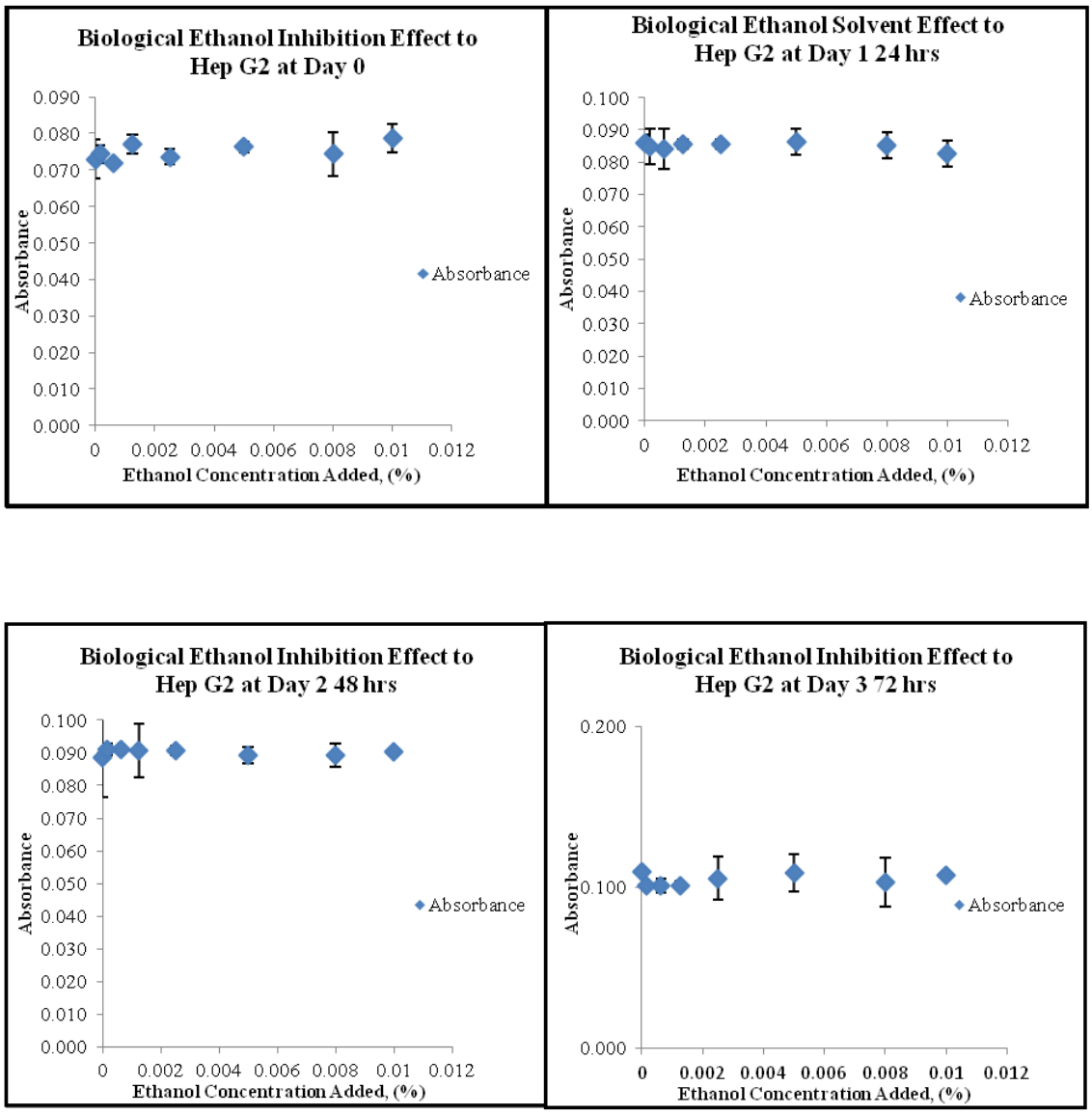

Figure 92: Biological ethanol inhibition effects on HEPG2 cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human hepatocellular carcinoma Hep G2 cells were treated biological ethanol with concentration at 1%, 0.75%, 0.5%, 0.25%, 0.0625%, 0.03125%, 0.007813%, and 0.001953%. Data are means ± standard errors (n=3).

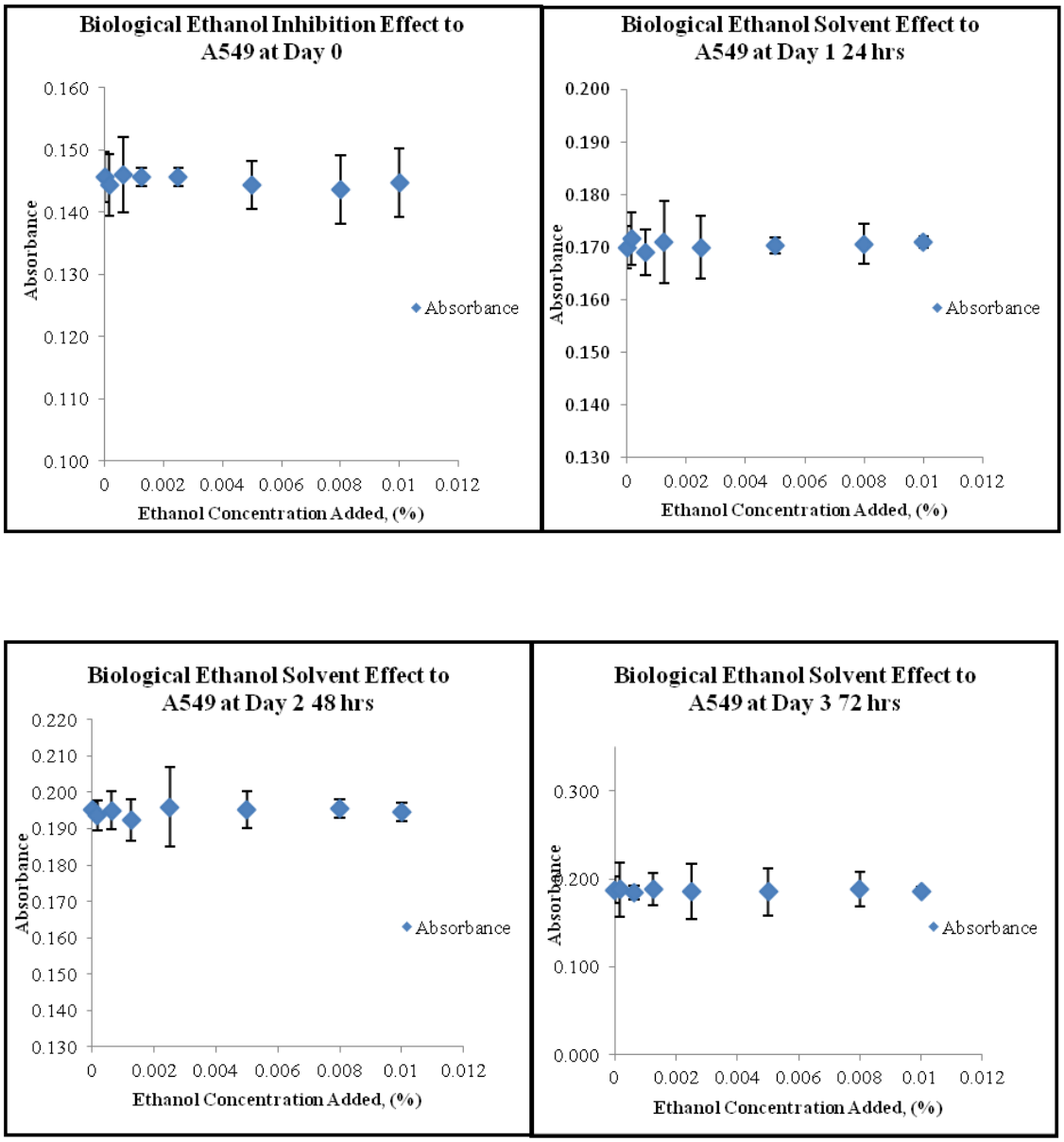

Figure 93: Biological ethanol inhibition effects on A549 cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human lung carcinoma A549 cells were treated biological ethanol with concentration at 1%, 0.75%, 0.5%, 0.25%, 0.0625%, 0.03125%, 0.007813%, and 0.001953%. Data are means ± standard errors (n=3).

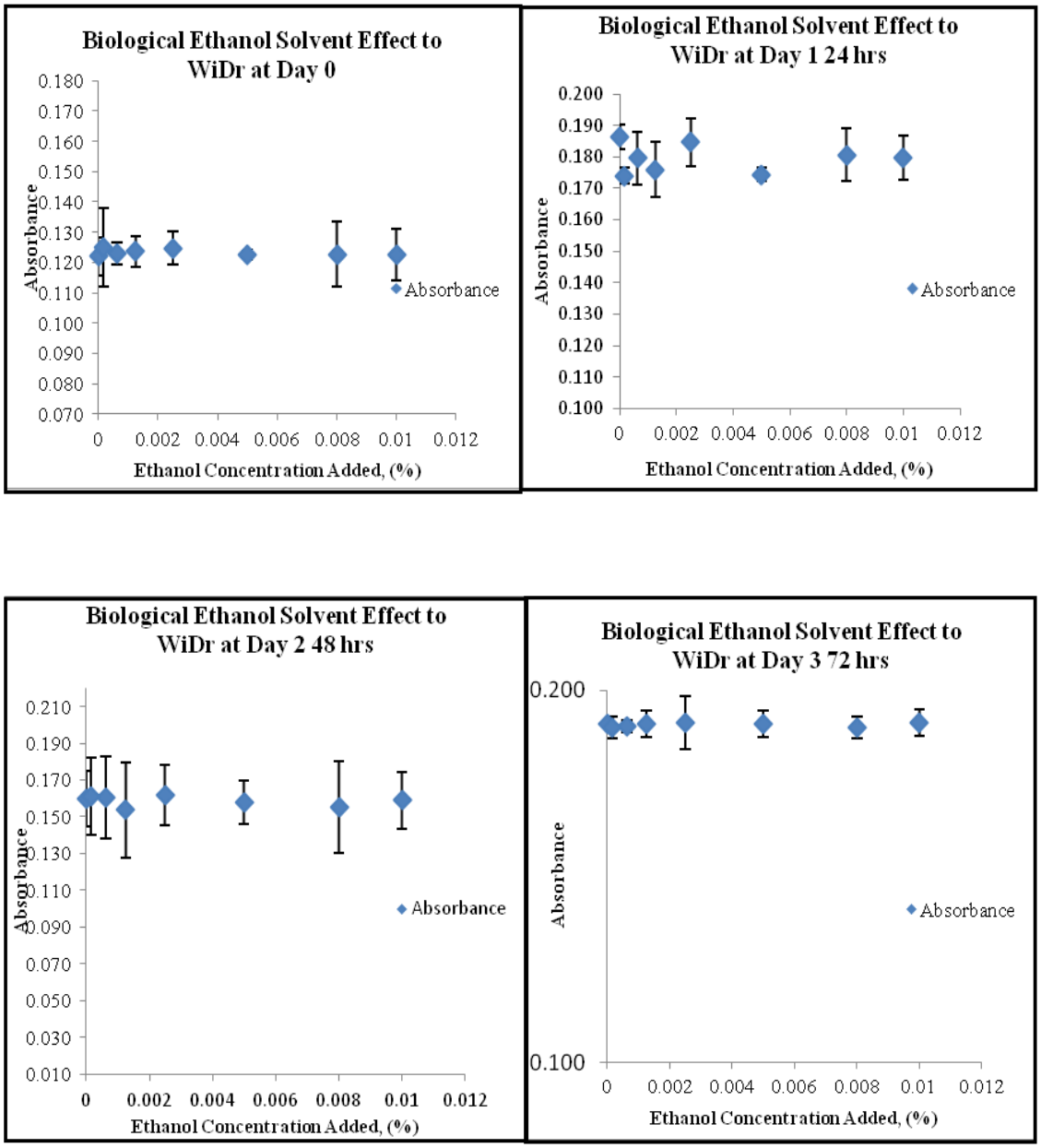

Figure 94: Biological ethanol inhibition effects on WIDR cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human colon adenocarcinoma WiDr cells were treated biological ethanol with concentration at 1%, 0.75%, 0.5%, 0.25%, 0.0625%, 0.03125%, 0.007813%, and 0.001953%. Data are means ± standard errors (n=3).

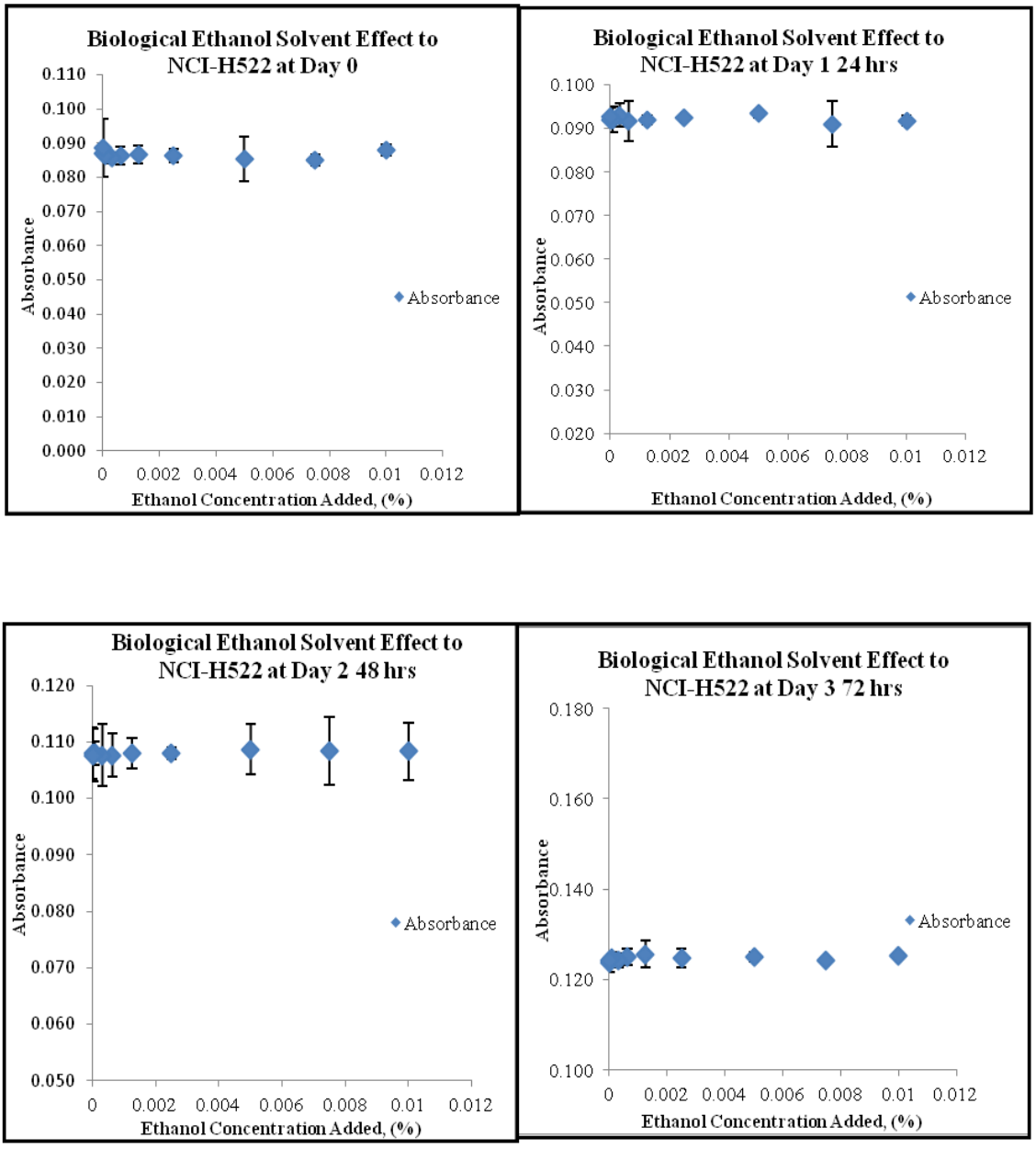

Figure 95: Biological ethanol inhibition effects on NCI-H522 cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human lung carcinoma NCI-H522 cells were treated biological ethanol with concentration at 1%, 0.75%, 0.5%, 0.25%, 0.0625%, 0.03125%, 0.007813%, and 0.001953%. Data are means ± standard errors (n=3).

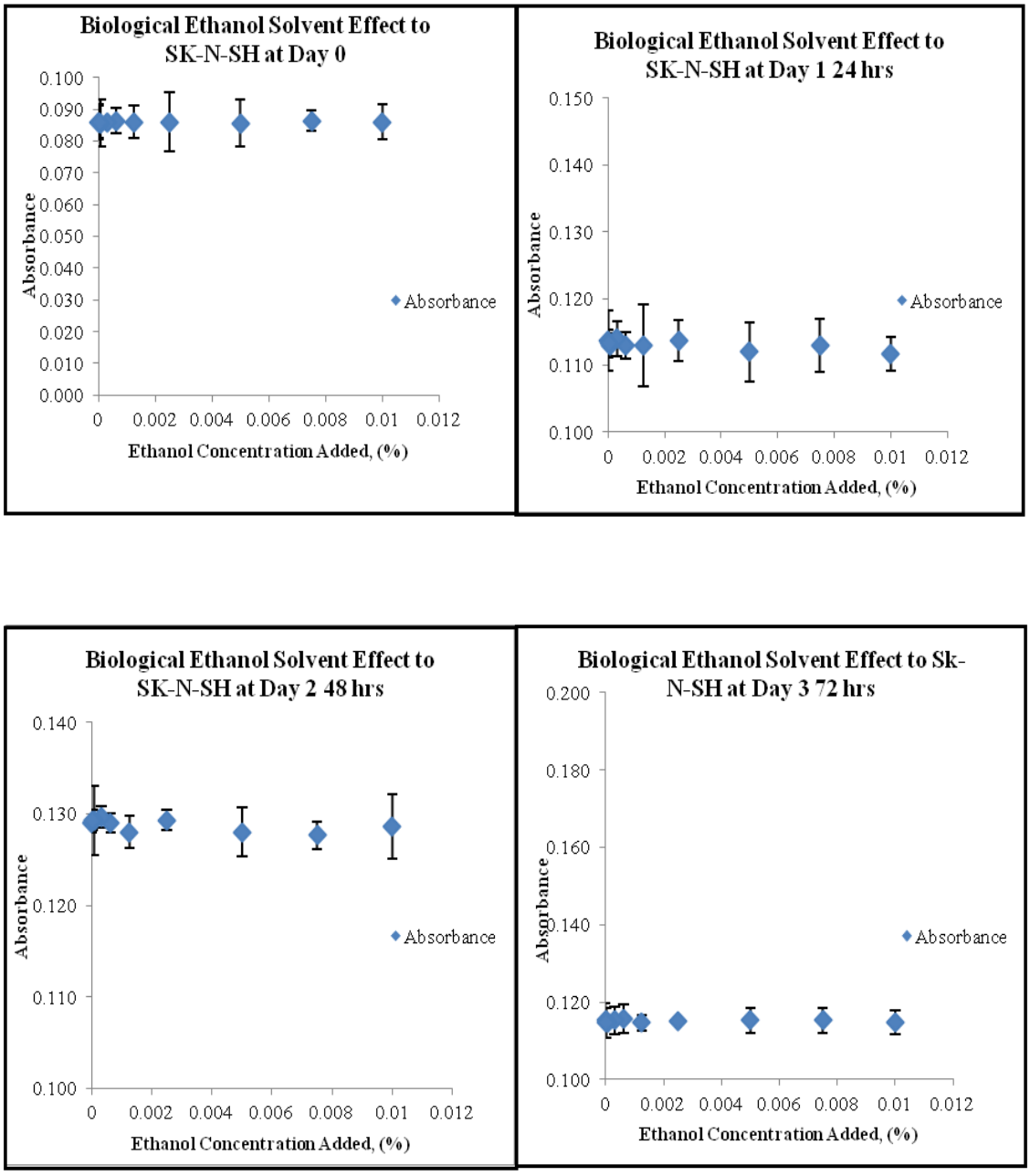

Figure 96: Biological ethanol inhibition effects on SK-N-SH cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human neuroblastoma SK-N-SH cells were treated biological ethanol with concentration at 1%, 0.75%, 0.5%, 0.25%, 0.0625%, 0.03125%, 0.007813%, and 0.001953%. Data are means ± standard errors (n=3).

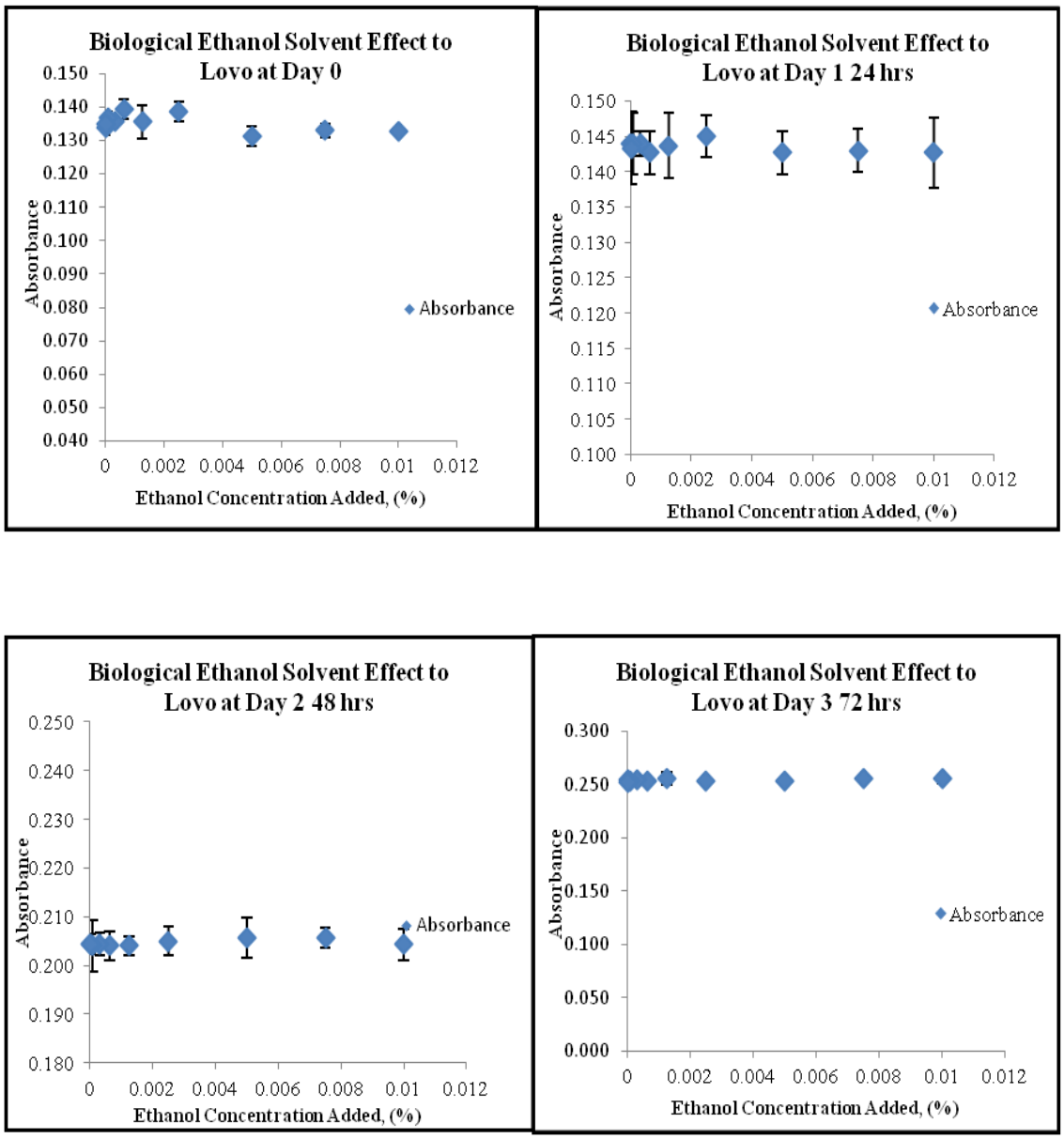

Figure 97: Biological ethanol inhibition effects on LOVO cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human colon adenocarcinoma Lovo cells were treated biological ethanol with concentration at 1%, 0.75%, 0.5%, 0.25%, 0.0625%, 0.03125%, 0.007813%, and 0.001953%. Data are means ± standard errors (n=3).

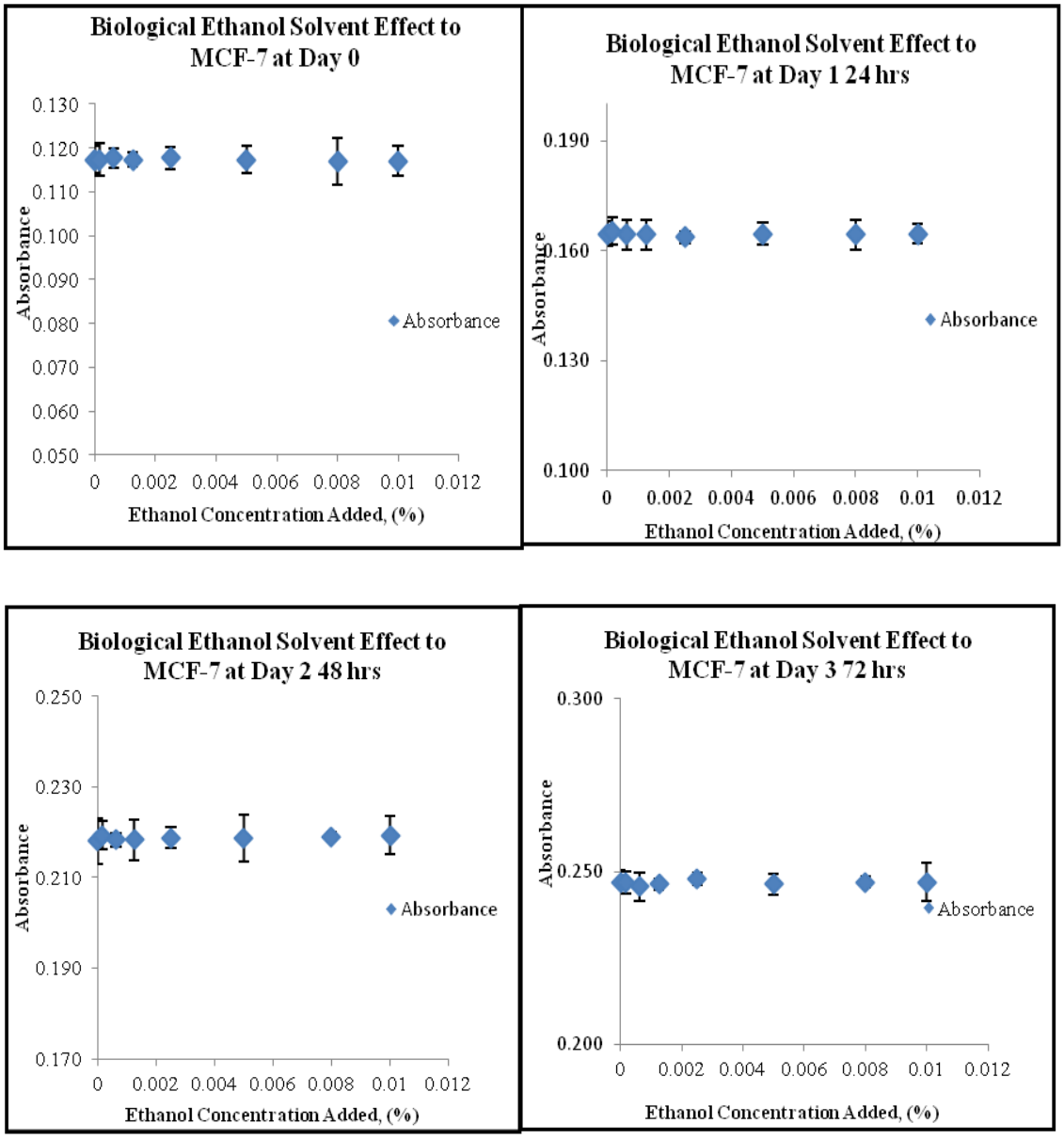

Figure 98: Biological ethanol inhibition effects on MCF-7 cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human breast carcinoma MCF-7 cells were treated biological ethanol with concentration at 1%, 0.75%, 0.5%, 0.25%, 0.0625%, 0.03125%, 0.007813%, and 0.001953%. Data are means ± standard errors (n=3).

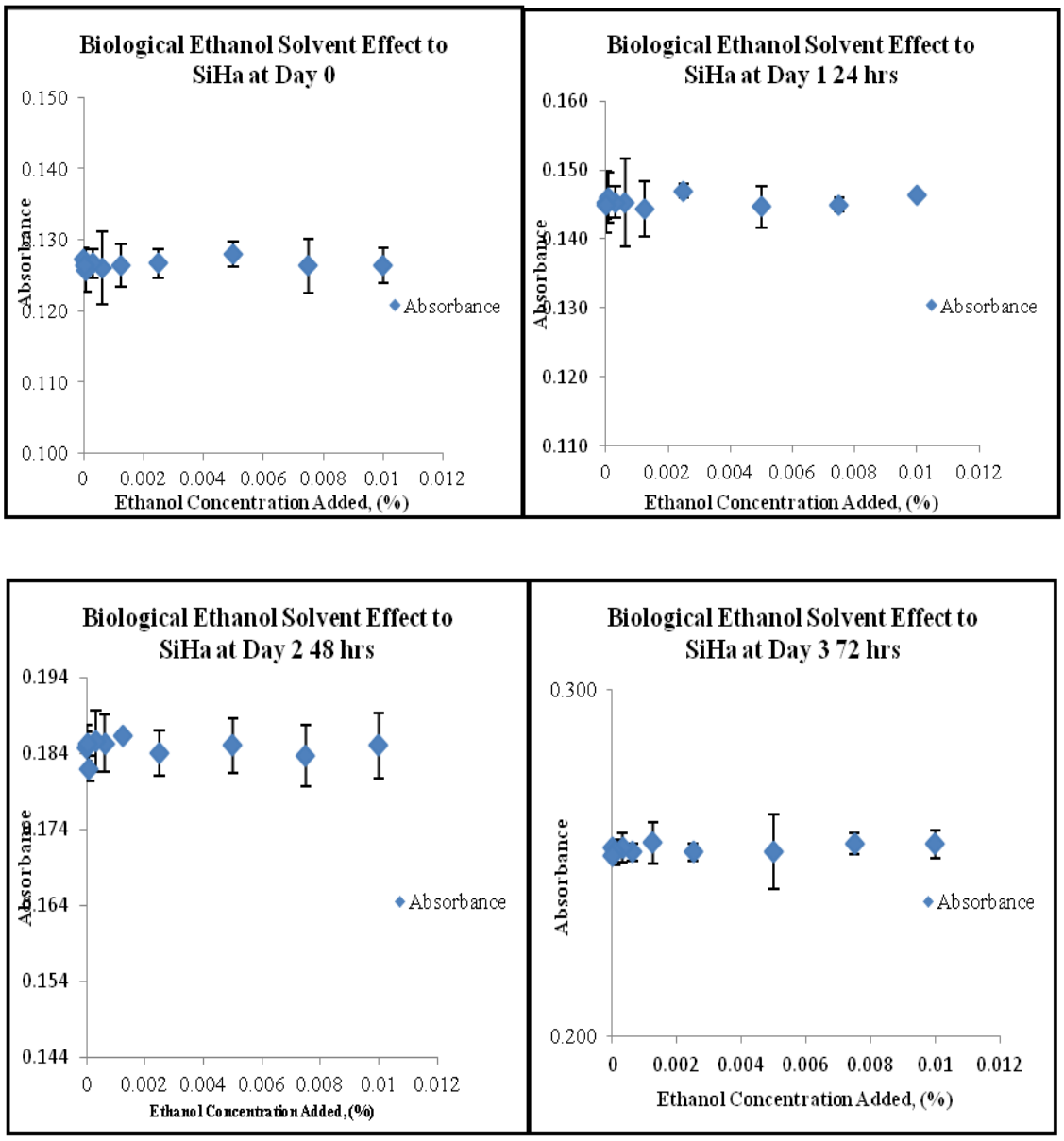

Figure 99: Biological ethanol inhibition effects on SIHA cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human cervix squmous carcinoma SiHa cells were treated biological ethanol with concentration at 1%, 0.75%, 0.5%, 0.25%, 0.0625%, 0.03125%, 0.007813, and 0.001953%. Data are means ± standard errors (n=3).

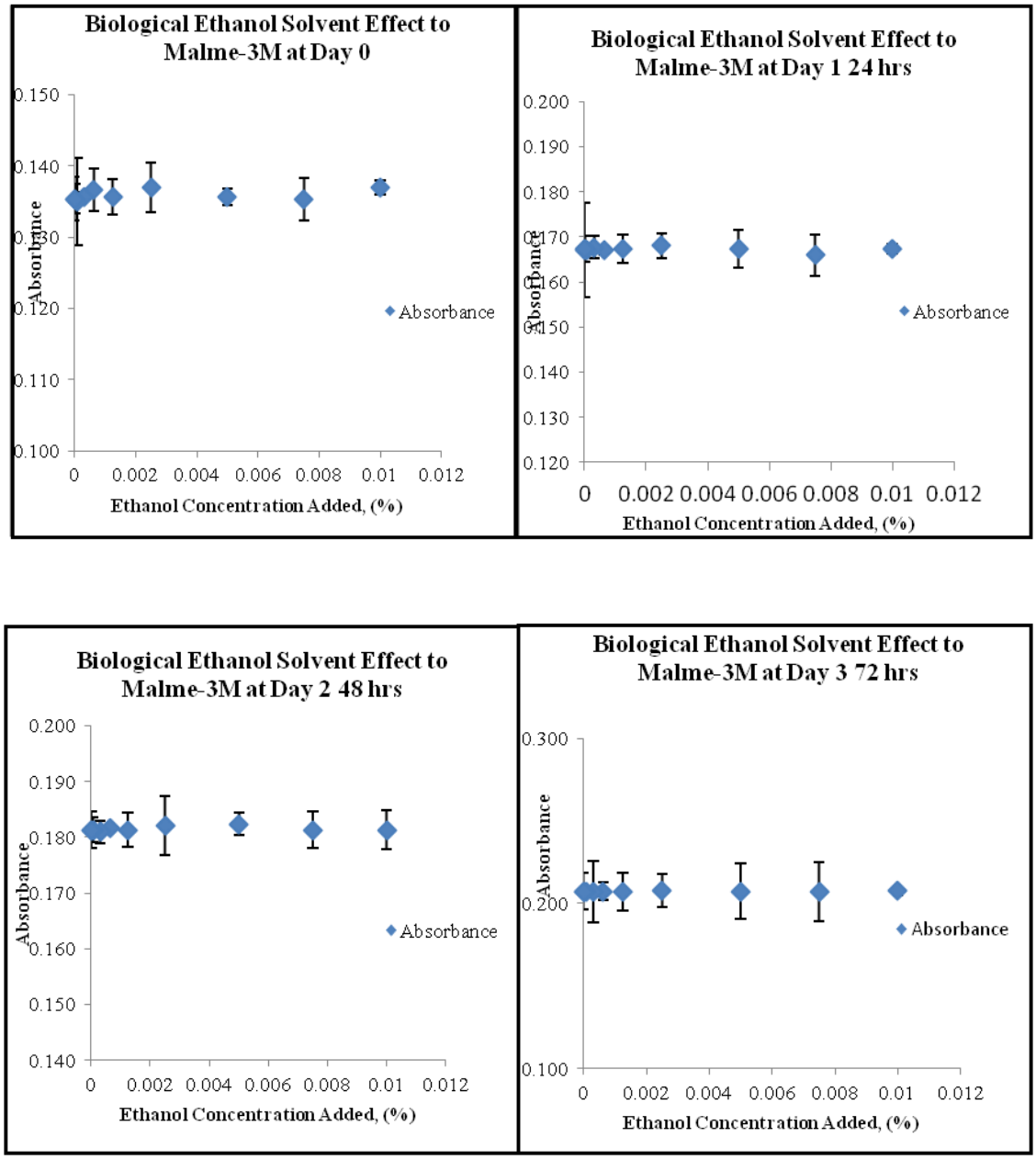

Figure 100: Biological ethanol inhibition effects on MALME-3M cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human malignant melanoma Malme-3M cells were treated biological ethanol with concentration at 1%, 0.75%, 0.5%, 0.25%, 0.0625%, 0.03125%, 0.007813%, and 0.001953%. Data are means ± standard errors (n=3).

A.2.2. The Second Ethanolic Concentration Range (2%, 1.6%, 1%, 0.5% 0.25%, 0.125%, 0.03125% and 0%)

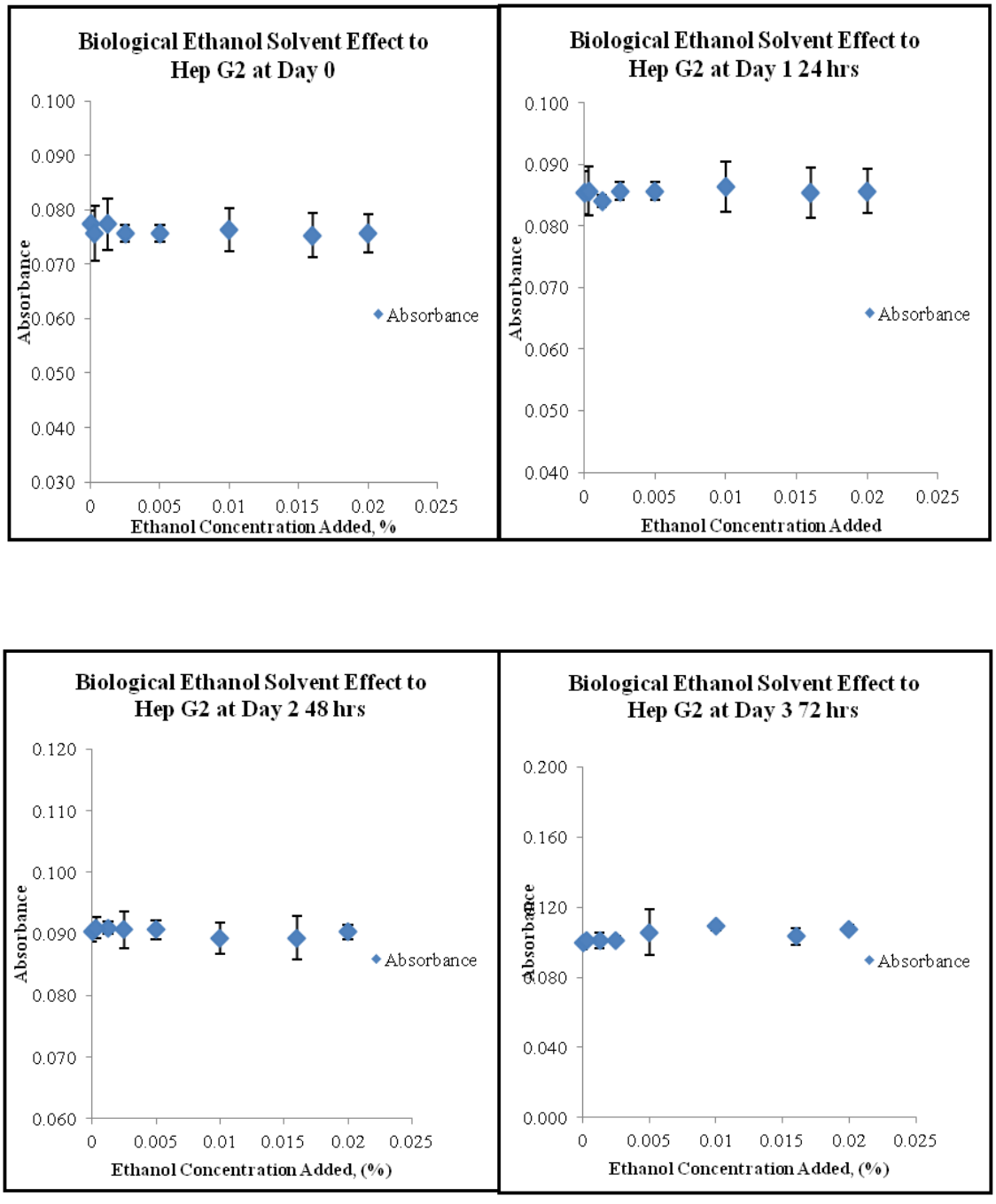

Figure 101: Biological ethanol inhibition effects on HEPG2 cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human hepatocellular carcinoma Hep G2 cells were treated biological ethanol with concentration at 2%, 1.6%, 1%, 0.5%, 0.25%, 0.125%, and 0.03125%. Data are means ± standard errors (n=3).

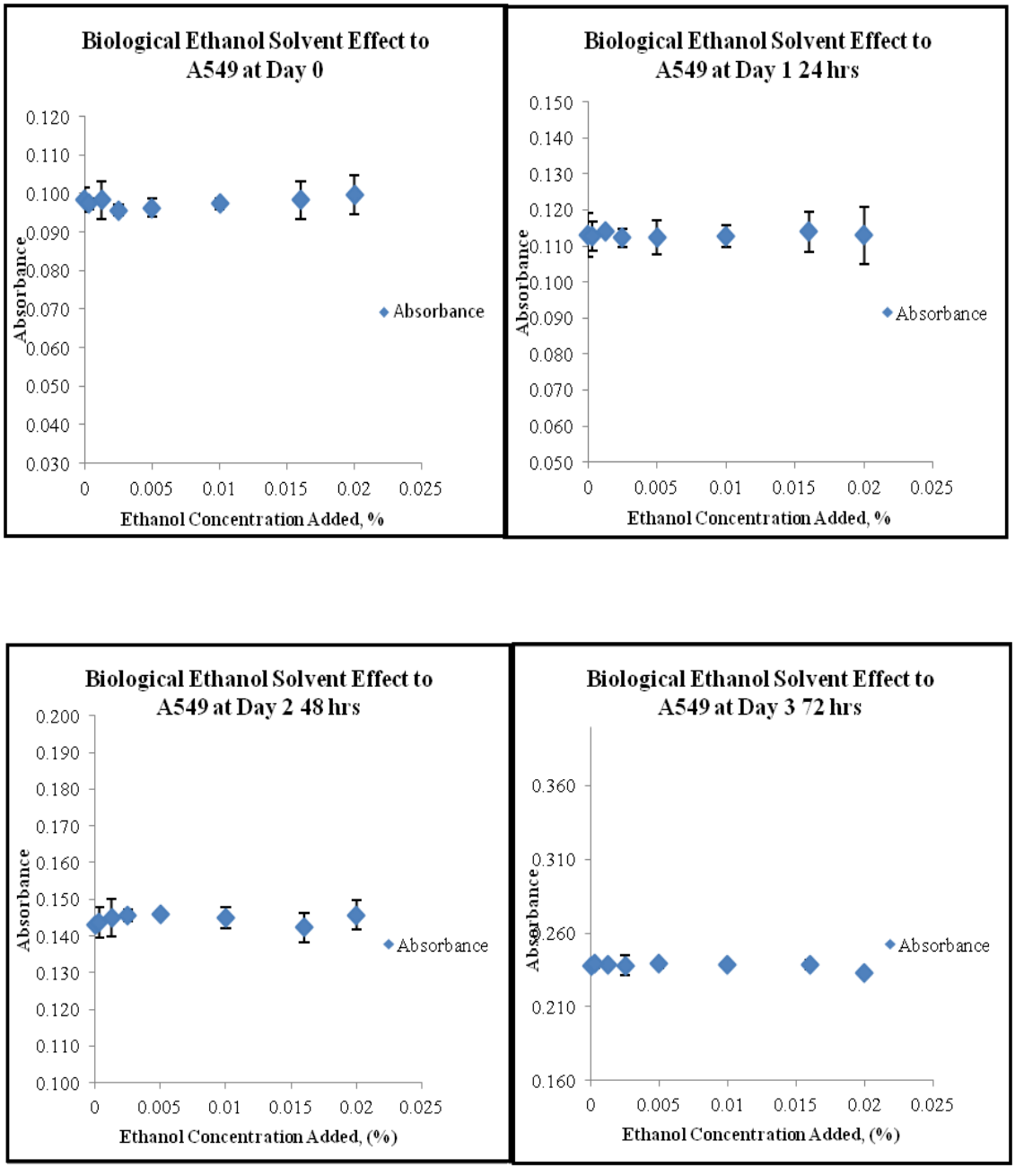

Figure 102: Biological ethanol inhibition effects on A549 cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human lung carcinoma A549 cells were treated biological ethanol with concentration at 2%, 1.6%, 1%, 0.5%, 0.25%, 0.125%, and 0.03125%. Data are means ± standard errors (n=3).

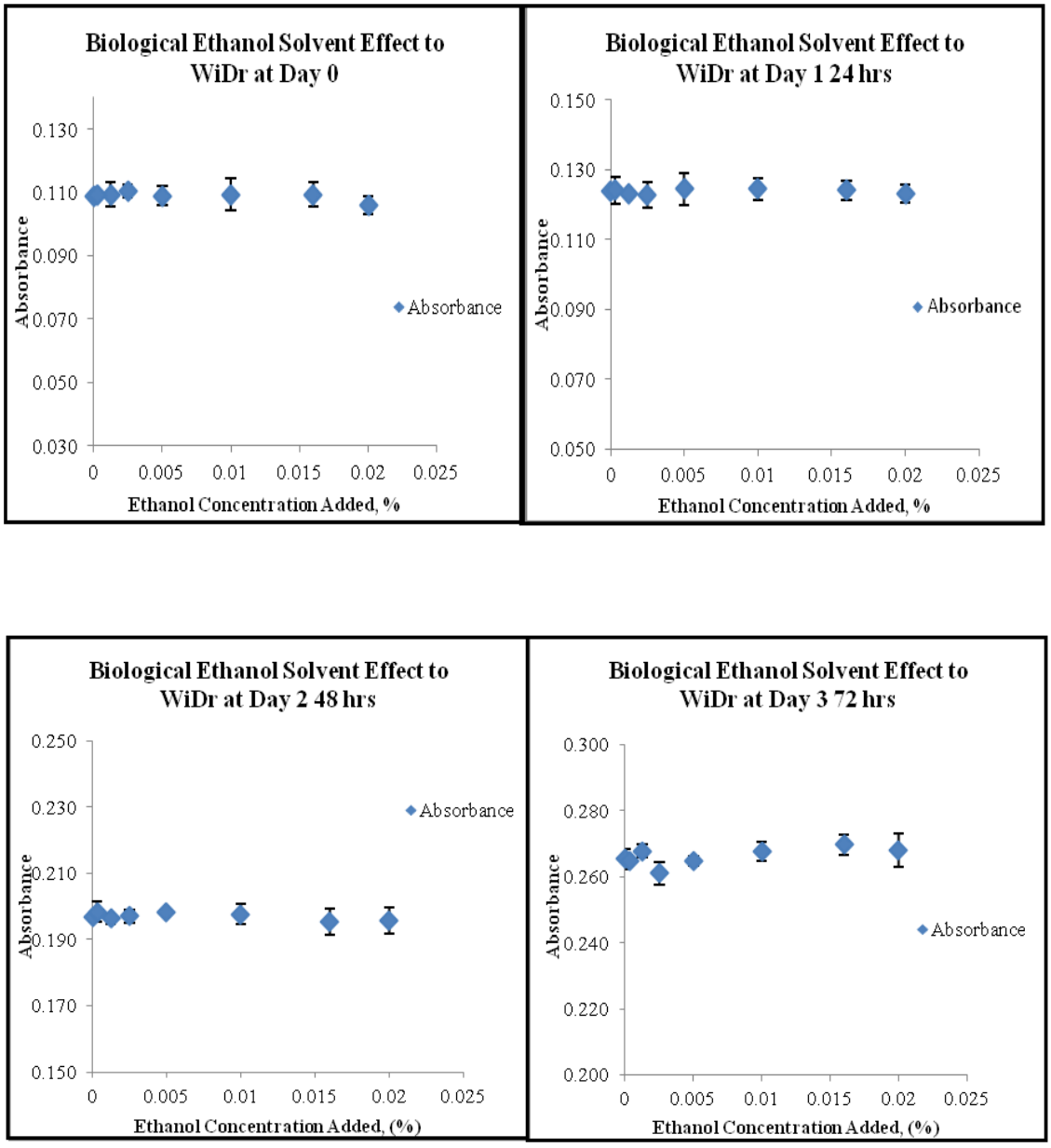

Figure 103: Biological ethanol inhibition effects on WIDR cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human colon adenocarcinoma WiDr cells were treated biological ethanol with concentration at 2%, 1.6%, 1%, 0.5%, 0.25%, 0.125%, and 0.03125%. Data are means ± standard errors (n=3).

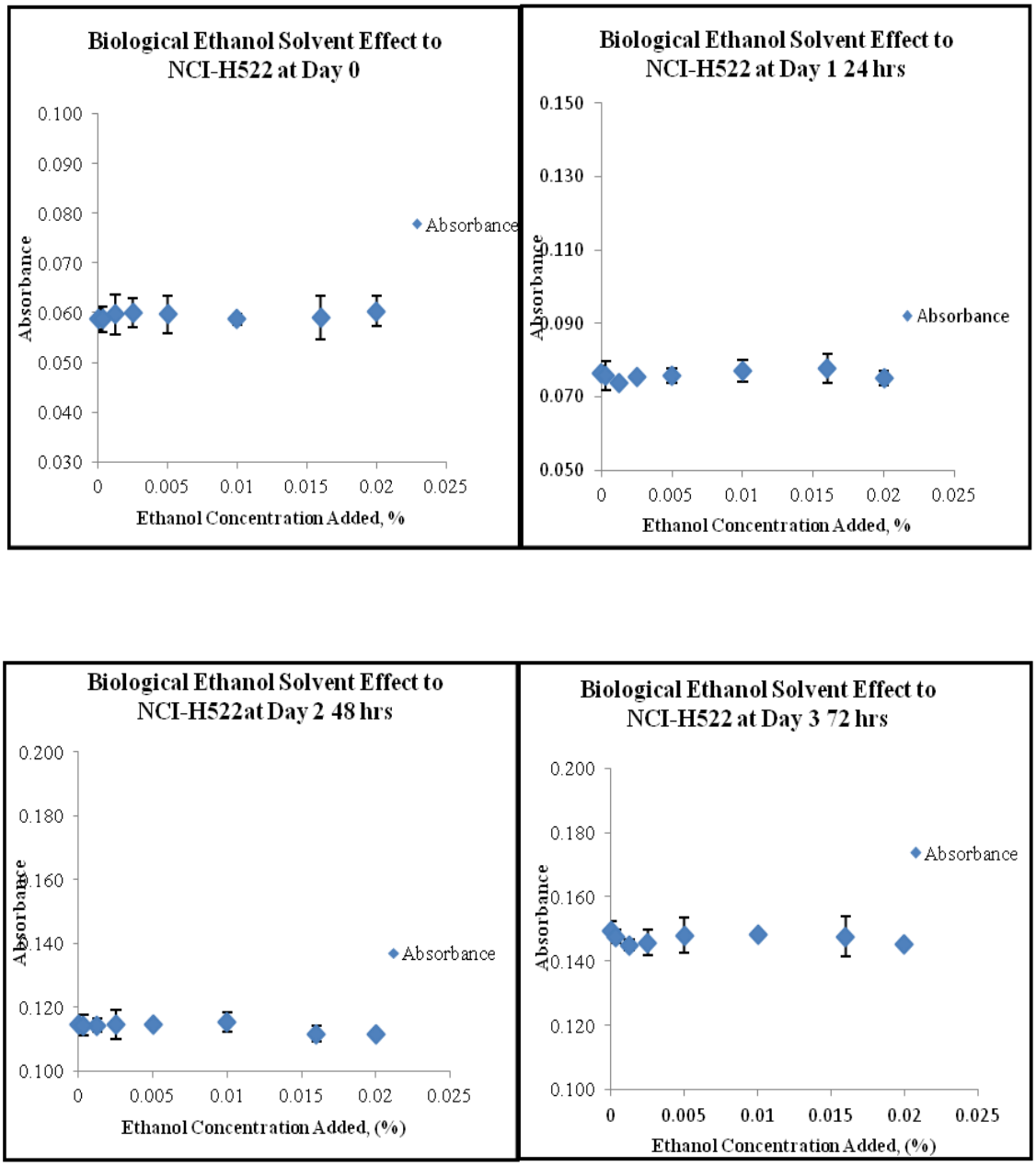

Figure 104: Biological ethanol inhibition effects on NCI-H522 cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human lung carcinoma NCI-H522 cells were treated biological ethanol with concentration at 2%, 1.6%, 1%, 0.5%, 0.25%, 0.125%, and 0.03125%. Data are means ± standard errors (n=3).

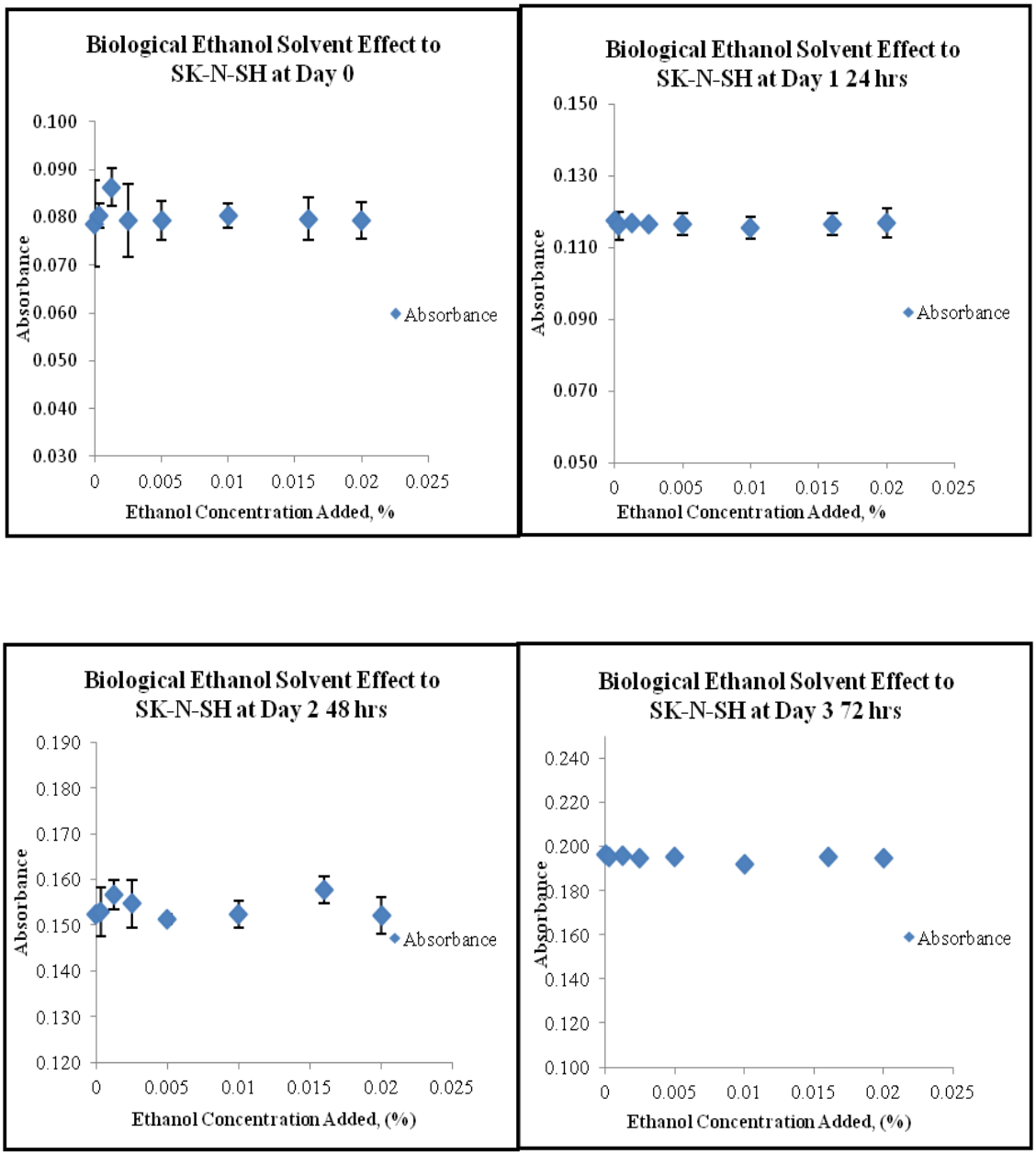

Figure 105: Biological ethanol inhibition effects on SK-N-SH cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human neuroblastoma SK-N-SH cells were treated biological ethanol with concentration at 2%, 1.6%, 1%, 0.5%, 0.25%, 0.125%, and 0.03125%. Data are means ± standard errors (n=3).

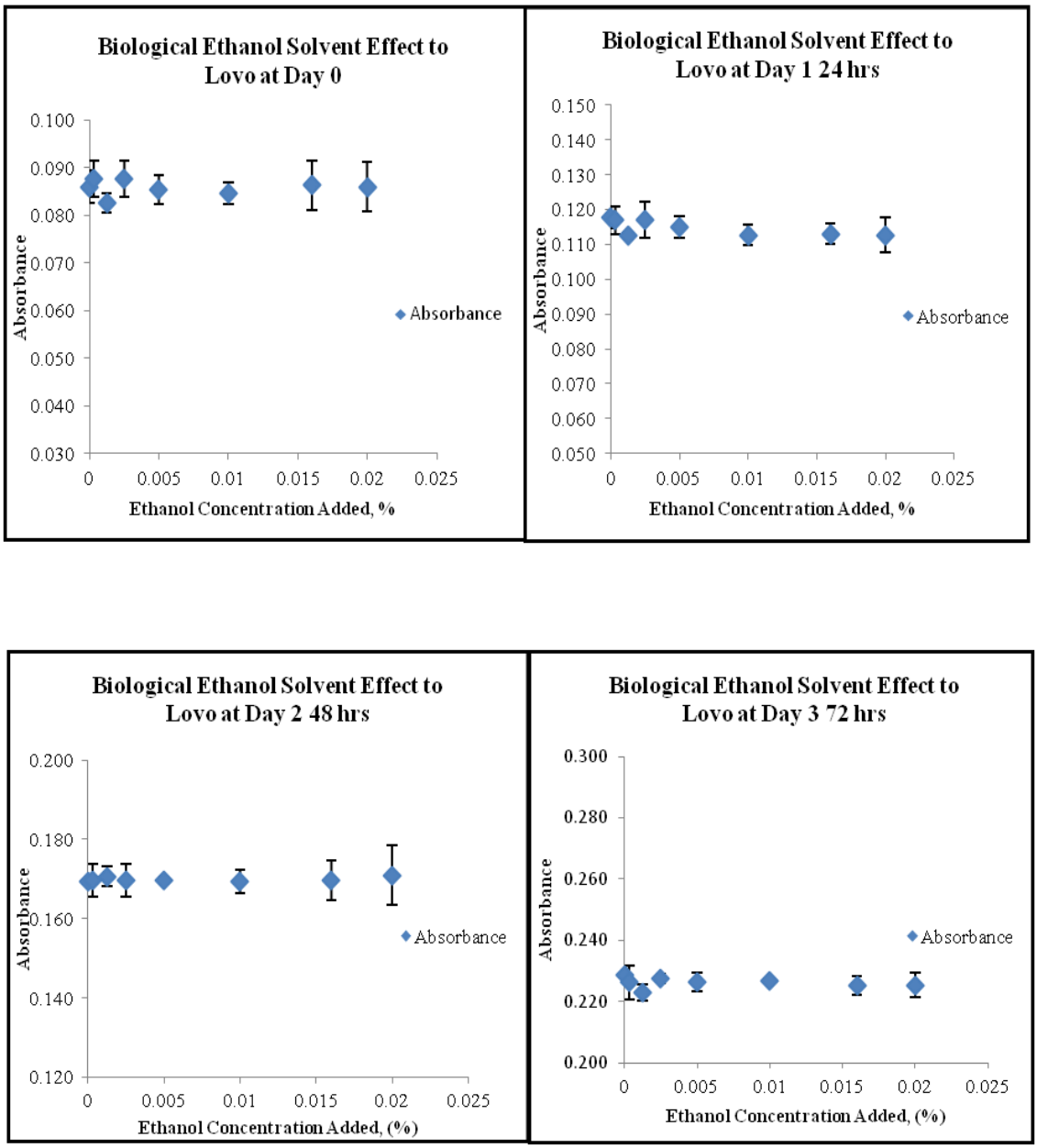

Figure 106: Biological ethanol inhibition effects on LOVO cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human colon adenocarcinoma Lovo cells were treated biological ethanol with concentration at 2%, 1.6%, 1%, 0.5%, 0.25%, 0.125%, and 0.03125%. Data are means ± standard errors (n=3).

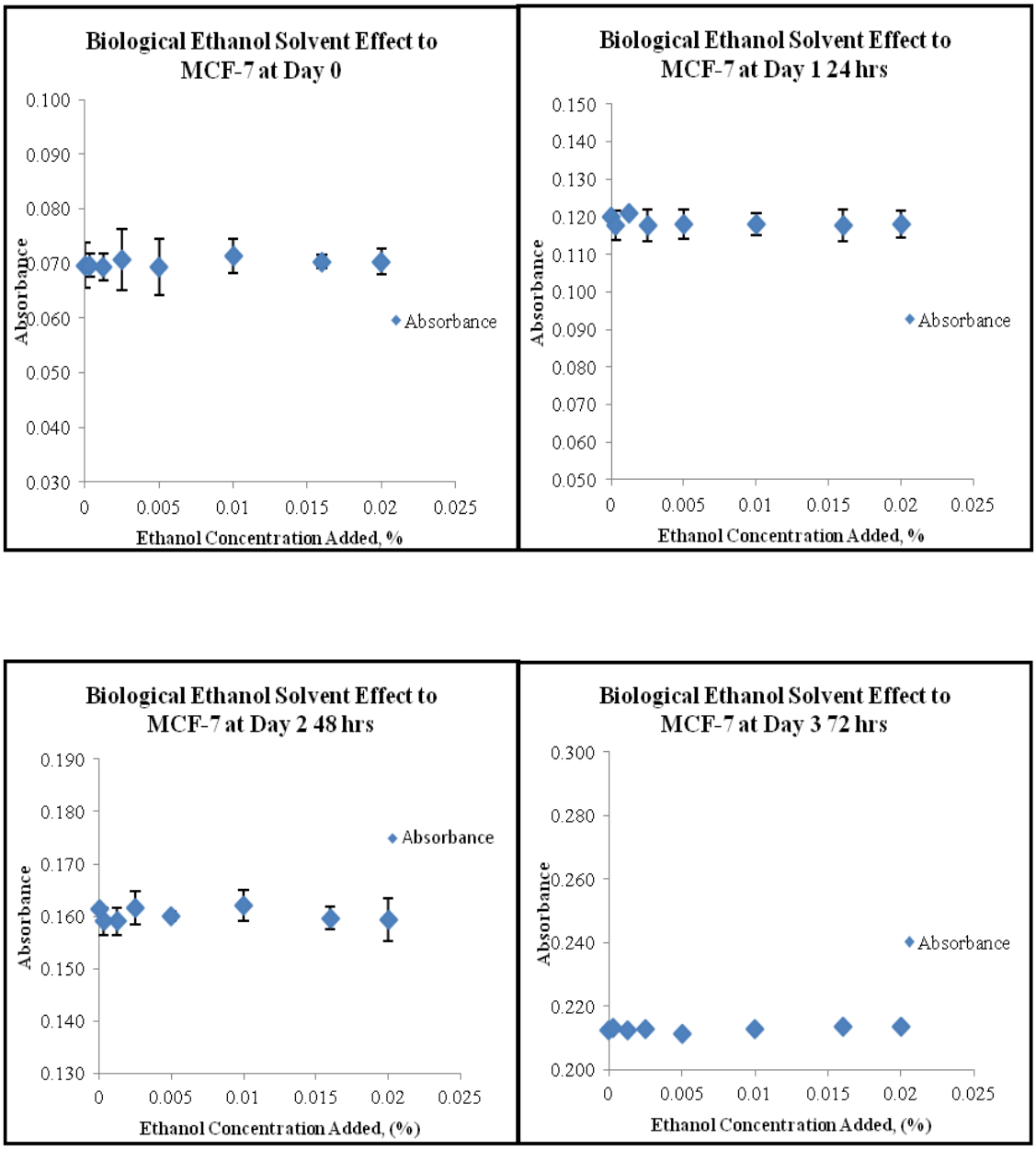

Figure 107: Biological ethanol inhibition effects on MCF-7 cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human breast adenocarcinoma MCF-7 cells were treated biological ethanol with concentration at 2%, 1.6%, 1%, 0.5%, 0.25%, 0.125%, and 0.03125%. Data are means ± standard errors (n=3).

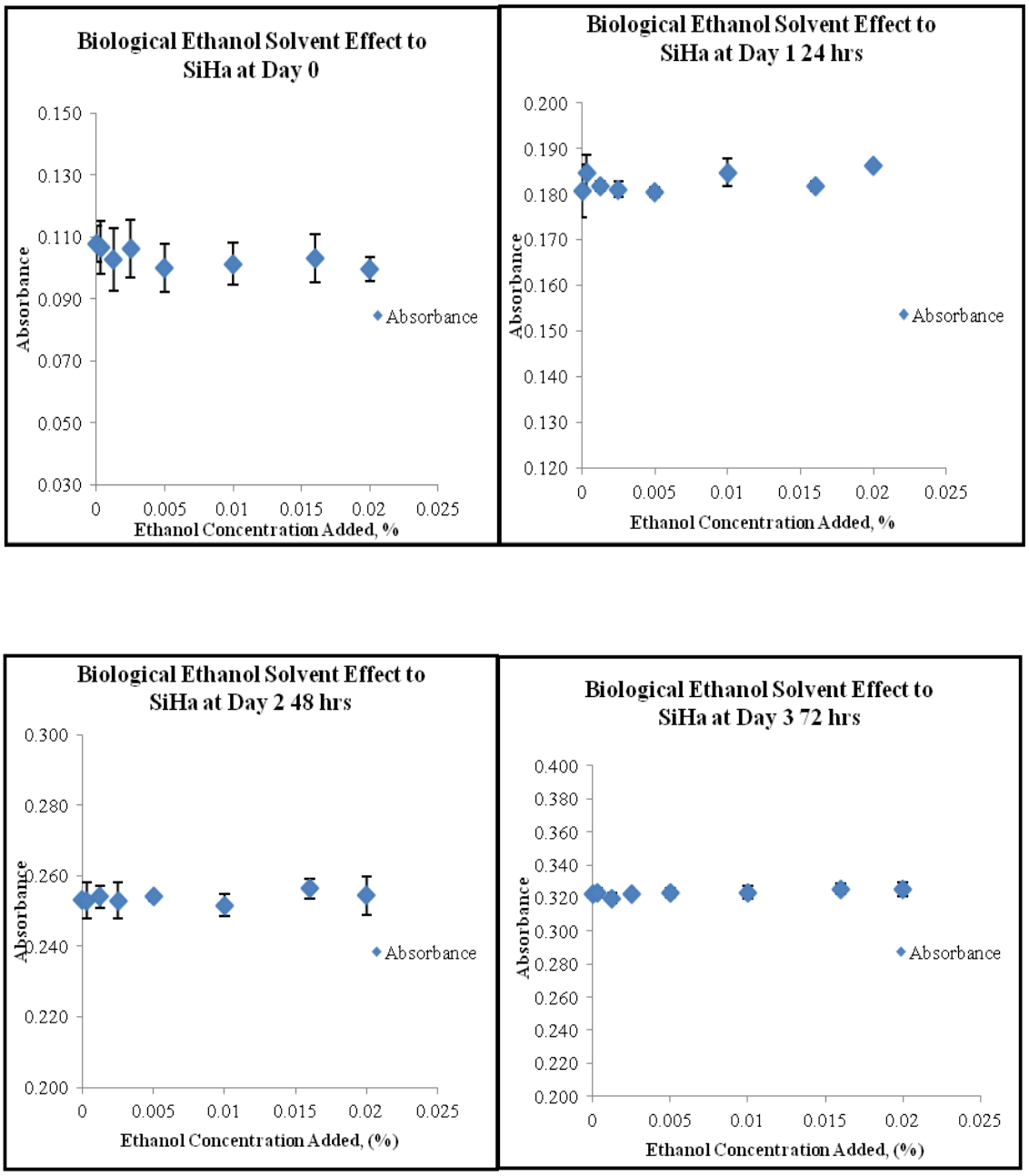

Figure 108: Biological ethanol inhibition effects on SIHA cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human cervix squamous carcinoma SiHa cells were treated biological ethanol with concentration at 2%, 1.6%, 1%, 0.5%, 0.25%, 0.125%, and 0.03125%. Data are means ± standard errors (n=3).

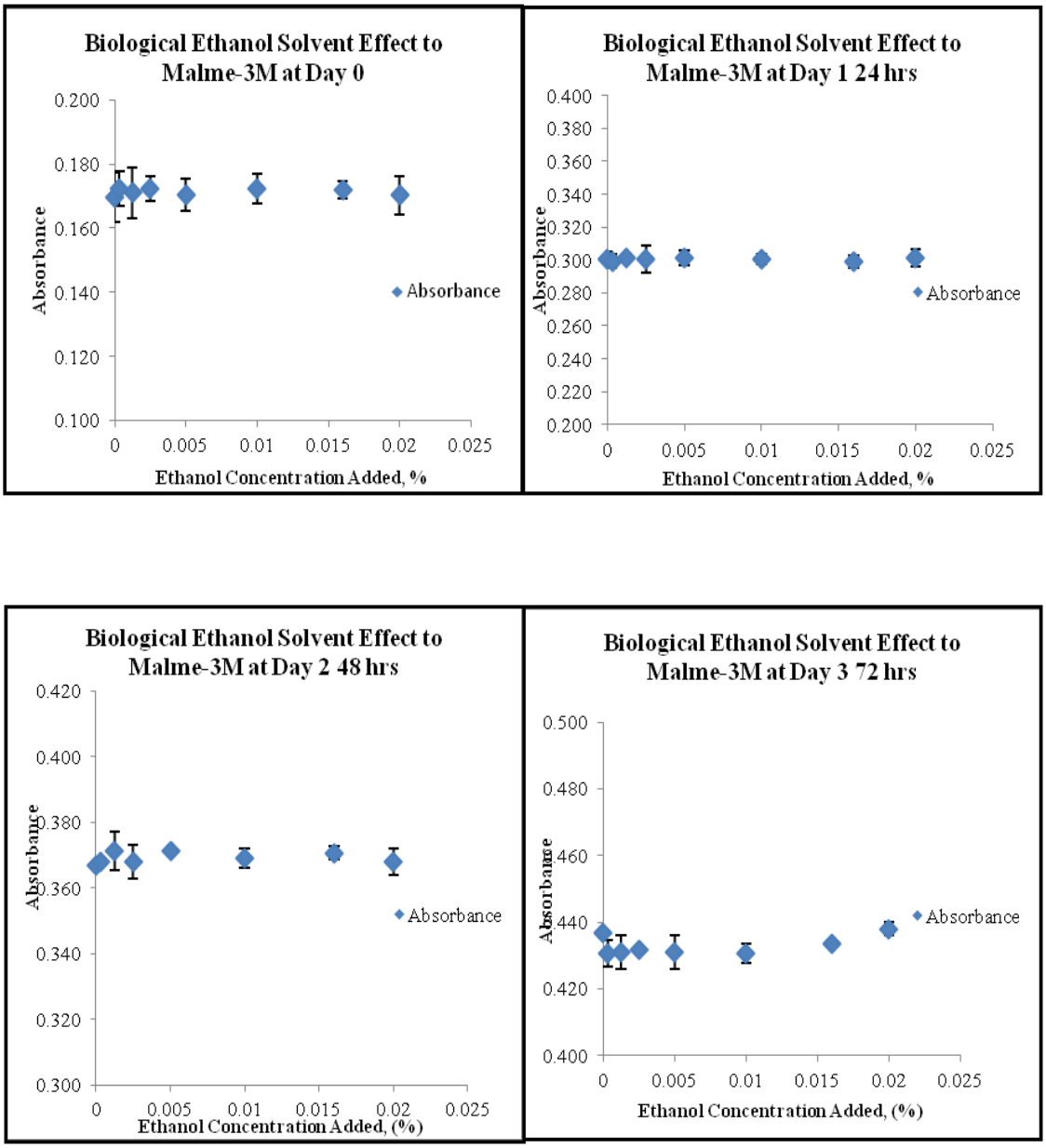

Figure 109: Biological ethanol inhibition effects on Malme-3M cells at Day 0, Day 1 24 hrs, Day 2 48 hrs & Day 3 72 hrs. Human hepatocellular carcinoma Hep G2 cells were treated biological ethanol with concentration at 2%, 1.6%, 1%, 0.5%, 0.25%, 0.125%, and 0.03125%. Data are means ± standard errors (n=3).

Appendix 3

A.3. Illustration of Minitab Output for Statistical Data Analysis

A.3.1. Illustration for Statistic Data Comparison about Anti-proliferative Effects between Pure Fucoxanthin Standard and First NZ Fucoxanthin Extract (containing 43.5% fucoxanthin)

The comparison about anti-proliferative effect to human heptacellular carcinoma Hep G2 between pure fucoxanthin standard and the first NZ fucoxanthin extract (containing 43.5% of fucoxanthin) was shown below, and this data comparison was illustrated and achieved by Minitab® (Version 16) software. This conclusion was confirmed with Tukey's Interval Test.

Step 1: State the Null and Alternative Hypothesis

H_0: Anti-proliferative effect to human heptacellular carcinoma Hep G2 between pure fucoxanthin standard and the first NZ fucoxanthin extract was the same

H_1: Anti-proliferative effect to human heptacellular carcinoma Hep G2 between pure fucoxanthin standard and the first NZ fucoxanthin extract was the different

Step 2: Select a level of significance

α=0.05

Step 3: Identify the test statistic

Test statistic is the *p*-value

Step 4: Formulate a decision rule

If *p*-value<0.05, reject H_0.

If *p*-value>0.05, accept H_0.

Step 5: Minitab output for this test

One-way ANOVA: Pure Fucoxanthin, First NZ Fucoxanthin Extract

```
Source  DF      SS      MS       F      P
Factor   1  836.15  836.15  188.24  0.000
Error    4   17.77    4.44
Total    5  853.92

S = 2.108   R-Sq = 97.92%   R-Sq(adj) = 97.40%



Level                     N    Mean  StDev
Pure Fucoxanthin          3  54.820  1.167
First NZ Fucoxanthin Ext  3  31.210  2.743

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level                     -----+---------+---------+---------+----
Pure Fucoxanthin                                    (----*---)
First NZ Fucoxanthin Ext  (---*---)
                          -----+---------+---------+---------+----
                            32.0       40.0       48.0       56.0

Pooled StDev = 2.108


Grouping Information Using Tukey Method

                             N    Mean  Grouping
Pure Fucoxanthin             3  54.820  A
First NZ Fucoxanthin Extract 3  31.210    B

Means that do not share a letter are significantly different.


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%


Pure Fucoxanthin subtracted from:

                           Lower   Center    Upper
First NZ Fucoxanthin Ext  -28.388  -23.610  -18.832

                          --------+---------+---------+---------+-
First NZ Fucoxanthin Ext  (---*----)
                          --------+---------+---------+---------+-
                               -20       -10         0         10
```

Step 6: Make a decision

Since *p*-value=0.000<0.05, reject H_0

Step 7: Write a Conclusion

Anti-proliferative effect to human heptacellular carcinoma Hep G2 between pure fucoxanthin standard and the first NZ fucoxanthin extract was the different.

A.3.2. Illustration for Statistic Data Comparison about Anti-proliferative Effects between Pure Fucoxanthin Standard and NZ *Undaria* Extract (containing 0.2% fucoxanthin)

The comparison about anti-proliferative effect to human colon adenocarcinoma Lovo between pure fucoxanthin standard and the NZ *Undaria* fucoxanthin extract (containing 0.2% of fucoxanthin) was shown below, and this data comparison was illustrated and achieved by Minitab® (Version 16) software. This conclusion was confirmed with Tukey's Interval Test.

Step 1: State the Null and Alternative Hypothesis

H_0: Anti-proliferative effect to human colon adenocarcinoma Lovo between pure fucoxanthin standard and the first NZ fucoxanthin extract was the same

H_1: Anti-proliferative effect to human colon adenocarcinoma Lovo between pure fucoxanthin standard and the first NZ fucoxanthin extract was the different

Step 2: Select a level of significance

α=0.05

Step 3: Identify the test statistic

Test statistic is the *p*-value

Step 4: Formulate a decision rule

If *p*-value<0.05, reject H_0.

If *p*-value>0.05, accept H_0.

Step 5: Minitab output for this test

One-way ANOVA: Pure Fucoxanthin, NZ Undaria Fucoxanthin Extract

Source	DF	SS	MS	F	P
Factor	1	74.131	74.131	234.96	0.000
Error	4	1.262	0.316		
Total	5	75.393			

S = 0.5617 R-Sq = 98.33% R-Sq(adj) = 97.91%

```
Level                     N    Mean  StDev
Pure Fucoxanthin          3  13.600  0.759
NZ Undaria Fucoxanthin E  3   6.570  0.233

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level                     -------+---------+---------+---------+--
Pure Fucoxanthin                                    (--*---)
NZ Undaria Fucoxanthin E  (--*---)
                          -------+---------+---------+---------+--
                               7.5       10.0       12.5       15.0

Pooled StDev = 0.562


Grouping Information Using Tukey Method

                                N     Mean  Grouping
Pure Fucoxanthin                3  13.6000  A
NZ Undaria Fucoxanthin Extract  3   6.5700    B

Means that do not share a letter are significantly different.


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%


Pure Fucoxanthin subtracted from:

                          Lower   Center    Upper
NZ Undaria Fucoxanthin E  -8.3033  -7.0300  -5.7567

                          ---+---------+---------+---------+------
NZ Undaria Fucoxanthin E  (----*----)
                          ---+---------+---------+---------+------
                          -7.5       -5.0       -2.5        0.0
```

Step 6: Make a decision

Since *p*-value=0.000<0.05, reject H_0

Step 7: Write a Conclusion

Anti-proliferative effect to human colon adenocarcinoma Lovo between pure fucoxanthin standard and the first NZ fucoxanthin extract was the different, and as the Tukey's Interval Test shown, the anti-proliferative effects from the NZ *Undaria* fucoxanthin extract (containing 0.2% fucoxanthin) was higher than the pure fucoxanthin standard.