Anti-proliferative Effect of Fucoxanthin on Human Pancreatic Cancer Cells

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A thesis submitted to

Auckland University of Technology

in partial fulfilment of the requirements for the degree of

Master of Applied Science (MAppSc)

2015

School of Applied Sciences

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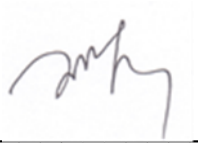
Signature: 

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to be the best of my knowledge and belief, 'Anti-proliferative effects of fucoxanthin on human pancreatic cancer cells', contains no material previously published or written by another person (except where explicitly defined 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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Acknowledgements

First of all, I greatly appreciate my primary supervisor A/Prof. Jun Lu, for giving me the opportunity to work on this subject. He is knowledgeable and possesses great knowledge about anti-cancer research. In the course of my study, he has guided me patiently, giving me a lot of good advice, so that I could avoid making too many detours in my research. He gave me careful and valuable guidance for the data analysis and thesis writing. He played an important role in the accomplishment of my project.

I also need to express my thanks to my secondary supervisor Dr. Yan Li. He gave me a lot of technical guidance about cell culture, cell mechanism studies and helpful advice with the results review. I am thankful for the support I received from him.

Additionally, I need to thank Kelvin Wang for teaching me the specific steps of cell culture, the cell cytotoxicity assay and cell cycle analysis. Not only that, but he also gave me a lot of advice about my research and thesis writing. I convey thanks to Dr. Michael Steiner, the engineer who has helped our lab with our of flow cytometry equipment. He gave lots of technical guidance about flow cytometry to me, including the operation of the machine, sample preparation and data analysis. I would not be able to finish the cell cycle analysis part without his help and support. I would like to thank Reza Nemati for helping me with the statistical analysis and giving me encouragement when my research was not proceeding well. I appreciate the help I received from the colleagues in my lab.

Special thanks should be given to Brid Lorigan and Sonya Popoff for helping me order all the experimental materials and supply a lot of other important lab stuffs. I am grateful to Dr. Chris Pook and Reachel Boyle for teaching me about the laboratory safety. Also, I give many thanks to all staff members in the laboratories of the Faculty of Health and Environmental Sciences in Auckland University of Technology.

Finally, I would like to thank my parents and other family members for their support.

Abbreviations

IC_{50}: The half maximal inhibitory concentration

IARC: International Agency for Research on Cancer

AIDS: Acquired Immune Deficiency Syndrome

WCRF: World Cancer Research Fund

WHO: World Health Organization

nt: Nucleotide

BMI: Body mass index

US: Ultrasonography

CT: Conventional computed tomography

DNA: Deoxyribonucleic acid

5-FU: 5-Fluorouracil

GEM (dFdC): Gemcitabine

hNTs: Human nucleoside transporters

hENT: Equilibrative nucleoside transporters

hCNT: Concentrative nucleoside transporters

dCK: Deoxycytidine kinase

UMP-CMP: Pyrimidine nucleoside monophosphate kinase

5'-NTs: 5'-nucleotidases

NF-κB: Nuclear factor-κB

FX: Fucoxanthin

HTLV-1: Human T-cell leukemia virus type 1

Cdk: Cyclin-dependent kinase

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

DMSO: Dimethyl sulfoxide

PBS: Phosphate buffered saline

hr(s): Hour(s)

mM: Millimoles per liter (mmol/L)

μM: Micromoles per liter (μmol/L)

nM: Nanomoles per liter (nmol/L)

RNAse: Ribonuclease

PI: Propidium iodide

S.D: Stand deviation

Log: Logarithm

Abstract

Cancer is one of the major public health problems in the world. Pancreatic cancer is the 8th most common cause of cancer death worldwide because of its poor prognosis. New Zealand is the fourth highest cancer incidence country worldwide, and pancreatic cancer is the leading cause of cancer mortality after diagnosis in New Zealand.

Gemcitabine a novel deoxycytidine analogue, first conceptualized and synthesized in 1980s, is used as the first line chemotherapeutic agent for the treatment of pancreatic cancer at present. However, gemcitabine alone is not satisfactory in the clinical treatment of pancreatic cancer: chemo-resistance is still found in the treatment process. Even combined with other chemo or radio-therapeutic agents it still shows limited efficacy, with severe side effects. According to cytotoxicity (MTT assay) analysis, gemcitabine showed its inhibition effect to human pancreatic cancer cell lines MIA PaCa-2, PANC-1 and human embryonic kidney cell line HEK 293 in a dose-dependent manner. Different cell lines response to gemcitabine with different sensibilities. The gemcitabine IC_{50} value after 72 hours exposure for MIA PaCa-2 cells was 16.00 ± 0.47 nM, PANC-1 cells was 48.55 ± 2.30 μM and for HEK 293 cells was 48.82 ± 3.27 nM. The anti-cancer activity of gemcitabine is primarily performed by impairing DNA synthesis. Gemcitabine showed cytostatic and cytotoxic effects to pancreatic cancer cells by blocking cell cycle in G_0-G_1 or S phase and further induced apoptosis.

Fucoxanthin is the most abundant natural carotenoid found in various marine algae, which is thought as a potential natural substance to be developed as a pharmaceutical anticancer agent. Fucoxanthin inhibited human pancreatic cancer cell lines MIA PaCa-2 and PANC-1 in a time- and dose- dependent manner. It also showed a selective inhibition effect towards human embryonic kidney cell line HEK 293. Fucoxanthin showed an anti-proliferative effect when treating human pancreatic cancer cell lines (MIA PaCa-2 and PANC-1) due to its cytostatic and cytotoxic properties. This was accomplished by

inducing cell cycle arrest at the G_0-G_1 phase and/or apoptosis. The fucoxanthin IC_{50} detected at 72 hours for MIA PaCa-2 was 8.74 ± 0.28 μM, for PANC-1 was 10.58 ± 0.56 μM and for HEK 293 was 8.28 ± 0.30 μM.

Fucoxanthin significantly improved the inhibitory effect of gemcitabine to pancreatic cancer cells even at a low concentration range (150,250 and 300 nM). Low doses of fucoxanthin even help enhance the cell viability of HEK 293 cells. The interaction of fucoxanthin and gemcitabine on inhibition of PANC-1 cells was significant ($P< 0.01$). Fucoxanthin (150, 250, 300 nM) simultaneously combined with gemcitabine (25 and 50 nM) showed significant anti-proliferation effect to MIA PaCa-2 cells in a concentration dependent manner ($P< 0.05$) in each gemcitabine group (GEM 25 nM group and GEM 50 nM group), compared to gemcitabine treatment alone. Fucoxanthin 10 μM combined with 500 nM gemcitabine, significantly decreased about 7% of PANC-1 cell viability compared to fucoxanthin 10 μM treatment alone ($P< 0.05$). In the fucoxanthin 20 μM group, the joint inhibitory effect of both gemcitabine 50 nM ($P< 0.05$) and 500 nM ($P< 0.01$) was significantly higher than 20 μM of fucoxanthin treating PANC-1 cells alone. Cell cycle results were consistent with the MTT assay results.

In summary, the *in vitro* study of gemcitabine and fucoxanthin on human pancreatic cancer cells showed additive inhibitory effects instead of synergistic effects when combining the two drugs to treat the cells for the designated time course. The findings also demonstrated that fucoxanthin effectively improved the cytotoxicity of gemcitabine even at low concentrations. This study is a beginning phase of research which investigates the anti-proliferative effect of fucoxanthin on pancreatic cancer. Fucoxanthin is considered as a potential candidate for the development of anti-cancer drugs for the treatment of pancreatic cancer as well as for further clinical applications.

Chapter 1 Introduction

1.1 Background

Pancreatic cancer is a digestive malignant tumour including exocrine and endocrine tumours of the pancreas (Vincent, Hruban, & Goggins, 2011). Pancreatic exocrine tumours account for upwards of 90% of the pancreatic tumours, of which 80% are derived from the pancreatic ductal epithelium (Bosetti et al., 2012). Pancreatic cancers are the 8th most common cause of cancer death worldwide with the feature of high-difficulty of diagnosis and treatment, and the incidence rate has a rising tendency in recent years. Pancreatic cancer is one of the most frequently diagnosed cancers ranked 13th in the world (Ferlay et al., 2010; Urrutia, Abbruzzese, & Neoptolemos, 2010). In New Zealand, it is a leading cause of cancer mortality after diagnosis (Ministry of Health, 2015). Chemotherapy is one of the major treatments not only for the 15% to 20% patients whose pancreatic tumours can be effectively removed surgically, but for the remaining pancreatic patients who present the disease, and it is not surgically resectable (Goodman & Hajj, 2013). Gemcitabine is a chemotherapy agent, which is now used as a standard of care for pancreatic cancer (Karak & Flechon, 2007). However, as a cytotoxic drug, gemcitabine inevitably has side effects (Anna, Ewa, & Jadwiga, 2012). Hence, it is crucial to improve gemcitabine treatment efficacy and reduce its drug toxicity, or develop a new anticancer drug with high inhibition effect and minor or no toxic effect to improve clinic efficiency of pancreatic cancer. In recent years, a considerable number of drug candidates have been yielded by marine natural product bioprospecting (Haefner, 2003). Marine resources are considered to be treasury of natural anticancer drugs.

Fucoxanthin is one of the most abundant natural carotenoids mainly derived from marine algae. Owing to its special molecular structure, it is thought as a potential natural substance to be developed as a pharmaceutical anticancer agent (Peng, Wu, & Wang,

2011). Fucoxanthin was found to be able to inhibit tumour growth with no apparent toxic performance in some animals (Ishikawa et al., 2008; K.-N. Kim et al., 2013). Fucoxanthin has also been stated to demonstrate anti-cancer effects as well as apoptotic effects in cancer cells in vitro (Ishikawa et al., 2008; Yamamoto, Yasumoto, & Mori, 2011; Zorofchian Moghadamtousi et al., 2014). Fucoxanthin was shown to have a selective cytotoxic activity, on account of its nontoxicity to some normal cells (Januar, Marraskuranto, & Wikanta, 2012). Additionally, fucoxanthin was proven to enhance the chemotherapeutic efficacy of some cytotoxic drugs such as cisplatin *in vitro* (Cheng-Ling, Yun-Ping, & Miao-Lin, 2013). For these reasons, the combination of gemcitabine, a cytotoxic drug, with fucoxanthin may present a great prospect in pancreatic cancer therapy. However, there is still not an established primary mechanism of action of fucoxanthin, though various mechanistic studies have been carried out by researchers. More studies need to be done. Moreover, little research has been done about the anti-cancer effects of fucoxanthin on pancreatic cancer cells. Thus, conducting some research about the inhibition effect of fucoxanthin on pancreatic cancer cells is of great significance and it is a good start to fill in the gaps of knowledge in this area.

1.2 Objectives of Study

The main purpose of the study is to find if the fucoxanthin has inhibitory effects on pancreatic cancer cells. If it does, finding whether fucoxanthin and gemcitabine have interactions with each other is the further objective. Hopefully, the results of this study may offer some references for treatment of pancreatic cancer patients. The specific goals in this study are summarized as the following points:

1: Testing if fucoxanthin can supress the growth of pancreatic cancer cell lines MIA PaCa-2 and PANC-1. If it does, try to find the optimal concentration range and action time as well as get IC_{50} value of fucoxanthin and gemcitabine.

2: Combining fucoxanthin and gemcitabine together to find the combination concentration and then find if they have interactions with each other.

3: Using the normal cell line HEK 293 to test if fucoxanthin has toxicity to the cells. If it does, get the IC_{50} value of fucoxanthin and gemcitabine.

4: Using flow cytometry to try to find the basic action mechanism of these two drugs on pancreatic cancer cells and human normal cells, especially fucoxanthin, by analysing the cell cycle and apoptosis of the cells.

1.3 Overview

This thesis consists of six chapters.

Chapter 2 is the literature review part, which comprehensively introduce the cancer, pancreatic cancer, gemcitabine and fucoxanthin. The introduction of cancer is followed by describing the definition of cancer and its pathogenesis. Besides, the incidence of cancer in the world and in New Zealand is discussed in this part. The epidemiology, aetiology, classification, diagnosis, staging and management of pancreatic cancer are described in the next. After that gemcitabine a chemotherapeutic agent in pancreatic cancer therapy and fucoxanthin a carotenoid found in marine algae are introduced. Their structure, mechanism, anti-cancer effect and toxicities are outlined.

Chapter 3 is the methodology part. The materials, equipment and methods used in this study are described, as well as experimental design, data analysis and statistical data analysis. Two major study methods are cytotoxicity assay and cell cycle assay.

Chapter 4 presents all the results obtained from the study, including the cytotoxicity assay results and cell cycle results.

Chapter 5 discusses the results shown in Chapter 4, and makes some assumptions about the mechanisms according to previous researches.

Chapter 6 is the final conclusion chapter, which summarises the findings obtained from this study, puts forward the weak points of current research and gives some suggestions for future study.

Chapter 2 Literature Review

2.1 Cancer

2.1.1 The Overview of Cancer

Cancer is broadly defined as a class of diseases characterized by out-of-control growth of abnormal cells. In terms of professional definition, cancers are called malignant tumours or malignant neoplasms. Cancers are phenomena associated with cell proliferation and differentiation. Cancer cells can constantly proliferate within a limited space, extruding and destroying normal cells by consuming large amounts of nutrients and generating toxic products, at the same time inhibiting the function of normal cells. The pathogenesis of cancer is not fully understood. Over 80% of cancers are related to environmental factors which include chemical factors, physical factors and biological factors. Genetic factors, endocrine factors and immunological factors are the three parts of the internal factors related to cancers. Environmental factors and internal factors lead to cancer in a synergetic or sequential manner (Danaei et al., 2005).

Essentially, mutation of healthy cells to cancer cells (cells cancerization) is recognized as a kind of genetic disease. Genetically, cell carcinogenesis is closely related to oncogenes and tumour suppressor genes. The normal expression of genes in cells will not cause cancer, but once the cells are stimulated by the environmental factors such as chemical and radioactive substances, oncogenes will change in a number of ways. Point mutations, genetic mutation or chromosomal rearrangement may result in the occurrence of cancer. Tumour suppressor genes play a negative regulatory role in normal cell growth, by means of suppressing cell proliferation and differentiation, as well as through inducing programmed cell death. The loss of one or more tumour-suppressor genes gives rise to cancerous cells. Disabled tumour suppressor genes make cells proliferate out of control (World Health Organization, 2013).

There are more than 100 types of cancer that threaten human health. Cancers are usually named after organs or tissues, which are the place of the original formation of cancer. Cancer is divided into five broad groups: carcinomas, sarcomas, lymphomas, leukaemias and adenomas. Surgery, radiotherapy and chemotherapy are the main methods for cancer treatment. Additionally, immunotherapy, hormone therapy and gene therapy are new types of ways used in cancer treatment (National Cancer Institute, n.d.).

2.1.2 Cancer in the World

Cancer is a major public health problem in the world. Currently, cancer has become the leading cause of human death, and the number of fatal cases caused by it exceeds the sum of deaths caused by AIDS, tuberculosis and malaria. In the past three decades, the incidence of the global cancer increased from 3% to 5% annually. According to the report from the International Agency for Research on Cancer (IARC), the specialized cancer agency of the World Health Organization, an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012. This result was achieved by investigating 28 types of cancer within 184 countries worldwide. However, comparing with 2012, the same cases were 12.7 million and 7.6 million, respectively, in 2008. The number of new cases is expected to rise by about 70% over the next two decades. More than 60% of the world's total new annual cases occur in Africa, Asia and Central and South America, which account for 70% of the world's cancer deaths (Danaei, Vander Hoorn, & Ezzati, 2011; World Health Organization, 2013).

Based on WHO statistics, the five most common types of cancer occurring in men in 2012 were lung, prostate, colorectal, stomach, and liver cancer. Meanwhile, the top five diagnosed cancers in women were breast, colorectal, lung, cervix, and stomach cancer (World Health Organization, 2013). Owing to the rising incidence of cancer in the population, it is urgent to develop effective and affordable approaches to the early detection, diagnosis, and treatment of cancer.

2.1.3 Cancer in New Zealand

New Zealand is the fourth highest cancer incidence country worldwide. Cancer is a principal threat for the health of New Zealanders, a leading cause of death (28.9%) and hospitalization, which likely occurs due to environmental factors and eating habits in the country. According to statistics from the World Cancer Research Fund (WCRF), about 309 people are diagnosed with cancer in every 100,000 New Zealanders yearly (Frizelle, 2009).

In 2010, 21,235 New Zealanders were diagnosed with cancer and 8593 deaths occurred due to this disease. In 2011, 21,050 new cancer-related cases were recorded in New Zealand, among which 52.5% were male. The incidence of cancer for women in New Zealand is second-highest in the world: 287 new cases appearing per 100,000 women each year, which is second only to Denmark. For men, the rate is 338 per 100,000 people, putting them eighth top on the list. In accordance with the statistical data, pancreatic cancer is the leading cause of cancer mortality after diagnosis in New Zealand (Ministry of Health, 2015).

2.2 Pancreatic Cancer

The pancreas is an inconspicuous small organ located in the epigastric and left hypochondriac regions, behind the stomach and peritoneum. The pancreas is one of the most important organs in the body because of its endocrine and exocrine functions, whose physiological effects and pathological changes are vital to life. It is not only a glandular organ, but also a digestive organ. The main compound of pancreatic exocrine is pancreatic juice, containing alkaline bicarbonate and digestive enzymes, both of which assist in neutralizing gastric acids, digesting and absorbing nutrients in the small intestine. The main products of the pancreas's endocrine secretions are insulin, glucagon, somatostatin, pancreatic polypeptide and gastrin (GAS) (Gronborg et al., 2004).

Pancreatic cancer is a malignant tumour of the digestive system, which is highly

difficult to diagnose and treat. It accounts for 1~2% of total malignant tumours, but its incidence rate has a rising tendency in recent years (Vincent et al., 2011). Because of the special physiological function of the organ, pancreatic cancer includes both exocrine and endocrine tumours of the pancreas. Over 90% of pancreatic tumours stem from duct adenocarcinoma of the gland epithelium so that pancreatic cancer usually indicates ductal adenocarcinoma, except where noted (Muniraj, Jamidar, & Aslanian, 2013).

2.2.1 Epidemiology and Aetiology

Pancreatic cancer is one of the most frequent cancers that is ranked 13th in the world. Additionally, it is the 8th most common cause of cancer death worldwide because of its poor prognosis rates. There were 278,684 new cases of pancreatic cancer and approximately 266,669 deaths in the world in 2008 (Ferlay et al., 2010; Urrutia et al., 2010). Pancreatic cancer occurs frequently in New Zealand Maoris, African-Americans and Jews. The highest incidence rates were found in Sweden, the United States, Italy and Japan, while Asia and Africa had the lowest incidence rates (Bosetti et al., 2012). The incidence of pancreatic cancer increases significantly with age, with people between the ages of 30 to 40 rarely at risk of pancreatic cancer. The high-risk population is in the age range of 60 to 65 years old and the age range between 70 to 80 years is the peak period of incidence (Michaud, 2002). In all recorded cases of pancreatic cancer, the mortality rate of male is slightly higher than females'. On a global basis, there are approximately 120,000 males which die from pancreatic cancer annually, in comparison to 107,000 females. In terms of the corresponding cumulative risk of death from the age of 0 to 64, males are 0.2% compared to 0.1% for females (Lowenfels & Maisonneuve, 2006).

The oncogenesis of pancreatic cancer is connected with the improvement of genetic predisposition caused by various genetic mutations. Some genetic mutations such as *BRCA1, BRCA2, MSH2, MSH6, MLH1, PMS, PM52, APC, CFTR, PRSS1, PRSS2, CDKN2A/P16, STK11/LKB1, FA, ATM* and *TP53* can lead to multiple germline mutations, inducing several genetic syndromes. Some syndromes can enhance the risk of getting

pancreatic cancer and are likely to have familial hereditary tendencies, including familial breast and ovarian cancer (FOBC), hereditary non-polyposis colorectal carcinoma (HNPCC), familial adenomatous polyposis (FAP), cystic fibrosis (CF), hereditary pancreatitis (HP), ataxia-telangiectasia (AT), Peutz-Jeghers syndrome (PJS) and familial adenomatous polyposis (FAP) (Hahn & Bartsch, 2005). About 5% to 10% of patients with pancreatic cancer have a family history of the disease. It is estimated that individuals whose first-degree relative was diagnosed with pancreatic cancer in the family have a 2 times higher possibility of getting the disease than an individual in the general population, and the risk increases significantly with the increasing number of first-degree relatives' cases (Gloria et al., 2006; Chanjuan et al., 2009). Wolpin and co-workers found that certain ABO blood types are associated with a statistically significantly higher risk of pancreatic cancer. People with blood groups A, B or AB are more likely to develop pancreatic cancer than people with blood group O (Wolpin et al., 2009). Aberrant expression of miRNAs (MicroRNAs, a group of small non-coding RNA molecules of 17–25 nucleotides (nt) in length, foretold to regulate about 30% of all protein-coding genes activities in mammals) may induce the occurrence of pancreatic cancer by participating in the process of cell proliferation, differentiation and apoptosis (Rachagani, Kumar, & Batra, 2010). Pancreatic cancer stem cells have the property of infinite proliferation and asymmetrical differentiation in the pancreas, playing an important role in the development of tumours, but more importantly they can tolerate the attack of chemotherapy and radiotherapy, which may be a reason for the poor prognosis in pancreatic cancer (Hermann et al., 2007). Exogenous carcinogens like tobacco, may damage DNA in the pancreas then promote oncogene activation and tumour suppressor genes inactivation, attending to the development of pancreatic cancer (Lin et al., 2011).

The occurrence of pancreatic cancer may also have a close relationship with environmental factors, of which smoking is the only one currently recognized (Hassan, 2007; Sara Raimondi, Patrick Maisonneuve, & Lowenfels, 2009; Yadav & Lowenfels,

2013). The relative risk (the ratio of the possibility of an event occurring in an exposed group to the possibility of the event occurring in a non-exposed group) of smoking is 1.75 and smokers have 2 times higher risk of pancreatic cancer than non-smokers, furthermore, a dose-response relationship was noticed (Hassan, 2007). Excessive amounts of alcohol can induce chronic pancreatitis and further cause cancer (Jeanine et al., 2009). Obesity generated by diets rich in fat and cholesterol may increase the probability of pancreatic cancer. There is a positive correlation potentially between the body mass index (BMI) and pancreatic cancer (Berrington de Gonzalez, Sweetland, & Spencer, 2003; Batty et al., 2009). High levels of vitamin D in the body promote the risk of pancreatic cancer (Stolzenberg-Solomon et al., 2010). In addition, pancreatic cancer may also be associated with climatic conditions, air pollution and risk occupation exposure.

Researchers suggested that chronic pancreatitis caused by cholelithiasis, excessive alcohol use and hereditary factors are established risk factors for pancreatic cancer, whose relative risk is 14. *K-ras*, *PRSS1*, *PRSS2*, *SPINK1*, *CFTR* or other gene mutations and chromosomal instability may contribute to its molecular mechanism (Volker, 2008; Whitcomb, 2010). People with rare chronic pancreatitis such as hereditary and tropical pancreatitis have the best chance of deteriorating into pancreatic cancer, 50 times greater than the general population (Raimondi, Maisonneuve, & Pezzilli, 2010). Diabetes is an early clinical symptoms of pancreatic cancer and about 60% to 81% of the patients with pancreatic cancer are characterized by impaired glucose tolerance or diabetes, in particular, type-II diabetes and late-onset diabetes have a certain connection with pancreatic cancer (Silverman et al., 1999; De Souza & Saif, 2014). The relative risk of type-II diabetes developing into pancreatic cancer is 1.84 and people with 4 years history of diabetes have 50% higher risk of pancreatic cancer than 5 to 10 years history or over 10 years (Huxley, Barzi, & Woodward, 2005). Other diseases, such as cholecystitis, cholecystectomy, helicobacter pylori infection, subtotal gastrectomy, appendectomy and immunodeficiency appear to be linked to pancreatic cancer (Silverman et al., 1999).

The oncogenesis of pancreatic cancer is a complicated process affected by several aspects, mainly including hereditary factors, environmental factors and disease factors with their own epidemiological characteristics.

Table 1: Risk factors for pancreatic cancer

Low increase (less than fivefold increase in risk)	**Moderate increase(five to ten- fold increase in risk)**
Alcohol use (≥ four drinks per day)	*BRCA2* gene carrier
Body mass index (≥ 30 kg per m^2)	Chronic pancreatitis
BRCA1 gene carrier	Cystic fibrosis
Chlorinated hydrocarbon exposure	Family history of pancreatic cancer in two first-degree relatives
Diabetes mellitus (type 2 for ≥ five years)	**High increase(more than a tenfold increase in risk)**
Familial adenomatous polyposis	Familial atypical multiple mole melanoma
Family history of pancreatic cancer in one first-degree relative	Family history of pancreatic cancer in at least three first-, second-, or third-degree relatives
Hereditary nonpolyposis colorectal cancer	Hereditary pancreatitis
Polycyclic aromatic hydrocarbon exposure	Peutz-Jeghers syndrome
Tobacco use	

2.2.2 Classification of Pancreatic Cancer

Pancreatic tumours are categorized into exocrine and endocrine tumours on the basis of the type of tissue from where the tumour originates in the gland. Exocrine and endocrine tumours have significant differences amongest each other. Both of them distinguish themselves mainly in terms of risk factors, causes, symptoms, diagnostic tests, treatments, and prognosis (Crosta, 2009).

Pancreatic *exocrine* tumours account for more than 90% of pancreatic tumours (Zhu,

2005). Malignant tumours, also called adenocarcinomas, account for 95% of exocrine pancreatic cancers. Among them *ductal* adenocarcinomas, which arise from the cells that line the ducts of the exocrine pancreas and, are characterized by insidious infiltration and rapid dissemination, account for 80% to 90% of all tumours of the pancreas. Invasive ductal adenocarcinomas are considered to be the most common variant (Basturk, Coban, & Adsay, 2010). Unless otherwise stated, pancreatic cancer usually refers to ductal adenocarcinoma. Besides, adenocarcinoma can also develop from pancreatic enzyme producing cells. Other types of exocrine pancreatic cancers such as adenosquamous carcinomas and giant cell carcinomas are named after their appearances under a microscope (Bond-Smith, Hammond, & Imber, 2012). Among the less common types of pancreatic exocrine tumours are the acinar cell carcinoma, cystic tumours that are typically benign but may become cancerous, and papillary tumours that grow with the pancreatic ducts.

Pancreatic endocrine tumours, also termed neuroendocrine or islet cell tumours, are known to be fairly uncommon. They are named after the category of originally affected hormone-producing cells. For instance: insulinomas (insulin), glucagonomas (glucagon), gastrinomas (gastrin), somatostatinomas (somatostatin), and VIPomas (vasoactive intestinal peptide or VIP). Most of the functioning neuroendocrine tumours, that still make hormones, are generally benign; while the non-functioning neuroendocrine tumours, who don't secrete any hormone, are more likely to be malignant (Crosta, 2009). Table 2 shows the new classification system for pancreatic tumours released by the World Health Organization (WHO) (Bosman Fred T, Theise Neil D, & Hruban, 2010).

Table 2: 2010 WHO classification of tumours of the pancreas

Epithelial tumours	Mixed acinar-ductal carcinoma
Benign:	Mixed acinar-neuroendocrine carcinoma
Acinar cell cystadenoma	Mixed acinar-neuroendocrine-ductal carcinoma
Serous cystadenoma	Mixed ductal-neuroendocrine carcinoma
Premalignant lesions:	Mucinous cystic neoplasm with an associated invasive carcinoma
Pancreatic intraepithelial neoplasia, grade 3 (PanIN-3)	Pancreatoblastoma
Intraductal papillary mucinous neoplasm with low- or intermediate-grade dysplasia	Serous systadenocarcinoma
Intraductal papillary mucinous neoplasm with high-grade dysplasia	Solid-pseudopapillary neoplasm
Intraductal tubulopapillary neoplasm	Neuroendocrine neoplasms
Mucinous cystic neoplasm with low- or intermediate-grade dysplasia	Pancreatic neuroendocrine microadenoma
Mucinous cystic neoplasm with high-grade dysplasia	Neuroendocrine tumour (NET)
Malignant	Nonfunctional pancreatic NET, G1, G2
Ductal adenocarcinoma	NET G1
Adenosquamous carcinoma	NET G2
Colloid carcinoma (mucinous noncystic carcinoma)	Neuroendocrine carcinoma(NEC)
Hepatoid carcinoma	Large cell NEC
Medullary carcinoma	Small cell NEC
Signet ring cell carcinoma	EC cell, serotonin-producing NET (carcinoid)
Undifferentiated carcinoma	Gastrinoma
Undifferentiated carcinoma with osteoclast-like giant cells	Glucagonoma
Acinar cell carcinoma	Insulinoma
Acinar cell cystadenocarcinoma	Somatostatinoma
Intraductal papillary mucinous neoplasm with an associated invasive carcinoma	VIPoma
	Mature teratoma
	Mesenchymal tumours
	Lymphomas
	Secondary tumours

2.2.3 Diagnosis and Staging

Owing to tumour growth, the symptoms of pancreatic cancer begin to appear, the most frequent of which are jaundice, weight loss and pain. The severity of pancreatic

cancer's clinical symptoms is related to tumour size, location and metastasis. The symptoms of pancreatic cancer in early stage are atypical, which then results in the diagnosis of pancreatic cancer in advanced stages (Michl, Pauls, & Gress, 2006). The early diagnosis and accurate staging of pancreatic cancer is of great importance on account of the very rare likelihood of being able to cure the disease at its advanced stage.

Pancreatic cancer is difficult for early diagnosis and the diagnostic value is limited by only one detection method. The combined application of various examination methods can improve the diagnosis rate (Germanos et al., 2006). Imaging modalities, tumour markers, gene molecular diagnosis, proteomics techniques and risk prediction model are the chief diagnostic methods. Ultrasonography (US) and conventional computed tomography (CT) are common imaging modalities employed in initial diagnosis of pancreatic cancer and spiral CT serves as a better means for making a definite diagnosis. Notably, tri-phasic pancreatic-protocol CT is the best method not only for initial diagnosis but also for staging of the disease (Vincent et al., 2011). Magnetic resonance imaging (MRI) presents ideal soft tissue contrast and simultaneously provides a perfect mode of imaging for soft tissue lesions. However, its diagnostic outcome is worse than CT in lymph node involvement (Soriano et al., 2004). Endoscopic ultrasonography (EUS) is an essential imaging modality for the diagnosis of pancreatic cancer, which can be employed when the disease is not diagnosed by using CT, but presents with suspicious clinical manifestations (Michl et al., 2006). EUS fine-needle aspiration (FNA) can undertake the diagnosis of primary tumour, lymph nodes and distant metastases. Endoscopic retrograde cholangiopancreatography (ERCP), an imaging modality, can detect the pathological changes of main pancreatic duct and its branches. Moreover, this method makes it possible to collect pancreatic juice for brush cytology, as well as genetic and biopsy analyses (Hailin Liu & Lei Wang, 2009). Laparoscopy and laparoscopic ultrasonography (LUS) play an important role in examination occult intra-abdominal metastatic disease and now they are mainly used in preoperative assessment of pancreatic cancer (Butturini

et al., 2007).

Tumour markers, gene molecular diagnosis, proteomics techniques and risk prediction model are used as adjuncts to imaging techniques. Carbohydrate antigen 19-9 (CA 19-9), a glycoprotein, is one of the most common tumour markers. Up to 80~90% of pancreatic cancer sufferers have a mutated K-ras gene, which presents valuable information for early diagnosis of the disease. Along with the development of proteomic technology, it's possible to find new tumour markers (Hailin et al., 2009). PancPRO stands for a start of the risk prediction model for pancreatic cancer, which is applied in the detection in high-risk populations (Wenyi et al., 2007).

The accurate staging for patients with pancreatic cancer is a precondition for them to get the best treatment. Primary tumour, lymph node metastasis and distant metastasis are the three principal aspects for staging. In clinical practice, pancreatic cancer can be divided into three categories: resectable cancer, locally advanced cancer and metastatic cancer (Li & Zhou, 2006). To date, there are three staging classification systems that are widely used: Japan Pancreas Society (JPS), Union for International Cancer Control (UICC) and American Joint Committee on Cancer (AJCC), respectively (Isaji & Kawarada, 2000; Bilimoria et al., 2007). The 7th edition of the AJCC tumour node metastasis (TNM) classification system is employed where the classification is done according to the assessment of pancreatic cancer resectability by means of helical CT (Table 3) (Bilimoria et al., 2007; Wasif et al., 2010). It is worth noting that EUS is also a primary imaging modality for pancreatic cancer staging (Michl et al., 2006). T1, T2, and T3 tumours have resectable possibilities, while T4 tumours covering the superior mesenteric artery or celiac axis are unresectable. Tumours located at superior mesenteric veins, portal veins or splenic veins can also be categorized into the T3 class, on account of the resectability and their reconstruction, given that they are patent (Manuel, 2010; Muniraj et al., 2013).

Table 3: AJCC 7th edition TNM staging system for pancreatic cancer

Definitions of TNM				
TX	Primary tumour cannot be assessed			
T0	No evidence of primary tumour			
Tis	Carcinoma in situ			
T1	Tumour limited to the pancreas, 2 cm or less in greatest dimension			
T2	Tumour limited to the pancreas, greater than 2 cm in greatest dimension			
T3	Tumour extends beyond pancreas but no involvement of celiac axis or superior mesenteric artery			
T4	Tumour involves the celiac axis or the superior mesenteric artery (unresectable primary tumour)			
NX	Regional lymph nodes cannot be assessed			
N0	No regional lymph node metastasis			
N1	Regional lymph node metastasis			
M0	No distant metastasis			
M1	Distant metastasis			
Staging grouping				
Stage 0	Tis	N0	M0	Localized within pancreas
Stage IA	T1	N0	M0	Localized within pancreas
Stage IB	T2	N0	M0	Localized within pancreas
Stage IIA	T3	N0	M0	Locally invasive, resectable
Stage IIB	T1,2, or 3	N1	M0	Locally invasive, resectable
Stage III	T4	Any N	M0	Locally advanced, unresectable
Stage IV	Any T	Any N	M1	Distant metastases

2.2.4 Management of Pancreatic Cancer

Surgical treatment is the most direct route for treating pancreatic cancer. However, more than 80% of patients present with disease that is not surgically resectable. It requires treatment for the locally advanced and the metastatic disease. Management methods for unresectable disease range from systemic chemotherapy alone to the combined forms of treatment with chemo-radiation therapy and chemotherapy. Chemo-radiation therapy and chemotherapy are two effective adjuvant ways for the common non-surgical treatments. Even patients with potentially resectable disease require multimodality treatments

including chemotherapy and (or) chemo-radiation therapy to improve resectability and reduce the recurrence (Goodman & Hajj, 2013).

2.2.4.1 Resectable Lesions

Surgical resection is the only potential approach which offers the curative chances for pancreatic ductal adenocarcinomas. After diagnosis, approximately 15 ~ 20% patients are those whose pancreatic tumours can be effectively removed surgically. But among them, less than 20% can survive five years (De La Cruz, Young, & Ruffin, 2014). Pancreatic resections are suggested to be performed at high-volume institutions, in which at least 15 pancreatic resections should be completed annually; owing to the lower mortality rates, shorter hospital stay, and lower overall cost in contrast to the low-volume institutions (Crist, Sitzmann, & Cameron, 1987; Ho & Heslin, 2003). Pancreaticoduodenectomy, also known as a *Whipple procedure*, is a typical surgery for resection of carcinoma at the head of the pancreas (De La Cruz et al., 2014). Tumours involving the body or tail of the pancreas are rarely resectable because of their advanced stage at diagnosis and presentation of initial symptoms later in the development of cancer (Park, Heo, & Choi, 2014). For resectable lesions, the surgery performed is typically a *Distal Pancreatectomy* with or without splenectomy. Negative margin status, tumour DNA content, tumour size, and absence of lymph node metastasis are the most dependable prognostic indicators for long-term survival. Large tumour size, high differentiation grade, and involvement of the lymph nodes are the main risk factors which promote recurrence of the disease (Manuel, 2010). Adjuvant chemotherapy with gemcitabine alone or gemcitabine combining with fluorouracil is considered the standard of care in this phase, which improves postoperative survival up to two to three months compared with results obtained by observation alone (Ghaneh, Costello, & Neoptolemos, 2007).

2.2.4.2 Locally Advanced Lesions and Metastasis

The primary goals of treatment for advanced pancreatic cancers are palliation and

improved survival. The median of overall survival is short, with patients only living an additional 9 to 10 months (De La Cruz et al., 2014). There are diverse treatment options to treat locally advanced pancreatic cancers including single or multi-agent chemotherapy, chemotherapy followed by chemo-radiation, or immediate concurrent chemo-radiation (Kaltsas, Syrigos, & Saif, 2014). Systemic chemotherapy followed by consolidation chemo-radiation therapy is recommended as a treatment of choice. Concurrent administration of gemcitabine or continuous fluorouracil with radiation is an adequate regimen (Ghaneh et al., 2007). Irinotecan (Camptosar), a new chemotherapeutic agent, is used for patients with metastatic pancreatic adenocarcinoma to decrease the progression and to improve the overall survival rate (De La Cruz et al., 2014). Currently, capecitabine plus radiation therapy is a frequently used regimen followed by either gemcitabine alone or with radiation which are the most commonly used regimens in this setting (Kaltsas et al., 2014). Gemcitabine monotherapy is regarded as a good method for patients with locally advanced lesions or metastasis, because it may relieve symptoms and can provide clinical and modest survival benefits. Besides that, gemcitabine combined with other agents such as fluorouracil, cisplatin, and oxaliplatin is a second-line therapy. According to disease response and patient tolerance, the decision of whether to choose up-front chemoradiation or induction chemotherapy followed by consolidation chemo-radiation will be made (De La Cruz et al., 2014). Nevertheless, each of these different approaches has its limitation in terms of efficacy and toxicity. The most appropriate therapy needs to be made according to the actual situation of the patients; keeping in mind the goals of care and palliation as well as survival.

2.2.4.3 Palliative Care

With the treatment of pancreatic cancer disease, patients will face many physical and emotional challenges. Palliative care for patients with locally advanced and metastatic pancreatic cancer should address symptoms from biliary obstruction, gastric outlet obstruction, cancer-related pain, malnutrition, thromboembolic disease, and depression

(De La Cruz et al., 2014). The goal of palliative medicine is to relieve suffering and to improve the quality of life by addressing each patient's particular requirements. Pillars of palliative care are control of pain and other non-pain related symptoms, clear and effective communication, and addressing of the psychosocial needs. Diagnosis provides a basis, upon which palliative care can be offered, on account of the poor relative survival rate and the symptom profile associated with pancreatic malignancies. Basic communication techniques and management of common symptoms are two basic primary palliative care skills for all oncologists (Erdek, King, & Ellsworth, 2013).

2.2.5 Chemotherapy

Chemotherapy is a type of cancer treatment dealing with usage of chemical substances, especially one or more anti-cancer drugs (chemotherapeutic agents) that are given as part of a standardized chemotherapy regimen (Lundqvist, 2012). Briefly, chemotherapy is to stop or slow the growth of cancer cells by using drugs and its three main functions are curing cancer, controlling cancer and easing cancer symptoms. Chemotherapy is the backbone treatment for pancreatic cancer, owing to the fact that most patients are being diagnosed with advanced disease (Xiong, Carr, & Abbruzzese, 2006). The modern chemotherapy for advanced pancreatic cancer began in the 1960s and 1970s, during which 5-fluorouracil (5-FU) was extensively used and investigated. 5-FU was first presented to be a beneficial component of adjuvant treatment for patients with surgically resected pancreatic cancer in 1985 (Wolff, 2007). 5-FU was the pillar of palliative treatment before the development of gemcitabine as first-line chemotherapy. 5-FU is an S-phase-specific fluorinated pyrimidine, which has been widely tested for efficiency when combined with other cytotoxic agents such as cisplatin. But the combination regiments increase the toxicity without showing any significant survival benefit compared with 5-FU alone (Glimelius et al., 1996; Palmer et al., 1994). However, more recent studies showed that 5-FU was only marginally active, using a more accurate assessment of objective response (Xiong et al., 2006).

Pemetrexed demonstrates modest single agent activity in patients with advanced pancreatic cancer. The associated significant hematologic toxicity, may limit the use, its dosage and schedule in a palliative setting (Miller et al., 2000).

Recent studies of pemetrexed have demonstrated its activity against mesothelioma but only a modest activity against pancreatic cancer. The efficacy of chemotherapy is limited. Gemcitabine is the only agent that improves symptoms and confers a modest survival advantage.

2.3 Gemcitabine

Gemcitabine (2',2'-difluoro-2'-deoxycytidine; dFdC) (Figure 1) was first conceptualized and synthesized at Lilly Research Laboratories in 1980s (Plunkett, Huang, & Gandhi, 1997). To date, gemcitabine is one of the most widely studied chemotherapeutic agents and it has been broadly employed in the therapy of pancreatic, ovarian, breast, bladder and non-small cell variant of lung cancers (Dasanu, 2008). The advent of gemcitabine as a standard of care represents a new era in the treatment of advanced pancreatic cancer. Single-agent gemcitabine regimen has been proposed as the standard first-line treatment in locally advanced or metastatic advanced pancreatic cancer by the US Food and Drug Administration, for many years (Karak & Flechon, 2007). The following molecular mechanisms associated with pharmacokinetics and pharmacodynamics of gemcitabine are reviewed from the view of pancreatic cancer.

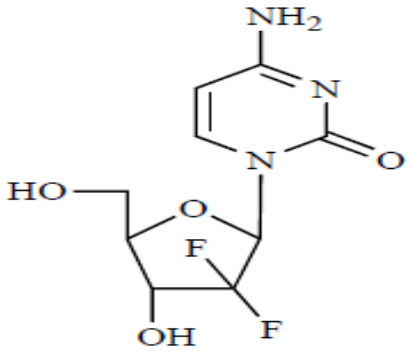


Figure 1: Chemical structure of gemcitabine

2.3.1 Uptake and Metabolism

Gemcitabine is a novel deoxycytidine analogue; a pyrimidine antimetabolite related to cytarabine, having miscellaneous modes of action in the cell (Karak & Flechon, 2007). Figure 2 shows the basics of gemcitabine metabolism. As a prodrug, gemcitabine must undergo a process of metabolism to become an active triphosphate form, dFdCTP (2',2'-difluoro-2'-deoxycytidine triphosphate). Human nucleoside transporters (hNTs), a family of integral membrane proteins, mediate the cellular uptake of gemcitabine by overcoming the inherent barrier to diffusion imposed by the hydrophilic nature of nucleosides and nucleoside analogues. Equilibrative (hENT) and concentrative (hCNT) nucleoside transporters are two recognized types of hNTs; being differentiated in terms of the mechanisms of transport (S. Jennifer et al., 2004). Among the four hENTs (hENT1, hENT2, hENT3, and hENT4) and three hCNTs (hCNT1, hCNT2, and hCNT3) which have been identified, hENT1 was demonstrated to be mainly acting to help mediate the gemcitabine uptake. Besides, hENT2, hCNT1 and hCNT3 play same roles but to a lesser extent (Sousa Cavalcante & Monteiro, 2014).

Once inside the cell, gemcitabine is first phosphorylated to monophosphate (dFdCMP) and then further phosphorylated to gemcitabine diphosphate (dFdCDP) by deoxycytidine kinase (dCK) and pyrimidine nucleoside monophosphate kinase (UMP-CMP) respectively, in the cytoplasm (An, Magnus, & Anna, 1999). The enzyme that is responsible for the final phosphorylation step (dFdCDP into the active metabolited FdCTP) remains unclear. The first phosphorylation by dCK is thought of as a rate-limiting step for dFdCDP and dFdCTP production (Sousa Cavalcante & Monteiro, 2014).

When gemcitabine is present in monophosphate form mediated by deoxycytidylate deaminase (dCTD), it may become inactivated via cytidine deaminase (CDA) mediated deamination. 2',2'-difluoro-2'-deoxyuridine (dFdU), the product of gemcitabine deamination by CDA, plays some intracellular roles which include the regulation of transport, accumulation and cytotoxicity of gemcitabine, as well as being cytotoxic itself

(Hodge, Taub, & Tracy, 2011). Besides, dFdUMP the deaminated product from dFdCMP, could inhibit thymidylate synthase activity, directly influencing the deoxynucleotide triphosphate (dNTP) pool (Bergman et al., 2000).

Gemcitabine can also become inactivated by 5'-nucleotidases (5'-NTs) which can catalyse the conversion of nucleotides back to nucleosides to oppose the nucleoside kinase action (Aksoy et al., 2009; Hunsucker, Mitchell, & Spychala, 2005). All the enzymes play a critical role in gemcitabine metabolism. By these means, the rate-limiting step of phosphorylation by dCK may be affected and the overall beneficial cytotoxicity of gemcitabine can also be compromised (Sousa Cavalcante & Monteiro, 2014).

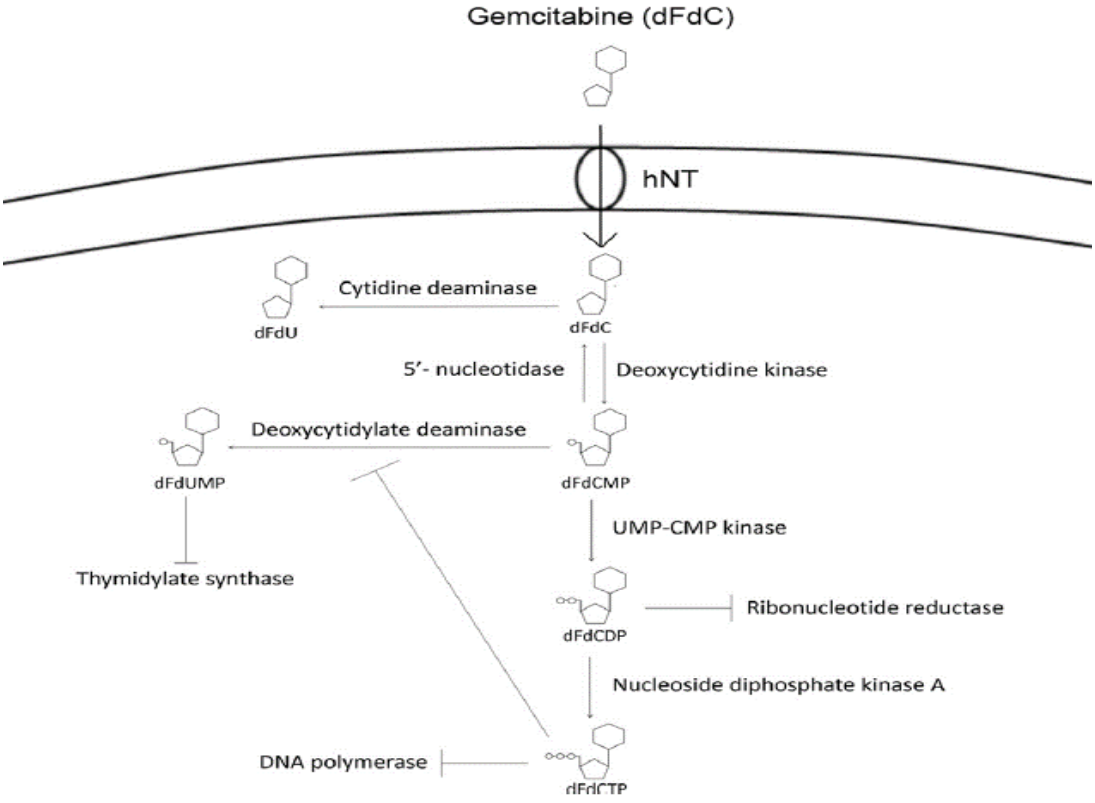


Figure 2: Gemcitabine cellular metabolism

hNT: human nucleoside transporter; dFdCMP: gemcitabine monophosphate; dFdCDP: gemcitabine diphosphate; dFdCTP: gemcitabine triphosphate; dFdU: 2',2'-difluoro-2'-deoxyuridine, dFdUMP: 2',2'-difluoro-2'-deoxyuridine monophosphate

2.3.2 Mechanism of Action

The major mechanism of action of gemcitabine is attributed to inhibition of DNA synthesis (Sousa Cavalcante & Monteiro, 2014). A single deoxynucleotide will be incorporated after the dFdCTP is incorporated into DNA. The incorporation of one more nucleotide prevents the chain elongation and leads to termination of DNA polymerization and single strand breakage (Plunkett et al., 1997). Besides, the extra nucleotide may keep the dEdCTP from DNA repair enzymes (Mini, Landini, & Mazzei, 2006).

Another important mechanism of gemcitabine action is self-potentiation by means of inhibitory enzymes connected with deoxynucleotide metabolism. The self-potentiation of gemcitabine action is the result of the inhibition of different enzymes by several gemcitabine metabolites (Mini et al., 2006). dCTD is one of the enzymes related to deoxynucleotide metabolism, which is inhibited directly by dFdCTP. Meanwhile, dFdCDP is able to inhibit dCTD indirectly owing to the reduction of the intracellular dNTP pool (Sousa Cavalcante & Monteiro, 2014). Ribonucleotide reductase (RR) is a catalyst in the reduction of ribonucleotides to deoxyribonucleotides, and it is inhibited by dFdCDP via formation of a covalent bond with the active site (H. Xu, Racca, & Dealwis, 2006). RR inhibition leads to the decrease in the dNTP pool as a result of lowering of dCTD activity (Mini et al., 2006). Furthermore, since dCTP is a potent feedback inhibitor of dCK, RR inhibition gives rise to the dCTP depletion which leads to a more efficient phosphorylation of gemcitabine, and subsequently making dFdCTP more likely to be incorporated into DNA (Dasanu, 2008; Sousa Cavalcante & Monteiro, 2014).

The third important mechanism of gemcitabine action is induction of apoptosis by caspase signalling (Habiro et al., 2004; Ferrandina et al., 2010). It has been illustrated that p38 mitogen-activated protein kinase (p38-MAPK) can be activated by gemcitabine to trigger apoptosis in response to cellular stress in tumour cells, but not in normal cells (Habiro et al., 2004). MARK-activated protein kinase (MK2) is an effector of p38-MAPK, whose activity was studied to be an indispensable part of gemcitabine-induced cell death

in vitro (Sousa Cavalcante & Monteiro, 2014). MK2 was suggested to be regulator in the cell cycle (Manke et al., 2005; Reinhardt et al., 2010). Some studies showed that gemcitabine resistance has a correlation with the heat shock protein 27 (Hsp27) expression levels and phosphorylation by employing the proteomic and Hsp27 inhibition approaches (siRNA and co-treatment with IFN-γ) (Kuramitsu et al., 2012; M. Nakashima et al., 2011). However, a study stated that gemcitabine is able to induce anti-proliferative effects of phosphorylated Hsp27 (H. Nakashima et al., 2006). Further research is needed to demonstrate the exact role of Hsp27. But an established fact is that caspase activation has an important role in gemcitabine action.

2.3.3 Gemcitabine Chemo-resistance and Sensitivity

Gemcitabine is included in a chemotherapy regimen which is the current standard of care for patients with advanced pancreatic cancer. However, the modest benefit of this treatment observed is an increase of only 5 weeks survival (Z. Wang, Ahmad, & Banerjee, 2011). Chemoresistance is a primary cause for not responding well to gemcitabine based chemotherapy for pancreatic adenocarcinoma. The resistance to gemcitabine is either intrinsic or acquired resistance (Andersson et al., 2009; Z. Wang et al., 2011).

The tumour environment is thought to be an important aspect of gemcitabine chemoresistance in pancreatic cancer. The main characteristics of pancreatic malignancy are deficient vascularization and dense stroma making it hard for drugs to penetrate inside. Pancreatic cancer is a good place for an extensive desmoplastic reaction, owing to which the drug delivery is poor and intrinsic chemo-resistance happens (Neesse et al., 2011). Hedgehog (Hh) signalling is able to promote tumourigenesis and pancreatic cancer desmoplasia, and thus, the extracellular matrix composition of stromal cells is modified (P & Magliano, 2003; M. B. Jennifer et al., 2008). Hh signalling plays an important role in pancreatic cancer, which is found among the most commonly altered pathways in this malignancy (Sian et al., 2008). It has been shown that synergistic effects can be seen in both pancreatic cancer cell lines and mouse xenografts, when using gemcitabine with

cyclopamine which is an inhibitor of Hh pathway (Bahra et al., 2012; M. Xu et al., 2013). These studies imply that combining Hh pathway inhibitors with gemcitabine is a promising therapy to overcome chemo-resistance.

Gemcitabine transporting is an important step for its activity. In other words, nucleoside transporters play a key role in sensitivity and resistance to gemcitabine. Genetic factors are closely related to intrinsic resistance to nucleoside analogs, including gemcitabine (Damaraju et al., 2003; J. Zhang et al., 2007). hENT1 is a main transporter of gemcitabine. Cells with insufficient hENT1 show high levels of gemcitabine resistance Levels of this protein have a positive correlation with the survival of pancreatic cancer patients (Giovannetti et al., 2006). But it is not sufficient to claim that hENT1 expression alone is linked to the acquired or intrinsic gemcitabine resistance (Nakano et al., 2007).

As a prodrug, gemcitabine needs to be phosphorylated by deoxycytidine kinase (dCK) so efficacy of gemcitabine is also closely related to dCK activity. Higher levels of dCK reflect the longer overall survival of pancreatic cancer patients (Valeria et al., 2006). Moreover, Human antigen (HuR), an RNA-binding protein, also affects gemcitabine efficacy. High levels of cytoplasmic HuR reveal the increasing expression of dCK (Costantino et al., 2009). Inhibiting the deamination of gemcitabine by CDA is another reported method to increase its efficacy. Tetrahydrouridine is an inhibitor of CDA activity, which was used to treat various cancer cell lines and a significant increase of gemcitabine cytotoxicity was observed (Eda et al., 1998). Besides, dFdCMP dephosphorylation might be another mechanism that is associated with gemcitabine sensitivity (Sousa Cavalcante & Monteiro, 2014).

As for the molecular targets linked with gemcitabine chemo-resistance, numerous studies have proven the important role of RR. The RR holoenzyme has two subunits, RRM1 and RRM2. Either of the two subunits shows positive relation with gemcitabine resistance. High levels of RRM1 promote the drug resistance in several cancer cell lines (Davidson et al., 2004; Eijk et al., 2005; Nakano et al., 2007; Ohtaka et al., 2008). On the

contrary, gemcitabine efficacy was found to be higher in patients having recurrent tumours with low RRM1 mRNA expression (Nakahira et al., 2007). And previous reports have shown that the median survival duration for lung cancer patients with low RRM1 mRNA expression was found to be longer than in those patients with a high expression of this gene (Rosell et al., 2003; Rafael et al., 2004). Analogously, RRM2 overexpression was correlative with gemcitabine chemo-resistance in pancreatic cancer cells both *in vitro* and *in vivo*, and silencing RRM2 by siRNA enhanced gemcitabine sensitivity (Duxbury, Ashley, & Whang, 2004). It is worth noting that siRNA-mediated silencing of RRM2 inhibited the growth of bladder cancer cell line significantly, without gemcitabine (Morikawa, Homma, & Fukayama, 2010). It can thus be seen that RRM2 silencing is a good target of anti-cancer drug.

Furthermore, gemcitabine resistance is also closely related to the activity of various transcription factors (Sousa Cavalcante & Monteiro, 2014). The over-expression of high mobility group A1 (HMGA1) proteins is usually observed in human malignant neoplasia. Gemcitabine sensitivity will increase *in vitro* when HMGA1 is inhibited (Maasch et al., 2010; Watanabe et al., 2012). Nuclear factor-κB (NF-κB) is another transcription factor that is associated with gemcitabine resistance (Arlt et al., 2003; Kong et al., 2010; M. K. Kim, Yim, & Kang, 2011). NF-κB could translocate into the cell nucleus freely, bind DNA and initiate transcription to promote the gemcitabine chemo-resistance, when it is activated (Holcomb et al., 2012). siRNA targeting the p65 subunit of NF-κB, plays a synergistic role in gemcitabine efficacy to inhibit tumour growth in vivo and also promotes apoptosis in different pancreatic cancer cell lines (Kong et al., 2010).

2.3.4 Toxicities

Gemcitabine is a cytotoxic drug, which inevitably has side effects. Gemcitabine is administered by 30-minute intravenous infusion in monotherapy or in combination with other drugs including 5-fluoro-uracil (5-FU), irinotecan, docetaxel, platinum, pemetrexed and others. But most frequently it is combined with cisplatin (O'Reilly & Abou-Alfa, 2007;

Anna et al., 2012). In addition, pharmacokinetic data advised achieving gemcitabine levels by doses of 350mg/m^2 frequently which were found good for optimal activity (Aapro, Martin, & Hatty, 1998). In preclinical and phase I studies the gemcitabine toxicity tended to be schedule dependent (Tonato, Mosconi, & Martin, 1995; Aapro et al., 1998). It is a phase-specific drug, acting mostly during the S phase of the cell cycle (Dasanu, 2008; Anna et al., 2012). Hence, the toxicities of gemcitabine are manifested mainly in actively replicating normal tissues such as bone marrow and gastrointestinal tract. The damage of bone marrow by using gemcitabine is manifested by neutropenia, thrombocytopenia and anemia. These symptoms may appear at 8 to 10 days after gemcitabine treatment (Dasanu, 2008). When gemcitabine is combined with 5-FU, docetaxel, platinum or other drugs, myelosuppression is more serious. Neutropenic fever is infrequent when gemcitabine is used alone, but the risk increases substantially when it is combined with platinum salts (Aapro et al., 1998). Besides, thrombocytosis is a very rare undesirable effect but can be observed in patients with pancreatic cancer treated with gemcitabine in clinic (Anna et al., 2012).

Other common side effects include mildly increased hepatic transaminases, hepatotoxicity, nephrotoxicity, constipation, lethargy, fever, oedema, stomatitis, balding, nausea, dyspnoea, vomiting, shank oedema, cutaneous hyperpigmentation, infusion-related maculopapular rash and radiation recall dermatitis (Dasanu, 2008).

In conclusion, the gemcitabine resistance leads to the low response rates in tumours. Although gemcitabine has acquired a great application in the treatment of several solid tumours, especially pancreatic cancer, the drug is toxic to many healthy cells and results in unpredictable severe toxic effects. Meanwhile, the inherent biological characteristics of pancreatic cancer and the blood pancreatic barrier in human give the unsatisfactory therapeutic effects of chemotherapy (Zorofchian Moghadamtousi et al., 2014). It is crucial to improve gemcitabine treatment efficacy and reduce its drug toxicity, or to develop a new anticancer drug with a high inhibition effect and minor toxic side effects

to improve clinic efficiency. With decreasing land resources, marine organisms are the new and ideal drug resource. Hence, it will be a giant leap to discover new natural products that are isolated from marine organisms. The features of high anticancer efficacy, with mild or no toxicity to normal cells, is the primary objective in cancer research.

2.4 Fucoxanthin

More than 70% of the earth's surface is covered by oceans and the oceans possess abundant resources. In recent years, a considerable number of drug candidates have been yielded by marine natural product *bioprospecting* (Haefner, 2003). Now, more emphasis is put on looking for potential anticancer drugs from the marine resources. Among the natural products identified from marine algae, fucoxanthin received particular interest (Gammone & D'Orazio, 2015). Fucoxanthin was first isolated by Willstätter and Page from the marine brown seaweeds *Fucus*, *Dictyota*, and *Laminaria* in 1914 (Peng et al., 2011). It is one of the most abundant carotenoids, which accounts for more than 10% of the estimated total production of carotenoids in nature, especially in the marine environment (Peng et al., 2011). It can be present in both macroalgae, such as *Undaria pinnatifida* or *Laminaria japonica*, and the microalgae such as *Phaeodactylum tricornutum* or *Cylindrotheca closterium* (Haefner, 2003).

2.4.1 Structure and Mechanism of Fucoxanthin

Carotenoids, a family of natural pigments with at least 600 members, are classified into two categories, carotenes and xanthophylls, on the basis of chemical structure (Ishikawa et al., 2008). Xanthophylls are less chemically hydrophobic because of their oxygen content. Xanthophylls are yellow pigments named after the formation of the yellow bands which are seen in early chromatography of leaf pigments. Xanthophylls and carotenes have similar molecular structure, except for the fact that xanthophylls contain oxygen atoms and carotenes are purely hydrocarbons. Polarity of xanthophylls is higher than those of pure hydrocarbon carotenes. Oxygen atoms which are present as either a

hydroxyl group or as a pair of hydrogen atoms in xanthophylls are substituted by oxygen atoms acting as a bridge in the epoxide (Gammone & D'Orazio, 2015).

Fucoxanthin is a xanthophyll. Besides, lutein, zeaxanthin, neoxanthin, canthaxanthin, violaxanthin, capsorubin, astaxanthin and α- and β-cryptoxanthin are also in the group of xanthophylls (McNulty, 2008). Fucoxanthin has an orange colour, and its chemical structure includes an unusual allenic bond and oxygenic functional groups, such as hydroxyl, epoxy, carbonyl, and carboxyl groups in addition to its polyene chain (Figure 3) (Zorofchian Moghadamtousi et al., 2014).

In recent years, considerable research has been performed concerning the absorption and metabolism of fucoxanthin in animals. Dietary fucoxanthin was first converted to fucoxanthinol and then metabolized to amarouciaxanthi A in mice (Sugawara, Tsuzuki, & Nagao, 2002; Sangeetha, Divakar, & Baskaran, 2010). The same metabolic conversion was also observed in the human hepatoma cell HepG2, requiring $NAD(P)^+$ as a cofactor (Das, Hashimoto, & Kanazawa, 2008). Digestive enzymes such as lipase and cholesterol esterase hydrolyse the fucoxanthin in the gastrointestinal tract to fucoxanthinol which is then converted to amarouciaxanthin A in the liver (Asai, Yonekura, & Nagao, 2008). Amarouciaxanthin A is stored in abdominal white adipose tissue and fucoxanthinol is stored in other tissues such as such as liver, lungs, kidney, heart, and spleen (Peng et al., 2011); both of which are supposed to be capable of carrying out their physiological functions in the body (Hashimoto et al., 2009). Fucoxanthinol, which comes from the de-acetylation of dietary fucoxanthin, enters the systemic bloodstream through the lymphatic duct. Some fucoxanthinol is reduced to amarouciaxanthin A in the liver and the remainder is gathered in the tissues. Hence, appropriate and safe usage of dietary fucoxanthin is essential for the best bioavailability of these metabolites (Matsumoto et al., 2010). Additionally, halocynthiaxanthin (Figure 3) is another metabolite of fucoxanthinol found in marine animals such as oysters and clams (Nishino et al., 1992).

However, very little data has been obtained about pharmacokinetics of fucoxanthin

and its metabolites in human subjects. Fucoxanthinol was reported to be detectable in human plasma after the daily intake of wakame and its bioavailability and metabolism is higher in humans than in mice (Hashimoto et al., 2012). Smaller, short-life animals were studied to eliminate drugs from their bodies faster than larger, longer-living animals. For example mice have the fastest elimination speed and human subjects have the least rapid among the species compared, due to the difference of pharmacokinetic profile in different species (Mordenti, 1986).

The composition of food matrix affects the absorption rate of fucoxanthin. Fucoxanthin has high solubility in medium-chain triacylglycerols or in fish oil (Maeda, Hosokawa, Sashima, & Miyashita, 2007). The absorption rate of fucoxanthin was found to be increased when dietary fucoxanthin is combined with edible oil or lipid in both KK-Ay mice and obese premenopausal women (Maeda, Funayama, & Miyashita, 2007; Maeda, Sashima, & Miyashita, 2007; Abidov, Seifulla, & Grachev, 2010).

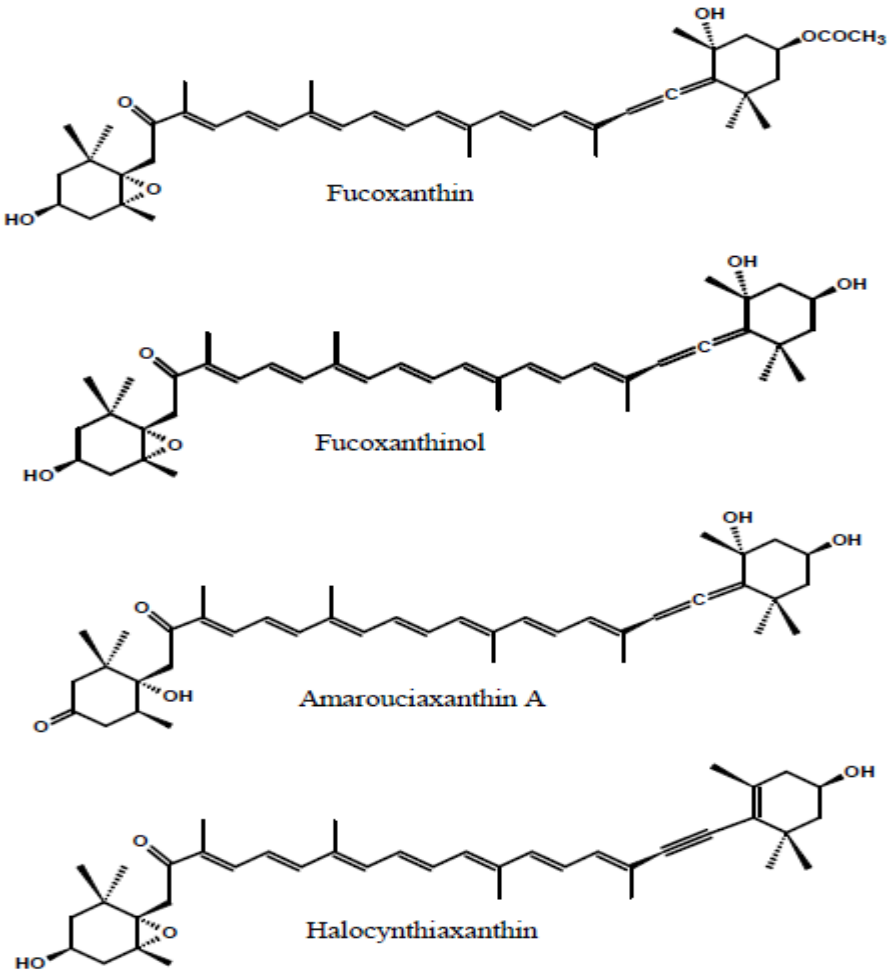


Figure 3: Chemical structure of fucoxanthin and its metabolites

2.4.2 Safety of Fucoxanthin

There was no apparent toxic performance in both mice and rats through intraperitoneal and oral administration of fucoxanthin extracted from *Fucus Vesiculosus*, even at the dose of 750 mg/kg daily for 4 weeks (Zaragozá et al., 2008). Researchers made a single (1000 and 2000 mg/kg) and repeated oral (500 and 1000 mg/kg for 30 days) dose toxicity study of purified fucoxanthin (93% purity) in ICR mice. According to the results, no mortality and no abnormalities in gross appearance were found in both studies. In the histological observations of the repeated doses study, fucoxanthin did not induce any abnormal changes of liver, kidney, spleen or gonadal tissues (Beppu, Hosokawa, & Miyashita, 2009). Some research was conducted to study about the sub-chronic toxicity

of fucoxanthin in rats and about genotoxicity in mice. There was no mortality and no change observed in the single oral dose study and the 50% lethal dose of fucoxanthin was over 2000 mg/kg body weight. The no-observed-adverse-effect level of fucoxanthin was 200 mg/kg body weight in the 13-week oral dose study, conducted in the tested sub-chronic dose condition (Iio, Okada, & Ishikura, 2011; Okada & Ishikura, 2011). Moreover, fucoxanthin was proven to have no genotoxicity to mice bone marrow cells and was non-toxic to human lymphocyte cells (Beppu, Niwano, Sato, et al., 2009). Additionally, fucoxanthinol, the metabolite of fucoxanthin, did not show any significant adverse effect *in vivo* (H. Zhang et al., 2015). Based on these studies, it was suggested that fucoxanthin was a safe compound, which was non-toxic and non-mutagenic under these experimental conditions.

Some carotenoids like canthaxanthin and astaxanthin may display the feature of increasing circulating cholesterol level in rodents (Murillo, 1992). Fucoxanthin was not only able to significantly increase plasma high-density lipoprotein: cholesterol levels (Beppu, Niwano, Tsukui, et al., 2009; Woo et al., 2010), but also remarkably improved the total cholesterol level in the blood of mice and rats by repeated daily intake (Beppu, Niwano, Tsukui, et al., 2009; Kumar, Hosokawa, & Miyashita, 2013). In short, it needs more studies to further assess the safety of fucoxanthin. The mechanisms, whereby fucoxanthin induces hypercholesterolemia and the fact that different results are seen in different species also require to be clarified.

2.4.3 Anti-Carcinogenic Effects of Fucoxanthin

Owing to the special molecular structure, fucoxanthin has remarkable biological properties including antioxidant, anti-inflammatory, anticancer, anti-obese, antidiabetic, antiangiogenic, and antimalarial activities and so on. Among all the biological activities, the anticancer activity of fucoxanthin attracts lots of attention. It has been found to play an inhibitory role by affecting the cellular processes in many kinds of cancer cells in dozens of studies. The potential of fucoxanthin has led scientists to study its molecular

mechanism(s) and estimate its worth in the treatment of cancer.

2.4.3.1 Decreased Incidence of Tumours

Fucoxanthin was supposed to have chemo-preventive effects. It was reported to completely suppress skin tumour formation and ENNG induced mouse duodenal carcinogenesis. The percentage of tumour-bearing mice in the fucoxanthin-treated group was significantly lower than the control group and fucoxanthin evidently decreased the mean number of tumours per mouse (Nishino, 1995). The same result can be seen in the study of Okuzumi and co-workers (J. Okuzumi et al., 1990; Junichi Okuzumi et al., 1993). Fucoxanthin was able to reduce the formation of aberrant crypt foci (ACF) induced by 1, 2-dimethylhydrazine dihydrochloride (DMH) in B6C3F_1 mice (J. M. Kim et al., 1998). Fucoxanthinol, the deacetylated metabolite of fucoxanthin, did not affect the incidence of combined immunodeficiency harbouring tumours induced by immunization of human T-cell leukemia virus type 1 (HTLV-1) infected T cells in mice, but distinctly decelerated the development of the transplanted tumours without any adverse events (Ishikawa et al., 2008). The anti-tumour effect of fucoxanthin can also be observed in melanoma tumour in vivo. The weight of melanoma tumour mass in Balb/c mice was distinctly lessened (5-fold reduction) with the application of fucoxanthin, by delaying the formation of tumour mass, compared with the B16F10 cells-injected mice group (K.-N. Kim et al., 2013). Fucoxanthin produced a significant drop in S180 sarcoma weight in mice in a dose-dependent manner and the induction of apoptosis in vivo is linked with down-regulating STAT3/EGFR signalling in S180 xenografts-bearing mice (J. Wang et al., 2012).

2.4.3.2 Anti-Proliferation of Cells

Fucoxanthin was found to have an inhibitory effect on various cancer cells such as GOTO, HL-60, Caco-2, HepG-2, Neuro2a, DU145, PEL, PC-3, HeLa, H1299, HT-29, and DLD-1 cells (Kumar et al., 2013). Fucoxanthin was first suggested to reduce the proliferation of 63% neuroblastoma (GOTO) cells at the concentration of 10 μg/mL, after

three days drug treatment. The flowcytometric analysis showed that fucoxanthin could arrest the cells at G_0-G_1 phase (J. Okuzumi et al., 1990). Fucoxanthin could significantly inhibit the proliferation of colon cancer cell lines including Caco-2, HT-29 and DLD-1. The mechanism proposed was that the fucoxanthin was able to induce the breakdown of colon cancer cellular DNA and lead to cellular apoptosis and inhibit the expression of Bcl-2 protein (Hosokawa et al., 2004). Swadesh found that fucoxanthin suppressed the growth of human colon cancer cell line WiDr in a dose-dependent manner along with cell cycle blocking in the G_0-G_1 phase. This was because of the fact that fucoxanthin boosted production of a cyclin-dependent kinase (cdk) inhibitory protein p21WAF1/Cip1 which was able to suppress the phosphorylation of retinoblastoma protein (pRb) site (Das et al., 2005). Fucoxanthin was proven to have the strongest effect to suppress the viability of prostate cancer lines especially PC-3. The 5, 6-monoepoxide in its molecule was considered to be associated with the bioactivity. Fucoxanthinol, the metabolite of fucoxanthin showed the same effect and can activate the caspase-3 in PC-3 cells to induce apoptosis (K.-N. Kim et al., 2013). Moreover, fucoxanthin was proposed to inhibit the proliferation of a hepatoma cell line SK-Hep-1 cells. Researchers discovered that fucoxanthin could strengthen the gap junctional intercellular communication (GJIC) of SK-Hep-1 cells and enhance the expressions of relevant protein, mRNA and calcium content in SK-Hep-1 cells, which were related to the cell cycle arrest at G_0-G_1 phase and cell apoptosis (Liu, Miyashita, & Hu, 2009). Fucoxanthin and fucoxanthinol inhibited the viability of primary effusion lymphoma (PEL) cell lines BCBL-1 and TY-1 cell in a dose-dependent manner and the inhibition effect of fucoxanthinol was more pronounced. They induced cell cycle arrest during G_1 phase and activated caspase-dependent apoptosis, and silenced nuclear factor-κB (NF-κB), activator protein 1 (AP-1) and Akt activation. Besides, they were conjunct with down-regulation of anti-apoptotic proteins and cell cycle regulators (Yamamoto et al., 2011).

The cytotoxic activity of fucoxanthin is selective (Januar et al., 2012). Fucoxanthin

is a potential natural substance to be developed as a pharmaceutical anticancer agent. However, there is still not an established primary mechanism of action of fucoxanthin; though various mechanistic studies were done by researchers. More studies should be carried out. Moreover, not enough research has been done regarding the anti-cancer effects of fucoxanthin on pancreatic cancer cells. Therefore, conducting research on the inhibitory effect of fucoxanthin on pancreatic cancer cells is of great significance, and will enhance the body of knowledge in this area of cancer research.

Chapter 3 Methodology

3.1 Cell Lines Used in this Study

Table 4: Cell line information

Cell Line Designation	Catalogue Number	Cell Line Description	Supplier
MIA PaCa-2	CRL-1420	Pancreas Carcinoma; Human	ATCC
PANC-1	CRL-1469	Epithelioid Carcinoma; Human/Pancreas/Duct	ATCC
HEK 293	CRL-1573	Embryonic Kidney; Human	ATCC

All three cell lines (Table 4) were stored at -80 °C freezer or in liquid nitrogen. After thawing the cell lines, they were maintained in 25 or 75 cm^2 tissue culture flasks containing 5 mL or 15 mL of completed growth culture medium in 37 °C incubator with 5% carbon dioxide humidified air.

3.2 Cytotoxicity Assay

To study the cytotoxicity of gemcitabine and fucoxanthin, cell viability of cell lines incubated in various concentrations of drugs for a certain period of time was determined by using the methylthiazol-diphenyl-tetrazolium (MTT) assay, an indirect method to measure cell growth. The MTT assay is a colorimetric assay. This method is able to measure the decrease of yellow MTT by mitochondrial succinate dehydrogenase. When MTT enters the cells and passes into the mitochondria, it will be reduced to an insoluble and dark purple formazan product. Using an organic solvent such as Dimethyl sulfoxide (DMSO) to solubilise the cells, the released and solubilised formazan reagent can be measured spectrophotometrically. Since the reduction of MTT can only occur in metabolically active cells, this method can indirectly reflect the viability of cells (Hansen,

Nielsen, & Berg, 1989).

3.2.1 Cell Culture and Cell Viability Assay Materials & Reagents

Table 5: Main materials in cell culture and cell viability assay

Number	Material	Supplier
1	Cell culture medium (RPMI 1640, no phenol red)	Life technologies
2	L-glutamine (200 mM; 100 mL)	Life technologies
3	Penicillin-Streptomycin (10,000 U/mL; 100 mL)	Life technologies
4	Trypan Blue Solution, 0.4%	Life technologies
5	TrypLE™ Express Enzyme (1X), no phenol red	Life technologies
6	PBS (Phosphate buffered saline), pH 7.2, no calcium magnesium and phenol red	Life technologies
7	Sterile filtered fetal bovine serum (FBS)	Medica Pacifica (Auckland, NZ)
8	MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (Cat No. M2128-1G)	Sigma- Aldrich (St Louis, USA)
9	DMSO (Dimethyl sulfoxide) (Cat No. 102952)	Merck-Chemicals
10	25 and 75 cm^2 cell culture flasks, 1, 5, 10 and 25 mL sterile disposable pipette tips, 15 and 50 mL centrifuge tubes, 96-well tissue culture plates, etc.	BD (Becton Dickinson) Bioscience (Auckland, NZ)

3.2.2 Preparation of Complete Growth Culture Medium

MIA PaCa-2, PANC-1 and HEK 293 were cultured in RPMI 1640 base medium with 1% Penicillin-Streptomycin, 1% L-glutamine and 10% fetal bovine serum.

3.2.3 Preparation of MTT Stock Solution

12 mM (5 mg/mL in PBS) of MTT solution was prepared by weighting 5 mg MTT powder dissolved with 1mL phosphate buffered saline (PBS). The powder was fully

dissolved by vortexing, and the 12 mM MTT phosphate buffered saline was filtered through a sterile Millex GV 0.22 μm syringe filter to remove any pathogens, undissolved MTT and any spontaneously formed formazan crystals. Once prepared, the MTT solution was stored at 4°C in the dark or at -20°C for long term storage.

3.2.4 Preparation of Fucoxanthin Stock Solution

Fucoxanthin used in this study was bought from Sigma-Aldrich (CAS Number 3351-86-8). The purity of the fucoxanthin was ≥ 95% as determined by HPLC. Fucoxanthin was dissolved in absolute ethanol to a final concentration of 5 mM as stock solution. Aliquots of fucoxanthin stock solution were separated in micro-tubes. The micro-tubes were wrapped up in aluminium foil and stored in the -80^{o}C freezer.

3.2.5 Preparation of Gemcitabine Stock Solution

Gemcitabine used in this study was purchased from Sigma-Aldrich (CAS Number 122111-03-9). The purity of the gemcitabine was ≥98% as determined by HPLC. Gemcitabine was dissolved in complete cell culture medium to a final concentration of 10 mM as stock solution. Aliquots of gemcitabine stock solution were separated in micro-tubes. The micro-tubes were wrapped in aluminium foil and stored in the -80^{o}C freezer.

3.2.6 Cell Culture Protocols

3.2.6.1 Thawing Frozen Cells

The cryovial containing frozen cells was removed from liquid nitrogen storage and immediately place it in a 37°C water bath. The cells were quickly thawed (< 1 minute) by gently swirling the vial in the 37°C water bath until there was just a small bit of ice left in the vial. Before opening, the outside of the vial was wiped with 70% ethanol. The desired amount of pre-warmed complete growth medium appropriate for each cell line was transferred dropwise into the centrifuge tube containing the thawed cells. Then the cell suspension was centrifuged. After the centrifugation, the clarity of supernatant and visibility of a complete pellet was checked. Then the supernatant was aseptically decant

without disturbing the cell pellet. At last the cells was gently re-suspended in complete growth medium, and then transferred into the 25 cm^2 tissue culture flask and kept in a 37°C, 5% CO_2, humidified incubator.

3.2.6.2 Changing Medium

If cells have been growing well for a few days but are not yet confluent then they required a medium change to replenish nutrients and maintain the pH correct. For medium changing, the spent cell culture medium was removed and discarded from the culture flask. PBS without calcium and magnesium (approximately 2 mL per 10 cm^2 culture surface area) was used to wash cells. Then the wash solution was removed and discarded. Finally, fresh pre-warmed culture medium (approximately 2 mL per 10 cm^2 culture surface area) was added and cells were returned to a 37°C incubator.

3.2.6.3 Passaging Adherent Cells

Cells were passaged when they were 70~80% confluent. The cells were washed first (the same step as section 3.2.5.2). The pre-warmed dissociation reagent TrypLE™ Express Enzyme was added to the side of the flask (approximately 0.5 mL per 10 cm^2). The culture flask was incubated in incubator for approximately 2 minutes (the actual incubation time varies with the cell line used). When more than 90% of the cells were detached, the equivalent of 2 volumes (twice the volume used for the dissociation reagent) of pre-warmed complete growth medium was added. Then the cells were transferred to a 15mL centrifuge tube and centrifuged for 5 to 10 minutes (the centrifuge speed and time vary based on the cell type). Re-suspend the cell pellet in 1mL pre-warmed complete growth medium and remove a sample for counting. An appropriate volume of cells were pipetted into a new cell culture flask, and return to the incubator. Note that most cells must not be split more than 1:10 as the seeding density would be too low for the cells to survive. As a general guide, from a confluent flask of cells:

1:2 split should be 70~80% confluent and ready for an experiment in 1 to 2 days；

1:5 split should be 70~80% confluent and ready for an experiment in 2 to 4 days；

1:10 split should be 70~80% confluent and ready for sub-culturing or plating in 4 to 6 days.

3.2.6.4 Freezing Cells

Freezing medium was prepared and stored at 2° to 8°C until use (The appropriate freezing medium depended on the cell line). The freezing medium should contain a cryoprotective agent such as DMSO or glycerol. For adherent cells, cells were gently detached from the tissue culture vessel (following the procedure used during the subculture). The cells were then re-suspended in complete medium and later determined the total number of cells. According to the desired viable cell density, the required volume of freezing medium was calculated. After centrifugation and decanting the supernatant, the cell pellet was re-suspended in cold freezing medium at the recommended viable cell density for the specific cell type. Aliquots of the cell suspension were dispensed into cryogenic storage vials. The cryovials containing the cells was placed in an isopropanol chamber and stored at -80°C overnight. At last the frozen cells were transferred to liquid nitrogen, and stored in the gas phase above the liquid nitrogen.

3.2.7 MTT cell proliferation assay protocols

The MTT assay used in this study was in light of the protocol first described by Mosmann (Mosmann, 1983).

3.2.7.1 Major Equipment and Materials Applied

Table 6: Major equipments and materials applied in MTT assay

Number	Equipment or Materials
1	Microtiter plate reader with 540 and 680 nm filters
2	Multi-functional orbital shaker
3	Inverted microscope
4	Multi-channel pipette
5	Haemocytometer
6	Centrifuge

3.2.7.2 Basic Steps for MTT Cell Proliferation Assay

Table 7: Basic steps for MTT assay

Step	Action
1	Basic cell culture and detaching cells from culture flask.
2	Determining the cell concentration.
3	Seeding cells on 96-well plates, 100 μL each well.
4	Adding treatments, 100 μL each well.
5	Replacing the original medium with 100 μL of fresh culture medium.
6	Adding MTT stock solution and incubating.
7	Adding DMSO and recording absorbance.

Step 1: Cell preparation

PBS and TrypLE™ Express Enzyme were used to wash and detach cells. The cells were collected in 15mL centrifuge tube to centrifuge (the same step as in section 3.2.6.3). After centrifugation, the supernatant was carefully removed and 1 mL of new completed culture medium was added into the tube to re-suspend cells gently but thoroughly.

Step 2: Cell counting

10 μL of the cell suspension was removed onto a piece of parafilm and mixed thoroughly with 10 μL of Trypan Blue. 10 μL of this mixture was placed to one side of the hemocytometer and the number of cells was determined under the microscope. The suspension was diluted enough so that the cells do not overlap each other on the grid, and was uniformly distributed on the hemocytometer. The total cell number counted (at least four squares) was recorded. The total number of cell in 1 mL culture medium was calculated by using the formula below:

$$\text{Total cells/mL} = \frac{\text{Total cells counted}}{\text{Number of squares}} \times \text{dilution factor} \times 10{,}000\text{cells/mL}$$

Here the sample was diluted 1:1 with Trypan blue, so the dilution factor is 2.

Step 3: Seeding cells

The cell density for all the cell lines in this study was 50,000 cells/mL. Based on this concentration, complete culture medium was applied to dilute the cell stock solution. After dilution, 100 μL of cells was seeded onto each well of 96-well plate. A column of wells had blank wells containing the medium only. The plates were kept in the incubator for 6 to 24 hours.

Step 4: Adding treatment

After incubation, all the cells attached to the wall of the wells. Different concentrations of drugs were prepared with pre-warmed complete culture medium before adding to each well. 100 μL of fresh complete culture medium containing various densities of drugs was added slowly to corresponding wells. A column of wells were set as a negative control group containing cells with no treatment. For the Day 0 plate, no treatment was added into the wells. The plates were kept in the incubator.

Step 5: Changing medium (Optional step)

After incubation for a set time (normally 0, 24, 48 and 72 hours), the old medium containing treatment was carefully removed and replaced with 100 μL of fresh complete

culture medium. Doing this step depends on the treatment. If the treatment was known to impact MTT assay results, then the treatment was removed before adding MTT solution.

Step 6: Adding MTT stock solution

An aliquot of 10 μL of MTT stock solution was added to each well and the plates were placed back in the 37°C incubator. After incubation for 2~4 hours, purple precipitate was clearly visible under an inverted microscope. The supernatant was gently removed from the wells.

Step 7: Quantification

An aliquot of 150 μL of DMSO was added to each well and mixed thoroughly using an orbit plate shaker. After incubating at 37°C for 20 to 30 minutes, the plate was shaken briefly and absorbance was measured by a plate reader (FLUOstar Omega, Alphatech) at the wavelength of 540 nm, with the reference wavelength at 680nm. The average absorbance value (OD value) was determined from sextuplicate readings, and the average values were subtracted from the average value from the blank readings in order to yield the final value.

3.2.8 Determination of MTT Assay Linearity Range

Suitable cell density and culture time are important to ensure that a very good linear relationship can be shown between the MTT formazan assay results and cell number. The cell number vs. absorbance standard curve is used to determine the linearity of the MTT assay and the cell number used in cell viability studies should fall within the linear portion of the curve.

The highest cell concentrations set in this study were 250,000 and 500,000 depending on different cell types. Cells were kept in 1.5mL micro-tubes and after a series of one in two (1:2) dilutions by cell culture medium, an aliquot of 100μL well mixed cells in each 1.5mL micro-tubes were seeded in each well of a 96-well plate. Each concentration was repeated six times. After seeding for 18 hours, step 7 (in section 3.2.7.2)

was undertaken to measure absorbance at 540nm. A linear relationship between absorbance value (OD value) and cell number was generated by plotting absorbance on the Y-axis against cell numbers a on the X-axis.

Table 8: 1:2 Dilution plan for making cell linearity standard curve

Number	Cell concentration (Cells/mL)	Cell concentration (Cells/mL)
1	250,000	500,000
2	125,000	250,000
3	62,500	125,000
4	31,250	62,500
5	15,625	31,250
6	7,812	15,625
7	3,906	7,812
8	1,953	3,906
9	976	1,953

3.2.9 Determination of Cells Doubling Time

Cells doubling time (or more accurately, cells doubling time in logarithmic growth period) is the period of time required for cells number to be double. Two methods were used to calculate the doubling time of cells in this study. One was obtained directly through the formula below and the second was calculated from the cell growth curve.

The cell doubling time calculation formula is shown below:

$$T_d = T \times Lg\ 2 / Lg\ (N / N_0)$$

T_d: Doubling time; T: Time interval; N: Final cell number, N_0: Initial cell number

To make a cell growth curve, 200 μL of cells from each well (density 5,000 cells/mL), was seeded onto six 96-well plates. The cell culture time points set were 0, 24, 48, 72, 96 and 120 hours. An MTT assay was conducted every day at the same time. Based on the experimental data, the relationship between absorbance and time was determined. According to the cell number vs. absorbance standard curve gotten previously, cell numbers at different time points were calculated and then the cell growth curve was drawn. Finally, the cell doubling time was calculated by cell growth curve fitting. The results were attached in Appendix A1.

According to the results cells doubling time in logarithmic growth period for MIA Paca-2 is 24.44 hours and for PANC-1 is 28.10 hours. And for HEK293 cells, N_0 is 5×10^4 cells/mL and N is 1.2×10^6 cells/mL after 72 hours. Thus the T_d of HEK293 is 15.70 hours.

3.2.10 Determination of the Inhibition Effect of Fucoxanthin

Step 1：Cell preparation

The cell density chosen in this study for MIA PaCa-2, PANC-1 and HEK 293 was 5, 000 cells/well. After proper dilution, a multi-channel pipette was used to seed the well mixed cells into the 96-well plate. Each well contained 100 μL of cells.

Step 2: Fucoxanthin solution preparation

The concentration of fucoxanthin ethanolic stock solution is 5 mM. All of the diluted treatment was kept in 1.5 mL micro-tubes. Fucoxanthin was diluted with cell culture medium. For different cancer cell line, the fucoxanthin concentration ranges were different. The dilution plans are shown in table 9, 11 and 12.

Step 3: Ethanol solution preparation

Because fucoxanthin was dissolved in ethanol, the effect of ethanol alone on cells

was tested. According to different fucoxanthin dilution plans, the corresponding ethanol dilution plans are shown in table 10 and 13.

Table 9: Fucoxanthin dilution Plan 1 for MIA PaCa-2

Number	Actual FX concentration (μM)	Prepared FX concentration (μM)	Dilution plan (Each 1.5 mL micro-tube has 1000 uL FX and complete culture medium mixture)
1	100	200	40 μL (5 mM FX stock solution) + 960 μL medium
2	80	160	32 μL (5 mM FX stock solution) + 968 μL medium
3	50	100	20 μL (5 mM FX stock solution) + 980 μL medium
4	25	50	10 μL (5 mM FX stock solution) + 990 μL medium
5	12.5	25	5 μL (5 mM FX stock solution) + 995 μL medium
6	6.25	12.5	500 μL (25 μM FX solution) + 500 μL medium
7	1.5625	3.125	125 μL (25 μM FX solution) + 875 μL medium

Table 10: Ethanol dilution plan for MIA PaCa-2

Number	Actual ethanol concentration (%)	Prepared ethanol concentration (%)	Dilution plan (Each 1.5 mL micro-tube has 1000 uL ethanol and complete culture medium mixture)
1	2.0	4	40 µL (100% ethanol) + 960 µL medium
2	1.6	3.2	32 µL (100% ethanol) + 968 µL medium
3	1.0	2	20 µL (100% ethanol) + 980 µL medium
4	0.5	1	10 µL (100% ethanol) + 990 µL medium
5	0.25	0.5	5 µL (100% ethanol) + 995 µL medium
6	0.125	0.25	500 µL (0.5% ethanol) + 500 µL medium
7	0.03125	0.0625	125 µL (0.5% ethanol) + 875 µL medium

Table 11: Fucoxanthin dilution Plan 2 for MIA PaCa-2

Number	Actual FX concentration (μM)	Prepared FX concentration (μM)	Dilution plan (Each 1.5 mL micro-tube has 1000 uL FX and complete culture medium mixture)
1	1	2	400 μL (5 μM FX solution) + 600 μL medium
2	0.5	1	200 μL (5 μM FX solution) + 800 μL medium
3	0.3	0.6	120 μL (5 μM FX solution) + 880 μL medium
4	0.25	0.5	100 μL (5 μM FX solution) + 900 μL medium
5	0.15	0.3	60 μL (5 μM FX solution) + 940 μL medium
6	0.05	0.1	20 μL (5 μM FX solution) + 980 μL medium
7	0.02	0.04	8 μL (5 μM FX solution) + 982 μL medium
5 μM FX solution (1000 μL) preparation			50 μM FX : 10 μL (5 mM FX stock solution) + 990 μL medium 5 μM FX : 100 μL (50 μM FX stock solution) + 900 μL medium

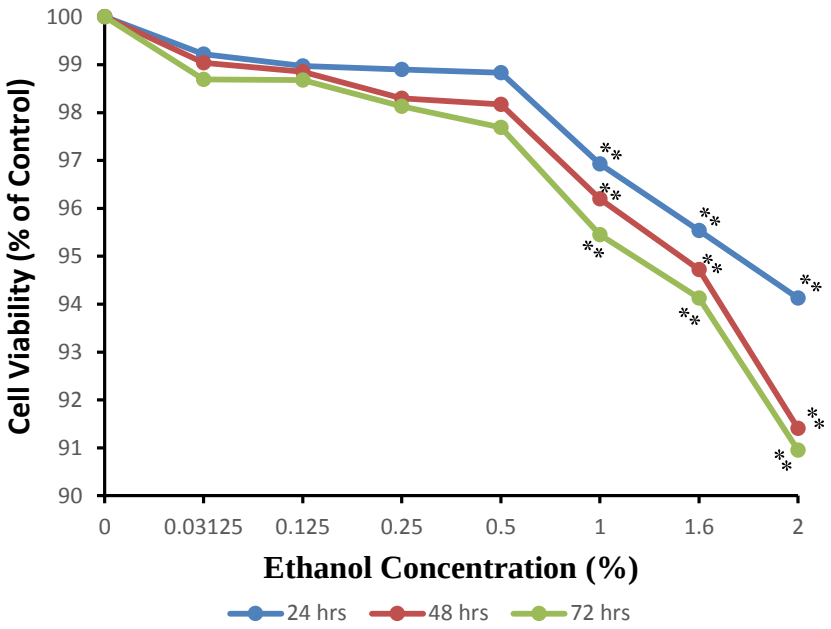


Figure 4: The effect of ethanol on the cell viability of MIA PaCa-2. Data are presented as means ± S.D, n=3. Two asterisks indicated a value significantly different from the control value (cell viability under 0% ethanol) in each day, $^{**}P < 0.01$ (Student's t test).

The result showed that 2%, 1.6% and 1.0% inhibited the growth of MIA PaCa-2 cells after incubating for 24, 48 and 72 hours. Ethanol at the concentration of 0.5% and lower did not significantly affect the growth of MIA PaCa-2 cells. The result was found in PANC-1 cells cultured in ethanol (Figure: Appendix-A1.1.3). Based on this result, no ethanol effect study was carried out in the following experiments, when the fucoxanthin concentration was under 25 µM.

Table 12: Fucoxanthin dilution plan for PANC-1

Number	Actual FX concentration (μM)	Prepared FX concentration (μM)	Dilution plan (Each 1.5 mL micro-tube has 1000 uL FX and complete culture medium mixture)
1	100	200	40 μL (5 mM FX stock solution) + 960 μL medium
2	50	100	20 μL (5 mM FX stock solution) + 980 μL medium
3	20	40	8 μL (5 mM FX stock solution) + 992 μL medium
4	10	20	500 μL (40 μM FX solution) + 500 μL medium
5	5	10	250 μL (40 μM FX solution) + 750 μL medium
6	2	4	100 μL (40 μM FX solution) + 900 μL medium
7	0.5	1	25 μL (40 μM FX solution) + 975 μL medium

Table 13: Ethanol dilution plan for PANC-1

Number	Actual ethanol concentration (%)	Prepared ethanol concentration (%)	Dilution plan (Each 1.5 mL micro-tube has 1000 uL ethanol and complete culture medium mixture)
1	2	4	40 μL (100% ethanol) + 960 μL medium
2	1	2	20 μL (100% ethanol) + 980 μL medium
3	0.4	0.8	8 μL (100% ethanol) + 992 μL medium
4	0.2	0.4	500 μL (0.8% ethanol) + 500 μL medium
5	0.1	0.2	250 μL (0.8% ethanol) + 750 μL medium
6	0.04	0.08	100 μL (0.8% ethanol) + 900 μL medium
7	0.01	0.02	25 μL (0.8% ethanol) + 975 μL medium

Step 4: Adding treatment

Once various fucoxanthin treatments were prepared, 100 μL of each diluted fucoxanthin solution were added to the well immediately. Each fucoxanthin concentration was performed in triplicate. The blank (200 μL cell culture medium only) and control (100 μL cell with 100 μL cell culture medium) groups were set in the same plate. Using MIA PaCa-2 as a sample, the design of the plate is shown in Figure 5. The next steps are the same in section 3.2.7.2 step 5, 6 and 7.

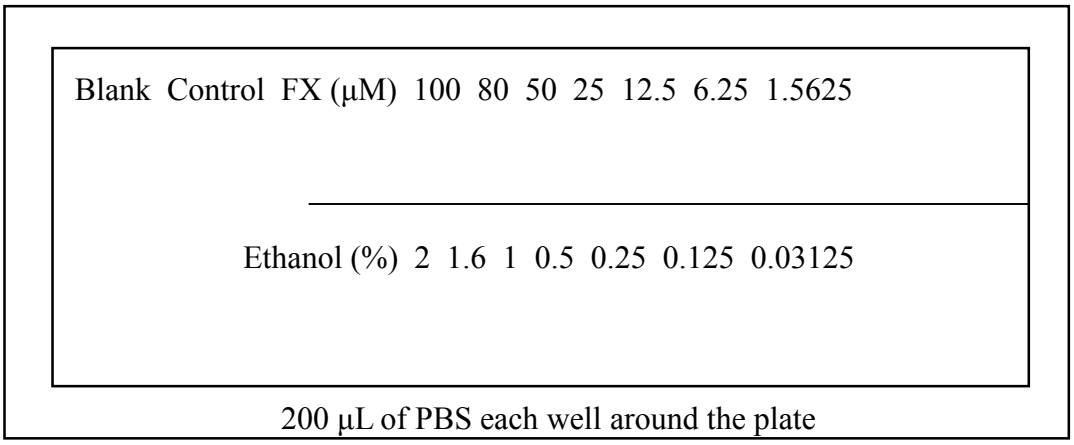


Figure 5: Plate design for MIA PaCa-2 treated with fucoxanthin

3.2.11 Determination of Fucoxanthin's Colour Effect on Absorbance Value (OD Value)

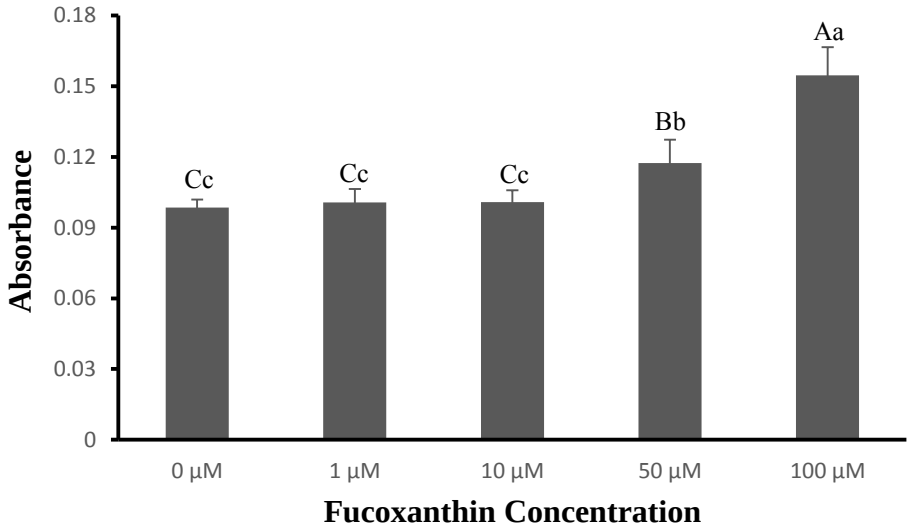
As fucoxanthin is an orange-coloured pigment, its colour may impact the final absorbance value of each well when performing MTT assays. Different concentrations of fucoxanthin solution were prepared to test respective absorbance values. The highest fucoxanthin concentration used in this study was 100 μM. Two test methods were used in this study. One was performed by testing 100 μL of each fucoxanthin solution under the plate reader directly; and the other was tested by following the MTT assay protocol.

10 μL MTT solution was added to each well and then 150 μL DMSO was added and finally the absorbance was read. The dilution plan of fucoxanthin is shown in Table 14.

Table 14: Fucoxanthin dilution plan for its colour influence test

Number	FX concentration (μM)	Dilution plan (Each 1.5 mL micro-tube has 1000 uL FX and complete culture medium mixture)
1	100	20 μL(5 mM FX stock solution) + 980 μL medium
2	50	10 μL (5 mM FX stock solution) + 990 μL medium
3	10	200 μL (50 μM FX solution) + 800 μL medium
4	1	20 μL (50 μM FX solution) + 980 μL medium

Test 1 (Following MTT assay protocol)



Test 2 (Testing under plate reader directly)

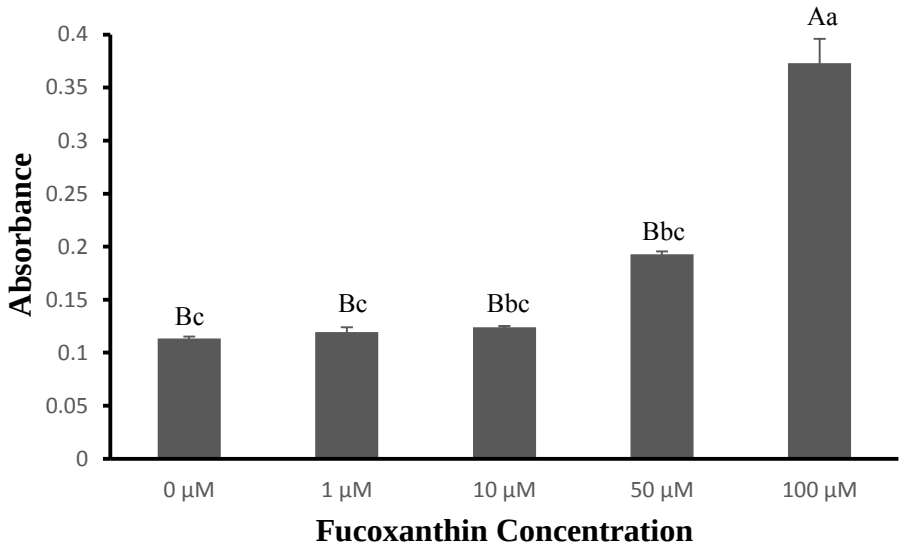


Figure 6: Absorbance of different concentrations of fucoxanthin. Absorbance was tested in the presence of various concentration of fucoxanthin (0, 1, 10, 50 and 100 µM). Data are presented as means ± S.D, n=6. In each column, the values with different capital letters indicated that the differences were very significant from each other, $P< 0.01$, and values with different small letters indicated that the differences were significant from each other, $P< 0.05$ (Post Hoc, Turkey's test).

Based on the two experimental results and analysis (Figure 6), there was a significant difference of the absorbance between fucoxanthin 100 μM with lower fucoxanthin concentration. In Test 1, 100 μL of 10 μM and 1μM fucoxanthin solution, as well as cell complete culture medium had no significant difference in the absorbance values. In Test 2, the absorbance value at fucoxanthin 50 μM, 10 μM and 1 μM has no significant difference with complete culture medium. But absorbance as fucoxanthin 100 μM is significantly different from the absorbance values. According to the results, fucoxanthin solution was replaced with fresh cell culture medium before adding MTT solution in this study.

3.2.12 Determination of Optimum Concentration of Gemcitabine

Procedures for the gemcitabine study were the same as described in the fucoxanthin experiments (Section 3.2.7.2). The dilution plans for MIA PaCa-2 and PANC-1 were different (see Table 15, 16 and 17). Since gemcitabine stock solution (10 mM) was prepared by cell culture medium, determination of the toxicity of dilution solvent was not required.

Table 15: Gemcitabine dilution plan for MIA PaCa-2

Number	Actual Gem concentration (μM)	Prepared Gem concentration (μM)	Dilution plan (Each 1.5 mL micro-tube has 1000 uL Gem and complete culture medium mixture)
1	1	2	40 μL (50 μM Gem solution) + 960 μL medium
2	0.5	1	20 μL (50 μM Gem solution) + 980 μL medium
3	0.1	0.2	4 μL (50 μM Gem solution) + 996 μL medium
4	0.05	0.1	500 μL (0.2 μM Gem solution) + 500 μL medium
5	0.025	0.05	250 μL (0.2 μM Gem solution) + 750 μL medium
6	0.01	0.02	100 μL (0.2 μM Gem solution) + 900 μL medium
7	0.005	0.01	50 μL (0.2 μM Gem solution) + 950 μL medium
8	0.001	0.002	10 μL (0.2 μM Gem solution) + 990 μL medium
50 μM GEM solution (1000 μL) preparation			5 μL (5 mM Gem stock solution) + 995 μL medium

Table 16: Gemcitabine dilution Plan 1 for PANC-1

Number	Actual Gem concentration (μM)	Prepared Gem concentration (μM)	Dilution plan (Each 1.5 mL micro-tube has 1000 uL Gem and complete culture medium mixture)
1	4	8	160 μL (50 μM Gem solution) + 840 μL medium
2	2	4	80 μL (50 μM Gem solution) + 920 μL medium
3	1	2	40 μL (50 μM Gem solution) + 960 μL medium
4	0.5	1	20 μL (50 μM Gem solution) + 980 μL medium
5	0.2	0.4	8 μL (50 μM Gem solution) + 992 μL medium
6	0.1	0.2	4 μL (50 μM Gem solution) + 996 μL medium
7	0.05	0.1	500 μL (0.2 μM Gem solution) + 500 μL medium
50 μM GEM solution (1000 μL) preparation			5 μL (5 mM Gem stock solution) + 995 μL medium

Table 17: Gemcitabine dilution Plan 2 for PANC-1

Number	Actual Gem concentration (μM)	Prepared Gem concentration (μM)	Dilution plan (Each 1.5 mL micro-tube has 1000 uL Gem and complete culture medium mixture)
1	100	200	20 μL (5 mM Gem solution) + 980 μL medium
2	50	100	10 μL (50 μM Gem solution) + 990 μL medium
3	10	20	400 μL (50 μM Gem solution) + 600 μL medium
4	1	2	40 μL (50 μM Gem solution) + 960 μL medium
5	0.5	1	20 μL (50 μM Gem solution) + 980 μL medium
6	0.1	0.2	4 μL (50 μM Gem solution) + 996 μL medium
7	0.05	0.1	500 μL (0.2 μM Gem solution) + 500 μL medium
50 μM GEM solution (1000 μL) preparation			5 μL (5 mM Gem stock solution) + 995 μL medium

3.2.13 Study on Joint Toxicity of Gemcitabine and Fucoxanthin

According to previous single treatment experiment results, the optimum concentrations of fucoxanthin and gemcitabine were determined. In the combination treatment study portion of the experiment, fucoxanthin at concentration 150, 250 and 300 nM, and gemcitabine, at concentration of 25 and 50 nM were concurrently combined with each other to treat MIA PaCa-2. Meanwhile, fucoxanthin concentrations (10 and 20 μM) were concurrently combined with gemcitabine (50 and 500 nM respectively) to treat PANC-1. The combination plan and dilution plan for MIA PaCa-2 are shown in Table 18 and 19. As for PANC-1, both plans can be seen in Table 20 and 21. Using PANC-1 as a sample, the design of the plate is shown in Figure 7. Other experimental steps were the same as described in single treatment study.

Table 18: Combination plan of gemcitabine and fucoxanthin for MIA PaCa-2

	GEM (nM)		
FX (nM)	0	25	50
0	Control	GEM 25 nM only	GEM 50 nM only
150	FX 150 nM only	25 + 150 GEM FX	50 + 150 GEM FX
250	FX 250 nM only	25 + 250 GEM FX	50 nM + 250 nM GEM FX
300	FX 300 nM only	25 + 300 GEM FX	50 nM + 300 nM GEM FX

Table 19: Dilution plan of combination treatment for MIA PaCa-2

Number	**Actual drug concentration (nM)**		**Prepared drug concentration (nM)**		**Dilution plan (Each 1.5 mL micro-tube has 1000 µL drug and complete culture medium mixture)**		
	GEM	FX	GEM	FX	0.2 µM GEM (µL)	5 µM FX (µL)	Medium (µL)
1	25	150	50	300	250	60	690
2	25	250	50	500	250	100	650
3	25	300	50	600	250	120	630
4	50	150	100	300	500	60	440
5	50	250	100	500	500	100	400
6	50	300	100	600	500	120	380

Table 20: Combination plan of gemcitabine and fucoxanthin for PANC-1

GEM (nM)	FX (μM)		
	0	10	20
0	Control	FX 10 μM only	FX 20 μM only
50	GEM 50 nM only	10 + 50 GEM FX	20 + 50 GEM FX
500	GEM 500 nM only	10 + 500 GEM FX	20 + 500 GEM FX

Table 21: Dilution plan of combination treatment for PANC-1

Number	**Actual drug concentration**		**Prepared drug concentration**		**Dilution plan (Each 1.5 mL micro-tube has 1000 μL drug and complete culture medium mixture)**		
	GEM (nM)	FX (μM)	GEM	FX	10 μM GEM (μL)	5 mM FX (μL)	Medium (μL)
1	50	10	100	20	10	4	986
2	50	20	100	40	10	8	982
3	500	10	1000	20	100	4	896
4	500	20	1000	40	100	8	892

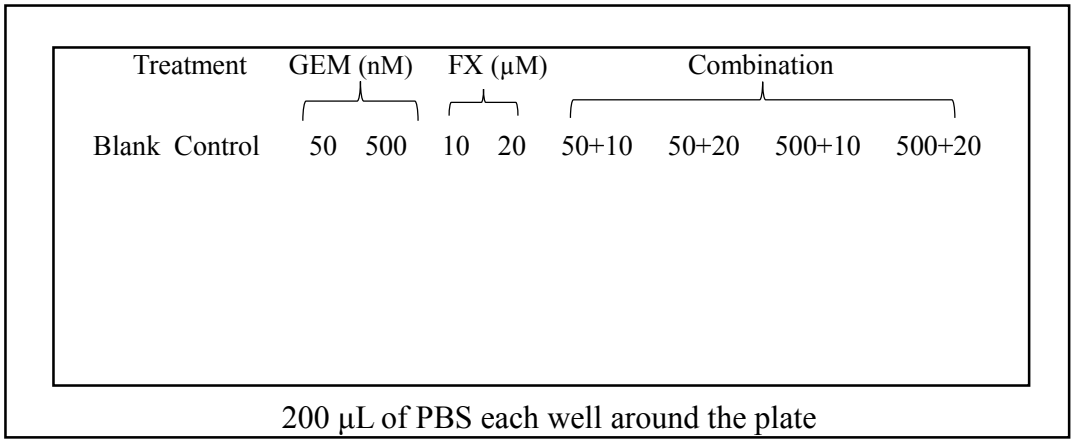


Figure 7: Plate design for PANC-1 treated with single and combination treatment. In each well of the 96-well plate 200 μL of solution was aliquotted according to the follow assignments. All groups other than blank group had 100 μL of medium and 100 μL of treatment as illustrated in Figure 7. The treatment were gemcitabine, fucoxanthin and combination groups.

3.3 Cell Cycle Assay

The cell cycle or cell-division cycle is a set of events that result in cell growth and division into two daughter cells. The cell cycle is a very orderly progression strictly following the sequence of G_1-S-G_2-M. The G_1 phase stands for "GAP 1", in which the cell size increases. The S phase represents "Synthesis" that is the stage for DNA replication. The G_2 phase stands for "GAP 2". Cells keep on growing in this phase. The M phase is abbreviation of "Mitosis", where chromosomes separate and cytokinesis occurs. Moreover, there is a G_0 phase, which is a resting phase for the cells which are not in the cycle and stop dividing (Vermeulen, Berneman, & Van Bockstaele, 2003).

Flow cytometry is an important technique applied in cell cycle studies. Cellular DNA content is often the single parameter measured by flow cytometry for cell cycle studies. Once fluorescent molecules are specifically and stoichiometrically used to bind DNA, a linear relationship between cellular fluorescence intensity and DNA amount can be

measured. The emitted fluorescence of the DNA specific dyes is proportional to DNA content present in different phases of the cell cycle. PI is the most commonly used dye to quantitatively assess DNA content. PI binds to DNA by intercalating between the bases with little or no sequence preference. Because PI also binds to RNA, nucleases such as Ribonuclease (RNAse), are necessary to distinguish between RNA and DNA (Hansen et al., 1989).

To explore the possible mechanisms of single and combined effects of fucoxanthin and gemcitabine, MIA PaCa-2, PANC-1 and HEK 293 cells were analysed for cell cycle alterations by staining with propidium iodide (PI).

3.3.1 Major Equipment and Materials Applied

Table 22: Major equipment and materials applied in cell cycle analysis

Number	Material / Equipment	Supplier
1	Ribonuclease A from bovine pancreas (Cat No. R4875- 100mg)	Sigma-Aldrich
2	Triton™ X-100 for molecular biology (Cat No.T8787-250ML)	Sigma-Aldrich
3	Ethanol, Anhydrous (Histological grade) (Cat No. 64-17-5)	Fisher scientific
4	PI (Propidium iodide) (Cat No. P4170-10MG)	Sigma-Aldrich
5	PBS (Phosphate buffered saline), pH 7.2, no calcium magnesium and phenol red	Life technologies
6	Flow cytometer	Beckman coulter
7	Six well plate	BD (Becton Dickinson) Bioscience (Auckland, NZ)
8	Test Tube, 12 x 75 mm, Polypropylene Blue (250/ pack) (Cat No.63058857)	Beckman coulter

3.3.2 Protocols for Cell Cycle Analysis

3.3.2.1 *80%* Ethanol Preparation

Absolute ethanol was diluted with distilled water to final value of 80%. The 80% ethanol solution was kept in a parafilm-sealed tube at -20ºC fridge.

3.3.2.2 RNAse A Solution Preparation

The amount of RNAse A (Ribonuclease A) powder in the manufacturer's vial was 100mg. RNAse A was dissolved in distilled water (DDW) at 1mg/mL as a stock solution. The total solution was separated in 1.5 mL micro-tubes and kept at -20ºC.

3.3.2.3 PI Stock Solution Preparation

The amount of PI powder in the vial was 10 mg. 10 mL distilled water (DDW) was used to dissolve PI powder and got 1mg/ml stock solution. The total solution was separated in 1.5 mL micro-tubes with aluminium-foil paper and kept in 4ºC fridge.

3.3.2.4 Cell Preparation and Drug Treatment

Step 1: Cell Seeding

After detaching, counting and diluting cells, cells were seeded onto 6-well plates. The seeding density for all cell lines in this study was 50,000 cell/mL and a 2 mL cell solution was contained in each well. The plates were kept in a 37 ºC incubator for 6 to 24 hours to ensure almost all of the cells attached to the walls of the wells.

Step 2: Cell (0% serum) starvation

When the cell attachment rate was the highest, cells were treated with 2 mL of 0% FBS medium (with 1% Penicillin and 1% L-glutamine) in each well, for synchronizing cell proliferation. One plate set in this study was a control group and other plates were designed as treatment groups (see Table 23). In Plate 1(no treatment group), Well Nos. A, B and C were treated with 2 mL of 0% FBS medium, and Well Nos. D, E and F were filled with 2mL of complete culture (10%) medium. Before adding the fresh medium (0% or 10% FBS medium), the entire old medium was removed first. The medium was added slowly and gently in circles along the wall of the well. For Plate 2 (treatment group), all the wells were added with 2 mL of 0% FBS medium. The plates were kept in 37 ºC incubator for 24 hours.

Table 23: Plate design for cell cycle assay

A s	B s	C s
D c	E c	F c

Plate 1 (No treatment group)

A C_T	B T	C T
D T	E T	F T

Plate 2 (Treatment group)

(In Plate 1, s means starvation; c means control with 2 mL complete culture medium inside the well; In Plate 2, CT means control with 2 mL complete culture medium; T means treatment with 2 mL treatment and complete culture medium mixture.)

Step 3: Addition of treatments

All the treatments were kept in 15 mL centrifuge tubes which were prepared with complete culture (10% FBS) medium. The old 0% medium was removed gently first and then 2 mL well mixed treatment solution was carefully added into designated wells. The control well was set in the plate of treatment group with 2 mL complete culture medium. All of the plates were kept in the incubator for 48 hours (day 2) and 72 hours (day 3) (Figure 8). Cell collection was needed for plate 1 at day 0 samples. The design of the 6-well plate is shown in Table 23-Plate 2. All the concentrations decided to be used in this part were the same as Section 3.2.12. Namely, fucoxanthin 150, 250 and 300 nM, gemcitabine 25 and 50 nM were used to treat MIA PaCa-2 solely and jointly. For PANC-1, fucoxanthin concentrations were 10 and 20 μM and gemcitabine were 50 and 500 nM.

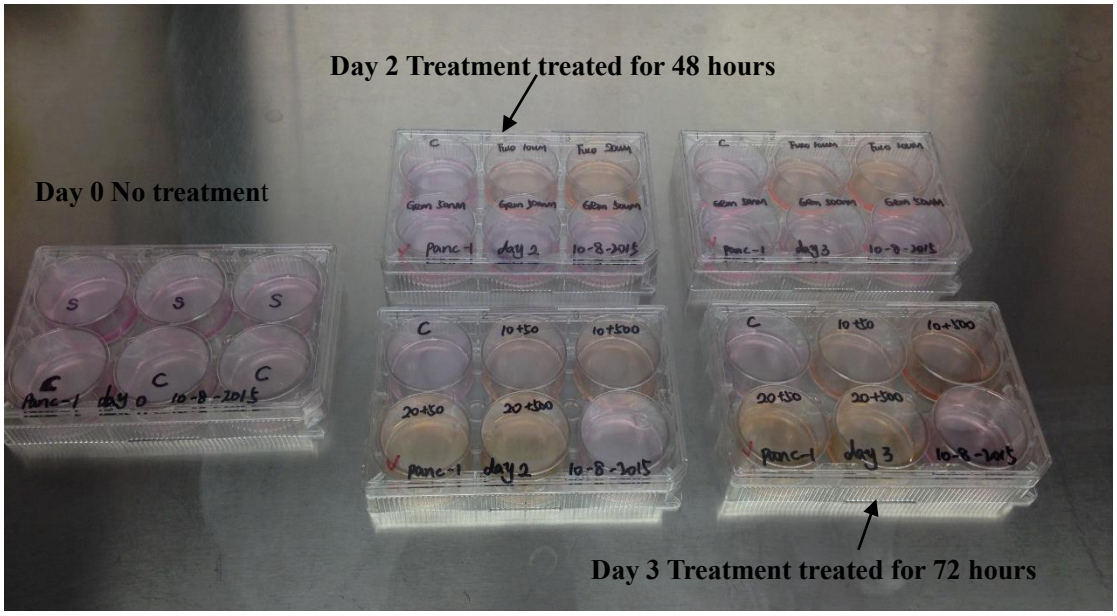


Figure 8: Cell cycle study of PANC-1 treated with gemcitabine and fucoxanthin

3.3.2.5 Cell Harvesting

After cells being cultured for the determined time, they were harvested and stored for the following analysis. The whole experiment process was conducted on ice.

Step 1: Cell detaching

In order to detach the cells from each of their wells, the entire medium in each well was removed into 15 mL marked centrifuge tubes. 500 µL PBS was added to wash cells and then transferred in relevant tubes. 500 µL TrypLE™ Express Enzyme was used to detach cells and the plate was kept in incubator for no more than 5 minutes. After detaching, the cell solution was transferred to relevant 15 mL tubes, and 500 µL PBS was added to wash each well, then collected.

Step 2: Cell centrifuging

The cells were centrifuged at 1200 RPM at 4°C for 5 minutes. After centrifuging, the supernatant in each tube was discarded. 1mL PBS was added and then cells were centrifuged again for 5 minutes.

Step 3: Cell mixing

After centrifugation, most of the supernatant in each tube was discarded and a little of PBS was left inside. The tube rack was used to mix the cells with the rest of PBS. Tubes were swiped left and right 3 times to re-suspend and mix cells.

Step 4: Cell fixing and storing

1 mL ice cold 80% ethanol was added into each tube. (Each tube was previously stored in the -20ºC freezer). The vortex was set at low speed and ethanol was added slowly. The tube was tilted diagonally so the ethanol was added to the sides and not directly onto the cells, thereby avoiding formation of aggregates. Each tube was sealed with parafilm and kept at -20^{o}C for at least overnight and not more than 10-14 days.

3.3.2.6 Cell Cycle Analysis

Step 1: Permeabilizing solution preparing

The permeabilizing solution of total 1ml per test tube: 0.1% Triton x100 (1µL for each 1 mL) + RNAse A (50 µg/mL from stock solution of 1 mg/mL). For example for 20 tubes, we made: 20 µL Triton-x100 +1000 µL RNAse +19,980 µL PBS.

Step 2: Ethanol removing

All tubes were centrifuged first at 1200RPM for 2 minutes after removing the parafilm. The ethanol was gently removed and 3 mL ice cold PBS was added to each tube before the second centrifugation step. After the second centrifugation, another 3 mL ice cold PBS was added to each tube to replace the initial wash. In total, the cells were washed twice with 3 mL ice cold PBS.

Step 3: PI staining

In the process of PI staining, the supernatant in each well was gently removed and then 1 mL well mixed permeabilizing solution was added to each tube. All cells in tubes were carefully mixed before transferred to test tubes and then incubated at 37^{o}C for 30~45 minutes. After permeabilization, 5 µg/mL of PI (5 µL to 1ml in each tube) was added to

each test tube and kept for 5 minutes. Finally all the tubes were run under flow cytometer (Figure 9). The optimal voltage for each sample running is attached in Appendix A3.

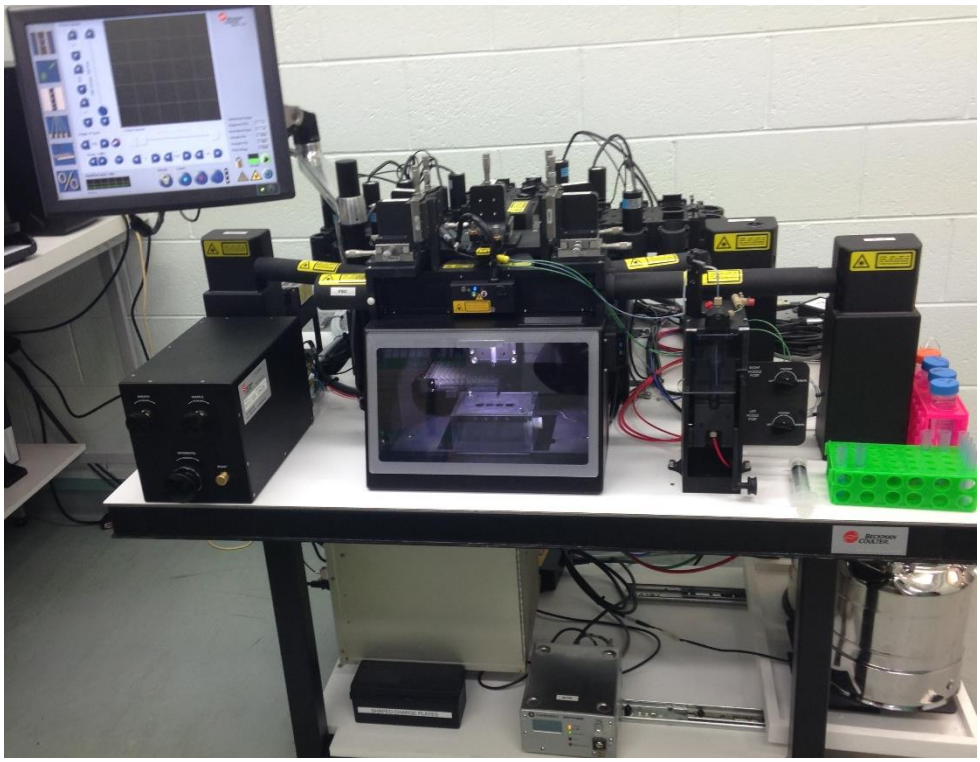


Figure 9: Photo of Flow cytometry (FCM) applied in cell cycle assay

3.3.2.7 Determination of the Fucoxanthin Fluorescence to Cell Cycle Analysis

Fucoxanthin is an allenic carotenoid, being well known to function efficiently as a main photosynthetic antenna pigment in marine algae, which has the property of fluorescence. It has been reported that the emission bands of all-*trans*-fucoxanthin isolated from a brown alga were around 630, 685 and 750 nm in CS_2 at 20°C (Katoh, Nagashima, & Mimuro, 1991). One group of PANC-1 cells treated with fucoxanthin 10 μM and 20 μM without PI staining was set in this study (other steps were same as Section 3.3.2.6), in order to determine whether fucoxanthin itself will affect the cell cycle result. The detailed results were attached in Appendix A2. The result shows that the fucoxanthin did not emit fluorescence under the excitation source (488 nM) used in this study. Fucoxanthin itself is not the interference factor in the cell cycle study.

3.4 Data Analysis

3.4.1 Analysis of MTT Assay Results

IC_{50}, the half maximal inhibitory concentration, is commonly used as a measure of drug effectiveness. IC_{50} was an important reference to measure the inhibitory effect of gemcitabine and fucoxanthin in this study. It was calculated by PRISM® software (Graphpad, Version 6.0) and the IC_{50} values were obtained by using dose-response inhibition, nonlinear regression (curve fit): Log (inhibitor) vs. Response-Variable slope (four parameters).

3.4.2 Analysis of Cell Cycle Assay Results

Kaluza® Flow Cytometry Analysis Software (Version 1.3) bought from Beckman Coulter was the software applied in cell cycle results analysis to measure the cell cycle distribution in this study.

3.5 Statistical Analysis

All experiments in this study were performed at least three times. Statistical differences in multiple groups was determined by one-way and two-way ANOVA (Analysis of Variance) with PRISM® software (Graphpad, Version 6.0) and SPSS (Version 22.0). Statistical comparisons were made by Post Hoc (Turkey's test). Analysis between two groups was determined by using unpaired Student's *t* test Data are expressed as means ± S.D (standard deviation). Differences with $P< 0.05$ were considered significant and $P< 0.01$ were considered very significant.

Chapter 4 Results

4.1 Single Inhibitory Effect of Gemcitabine and Fucoxanthin on MIA PaCa-2 Pancreatic Cancer Cell Line

4.1.1 Linearity of MTT Assay for MIA PaCa-2

A standard (linearity) curve, comparing cell number vs. absorbance was important in establishing a starting point in the MTT assay portion of the study. A good linearity curve reflects a good growing status of the cells. Cell number used in cell viability studies should fall within the linear portion of the curve. Cell seeding concentration can neither be too high nor too low, so that a good growing condition for cells can be guaranteed. Figure 10 shows the linearity curve of MIA PaCa-2. It shows a good linear relationship ($R^2 = 0.986$) between the absorbance, determined at 540nm wavelength and different cell densities (from 250,000 cells/mL to 976 cells/mL).

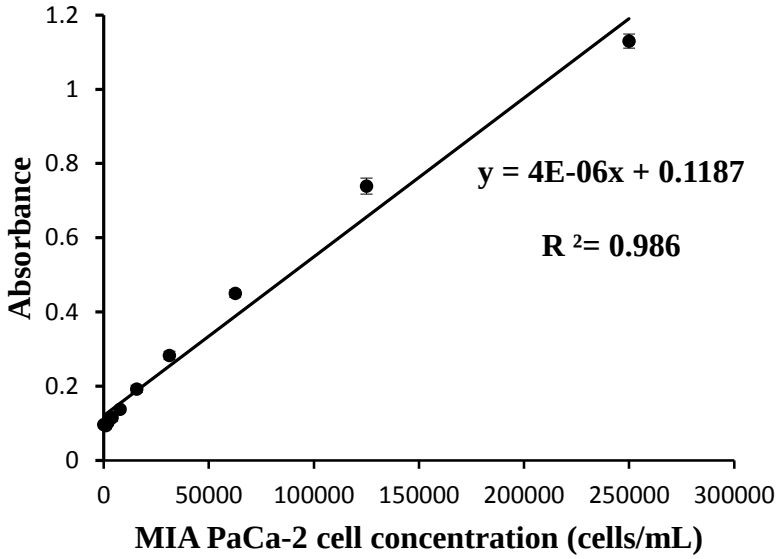


Figure 10: Linearity between MIA PaCa-2 cell numbers and absorbance values

Data are presented as means ± S.D, n=6.

4.1.2 Single Inhibitory Effect of Gemcitabine on MIA PaCa-2 Cells

In this study, gemcitabine was first examined to explore its effect on the cell viability of MIA PaCa-2 cells. Culture of cells with different concentrations of gemcitabine for 72 hours resulted in very significant suppression of cell viability in a dose-dependent manner ($P< 0.01$) and its IC_{50} was 25.00 ± 0.47 nM. In addition, observations under an inverted microscope showed that in the previous two days (culturing for 24 and 48 hours), there were no remarkable morphological changes. However, after 72 hours cell density, the cell attachment rate was reduced with the increase of the gemcitabine concentration, and the cell volume was bigger than the control group. Figure 11 shows that a cell viability vs. Log gemcitabine concentration curve fits well with a sigmoidal model of negative exponential distribution. Additionally, cell viability at 5 nM and 100 nM almost reaches a plateau, which is neither increased nor reduced any more. According to one-way ANOVA testing, there was no significant difference (P=0.26>0.05) of cell viability between gemcitabine 100 nM and 1000 nM, or between 1 nM and 5 nM (P =0.08>0.05), which means gemcitabine at 5 nM and 100 nM were the two split points of the curve. Gemcitabine at 25 and 50 nM showed about 48 % and 58 % cell death of MIA PaCa-2 cells. These two concentration points were around 50 % of cell death, thus they were chosen in the following combination study.

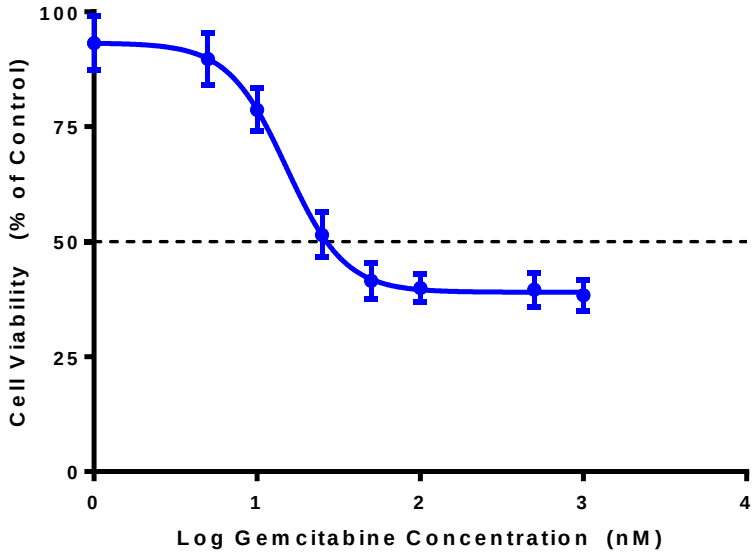


Figure 11: Inhibitory effect of gemcitabine on the growth of MIA PaCa-2 cells at 72 hours. Cells were incubated in the presence of various concentrations of gemcitabine (1, 5, 10, 25, 50, 100, 500 and 1000 nM). A relative viability of 100% was designated as the total number of cells that grew in 72 hours cultures in the absence of gemcitabine. Data are presented as means ± S.D, n=6.

4.1.3 Single Inhibitory Effect of Fucoxanthin on MIA PaCa-2 Cells

The effect of fucoxanthin on MIA PaCa-2 cells was investigated. Fucoxanthin inhibited the proliferation of MIA PaCa-2 cells depending upon its time and concentration (Figure 12). The IC_{50} value at each time point was 17.72 ± 1.04, 10.68 ± 0.64 and 8.74 ± 0.28 µM respectively (Table 24). Morphological changes observed under the inverted microscope were clearer with the extension in culture time, in the presence of fucoxanthin concentrations which were higher than 6.25 µM. Cells diminished in size and were scattered, detaching easily from the flask wall. After 72 hours, the volume of cells was significantly reduced and the edges of the cells were rough. As in Figure 12, cell viability at 12.5 µM decreased sharply with the increase in treated time, especially from 24 hours to 48 hours and until 72 hours cell proliferation was reduced by 78%. MIA PaCa-2 cell viability was dropped below 50 % upon 24 hours exposure to 25 µM and higher

concentrations of fucoxanthin. It was interesting to note that the cell viability at 72 hours of fucoxanthin 1.5625 and 3.125 μM was higher than that observed at 48 hours. This implied that MIA PaCa-2 cells grew at lower concentrations of fucoxanthin.

A small fucoxanthin concentration range (20 ~ 1000 nM) was tried in MIA PaCa-2 cells for 72 hours, to further study the effect of low concentrations of fucoxanthin (Figure 13). Low doses of fucoxanthin neither suppressed nor improved the growth of MIA PaCa-2 cells. The cell viability value determined from each fucoxanthin concentration was around 100%. Thus, lower doses of fucoxanthin did not change cell proliferation.

Table 24: Cytotoxicity of fucoxanthin in MIA PaCa-2 pancreatic cancer cell line detected at various time points (24, 48, and 72 hours). Data are presented as means ± S.D, n=6.

Time	24 hours	48 hours	72 hours
IC_{50} (µM)	17.72 ± 1.04	10.68 ± 0.64	8.74 ± 0.28

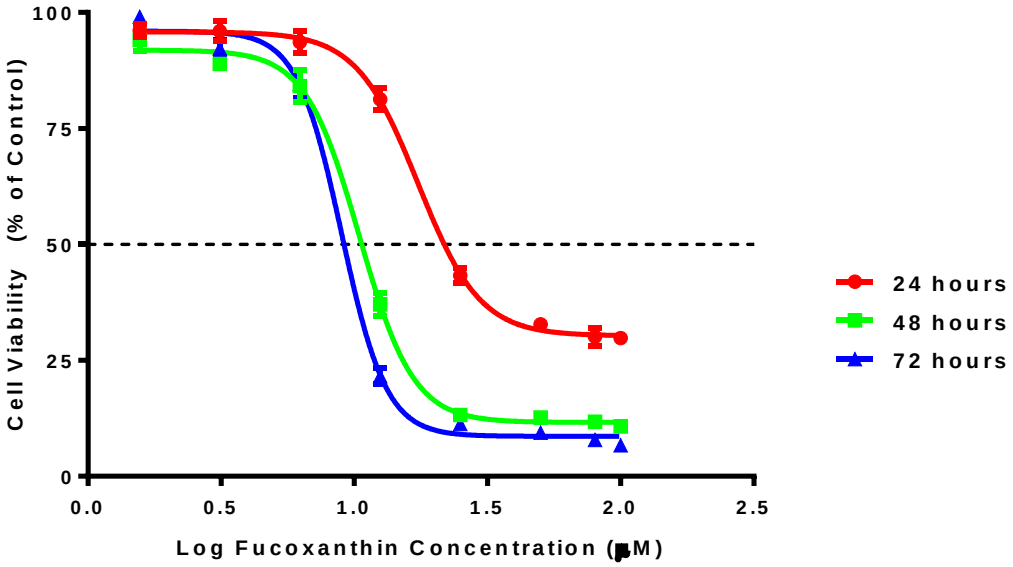


Figure 12: Inhibitory effect of fucoxanthin on the growth of MIA PaCa-2 cells at 24, 48 and 72 hours. Cells were incubated in the presence of various concentrations of fucoxanthin (1.5625, 3.125, 6.25, 12.5, 25, 50, 80 and 100 µM). A relative viability of 100% was designated as the total number of cells that grew in 24, 48 and 72 hours cultures in the absence of fucoxanthin. Data are presented as means ± S.D, n=6.

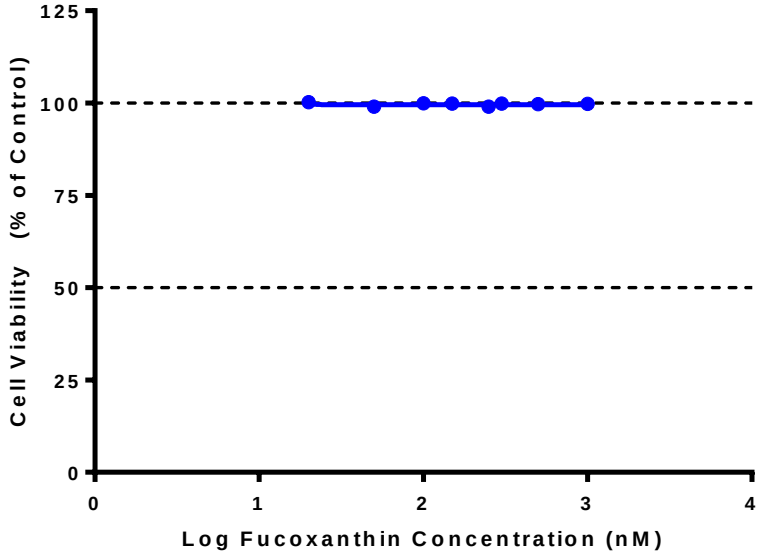


Figure 13: Inhibitory effect of fucoxanthin on the growth of MIA PaCa-2 cells at 72 hours. Cells were incubated in the presence of various concentrations of fucoxanthin (20, 50, 100, 150, 250, 300, 500 and 1000 nM). A relative viability of 100% was designated as the total number of cells that grew in 72 hours cultures in the absence of fucoxanthin. Data are presented as means ± S.D, n=6.

4.2 Single Inhibitory Effect of Gemcitabine and Fucoxanthin on PANC-1 Pancreatic Cancer Line

4.2.1 Linearity of MTT Assay for PANC-1

The cell number vs. absorbance linearity curve of PANC-1 is given in Figure 14. The linear relationship between the absorbance determined at 540nm wavelength and different cell densities (from 250,000 cells/mL to 976 cells/mL) is very good ($R^2 = 0.994$). This result indicates the PANC-1 cells grew very well in the culture conditions and they were ready for the subsequent experiments. This curve shows that cell concentration at 50,000 cells/mL fits the curve very well, which is neither too crowed nor too sporadic and falls within the linear portion of the curve. Thus, a cell density of 50,000 cells/mL used in MTT and cell cycle assays was determined to be reasonable.

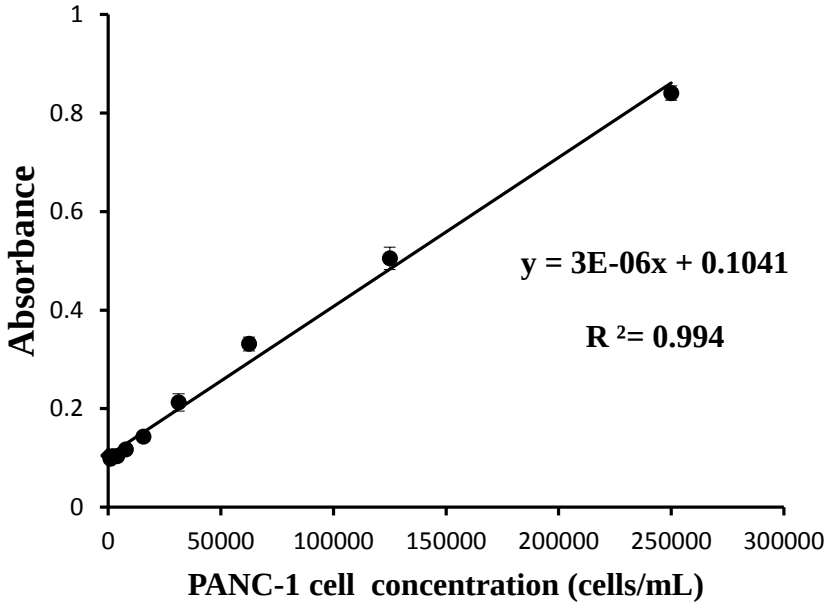


Figure 14: Linearity between PANC-1 cell numbers and absorbance values. Data are presented as means ± S.D, n=6.

4.2.2 Single Inhibitory Effect of Gemcitabine on PANC-1 Cells

Two different gemcitabine concentration ranges were tried to treat PANC-1 cells in this study part. Even though gemcitabine is one of the common drugs used in the treatment of pancreatic cancer patients, the highest concentration of 4,000 nM gemcitabine could only induce 31 % cell death in PANC-1 cells as compared to control, under a 72 hours treatment schedule (Figure 15). Gemcitabine was decreased by 80 fold from 4,000 to 50 nM, while the cell viability was only 14% lower than that observed in the highest drug concentration. In the previous 24 and 48 hours, there were no numerous morphological changes under the microscope. Based on this result, a higher gemcitabine concentration range was tried keeping the action time 72 hours only (Figure 16). The IC_{50} value calculated was 48.55 ± 2.30 µM. Moreover, in the higher concentration groups, numerous morphological changes were observed.

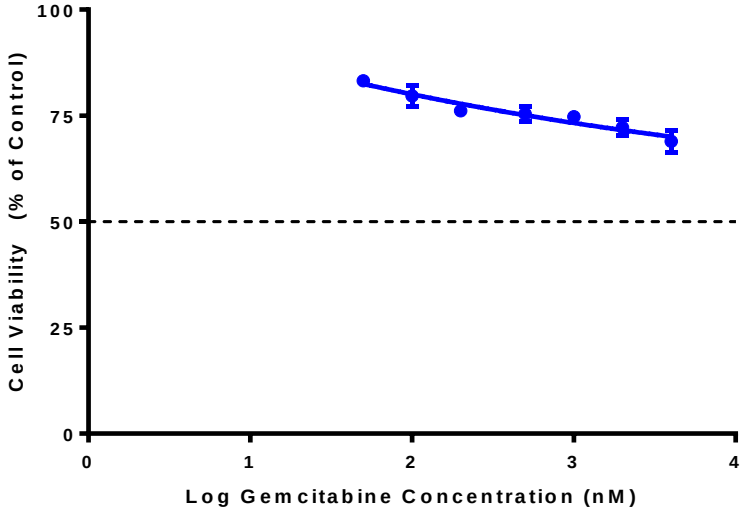


Figure 15: Inhibitory effect of gemcitabine on the growth of PANC-1 cells at 72 hours. Cells were incubated in the presence of various concentrations of gemcitabine (50, 100, 200, 500, 1000, 2000, and 4000 nM). A relative viability of 100% was designated as the total number of cells that grew in 72 hours cultures in the absence of gemcitabine. Data are presented as means ± S.D, n=6.

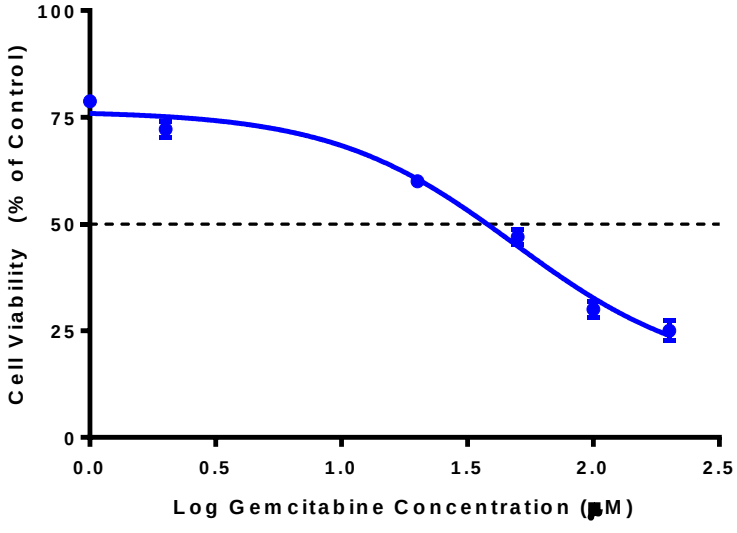


Figure 16: Inhibitory effect of gemcitabine on the growth of PANC-1 cells at 72 hours. Cells were incubated in the presence of various concentrations of gemcitabine (1, 2, 20, 50, 100 and 200 µM). A relative viability of 100% was designated as the total number of

cells that grew in 72 hours cultures in the absence of gemcitabine. Data are presented as means ± S.D, n=6.

4.2.3 Single Inhibitory Effect of Fucoxanthin on PANC-1 Cells

Figure 17 shows the cell viability of PANC-1 cells in the presence of fucoxanthin. Fucoxanthin inhibited proliferation of PANC-1 cells in a dose dependent manner. Cell viability at fucoxanthin concentrations of 5, 10, 20, and 50 µM was quite different. These four concentration points with the corresponding cell viability were 77%, 46%, 22%, and 8.4% respectively. From Figure 17, it seems that the decrease of cell viability followed a linear trend within the four points range, with the increase in the fucoxanthin concentration. In this study, the IC_{50} value of fucoxanthin on PANC-1 cells was 10.58 ± 0.56 µM. As for numerous morphological observations, there were not any marked changes in the low concentration groups as in 5 µM or lower.

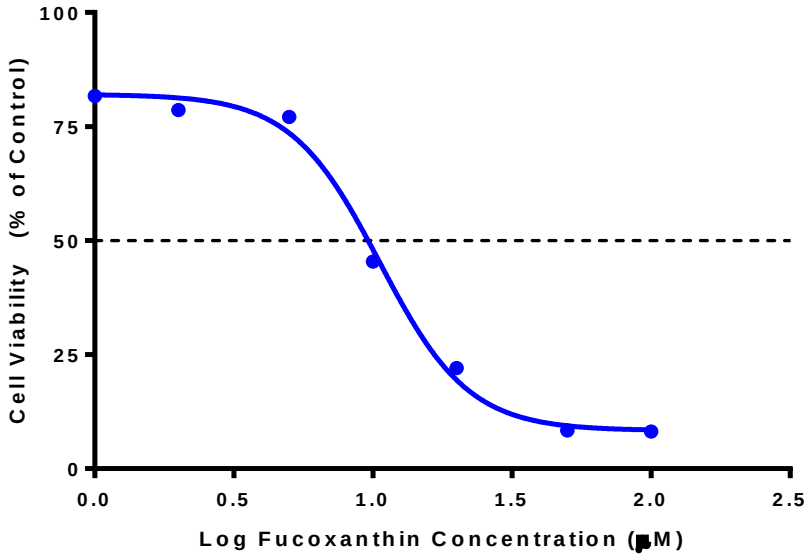


Figure 17: Inhibitory effect of fucoxanthin on the growth of PANC-1 cells at 72 hours. Cells were incubated in the presence of various concentrations of gemcitabine (0.5, 1, 2, 5, 10, 20, 50 and 100 µM). A relative viability of 100% was designated as the total number of cells that grew in 72 hours cultures in the absence of fucoxanthin. Data are presented as means ± S.D, n=6.

4.3 Single Inhibitory Effect of Gemcitabine and Fucoxanthin on HEK 293 Human Normal Cell Line

The toxic effects of drugs at cellular levels are first evaluated by growth inhibition on cancer cell lines and then they need to be tested on human normal cell lines. In this study, HEK 293 (human embryonic kidney) cells were utilized in efficacy testing as representative of a normal cell line. The HEK 293 cell line is commonly used in biological research on account of its high metabolic rate, sensitivity to treatments and ease of growth (Keter et al., 2008).

4.3.1 Linearity of MTT Assay for HEK 293

Figure 18 shows a very good linear relationship between various cell densities (from 500,000 to 1,953 cells/mL) and corresponding absorbance ($R^2 = 0.991$). At the same time, this figure proves that a HEK 293 cell density of 50,000 cells/mL, selected to be used in the following MTT and cell cycle assays, was reasonable.

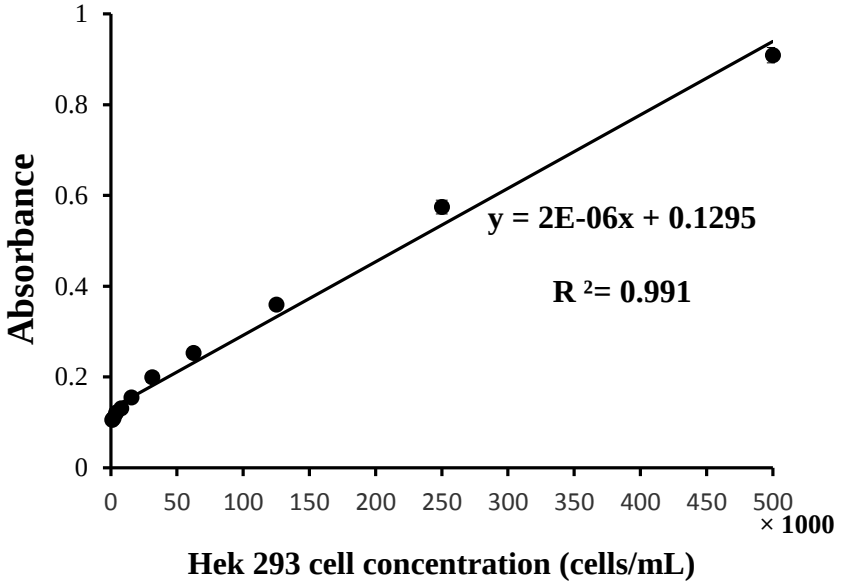


Figure 18: Linearity between HEK 293 cell numbers and absorbance values. Data are presented as means ± S.D, n=6.

4.3.2 Single Inhibitory Effect of Gemcitabine on HEK 293 Cells

Figure 19 shows the result of HEK 293 cells treated with the same gemcitabine concentration as was used for MIA PaCa-2 cells. Likewise Figure 20 depicts the result for HEK 293 cells treated with the same concentration range as tried on PANC-1 cells previously. Gemcitabine significantly inhibited the cell viability of HEK 293 cells in a concentration dependent manner, upon exposure to gemcitabine concentration of 1~1000 nM for 72 hours as compared to control ($P < 0.05$). The IC_{50} value determined in this study was 48.82 ± 3.27 nM. Cell viability was higher than 50% in the presence of gemcitabine for which the concentration was in the range of 1~50 nM. The gemcitabine concentration range tried on PANC-1 cells (50~4000 nM) was too high for HEK 293 cells. As shown in Figure 20, all cell viability was below 50 % except at the point of a gemcitabine concentration of 50 nM. In addition, when the gemcitabine concentration was above 100 nM, the cell viability reached a plateau of 20~30% of control, no matter how the gemcitabine concentration changed.

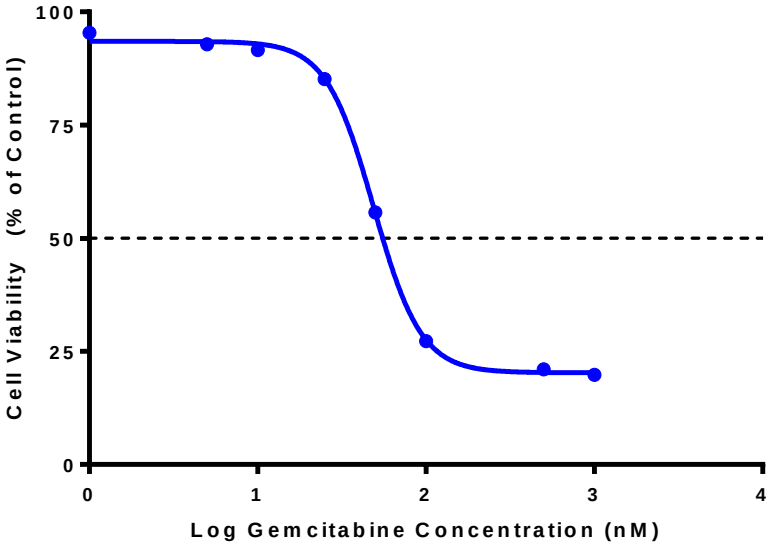


Figure 19: Inhibitory effect of gemcitabine on the growth of HEK 293 cells at 72 hours. Cells were incubated in the presence of various concentrations of gemcitabine (1, 5, 10, 25, 50, 100, 500 and 1000 nM). A relative viability of 100% was designated as the total number of cells that grew in 72 hours cultures in the absence of gemcitabine. Data

are presented as means ± S.D, n=6.

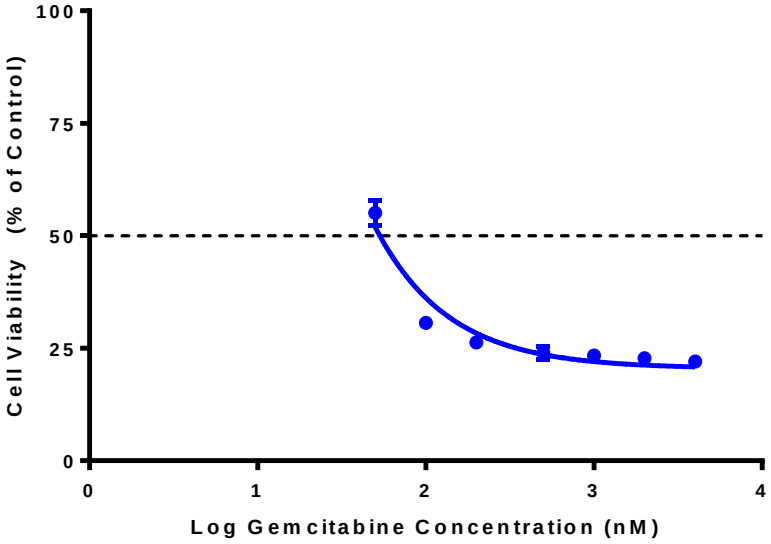


Figure 20: Inhibitory effect of gemcitabine on the growth of HEK 293 cells at 72 hours. Cells were incubated in the presence of various concentrations of gemcitabine (50, 100, 200, 500, 1000, 2000, and 4000 nM). A relative viability of 100% was designated as the total number of cells that grew in 72 hours cultures in the absence of gemcitabine. Data are presented as means ± S.D, n=6.

4.3.3 Single Inhibitory Effect of Fucoxanthin on HEK 293 Cells

Three different fucoxanthin concentration ranges, tested on both MIA PaCa-2 and PANC-1, were applied to treat HEK 293 cells. Fucoxanthin reduced the cell viability of this human normal cell line in a dose- and time- dependent manner (Figure 21 & 22). HEK 293 cells were able to proliferate in lower fucoxanthin concentration group, when exposed to 6.25 μM and lower concentrations of fucoxanthin for 24 hours. Meanwhile, cells can even grow under 3.125 μM of fucoxanthin up to 48 hours. Figure 21 shows that cell viability at both 1.5625 and 3.125 μM concentrations, in the curves of 24 hours, 48 hours and 72 hours, is higher than 100%. But at the point of fucoxanthin 6.25 μM, the cell viability was remarkably decreased after being treated for 48 hours. After treating HEK 293 cells for 72 hours, except at the points of 1.5625 and 3.125 μM, all cell viability

values were reduced. IC_{50} values detected in 24, 48 and 72 hours were 28.97 ±1.58, 8.70 ±1.17 and 8.28 ±0.30 µM, respectively.

Another small fucoxanthin concentration range (20~1000 nM), tried on MIA PaCa-2 cells, was tested on HEK 293 cells as well (Figure 23). Cells cultured in different fucoxanthin concentrations for 72 hours did not show any significant difference of cell viability (P> 0.05). Cell viability at all the concentration points was almost around 100%. Fucoxanthin 1 µM even promoted the growth of the cells, resulting in cell viability values higher than 100%. This result showed that in small concentrations, fucoxanthin did not inhibit the proliferation of HEK 293 cells, as compared to control.

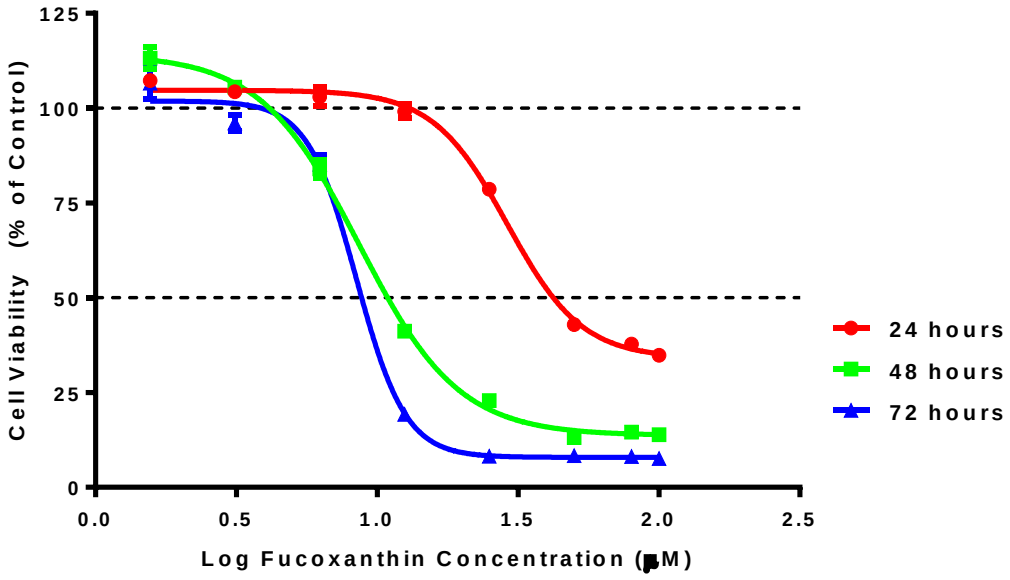


Figure 21: Inhibitory effect of fucoxanthin on the growth of HEK 293 cells at 24, 48 and 72 hours. Cells were incubated in the presence of various concentrations of fucoxanthin (1.5625, 3.125, 6.25, 12.5, 25, 50, 80 and 100 µM). A relative viability of 100% was designated as the total number of cells that grew in 24, 48 and 72 hours cultures in the absence of fucoxanthin. Data are presented as means ± S.D, n=6.

Table 25: Cytotoxicity of fucoxanthin in HEK 293 human normal cell line detected at various time points (24, 48, and 72 hours). Data are presented as means ± S.D, n=6.

Time	24 hours	48 hours	72 hours
IC_{50} (µM)	28.97 ± 1.58	8.70 ± 1.17	8.28 ± 0.30

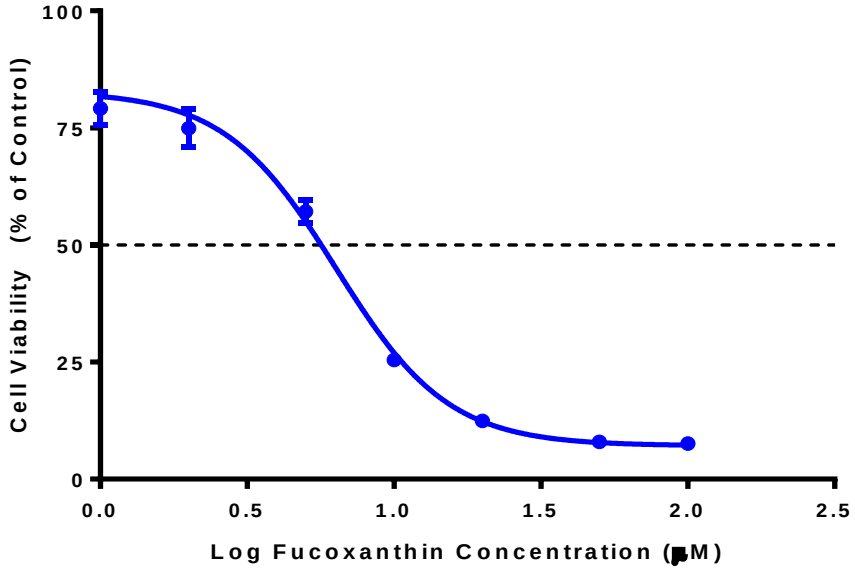


Figure 22: Inhibitory effect of fucoxanthin on the growth of HEK 293 cells at 72 hours. Cells were incubated in the presence of various concentrations of fucoxanthin (0.5, 1, 2, 5, 10, 20, 50 and 100 µM). A relative viability of 100% was designated as the total number of cells that grew in 72 hours cultures in the absence of fucoxanthin. Data are presented as means ± S.D, n=6.

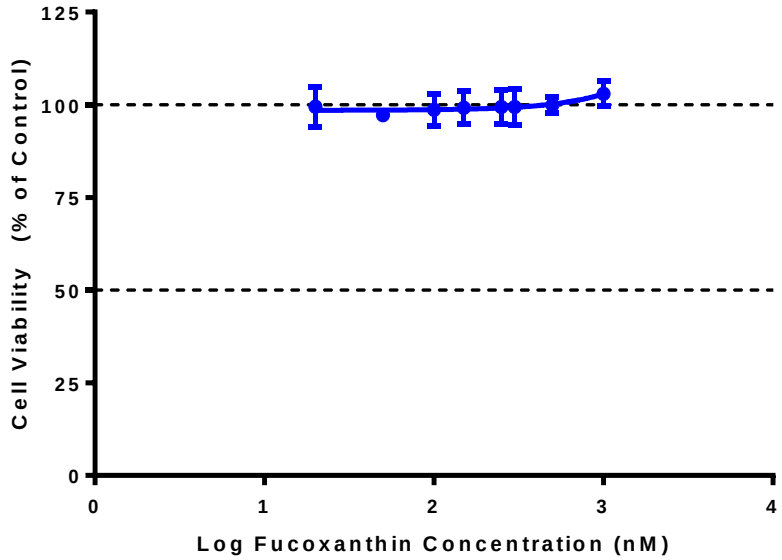


Figure 23: Inhibitory effect of fucoxanthin on the growth of HEK 293 cells at 72 hours. Cells were incubated in the presence of various concentrations of fucoxanthin (20, 50, 100, 150, 250, 300, 500 and 1000 nM). A relative viability of 100% was designated as the total number of cells that grew in 72 hours cultures in the absence of fucoxanthin. Data are presented as means ± S.D, n=6.

4.4 Joint Inhibitory Effect of Gemcitabine and Fucoxanthin

From previous single treatment studies, it's known that both fucoxanthin and gemcitabine showed potent cytotoxicity at higher levels of concentration. However, high concentrations of these two drugs killed large numbers of human normal cells. Hence, a joint inhibitory effect study of these two drugs was conducted in order to investigate whether fucoxanthin and gemcitabine can make a stronger inhibitory effect at lower concentration ranges when combined together.

Some reasonable gemcitabine and fucoxanthin concentrations were chosen to treat pancreatic cancer cell lines MIA PaCa-2 and PANC-1, based on previous single treatment results. For MIA PaCa-2 cells, chosen gemcitabine concentrations were 25 and 50 nM, whereas, fucoxanthin concentrations were 150, 250, and 300 nM. For the other cell line

i.e. PANC-1, applied gemcitabine concentrations were 50 and 500 nM whereas 10 and 20 μM were fucoxanthin concentrations. These two drugs, with different concentrations were concurrently combined with each other to treat the cancer cells and the control, whereas single drug treatment groups were set at the same time. Thus, there were eleven treatment groups and one control group in MIA PaCa-2 cell line cytotoxicity assay, and eight treatment groups and one control group in the PANC-1 cell line study. Similar to the single treatment inhibitory effect study, HEK 293, the human normal cell line was also cultured, to undertake efficacy testing for all the combination treatments.

4.4.1 Joint Inhibitory Effect of Gemcitabine and Fucoxanthin on MIA PaCa-2 Cells

Based on single drug experiments, the reasonable effect time of the combination drug treatments for MIA PaCa-2 cells, in this study was 72 hours. Figure 24 shows the joint inhibitory effect of fucoxanthin and gemcitabine on MIA PaCa-2 cells. Cells incubated with fucoxanthin (150,250 and 300 nM) alone for 72 hours, did not show any inhibitory effect on the cells. As for cells treated with gemcitabine (25 and 50 nM) alone for 72 hours incubation, the cell viability was about 63% and 42%, respectively. The cell viability in groups treated with combination of gemcitabine and fucoxanthin for 72 hours, is shown in Table 26. Gemcitabine 25 nM combined with 150, 250 and 300 nM fucoxanthin significantly reduced the cell viability by 4%, 10% and 13% respectively, as compared with gemcitabine 25 nM applied to treat cells alone. Likewise, in gemcitabine 50 nM groups, the enhanced inhibitory effect of combining these two drugs was also observed. With the increase of fucoxanthin concentration from 150 to 300 nM, the inhibitory rate increased by 2 %, 4 %, 6 % respectively, in comparison to gemcitabine 50 nM alone. The joint inhibitory effect in gemcitabine 25 nM group was slightly higher than gemcitabine 50 nM group, but in 50 nM group, the proliferation rates of MIA PaCa-2 cells were all over 50%. According to two-way ANOVA statistical analysis, the interaction between fucoxanthin and gemcitabine was very significant ($P< 0.01$). In all

groups, with increasing the concentration of fucoxanthin combined with gemcitabine, the cell viability of MIA PaCa-2 cells was reduced gradually. Hence, fucoxanthin was able to enhance the inhibitory effect of gemcitabine on proliferation of MIA PaCa-2 cells in a concentration dependent manner, even in a very small concentration range.

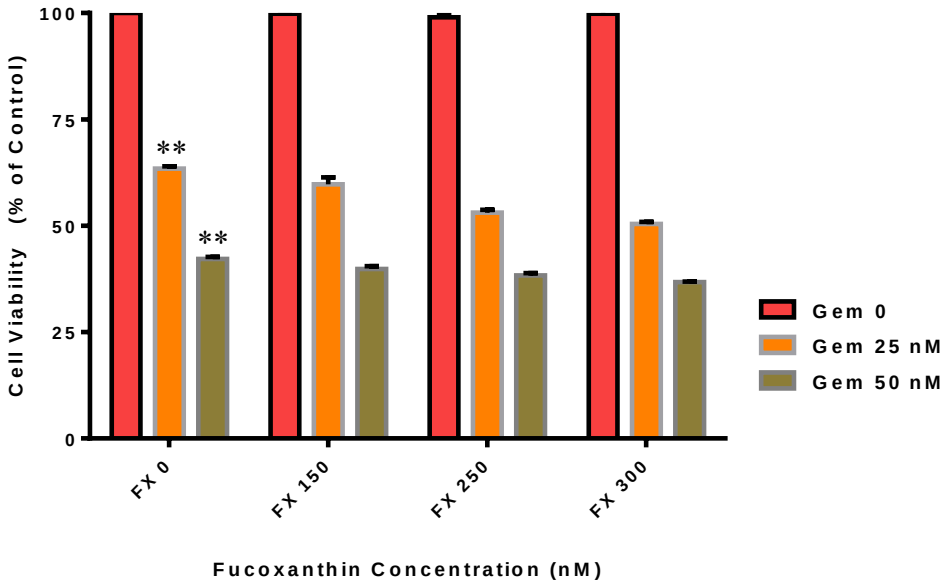


Figure 24: Joint inhibitory effect of gemcitabine and fucoxanthin on the growth of MIA PaCa-2 cells at 72 hours. Data are presented as means ± S.D, n=6. Two asterisks indicated a value significantly different from the control value, $^{**}P < 0.01$ (Student's t test).

Table 26: Cell viability of MIA PaCa-2 cells incubated in the presence of single and combination treatment. Data are presented as means ± S.D, n=6.

Fucoxanthin (nM)	Gemcitabine (nM)		
	0	25	50
0	100 ^{A}	63.45 ± 0.54 ^{Aa}	42.18 ± 0.48 ^{Aa}
150	99.83 ± 0.19 ^{A}	59.76 ± 1.63 ^{Bb}	39.81 ± 0.69 ^{Bb}
250	98.99 ± 0.89 ^{A}	53.06 ± 0.70 ^{Cc}	38.32 ± 0.52 ^{BCc}
300	99.83 ± 0.61 ^{A}	50.40 ± 0.50 ^{Cd}	36.72 ± 0.12 ^{Cd}

In each column, the values with different capital letters indicated that the differences were very significant from each other, $P< 0.01$, and values with different small letters indicated that the differences were significant from each other, $P< 0.05$(Post Hoc, Turkey's test).

4.4.2 Joint Inhibitory Effect of Gemcitabine and Fucoxanthin on PANC-1 Cells

From the above single treatment results, a conclusion could be made that PANC-1 cells were not sensitive to gemcitabine as compared to MIA PaCa-2 cells. Therefore, small concentrations of gemcitabine (50 and 500 nM) and relatively higher concentrations of fucoxanthin (10 and 20 μM) were taken to test PANC-1 cells. The treatment time of these drugs was 72 hours.

The joint inhibitory effect of fucoxanthin and gemcitabine on PANC-1 cells is shown in Figure 25 and the specific cell viability values under different concentrations of drugs are listed in Table 27. After 72 hours of incubation, with 10 and 20 μM fucoxanthin alone, the number of viable cells decreased to 57% and 19% respectively, as compared to the control. However, when PANC-1 cells were cultured in 50 and 500 nM of gemcitabine alone for the same time, only around 10% and 18% cells were killed. Cell viability values

measured from each single treatment group were very significant from one another ($P<$ 0.01). In the combination treatment group (FX 10 μM), there was no significant difference of the cell viability between fucoxanthin 10 μM treatment alone and fucoxanthin 10 μM joint with 50 nM gemcitabine. But when the same concentration of fucoxanthin combined with 500 nM gemcitabine, cell viability significantly decreased by about 7%, as compared to fucoxanthin 10 μM acting alone ($P<$ 0.05). In the fucoxanthin 20 μM group, the joint inhibitory effect of both gemcitabine 50 and 500 nM was significantly higher than 20 μM of fucoxanthin treating cells alone. However, there were no significant differences in cell viability values between 50 and 500 nM gemcitabine combined with fucoxanthin. Besides all this, according to the analytical result of two-way ANOVA, the interaction of fucoxanthin and gemcitabine on inhibition of PANC-1 cells was very significant ($P<$ 0.01). Hence, the combined inhibitory effects of fucoxanthin and gemcitabine can be shown in PANC-1 cells.

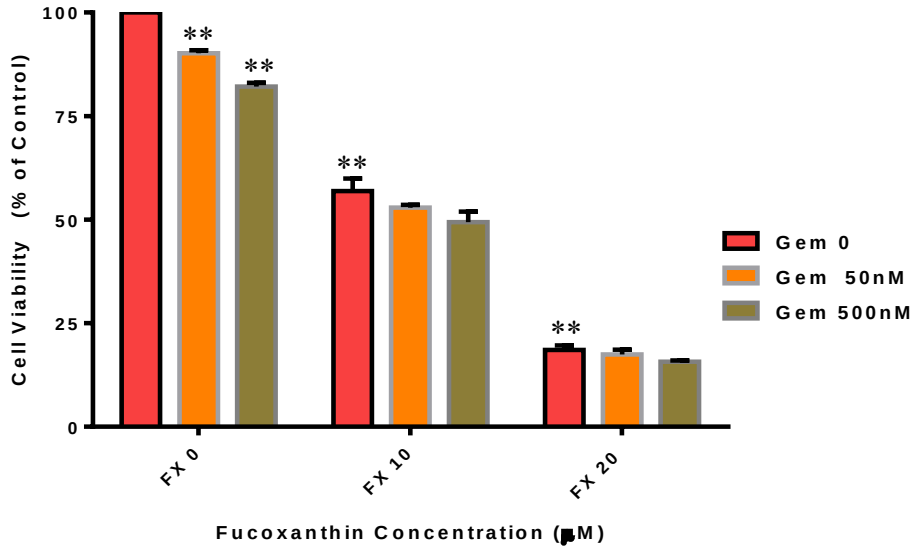


Figure 25: Joint inhibitory effect of gemcitabine and fucoxanthin on the growth of PANC-1 cells at 72 hours. Data are presented as means ± S.D, n=6. Two asterisks indicated a value significantly different from the control value, $^{**}P<$ 0.01 (Student's t test).

Table 27: Cell viability of PANC-1 cells incubated in the presence of single and combination treatment. Data are presented as means ± S.D, n=6.

Gemcitabine (nM)	Fucoxanthin (µM)		
	0	10	20
0	100 ^{A}	56.91 ± 3.00 ^{Aa}	18.54 ± 1.10 ^{Aa}
50	90.20 ± 0.67 ^{B}	52.92 ± 0.63 ^{Aab}	17.42 ± 1.20 ^{ABb}
500	82.15 ± 0.88 ^{C}	49.42 ± 2.51 ^{Ab}	16.01 ± 0.36 ^{Bb}

In each column, the values with different capital letters indicated that the differences were very significant from each other, $P< 0.01$, and values with different small letters indicated that the differences were significant from each other, $P< 0.05$ (Post Hoc, Turkey's test).

4.4.3 Joint Inhibitory Effect of Gemcitabine and Fucoxanthin on HEK 293 Cells

Two groups of fucoxanthin and gemcitabine combination concentrations were tested on HEK 293 cells. The first group was the same as MIA PaCa-2 (Fucoxanthin 150, 250, and 300nM; Gemcitabine 25 and 50 nM) and the other was identical to the concentrations tried on PANC-1 cells (Fucoxanthin 10 and 20 µM; Gemcitabine 50 and 500 nM).

The first group is shown in Figure 26 and Table 28. Gemcitabine significantly inhibited the cell proliferation of HEK 293 cells by 23 % and 49 % after incubation with 25 and 50 nM gemcitabine alone for 72 hours respectively, as compared to control ($P<$ 0.01). Whereas, in HEK 293 cells treated with fucoxanthin 150, 250, and 300 nM, there was no significant suppression by fucoxanthin, as compared to control. The cell viability values were around 100%. Cell viability values, measured under these three fucoxanthin concentrations had no differences. To investigate whether the combination of fucoxanthin and gemcitabine increases the inhibitory effects in HEK 293 cells, fucoxanthin 150, 250

and 300 nM were combined simultaneously to treat cells. Among gemcitabine 25 nM groups (gemcitabine 25 nM alone and combined with 150,250 and 300 nM fucoxanthin), there was no difference between one another. This result meant that the joint inhibitory effect of fucoxanthin (150,250 and 300nM) and gemcitabine (25nM) was the same as gemcitabine alone. Among gemcitabine 50 nM groups (gemcitabine 50 nM alone and combined with 150, 250 and 300 nM fucoxanthin), the joint inhibitory can be seen in gemcitabine combined with fucoxanthin 250 and 300 nM. Cell viability rate was significantly increased ($P< 0.05$) to 4% and 5%, after incubation with 50 nM gemcitabine combined with fucoxanthin 250 and 300 nM for 72 hours, respectively, but fucoxanthin 150 nM did not enhance the cell viability rate, as compared to 50 nM gemcitabine incubated alone. At the same time, there was no difference between each condition when comparing cell viability values, measured among groups with gemcitabine 50 nM in combination with 150, 250 and 300 nM fucoxanthin. This result showed that, fucoxanthin at a low concentration range (150, 250 and 300 nM) combined with gemcitabine (25 and 50 nM), could not inhibit the cell proliferation. Conversely, when 50 nM gemcitabine was combined with fucoxanthin 250 and 300 nM, the cell viability was enhanced.

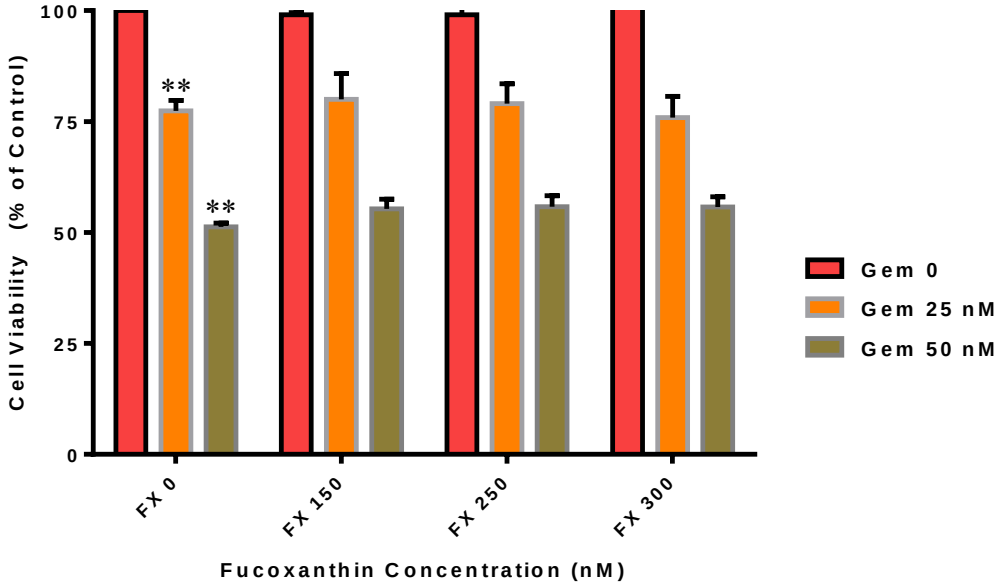


Figure 26: Joint inhibitory effect of gemcitabine and fucoxanthin on the growth of HEK 293 cells at 72 hours (Fucoxanthin 150, 250, and 300nM; Gemcitabine 25 and 50 nM). Data are presented as means ± S.D, n=6. Two asterisks indicated a value significantly different from the control value, $^{**}P< 0.01$ (Student's t test).

Table 28: Cell viability of HEK 293 cells incubated in the presence of single and combination treatment (Fucoxanthin 150, 250, and 300nM; Gemcitabine 25 and 50 nM). Data are presented as means ± S.D, n=6.

Fucoxanthin (nM)	Gemcitabine (nM)		
	0	25	50
0	100 [a]	77.42 ± 2.34 [a]	51.27 ± 0.88 [b]
150	99.08 ± 1.71 [a]	80.00 ± 5.80 [a]	55.32 ± 2.20 [a]
250	99.06 ± 1.23 [a]	79.02 ± 4.49 [a]	55.80 ± 2.49 [a]
300	100.04 ±1.99 [a]	75.92 ± 4.77 [a]	55.73 ± 2.31 [a]

In each column, the values with different capital letters indicated that the differences were very significant from each other, $P< 0.01$, and values with different small letters indicated that the differences were significant from each other, $P< 0.05$ (Post Hoc, Turkey's test).

The results for the other combination test group (Fucoxanthin 10 and 20 µM; Gemcitabine 50 and 500 nM), are shown in Figure 27 and Table 29. Fucoxanthin alone indicated a significant inhibitory effect on the proliferation of HEK 293 cells, with an inhibition rate of 70% and 87% at 10 µM and 20 µM, respectively, as compared to the control ($P< 0.01$). The cell viability of HEK 293 cells was significantly decreased, when they were exposed to 50 and 500 nM gemcitabine for 72 hours (47% at gemcitabine 50nM, 24% at gemcitabine 500 nM, $P< 0.01$). Among gemcitabine 50 nM groups (gemcitabine 50 nM alone and combined with 10 and 20 µM fucoxanthin), fucoxanthin significantly enhanced the inhibitory rate of HEK 293 cells, 25% at fucoxanthin 10 µM and 36% at 20 µM combined with 50 nM gemcitabine respectively, in relation to gemcitabine 50 nM treating cells alone ($P< 0.01$). The cell viability values between fucoxanthin 10 and 20 µM combined with gemcitabine were significantly different ($P< 0.01$). The joint

inhibitory rate at fucoxanthin 20 µM was about twofold that of fucoxanthin 10 µM. Among gemcitabine 500 nM groups (gemcitabine 500 nM alone and combined with 10 and 20 µM fucoxanthin), about 4% cell viability was significantly decreased when cells were treated with gemcitabine 500 nM and fucoxanthin 10 µM concurrently for 72 hours, as compared to the cells with gemcitabine 500 nM incubated solely ($P< 0.05$). The cell proliferation at fucoxanthin 20 µM combined with gemcitabine 500 nM (10%) was significantly lower than the same concentration of gemcitabine incubated alone ($P< 0.01$). Moreover, the cell viability at fucoxanthin 10 µM versus fucoxanthin 20 µM combined with gemcitabine 500 nM, was significantly different when compared with each other ($P< 0.01$). The results indicated that in a high concentration range, fucoxanthin can enhance the inhibitory effect of gemcitabine on the proliferation of HEK 293 cells, in a concentration dependent manner.

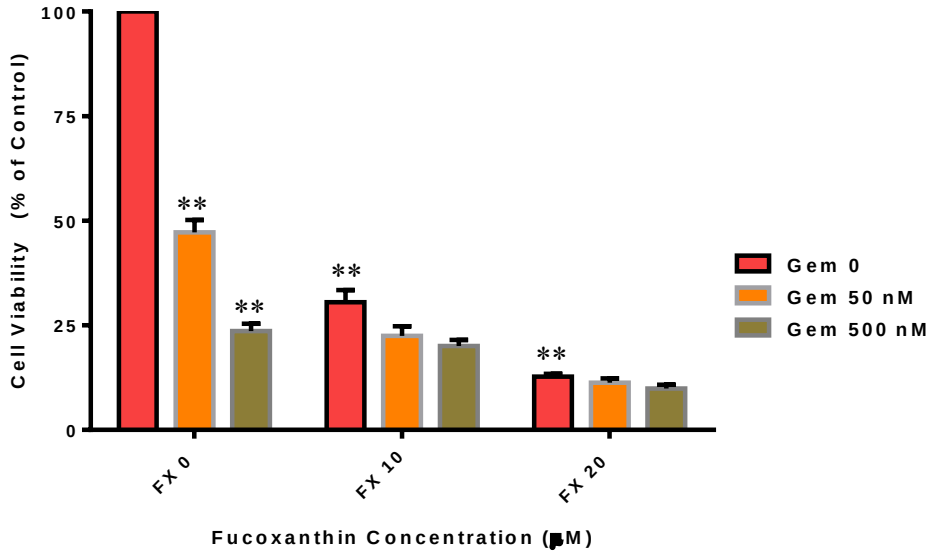


Figure 27: Joint inhibitory effect of gemcitabine and fucoxanthin on the growth of HEK 293 cells at 72 hours (Fucoxanthin 10 and 20 µM; Gemcitabine 50 and 500 nM)**.** Data are presented as means ± S.D, n=6. Two asterisks indicated a value significantly different from the control value, $^{**}P< 0.01$ (Student's t test).

Table 29: Cell viability of HEK 293 cells incubated in the presence of single and combination treatment (Fucoxanthin 10 and 20 µM; Gemcitabine 50 and 500 nM). Data are presented as means ± S.D, n=6.

Fucoxanthin (µM)	Gemcitabine (nM)		
	0	50	500
0	100 ^{A}	47.23 ± 3.00 ^{A}	23.60 ± 1.79 ^{Aa}
10	30.51 ± 2.92 ^{B}	22.47 ± 2.30 ^{B}	20.04 ± 1.45 ^{ACb}
20	12.72 ± 0.66 ^{C}	11.25 ± 1.03 ^{C}	9.89 ± 0.93 ^{Bc}

In each column, the values with different capital letters indicated that the differences were very significant from each other, $P< 0.01$, and values with different small letters indicated that the differences were significant from each other, $P< 0.05$ (Post Hoc, Turkey's test).

4.5 Joint Effects of Gemcitabine and Fucoxanthin on the Alterations of Cell Cycle

Single or combination effects of gemcitabine and fucoxanthin on cell cycle progression of human pancreatic cancer cell lines MIA PaCa-2 and PANC-1 were studied. The cells were treated with either gemcitabine or fucoxanthin alone, or both combined, for 48 hours and 72 hours. Flow cytometry was used to analyze the DNA content in each phase of the cell cycle. Each experiment was performed in triplicate.

4.5.1 Effects of Gemcitabine and Fucoxanthin on MIA PaCa-2 Cell Cycle Progression

Figure 29 shows that 24 hours cell (0% serum) starvation did have effect on MIA PaCa-2 cells, the percentage of G_0-G_1 phase increased about 19%. The S-phase dropped

from 17.28% to 10.62%. And the percentage of G_2-M phase decreased about 11%. The cells synchronized and blocked in G_0-G_1 as a result of starvation.

In single treatment groups, it was found that the distribution of the cell cycle was not changed by incubation with 150 and 250 nM fucoxanthin for either 48 or 72 hours. Fucoxanthin 300 nM did have some effect: the percentage of G_0-G_1 phase increased about 3~4% at both 48 hours and 72 hours, related to the control. Treatment with fucoxanthin 10 μM for 48 hours, resulted in enrichment of MIA PaCa-2 cells in G_0-G_1 phase (from 48.78% to 59.51%), and the percentage of sub-G_1 was increased (from 8.00% to 12.83%), as compared to the control. Here, for fucoxanthin 10 μM was the IC_{50} value, measured from MTT assay. While at 72 hours, fucoxanthin 10 μM not only increased the cells in sub-G_1 phase, but also elevated the percentage of G_2-M phase with a concomitant decrease in number of cells in S phase. Gemcitabine (25 and 50 nM) caused a significant accumulation of cells in S phase, as compared to the control both at 48 hours and 72 hours. The number of cells in G_0-G_1 phase was reduced by gemcitabine. Additionally, sub-G_1 phase accumulation decreased in a time- and dose- dependent manner, and after 72 hours gemcitabine 50 nM treatment, the percentage was even up to 39.32%.

In combination treatment groups, the results indicated that fucoxanthin (150, 250 and 300 nM) combined with gemcitabine treated MIA PaCa-2 cells, for 48 hours, helped the increase in percentage of cells in S phase, but the sub-G_1 phase was not altered, as compared to gemcitabine (25 and 50 nM) alone (Figure 28-A). After 72 hours of the combination treatment, sub-G_1 percentage was enhanced with the corresponding increase in fucoxanthin concentration, with respect to gemcitabine alone. But sub-G_1 phase accumulation in gemcitabine 25 nM combination groups was more distinct than gemcitabine 50 nM groups (Figure 28-B).

Table 30: Cell cycle distribution of MIA PaCa-2 cells after treatment with gemcitabine in the presence and absence of fucoxanthin

Treatment (nM)	G_0-G_1	G_2-M	S	sub-G_1
Control	48.78 ± 0.77/	20.40 ± 0.84/	19.40 ± 0.42/	8.00 ± 0.52/
	65.31 ± 0.56	14.37 ± 0.36	10.56 ± 0.46	7.07 ± 0.81
FX 150	47.82 ± 4.05/	18.70 ± 0.46/	20.41 ± 0.22/	8.18 ± 0.53/
	65.61 ± 0.49	14.92 ± 0.63	8.36 ± 0.36	7.73 ± 0.29
FX 250	48.28 ± 2.61/	18.26 ± 1.79/	19.77 ± 0.35/	8.70 ± 0.14/
	66.61 ± 0.67	14.97 ± 0.39	7.13 ± 0.45	8.35 ± 0.37
FX 300	52.53 ± 2.22/	19.24 ± 1.74/	16.89 ± 1.31/	7.46 ± 0.11/
	67.21 ± 1.09	12.77 ± 0.31	6.49 ± 0.17	10.08 ± 0.37
GEM 25	6.85 ± 0.52/	8.64 ± 0.65/	62.73 ± 0.81/	16.20 ± 1.51/
	37.07 ± 1.04	7.62 ± 4.06	29.40 ± 1.44	25.09 ± 0.78
25+150	6.91 ± 0.61/	9.85 ± 0.60/	65.56 ± 1.51/	15.63 ± 0.69/
	36.80 ± 1.10	6.95 ± 0.45	28.84 ± 1.42	27.03 ± 0.59
25+250	6.66 ± 0.30/	7.76 ± 0.32/	67.88 ± 3.09/	15.79 ± 0.18/
	33.16 ± 0.54	8.84 ± 0.45	28.97 ± 0.96	29.97 ± 0.84
25+300	6.85 ± 0.67/	8.01 ± 0.62/	69.34 ± 0.10/	15.86 ± 0.89/
	36.79 ± 0.61	1.12 ± 0.08	25.01 ± 0.81	33.69 ± 0.22
GEM 50	8.39 ± 0.36/	4.73 ± 0.25/	64.73 ± 2.74/	18.68 ± 0.13/
	11.96 ± 1.09	5.01 ± 0.34	35.60 ± 2.01	39.32 ± 0.65
50+150	9.05 ± 0.88/	3.29 ± 0.34/	69.96 ± 2.56/	14.16 ± 0.84/
	13.84 ± 1.08	7.31 ± 0.20	34.38 ± 0.40	40.93 ± 0.56
50+250	7.12 ± 0.40/	4.58 ± 0.28/	70.46 ± 3.68/	15.32 ± 1.03/
	12.29 ± 1.38	7.61 ± 0.18	35.57 ± 1.82	42.53 ± 0.21
50+300	5.44 ± 0.15/	4.39 ± 0.12/	71.94 ± 3.33/	16.64 ± 1.20/
	15.28 ± 1.52	7.01 ± 0.68	33.56 ± 2.53	43.87 ± 0.59
FX 10 µM	59.51 ± 2.08/	12.72 ± 0.33/	12.12 ± 0.51/	12.83 ± 0.14/
	63.49 ± 1.86	17.85 ± 1.04	4.94 ± 0.15	11.80 ± 0.14

Cells treated with gemcitabine, fucoxanthin or combined of two drugs were harvested, stained with propidium iodide (PI) and analysed at 48 and 72 hours. Data show the percentage of each phase (%) after 48 hours treatment/72 hours treatment, and are

expressed as means ± S.D (n = 3).

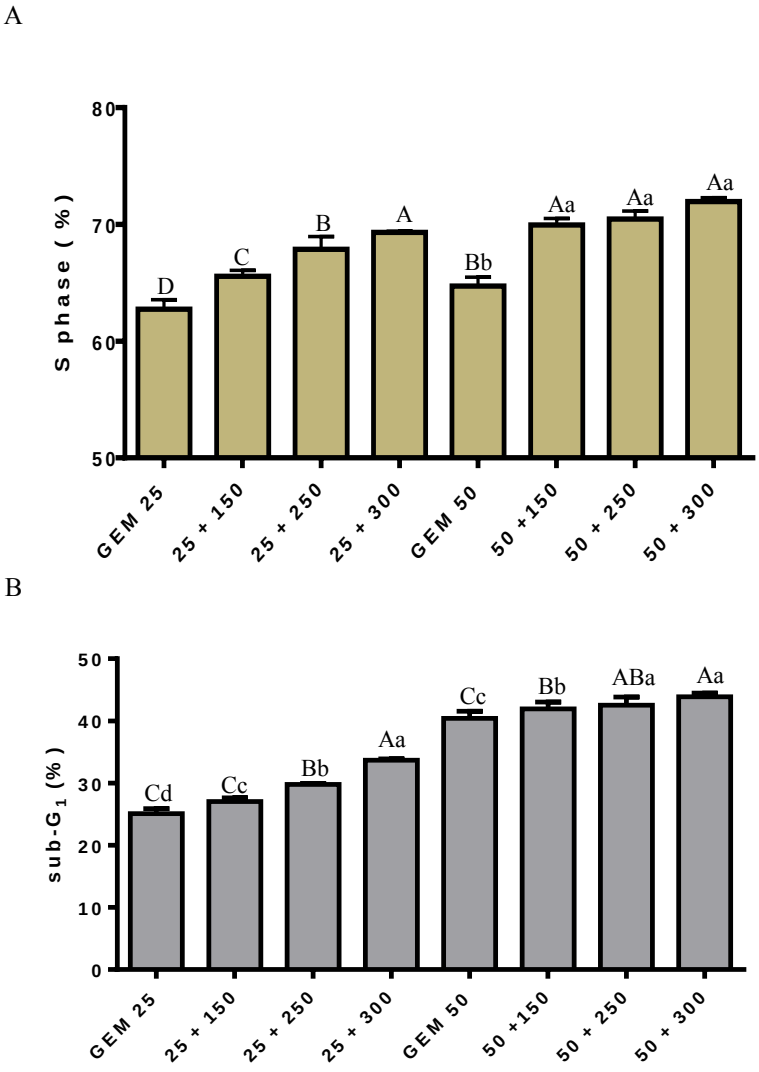
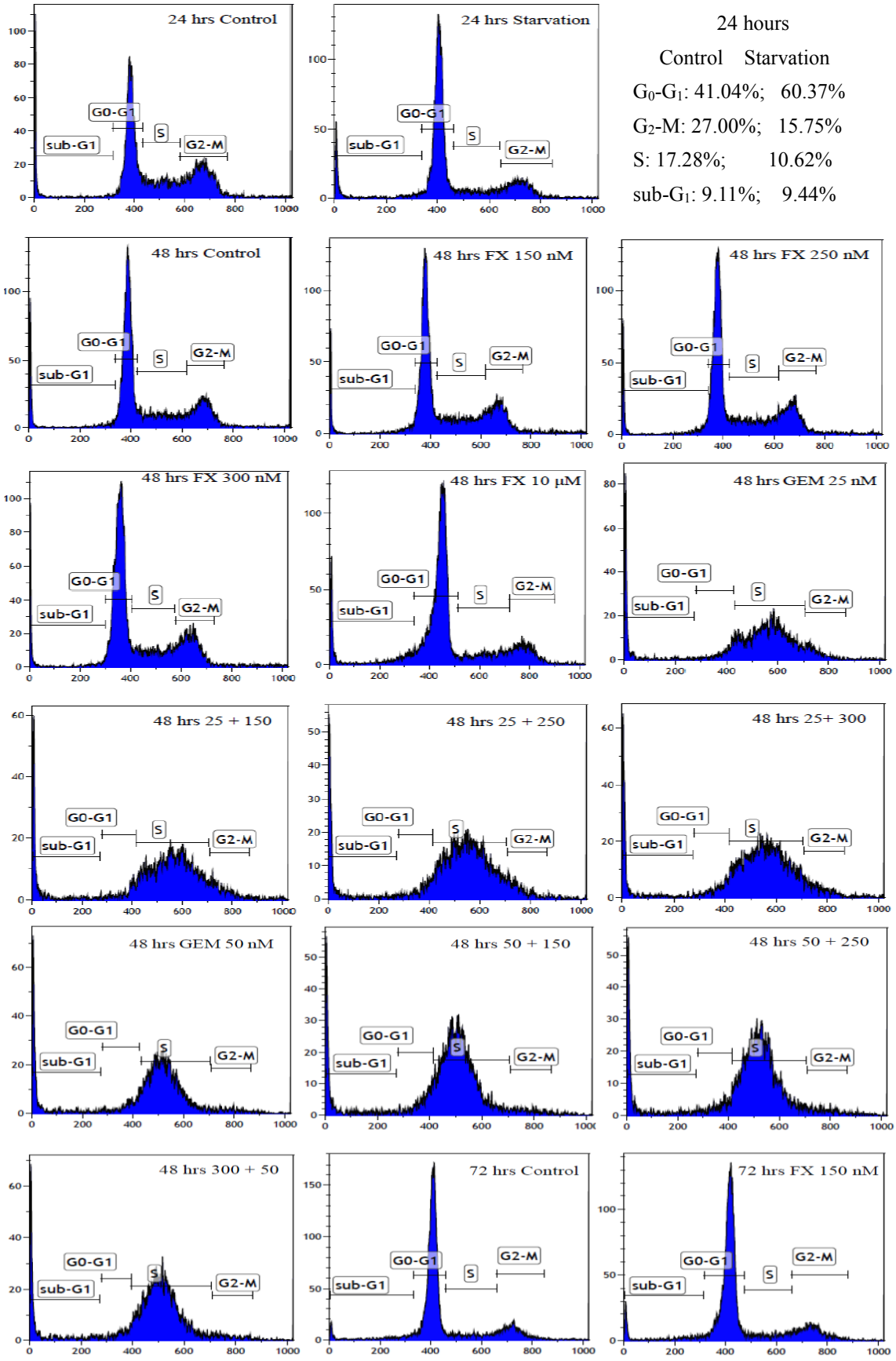


Figure 28: Effects of fucoxanthin on gemcitabine induced cell cycle arrest in MIA PaCa-2 cells. Data are presented as means ± S.D, n=3. Figure 28-A shows percentages of MIA PaCa-2 cells in S phase after treatment for 48 hours; Figure 28-B shows percentages of MIA PaCa-2 cells in sub-G_1 after treatment for 72 hours. There are two groups, namely GEM 25nM group and GEM 50 nM group. In each group, the values with different capital letters indicated that the differences were very significant from each other, $P< 0.01$, and values with different small letters indicated that the differences were significant from each other, $P< 0.05$(Post Hoc, Turkey's test).



24 hours

	Control	Starvation
G_0-G_1:	41.04%;	60.37%
G_2-M:	27.00%;	15.75%
S:	17.28%;	10.62%
sub-G_1:	9.11%;	9.44%

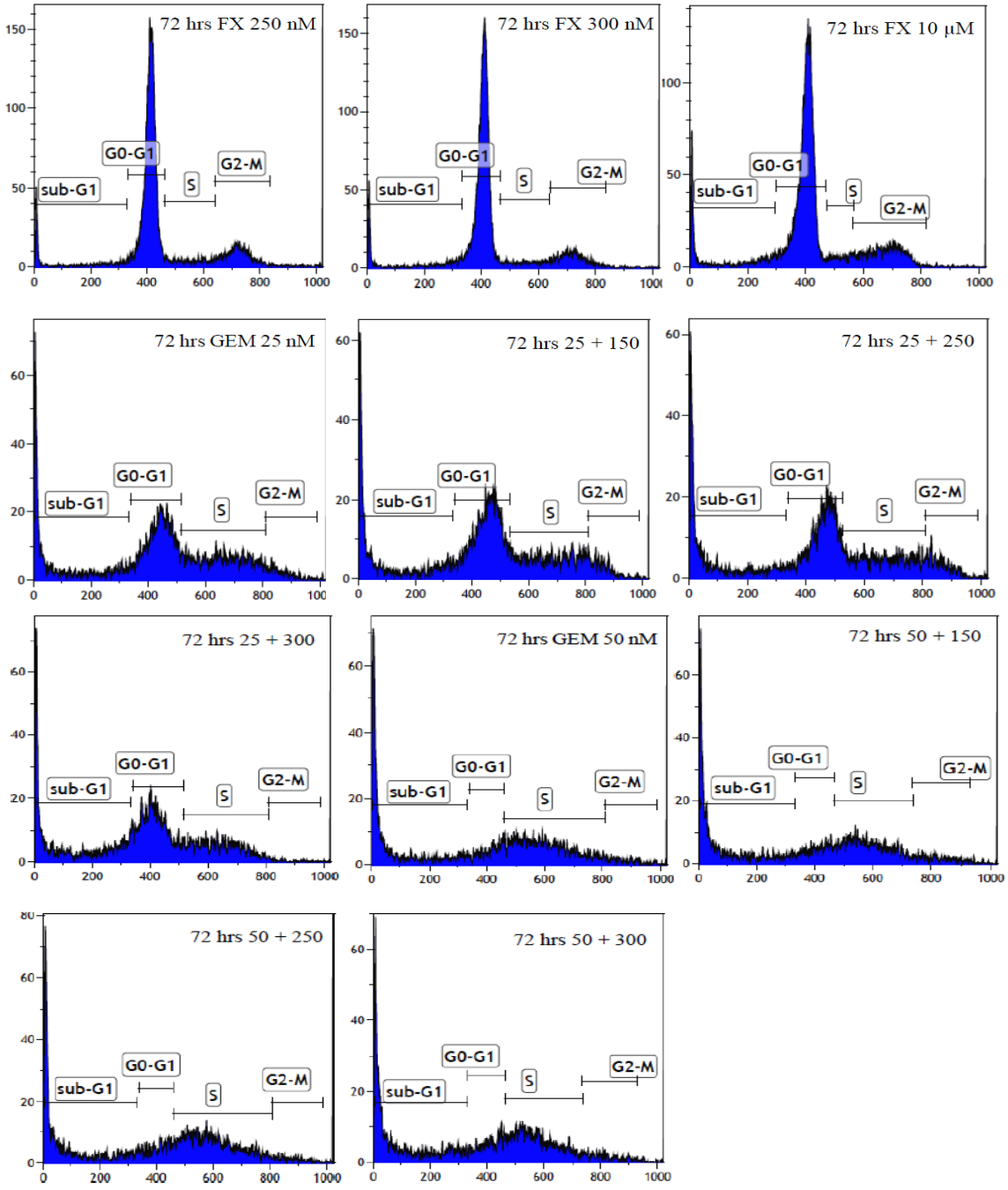


Figure 29: Cell cycle distribution of MIA PaCa-2 cells after treatment with gemcitabine in the presence and absence of fucoxanthin

4.5.2 Effects of Gemcitabine and Fucoxanthin on PANC-1 Cell Cycle Progression

The results showed that cell (0% serum) starvation for 24 hours, synchronized and blocked the PANC-1 cells in G_0-G_1. The percentage of G_0-G_1 phase increased about 19.18%, the cell number in S-phase decreased from 20.73% to 9.62% and G_2-M dropped to 18.03% (Figure 31).

In single treatment groups, the results showed that the accumulation of G_0-G_1 phase was significantly increased by the incubation with fucoxanthin 10 μM and 20 μM for 48 hours as compared to the control, and the S phase decreased with the increase of fucoxanthin concentration. At 72 hours, fucoxanthin 20 μM did not block the cells in G_0-G_1 phase, but induced the increase of sub-G_1 phase up to 24.70%. Fucoxanthin abated the proportion of cells in G_2-M and the percentage of sub-G_1 increased in a time- and dose-dependent manner. Gemcitabine 50 nM, 500nM and 50 μM blocked the cells in G_0-G_1 after 48 hours, and the percentage of sub-G_1 improved in a time- and dose- dependent manner, the same results were shown at 72 hours, except for gemcitabine 50 μM which induced 49.43% cell apoptosis.

In combination treatment groups, with comparison of fucoxanthin 10 μM treated cells alone for 48 hours, gemcitabine 50 nM and 500 nM combined with fucoxanthin 10 μM accumulated more cells in G_0-G_1 phase, and the percentage of sub-G_1 phase was higher. The accumulations of S phase increased with the higher gemcitabine concentrations (50 nM to 500 nM), and G_2-M phase was almost disappeared. However, cells treated with the combination drugs for 72 hours did not show the increased arrest of G_0-G_1 phase, with respect to fucoxanthin 10 μM alone, instead the percentage of sub-G_1 increased at more than 48 hours. As for fucoxanthin 20 μM alone and combined with gemcitabine (50 nM and 500 nM), the combined treatment induced more accumulations of S phase and cell apoptosis, as compared to fucoxanthin 20 μM treating cells alone, at

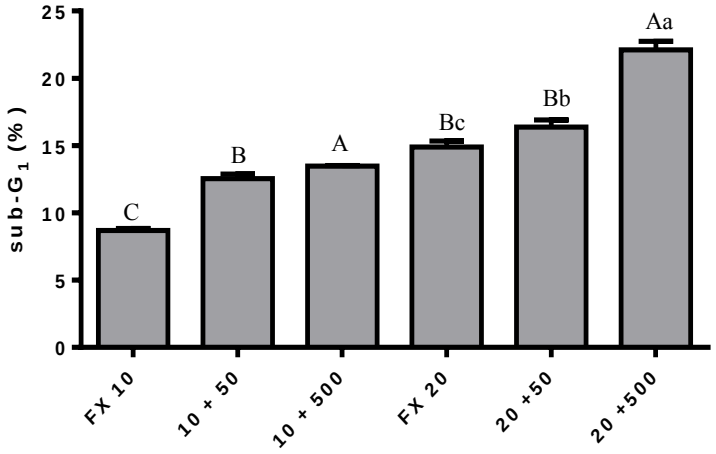
both at 48 hours and 72 hours. Again, G_2-M phase detection was negligible with the exposure of combination treatments across the time points.

Table 31: Cell cycle distribution of PANC-1 cells after treatment with gemcitabine in the presence and absence of fucoxanthin

Treatment	G_0-G_1	G_2-M	S	sub-G_1
Control	51.08 ± 1.47/ 56.65 ± 0.39	23.21 ± 0.80/ 18.83 ± 0.15	16.71 ± 0.62/ 11.61 ± 0.18	5.79 ± 0.87/ 9.00 ± 0.51
GEM 50 nM	72.05 ± 0.26/ 64.57 ± 0.48	2.72 ± 0.12/ 1.96 ± 0.11	11.57 ± 0.35/ 13.73 ± 0.38	11.83 ± 0.37/ 20.22 ± 1.07
GEM 500 nM	70.32 ± 0.14/ 63.82 ± 0.82	3.68 ± 0.30/ 0.57 ± 0.03	13.75 ± 0.14/ 10.65 ± 0.45	11.21 ± 0.34/ 24.42 ± 0.36
FX 10 μM	70.65 ± 1.90/ 70.70 ± 0.66	13.49 ± 0.81/ 8.84 ± 0.33	5.45 ± 0.31/ 3.66 ± 0.39	8.7 ± 0.13/ 15.22 ± 0.43
10+50	76.51 ± 0.37/ 64.98 ± 0.55	2.41 ± 0.20/ 2.74 ± 0.16	7.82 ± 0.68/ 8.60 ± 0.82	12.55 ± 0.34/ 23.51 ± 0.82
10+500	74.23 ± 0.85/ 58.44 ± 0.19	3.30 ± 0.19/ 1.52 ± 0.34	9.78 ± 0.56/ 8.71 ± 0.26	13.48 ± 0.35/ 31.65 ± 0.26
FX 20 μM	63.11 ± 0.94/ 52.29 ± 0.11	14.74 ± 0.56/ 15.90 ± 0.62	4.81 ± 0.57/ 3.44 ± 0.12	14.89 ± 0.44/ 24.70 ± 1.01
20+50	65.25 ± 0.33/ 49.58 ± 0.21	6.06 ± 0.61/ 3.60 ± 0.35	12.45 ± 0.12/ 15.68 ± 0.14	16.37 ± 0.53/ 30.04 ± 0.56
20+500	59.39 ± 0.76/ 49.51 ± 0.30	5.23 ± 0.85/ 3.61 ± 0.45	12.82 ± 0.47/ 13.36 ± 0.33	22.11 ± 0.63/ 33.66 ± 0.53
GEM 50 μM	65.41 ± 0.16/ 25.99 ± 1.08	2.66 ± 0.12/ 5.34 ± 0.24	13.41 ± 0.43/ 17.43 ± 0.23	18.72 ± 0.19/ 49.43 ± 0.92

Cells treated with gemcitabine, fucoxanthin or combined of two drugs were harvested, stained with propidium iodide (PI) and analysed at 48 and 72 hours. Data show the percentage of each phase (%) after 48 hours treatment/72 hours treatment, and are expressed as means ± S.D (n = 3).

A



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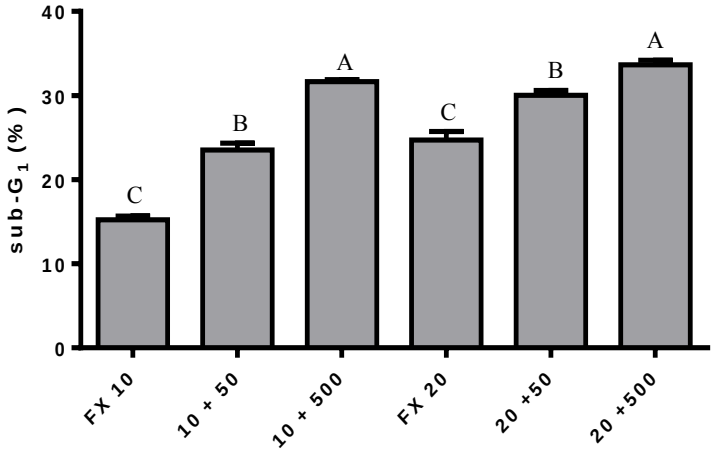
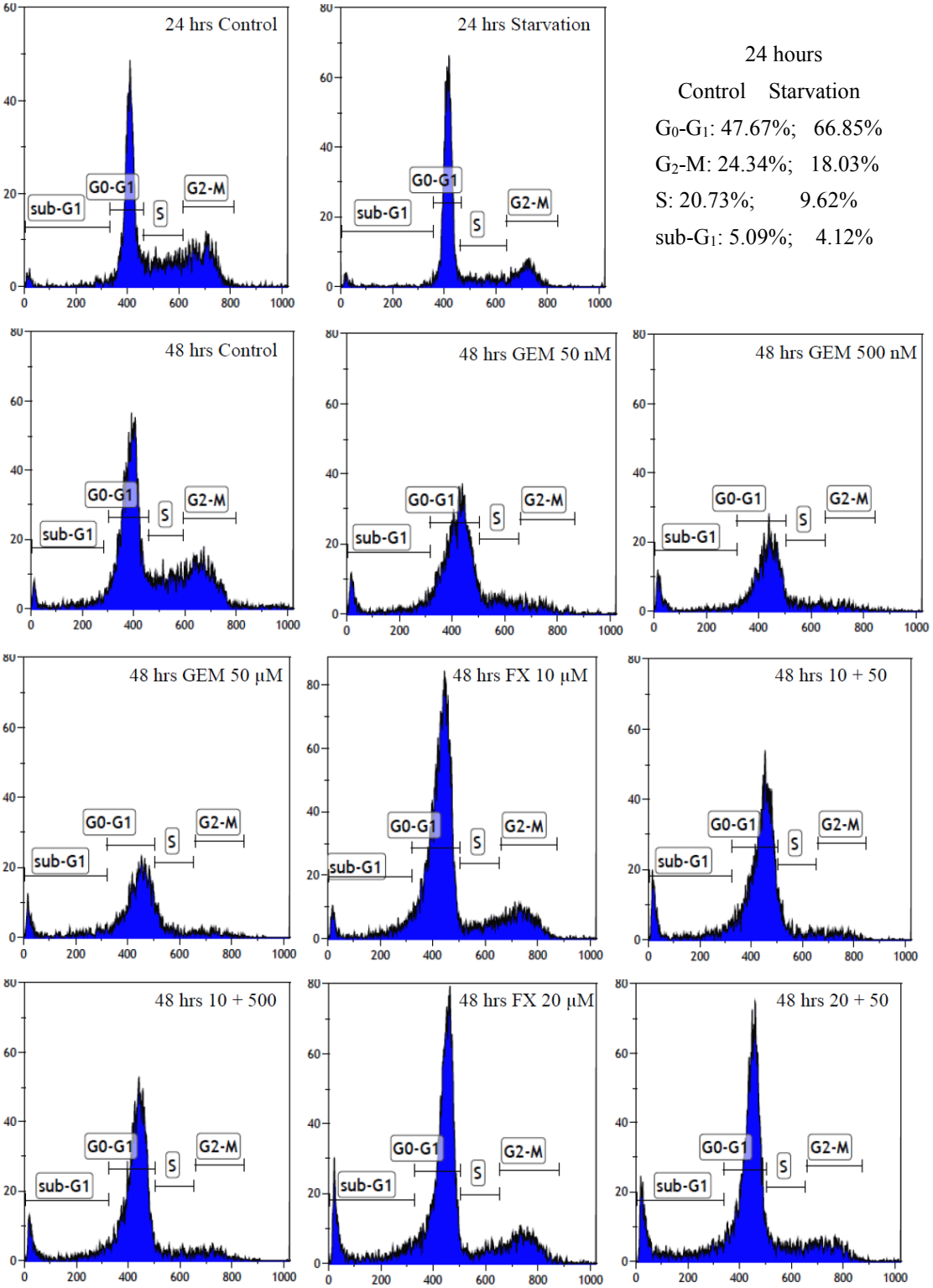


Figure 30: Effects of fucoxanthin on gemcitabine induced cell cycle arrest in PANC-1 cells. Data are presented as means ± S.D, n=3. Figure 30-A shows percentages of PANC-1 cells in sub-G_1 after treatment for 48 hours; Figure 30-B shows percentages of PANC-1 cells in sub-G_1 after treatment for 72 hours. There are two groups FX 10 μM group and FX 20 μM group. In each group, the values with different capital letters indicated that the differences were very significant from each other, $P< 0.01$, and values with different small letters indicated that the differences were significant from each other, $P< 0.05$(Post Hoc, Turkey's test).



24 hours

	Control	Starvation
G_0-G_1:	47.67%;	66.85%
G_2-M:	24.34%;	18.03%
S:	20.73%;	9.62%
sub-G_1:	5.09%;	4.12%

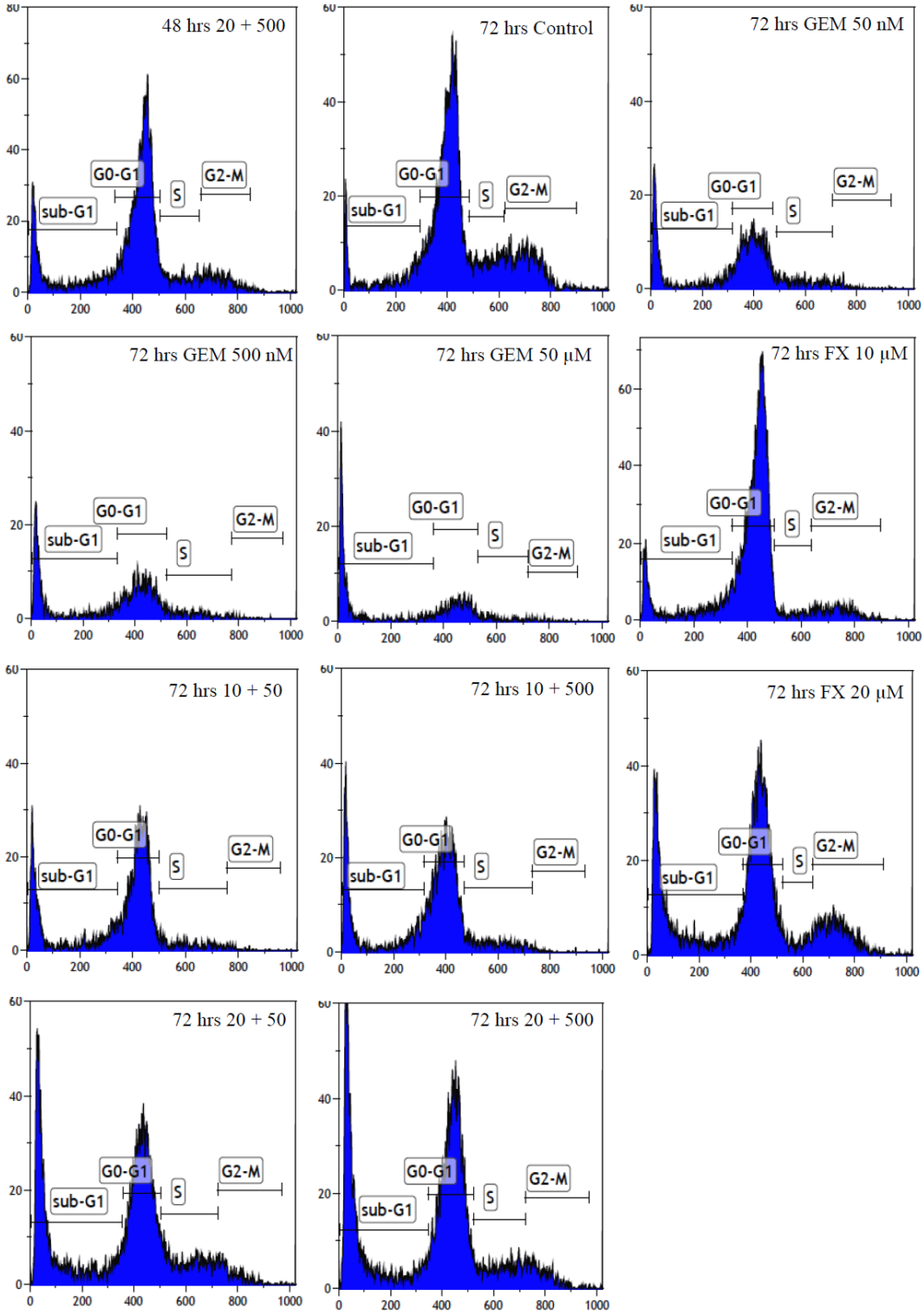


Figure 31: Cell cycle distribution of PANC-1 cells after treatment with gemcitabine in the presence and absence of fucoxanthin

4.5.3 Effects of Gemcitabine and Fucoxanthin on HEK 293 Cell Cycle Progression

Results show that 24 hours (0% serum) starvation did not help arrest the HEK 293 cells in G_0-G_1 phase, conversely the percentage of G_0-G_1 phase was a little lower than control (Figure 32).

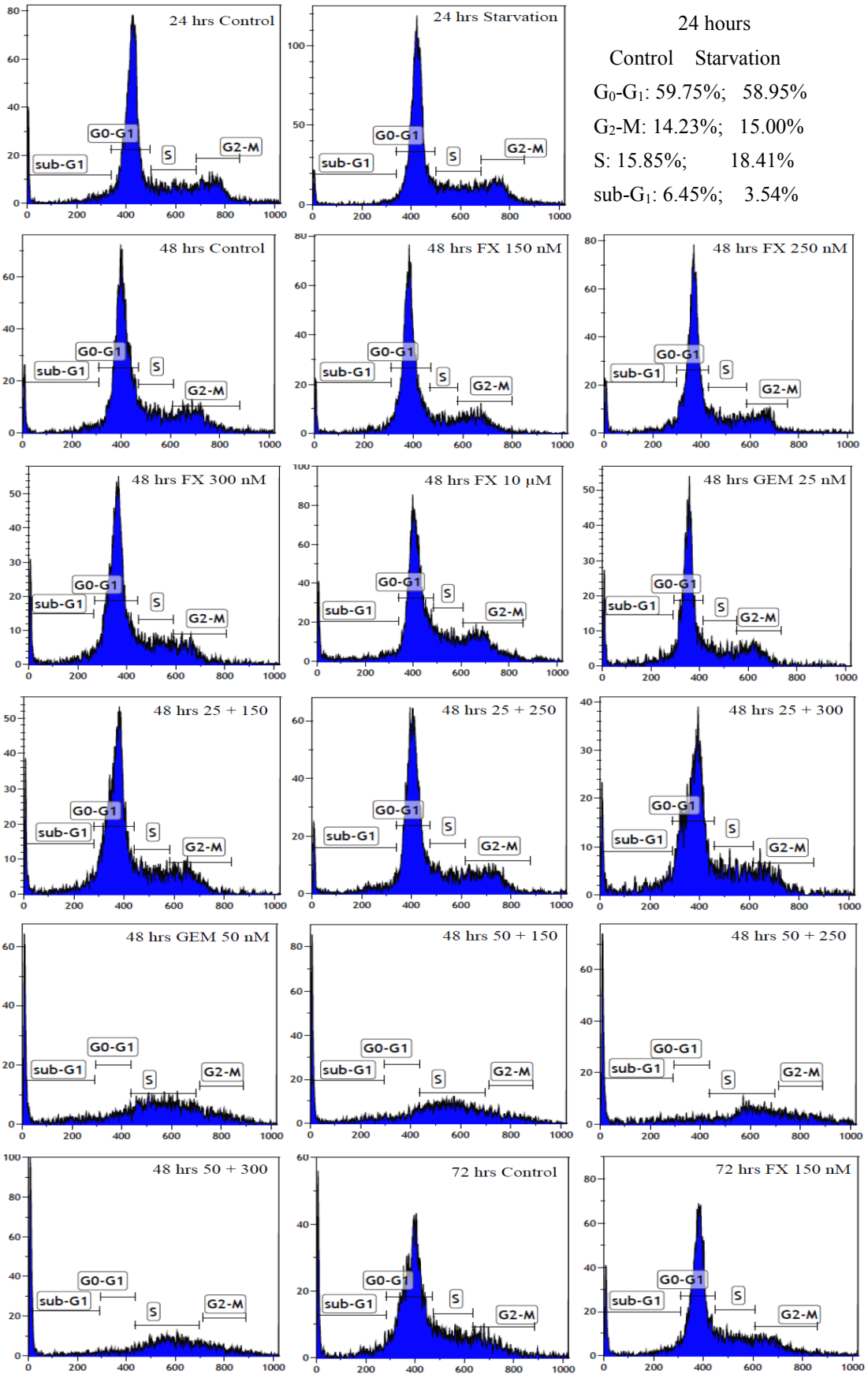
The cell cycle distribution of HEK 293 is shown in Table 32. In single treatment groups, the distribution of cell cycle phases in fucoxanthin (150, 250 and 300 nM) treated HEK 293 cells was similar to that in control cells at both 48 hour and 72 hours after treatment. Fucoxanthin 10 μM treating HEK 293 cells for 48 hours only induced about 4% higher cell apoptosis values than control, and the percentage of G_0-G_1 was even lower than that of controls. G_2-M and S phase were not altered too much in relation to the control. However at 72 hours, fucoxanthin 10 μM was responsible for higher accumulation of cells in G_2-M phase than that of control, and its percentage of sub-G_1 was only about 2% higher than the same concentration treated cells for 48 hours. Gemcitabine 25 nM treating HEK cells for 48 hours could not make any huge difference of the cell cycle distribution in relation to the control, and apoptosis was increased by only about 6%. But gemcitabine 50 nM with the cells cultured for 48 hours, did induced apoptosis (25.28%), and most of the cells were arrested at S phase. At 72 hours, gemcitabine 25 nM increased the percentage of sub-G_1 over a very small range. Gemcitabine 50 nM induced the cell apoptosis up to 38.57% and no cell phase distribution could be observed, implying high level of apoptotic induction in these cells.

In combination treatment groups, fucoxanthin (150, 250 and 300 nM) combined with gemcitabine (25 and 50 nM) for both 48 hours and 72 hours did not make any difference in the distribution of all phases in the cell cycle, as compared to gemcitabine treating cells alone. But the combination treatments induced a slightly lower percentage of cell apoptosis compared to that observed with single gemcitabine treatment.

Table 32: Cell cycle distribution of HEK 293 cells after treatment with gemcitabine in the presence and absence of fucoxanthin

Treatment (nM)	G_0-G_1	G_2-M	S	sub-G_1
Control	57.25 ± 2.06/ 57.98 ± 1.87	13.64 ± 0.93/ 13.53 ± 0.60	15.29 ± 0.18/ 15.34 ± 0.54	7.38 ± 0.32/ 7.14 ± 0.58
FX 150	56.99 ± 0.49/ 57.32 ± 2.95	12.32 ± 1.01/ 12.49 ± 0.35	12.68 ± 0.73/ 15.58 ± 0.93	7.73 ± 0.40/ 7.93 ± 0.81
FX 250	58.21 ± 0.62/ 56.76 ± 3.14	10.83 ± 0.79/ 11.68 ± 0.38	14.75 ± 0.46/ 15.60 ± 0.59	8.35 ± 0.27/ 8.29 ± 0.31
FX 300	56.70 ± 1.45/ 58.83 ± 0.52	8.19 ± 0.94/ 11.63 ± 0.53	16.64 ± 0.34/ 15.51 ± 0.52	9.41 ± 0.40/ 9.03 ± 0.71
GEM 25	56.59 ± 1.87/ 57.39 ± 3.13	11.63 ± 1.10/ 12.00 ± 0.94	13.25 ± 0.07/ 13.91 ± 0.83	13.36 ± 0.16/ 14.33 ± 0.85
25 + 150	56.26 ± 2.12/ 57.00 ± 0.49	12.37 ± 0.29/ 12.82 ± 0.56	14.68 ± 0.42/ 14.17 ± 0.93	12.04 ± 0.03/ 13.33 ± 0.85
25 + 250	57.24 ± 3.23/ 57.82 ± 1.96	9.70 ± 1.13/ 11.92 ± 0.34	14.55 ± 0.73/ 15.64 ± 0.92	12.52 ± 0.01/ 10.44 ± 0.52
25 + 300	56.56 ± 0.55/ 57.44 ± 1.98	4.89 ± 0.14/ 12.17 ± 0.93	14.24 ± 0.19/ 15.73 ± 1.38	12.51 ± 0.45/ 9.88 ± 0.17
GEM 50	11.06 ± 1.04/ -	10.21 ± 0.88/ -	49.43 ± 1.68/ -	25.28 ± 0.11/ 38.57 ± 0.52
50 + 150	10.26 ± 0.98/ -	9.40 ± 0.33/ -	49.42 ± 0.67/ -	24.97 ± 0.95/ 35.60 ± 1.05
50 + 250	9.23 ± 0.72/ -	13.05 ± 0.77/ -	49.64 ± 0.24/ -	24.90 ± 0.30/ 36.42 ± 0.47
50 + 300	7.98 ± 0.77/ -	12.19 ± 0.91/ -	50.34 ± 0.16/ -	24.60 ± 0.28/ 35.36 ± 0.52
FX 10 µM	55.72 ± 1.22/ 57.33 ± 4.90	16.59 ± 0.90/ 16.15 ± 0.17	12.67 ± 0.44/ 9.46 ± 1.71	11.35 ± 0.19/ 13.51 ± 0.95

Cells treated with gemcitabine, fucoxanthin or combined of two drugs were harvested, stained with propidium iodide (PI) and analysed at 48 and 72 hours. Data show the percentage of each phase (%) after 48 hours treatment/72 hours treatment, and are expressed as means ± S.D (n = 3).



24 hours

	Control	Starvation
G_0-G_1:	59.75%;	58.95%
G_2-M:	14.23%;	15.00%
S:	15.85%;	18.41%
sub-G_1:	6.45%;	3.54%

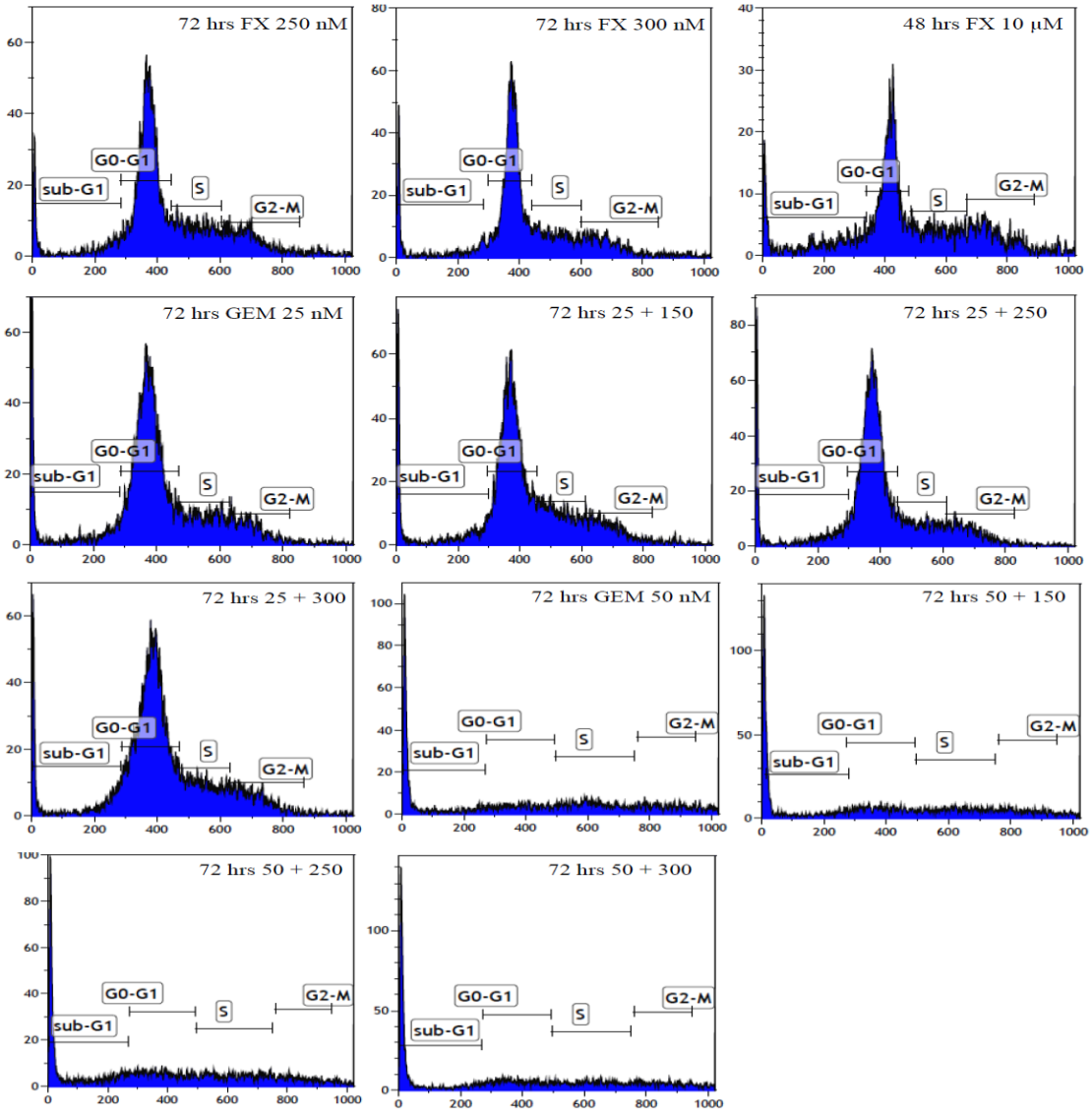


Figure 32: Cell cycle distribution of HEK 293 cells after treatment with gemcitabine in the presence and absence of fucoxanthin

Chapter 5 Discussion

In the current study, study about the inhibitory effect of fucoxanthin alone as well as in combination with gemcitabine to pancreatic cancer cell lines was carried out for the very first time, to the best of my knowledge. Two human pancreatic cancer lines namely MIA PaCa-2 and PANC-1 were employed as a study model to examine the toxicity of fucoxanthin on pancreatic cancer cells and also one human normal cell line, HEK 293, was applied to test the efficacy of this drug on normal cells.

5.1 Cytotoxicity

The MTT assay is a representative method of cytotoxicity assays, which is often used to assess cytotoxicity of substances by exposure to cells. This method is widely used in *in vitro* cell cytotoxicity studies in routine laboratories, with the advantages of reproducibility, ease of performance, economy and safety (Hussain, Nouri, & Oliver, 1993; Fotakis & Timbrell, 2006). Thus, the MTT assay was utilized in this study to assess the cytotoxicity of gemcitabine and fucoxanthin to MIA PaCa-2, PANC-1 and HEK 293 cells.

5.1.1 Cell Seeding Density & Ethanol Effect

According to the cell linearity curve, the cell seeding density used in this study was 50,000 cells/mL. The seeding density was reasonable, owing to this point falling within the linear portion of the curve. Low concentrations of ethanol did not make any effect to the cell viability as compared to the control. The optimal fucoxanthin concentrations used in the study were 20 μM, 10 μM, 150 nM, 250 nM and 300 nM. Thus ethanol being very low in concentration, did not affect the results of the fucoxanthin study part.

5.1.2 Gemcitabine

At present, gemcitabine is used as the first line chemotherapeutic agent for the treatment of pancreatic cancer. However, gemcitabine even combined with other chemo

or radio-therapeutic agents still shows limited efficacy in the alleviation of pancreatic cancer in addition to its severe side effects (Reddy & Patrick, 2008).

The results obtained from this study indicate that the inhibitory effect of gemcitabine on MIA PaCa-2, PANC-1 and HEK 293 cells occurs in a dose-dependent manner. Among these three cell lines, the most remarkable cell morphological changes observed under the inverted microscope was at 72 hours gemcitabine treatment. In the cell line of MIA PaCa-2, gemcitabine treatments at concentrations higher than 100 nM did not further decrease the cell viability, indicating that gemcitabine 100 nM is thought of as the viability plateau. The same results were shown with gemcitabine treating the human normal cell line HEK 293. These two results were consistent with the previous study of gemcitabine on the human cell lines MCF-7, breast adenocarcinoma cell line MDA-MB-231, colorectal adenocarcinoma cell line HT-29, and cervix carcinoma cell line KB-3-1 (Bildstein et al., 2011). The reason of this time consumption by gemcitabine to exert its cytotoxicity may be due to the fact that gemcitabine is a hydrophilic compound, it requires nucleoside transporters (NTs) for its transport into the cells. Once gemcitabine is transported into the cells, it undergoes a series of phosphorylation reactions, converting it to gemcitabine triphosphate (dFdCTP). dFdCTP is the active form of gemcitabine which can then be incorporated into DNA as a false nucleoside, inhibiting DNA polymerase and preventing the detection and repair of DNA repairing enzymes (Reddy & Patrick, 2008). Based on these reasons, the most significant cytotoxicity of gemcitabine against the cell lines used in this study was observed after an incubation for 72 hours. However, a previous study on the human leukaemia cell line CCRF-CEM showed that gemcitabine was able to fully display its cytotoxic effect after only a 48 hour exposure (Bildstein et al., 2011). This difference of time consumption for the complete play of the gemcitabine efficacy reflects the sensibility of different cell lines to gemcitabine. The major mechanisms of the chemo-resistance in human cells include the dysregulated apoptosis and ineffective drug concentrations at the intracellular target sites (Hagmann & Jesnowski, 2009). The reason

for the later mechanism is either lack of sufficient drug uptake into cells or enhanced drug efflux from cells. The reason of the difference of drug uptake maybe different expression levels of the nucleoside transporters (NTs) and enzymes such as deoxycytidine kinase (dCK), which are necessary for the activation of gemcitabine within different cell lines (Mackey et al., 1998). The ATP-binding cassette (ABC) family of transporters is the major transporter responsible for the drug efflux across cellular plasma membranes (Kruh & Belinsky, 2003). Thus the cytotoxicity of gemcitabine is strongly correlated with the expression levels of transporters and enzymes within the cells. In addition to time, the effectiveness of drug concentration is another point of the drug sensitivity measurement. The gemcitabine IC_{50} value determined in this study for MIA PaCa-2 cells was 16.00 ± 0.47 nM, PANC-1 cells was 48.55 ± 2.30 μM and for HEK 293 cells was 48.82 ± 3.27 nM. These three different values indicated a different sensitivity of three cell lines to gemcitabine. In which MIA PaCa-2 cell line was the most sensitive to gemcitabine, HEK 293 was the second, whereas the PANC-1 cell line showed the strongest chemo-resistance than. Previous studies also showed the same result as concluded in this study (Elisa & Valentina, 2004; Hering et al., 2007). PANC-1 was found to have low sensitivity to gemcitabine in an earlier study (Christopher et al., 2004). MIA PaCa-2 and PANC-1 are both primary tumour cells (Moore et al., 2001). The difference of IC_{50} value between these two cell lines could be due to the fact that PANC-1 cells are more resistant and advanced as compared to MIA PaCa-2 cells. And the previous studies reported that even at very low doses (5~10 nM), gemcitabine was able to induce nuclear factor-kappaB (NF-κB) activity, which can promote gemcitabine chemo-resistance (Holcomb et al., 2012). Hence, it can be assumed that the doses (higher than 50 nM) used in this study could significantly activate the NF-κB activity. The expression levels of the transporters and enzymes could be one of the reason for difference between PANC-1 and HEK 293 cells to gemcitabine sensitivity, further work needs to be done to evaluate the role of the transporters and enzymes.

5.1.3 Fucoxanthin

Fucoxanthin is the most abundant carotenoid found in various classes of microalgae and brown macroalgae, which has the benefits of being anti-mutagenic, anti-diabetic, anti-obesity, anti-inflammatory and anti-cancer (Martin, 2015). The special structure of fucoxanthin might be important in reducing the growth and also inducing the apoptosis in cancer cells. The main isomer of fucoxanthin that is found in natural sources, particularly in brown seaweeds, is *trans*-fucoxanthin (Nakazawa & Sashima, 2009). In the current study, fucoxanthin inhibited the viability of human pancreatic cancer cell lines, MIA PaCa-2, PANC-1 and human normal cell line HEK 293 in a dose-dependent manner. Moreover, fucoxanthin time dependently suppressed the proliferation of MIA PaCa-2 and HEK 293 cells. In higher fucoxanthin concentration treatment groups of each cell line, formation of nuclear condensation was evidently clear when observed under the inverted microscope. In the cell line of MIA PaCa-2, the viability of cells incubated in low concentration of fucoxanthin (1.5625 and 3.125 μM) for 72 hours was higher than cell viability under the same concentration at 48 hours. And these two points at 72 hours were almost overlapped with the same concentration points at 24 hours. One possible reason for this might be that the cellular uptake of fucoxanthin at 24 hours is not enough to inhibit the cell proliferation, owing to the fact that most of the fucoxanthin is still in the cell culture medium. However at 48 hours, most of the fucoxanthin has entered the cells and then plays its role inside the cells. Since the quantity of fucoxanthin is limited, only some of the cells are inhibited, and the rest of the cells continue to proliferate. Additionally, fucoxanthin and *trans*-fucoxanthin have been proven to be degraded under the conditions of heating from 25 to 100 ^{o}C in the absence of light and air (Zhao, Kim, & Pan, 2014). All *trans*-fucoxanthin levels have been demonstrated to decrease owing to degradation, after the incubation for 12~24 hours (Nakazawa & Sashima, 2009). The formation of *cis*-isomers of fucoxanthin was promoted with the increase in heating temperature, which resulted in the decrease in fucoxanthin antioxidant activity (Kawee-ai, Kuntiya, & Kim,

2013). The antioxidant property was thought to be one of the major reasons of the anticancer effect of fucoxanthin (Kumar et al., 2013). NF-κB activity has been demonstrated to be inhibited by antioxidants (Campo et al., 2008). Thus another reason of overlapping of the cell viability points between 24 and 72 hours might be that fucoxanthin kept in the 37^{o}C incubator, leads to some of its proportion being degraded to its *cis*-isomers. This also happened in high fucoxanthin concentration groups, since its content is higher than low fucoxanthin concentrations, the remaining portion of fucoxanthin is still stable and could exert its effect normally. Moreover, although the *cis*-isomers of fucoxanthin did inhibit the growth of many cell lines, the incorporation rate of these forms into cells was found to be slower than the all-*trans* (Nakazawa & Sashima, 2009). Hence, when the *cis*-isomers are formed in the culture medium, it takes time to enter into the cells. This might be one of the reason to explain the big difference in cell viability at the same concentration of fucoxanthin, between 24 hours and 48 hours treatment incubation, due to the fact that most of the *cis*-isomers might incorporate into the cells after over 24 hours culture.

It has been suggested that some part of fucoxanthin is hydrolysed to fucoxanthinol by the extracellular enzymes (might be lipase and cholesterol esterase) during the uptake by human colonic cancer cell line Caco-2 (Sugawara et al., 2002). Then fucoxanthinol is further converted into amarouciaxanthin A in HepG2 cells. These two fucoxanthin metabolites were found to reduce the viability of human prostate cancer cell line PC-3 (Akira & Tatsuya, 2004). Fucoxanthinol was also found to have more efficient anti-proliferative effects than fucoxanthin (Akira & Tatsuya, 2004; H. Zhang et al., 2015). Therefore, fucoxanthin might also be further deacetylated into fucoxanthinol and amarouciaxanthin A inside the cells to suppress cell growth. The IC_{50} values of fucoxanthin at 72 hours on the proliferation of the three cell lines (MIA PaCa-1, PANC-1 and HEK 293) were 8.74, 10.58, and 8.28 μM. This result indicated the level of sensitivity of these three cell lines against fucoxanthin (HEK 293 > MIA PaCa-2 >

PANC-1). The difference in sensitivity for different cell lines might be due to the different content of hydrolytic enzymes in the different cells. Since fucoxanthinol was thought to have a stronger anti-cancer effect than fucoxanthin. Meanwhile, the degradation degree of fucoxanthin on account of temperature, light and other factors, maybe additional reasons that fucoxanthin is not a stable compound.

In the current study, ethanol was used as the delivery vehicle of fucoxanthin. Because ethanol is easy to volatilize, in the process of adding fucoxanthin treatment, some of the fucoxanthin might be lost during the volatilization process of ethanol. This is a factor influencing the efficacy of fucoxanthin. A previous study found that fucoxanthin delivered by ethanol/FBS (1:9) had a higher cellular uptake than by ethanol alone, in the murine embryonic hepatic BNL CL.2 cells with incubation for 72 hours (Liu, Liang, & Hu, 2011).

The present study results indicated that fucoxanthin concentration dependently suppressed the proliferation of cells after 24 hours of treatment. The morphologic changes were simultaneously observed in most of the treatment groups. However, gemcitabine was found to have significant cytotoxic effects on these three cell lines only after 72 hours incubation. The morphological changes observed under the inverted microscope were not remarkable on the previous two days' time points. This difference in effective time for these two drugs against the same cell lines, might be due to the diverse cellular intake methods of the cells. Additionally, owing to their various chemical properties and mechanisms of action. Gemcitabine is a hydrophilic compound while fucoxanthin is easy to be absorbed by cellular lipids. In order to complete the uptake of gemcitabine, it should undergo a transportation process by means of specific transporters, and then further being activated by enzymes, to its recognized active form (dFdCTP). And even gemcitabine efflux from cells happens in this process. However, fucoxanthin itself plays the role of inhibition, and its conversion to fucoxanthinol and amarouciaxanthin A does not take too much time, with reference to fucoxanthin has been found to hydrolyse to fucoxanthinol

in PC-3 cells within 4 hours of culture (Akira & Tatsuya, 2004).

5.1.4 Gemcitabine Combined with Fucoxanthin

Gemcitabine alone is not a satisfactory option in the clinical treatment of pancreatic cancer. Gemcitabine in combination with other drugs has been demonstrated to reduce the resistance of pancreatic cancer cells to gemcitabine. However, combination chemotherapy did not considerably increase the survival rates of pancreatic cancer patients and was accompanied by numerous side effects in most of the clinical trials (Jin, Sun, & Miao, 2013). Thus gemcitabine combined with natural compounds with low or no cytotoxicity is worthy of attention. In the present investigation, gemcitabine was used with fucoxanthin simultaneously, to explore their combined effects on pancreatic cancer cells. According to the single treatments, the optimal incubation time decided for combination treatments was 72 hours. At this point, gemcitabine well exerted its significant inhibition effect, and fucoxanthin also showed a time-dependent inhibition manner after incubation in this study.

While studying the reduction in MIA PaCa-2 cell viability through the combined effect of fucoxanthin and gemcitabine, fucoxanthin 150 nM and 250 nM alone did not significantly increase or decrease the inhibition rate of the cells. Thus a small concentration range of fucoxanthin does not affect too much of the cell viability of MIA PaCa-2 cells, as compared to control. To the best of my knowledge, applying a fucoxanthin concentration range under 1 μM in an anti-cancer research was performed for the first time (Kumar et al., 2013). The aim of using small fucoxanthin concentrations is to find whether fucoxanthin is able to effectively improve the cytotoxicity of gemcitabine even at low concentration doses. Moreover, there is a consideration of cytotoxicity of fucoxanthin to the cells, though it is a natural compound and its cytotoxicity is selective. The result demonstrates that the combination effect of fucoxanthin and gemcitabine on the reduction of MIA PaCa-2 cell viability is significantly higher than either gemcitabine (25 and 50 nM) or fucoxanthin (150, 250 and

300 nM) treated cells alone. A synergistic effect was observed in the group of gemcitabine 25 nM combined with fucoxanthin. However, in the results of gemcitabine 50 nM joint with fucoxanthin, only an additive effect could be observed. A possible explanation for this observation might be that gemcitabine at the concentration of 50 nM is too effective and it is more cytotoxic. So that low doses of fucoxanthin cannot provide additional effect on top of it.

PANC-1 cells were found to be resistant to gemcitabine. Relatively, PANC-1 cells were sensitive to fucoxanthin treatment. Thus, fucoxanthin (10 and 20 μM) played a leading role in the combination effect, as compared to gemcitabine, in the suppression of PANC-1 cells. Fucoxanthin was shown to significantly enhance the inhibitory effect of gemcitabine. The cell viability under the combination treatment was significantly lower than fucoxanthin and gemcitabine treated the cells alone. But the combination effect was not improved synergistically by these two drugs. And even gemcitabine did not dose-dependently increase the inhibitory effect of fucoxanthin in both of the combination groups. There was no significant difference between the cell viability values when gemcitabine 50 nM and 500 nM was mutually combined with fucoxanthin 10 μM or 20 μM.

In the MIA PaCa-2 combination treatment study, even fucoxanthin 150 nM, 250 nM and 300 nM made significant differences of the cell viability among one another when combined with gemcitabine respectively. However, gemcitabine 500 nM, tenfold of the gemcitabine 50 nM, did not make any significant difference between each other when combined with fucoxanthin separately. One of the reasons to explain this result might be because of the chemo-resistance of PANC-1 cells to gemcitabine. Gemcitabine 500 nM solely treated the PANC-1 cells for 72 hours, led to 8 % increase of cell inhibition rate only, in comparison to gemcitabine 50 nM. And even fucoxanthin 10 μM induced almost half percentage of the cell death when cultured with the cells for 72 hours, not to mention fucoxanthin 20 μM kept less than 20% of cell viability compared with control. Meanwhile

PANC-1 cells are resistant to gemcitabine, owing to the activity of some transcription factors like NF-κB, that might be promoted during the incubation process. Thus the effect of gemcitabine was not much obvious when combined with fucoxanthin. However, fucoxanthin and fucoxanthinol have been known to downregulate the activity of NF-κB (K.-N. Kim & Heo, 2010). Based on this finding, it can be speculated that fucoxanthin combined with gemcitabine inhibits the activity of NF-κB and helps reduce the chemo-resistance of cells to gemcitabine. It is possible that the number of gemcitabine transporters and enzymes are limited, and therefore the inhibition efficacy of gemcitabine 50 nM and 500 nM is at its plateau. Hence their combination effects were not significant in this study.

Conversely, MIA PaCa-2 cells are sensitive to both gemcitabine and fucoxanthin. Thus even the major effect is reflected by gemcitabine, the impact of fucoxanthin can still be observed. Another hypothesis is that high concentration of fucoxanthin might inhibit the activity of gemcitabine transporters and deacetylation enzymes. Some part of the fucoxanthin will be hydrolyzed by extracellular enzymes and most of the fucoxanthin will be absorbed by cellular lipids. Meanwhile, the cellular intake of gemcitabine also needs transporters, but the concentration of fucoxanthin is extremely higher than gemcitabine. Thus it can be supposed that the activity of gemcitabine transporters and deacetylase enzymes will be inhibited by the extracellular enzymes and cellular lipids which are responsible for the transportation and hydroxylation of fucoxanthin to some degree.

In the current study, it was found that low doses of fucoxanthin treatment alone (150, 250, 300 nM) could not inhibit the growth of HEK 293 cells with exposure for 72 hours. However, high concentrations of the fucoxanthin (10 and 20 μM) significantly suppressed the proliferation of HEK 293 cells up to more than a half of the total cell number, as compared to control. Low concentrations of fucoxanthin did not promote the inhibitory effect of gemcitabine 25 nM. Interestingly, in gemcitabine 50 nM groups, low doses of fucoxanthin helped reduce the cytotoxicity of gemcitabine. More HEK 293 cells survived

under the incubation with combination treatment for 72 hours in relation to gemcitabine 50 nM cultured the cells alone. However, this result was not found in high doses fucoxanthin combination groups. High concentration levels of fucoxanthin could enhance the inhibitory effect of gemcitabine to HEK 293 cells depending upon its concentration. Fucoxanthin has been demonstrated to have no inhibitory effect on human lymphocyte cells, uninfected leukaemia cell lines and PBMCs (human peripheral blood mononuclear cells), over a certain concentration range (Ishikawa et al., 2008). The results indicated that fucoxanthin still has toxicity to normal cells when used in high doses, though its cytotoxicity is selective. Moreover, fucoxanthin and gemcitabine when combined with each other must be in a proper concentration range in order to create synergistic effects against cancer cells but to exert toxicity to normal cell lines as little as possible.

5.2 Cell Cycle Distribution

The arrest of cell cycle progression, inducing of apoptosis, or both, are the three reasons of the inhibition of cancer cell proliferation (Hsiao, Hsieh, & Kuo, 2007). The effects of gemcitabine and fucoxanthin on the cell cycle were examined in this study.

5.2.1 Cell Synchronization by Serum Starvation

Cell synchronization induced by serum starvation has been widely used in cell cycle studies from the time when Pardee established the concept of the restriction point (Pardee, 1974). Serum elimination from culture medium is supposed to reduce basal cellular activity. Cells cultured after starvation are more homogenous, owing to their withdrawing from the cell cycle and entrance into quiescent G_0-G_1 phase. G_1 phase plays an important part in the cell cycle and determines whether a cell commits to division or to leave the cell cycle (Corinne & Erik, 1996). Moreover, serum elimination helps reduce the analytical interference and offers more reproducible experimental conditions (Sergej & Alexander, 2011). Cell starvation carried out for the three cell lines in the present study was FBS (fetal bovine serum) free in culture medium and the time set was 24 hours. For

the two pancreatic cancer cell lines, 24 hours serum-free starvation resulted in good synchronization. However, for HEK 293 cells, the same starvation conditions did not affect the cell line, with the percentage of cells in G_0-G_1 after starvation lower than that of control (normal culture in complete medium). This result confirms the high level of aggressiveness of HEK 293 cells and low level of response to the growth factors in the medium. Appropriate serum concentration is important for starvation. High serum concentration results in imperfect starvation. If the starvation is not absolute, some leakage will occur. The cells will be able to slowly accumulate material and lead to an initiation of S phase during the process of low serum starvation (Cooper, 2003). But a previous study indicated that a certain concentration of serum which could result in a slight cell leakage under the period of serum starvation would be the most appropriate serum concentration (Song & Lu, 2003). Moreover, the starvation time is another important factor. Long starvation time leads to the deleterious effects on cell survival and results in massive DNA fragmentation (apoptosis) (Kues et al., 2000). However, if the time is not enough, it could not help the leakage cells complete the second time cycle and may then block them in G_0-G_1 phase. For this result, it can be speculated that the starvation time and the serum concentration are not suitable for HKE 293 cells. The serum concentration might be too harsh and the starvation time might be too short for the cells. Additionally, the dependence on serum varies with different cell lines. For most of the cancer cells, their dependence on serum is low (Song & Lu, 2003). Hence, this maybe one of the reasons to explain the difference of the starvation effect under the same conditions between pancreatic cancer cells (MIA PaCa-2 and PANC-1) and human normal cells (HEK 293).

5.2.2 Effects of Gemcitabine on Cell Cycle Distribution

The anti-cancer activity of gemcitabine is primarily performed by impairing DNA synthesis. It results in the cytostasis owing to the block of cell cycle in G_0-G_1 or S phase (Lorusso & Di Stefano, 2006). Subsequently, cells may go through apoptosis or undergo

mitotic catastrophe upon escaping the cell cycle blockage, which will finally lead to cell death (Mose et al., 2003). Gemcitabine has been demonstrated to block some human solid tumour cells in S phase, relying on the exposure time and gemcitabine concentration (Cronauer et al., 1996). However, some studies suggested that gemcitabine arrest cells in G_0-G_1, G_1, early S or S phase only depending upon the gemcitabine concentration (Mose et al., 2003).

In the present study, gemcitabine was found to dose-dependently arrest the MIA PaCa-2 cells in S phase after both 48 and 72 hours exposure. However, after the exposure for 72 hours, the accumulations of cells decreased and the percentage of sub-G_1 increased in a dose-dependent manner. Sub-G_1 is an index of the apoptotic DNA fragmentation (Yu et al., 2011). This result indicated that gemcitabine first blocked MIA PaCa-2 cells in S phase and then induced the cell apoptosis. The same result was found in HEK 293, but gemcitabine at 25 nM only induced minor cytotoxic effects on HEK 293 cells. Whereas, gemcitabine was found to arrest PANC-1 cells in G_0-G_1 phase after culture for 48 and 72 hours. Gemcitabine induced the apoptosis of PANC-1 cells in a time- and dose- dependent manner. Taken together, the current results in MIA PaCa-2, PANC-1 and HEK 293 cells are consistent with the previous reports, namely gemcitabine induces G_0-G_1 and S phase arrest and subsequently undergoes apoptosis. Moreover, all these results match well with the cytotoxicity analysis results.

Gemcitabine time and dose dependently induced cell apoptosis potentially because of the accumulation of dFdCTP (gemcitabine triphosphate), which is the active metabolite of gemcitabine. dFdCTP has been demonstrated correlating with the toxicity of gemcitabine. It leads to a (deoxy)ribonucleotide pool imbalance and thereby induces cell apoptosis (Van Moorsel et al., 2000).

The cell cycle is regulated by a complex regulation network of cyclins, cyclin-dependent kinases (Cdks) and Cdks inhibitors (Hunter & Pines, 1994). Cyclins are a family of cell cycle oscillator proteins, repeatedly express and degrade during the cell

cycle process. Cyclins binding with CDKs acts as the engines of the cell cycle. CDK inhibitors are able to bind complexes of cyclins and CDKs and inhibit their cell cycle accelerator function (Arora et al., 2011). Thus, in this study gemcitabine blocks the cells in G_0-G_1 or S phase, that might be because of the downregulation of cyclins (e.g., cyclin D1, cyclin E) and Cdks (e.g., Cdk2 and Cdk4) as well as due to the upregulation of the Cdks inhibitors (e.g., p21 and p27). Because of the various regulation levels in different cell lines, the effects of gemcitabine are different.

The resistance of cancer cells to gemcitabine is known to be determined by the involvement of many factors instead of a single factor (Masanori et al., 2005). Among them, the alteration of apoptosis regulating genes is a key factor. COX-2 has been found to be strongly expressed in several pancreatic cancer cell lines and it is related to the resistance to the cytotoxic effect by gemcitabine. Additionally, p8 (an anti-apoptotic protein) has been shown to have high expression in PANC-1 cells (Rathos & Joshi, 2012). Meanwhile, pro-apoptotic proteins like BNIP3 are also known to be important in gemcitabine sensitivity in pancreatic cancer cells *in vitro*. BNIP3 is a member of the BH3-only subfamily of Bcl-2 family proteins, which antagonizes the activity of pro-survival proteins such as XIAP, cIAP2, Bcl-2 and Bcl-xl, to promote apoptosis (Masanori et al., 2005). The different suppression levels of the related proteins might be one of the mechanisms explaining different gemcitabine sensitivity against these three cell lines in this study.

In short, gemcitabine confers cytostatic and/or cytotoxic effects on MIA PaCa-2, PANC-1 and HEK 293 cells. dFdCTP accumulation, cell cycle proteins and apoptosis regulating genes may be the mechanisms to explain the sensibility of cells to gemcitabine. But there is no clear result in this study that confirm whether gemcitabine arrests the cells based on the gemcitabine concentration or also affected by the exposure time of the treatments.

5.2.3 Effects of Fucoxanthin on Cell Cycle Distribution

Carotenoids have been proven to inhibit tumour cell growth by inducing cell cycle arrest at the G_1 phase and/or apoptosis (Das et al., 2006; Satomi & Nishino, 2007). Fucoxanthin has been suggested to accumulate the cells in G_0-G_1 of different cell lines in various studies (Kumar et al., 2013). In the present study, higher concentrations of fucoxanthin were found to arrest the cells in the G_0-G_1 phase. Fucoxanthin (10 μM) induced the arrest of MIA PaCa-2 and PANC-1 cells in G_0-G_1 in a time-dependent manner. The effect of fucoxanthin (20 μM) was mainly shown in the induction of PANC-1 cells apoptosis instead of G_0-G_1 arrest, after treating the cells for 72 hours. In HEK 293 cells however, fucoxanthin 10 (μM) was found to arrest some of the cells in G_2-M phase. Fucoxanthin has been observed to block the human gastric adenocarcinoma cell line MGC-803 in G_2-M phase (Yu, Hu, & Xu, 2011). In contrast, it has been suggested that carotenoids could not arrest the cells in G_2-M phase (Satomi & Nishino, 2009). Thus, whether fucoxanthin induces the arrest of cells in G_2-M phase needs further research. Low doses of fucoxanthin (150, 250 and 300 nM) could not alter too much of the cell cycle distribution. This is consistent with the cytotoxicity analysis result. Like gemcitabine, fucoxanthin inducing G_0-G_1 cell cycle arrest rather than apoptosis in some cancer cells appeared to contribute to its cytostatic effects in these cells (Satomi & Nishino, 2009). And an appropriately high dose of fucoxanthin (such as 20 μM) is cytotoxic to cells in the current study. The apoptosis observed at higher concentrations may be caused by partial conversion of fucoxanthin to its metabolites such as fucoxanthinol and amarouciaxanthin A.

Fucoxanthin induced the G_0-G_1 cell cycle arrest in this study is speculated to be associated with the downregulation of the proteins expressions in G_1/S transition such as cyclin D1, cyclin D2, CDK4 and CDK6. Besides, the upregulation of GADD45α might occur to inhibit the entry of cells into S phase. Moreover, a previous study indicated that fucoxanthin and fucoxanthinol inducing cell apoptosis is associated with activation of

caspase-3, -8 and -9, along with downregulation anti-apoptotic protein expressions, XIAP, cIAP2, Bcl-2 and survivin (Ishikawa et al., 2008). In a nut shell, the present study demonstrated that fucoxanthin is effective on the anti-proliferation of human pancreatic cancer cell lines (MIA PaCa-2 and PANC-1) due to its cytostatic and cytotoxic properties.

5.2.4 Combined effect of Gemcitabine and Fucoxanthin on Cell Cycle Distribution

Fucoxanthin combined with gemcitabine was found to help induce the pancreatic cancer cell arrest in G_0-G_1 or S phase. The lack of cell cycle arrest by fucoxanthin on HEK 293 cells implicated that fucoxanthin has relatively selective toxicity. However, the combination of fucoxanthin and gemcitabine did not result in any synergistic effect, only an additive effect was observed. Both gemcitabine and fucoxanthin block of cells in G_1/S phase in the cell cycle distribution and effects of each drug on cellular metabolism are different. The combination of these two drugs is supposed to make synergistic effects. A plausible explanation for this observation might be because of the schedule sequence. The schedule sequence has been demonstrated to be very important for the antitumor effect of drug association. The same drugs with different treatment sequences was found to obtain opposite results (Mortara et al., 2013). Gemcitabine and fucoxanthin were added simultaneously to the three cell lines in this study. Therefore, pre-treating the cells with one of the compounds and later treating with the second one should be tried in the further studies, namely gemcitabine-fucoxanthin and fucoxanthin-gemcitabine. Additionally, the interval time of the second drug addition should be determined. Another possible reason for such results, might be due to the drug concentrations set for this study. Fucoxanthin concentrations (150, 250 and 300 nM) set for MIA PaCa-2 might be too low, as compared to the gemcitabine (25 and 50 nM) chosen to treat the cells. As for the gemcitabine concentration used to treat PANC-1 cells, the major effect was shown by fucoxanthin rather than gemcitabine, since PANC-1 cells are highly resistant to gemcitabine. Nevertheless, low doses of fucoxanthin help improve the anti-proliferative efficacy of

gemcitabine by inducing growth arrest and high doses of fucoxanthin inhibited proliferation by inducing apoptosis, was found in this study. The synergistic effect could be observed by using the best combination concentrations and best treatment sequence of fucoxanthin and gemcitabine.

Chapter 6 Conclusion

6.1 Overall Conclusion

The present study describes the effect of fucoxanthin and gemcitabine on the proliferation of human pancreatic cancer cell lines MIA PaCa-2 and PANC-1. Fucoxanthin inhibited the growth of the cancer cells in a time- and dose- dependent manner in the present study. Simultaneous combination of fucoxanthin and gemcitabine made an additive inhibitory effect on pancreatic cancer cells. Fucoxanthin has the potential to reverse gemcitabine resistance and by this means to improve the response of pancreatic cancer cells to gemcitabine. Cell cycle analysis conducted by using flow cytometry was applied to study the mechanism of action of these two drugs. This study provides further evidence that gemcitabine blocks the cell cycle in G_0-G_1 or S phase and fucoxanthin blocks cells in G_0-G_1 phase.

Gemcitabine is the first line chemotherapeutic agent for the treatment of pancreatic cancer at present. However, chemo-resistance to gemcitabine was found in pancreatic cancer cells. And gemcitabine has also been demonstrated to have severe side effects. In this study, even at low doses, fucoxanthin significantly improved the anti-proliferative effect of gemcitabine. Meanwhile, fucoxanthin used in this study was selectively toxic to HEK 293, a cell line used to represent normal human cells. Moreover, fucoxanthin is shown to have mild adverse side effects. Fucoxanthin, the most abundant carotenoid found in marine algae, is considered as a potential candidate for the development of anti-cancer drugs for the treatment of pancreatic cancer and further applied in the clinical cancer chemotherapy. In summary, this study is a beginning of the research to see the anti-proliferative activity of fucoxanthin against pancreatic cancer cells.

6.2 Future Directions

The *in vitro* study of gemcitabine and fucoxanthin on human pancreatic cancer cells showed additive inhibitory effects instead of synergistic effects, when used in combination over a designated time. This result might closely relate to the designation of drug concentrations, treatment sequence and exposure time. Moreover, only cell cycle analysis was applied to study the mechanism of action of drugs in this study. A number of studies for deep investigation of the cell cycle inhibition and apoptosis mechanisms such as western blot, apoptosis and cell death analysis are not carried out in this study. In order to deeply explore the mechanism of action of gemcitabine and fucoxanthin, further researches are imperative. Meanwhile, a better experimental design is necessary. For example, the delivery solution of fucoxanthin should consider to do some change to ensure the best efficacy of fucoxanthin, because of the volatility of ethanol. Additionally, some adjustments can be made to the drug concentrations, treatment sequence and exposure times used in the initial studies.

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Appendix

A1: Cell Doubling Time

A1.1 Cell Growth Fitting for MIA PaCa-2 and PANC-1 Cells

A.1.1.1 MIA PaCa-2 Cells

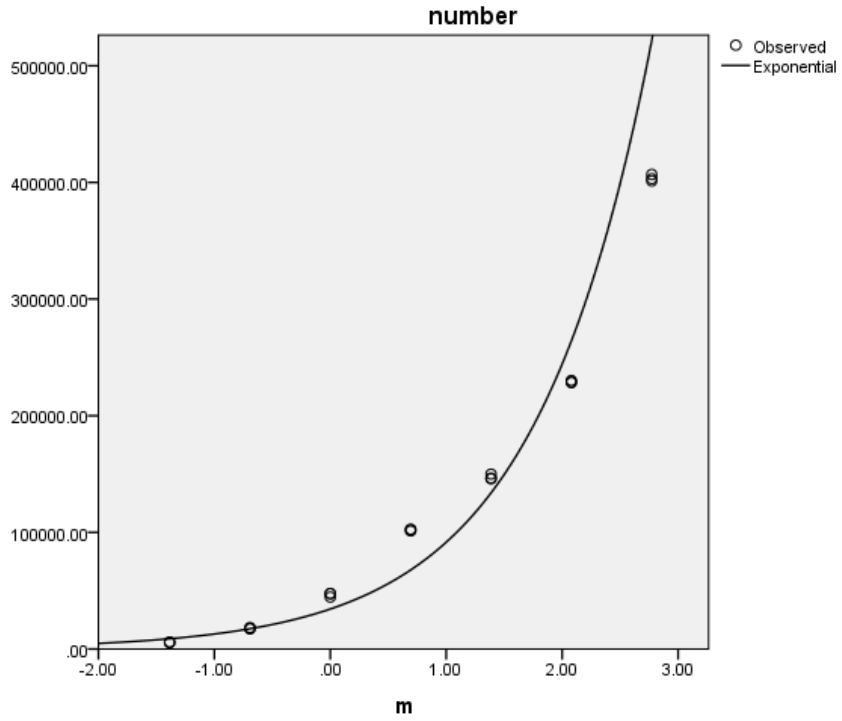

Figure 33: Cell Growth Fitting for MIA PaCa-2 cells

Model Summary and Parameter Estimates

Dependent Variable: number. The independent variable is m.

	Model Summary					Parameter Estimates	
Equation	R Square	F	df1	df2	Sig.	Constant	b1
Exponential	.959	449.734	1	19	.000	8800.047	.982

The doubling time of MIA PaCa-2 cells: T_d= (24/0.982) hrs=24.44 hrs

A.1.1.2 PANC-1 Cells

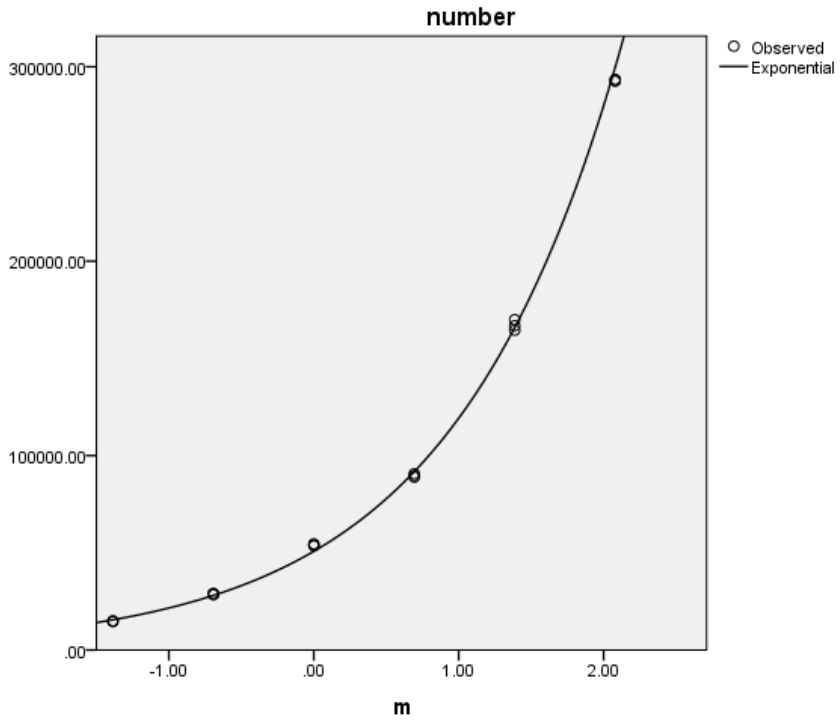

Figure 34: Cell Growth Fitting for PANC-1 cells

Model Summary and Parameter Estimates

Dependent Variable: number. The independent variable is m.

	Model Summary					Parameter Estimates	
Equation	R Square	F	df1	df2	Sig.	Constant	b1
Exponential	.999	11430.141	1	16	.000	50767.258	.854

The doubling time of MIA PaCa-2 cells: T_d= (24/0.854) hrs=28.10 hrs

A.1.1.3 The Effect of Ethanol on the Cell Viability of PANC-1

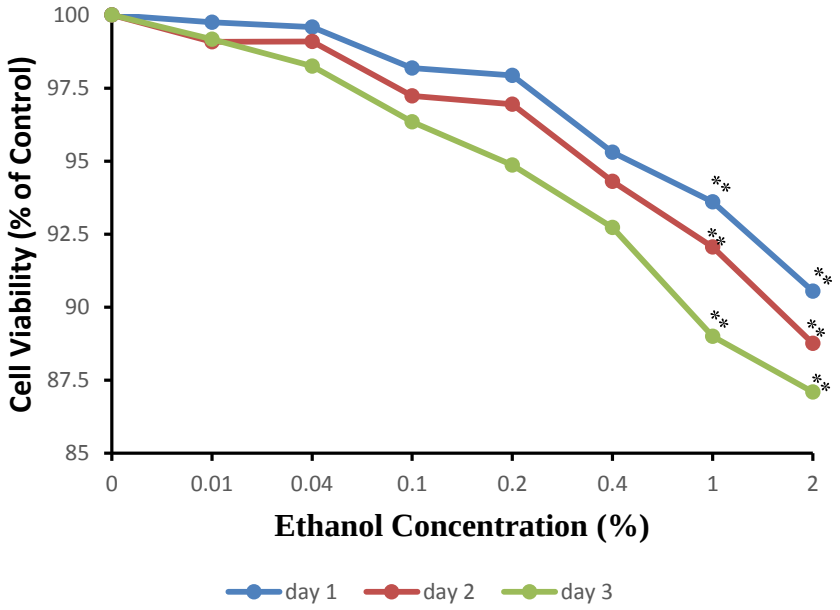

Figure 35: The effect of ethanol on the cell viability of PANC-1. Data are presented as means ± S.D, n=3. Two asterisks indicated a value significantly different from the control value (cell viability under 0% ethanol) in each day, $^{**}P< 0.01$ (Student's t test).

A2: The Fucoxanthin Fluorescence to Cell Cycle Analysis

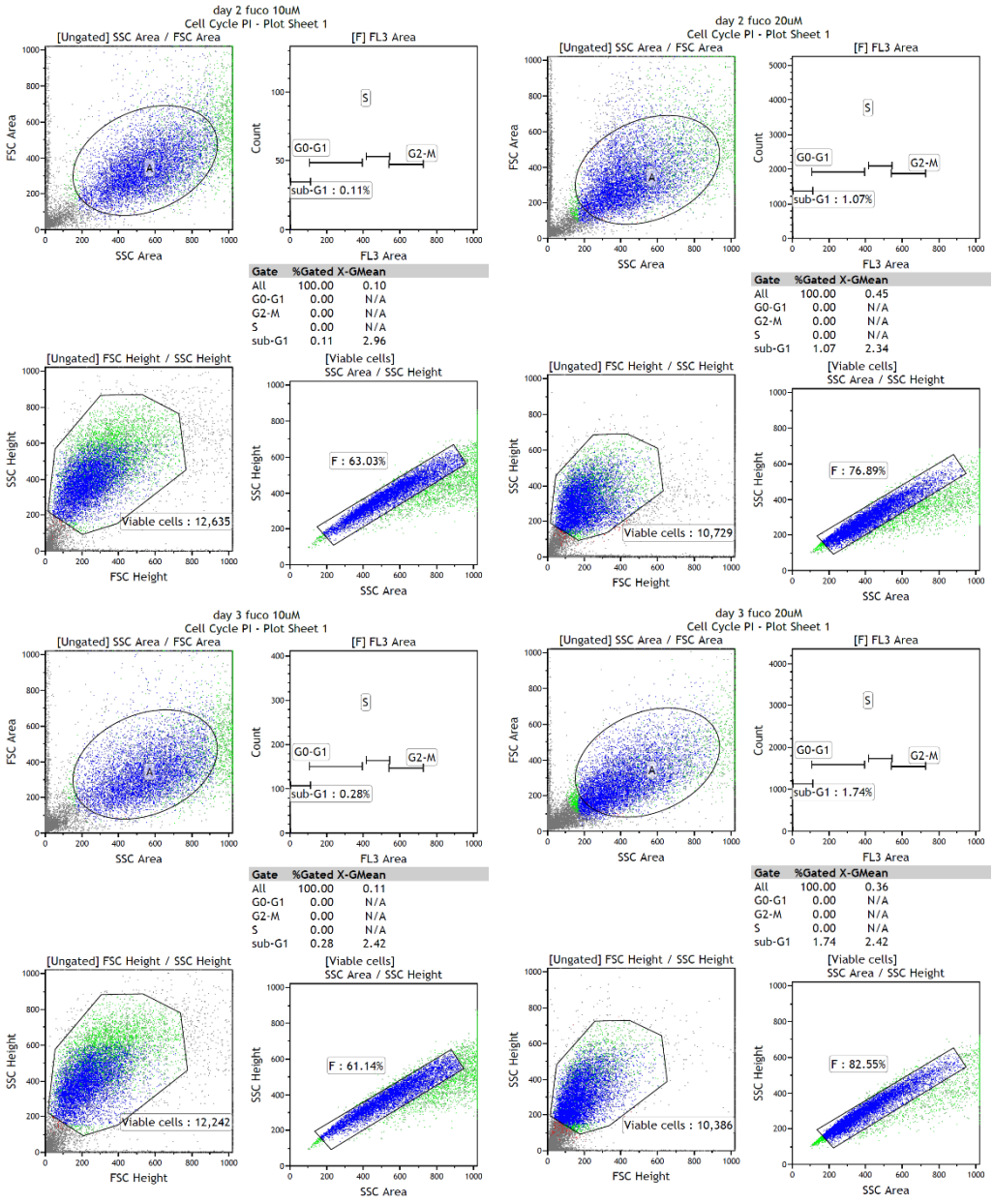

Figure 36: Cell cycle results of PANC-1 cells treated with fucoxanthin (10µM and 20 µM) for 48 hours and 72 hours without PI staining

A3: The Voltage Used for Sample Running

Table 33: The FL3 voltage set for each time sample running

	Voltage (Volts)		
Cell line	**Starvation**	**48 hours**	**72 hours**
MIA PaCa-2	455	475	480
PANC-1	460	465	470
HEK 293	480	485	500