Anti-proliferative potential of Fucoidan on Human Prostate Cancer Cells

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no 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.

Signed 

Name Bhawana Prakash

Date 30th March 2016

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Abstract

Globally, prostate cancer is the second most frequently diagnosed cancer and the fifth most common cause of cancer death among men. Long-term clinical trials have shown that, eventually prostate cancer becomes resistant to treatment options available and progresses to a more severe and dreadful form which is said to be increasing at an alarming rate in industrialized countries (in New Zealand, for example, prostate cancer is the most common cancer in men). Hence, there is an urgent need to identify novel chemotherapeutic agents against advanced prostate cancer, with selective cytotoxic effect (i.e. causes minimal damage to healthy tissues and have maximum cytotoxic nature toward tumour cells).

Fucoidan is a sulphated polysaccharides from seaweed. In the present study, commercial crude and pure fucoidan from *F. vesiculosus* (CCF, CPF; purchased from sigma), crude fucoidan from New Zealand *U. pinnatifida* extracted at AUT (ACF) and commercial pure fucoidan from *U. pinnatifida* (UPF; purchased from sigma) were investigated for their antiproliferative effect on human prostate cancer cell lines (PC-3 and DU-145; which are analogous to androgen-independent cancer cells).

Two types of bioassays were employed to test the anticancer potentials of CCF, ACF, UPF and CPF. The MTT cell proliferation assay was employed to determine the viability of cells after treatment with various concentration of fucoidans, and the apoptosis assay using Flow Cytometer to determine the proportion of apoptotic sub-G1 hypodiploid cells.

Results reveal that crude fucoidan from either of the species (CCF and UPF) were more effective in inhibiting growth of prostate cancer cells in a time and dose dependent manner than pure forms of fucoidan (CPF and UPF). Once the antiproliferative effect of fucoidans were evaluated, the mechanism behind this effect was elucidated by flow cytometric analysis of apoptosis. Crude fucoidan were found to induce more cell death in a dose dependent manner after 72 hours of treatment.

Cytotoxic effect of various fucoidan tested in this study were analysed on normal cells (HEK-293) as well. It was demonstrated that CCF was most toxic as it inhibited the growth of normal cell to the greatest extent (IC_{50} value, 268.2 µg/ml; sub-G1, 27.40%),

whereas ACF was comparatively less cytotoxic (IC_{50} value, 958.4 µg/ml; sub-G1, 11.73%) than CCF and exhibited lower apoptosis inducing activity on normal cells.

Even though ACF was less capable of inhibiting the growth of prostate cancer cells in comparison to CCF (the most effective), owing to its lesser toxicity towards normal cells, ACF would be a more apt option to be investigated further for its activity against prostate cancer. Thus this study highlights that fucoidan form New Zealand *U.pinnatifida* might have therapeutic potential for advanced prostate cancer treatment with milder side effect.

CHAPTER ONE

1. INTRODUCTION

1.1 Cancer

1.1.1 Cancer overview

Cancer is a diverse family of diseases, consisting of over 100 forms that spans from almost every cell type in the body. Each cell type gives rise to distinct form of cancer, and the diversity is greatly increased by the fact that multiple forms of cancer can develop from each cell type, depending on both location of the cell and the genetic aberration. Despite broad diversity, several features are common to all cancers (Hanahan & Weinberg, 2000), these includes:

- unrestricted cellular proliferation
- circumvention (evasion) of cell cycle control
- growth without appropriate signal
- escape from programmed cell death
- altered interaction between cells and the surrounding environment
- evasion of immune mediated eradication
- invasiveness into normal tissue and metastasis

Cancer manifests itself as either a solid tumour or a nonsolid leukemia in the circulatory systems. The term tumour is nonspecific for a lump or swelling and tumour are characterized as either benign or malignant. The differences between benign and malignant tumour are tabulated below.

Table 1: Comparison of benign and malignant tumours

Factor	Benign tumour	Malignant tumour
Invasiveness	Non-invasive, often encapsulated	Invasive
Rate of growth	Slow, often static	Rapid
Differentiation	Well differentiated; often resembles tissue of origin	Undifferentiated
Metastasis	Never	Often metastatic

Several criteria or factors like rate of growth, degree of differentiation, extent of invasiveness, and metastatic potential are used to determine the degree of tumour malignancy. Never-the-less in most cases, metastasis characterizes the highest degree of tumour malignancy, as it is usually the cause of death in cancer patients.

Alternative Pathways to Cancer

The routes or pathways cells take on their way to become malignant are highly variable (Colman, 2006). It is a well-studied fact that cancer is a genetic disease (Vogelstein & Kinzler, 1993; Vogelstein, Lane, & Levine, 2000) as it is caused by the acquired changes (mutation) in genes which are involved in the development and maintenance of normal cells and tissues. Genetic changes that cause cancer can be inherited from our parents or they can also arise during a person's lifetime as a result of errors that occur as cells divide (or because of damage to DNA) caused by certain environmental exposures. These genes fall in three categories: proto-oncogenes, tumour suppressor genes, and DNA repair genes (Vogelstein & Kinzler, 2004).

Proto–oncogenes contribute to cellular replication during normal growth and maintenance of tissues. When mutation takes place in proto-oncogene, they transform into oncogenes (cancer causing genes) and become hyperactive, hence leading to uncontrolled cellular replication.

Tumour-suppressor genes prevent cell growth in the absence of proper mitogenic (growth stimulatory) signals. Mutation in tumour-suppressor gene results in the loss of growth inhibitory mechanisms and leads to unregulated (without appropriate signal) growth.

DNA repair genes do not stimulate or inhibit cellular growth. The function of DNA repair genes is sensing and fixing DNA mutation and hence are called caretakers of genome (Kinzler & Vogelstein, 1998). Mutation in DNA repair gene causes accumulation of proto –oncogenes and tumour-suppressor genes, hence leading to cancer ignition and progression (Jackson & Loeb, 1998).

Mutation resulting in the hyper activation of a single proto-oncogene or the functional inactivation of a single tumour-suppressor gene are typically insufficient for cancer initiation. Cells uses numerous checks and balances that prohibit uncontrolled growth in response to a signal activating signal. Multiple genetic lesions in various signalling pathways are necessary for cancer to arise. The continuous accumulation of genetic lesions mandatory for cancer genesis is called the multiple theory of carcinogenesis (Vogelstein & Kinzler, 1993). The accumulation of mutagenic hits results not only in the transformation of a cell from a normal to a cancerous state, but also in the severity or escalation of tumour malignancy.

1.1.2 World cancer burden

Cancer can truly said to be “The Emperor of All Maladies” (Mukherjee, 2010). As documented by The International Agency for Research on Cancer (IARC), cancer is one of the leading cause of disease and mortality worldwide with an estimated 14.1 million new cases and 8.2 million deaths in 2012. It was also emphasised in the report that the rising number of cancer incidences around the world is mainly because of ageing of the populations and population growth. Based on World Health Organization (WHO) statistics, the five most common types of cancer detected in men were lung, prostate, colorectal, stomach, and liver cancer. Meanwhile, the top five diagnosed cancers in women were breast, colorectal, lung, cervix, and stomach cancer.

It is apparent from the report that, there is a disproportionate or unequal cancer trend observed in developing countries. Africa, Asia, Central and South America accounts for

60% of world's total cancer incidences and 70 % of world's total cancer death (J Ferlay et al., 2015; Siegel, Naishadham, & Jemal, 2013). The Report has also proposed that the world cancer burden would rise by 75% and reach close to 25 million over next two decades (WHO, 2013).

The alarming rate of cancer incidences and associated mortality, calls for an immediate action plan against it. Hence it is urgent to develop an effective and affordable mode of early detection, diagnosis and treatment of cancer.

1.1.3 The Cancer Burden in New Zealand

From 1960 onwards, New Zealand's cancer mortality rate has been increasing considerably faster than those of Australia, Canada, the USA, and the United Kingdom (Ministry of Health, 1999). It has been a major health concern for New Zealanders ever since. Out of 175 countries studied, New Zealand women have the sixth highest cancer mortality rate in the world, and men have the 33rd highest cancer mortality (Parkin, Pisani, & Ferlay, 1999).

According to the latest health statistic reports released by Ministry of Health, New Zealand, in 2015, there were 21,814 new cases of cancer registered in New Zealand. Out of which, the most commonly registered cancers were prostate (3129 cases), breast (3054), colorectal (3016), melanoma (2324) and lung (2027). People aged 65 years and older accounted for nearly 6 out of 10 new cancer cases.

Out of registered cases, 8905 people died due to cancer in New Zealand, from which more than half of cancer deaths were male (4735 cases, 53.2%). The most common cancer deaths were from lung (1628 deaths), colorectal (1283), breast (618), prostate (607) and pancreatic cancer (463). For males the most common cancer deaths were from lung (891 deaths), colorectal (664), prostate (607) and pancreatic (229) cancer, and melanoma (222) and for females the most common cancer deaths were from lung (737 deaths), colorectal (619), breast (617), pancreatic (234) and ovarian (175) cancer.

The incidences of cancer has increased at such a fast rate that one in three New Zealanders is said to have some experience of cancer, either personally or through a relative or friend. Cancer is the country's prominent cause of death (28.9 percent) and hospitalisation. Increasing numbers of New Zealanders are likely to suffer from cancer over the next two decades (Ministry of Health, 2015).

1.2 Prostate Cancer

The Prostate is a walnut-sized gland in men that surrounds the top of the urethra; it produces seminal fluid. Androgens, particularly testosterone and dihydrotestosterone are essential for the normal growth and functioning of the prostate (Kufe et al., 2003).

Several types of cells are found in the prostate, but almost all (99%) prostate cancers are adenocarcinomas derived from the glandular epithelial cells. Other types of cancer can also start in the prostate gland, including sarcomas, small cell carcinomas, and transitional cell carcinomas but are documented to be very rare (Network, 2004).

1.2.1 Incidence and Mortality

Globally, prostate cancer is the second most frequently diagnosed cancer and the fifth most common cause of cancer death among men, with an estimated 1.1 million new cases (15% of all cancers in men) and 0.3 million cancer deaths (7% of all cancer deaths in men) in 2012. In 2012, 60% of the estimated new cases and 41% of the deaths occurred in Europe and North America (WHO, 2013).

Mortality rates are highest in countries and areas with predominantly Black populations – in the Caribbean and in parts of sub-Saharan Africa (Parkin et al., 1992)– but mortality rates are also high in certain northern European countries, such as the Nordic countries (Hsing & Devesa, 2001). Incidence rates of prostate cancer vary by more than 25-fold in different parts of the world. The highest rates are in Australia and New Zealand (111.6 per 100 000), northern and Western Europe, and North America (Jacques Ferlay et al., 2010).

This was largely because of the increased availability of screening for prostate specific antigen (PSA) in men without the symptoms of the disease. This test leads to the detection of many prostate cancers that are small and/or would otherwise remain unrecognised, and which may or may not develop further into higher stage disease. However, rates were already increasing before the availability of PSA testing, and have continued to increase in middle-income countries where screening is still not widely available. This suggests that prostate cancer is influenced by environmental factors. Although screening is increasingly popular in many high-income countries, its value, for example in reducing mortality, is controversial.

1.2.2 Symptoms

Prostate cancer is documented to be asymptomatic (Miller, Hafez, Stewart, Montie, & Wei, 2003) until the condition is quite advanced. The general clinical symptoms which cannot be distinguished from those caused by benign prostatic hyperplasia are urinary frequency, nocturia and urgency caused by obstruction of the urethra (van der CRUIJSEN-KOETER et al., 2005).

1.2.3 Risk Factors

Despite prostate cancer's high morbidity, its etiology remains obscure (Chokkalingam, Stanczyk, Reichardt, & Hsing, 2006). The oncogenesis of prostate cancer is a complicated process affected by several aspects, mainly including demographic, hormonal, behavioural, and lifestyle associated risk factors.

1.2.3.1 Demographic risk Factors

a. Age

Age is the most well-documented risk factor for prostate cancer and the incidence of prostate cancer increases exponentially with advancing age (beginning at approximately age 50 to 55) – an increase that is faster than that for any other malignancy (Hankey et al., 1999). This pattern is less evident in Asian countries, but in western countries it represents the steepest age-dependent incline of any cancer (Brawley, Knopf, & Thompson, 1998).

b. Racial/ethnic variation

This is another consistently observed but poorly explained factor. It has been documented that African – Americans have the highest prostate cancer incidence rates in the world, which is roughly 60 times that of men in Shanghai, China, where the rates are the lowest in the world (Hsing, Tsao, & Devesa, 2000). This disparity between populations can just not be because of difference in detection or screening.

Migrant studies have demonstrated that Japanese men (low risk country) manifest some excess prostate cancer incidence by moving to America but they typically do not assume the full risk profile of American natives (high risk country) (Shimizu et al., 1991). This variation also, can just not be attributed to the screening process alone. This supports the results of migrant studies suggesting that ethnic factors, including genetic, lifestyle,

or environmental factors, may affect prostate cancer risk and explain much of the difference in risk between high and low-risk populations (Cook, GOLDOFT, Schwartz, & Weiss, 1999).

1.2.3.2 Genetic factors

Various data suggest that prostate cancer has one of the strongest heritable components of any cancer, with an estimate of 30% to 40% of cancer risk explained by genetic factors (Baker, Lichtenstein, Kaprio, & Holm, 2005; Lichtenstein et al., 2000).

It has also been documented that men with a first degree relative with prostate cancer has a two to four fold increase risk of the disease (Thompson et al., 2006). Another notable finding in some studies states is that having an affected brother confers a stronger elevation in risk than does having an affected father (Cerhan et al., 1999; Whittemore et al., 1995).

According to Cancer Research UK scientists, there are more than one gene responsible for prostate cancer. Mutations in BRCA1 and BRCA2, which are important risk factors for ovarian cancer and breast cancer in women, have also been suspected in prostate cancer progression (Struewing et al., 1997). Some evidences are also documented which support, role of chronic inflammation in the pathogenesis and progression of prostate cancer (Carpten et al., 2002; Xu et al., 2002).

1.2.3.3 Hormonal, behavioural, and lifestyle associated risk factors

a. Hormone and growth factors

The prostate gland depends on androgen, both for development within the foetus and for growth, maintenance, and functioning in mature adults (Coffey, 1979; Niu et al., 2001). However, the precise role of androgens in the etiology of prostate cancer is unclear. Prostate cancer is notably absent in castrated men and androgen ablative therapy is the treatment option with a curative intent for advanced prostatic cancer.

Laboratory studies show that androgen can induce prostate cancer in experimental animals (Huggins & Hodges, 2002). Hence, amalgamation of the facts that androgen is needed for normal prostatic growth and functioning, testes is indirectly essential for prostate cancer; all these findings are implicating towards the fact that androgen is an important sensitive factor for prostate cancer.

b. Vitamin D

Vitamin D, is a steroid hormone, which is obtained primarily from dermal synthesis in response to sunlight exposure. Vitamin D and its analogues are said to have potent antiproliferative, pro-differentiative, and pro apoptotic effects on prostate cancer cells. In addition, vitamin D inhibits prostate tumour growth *in vivo*. In general, laboratory data are consistent and support the hypothesis that vitamin D may protect against prostate cancer. However, results from epidemiologic studies investigating serum levels of vitamin D have been inconsistent (Garland et al., 2006). The reasons for these conflicting results are unclear.

c. Insulin like growth factor – I

Insulin like growth factor – I, is a major growth regulating molecule which is known to be a potent mitogen and has anti apoptosis effect on prostate epithelial cells. Accumulating epidemiological evidence supports a role of the insulin like growth factor-I (IGF) in prostate cancer carcinogenesis (Renehan et al., 2004).

d. Diet

Studies attempting to elucidate the nutritional etiology of prostate cancer are many, yet no definitive answer have been emerged.

It has been documented that dietary animal fat (from meat and dairy products) acts as a positive risk factor for prostate cancer as it increases the production and availability of both androgens and estrogens (Kolonel, 2001); but the interpretation of this finding remains controversial.

Increased intake of dairy products has been associated with prostate cancer risk in several case control and cohort studies (Chan et al., 1998; Rodriguez et al., 2003; Tseng, Breslow, Graubard, & Ziegler, 2005).

Calcium has been proposed to increase the risk of prostate cancer by suppressing circulating levels of dihydroxyvitamin D (1,25(OH)2D), a possible protective factor for prostate cancer (Giovannucci et al., 1998).

The relationship between tomatoes, lycopene -a carotenoid consumed principally from tomato products and prostate cancer has received great attention (Giovannucci, 1999). Although not definitive, the available data (Gärtner, Stahl, & Sies, 1997; Giovannucci,

1999; Tonucci et al., 1995) suggests that increased consumption of tomato and tomato based products may be associated with lower prostate cancer risk and progression (Chan et al., 2006; Tseng et al., 2005).

Although the genetic component may be larger for prostate cancer than for most other malignancies, the evidence of environmental, hormonal and lifestyle associated risk factors are important and cannot be over looked.

1.2.4 Pathogenesis of prostate cancer

Even though, several types of cells are found in the prostate, almost all (99%) prostate cancers are adenocarcinoma derived from the glandular epithelial cells (Coffey, 1979). Despite the high incidence of prostate cancer, its etiology is not entirely elucidated. The induction of prostate cancer in humans has been observed as a multistage process (as outlined in Figure. 1 involving progression from low histologic grade small latent carcinoma to higher-grade large metastasizing carcinoma. A number of pathogenetic pathways have been documented and inferred to be involved in the origin of prostate cancer.

Prostate cancer begins when normal semen secreting prostate gland cells mutate into cancer cells. Initially, small clumps of cancer cells remain confined to otherwise normal prostate glands, a condition known as carcinoma in situ or prostatic intraepithelial neoplasia (PIN). Although it is uncertain, whether PIN is a cancer precursor or not, it is closely associated with cancer. Over time, these cancer cells begin to multiply and spread to the surrounding prostate tissue (the stroma) forming a tumour.

Carcinoma of prostate spreads by: (a) local invasion (b) vascular invasion and (c) lymphatic spread. Locally, the tumour may penetrate the prostatic capsule along the perivesical sheath and bladder base, where the ureters may become obstructed. This occurs in 10-35% of patients. Metastases to bone occurs in 85% of patients dying of prostate cancer.

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Figure 1. Proposed origin of human prostate cancer is a multistep process (source (Nakayama et al., 2000).

1.2.5 Diagnosis

Traditionally, prostate cancers were diagnosed by digital rectal examination (DRE) alone (Schröder et al., 1998). Since the late 1980s, an increasing proportion of prostate cancers in asymptomatic men has been detected through screening with prostate specific antigen (PSA) in the blood (Petricoin et al., 2002). Modern individualized treatment, however, requires morphologic confirmation by core biopsy, fine needle aspiration biopsy, or transurethral resection and staging.

1.2.6 Treatment of Prostate Cancer

Major factors which need to be thoroughly examined before deciding upon various treatment options available are, stage of cancer, the Gleason score, and the PSA level. Other important aspects are age, general health, and a person's views about potential treatments and their possible side effects.

Depending upon the situation (i.e. severity of disease), various treatment options available are briefly described below.

1.2.6.1 Treatment of clinically localised prostate cancer

Localised prostate cancer is the most commonly diagnosed stage. The choice of treatment (active monitoring, radical prostatectomy, or any type of radiotherapy) is based on factors such as tumour characteristics and the patient’s life expectancy. It is

very important to note that the curative intent of treatment is not used for all patients, who are suffering from prostate cancer, as in many cases of highly to moderately differentiated prostate cancers the disease is indolent (15% progression rate at 10 year) when left without active treatment (Albertsen, Hanley, & Fine, 2005; Johansson et al., 2004). Doctors often recommend Active surveillance or watchful waiting (Heidenreich et al., 2008), if men have been detected with early stage prostate cancer and have a life expectancy of less than 10 years. This approach is also an option for all men in stage T1C OR T2a (low risk) group.

Treatment with a curative intent (radical prostatectomy or local irradiation) is an option for men with poorly differentiated tumours and a long life expectancy (i.e. younger men). It should be noted that there is no absolute cut off limit for age, as to when active treatment will be of value. If life expectancy of older men is long enough treatment with curative intent can be their option (Wong et al., 2006).

Of the treatments available radical prostatectomy (removal of the prostate) have documented to be having a survival advantage over watchful waiting (Bill-Axelson et al., 2005). Radical surgery can be done by laparoscopic techniques which might be either done conventionally or robotic-assisted laparoscopic radical prostatectomy (RALRP). During the past decade, the methods for delivery of radiotherapy in a sufficiently high dose to a precise target (thus minimising damage to surrounding tissue) has improved.

Some randomised trials support the use of high radiation doses (Dearnaley et al., 2007; Peeters et al., 2006). Whether there should be a combination of high-dose radiation with hormonal therapy to treat clinically localised disease is not clear but there seems to be some benefit from the combination (Dearnaley et al., 2007).

1.2.6.2 Treatment with curative intent for locally advanced prostate cancer

For men with extracapsular extension of tumour, radiotherapy is the standard option. Radiation therapy uses high doses of radiation energy to treat cancer. It exploits the fact that when ionizing radiation are absorbed in tissue, it damages the DNA in cancer cells and increases the chances of apoptosis (cell death). Compared to the normal cells, cancer cells are not able to recover from the damaging effect of radiation.

Effectiveness of radiotherapy, in locally advanced prostate cancer can be increased either by dose escalation (Nilsson, Norlén, & Widmark, 2004) or by combination of radiation with adjuvant hormonal therapy (any treatment given after primary therapy) or neoadjuvant hormonal therapy (any treatment given before primary therapy) (Aus et al., 2005). Documentation regarding use of surgery for patients with small tumours that extend outside the gland are also present (Aus et al., 2005; C.-Y. Hsu, Joniau, Oyen, Roskams, & Van Poppel, 2007).

1.2.6.3 Second-line treatment after failed primary surgery or radiotherapy

A persistent elevated serum level of prostatic specific antigen (occurring from months to year), without clinical or radiographic metastases, after treatment with a curative intent is the first sign of recurrent prostate cancer. This biochemical recurrence (BCR) is indicative of the presence of prostatic epithelial tissue, and is expected to represent cancer.

The clinical manifestation of patients with BCR is very variable. After surgery, some experience slow clinical progression (like late PSA increase, a long PSA doubling time, and a not too aggressive disease at diagnosis; where as some might exhibit rapid clinical progression to metastases (like early PSA increase, a short PSA doubling time and adverse pathological changes) (Aus et al., 2005; Simmons, Stephenson, & Klein, 2007).

For men with comparatively long life expectancy (i.e. more than 5-10 years), treatment options available after recurrent prostate cancer is external beam radiotherapy (if surgery was the first choice before recurrent cancer). New experimental therapies like, high-intensity focused ultrasound or cryotherapy, is not preferred as it is accompanied with high complication rates and a slim chance of permanent success (Aus et al., 2005; Simmons et al., 2007).

For men with short life expectancy and also for those men who have received primary radiotherapy, options are watchful waiting with delayed hormonal therapy. It has been documented that most patients with high risk of systemic failure, some form of hormonal therapy is recommended, till the metastatic disease can be detected using available imaging methods(Simmons et al., 2007).

1.2.6.4 Treatment of metastatic prostate cancer

The main therapy practiced for the management of an advanced prostatic cancer is androgen–ablative therapy (Hodges, 1941). Prostate cancer patients, with extra prostatic growth, with or without lymph-node metastases, or cancer with distant metastases are advised to take this therapy. This is done either by surgically removing the source of androgen the testicles (orchiectomy); hence the prostate not only lacks the testosterone stimulus to produce DHT but also does not have enough testosterone to transform into DHT. Orchiectomy considered the gold standard of treatment. Or by using anti-androgens – drugs like flutamide (Eulexin®), bicalutamide (Casodex®), and nilutamide (Nilandron®) which binds to androgen receptors instead of androgen and hence stops androgen from working.

70 to 80 % of patients suffering with metastatic prostate cancer are said to have reduced bone pain, improved performance status, and a general improvement with an increased sense of wellbeing after androgen ablation (Hodges, 1941).

1.2.6.5 Treatment of metastatic androgen-independent prostate cancer

Long-term clinical trials have shown that, eventually prostate cancer becomes resistant to androgen-deprivation therapy and progresses (Feldman & Feldman, 2001). This is considered to be the final stage of prostate cancer and constitutes a substantial threat of morbidity and mortality (Taplin et al., 1995). For long, chemotherapy, was thought to be ineffective. Yet two large clinical trials, have put forward the importance of chemotherapy in improving the survival rate and quality of life of hormone refractory prostate cancer patients (Petrylak et al., 2004; Tannock et al., 2004). Chemotherapeutics docetaxel, alone or in combination with estramustine, improved the survival of men in comparison with mitoxantrone and corticosteroid (Petrylak et al., 2004; Tannock et al., 2004). Another FDA approved drug cabazitaxel is used when advanced prostate cancer has progressed during or after treatment with docetaxel.

1.2.6.6 Drawbacks associated with treatment available for prostate cancer

Primary endpoints of medical treatment according to the phase of the disease are safety, activity and efficacy. Different options of medical treatment for prostate cancer have their own side effects as summarized below:

Side effects of radical prostatectomy

- Erectile dysfunction (20–100%)
- Urinary incontinence (any 0–70%; severe 0–4%)
- Stricture (0–12%)
- Mortality (<1%)

Side effects of radiotherapy

- Gastrointestinal toxic effects (any 2–100%, severe 0–20%)
- Genitourinary toxic effects (any 0–70%, severe 0–20%)
- Urinary incontinence (any 0–60%, severe 2–15%)
- Erectile dysfunction (10–85%)
- Mortality (<1%)

Side effects of hormonal therapy

Castration

- Loss of libido
- Erectile dysfunction
- Hot flushes (55–80% of patients during androgen deprivation therapy)
- gynaecomastia and breast pain (49–80% diethylstilboestrol, 50% CAB, 10–20% castration)
- Increase in body fat
- Muscle wasting
- Anaemia (severe in 13% CAB)
- Decrease in bone mineral density
- Cognitive decline

Oestrogens

- Cardiovascular toxic effects
- Antiandrogens
- Pharmacological side-effects are loss of libido, erectile dysfunction, but rarely gynaecomastia
- Non-pharmacological side effects are related to individual drugs

Side effects of chemotherapy

- Common side effects of chemotherapy are hair loss, mouth sores, loss of appetite, nausea and vomiting, diarrhoea, increased chance of infections (from having too few white blood cells), easy bruising or bleeding (from having too few blood platelets), fatigue (from having too few red blood cells)
- Docetaxel and cabazitaxel sometimes cause severe allergic reactions
- Mitoxantrone can rarely cause leukemia several years later
- Doxorubicin can weaken the heart muscle over time

Even though there are many options for treatment of prostate cancer, these treatments or therapies cause severe side effects. They might increase the life expectancy by adding marginal years to a patient's life but does not improve the quality of life significantly.

The concerns over toxicity, lack of substantial treatment for hormone refractory prostate cancer, tumour cell resistance and development of secondary cancers from chemotherapeutic chemicals have generated interest in exploiting natural products for prostate cancer treatment.

1.3 Ocean as a source of prospective anticancer drugs

More than 70% of the earth's surface is covered by oceans and the oceans possess abundant resources as it is particularly rich in biodiversity. The growing amount of research work and epidemiological proofs supporting the preventive role of marine products in controlling chronic diseases like cancer has led to significant scientific interest in evaluating the pharmacological properties of marine products.

Marine algae or seaweed have known to be used extensively in Asian countries (Yang, Zeng, Dong, Liu, & Li, 2010) as food and traditional remedies. Because of the unique living environment, marine algae are not only rich in minerals and certain vitamins but also contains bioactive substances like polysaccharides, proteins, polyphenols, phycocyanins and terpenes with anticancer, antitumour, anti-inflammatory and other biological activity (Chandini, Ganesan, & Bhaskar, 2008; O'Sullivan et al., 2011).

Even though there are different types of marine algae, depending upon their pigment, Phaeophyceae or brown algae are studied most widely and forms an important group

of mostly marine multicellular algae (Murata & Nakazoe, 2001). They are documented to be important for marine environment, as food and also for the habitat they form. Because of extensive beneficial health effects of brown algae, its active constituent has been widely studied in recent years and they are documented to be potential candidates for the development of new pharmaceutical agents (Bhadury, Mohammad, & Wright, 2006).

1.4 Fucoidan

Fucoidan is a type of complex, fucose rich sulphated polysaccharides, mainly found in the fibrillary cell walls and intercellular spaces of various brown seaweed species (K.-J. Kim, Lee, Lee, & Lee, 2010; O'Connell, Murray, Piggott, Hennequart, & Tuohy, 2008). It may also contain galactose, mannose, xylose, glucose and/or glucuronic acid, usually in minor amounts depending upon the seaweed species from which it has been extracted (Jiao, Yu, Zhang, & Ewart, 2011). It was first isolated in 1913 from marine phaeophyceae by Kylin (Kylin, 1913) and was named "fucoidin". Now according to IUPAC nomenclature rules, it is named "fucoidan"(Berteau & Mulloy, 2003).

1.4.1 Different sources of fucoidan

Fucoidan are mainly found in various species of brown algae and some marine invertebrates (O'Neill, 1954). In this study, the description about fucoidan is limited to fucoidan obtained from various species of brown algae.

1.4.2 Structure of fucoidan

Structure of fucoidan is highly contrasting, largely depending upon different factors, such as the source of fucoidan, species of brown algae, the time and location of harvesting and the extraction method (Ale, Mikkelsen, & Meyer, 2011; B. Li, Lu, Wei, & Zhao, 2008). Basically fucoidan is majorly composed of L-fucose and sulphate. Besides fucose and sulfate they also contains contain other monosaccharides (mannose, galactose, glucose, xylose, etc.) and uronic acids, even acetyl groups and protein (Patankar, Oehninger, Barnett, Williams, & Clark, 1993). Figure 2, shows the general structure of fucoidan, but it should be noted that chemical composition of fucoidan varies with species (Synytsya et al., 2010).

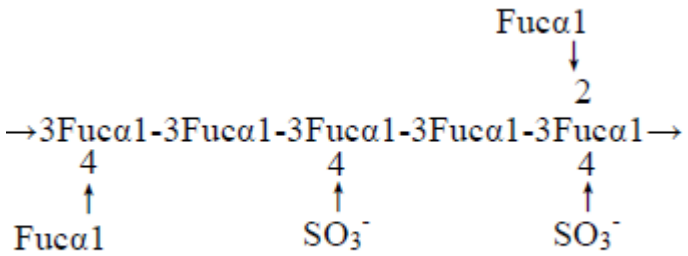

Figure 2: Pankter model for the general structure of fucoidan (Source: Li, Lu et al., 2008)

For example, fucoidan found in *Fucus vesiculosus* are documented to have a backbone built of (1→3)-linked α-l-fucopyranosyl residues or of alternating (1→3)- and (1→4)-linked α-l-fucopyranosyl residues ((Patankar et al., 1993; Percival & McDowell, 1967). Fucoidan found in *F. vesiculosus* are said to be relatively simpler. In *Undaria pinnatifida*, where the chemical composition is complex, it is documented that it includes sulfated galactofucans with backbones built of (1→6)-β -d-galacto- and/or (1→2)-β -d-mannopyranosyl units (Duarte, Cardoso, Noseda, & Cerezo, 2001). Notable differences in the percentage of fucose (12.6 to 36.0%) and sulphate content (8 to 25%) has been observed in fucoidan extracted from a number of brown seaweed algae like *Fucus vesiculosus* (Aisa et al., 2005), *Ascophyllum nodosum (Riou et al., 1995)*, *Sargassum kjellmanianum* (Yamamoto, Takahashi, Suzuki, Seino, & Mori, 1984), *Sargassum thunbergii* (Zhuang, Itoh, Mizuno, & Ito, 1995), *Cladosiphon okamuranus Tokida* (Haneji et al., 2005).

1.4.3 Uptake study of fucoidan

Fucoidan can either be administered by oral ingestion or by systemic delivery (by intraperitoneal injection or intravenously or subcutaneously). Although there is much literature available about the biological effect of fucoidan (discussed later in the thesis) after its administration in body, but barely any research information is available regarding uptake and fate of fucoidan. The fact that fucoidan is a highly branched molecule and hence difficult to be orally absorbed, caused problems in understanding as to how then systemic effects or absorption occur. Initially, researchers suggested that fucoidan was not modified by human bacterial flora and was excreted in its native state

(Michell, Lahaye, Bonnet, Mabeau, & Barry, 1996), supporting assumption that it might be possible that systemic observation of fucoidan is because of prebiotic effects.

Recently it was proposed that small amounts of dietary fucoidan can be endocytosed and cross the intestinal wall directly without breaking down (Irhimeh, Fitton, Lowenthal, & Kongtawelert, 2005). Further to investigate the fucoidan uptake process by cell, the internalization of low molecular weight fucoidan into rabbit smooth muscle cells have been studied. This study proposed a time- dependent accumulation of fucoidan in the perinuclear region of cells after 6 and 12 hours of incubation; however fucoidan was not detected inside the nucleus (Deux et al., 2002). However when native fucoidan from *Cladosiphon okamuranus*, with comparatively high molecular weight was examined for internalization, it was observed that there is poor permeation of fucoidan across the human colon adenocarcinoma Caco-2 cell monolayer (Kimura, Rokkaku, Takeda, Senba, & Mori, 2013).

Hence it can be concluded that the distribution of fucoidan in body fluids and tissues may be influenced by molecular weight, branching and sulphate group position, as well as on the monosaccharide residues and their arrangement. Further study is required to understand the fate of fucoidan after its *in vivo* administration. Hence, to better understand the potential pharmacologic importance of fucoidan, it is vital to study or elucidate the mechanism behind its digestion, absorption metabolism and excretion.

1.4.4 Fucoidan Toxicity

Several *in vitro* and *in vivo* studies have documented that there is no significant toxicity associated with fucoidan administration (refer to Table 2 for details). For *in vitro* studies, researchers have utilized normal cells such as normal fibroblasts alongside tumour cell lines and reported that fucoidan did not induce apoptosis within normal cells at the dose which were toxic for cancer cell lines. For example, a very high dose of 3 mg/ml fucoidan suppressed the viability of peripheral blood mononuclear cells from healthy donors by 25% compared to 60%-90% in five different leukemic T cells (Haneji et al., 2005). During *in vivo* studies it was shown that oral administration of up to 1 g/ml/body weight of *Undaria* species fucoidan was non-toxic in mice. Similarly, during another study involving daily administration of 3000 mg/kg/body weight of fucoidan from *Laminaria japonica* species in rats for over 6 months did not affect the rats adversely (N. Li, Zhang,

& Song, 2005). Based on the above mentioned studies and detailed information presented in Table 3, it can be safe to say that fucoidan is a harmless compound, which is non-toxic and non-mutagenic and a potential candidate for clinical studies in the fight against cancer.

Table 2: Toxicology of fucoidan

Fucoidan source	Chemical composition	Studies	Dose	Species	Results	References
Undaria pinnatifida	53% total sugar 7.4% sulfate 27% Uronic acid 54% fucose 35% galactose	Acute *in vivo* Ames test Bone marrow micronucleus	1000 mg/kg Body weight per day for 28 days	Sprague Dawley rats	Not toxic to 1000 mg/kg bw; Increase in ALT at 200 mg/kg	(Chung et al., 2010)
Laminaria japonica	Fucoidan Fraction MW average "10–300 kD"	Oral dosing in experimental model; Toxicity by clinical observation	Escalation doses up to 20 mg/kg	Dogs with haemophilia A; Rats	No clinical toxicity	(Prasad et al., 2008)
Laminaria japonica	Fluorescent labelled fucoidan	Subcutaneous	5 mg/kg	Rat	Half-life 83 min	(Prasad et al., 2008)
Laminaria japonica	MW average 189 kda Fucose 28% sulfate 29%	Acute and sub-chronic toxicity	300,900 and 2500 mg/kg bw to 6 months	Wistar Rats	Not toxic to 300 mg/kg bw Prolonged clotting	(N. Li et al., 2005)

	total sugar 48%				at 900 and 1200 mg/kg bw	
Undaria pinnatifida	64.4 ± 6.0% fucose, 31.9 ± 4.7% galactose, 3.6 ± 1.3% mannose, and 31.7 ± 2.2% sulfate	Genotoxicity Bacterial mutation Bone marrow Micronucleus formation	Up to 2000 mg/kg/bw orally	Sprague Dawley rats	Fucoidan presents no significant genotoxic concern	(K.-J. Kim, Lee, & Lee, 2010)
Undaria pinnatifida	75% fucoidan	Full blood count, clinical biochemistry	3 g per day for 12 days	Human	No toxicity	(K.-J. Kim, O.-H. Lee, H.-H. Lee, et al., 2010)
Fucus vesiculosus Macrocystis pyrifera Laminaria japonica	75% total fucoidan	Full blood count, clinical biochemistry	0.1 and 1g for 84 days	Human	No toxicity	(Myers et al., 2010)

Fucus vesiculosus Macrocystis pyrifera Laminaria japonica	75% total fucoidan	Full blood count, clinical biochemistry	0.1 and 1g for 84 days	Human	No toxicity	(Myers et al., 2011)
Cladisiphon sp.	75% total fucoidan	Patients with HTLV1 were treated clinically	6 g per day up to 13 months	Human	Some patients suffered from diarrhoea; No other toxicity noted;	(Araya et al., 2011)

1.4.5 Fucoidan Anti-Cancer Potential

Anticancer potential of fucoidan against different types of cancer have been documented in several *in vitro* and *in vivo* studies. Still, research supporting fucoidan anticancer properties in clinical trials is very rare. Even though, the mode of action of fucoidan is not fully established, various mechanisms like activation of apoptosis, induction of cell cycle arrest, activation of immune response are proposed for its anticancer property. Several other mechanisms (comparatively less widely studied) like induction of inflammation through oxidative stress, stem cell mobilization etc. are also suggested to be responsible for anticancer properties of fucoidan.

1.4.5.1 Antiproliferative or cytotoxic effect of fucoidan

Fucoidan from distinct sources, was found to have antiproliferative effect on various cell lines (listed in the Table 3). These studies support the fact that fucoidan inhibit the proliferation of various cancer cell lines, and it would be very promising to establish fucoidan anticancerous nature further.

Table 3: List of fucoidan isolated from marine brown seaweed species and its antiproliferative effect on various cancer cell lines.

Species (brown seaweed)	Cancer types and cell lines	Reference
Fucus vesiculosus	Lymphoma (HS-Sultan cells)	(Araya et al., 2011)
Cladosiphon novae-caledoniae	Breast (MCF-7 and MDA-MB-231), Cervical carcinoma (HeLa), Fibro sarcoma (HT1080)	(Z. Zhang, Teruya, Eto, & Shirahata, 2011)
Cladosiphon novae-caledoniae	Breast (MDA-MB-231)	(Senthilkumar & Kim, 2014)
Saccharina japonica and Undaria pinnatifida	Bladder and Melanoma (T-47D and SK-MEL-28)	(Vishchuk, Ermakova, & Zvyagintseva, 2011)

Cladosiphon okamuranus	Liver (Huh7 and HepG2 cell line)	(Nagamine et al., 2009)
Fucus vesiculosus	Lung (A549 cells) Leukemia (HL-60 and THP-1 cells)	(Jin, Song, Kim, Park, & Kwak, 2010)

1.4.5.2 Fucoidan and cell cycle

The cell cycle is a series or set of events which is responsible for cell division or duplication. It is an energy dependent, highly regulated process, which ensures the correct duplication and segregation of the genome. This process involves four sequential phases, that span from senescent (G0) to proliferation (GI,S,G2 and M phase) and back to senescent (Norbury & Nurse, 1992). Deregulation in cell cycle is the most prominent cause of cancer. Hence, therapeutics or potential anticancer candidate (fucoidan) which can either modulate signalling pathways (in cancer cells) leading to cell cycle regulation or can directly alter cell cycle regulatory molecule is being investigated. Fucoidan treatment is documented to regulate the cell cycle of cancerous systems *in vivo*. Inhibitory effect of fucoidan on cell cycle from different sources at single or range of concentrations with respective references is listed in Table 4.

Table 4: Apoptosis inducing effects of fucoidan on various cancerous cell lines.

Fucoidan Sources	Cancer cell type	Dose (µg/ml)	Effect on cell cycle	References
Fucus vesiculosus	Human lymphoma (HS-sultan cells)	100	• ↑ sub-/G1 • No G0/G1 or G2/M arrest	(Aisa et al., 2005)
Okinawa mozuku	Human hepatocellular carcinoma cells (HAK-1A, KYN-2, KYN-3 cells)	22.5	• ↑ G2/M phase	(Fukahori et al., 2008)

Fucus vesiculosus	Human acute leukemia (NB4 and HL-60 cells)	150	• ↑ sub-G1	(Jin et al., 2010)
Undaria pinnatifida	Human lung cancer(A549 cells)	50, 100, 200	• ↑ sub-G1	(H. J. Boo et al., 2011)
Cladosiphon novae-caledoniae	Human breast cancer (MCF-7 cells)	82, 410, 820	• ↑ sub-G1 • No significant changes in cell cycle distribution	(Z. Zhang et al., 2011)
Undaria pinnatifida	Human prostate cancer (PC-3 cells)	100	• ↑ sub-G1	(H.-J. Boo et al., 2013)
Fucus vesiculosus	Human bladder carcinoma (5637 and T-24 cells)	100	• ↑ G1 phase	(Cho, Kim, & Moon, 2014)

1.4.5.3 Fucoidan and Apoptosis

Apoptosis is a process of programmed cell death, which is very critical for the survival of multicellular organisms (Lockshin & Zakeri, 2007). It is characterised by distinct morphologic characteristics and energy dependent biochemical mechanisms, which are highly regulated and controlled processes. Deregulation of this inherent defence mechanism of body, leads to abnormal cellular proliferation and accumulation of genetic defects. This ultimately leads to genetic mutation and carcinogenesis (Johnstone, Ruefli, & Lowe, 2002). Many researchers have shown that fucoidan has time and dose dependent cytotoxic effect on different types of cancer cells (hepatocellular carcinomas, cholangiocarcinoma, gallbladder cancer, ovarian cancers) *in vitro* (Fukahori et al., 2008; Z. Zhang et al., 2011). This seems to be indicative of the fact that fucoidan induces the characteristic changes associated with apoptosis and brings drug induced cell death (Fukahori et al., 2008).

Apoptosis is mediated through either, the extrinsic (cytoplasmic) pathway whereby death receptors trigger the apoptosis, or intrinsic (mitochondrial) pathway in which

changes in mitochondrial membrane potential (MMP) lead to cytochrome C release and death signal activation. These pathways are said to activate caspases that cleave regulatory and structural molecules (Ghobrial, Witzig, & Adjei, 2005). Studies showed that fucoidan extracted from Ecklinia cava, dose dependently enhanced the DNA fragmentation on human leukemia monocyte lymphoma (U-937) and had a clear effect on the caspase-7, caspase 8 which cleave protein substrates, including PARP, an inducer of apoptosis responsible for DNA cleavage (Athukorala et al., 2009). Similarly, fucoidan from *Fucus vesiculosus* induced apoptosis of human hepatoma cell (HepG2) by down regulation of P-Stat3 (Roshan & Banafa, 2014). Fucoidan also affects other components of extrinsic and intrinsic pathways. There are several other studies supporting the drug induced cell death property of fucoidan and suggesting the probable mechanism it follows while inducing cell death (Haneji et al., 2005; Z. Zhang et al., 2011; Z. Zhang, Teruya, Eto, & Shirahata, 2013). Figure 3 schematically represents the proposed mechanism behind cell cycle inhibition or arrest and apoptosis inducing effect of fucoidan.

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Figure 3: Schematic representation of cell cycle arrest and apoptosis mechanisms of fucoidan. Source: (Senthilkumar, Manivasagan, Venkatesan, & Kim, 2013)

1.4.5.4 Fucoidan and Metastasis

Metastasis is one of the important hallmarks of cancer. 90% of cancer related death is because of metastasis. It is the process by which cancer spreads from its primary site (place of origin) to distant locations in the body (development of secondary malignant growths) (Fidler & Ellis, 1994). Other than acquired abilities of increased motility and invasiveness (by cancer cells), Matrix metalloproteinases (MMPs) is also documented to play a key role in tumour metastasis (Price, 1989). For invasiveness, the degradation of extracellular matrix (ECM) is important, which emphasises the need of matrix degrading proteinases. Fucoidan is documented to suppress MMP-2 activity in A549 (lung) cancer cells with increasing concentration of fucoidan(Lee, Kim, & Kim, 2012). Fucoidan anti-metastasis property has also been proven in animal model of transplanted Lewis lung carcinoma (LLC) cells (Alekseyenko et al., 2007). There are several cell surface proteins

involved in migration and cell adhesion essential for metastasis, fucoidan has been shown to have an adverse effect (Lake et al., 2006; Rouzet et al., 2011).

1.4.5.5 Fucoidan and Angiogenesis

Angiogenesis is the process in which new blood vessels are formed. It is a highly regulated process and is essential for embryonic development and wound healing as it supplies nutrient and oxygen (Folkman, 1995). Tumour progression requires persistent supply of nutrients for manifestation of cancer, hence, uncontrolled and persistent angiogenesis is inevitable. Inhibiting angiogenesis during tumour progression is one of the possible therapies against cancer (Nelson, 1998). Fucoidan from Undaria pinnatifida has shown to dose dependently inhibit angiogenesis against human umbilical vein endothelial cell (HUVEC) (Liu et al., 2012). Recent studies have suggested that fucoidan significantly reduced the tumour growth in 4T1-bearing mice, by suppressing cell proliferation, colony formation, and blocking cell migration and cell invasion (H.-Y. Hsu et al., 2012).

1.4.5.6 Fucoidan and growth signalling pathways

For human biology and also for medicine, regulated cell signal transmittances is important. Although it is a very complex process, what really happens is this - there is transmittance of information from extra-cellular environment into cells so that cells can respond appropriately to the signals. For communication between and within cells, mechanisms like phosphorylation and proteolysis, are put into work. Herein, signalling proteins experience, a switch like activation from an inactive to an active state and vice versa (Tonks & Neel, 1996). Published documents indicate that various compounds exert chemo preventive and chemotherapeutic effects through the inhibition of phosphorylation of membrane receptors, including receptor tyrosine kinases (RTKs), epidermal growth factor (EGFR) and platelet-derived growth factor receptor (PDGFR), which are involved in the transduction of mitogenic signals across the plasma membrane and in the regulation of cell growth and proliferation.

Marine compounds have been shown to interrupt growth factor stimulated cell signalling pathways (Figure 3), like Ras activate MAP kinase cascade (pathway involves: Ras → Raf → extracellular regulated kinas (ERK) →mitogen activated kinase/ERK-kinase (MEK) →activator protein (AP)-1 pathway), which is important for targeting

transcription factors to stimulate the entry of cell into the cell cycle and maintain cell proliferation, differentiation and apoptosis (Smalley, 2003; Whitmarsh & Davis, 2007). Faulty ERK1/2 pathway is involved in the malignancy manifestation of many types of cancer (Suthiphongchai, Promyart, Virochrut, Tohtong, & Wilairat, 2003; Whitmarsh & Davis, 2007). Other than ERK1/2 pathways, P13K –AKT pathway is also related to invasiveness and metastasis of non-small – cell lung cancer (NSCLC) and other tumours (Liao, Wang, Zhang, & Liu, 2006).

Fucoidan was proposed to inactivate ERK1/2 pathways, in human lung cancer cells (A549) and inhibit its apoptosis and hence, implicate its role in modulating signalling pathway (H.-Y. Hsu et al., 2014). Cancer therapeutics involving fucoidan and signalling pathways are still in very early phase of development. More experimentations are needed to support and validate fucoidan effects on signalling pathways in cells. A schematic representation of proposed involvement of fucoidan on growth signalling pathways in cells is illustrated in Figure 4.

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Figure 4: Schematic representation of proposed involvement of fucoidan on growth signalling pathways in cells. Source: (Senthilkumar et al., 2013)

Despite tremendous progress in medicine, the treatment options available for cancer is limited by their side effects. Hence, the quality of life of people, suffering from cancer has remained questionable. Therefore, there is a need to establish a therapeutics, whose cytotoxic activity is selective (i.e. causes minimal damage to heathier tissues and eradicated tumour cells). Natural substances with minimal toxicity is proposed to be a solution to the problem.

Fucoidan has been documented to possess strong anticancer bioactivity, with minimal or negligible toxicity on healthy tissues. Owing to the selective nature of fucoidan, it is a potential natural substance, to be developed as a cancer therapeutics. Although fucoidan has been tested against different types of cancer, not enough research has been done regarding the anti-cancer effect of fucoidan on prostate cancer. Hence, it is

imperative to establish the cytotoxic effect of fucoidan along with its mechanism of action (as an anti-cancer agent) against prostate cancer, which is a leading cause of cancer mortality among men around the world.

1.5 Anticancer Bioassays

Oncological processes are characterized by an unregulated increase in cellular proliferation (Hanahan & Weinberg, 2000); hence, the availability of a suitable *in vitro* cytotoxicity assay in the early discovery efforts of screening number of promising chemicals would provide an important advantage in identifying novel agents. Cell assays are based on various cell functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity. Some of the major bioassays available for screening number of compounds and to determine if the test molecules have effects on cell proliferation or show direct cytotoxic effects are discussed below.

1.5.1 Trypan Blue Exclusion Assay

Trypan Blue is a commonly used dye for assessing cell viability by dye exclusion assays. Dye exclusion assays are based on the principle that an intact cell membrane is necessary for the exclusion of certain dyes (Allison & Ridolpho, 1980). Hence trypan blue selectively colours the dead cells blue and leaves the viable cells unstained. In this method cell viability is determined by counting the unstained cells under an inverted microscope using Neubauer chamber. Even though it is widely used the trypan blue (TB) exclusion assay has limitations. Firstly the dye can be incorporated by live cells after a short exposure time, and secondly, trypan blue staining cannot be used to differentiate between healthy cells and cells which are alive but losing cell functions. If there are large numbers of test compounds to be analysed, it can provide low precision results because of the lengthy run time and intensive microscopic examination needed (Avelar-Freitas et al., 2014).

1.5.2 Clonogenic assay or Colony formation assay

Clonogenic assay is an in vitro cell survival assay which is based on the ability of a single cell to grow into a colony. The assay fundamentally analysis every cell in the population for its “infinite” division capability (Franken, Rodermond, Stap, Haveman, & Van Bree,

2006). It is routinely used to deduce cell reproductive death after treatment with ionizing radiation. It is considered to be the "gold standard assay" in Radiobiology (Algan, Stobbe, Helt, Hanks, & Chapman, 1996). However, this technique has its drawbacks too. The assay takes a number of weeks to yield useful data. Quality control has also been a problem with the cloning assays, in particular difficulties of distinguishing clones from cell clumps.

1.5.3 Thymidine Incorporation Assay

Thymidine incorporation assay is based on nucleotide uptake activity. It utilizes a strategy wherein a radioactive nucleoside, 3H-thymidine, is incorporated into new strands of chromosomal DNA during mitotic cell division. A scintillation beta-counter is used to measure the radioactivity in DNA recovered from the cells in order to determine the extent of cell division that has occurred in response to a test agent (Ahmed, Gogal, & Walsh, 1994). As it provides a direct measurement of proliferation, it is considered to be a very sensitive method, but the use of radioisotope is a big health concern.

1.5.4 MTT Cell Proliferation Assay

The MTT cell proliferation assay was the first homologous assay developed for a 96-well format that was suitable for high throughput screening (HTS). This assay was first described by Mossman in 1983 (Mosmann, 1983). It utilizes the ability of viable cells to convert a water soluble tetrazolium salt, 3-4, 5 dimethylthiazol-2, 5 diphenyl tetrazolium bromide (MTT), into an insoluble formazan precipitate (Slater, Sawyer, & Sträuli, 1963). The reduction is aided by mitochondrial succinate dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH). The purple-coloured formazan crystals (formed after addition of MTT) can be dissolved in a variety of organic solvents (DMSO, isopropanol) and the quantity of formazan (presumably directly proportional to the number of viable cells) is measured by recording the absorbance at 540 nm, on a multiwall spectrophotometer (ELISA plate reader). A references wavelength of 680 nm is also used.

The amount of signal generated is dependent on several parameters including:

- the concentration of MTT,
- the length of the incubation period,

- the number of viable cells and their metabolic activity.

All of these parameters should be considered when optimizing the assay conditions for particular cell line, so that it generates enough amount of product, which can be detected above background.

1.5.4.1 Pros and cons of MTT Cell Proliferation Assay

The MTT tetrazolium assay has been widely accepted and remains popular in academic labs for screening compounds (for its antiproliferative property) against various cell lines (Carmichael et al., 1988; Lee et al., 2012; Sordella, Bell, Haber, & Settleman, 2004). As MTT assay requires reagent which are very economical, and easy to store and maintain, it is considered to be the most economic, convenient and rapid methods available (Berg, Zhai, Chen, Kharazmi, & Owen, 1994). In comparison to other anticancer bioassay discussed above, MTT assay estimates the number of viable cells after treatment with the drug in question for its potential anticancer property, in a multi-well plate system using a spectrophotometer (ELISA plate reader). Hence it is easy to analyse a large number of potential anticancer compounds (with different concentrations plan for each drug) in a plate, which allows MTT assay to be high through put and semi-automated screening assay.

In 1997, Malich and his colleagues (Malich, Markovic, & Winder, 1997) analysed, twenty chemicals for their cytotoxicity against epithelial carcinoma of the cervix (HeLa) and normal epithelial cell lines (F1-73). The results from this study showed that there is a good correlation between data obtained from MTT assay and published data. Hence, this finding corresponds to the specificity of MTT assay.

But it has its limitations too. MTT assay is limited by its cytotoxic nature. As documented, the conversion of MTT substrate to formazan by cells is a time dependent process, which in fact accounts for the sensitivity of this assay, but if cells are incubated for longer period than optimized (for each cell line), it may lead to a false positive result (T. Riss et al., 2004). It has also been documented that owing to the cytotoxic nature of MTT, the morphology of the certain types of cells changed drastically, after subsequent addition of MTT (Squatrito, Connor, & Buller, 1995).

A false increase in absorbance values in MTT assay has been said to occur when certain reducing compounds, like, ascorbic acid, or sulfhydryl-containing compounds interact

with tetrazolium salts, and reduce it non-enzymatically (Ulukaya, Colakogullari, & Wood, 2004). Prolong exposure to direct sunlight and an elevation in pH of culture medium also seems to interfere with tetrazolium salts and gives a false increase in absorbance values (T. Riss et al., 2004).

1.5.5. Flow Cytometric Analysis of Apoptosis by Propidium Iodide

Apoptosis is a process of programmed cell death, which is very critical for the survival of multicellular organisms (Lockshin & Zakeri, 2007). It is characterised by distinct morphologic characteristics and energy dependent biochemical mechanisms, which are highly regulated and controlled process. Apoptosis is mediated through either, the extrinsic (cytoplasmic) pathway whereby death receptors trigger the apoptosis, or intrinsic (mitochondrial) pathway in which changes in mitochondrial membrane potential (MMP) lead to cytochrome C release and death signal activation. These pathways are said to activate a family of protein known as caspases that cleave regulatory and structural molecules, which are mandatory for normal cellular function (Ghobrial et al., 2005). The caspases also regulate degradation enzymes like, deoxyribonucleases - DNases (one type of nuclease). This enzyme catalyses the hydrolytic cleavage of phosphodiester linkages in the DNA backbone between oligonucleosomes (Arends, Morris, & Wyllie, 1990). The products of DNA cleavage are nucleosomal and oligonucleosomal DNA fragments (180 base pairs and multiples of 180 base pairs). It is important to note here that this fragmentation of the genomic DNA, is the biochemical hallmark of apoptosis and its quantification in apoptotic cells is the principle behind this assay.

As the DNA in the apoptotic cells are partially degraded, the fraction of low-molecular-weight DNA can be extracted, whereas the non-degraded DNA remains in the cell nucleus (Gong, Traganos, & Darzynkiewicz, 1994). It is documented that as the DNA fragments are lost from apoptotic nuclei and nuclear DNA content can be easily measured by flow cytometry, after it has been stained with fluorochrome, like Propidium iodide (PI). Hence, this method can be used for detection and quantification of apoptotic nuclei.

PI is a fluorogenic compound that bind stoichiometrically to nucleic acid (Ormerod, Collins, Rodriguez-Tarduchy, & Robertson, 1992), so that fluorescence emission is

proportional to DNA (and RNA, which has to be removed if DNA is to be measured) content of a cell. As described by Ormerod (Ormerod, 2002), during experiments when apoptotic cells are stained with PI and analysed with flow cytometry, the DNA histogram display a somewhat broad hypodiploid (sub-G1) peak, which are discriminated from proportion of cells in G0/G1, S and G2/M phases as narrow peak of cells with normal (diploid) DNA content. Hence, apoptotic cells with hypodiploid DNA would be measured by quantifying the sub-G1 peak in the cell cycle pattern.

1.5.5.1 Pros and cons of Flow Cytometric Analysis of Apoptosis by Propidium Iodide

There are several advantages of flow cytometric assay used for estimation of apoptosis; primarily being, rapid, reliable and reproducible in quantifying apoptosis. The second important advantage of this assay is that it allows the simultaneous analysis of the cell cycle parameter of surviving cells in the DNA histogram (which is obtained during analysis of apoptosis) (Telford, King, & Fraker, 1994). In addition to its rapidity, it also allows the fixed samples (using 80% ethanol) to be prepared and analyzed at a later time.

However, it should be noted that there are many types of apoptosis and extensive DNA fragmentation and loss of DNA is not central in all apoptotic death. Another important fact is that DNA fragmentation and hence hypodiploid nuclei may be displayed in necrosis (Darzynkiewicz, Bedner, & Smolewski, 2001). Hence optimization of this assay for different cell lines is important.

Nevertheless, when used appropriately, the PI flow cytometric assay is a rapid and easily reproducible method that can be adapted for apoptosis evaluation in different kinds of cells. It also offers a good start for screening, potential apoptotic compounds for cancer therapeutics by demonstrating, that a potential compound is increasing the number of dead cells by inducing apoptosis in different types of cancers. Once a compound is established for its apoptosis inducing ability (in *in vitro* studies) it can be further cross checked with other methods available and further analysed in *in vivo* studies and clinical trials.

1.6 Objectives

The aims of this thesis were as follows:

1) To explore the cancer chemotherapeutics potential of crude and pure forms of fucoidan from *F. vesiculosus* and *U. pinnatifida*.

2) To investigate the antiproliferative effect of fucoidan on prostate cancer cell lines PC-3 and DU-145 and to estimate the IC_{50} values for each type of fucoidan tested in this study.

3) To compare and identify which form of fucoidan has the highest anticancer potential against prostate cancer cell lines.

4) To estimate the cytotoxic effect of fucoidans tested in this study on noncancerous or normal cell line HEK-293. `

5) To propose the basic mechanism of action of fucoidan as an anti-cancer agent against prostate cancer, using flow Cytometer.

CHAPTER TWO

2. MATERIALS AND METHODS

Overview of methodologies applied in this research

The major goals behind carrying out this project was to estimate and compare the anticancer therapeutic potential of different types of fucoidan (crude and pure forms) on prostate adenocarcinoma (PC-3 and DU-145) and on noncancerous HEK--293 cells. Cell-based assays are often used to screen compounds in order to determine if the test molecules have effects on cell proliferation and understand the mechanism behind cytotoxic effects that eventually lead to cell death. The first assay employed in this study is MTT Cell viability Assay (the principle, mechanism and reasons for choosing this assay over others are reviewed in section 1.5).

MTT assay helps to identify the dose and time-dependency of different types of fucoidan on cancer cells (PC-3 and DU-145) over normal cells (HEK-293).

Once the antiproliferative or cytotoxic potential was established it was important to elucidate the mechanism by which our experimental drugs does so. It was proposed that fucoidan induces apoptosis in cells to support its cytotoxic effect on different cell lines. Hence apoptosis inducing effect of different type of fucoidan in a dose dependent manner were analysed by Flow Cytometric (Beckman Coulter MoFlo™ XDP) analysis of Apoptosis. The principle, mechanism and reasons for choosing this assay over other are reviewed in section 1.5.5.

To have a clear or stepwise idea of methodologies involved in this research, I have separated the methodologies under three headings.

A. Cell culture
B. Cell viability Assay
C. Apoptosis Assay

Herein, it is very important to note that, all solutions and equipment that came in contact with the cells were sterile and followed proper sterile techniques and experiments were carried out in a biological safety cabinet Class I.

2.1 Cell Culture

Two prostate carcinoma cell lines (PC-3 and DU-145) and one non-cancerous (HEK-293) cell lines were considered to evaluate the proposed antiproliferative and apoptosis inducing potential of fucoidan from *Fucus vesiculosus* and *Undaria pinnatifida*. The cell lines were obtained from American Type Culture Collection (ATCC), USA, and once cells vials were received from ATCC, they are stored in liquid nitrogen freezer. Table 5 lists all the general information about cell lines tested in this study.

Table 5: General Information regarding ATCC cell line analysed (source: ATCC, USA)

Cell lines	General description	Culture properties	Morphology
PC-3 (ATCC® CRL-1435™)	Prostate carcinoma (grade IV, adenocarcinoma); derived from metastatic site: brain; Human	Adherent	Epithelial
DU 145 (ATCC® HTB-81™)	Prostate carcinoma; derived from metastatic site: brain; Human	Adherent	Epithelial
HEK-293 (ATCC® CRL-1573™)	Embryonic kidney; Human	Adherent	Epithelial

2.1.1. Materials and equipment required for cell culture

The materials reagents which were required for culturing cells *in vitro* are listed in table 6.

Table 6: General requirements for cell culture

Materials and equipment	Suppliers
Roswell Park Memorial Institute (RPMI) 1640 Medium, no phenol red	Life technologies (NZ)
Eagle's Minimum Essential Medium (EMEM)	ATCC (USA)
Foetal Bovine Serum (FBS)	Medica Pacifica (NZ)
L-Glutamine (200 mM; 100 ml)	Life technologies (NZ)
Penicillin-Streptomycin (10,000 U/mL; 100 mL)	Life technologies (NZ)
TrypLE™ Express (1X), no phenol red	Life technologies (NZ)
Dulbecco's Phosphate Buffered Saline (D-PBS), pH 7.2, no calcium magnesium and phenol red	Life technologies (NZ)
Trypan Blue Stain (0.4%)	Life technologies (NZ)
Synth-a-Freeze Medium	Life technologies (NZ)
Biological Safety Cabinet	Airpro Scientific LTD
Inverted Phase Contrast Microscope	Olympus
Haemocytometer	Life technologies (NZ)

2.1.2 Preparation of complete growth media

The base medium used for culturing PC-3 and HEK-293 was RPMI-1640. ATCC cell culture guidelines strongly recommended usage of ATCC-formulated EMEM base medium for culturing DU-145 cells. To make the complete growth medium for PC-3 and HEK-293, the following components were added to the RPMI -1640 base medium.

- Fetal bovine serum to a final concentration of 10%.
- 1 % pen-step
- 1 % L-glutamine

To make the complete growth medium, for DU-145, the following component was added to the base medium.

- Fetal bovine serum to a final concentration of 10%.

2.1.3 Initiating frozen culture

Once the vials with respective frozen cells were received from ATCC, they were stored in liquid nitrogen freezer. After preparation of complete mediums (RPMI 1640 for PC-3 and EMEM for DU-145), the respective cells were revived in a step wise manner as described below.

- The cryovial containing frozen cells was removed from liquid nitrogen storage and placed in an ice box for about 10 minutes, as sudden change in temperature (from too cold to room temperature) would cause the vial to bust.
- Afterwards, the vial was thawed rapidly by briefly immersing it in 37°C water bath for 2–3 min with constant agitation.
- The vial was removed from the water bath and decontaminated by dipping in or spraying with 70% ethanol. Hereafter, further process involved in unthawing of cells were done in a biological safety cabinet, with strict aseptic techniques.
- The vial was unscrewed and content was transferred to a sterile 15 ml centrifuge tube (which already has 4 ml of the prewarmed complete growth medium).
- The centrifuge tube was then subjected to centrifugation at 500 x g for 5 minutes (this step helps to remove the cryoprotectant agent (DMSO) which is present along with the cells in the vial before unthawing them).
- After centrifugation, the supernatant was discarded, without disturbing the cell pellet and then the pellet was resuspended in 1 or 2 ml of complete growth medium.
- The cell suspension was transferred into T25 culture flask containing prewarmed complete growth medium. To avoid clumping of cells and to facilitate even distribution of cells in the flask, the cell suspension was mixed thoroughly by gentle pipetting. Then the culture flasks were placed in a 37°C, 5% CO_2, humidified incubator.

2.1.4 Maintaining the cell culture

In order to avoid 100% cell confluency, and possess healthy cell population in culture flask, the cells need to be monitored at regular interval of time. If it was inferred after observation of culture flask under microscope that cells have been growing well for a few days but have not attained around 80% confluency, then medium has to be changed to replenish nutrients and maintain correct pH. For resuspending the cells in fresh complete medium, the spent medium was discarded from the culture flask with help of a pipette. Prewarmed PBS (approximately 1mL for T25 culture flask) was used to wash cells. After washing the cells, the suspension was discarded. Finally, the cells were resuspended in fresh prewarmed culture medium (approximately 5 mL for T25 culture flask). The culture flask was then returned to the incubator.

2.1.5 Passaging or sub culturing adherent Cells

Cells growing in a controlled condition needs to be subcultured at regular intervals so that the cells remain in their exponential growth phase. When the cells are roughly 70% to 80% confluent they are said to have reached near the end of exponential phase and apt to be subcultured.

Subculture involves the dissociation of the cells from each other and the substrate to generate a single-cell suspension that can be further cultured and quantified. Reseeding this cell suspension at a reduced concentration into a flask or dish generates a secondary culture, which can be maintained and subcultured further as needed for subsequent planned experiments. The steps involved in sub culturing are as followed:

- To start with, spent media from culture flask was removed and washing step was performed as discussed in section 2.1.4
- The prewarmed dissociating agent, TrypLE™ Express, approximately 1ml for T25 flask and 2ml for T75 (basically to cover the cell layer) was added to the side of the flask. The culture flask was gently swirled so that the entire surface of flask is covered with dissociation agent and incubated at room temperature to facilitate detachment.
- The progress of cell dissociation was checked under microscope.

- The time period required (2 to 10 minutes for most cell lines) for which cells would be incubated with TrypLE™ Express, for cell detachment depends upon cell line tested.
- In this study, it was deduced that HEK-293 cells detached from the culture flask almost immediately (around a minute) after TrypLE™ Express was added to the flask. Whereas the incubation required for cells detachment was longer for PC-3 and DU-145 cells (8 to 10 minutes).
- After it is confirmed that ≥ 90% of the cells have detached (cells appear rounded and refractile under microscope), the culture flask was tilted to allow the cells to drain and collect at one end of the corner of the flask.
- Around 2ml (twice the volume of the dissociation reagent) of pre-warmed complete growth medium was added to the cell suspension to inactivate the action of TrypLE™ Express. The suspension was mixed properly by gentle pipetting to disperse the cells.
- The suspension was transferred into a 15 ml centrifuge tube and centrifuged at 500 x g for 5 minutes.
- The supernatant was discarded and the pellet was resuspended in 1ml of prewarmed complete medium and 10 µL sample was removed for counting using a haemocytometer (described in Section 2.1.6).
- An appropriate volume of cells were dispensed into newly prepared flask (with prewarmed complete medium) and was placed back into the incubator for further growth.

Note:

Split ratio is an important factor which helps a researcher to ensure that cells would be ready for an experiment on a particular day or to just keep culturing cells for future use or as a backup. It is recommended that slow growing cells should have a low split ratio and fast growing cells should have a high split ratio to avoid overcrowding of cells. It should also be noted that most cells must not be split more than 1:10 as the seeding density will be too low for the cells to survive. General guidelines to be followed for a, confluent flask of cells are as follows:

- 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.

2.1.6 Counting Cells using a Haemocytometer

Preparing Sample

- After harvesting the cells, the cell suspension was resuspended in 1 ml of prewarmed complete medium and mixed gently. Care was taken so as to not generate any air bubble while mixing cell suspension, as this might lead to pipetting error.
- 10 µL of above cell suspension was removed using a micropipette and mixed with 10 µL (0.4%) Trypan Blue Stain (Trypan Blue selectively stains dead cells blue over live cells, hence useful in differentiation and counting of cells and estimating cell viability) to obtain 20 µL of cell mixture.

Loading Sample

- It should be noted that, it is very important to have an even distribution of cells on the haemocytometer slide. Firstly haemocytometer and cover slip were cleaned with 70% ethanol and then dried completely. Coverslip was placed on the counting chamber.
- Using a micropipette, smoothly, 10 µL of mixture (10 µL of cell suspension + 10 µL of (0.4%) Trypan Blue Stain) was added to the v-shaped groove of the haemocytometer counting chamber.
- The haemocytometer was placed in the inverted microscope under a 10X objective and cells within each of the four large quadrants was counted.
- The cell density (or concentration of cells in the mixture) was estimated using the following formula.

Concentration (Cells/ml) = Average number of cells x dilution factor x 10,000

- Here the dilution factor is 2 because 10 µL of cell suspension was diluted with 10 µL Trypan Blue.
- After the cell density was determined for each cell line, cells were either simply divided according to a routine split ratio and dispensed into the medium of the newly prepared flask or were seeded in microtiter plates (96 – well plate for MTT assay and 6-well plate for Apoptosis assay) to perform planned experiments.

2.1.7 Cryopreservation of Cells

Because an established cell line is a valuable resource and its replacement is expensive and time consuming, it is vitally important that they are frozen down and preserved for long-term storage. As soon as a small surplus of cells becomes available after sub culturing, they are frozen as a seed stock, and stored for further use. Working stocks can be prepared and replenished from frozen seed stocks. The protocol for cryopreservation of cells is as followed.

- Culture flask containing cells in log-phase of growth are harvested in the same manner as described in the Section 2.1.5.
- After centrifugation, the supernatant was disposed and the pellet was resuspended in Synth-a-Freeze® (cryopreservation medium) (the volume of cryopreservation medium depends upon the density of pellet, usually 1 ml of cryopreservation medium for each cryovial).
- The cryovials were stored in liquid nitrogen freezer for further use in future.

Note:

Synth-a-Freeze® is a liquid cryopreservation medium containing 10% dimethyl sulfoxide (DMSO) without antibiotics, hormones, growth factors, serum, or protein, and buffered with HEPES and sodium bicarbonate.

2.2 Cell Viability Assays

In this thesis, the antiproliferative effect of fucoidan from *Fucus vesiculosus* (both pure and crude forms) and *Undaria pinnatifida* (both pure and crude forms) on the growth of PC-3, DU-145 and HEK - 293 were evaluated using MTT Cell Proliferation Assay

(Mosmann, 1983). The principle, advantages and drawback associated with MTT Cell Proliferation Assay have been reviewed in Section 1.5.4.

2.2.1 MTT Cell Proliferation Assay

2.2.1.1 Materials and equipment required for MTT Cell Proliferation Assay

Other than cell culture requirements, the following equipment and materials are required for proper functioning of MTT assay.

Table 7: Requirements for MTT Cell Proliferation Assay

Materials and equipment	**supplier**
MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) Formazan powder;	Sigma Aldrich (USA)
DMSO (Dimethyl sulfoxide);	Merck Chemicals (NZ)
Microtiter plate reader (with 540 nm and 680 nm filters)	FLUOstarOmega, Alphatech (NZ)
Inverted microscope	Olympus Life Science
Millex GV 0.22 µM filter and a 10 ml syringe	Life Technology (NZ)
Multi-channel pipette; Haemocytometer	Life Technology (NZ)
96-well plate; sterile pipette tips; serological pipettes; centrifuge tubes etc.	ThermoFisher Scientific (NZ)

2.2.1.2 MTT Reagent Preparation

The steps involved in the preparation of MTT reagent is as follows:

- 5 mg of MTT powder was dissolved in 1ml of Dulbecco's Phosphate Buffered Saline (pH=7.4) by vortexing to obtain 12 mM MTT stock solution.
- The MTT solution was filter-sterilized through a 0.22 µM filter into a sterile, light protected container.

- The MTT solution was stored, protected from light, at 4°C, for frequent use or at -20°C for long term storage.

2.2.1.3 Basic Protocol for MTT Assay

The basic steps for MTT cell proliferation assay are as outlined.

1. Routine cell culture and detachment of cells from culture flask were performed as outlined previously.
2. After estimating the optimal cell count for each cell line (preferably between 1,000 and 100,000), cells were plated in 100µl of complete medium per well in 96-well (flat bottom).
3. The plates were incubated overnight at 37 °C in 5% CO_2.
4. A total of 10 µl of MTT stock solution was added and incubated until (usually 4 hours) purple precipitate was visible.
5. A total of 80 µl aliquot was removed from each well and then followed by the subsequent addition of 100 µl of DMSO.
6. Absorbance was read using a microtiter plate reader at 540nm (and background wavelength was 680nm).

2.2.1.4 Determination of MTT assay linearity range and optimal cell count

Before proceeding with the MTT cell viability assay to determine the antiproliferative potential of fucoidan (from different sources and form) against different cell lines, it was important to optimize the assay for each cell type used in this study. This was done by determining the optimal cell seeding density and incubation time for each cell type individually as follows:

- After cells in culture flask have attained 70% to 80% confluency, they were washed and detached with prewarmed PBS and TrypLE™ Express respectively (same instruction followed as mentioned in Section 2.1.5). The cell suspension obtained was subjected to centrifugation.
- The supernatant was discarded and pellet was resuspended in 1 ml of prewarmed complete medium.
- The cells are resuspended at 500,000 and 250,000 per mL (two dilution plans-listed in table 8) in prewarmed complete medium.

- Two sets of serial dilutions of cells in culture medium from 500000 to 1,953 cells per mL and 125,000 to 976 were found to be sufficient for determining the optimum cell seeding density.
- A total of 100 µL of each dilutions was plated into wells of a microtiter plate in triplicate.
- Three control wells of medium alone was included to provide the blanks for absorbance readings.
- The cells were incubated overnight (around 18 hours) for PC-3 and DU-145 and around 24 hours for HEK-293 (more incubation time was needed as it requires more time for reattachment to the bottom of 96 well plate).
- A total of 10 µL of MTT Reagent was added to each well, including controls.
- The plate was placed back into the incubator for 4 hours.
- Periodically the cells were viewed under an inverted microscope for presence of intracellular punctate purple precipitate. This step helped to determine the incubation time (around 4 hours) required for each of the different cell line used in this study, and assisted to optimize the MTT protocol for better results.
- When the purple precipitate was clearly visible under the microscope, 80 µL of the supernatant was removed from each well and 100 µL of DMSO (detergent reagent) was added to each wells, including controls.
- It was made sure that no bubbles were generated during the process.
- The microtiter plate was placed on a plate shaker for 5 minutes to completely dissolve the purple precipitate.
- Plate cover was removed and the absorbance in each well, including the blanks was measured, at 540 nm in a microtiter plate reader (FLUOstar Omega, Alphatech).
- The reference wavelength considered in this experiment was 680 nm.
- A linear relationship between absorbance value (OD value) and cell number was generated by plotting absorbance on the Y-axis against cell numbers on the X-axis. An optimum cell seeding density to be used in MTT assay (cell seeding density) should lie within the linear portion of the plot.

Note:

The incubation time of 4 hours was determined after careful observation of formazan formation for each cell line under the microscope. The formazan development was found to be time dependent, and that after reaching a maximal absorbance values a 4 hour incubation period with the MTT concentration of 5mg/ml was found to be necessary for all the three cell lines used here.

Suitable cell seeding 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.

Table 8: Serial dilution plan for making cell linearity standard curve

Plate labels	**Cell concentration Set I**	**Cell concentration Set II**
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

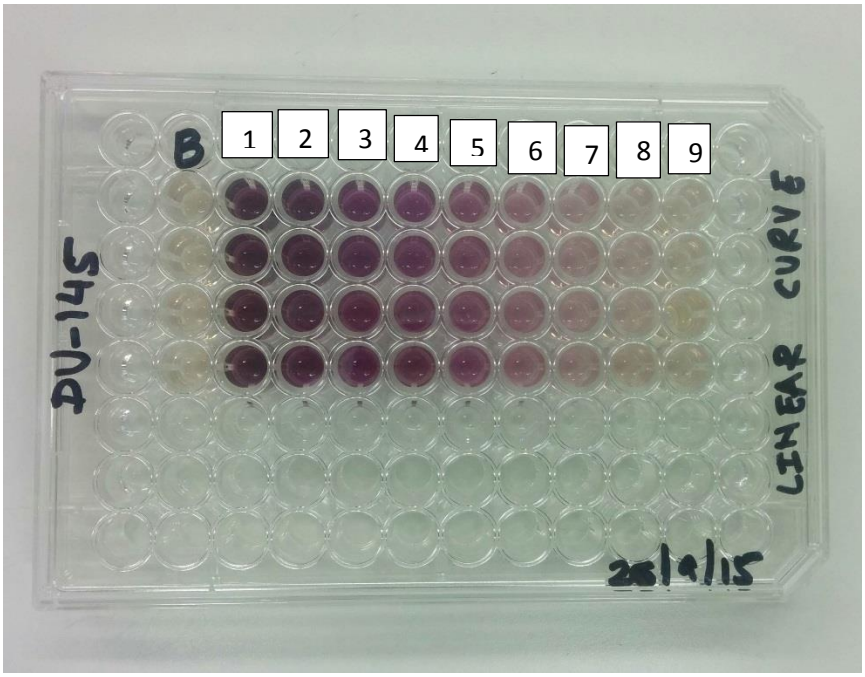


Figure 5: MTT assay performed after serially diluting cells and incubating overnight.

2.2.1.5 Estimation of antiproliferative potential of Fucoidan

The antiproliferative effect of fucoidan from different sources (Refer Table 10) was determined by MTT cell proliferation Assay. The cytotoxic effect was estimated against 2 prostate cancer cell line (PC-3 and DU-145) and 1 non-cancerous cell line (HEK-293), in a time response (day 1 i.e. 24 hours; day 2 i.e. 48 hours and day 3 i.e. 72 hours) and dose response manner. The procedure is explained in detail as followed.

2.2.1.5.1 Harvesting and plating of cells

- Cells were harvested using the procedure mentioned in Section 2.2.1.4. After the optimal cell seeding density was determined for each cell line (which is 5000 cells/well for all the three cell lines in this study) cells were seeded in 96 well plates at densities of 5000 cell/well.
- Herein four plates were seeded for each cell line to evaluate, time (day 0, day 1 i.e. 24 hours; day 2 i.e. 48 hours and day 3 i.e. 72 hours) and dose dependent effects of fucoidan.
- After cell seeding, the microtiter plates were incubated at 37°C, 5% CO_2, overnight prior to addition of fucoidan.

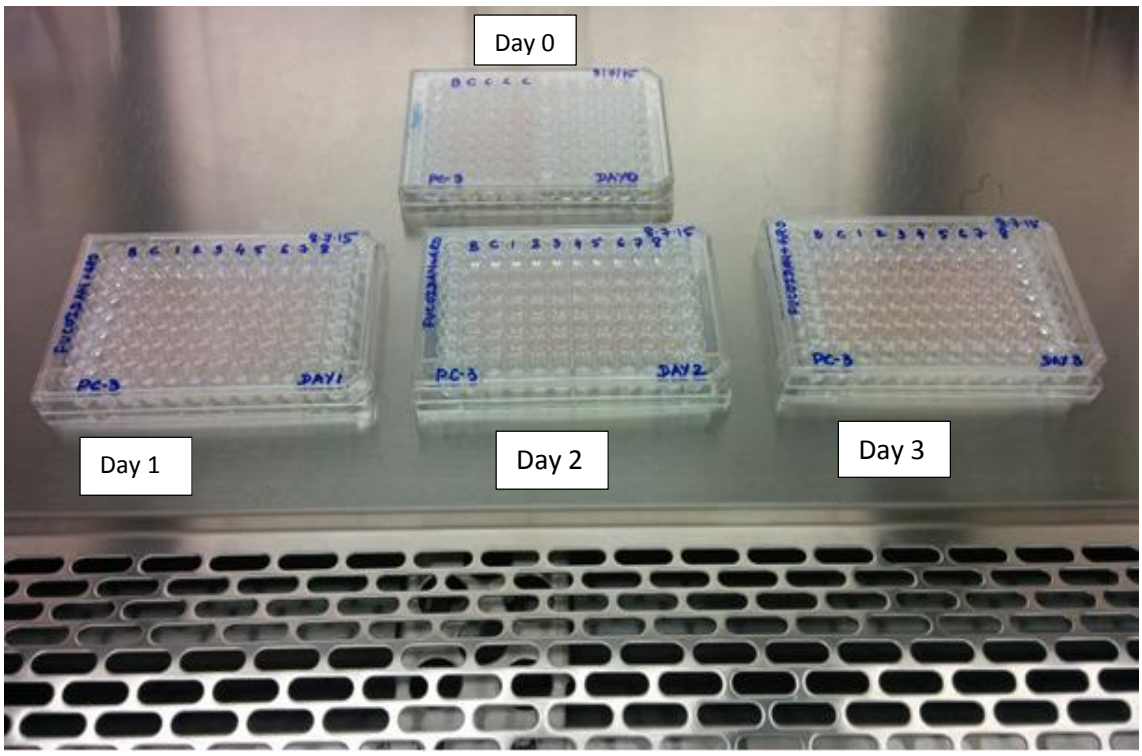


Figure 6: Cell seeding plan in 96-well plates for estimation of antiproliferative effect of fucoidan in a time and dose dependent manner.

2.2.1.5.2 Preparation of stock of fucoidan and desired dilutions tested

General information regarding various types fucoidan used in this study is mentioned in Table 9.

Table 9: General description of fucoidan used in this study

Type of fucoidan	Quality	Supplier
Fucoidan from *Fucus vesiculosus;* F5631; (CCF)	Crude	Sigma Aldrich (USA)
Fucoidan from *Fucus vesiculosus;* F8190; (CPF)	≥95% pure	Sigma Aldrich (USA)
Fucoidan from New Zealand *Undaria pinnatifida;* (ACF)	Crude	Extracted at AUT (NZ)
Fucoidan from *Undaria pinnatifida;* F8315; (UPF)	≥95% pure	Sigma Aldrich (USA)

- 16 mg of each type of fucoidan (listed in Table 10 above) was dissolved in 1 ml of pre warmed complete medium to have a final concentration of 16mg/ml. This served as our stock. Fresh stock was prepared every time treatment was supposed to be added to the 96-well microtiter plate.
- It is very important to note that for PC-3 and HEK-293 cells complete RPMI-1640 medium was used, and for DU-145, complete EMEM medium was used for culturing, preparation of different types of fucoidan stock and for preparation of different dilutions of fucoidan evaluated in this study.
- For each type of fucoidan, respective stock was diluted to prepare different concentration of fucoidan which were tested for its antiproliferative potential against the three cell lines. The dilution plan remains the same for all the four types of fucoidan. The dilution plan for fucoidan is listed in Table 10 below.

Table 10: Various concentrations of fucoidan tested in this study

Tube labels	**Test concentration (µg/ml)**	**Medium concentration (µg/ml)**	**Dilution plan (volume of stock + volume of complete medium)**
1	5	25	5 µl of stock of fucoidan + 3195 µl of complete medium
2	25	50	10 µl of stock of fucoidan + 3190 µl of complete medium
3	50	100	20 µl of stock of fucoidan + 3180 µl of complete medium
4	100	200	40 µl of stock of fucoidan + 3160 µl of complete medium
5	200	400	80 µl of stock of fucoidan + 3120 µl of complete medium
6	400	800	160 µl of stock of fucoidan + 3040 µl of complete medium

7	800	1600	320 µl of stock of fucoidan + 2880 µl of complete medium
8	1000	2000	400 µl of stock of fucoidan + 2800 µl of complete medium

Test concentration: Fucoidan inhibition test concentration in 96-well plate; Medium concentration: Fucoidan diluted concentration in complete culture medium

2.2.1.5.3 Addition of treatment

After cells were seeded in 96 well plates and incubated overnight (for proper attachment of cells), for the microtiter plate marked as day 0, basic protocol of MTT which is discussed in Section 2.2.1.3 was performed. Day 0 plate is required if time point based study has to be undertaken as it provide the references point from which cell proliferation is estimated.

The very day, after different concentration of each type of fucoidan (refer to Table 11) was prepared, 100 µl of each dilution was added to each well of respective column. There are six replicates for each concentration. 100 µl of prewarmed complete medium was added to column marked as blank and control.

After addition of different dilutions of treatment (to respective column) to plates marked as Day 1, 2 and 3; they are incubated for 24 hours, 48 hours and 72 hours respectively. Subsequently when the required amount of incubation period is over for each plate, cytotoxic effect of different concentrations of fucoidan was estimated by MTT assay (as discussed in Section 2.2.1.3). For example, plate marked as Day 1 was analysed (by MTT assay) for the cytotoxic effect of fucoidan after 24 hours of incubation. The general plan for addition of various dilutions of fucoidan in a 96-well plate for MTT assay is depicted in Figure 7. Each plate had six replicates for each test concentration and each experiment was repeated three times.

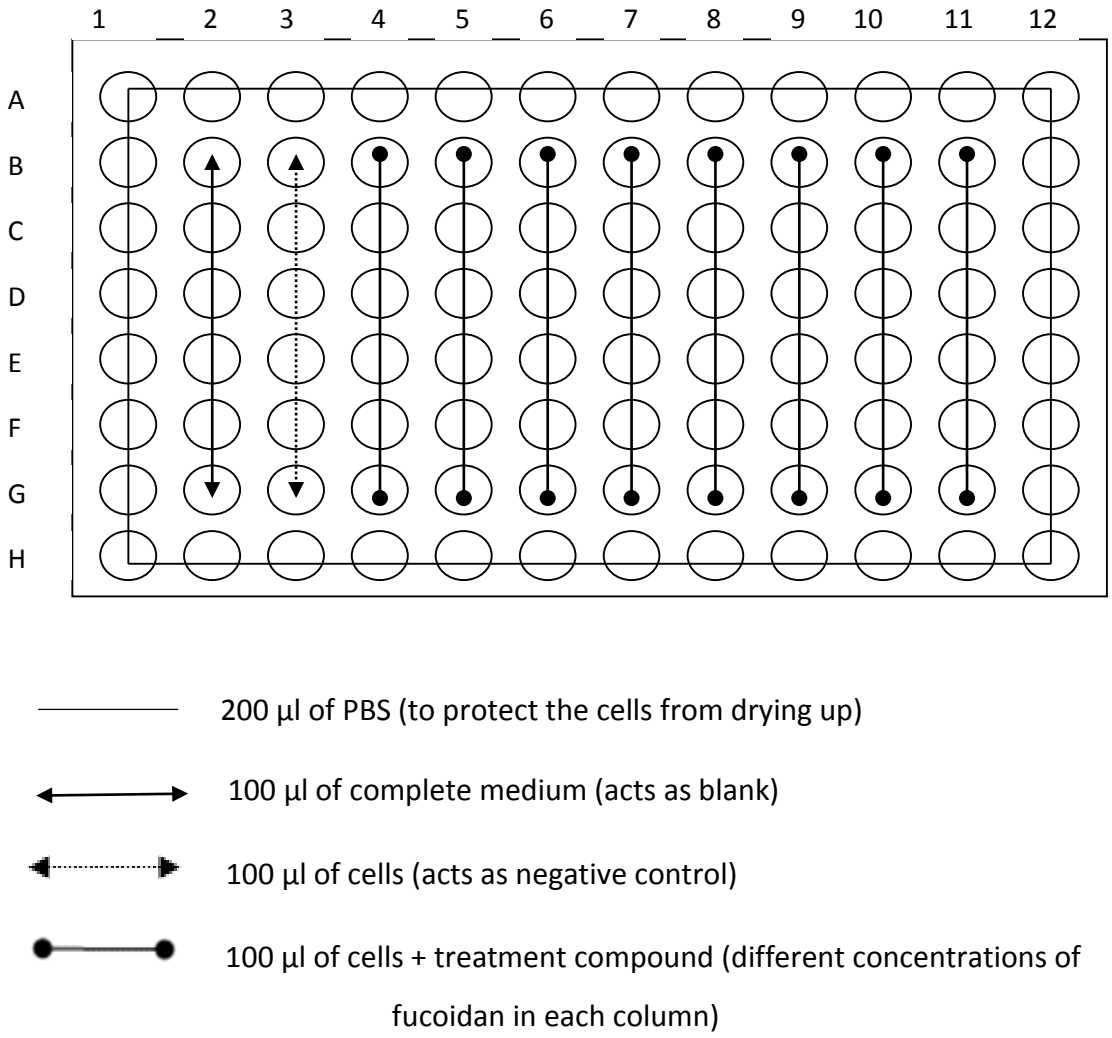


Figure 7: The general plan for addition of various dilutions of fucoidan in a 96-well plate for MTT assay

2.3 Apoptosis Assay: Flow Cytometric Analysis of Apoptosis by Propidium Iodide

Flow cytometry analysis was conducted in order to determine the proportion of apoptotic sub-G1 hypodiploid cells for each cell line after treatment with different dilutions of fucoidan (Riccardi & Nicoletti, 2006).

2.3.1 Materials and equipment required for apoptosis assay

Other than cell culture requirements, following equipment and materials were required for proper functioning of apoptosis assay.

Table 11: Requirements for Apoptosis Assay

Materials and equipment	Supplier
Ribonuclease A (RNAse A) from bovine pancreas	Sigma-Aldrich (USA)
Triton™ X-100	Sigma-Aldrich (USA)
Absolute Ethanol	Fisher scientific (NZ)
Propidium iodide (PI)	Sigma-Aldrich (USA)
Dulbecco's Phosphate Buffered Saline (D-PBS), pH 7.2, no calcium magnesium and phenol red	Life technologies (NZ)
Cooling centrifuge	Hitachi Centrifuge
Beckman Coulter MoFlo™ XDP-Flow cytometer	Beckman coulter (USA)
Six well plates; 15 ml centrifuge tubes; 50 ml falcon tubes; polypropylene flow cytometer tubs, micropipettes, tips; serological pipettes	Life technologies (NZ)

2.3.2 Preparation of Reagents associated with Apoptosis assay

2.3.2.1 Preparation of 80% Ethanol

Absolute ethanol was diluted with distilled water to get 80% ethanol. It was stored in a parafilm-sealed tube at -20˚C.

2.3.2.2 Preparation of RNAse A stock

RNAse A powder was dissolved in double distilled water (DDW) to get a stock solution of 1mg/ml and stored at –20°C.

2.3.2.3 Preparation of PI stock

PI powder was dissolved in double distilled water (DDW) (10 mg into 10 ml) to get a stock solution of 1mg/ml and stored 4°C in the dark.

2.3.2.4 Preparation of serum Starvation (0% serum) medium

For cell synchronization serum starvation medium was prepared for the cell lines. For PC-3 and HEK cells, 1% Penicillin and 1% L-glutamine were added to 450 ml of base RPMI 1640 medium.

For DU-145, the ATCC formulated EMEM medium (which was highly recommended by ATCC) without any serum was used.

Note:

Gloves were worn while preparing and using PI as it is mutagenic.

2.3.3 Protocol for Apoptosis Assay

The apoptosis inducing effect of different types of fucoidan, was analysed by flow cytometer after staining the cells (PC-3, DU-145 and HEK-293) with propidium iodide (PI).

2.3.3.1 Harvesting and plating of cells

- After detaching, counting and diluting cells, cells were seeded into 6-well plates at a density of 50,000 cells/ml and incubated at 37°C at 5% CO_2 overnight.
- Two plates were seeded for each cell line, one which acts as control (plate labelled as day 0) and the other plate (plate labelled as day 3) to study the dose dependent effect of fucoidan after 72 hours.

2.3.3.2 Serum Starvation

- To study the apoptosis inducing effect of fucoidan, cells have to be synchronized prior to addition of fucoidan. Hence the following day (of seeding the plate), starvation medium was added according to plate design.
- The plate design can be better understood as follows; one plate set in this study acts as control group (day 0) and other plate acts as a treatment group (day 3) (see Figure 8). In Plate 1 (control group), well labelled as A, B and C were replaced with 2 mL of starvation medium, and wells labelled as D, E and F were replaced with complete medium. For Plate 2 (treatment group), all the wells were

replaced with 2 mL of starvation medium; C_T acts as control (without treatment) for that particular day when the cells are collected for flow cytometer analysis.

- Then the plates were incubated for 24 hours.

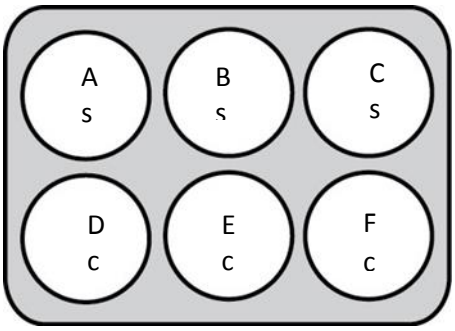


Plate 1 (Control or no treatment group)

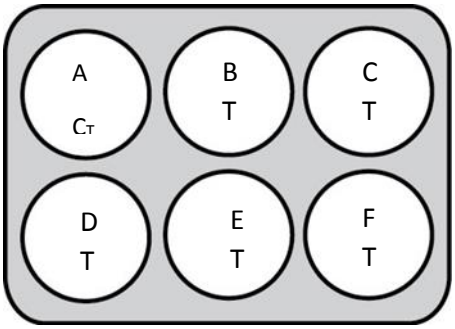


Plate 2 (treatment group)

Figure 8: Plate Design for Apoptosis Analysis

2.3.3.3 Addition of treatment

- After incubating the cells for 24 hours, plate labelled as Day 0, was processed as mentioned in section
- For plate labelled as Day 3, cells (cells in all wells except one, which acts as control for that plate) were incubated with different dilutions of fucoidan for 72 hours.

2.3.3.4 Harvesting of cells for flow cytometric analysis of Apoptosis

- From herein, all procedures were consummated on ice.
- After the cells have been incubated for the predecided time period, the spent medium containing the floating cells were collected in a 15 ml tube and washed with 500 µl of ice-cold PBS.

- The cells were then detached with 1ml of prewarmed TrypLE™ Express enzyme and again washing steps were performed with 500 µl of ice-cold PBS.
- The tubes were then centrifuged at 500×g for 5 minutes at 4 °C.

2.3.3.5 Washing cells

- After centrifugation the supernatant was discarded and cell sample were washed with 3ml of ice-cold PBS.

2.3.3.6 Fixation of cells with 80% ethanol

- It is very important to note that cells need to be mixed evenly before cell samples are fixed with 80% ethanol.
- For even mixing of cells samples, the centrifuge tube (containing the cell samples) is run over the eppendorf tube rack thrice. This step ensures proper mixing of cell samples and prevents blockage when cells are analysed by flow cytometer.
- 80% ethanol was prepared and stored at -20˚C beforehand at least a day before cell samples were intended to be fixed.
- For each tube, 1 ml of ice-cold 80 % ethanol was added onto the cell sample by continuous vortexing throughout, avoiding aggregates formation.
- The cells were incubated at least overnight and not more than 10-14 days in -18°C, before further analysis.

2.3.3.7 Flow cytometric analysis

Before samples preparation procedure commenced, Permeabilization solution needs to be prepared beforehand. The Permeabilization solution was prepared as follows:

Preparation of permeabilizing solution

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

- The 80% ethanol was removed from cell samples by spinning down the tubes for 2min at 600×g and then after discarding the supernatant, pellet was washed twice (to remove any trace of ethanol) with ice-cold 3ml of PBS.

Note that ethanol-fixed cells require higher centrifugal speeds to pellet compared to unfixed cells since they become more buoyant upon fixation.

- After washing the cell samples, 1ml of permeabilization solution (formulation of PS as described before) was added to each tube and the tubes were incubated at room temperature for 20 minutes.
- After incubation of tubes with PS, 5 µl of propidium iodide (PI) was added to each tube and then cells were incubated for 5 to 10 minutes at room temperature.

Note: As PI is light sensitive, all the tubes were stored in dark condition until analysed.

- After PI incubation was over, cell samples were analysed by Beckman Coulter MoFlo™ XDP flow cytometry (Figure 8).
- The optimal voltage for each sample running is listed in Appendix A5.

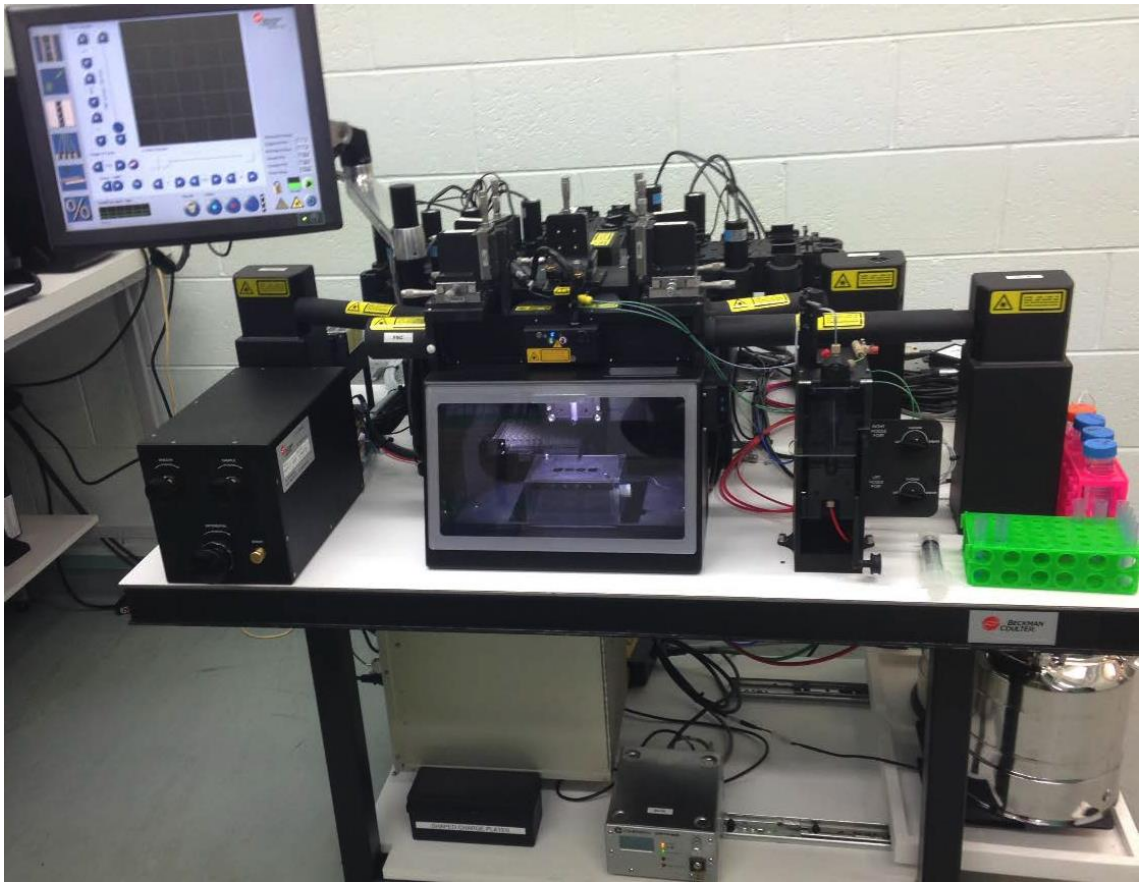


Figure 9: Beckman Coulter MoFlo™ XDP flow cytometer used for analysis of apoptosis

Fluorescence

Propidium iodide

Absorbs at 370 – 550 nm

Emits at 560 – 680 nm

2.4 Data Analysis

2.4.1 Analysis of MTT Assay Results

IC50, the half maximal inhibitory concentration, is commonly used as a measure of drug effectiveness. IC50 is an important reference to measure the inhibitory effect of crude and pure fucoidan from *Fucus vesiculosus* and *Undaria pinnatifida* against prostate carcinoma 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).

2.4.2 Analysis of Apoptosis Assay Results

Kaluza® Flow Cytometry Analysis Software (Version 1.3) provided by Beckman Coulter was applied for apoptosis assay results analysis. Kaluza software helped to determine the proportion of apoptotic sub-G1 hypodiploid cells for each cell line before and after treatment with different dilution of fucoidan.

2.5 Statistical Analysis

All experiments in this study were performed at least three times. Data analysis was done using one-way ANOVA (Analysis of Variance) followed by Dunnett's multiple comparison test. Data are expressed as means ± S.D (standard deviation). Values were considered significant at $P< 0.05$ and $P< 0.01$ were considered very significant.

3. RESULTS

3.1 Antiproliferative activity

Crude and pure fucoidan from *F. vesiculosus* and *U. pinnatifida* were tested for their inhibitory effect on PC-3, DU-145 (prostate carcinoma) and HEK-293 (non-cancerous) cells using MTT cell proliferation assay. The experimental results for the assay for each cell line are presented in the figures below. For each cell line the linearity was established first, followed by determining the inhibitory effect (i.e. anticancer potential) of each type of fucoidan against each cell line tested in this study.

Inhibitory effect of different fucoidan (CCP, CPF, ACF and UCP) on Prostate cancer cell lines (PC-3 and DU-145)

3.1.1 MTT assay linear curve for PC-3 cells

Before determining or evaluating the inhibitory effect of crude and pure fucoidan from *F. vesiculosus* and *U. pinnatifida* on the proliferation of various cancerous and non-cancerous cell lines, it is very important to first establish the correlation between the number of cells and absorbance, (in absences of any potential antiproliferative drug), for each cell line tested in this study. The linear correlation between number of cells and absorbance helps to optimize the cell seeding density and incubation period for each cell line. This in fact standardises the MTT procedure, which is essential for proper functioning of the assay and to obtain a reliable result. A good linear curve implies that the cells have been provided with optimal growing condition in the laboratory. It has been reported that the optimal seeding density for the assay should fall within the linear portion of the curve (Ciapetti, Cenni, Pratelli, & Pizzoferrato, 1993). This density should neither be too high nor be too low and ensures a proper cell culture condition. Figure 10 represents the linear curve for PC-3 cells. Herein PC-3 cells were serially diluted from 500, 000 cells/ml to 1,953 cells/ml for obtaining a cell linearity standard curve.

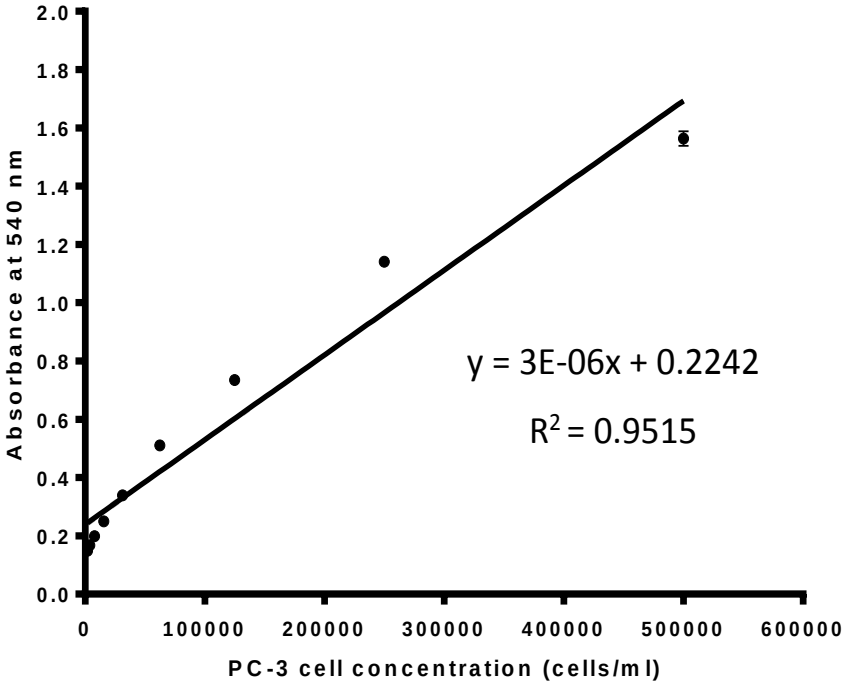


Figure 10: Linear curve of PC-3 cells; data are presented as mean ± SD, n=6.

Here linearity is expressed in R^2 value, which is a measure of goodness-of-fit of linear regression. The R^2 value is considered to be perfect when it is 1; which means that all points lie exactly on a straight line with no scatter. R^2 value for PC-3 cells was 0.9515, hence the absorbance value is falling very close on the linear portion of the curve. After careful observation of each well (with serial dilution of cells) under microscope, it was established that at 50,000 cells/ml, cells are neither too crowed nor too sporadic and falls within the linear portion of the curve. Hence an optimal seeding density of 50,000 cells/ml (for PC-3 cells) was chosen to determine the anticancer potential of drugs (fucoidan) by MTT and apoptosis assay.

3.1.2 Inhibitory effect of crude and pure fucoidan from *F. vesiculosus* and *U. pinnatifida* on the growth of PC-3 cells

To examine the effect of fucoidan on growth of PC-3 cells, cell viability was evaluated using the MTT assay. The different types of fucoidan tested in this study are commercial crude and pure fucoidan from *F. vesiculosus (*purchased from sigma; henceforth would be referred to as CCF and CPF respectively), crude fucoidan from New Zealand *U.*

pinnatifida (extracted from New Zealand *U. pinnatifida* at AUT; henceforth would be referred to as ACF) and commercial pure fucoidan from *U. pinnatifida (*purchased from sigma; hence forth would be referred to as UPF).

Different concentrations of fucoidan (CCF, CPF, ACF and CPU) were tested for its effect on PC-3 cells for day 1 (24 hours), day 2 (48 hours) and day 3 (72 hours). Figure 11, shows time course analysis to estimate the degree of cell growth inhibition at various time (day 1, 2 and 3) periods following treatment. As depicted in the figure, out of the four types of fucoidan analysed, crude fucoidan from *F. vesiculosus* (sigma) was found to inhibit the PC-3 cells growth the most, followed by crude fucoidan from *Undaria pinnatifida* (extracted at AUT). Comparatively, the inhibition of the growth of PC-3 cells by CPF and UPF was less, as is evident from the figure 11.

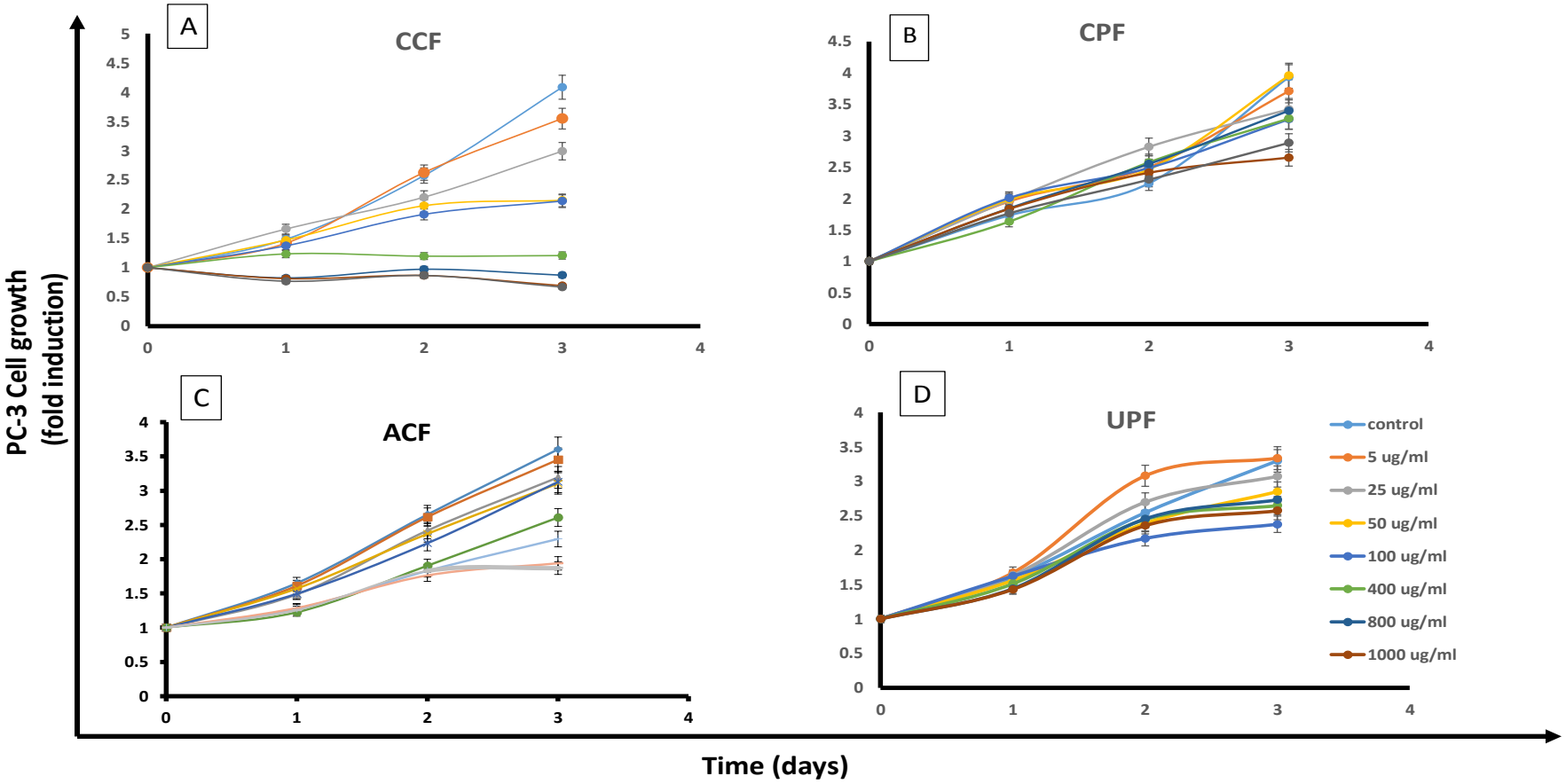


Figure 11: **Inhibitory effect of CCF, CPF, ACF and UPF fucoidan on PC-3 cell growth in a time dependent manner.** PC-3 cells were treated with 5, 25, 50, 100,200, 400, 800 and 1000 µg/ml of CCF, CPF, ACF and UPF. MTT assay was performed 1, 2, and 3 days later. The Results are presented as fold induction of the control and are presented as the mean ± S.D. from three independent experiments.

Following the establishment of how fucoidan affects the PC-3 cells growth in a time course (days 1, 2, and 3), it is important to evaluate the dose dependent response at particular time points. Herein, the dose response curve is depicted after 72 hours of drug treatment, as at 72 hours there was significant suppression of cell viability in a dose-dependent manner and the results are also much clearer and easier to understand and comprehend. It was estimated that after 72 hours, CCF compared to CPF, ACF and UPF resulted in significant suppression of cell viability in a dose-dependent manner and was the most effective against PC-3 cells growth. Figure 12, depicts the comparative antiproliferative effects of fucoidan (CCF, CPF, ACF and UPF) in a dose dependent manner on PC-3 cells at 72 hours.

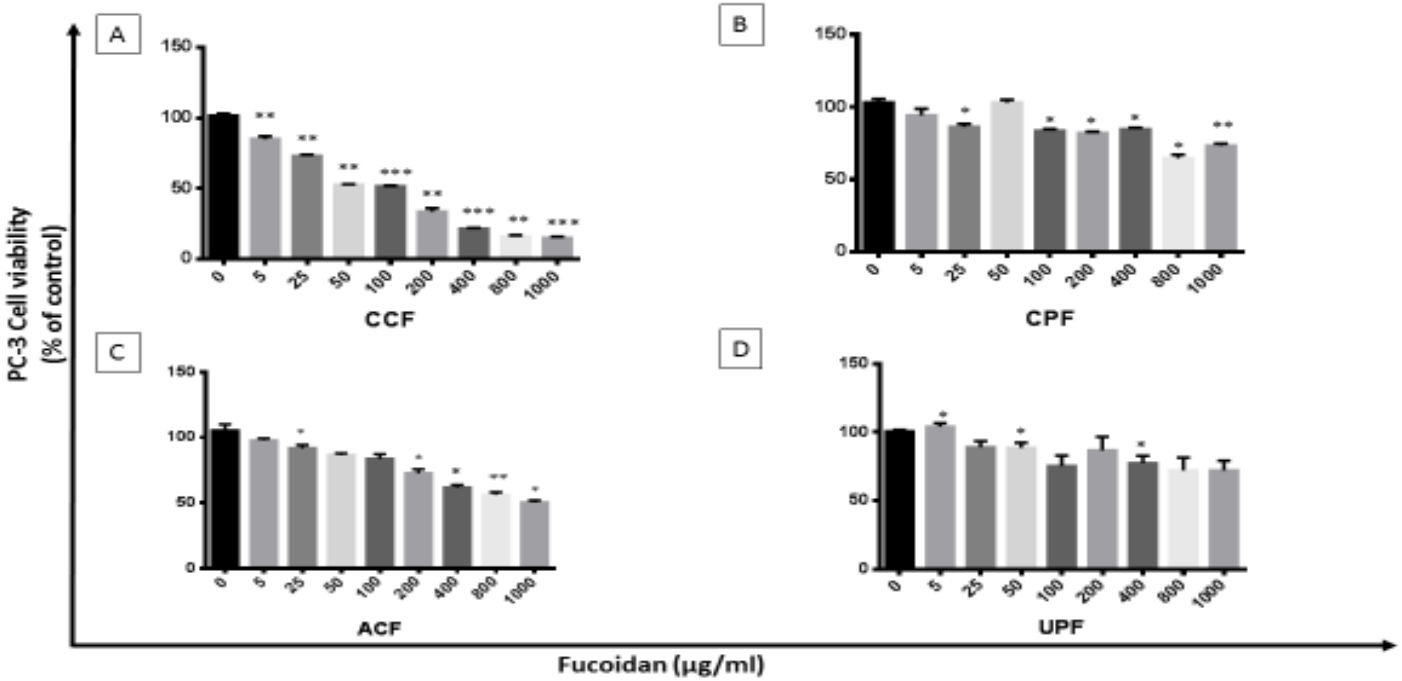


Figure 12: Comparative antiproliferation effect of fucoidan (CCF, CPF, ACF and UPF) in a dose dependent manner on PC-3 cells at 72 hours. PC-3 cells were treated with 5, 25, 50, 100,200, 400, 800 and 1000 µg/ml of CCF, CPF, ACF and UPF for 72 hours. The Results are presented as fold induction of the control and are presented as the mean ± S.D (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, each treatment compared to the control) from three independent experiments.

Table 12, compares the fucoidan induced dose-dependent cell death by each type of fucoidan tested in this study on PC-3 cells growth. Herein, the columns represents the dose tested in this study and the rows headings represent the type of fucoidan evaluated for its anticancer property against PC-3 cell growth. It needs to be emphasised that at some particular dose, an increase in the PC-3 cell growth (for example 50 µg/ml of CPF by 0.51%) was observed, instead of decreasing the PC-3 cells growth. Wherever an increase (in PC-3 cells) was noticed after treatment, it is illustrated with an upward arrow in the table. Dose-dependent effects of cell death by CCF, CPF, ACF and UPF fucoidan on HEK-293 cells growth.

Table 12: Dose-dependent effects of CCF, CPF, ACF and UPF fucoidan on PC-3 cells growth

	5 µg/ml	**25 µg/ml**	**50 µg/ml**	**100 µg/ml**	**200 µg/ml**	**400 µg/ml**	**800 µg/ml**	**1000 µg/ml**
CCF	13.08 %	27.75 %	47.34 %	47.66 %	68.55 %	78.48 %	83.56 %	85.68 %
CPF	5.71 %	13.07 %	↑ cell growth by 0.51 %	17.11 %	16.84 %	13.63 %	32.61 %	26.66 %
ACF	4.19 %	11.39 %	13.83 %	13.21 %	37.64 %	46.32 %	49.16 %	50.1 %
UPF	↑ cell growth by 6.47 %	15.91 %	7.21 %	16.38 %	25.19 %	29.06 %	31.46 %	32.94 %

In addition, Figure 13, illustrates the effectiveness of CCF, CPF, ACF and UPF in references to IC_{50} after 72 hours of treatment addition. IC_{50} is defined as how much of a compound or inhibitor is required to inhibit a biological process by half (i.e. 50%). Hence the ability to accurately measure the concentration of the each type of fucoidan (i.e. inhibitor) which is required to inhibit the growth of PC-3 cells by half, facilitates the ranking of each type of fucoidan (evaluated in this study) in order of their anticancer

potential. IC_{50} value for each type of fucoidan analysed in this study was calculated using Graphpad Prism software. To determine the IC_{50} value, the cell viability on Y axis was plotted against the logarithm of the dose on X axis.

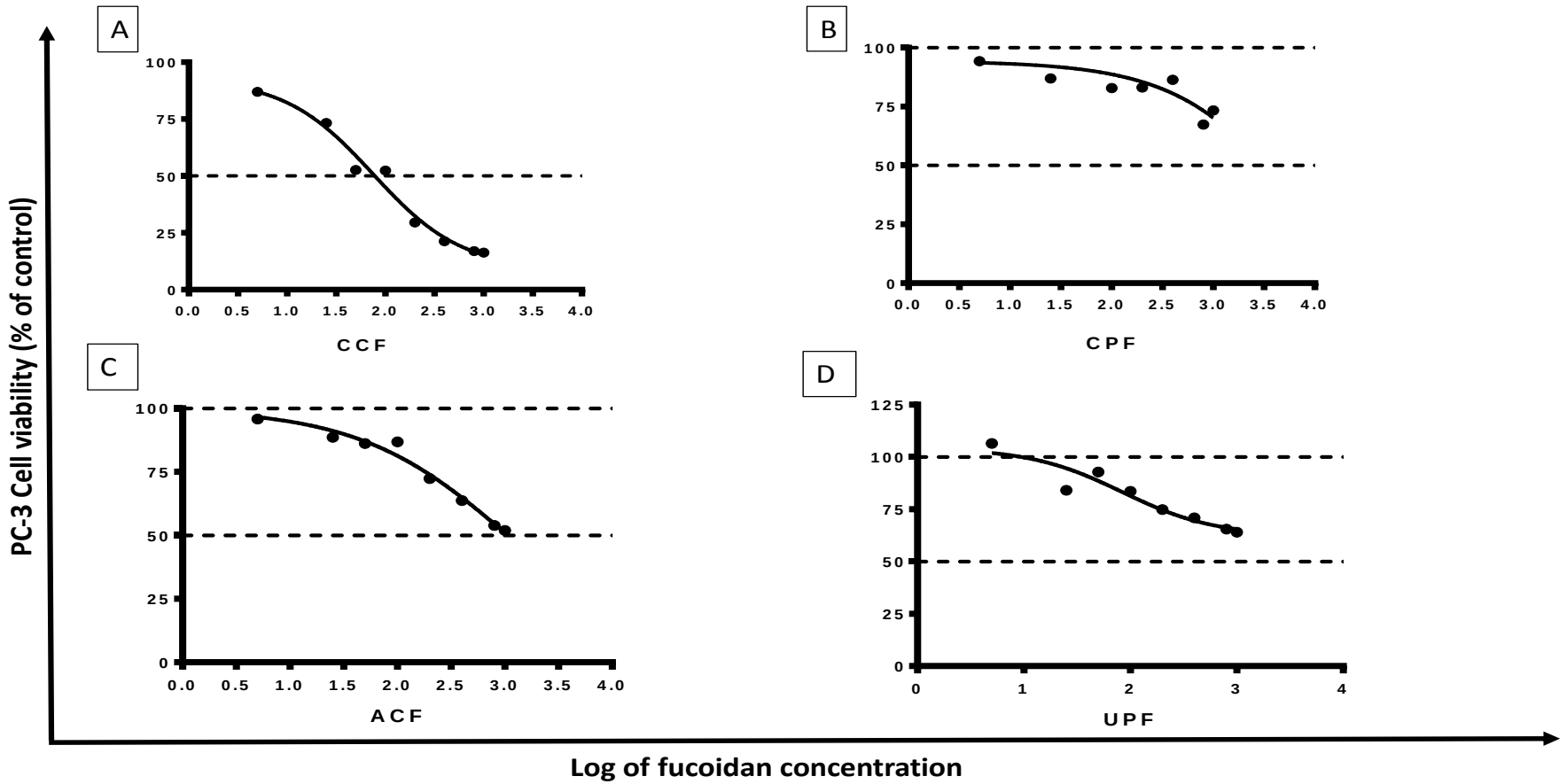


Figure 13: Comparative effectiveness of CCF, CPF, ACF and UPF on PC-3 cells, in references to IC_{50} after 72 hours of treatment addition. PC-3 cells were incubated in the presence of various concentrations of fucoidan (5, 25, 50, 100,200, 400, 800 and 1000 µg/ml of CCF, CPF, ACF and UPF). A relative viability of 100% was designated as the total number of cells that grew in 72 hours cultures without any fucoidan.

The IC_{50} value for CCF is 74.88 µg/ml and for all other types of fucoidan, the IC_{50} is more than 1000 µg/ml after 72 hours of treatment. The prism software also provides a tentative IC_{50} value for ACF, CPF and UPF; which is 903 µg/ml for ACF, 1964 µg/ml for CPF and 1247 µg/ml for UPF. Table 13 compares the effectiveness of CCF, CPF, ACF and UPF against PC-3 cells, on the basis of its IC_{50} values.

Table 13: The *in vitro* anticancer activities of CCF, CPF, ACF and UPF fucoidans are reported as IC_{50} values (in µg/ml) on PC-3 cells at 72 hours. The half maximal inhibitory concentration (IC_{50}) for each type of fucoidan presented below was determined by MTT colorimetric assay and estimated using Graphpad Prism software from three independent experiments.

Types of fucoidan evaluated for its anticancer potential	IC_{50} values (µg/ml)
CCF (Crude fucoidan from *F. vesiculosus*, purchased from sigma)	74.88
ACF (Crude fucoidan extracted from New Zealand *U. pinnatifida*, supplied by AUT)	903
UPF (Pure fucoidan from *U. pinnatifida*, purchased from sigma)	1247
CPF (Pure fucoidan from *F. vesiculosus*, purchased from sigma)	1964

Based on the IC_{50} values (table 13), CCF is proposed to be the most effective in inhibiting the growth of PC-3 cells, followed by ACF, UPF and CPF.

3.1.3 MTT assay linear curve for DU-145 cells

To establish the optimum number of cell count and incubation period for DU-145 cells, it is important to determine the correlation between the number of cells and absorbance first. DU-145 cells were serially diluted from 250,000 cells/mL to 976 cells/mL to obtain a cell linearity standard curve. When absorbance at 540 nm is read and plotted against number of cells/ml, a linearity is expected (i.e. a curve with linear portion). The optimal number of DU-145 cells for the MTT assay and further experiments should fall within the linear portion of the curve. Figure 14 represents the linear curve for DU-145 cells.

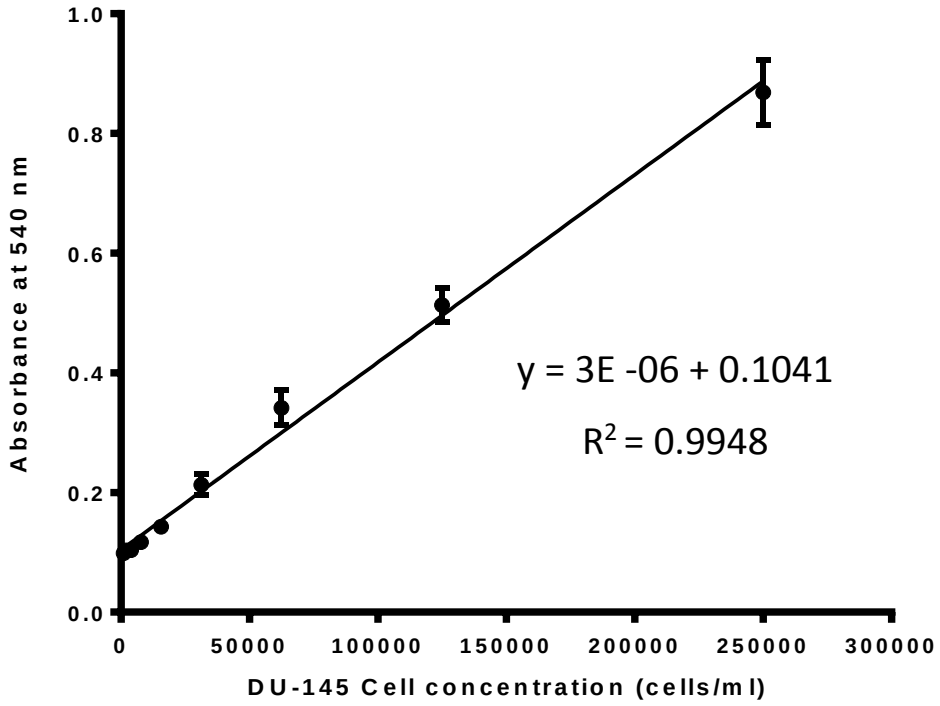


Figure 14: **Linear curve of DU-145 cells**; data are presented as mean ± SD, n=6.

Here linearity is expressed in R^2 value, which is a measure of goodness-of-fit of linear regression. The R^2 value is considered to be perfect when it is 1; which means all points lie exactly on a straight line with no scatter. R^2 value for DU-145 cells was 0.9948, showing almost all the absorbance value fell on the linear portion of the curve.

After careful observation of each well (containing serially diluted cells) under microscope, it was established that at 50,000 cells/ml, cells are neither too crowed nor too sporadic and falls within the linear portion of the curve. Hence an optimal seeding density of 50,000 cells/ml (for DU-145 cells) was chosen to determine the inhibitory effect of potential anticancer drugs (fucoidan) by MTT and apoptosis assay.

3.1.4 Inhibitory effect of crude and pure fucoidan from *F. vesiculosus* and *U. pinnatifida* on the growth of DU-145 cells

Other than PC-3 cells, another prostate carcinoma cell line explored or analysed in this study was DU-145 cells. Various concentrations of fucoidan (CCF, CPF, ACF and CPU) were tested for their antitumour genic effect on DU-145 cells for day 1 (24 hours), day

2 (48 hours) and day 3 (72 hours). Figure 15, exhibits time course analysis to estimate the degree of cell growth inhibition at various time (day 1, 2 and 3) periods following treatment. Here, the anticancer potential was estimated for crude and pure fucoidan from *F. vesiculosus* (Sigma) and crude (extracted from New Zealand *U. pinnatifida* at AUT) and pure fucoidan from *U. pinnatifida* (Sigma). MTT assay results at definite time point are depicted and compared for all the three mentioned fucoidan (CCF, CPF and UPF) except crude fucoidan supplied by AUT (ACF), which was extracted from New Zealand *U. pinnatifida*.

The MTT assay results for ACF fucoidan could not be depicted in the form of graph as the absorbance readings at various concentration of AUT fucoidan (designated as ACF) were too high. This unusual result development was reported to my supervisor. After careful deliberation the initial plate design was modified so that the interaction of ACF with EMEM medium could be studied and then accordingly propose the reason for very high absorbance reading.

It should be noted here that very high absorbance reading was not obtained when ACF was tested for its anticancer potential on PC-3 cells growth, thus ACF alone was not responsible for high OD reading. Here the culture medium used for DU-145 cells was EMEM, which was different from RPMI-1640 culture medium used for PC-3 cells. Hence the 96 well plate was designed to find out the interaction between MTT reagent and cells; EMEM medium alone and in combination with ACF.

To check possible reasons behind high OD values, plates were designed (Figure 15) as follows:

- First column having EMEM medium alone (i.e. blank)
- DU-145 cells + EMEM medium (negative control)
- DU-145 cells + EMEM medium + ACF at various concentration (ACF with cells)
- EMEM medium + ACF at various concentration (ACF without cells)

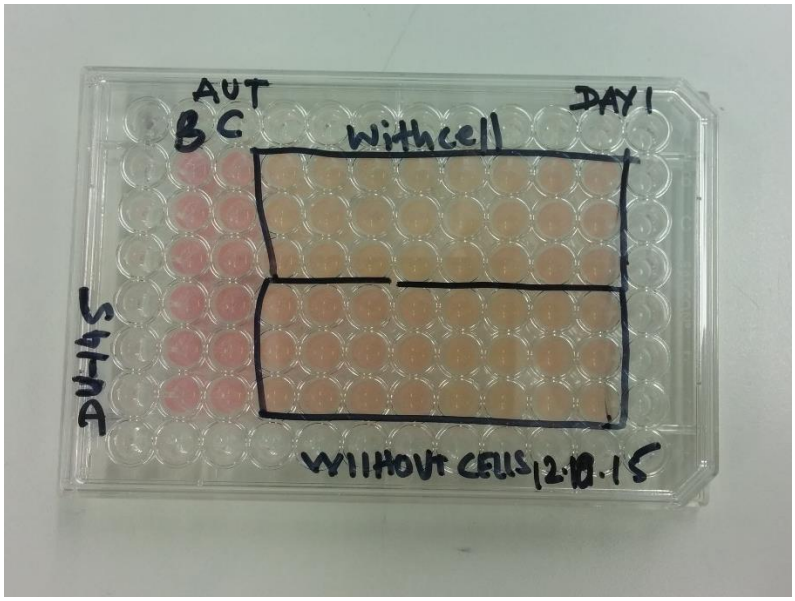


Figure 15: 96-well plate design to identify the reason for very high OD reading, before MTT assay. Here the DU-145 cell seeding density was 5000 cells/well and after 24 hours incubation, various concentrations of ACF (5 µg/ml to 1000 µg/ml) was added. Here the set of columns with DU-145 cells and without DU-145 cells have various concentration of ACF fucoidan.

After the DU-145 cells were seeded and incubated for 24 hours for cell attachment, the treatment compound ACF, was added at various concentrations (5, 25, 50, 100, 200, 400, 800 and 1000 µg/ml) and then, the MTT cell proliferation assay was performed at various time point, according to procedure mentioned in Section 2.2.1.5. Figure 16, depicts the 96-well plate after MTT assay was performed.

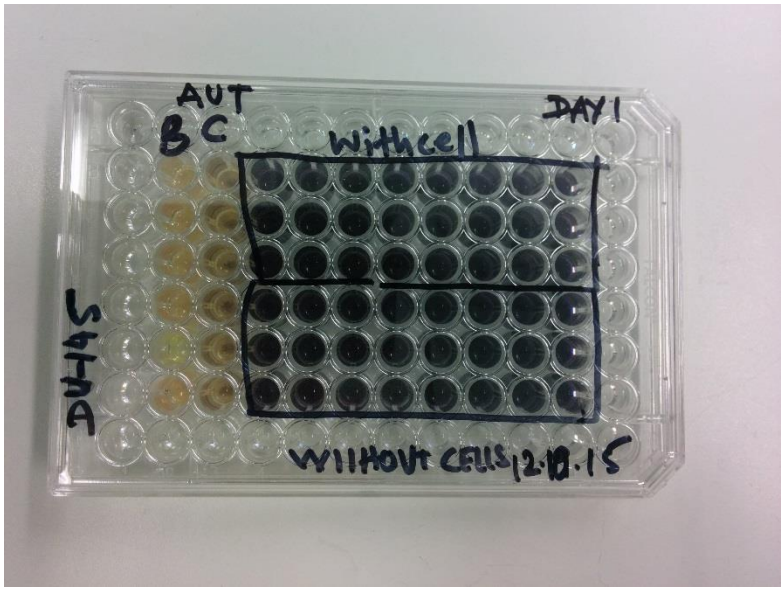


Figure 16: Observation noted in 96-well plate after MTT assay was performed.

After MTT assay was performed, it was observed that the OD reading for first two columns (EMEM medium alone i.e. blank and DU-145 cells along with medium i.e. negative control) were as anticipated and in range (i.e. neither too low nor too high). But in the other two sets of columns (containing DU-145 cells with various concentration of ACF and EMEM medium with various concentration of ACF) the OD reading was way too high.

It was inferred from the above observation that OD values were in normal range when EMEM medium and DU-145 cells alone interacted with MTT reagent. High OD readings were observed when different concentrations of ACF along with EMEM medium interacted with MTT reagent. Hence, as high OD values were only observed when MTT reagent interacted with ACF along with EMEM medium and not with DU-145 cells or EMEM medium alone, some chemical interactions between EMEM medium and ACF fucoidan could be the reason behind very high OD readings.

Thus, it was proposed that some components of EMEM medium was interacting with ACF to bring about very high OD readings. The probable reasons behind very high OD reading are explained in details in the Discussion section (4.4) of this thesis.

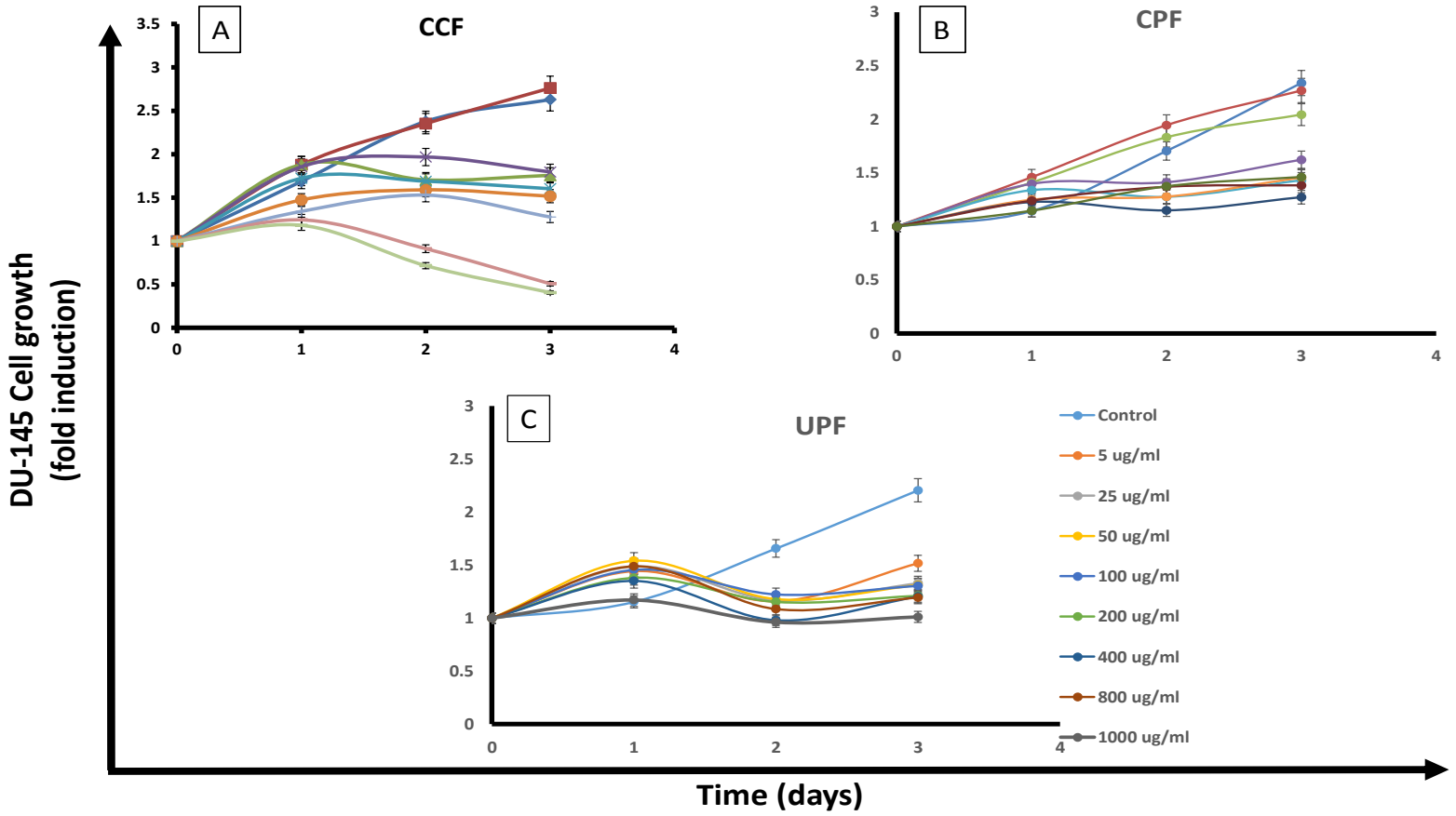


Figure 17: Inhibitory effect of CCF, CPF, and UPF fucoidan on DU-145 cell growth in a time dependent manner. DU-145 cells were treated with 5, 25, 50, 100, 200, 400, 800 and 1000 µg/ml of CCF, CPF, and UPF. MTT assay was performed 1, 2, and 3 days later. The results are presented as fold induction of the control and are presented as the mean ± S.D. from three independent experiments.

Figure 18 illustrates the antiproliferative response of CCF, CPF and UPF on DU-145 cells after 72 hours of treatment. The dose response results for ACF fucoidan at 72 hours could not be depicted as the absorbance readings at various concentrations of AUT fucoidan (designated as ACF) were too high.

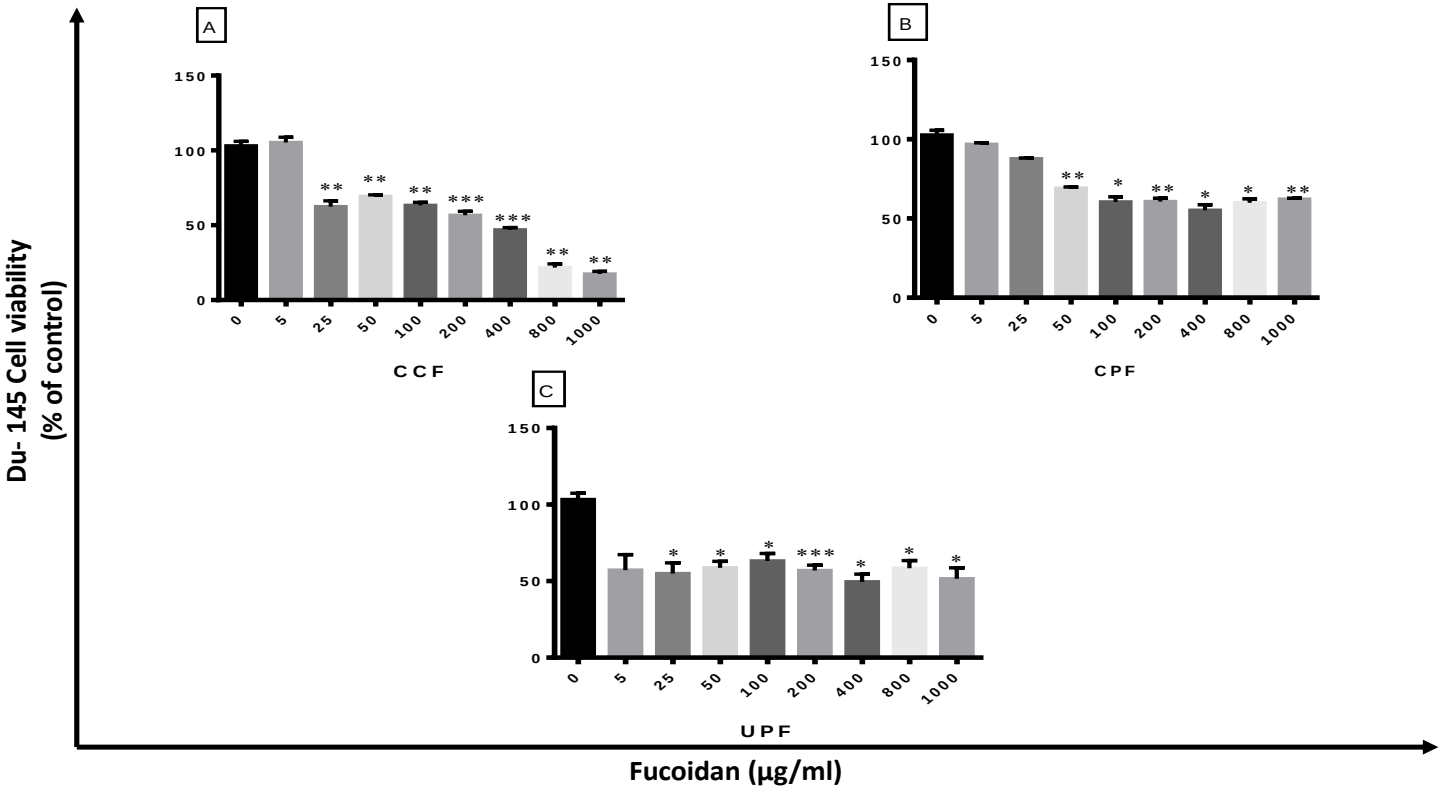


Figure 18: Comparative antiproliferation effect of fucoidan (CCF, CPF, and UPF) in a dose dependent manner on DU-145 cells at 72 hours. DU-145 cells were treated with 5, 25, 50, 100,200, 400, 800 and 1000 µg/ml of CCF, CPF, and UPF for 72 hours. The results are presented as fold induction of the control and are presented as the mean ± S.D (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; each treatment compared to the control) from three independent experiments.

Table 14, compares the fucoidan induced dose-dependent cell death by different types of fucoidan (other than ACF) tested in this study on DU-145 cells growth. It represents the antiproliferative effect of fucoidan on DU-145 cells at various dose. For example, CCF treatment induced dose-dependent cell death (i.e. at 25 µg/ml, 33.2 % of DU-145 cells were dead; 50 µg/ml, 31.7 %; 100 µg/ml, 39.06 %; 200 µg/ml, 42.35%; 400 µg/ml, 51.44 %; 800 µg/ml, 80.68% and at 1000 µg/ml, 84%) other than at 5 µg/ml where there was increase in the growth of DU-145 cells by 5.02 %.

Table 14: Dose-dependent effects of CCF, CPF and UPF fucoidan on DU-145 cells growth.

	5 µg/ml	**25 µg/ml**	**50 µg/ml**	**100 µg/ml**	**200 µg/ml**	**400 µg/ml**	**800 µg/ml**	**1000 µg/ml**
CCF	↑ cell growth by 5.02 %	33.2 %	31.7 %	39.06 %	42.35 %	51.44 %	80.68 %	84.56 %
CPF	3.01 %	12.61 %	30.62 %	38.79 %	37.73 %	45.58 %	40.84 %	37.51 %
UPF	21.94 %	31.74 %	37.49 %	40.84 %	45.08 %	47.38 %	43.82 %	51.11 %

In addition, Figure 15, illustrates the effectiveness of CCF, CPF, and UPF in references to IC_{50}, showing how much of fucoidan is required to inhibit the DU-145 cells by half, after 72 hours of treatment addition.

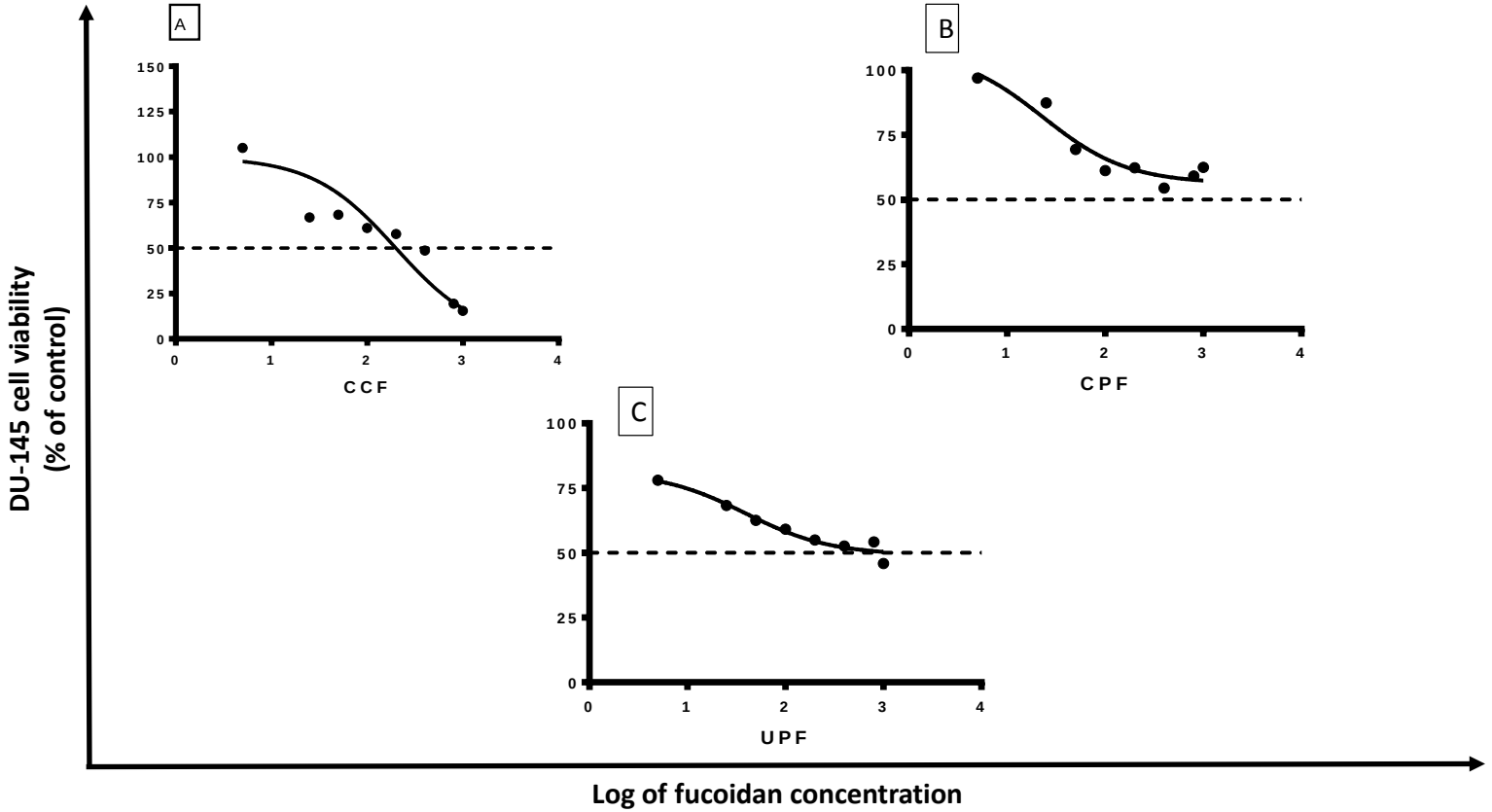


Figure 19: Comparative effectiveness of CCF, CPF, and UPF on DU-145 cells in references to IC50 after 72 hours of treatment addition. DU-145 cells were incubated in the presence of various concentrations of fucoidan (5, 25, 50, 100,200, 400, 800 and 1000 µg/ml of CCF, CPF, and UPF). A relative viability of 100% was designated as the total number of cells that grew in 72 hours culture without any fucoidan.

The IC_{50} value obtained when the data were analysed by Graphpad Prism software are tabulated in Table 15. The IC_{50} value for CCF and UPF are 331.9 and 995 µg/ml. The tentative IC_{50} value for CPF is 1587 µg/ml.

Table 15: The *in vitro* anticancer activities of fucoidans are reported as IC_{50} values (in µg/ml) against DU-145 cells at 72 hours. The half maximal inhibitory concentration (IC_{50}) for each type of fucoidan was determined by MTT colorimetric assay and estimated using Graphpad Prism software from three independent experiments.

Types of fucoidan evaluated for its anticancer potential	IC_{50} values (µg/ml)
CCF (Crude fucoidan from *F. vesiculosus*, purchased from sigma)	331.9
UPF (Pure fucoidan from *U. pinnatifida*, purchased from sigma)	995
CPF (Pure fucoidan from *F. vesiculosus*, purchased from sigma)	1587

Hence on the basis of IC_{50} values, CCF is the most effective in inhibiting the growth of DU-145 cells, followed by UPF and then by CPF.

Inhibitory effect of different fucoidan (CCP, CPF, ACF and UCP) on HEK-293, human embryonic kidney cells (noncancerous or normal cell lines)

The two prostate cancer cell lines chosen and analysed in this study were PC-3 cells and DU-145 cells. Once the anticancer potential of different types of fucoidans were established for their antiproliferative potential (on PC-3 and DU-145 cells), it becomes imperative that the cytotoxic nature of fucoidan is established on normal cell lines also, as we want a prospective drug that has maximum cytotoxic effect on cancerous cells and minimum cytotoxic effect on non-cancerous or healthy cells.

In this study, HEK-293 (human embryonic kidney cells) 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).

3.1.5 MTT assay linear curve for HEK-293 cells

Figure 20 displays a very good linear relationship between various cell densities (from 500,000 to 1,953 cells/mL) and corresponding absorbance (R^2 = 0.991). After careful observation of each well (containing serially diluted cells) under microscope, it was established that at 50,000 cells/ml, cells are neither too crowed nor too sporadic and falls within the linear portion of the curve. Hence an optimal seeding density of 50,000 cells/ml (for HEK-293) was chosen to determine the inhibitory effect of potential anticancer drugs (fucoidan) by MTT and apoptosis assay.

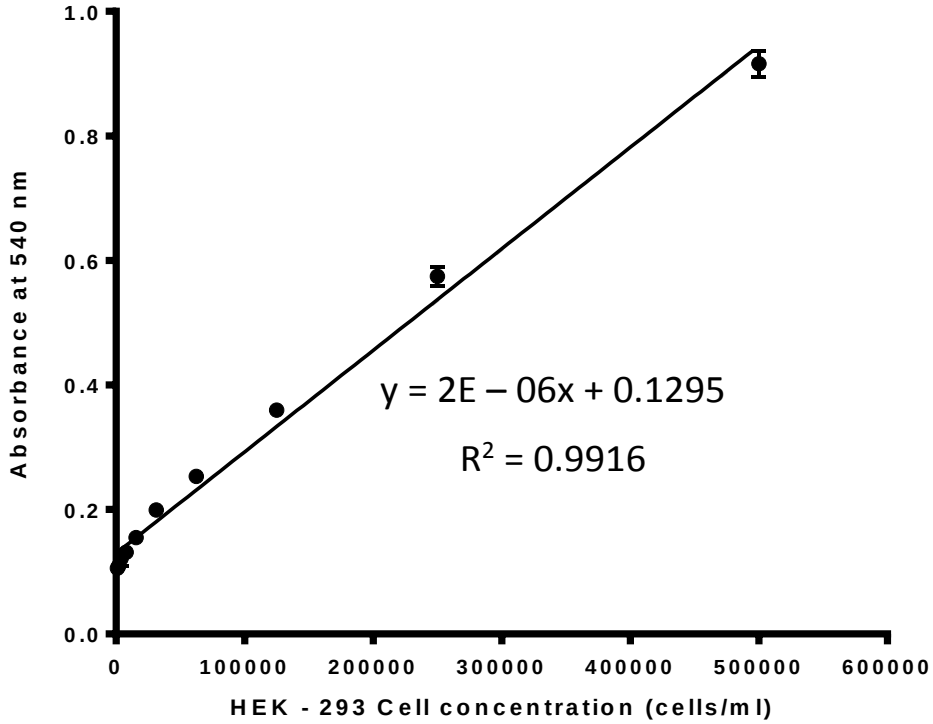


Figure 20: **Linear curve of HEK-293 cells**; data are presented as mean ± SD, n=6.

3.1.6 Inhibitory effect of crude and pure fucoidan from *F. vesiculosus* and *U. pinnatifida* on the growth of HEK-293 cells

Figure 21, exhibits time course analysis to estimate the degree of cell growth inhibition at various time (day 1, 2 and 3) periods following treatment. Time course analysis to estimate the degree of cell growth inhibition at various time periods following treatment showed that CCF was the most cytotoxic, followed by ACF.

Figure 22, demonstrates the antiproliferative response of CCF, CPF, ACF and UPF on HEK-293 cells after 72 hours of treatment. Table 16 summaries the cytotoxic effect of CCF, CPF, ACF and UPF on HEK-293 cells growth at 72 hours in the presence of various dose of fucoidan (i.e. the % of cell death of HEK-293 cells at each concentration of different fucoidan analysed). The cytotoxic effect is also represented in the form of IC_{50} graph representation (Figure 23) obtained by prism software along with the IC_{50} values (Table 17) of each type of fucoidan against HEK-293 cells.

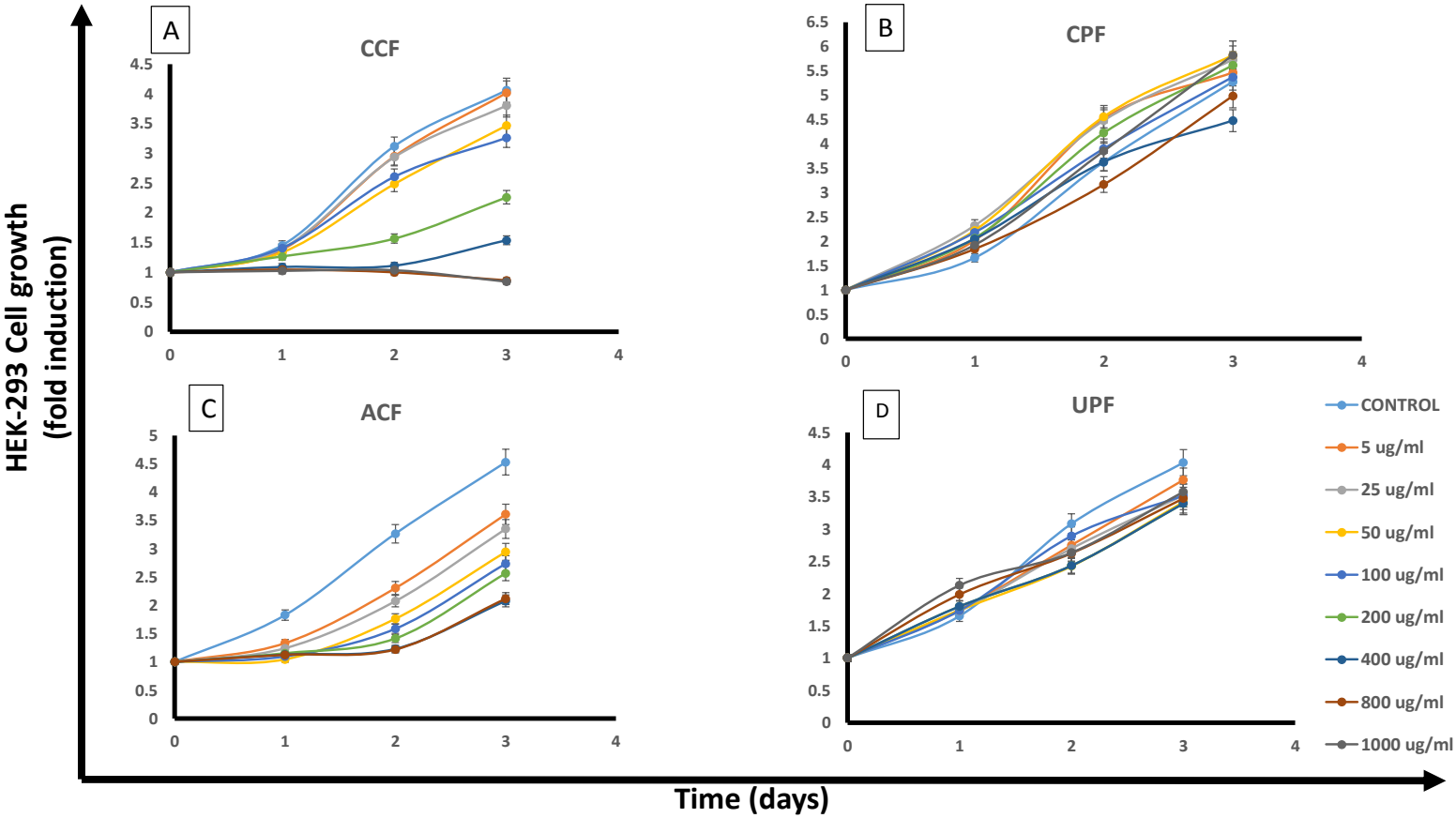


Figure 21: Inhibitory effect of CCF, CPF, ACF and UPF fucoidan on HEK-293 cell growth in a time dependent manner. HEK-293 cells were treated with 5, 25, 50, 100, 200, 400, 800 and 1000 µg/ml of CCF, CPF, and UPF. MTT assay was performed 1, 2, and 3 days later. The results are presented as fold induction of the control and are presented as the mean ± S.D. from three independent experiments.

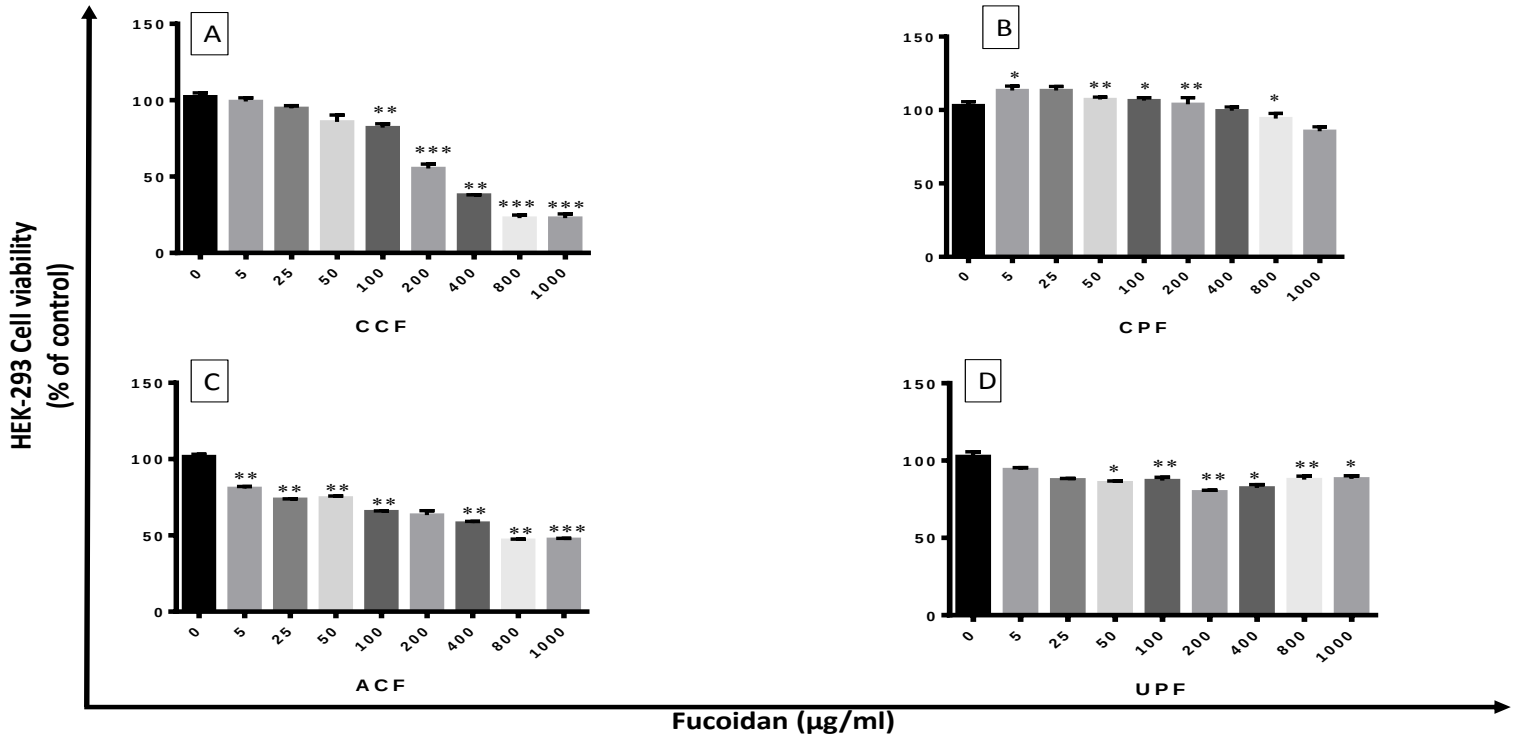


Figure 22: Comparative antiproliferation effect of fucoidan (CCF, CPF, and UPF) in a dose dependent manner on HEK-293 cells at 72 hours. HEK-293 cells were treated with 5, 25, 50, 100,200, 400, 800 and 1000 µg/ml of CCF, CPF, and UPF for 72 hours. The results are presented as fold induction of the control and are presented as the mean ± S.D (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, each treatment compared to the control) from three independent experiments.

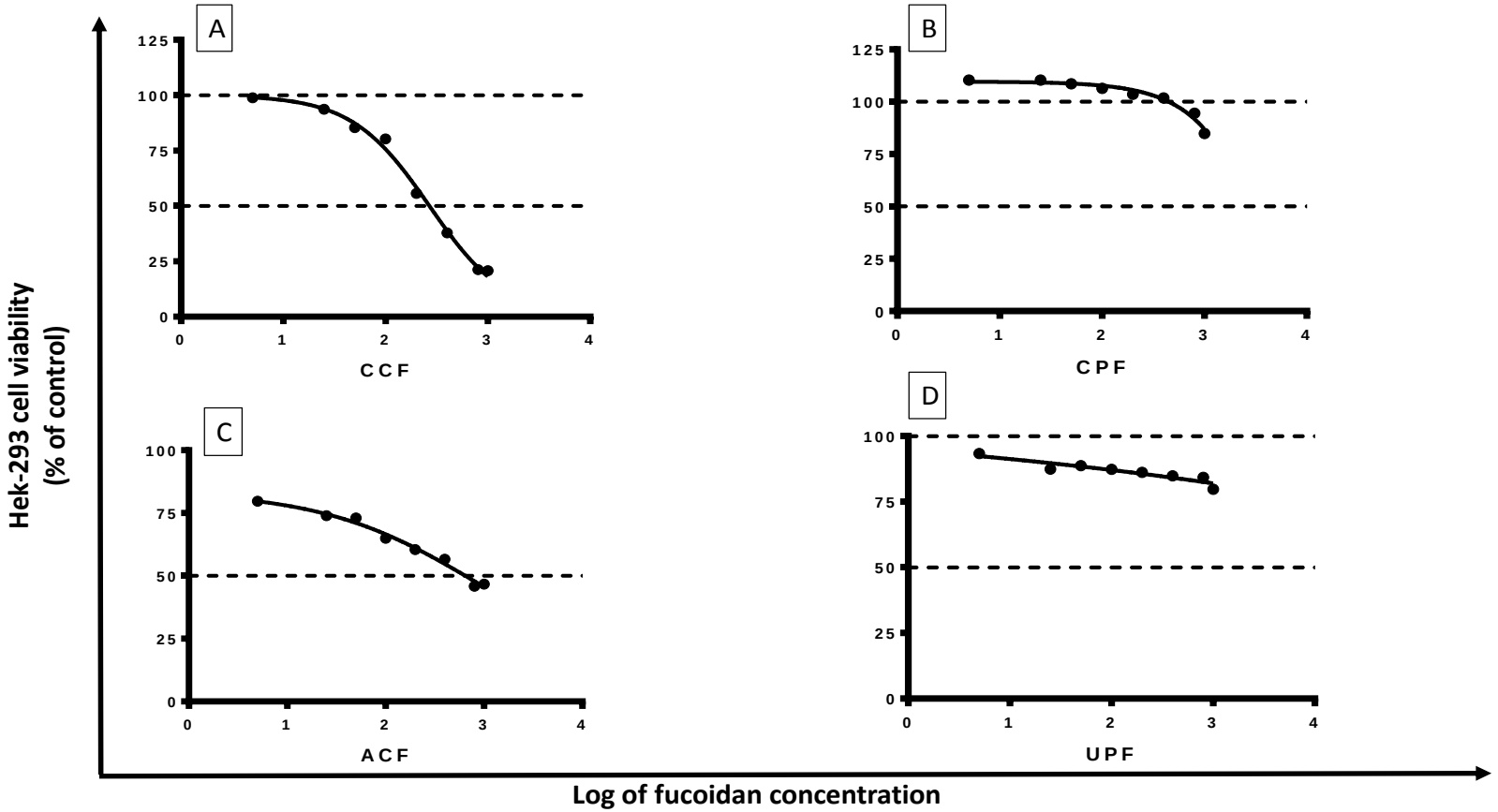


Figure 23: Comparative effectiveness of CCF, CPF, ACF and UPF on HEK-293 cells in references to IC50 after 72 hours of treatment addition. HEK-293 cells were incubated in the presence of various concentrations of fucoidan (5, 25, 50, 100,200, 400, 800 and 1000 µg/ml of CCF, CPF, and UPF). A relative viability of 100% was designated as the total number of cells that grew in 72 hours culture without any fucoidan.

Table 16: Dose-dependent effects of CCF, CPF, ACF and UPF fucoidan on HEK-293 cells growth.

	5 µg/ml	**25 µg/ml**	**50 µg/ml**	**100 µg/ml**	**200 µg/ml**	**400 µg/ml**	**800 µg/ml**	**1000 µg/ml**
CCF	1.15 %	6.38 %	14.67 %	19.73%	44.37 %	62.12 %	78.73 %	79.25 %
CPF	↑ cell growth by 10.33 %	↑ cell growth by 10.36 %	↑cell growth by 8.56 %	↑cell growth by 6.4 %	↑cell growth by 3.59 %	↑cell growth by 1.87 %	5.5 %	15.12 %
ACF	10.33 %	16.04 %	16.96 %	22.01%	26.58 %	30.35 %	50.16 %	51.28 %
UPF	6.68 %	12.57 %	11.25 %	12.68 %	13.8 %	15.15 %	15.73 %	20.23 %

Table 17: The *in vitro* anticancer activities of fucoidans are reported as IC_{50} values (in µg/ml) against HEK-293 cells at 72 hours. The half maximal inhibitory concentration (IC_{50}) for each type of fucoidan was determined by MTT colorimetric assay and estimated using Graphpad Prism software from three independent experiments.

Types of fucoidan evaluated for its anticancer potential	**IC_{50} values (µg/ml)**
CCF (Crude fucoidan from *F. vesiculosus*, purchased from sigma)	268.2
ACF (Crude fucoidan extracted from New Zealand *U. pinnatifida*, at AUT)	958.4
UPF (Pure fucoidan from U. pinnatifida, purchased from sigma)	>2000
CPF (Pure fucoidan from F. vesiculosus, purchased from sigma)	>5000

Hence on the basis of IC_{50} values, it can be proposed that CCF was inhibiting the HEK-293 cells to the maximum extent, followed by ACF.

3.2 Flow cytometric Analysis of Apoptosis

Investigation was carried out to ascertain whether the inhibitory effect of fucoidan (CCF, ACF, UPF and CPF) on the growth of PC-3, DU-145 and HEK-293 cells resulted from apoptosis induction. Flow cytometry analyses were conducted in order to determine the proportion of apoptotic sub-G1 hypodiploid cells after staining the cells with PI (Telford et al., 1994). The size of sub-G1 peak of the histogram is directly proportional to the extent of apoptosis of a given treatment (Riccardi & Nicoletti, 2006). Thus, apoptotic cells with hypodiploid DNA were measured by quantifying the sub-G1 peak in the cell cycle pattern. For each experiment 20,000 events per samples were analysed and experiments were repeated 3 times. The apoptotic assay implemented in this study facilitates simultaneous analysis of cell-cycle parameters of surviving cells also, which were quantified as the proportion of cells in G0/GI, S and G2/M phases in DNA histogram (Riccardi & Nicoletti, 2006).

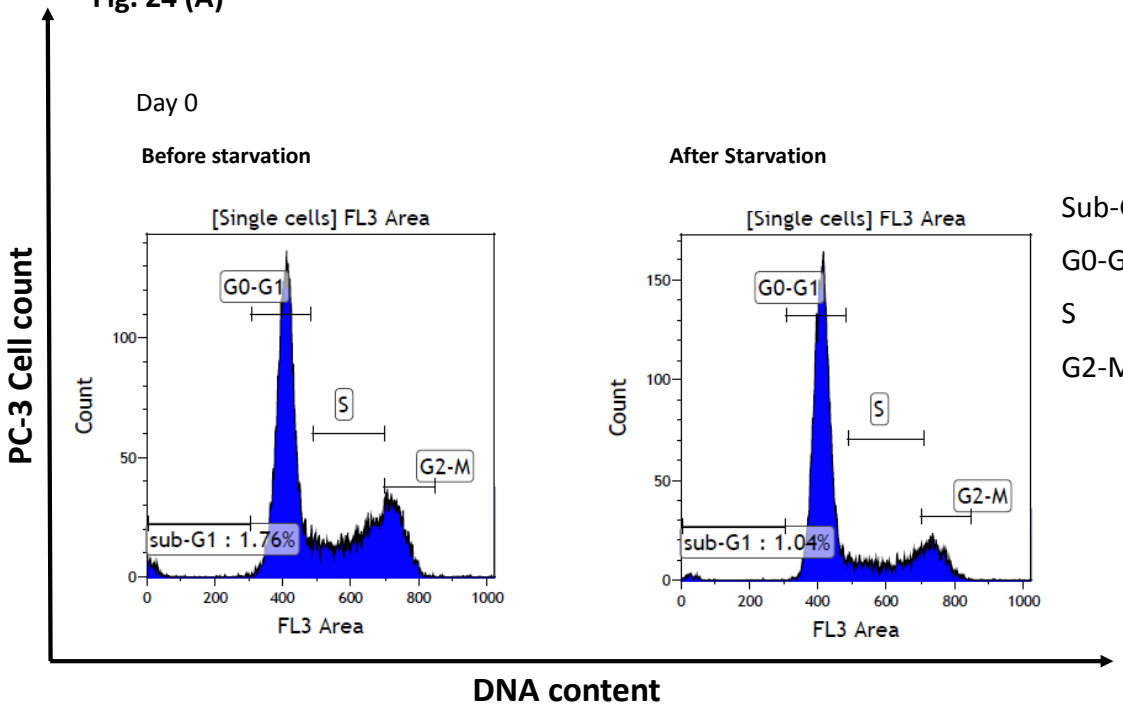
3.2.1 Fucoidan (CCF, ACF, UPF and CPF) induced apoptosis in PC-3 cells

To determine the degree of cell death induced by fucoidan, PC-3 cells were treated with CCF, CPF, ACF and UPF for 72 hours at various concentrations (100, 200,400 and 800 µg/ml). Figure 24, depicts the DNA histogram of PC-3 cells. Figure 24 (A), represents the cell percentage of PC-3 cells before and after starvation. The data shows that after 24 hours of cells starvation (i.e. with 0% serum), there was an increase of G0-GI phase from 59.16% to 72.10% (i.e. G0-G1 phase increased by 12.94%). The S-phase dropped from 8.59% to 6.23%, and the percentage of G2-M phase decreased by 9.77%. The cells were synchronized and arrested in G0-G1 phase as a result of starvation.

Figure 25 (B) and (C) represents apoptosis inducing effect of CCF, ACF, UCF and CPF on the phases of cell cycle in form of DNA histogram after 72 hours of drug treatment. Here, cells which acts as control are without any fucoidan treatment (depicted as 0 in the figure). Figure 24 shows the percentage of sub-G1 fraction (which is proportional to degree of apoptosis) of PC-3 cells for various concentration of CCF, ACF, UPF and CPF analysed in this study. The data shows here that percentage of sub-G1 fraction increased in a dose dependent manner for all the four types of fucoidan tested for its apoptosis inducing effect. Here statistical analysis of three typical results was presented with p-

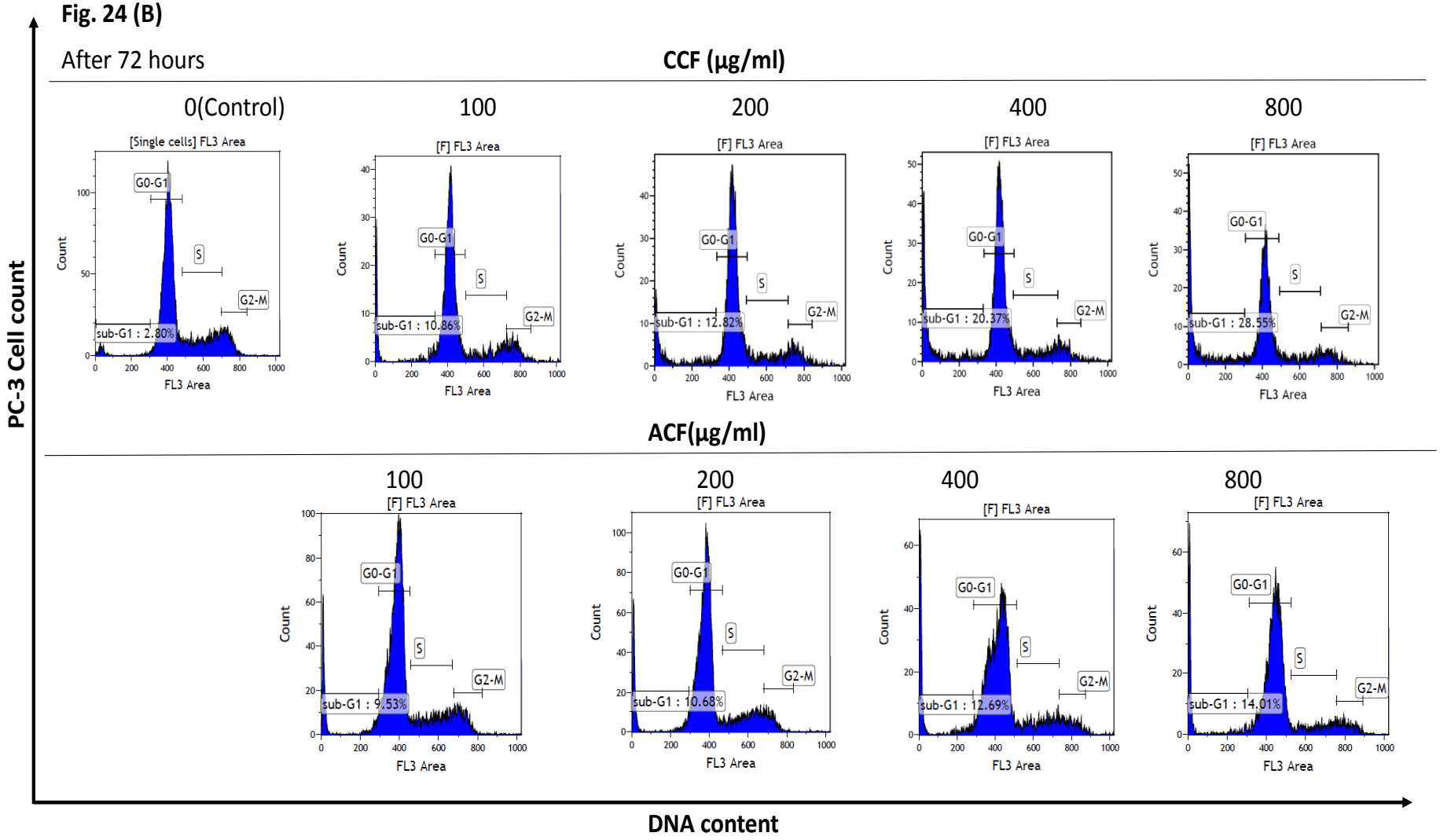
values: * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ each treatment compared to the control (control; without treatment). CCF significantly increased the sub-G1 percentage and hence is the most potent to induce apoptosis in PC-3 cells (at 100 µg/m, 10.86%; at 200 µg/ml, 20.37%; at 400 µg/ml, 20.37% and at 800 µg/ml, 28.55% of induced cell death). ACF was ranked as second most effective in inducing apoptosis with 9.53%; 10.68%; 12.69% and 14.01% of cell death recorded at 100 µg/ml, 200 µg/ml, 400 µg/ml, and 800 µg/ml respectively. ACF was followed by UPF and CPF. Hence, on the basis of percentage of sub-G1, the decreasing order of apoptosis inducing effect of different type of fucoidan tested in this study are CCF > ACF > UPF > CPF.

Fig. 24 (A)



	Before Starvation	After starvation
Sub-G1 :	1.76 %	1.04 %
G0-G1 :	59.16 %	72.10 %
S :	8.59 %	6.23 %
G2-M :	29.58 %	19.81 %

Fig. 24 (B)


After 72 hours
CCF (µg/ml)
0(Control)
100
200
400
800
[Single cells] FL3 Area
[F] FL3 Area
G0-G1
S
G2-M
sub-G1 : 2.80%
sub-G1 : 10.86%
sub-G1 : 12.82%
sub-G1 : 20.37%
sub-G1 : 28.55%
Count
FL3 Area
ACF(µg/ml)
100
200
400
800
sub-G1 : 9.53%
sub-G1 : 10.68%
sub-G1 : 12.69%
sub-G1 : 14.01%
PC-3 Cell count
DNA content

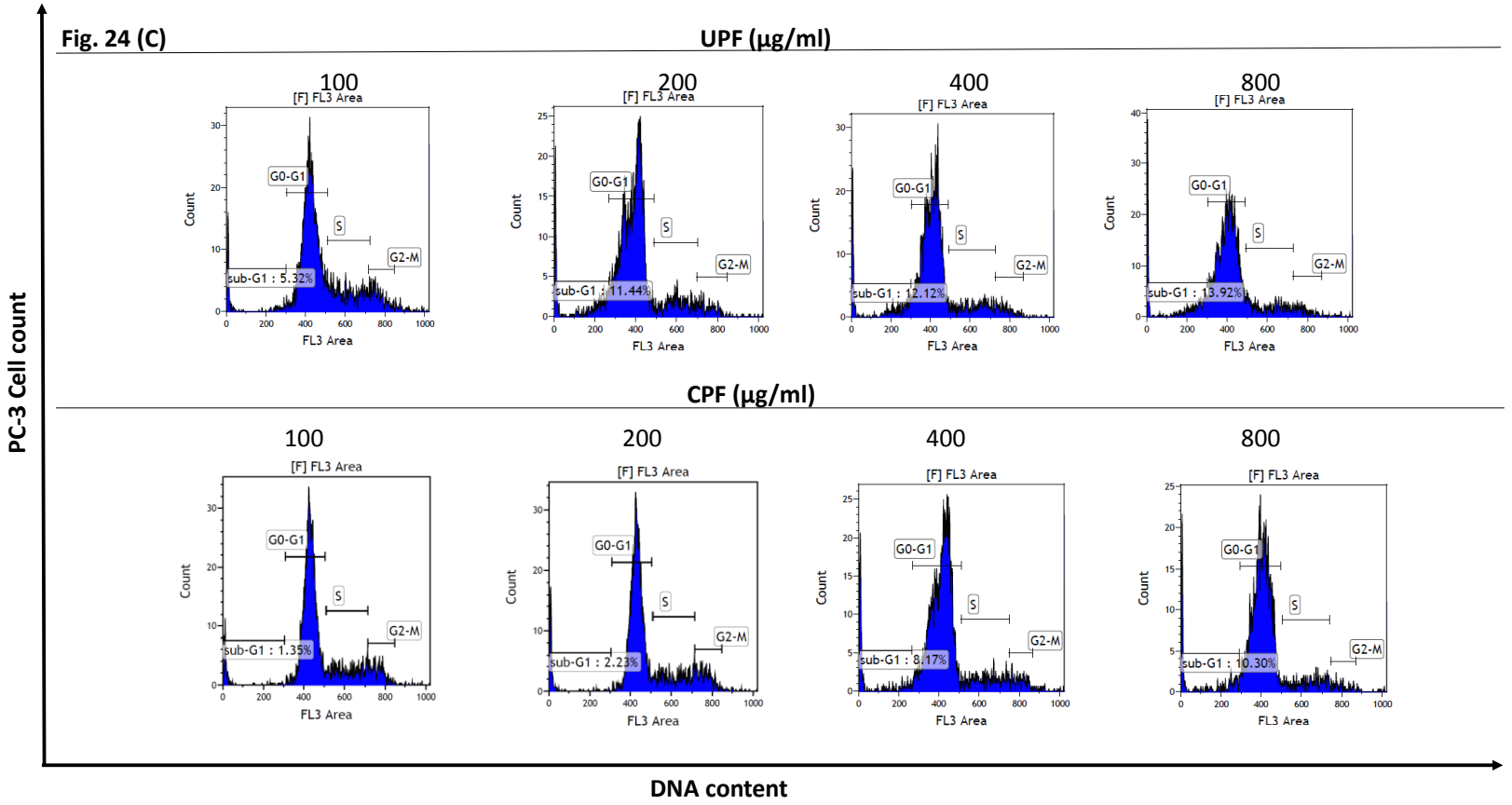


Figure 24: Apoptosis inducing effect of CCF, ACF, UPF and CPF represented as DNA histogram. (A) Represents the cell percentage of PC-3 cells before and after starvation. PC-3 cells were treated for 72 hours with CCF, ACF, UPF and CPF (100, 200,400 and 800 µg/ml). The cells were then harvested and analyzed for their DNA content by flow cytometry. Figure 24 (B) and (C) represents sub-G1 fraction, of PC-3 cells after treatment with CCF, ACF, UPF and CPF.

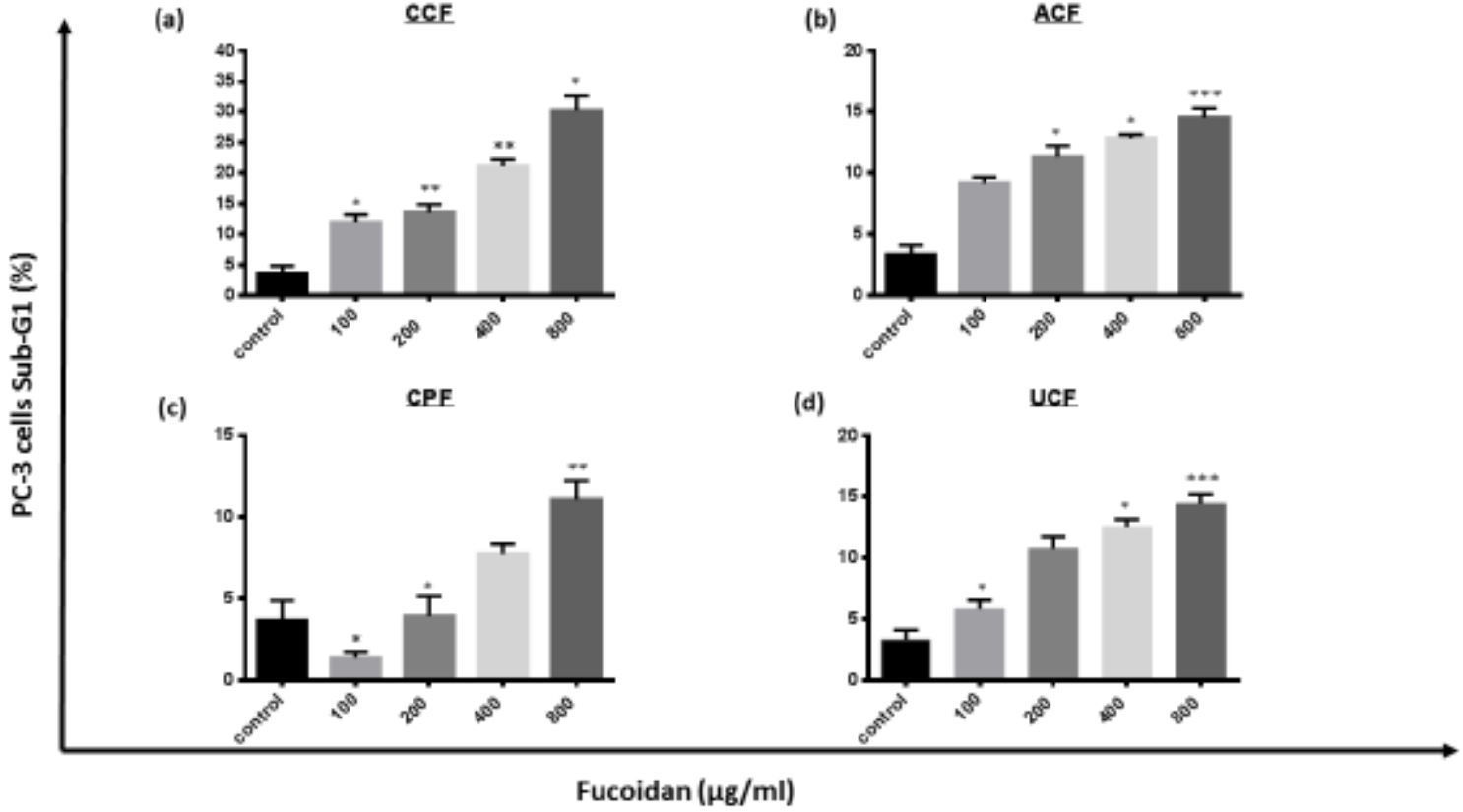


Figure 25: The PC-3 cell percentage of sub-G1 fraction in the cell cycle after 72 hours of treatment with fucoidan (CCF, ACF, UPF and CPF). Data are presented as mean ± SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the control (control; without fucoidan).

3.2.2 Fucoidan (CCF, ACF, UPF and CPF) induced apoptosis in DU-145 cells

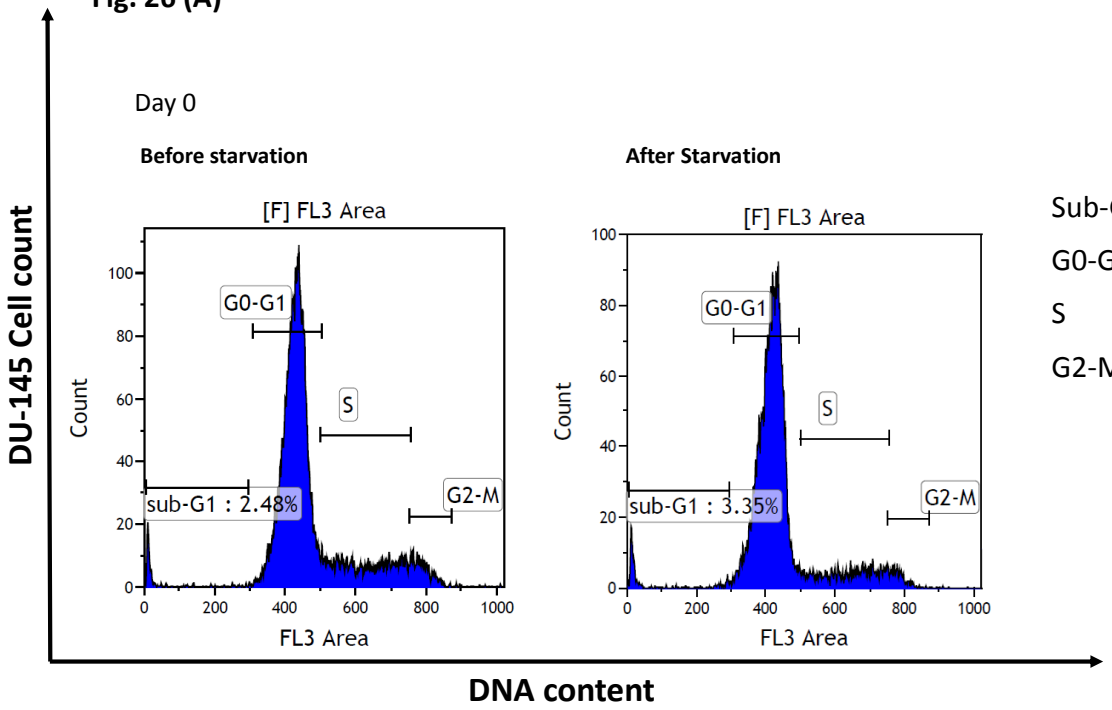
DU-145 cells were treated with CCF, CPF, ACF and UPF for 72 hours at various concentrations (100, 200,400 and 800 µg/ml). Figure 26 (A) represents the cell percentage of DU-145 cells before and after starvation. The data shows that after 24 hours of cells starvation (i.e. incubating the cells with 0% serum), there was an increase of G0-GI phase from 74.12% to 80.15% (i.e. G0-G1 phase increased by 6.03%). Along with an increase in G0-G1%, the S-phase dropped from 18.22% to 12.17%, and the percentage of G2-M phase decreased by 1.5%. The cells were synchronized and arrested in G0-G1 phase as a result of starvation.

Figure 26 (B) and (C), represents apoptosis inducing effect of CCF, ACF, UCF and CPF on the phases of cell cycle of DU-145 cells after 72 hours of drug treatment. Figure 25 shows the percentage of sub-G1 fraction (which is proportional to the degree of apoptosis) of DU-145 cells for various concentration of CCF, ACF, UPF and CPF analysed in this study. Here there was an increase in percentage of sub-G1 fraction in a dose dependent manner for CCF, UPF and CPF. CCF significantly increased the sub-G1 percentage and hence induced apoptosis most effectively (at 100 µg/ml, 9.37%; at 200 µg/ml, 13.19%; at 400 µg/ml, 14.95% and at 800 µg/ml, 31.08% of induced cell death) in DU-145 cells. There was not much difference between UPF and CPF in inducing cell death after 72 hours of treatment as at 800 µg/ml the % of sub-G1 fraction was 11.30% and 10.64 % respectively. Here data are presented as mean ± SD from three independent experiments. The statistical significance of each treatment was compared to the control (control; without treatment). Hence, on the basis of percentage of sub-G1, the decreasing order of apoptosis inducing effect of different type of fucoidan tested in this study are CCF > UPF and CPF.

As mentioned previously (in Section 3.1.4), the crude fucoidan (ACF) extracted from New Zealand seaweed, *U. pinnatifida* at AUT was found to be interacting with EMEM medium (medium used for culturing DU-145 cells) and hence, no appropriate results were obtained after analysing the apoptosis inducing effect of ACF on DU-145 cells. It was inferred from the Figures 26 (B), 27 (b), 31, 32, 33 and 34 that the cell counts were

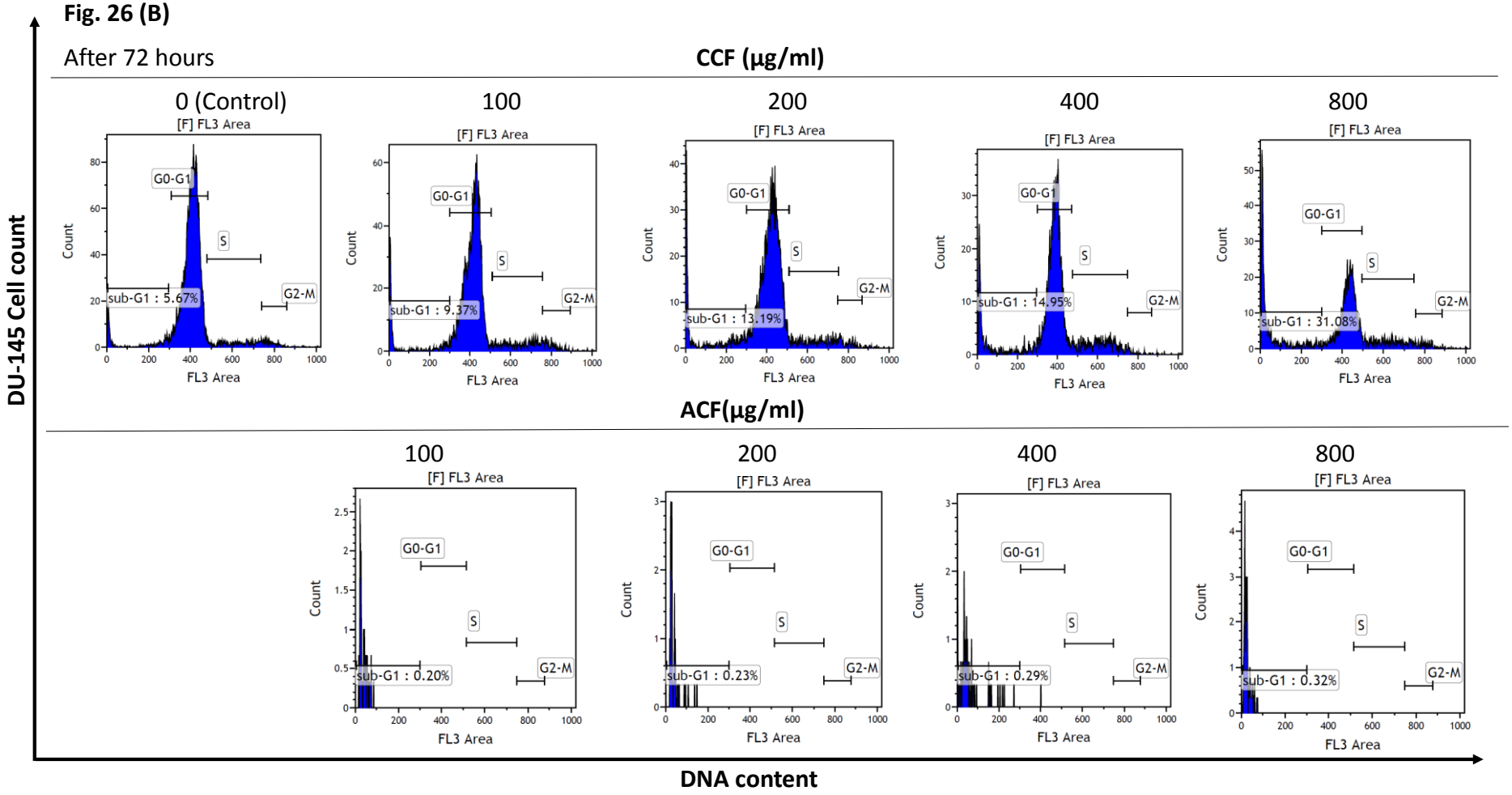
unseemly very low and difficult to comprehend. The possible reasons behind this result are discussed in details in discussion.

Fig. 26 (A)



	Before Starvation	After starvation
Sub-G1 :	2.48%	3.35%
G0-G1 :	74.12%	80.00%
S :	18.22%	12.87%
G2-M :	5.47%	3.07%

Fig. 26 (B)


After 72 hours
CCF (µg/ml)
0 (Control)
100
200
400
800
[F] FL3 Area
G0-G1
S
G2-M
sub-G1 : 5.67%
sub-G1 : 9.37%
sub-G1 : 13.19%
sub-G1 : 14.95%
sub-G1 : 31.08%
Count
FL3 Area
ACF(µg/ml)
100
200
400
800
sub-G1 : 0.20%
sub-G1 : 0.23%
sub-G1 : 0.29%
sub-G1 : 0.32%
DU-145 Cell count
DNA content

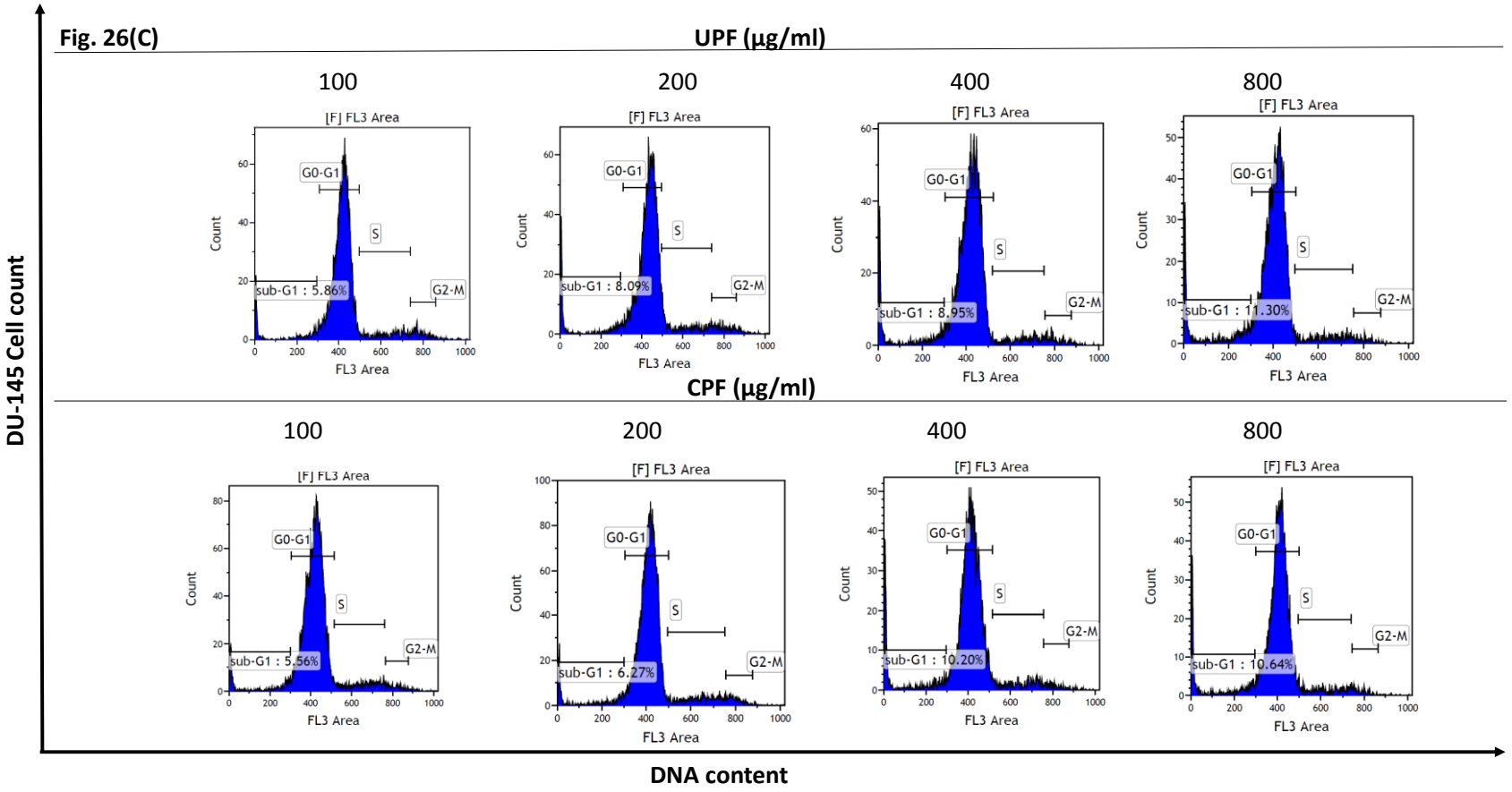


Figure 26: Apoptosis inducing effect of CCF, ACF, UPF and CPF represented as DNA histogram. (A) Represents the cell percentage of DU-145 cells before and after starvation. DU-145 cells were treated for 72 hours with CCF, ACF, UPF and CPF (100, 200,400 and 800 µg/ml). The cells were then harvested and analysed for their DNA content by flow cytometry. Figure 26 (B) and (C) represents sub-G1 fraction, of DU-145 cells after treatment with CCF, ACF, UPF and CPF.

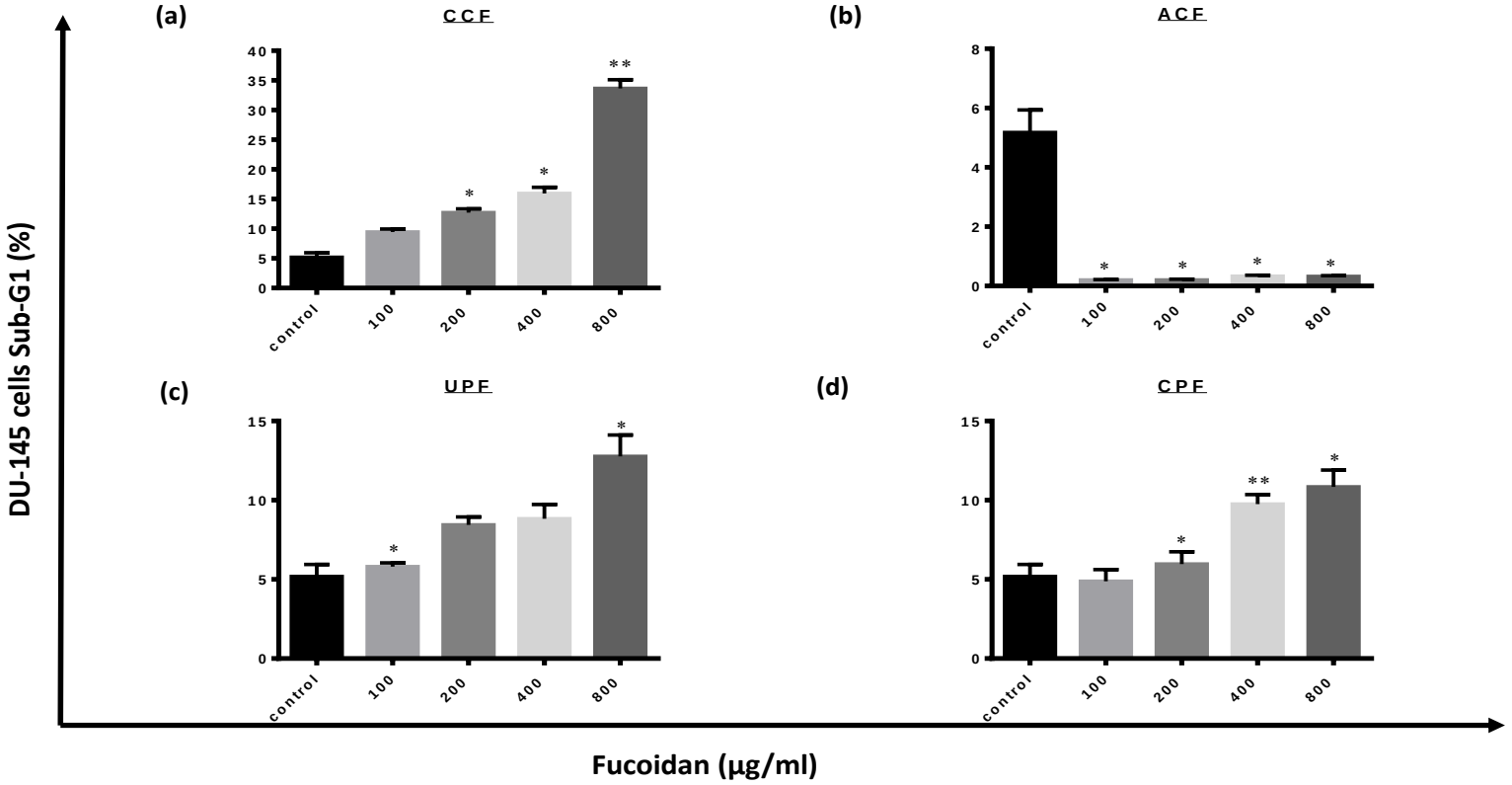


Figure 27: The DU-145 cell percentage of sub-G1 fraction in the cell cycle after 72 hours of treatment with fucoidan (CCF, ACF, UPF and CPF). Data are presented as mean ± SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the control (control; without fucoidan).

3.2.3 Fucoidan (CCF, ACF, UPF and CPF) induced apoptosis in HEK-293 cells

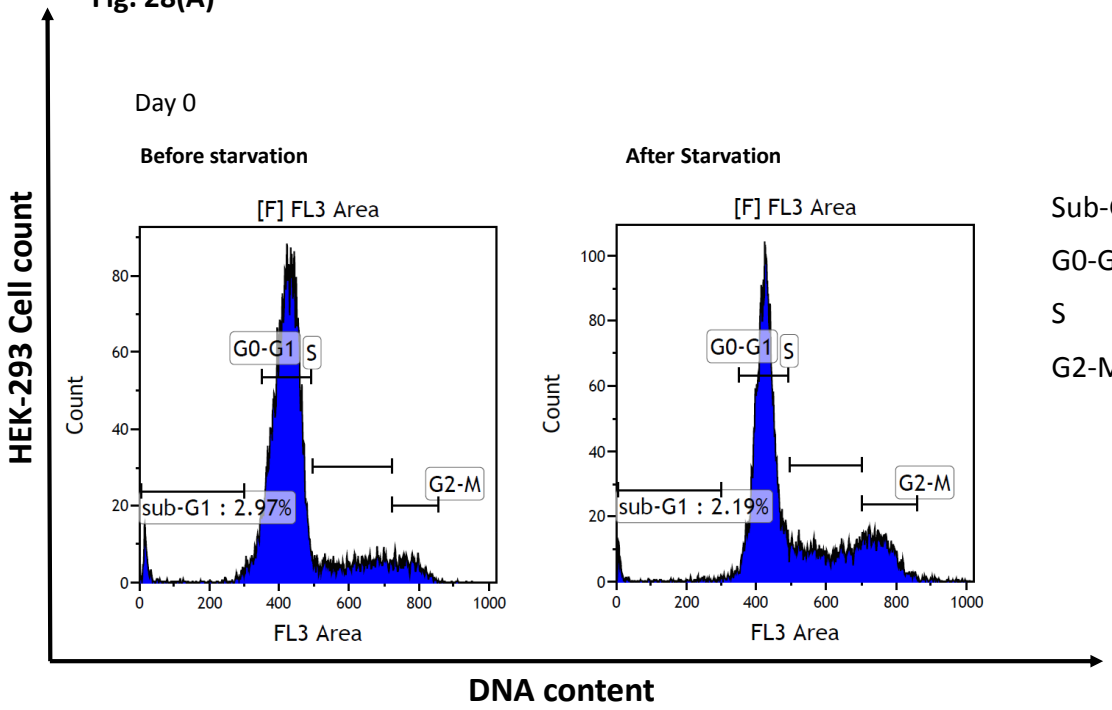
HEK-293 was the noncancerous or normal cell line analysed in this study to estimate the apoptosis inducing effect of CCF, ACF, UPF and CPF. Apoptosis assay was performed (by PI staining) for various concentrations of fucoidan (100, 200, 400 and 800 µg/ml) on HEK-293 after 72 hours of treatment addition. Figure 28 (A) represents the cell percentage of DU-145 cells before and after starvation. The data shows that after 24 hours of cells starvation (i.e. incubating the cells with 0% serum), there was an increase of G0-GI phase from 60.69% to 74.48 5 (i.e. G0-G1 phase increased by 13.79%). Along with an increase in G0-G1%, the S-phase dropped from 19.82% to 11.86%, and the percentage of G2-M phase decreased by 9.10%. The cells were synchronized and arrested in G0-G1 phase as a result of starvation.

Figures 28 (B) and (C), represent apoptosis inducing effect of CCF, ACF, UCF and CPF on the phases of cell cycle of HEK-293 cells after 72 hours of drug treatment. Figure 29 shows the percentage of sub-G1 fraction (which is proportional to degree of apoptosis) of HEK-293 cells for various concentration of CCF, ACF, UPF and CPF analysed in this study.

For CCF, there was a considerable increase in apoptosis inducing activity as the sub-G1 fraction increased from 9.40% to 27.40% for 100 to 800 µg/ml of fucoidan. At the highest concentration of CCF (i.e. 800 µg/ml) apoptosis inducing effect of CCF increased by 23.2% in comparison to control (without CCF).

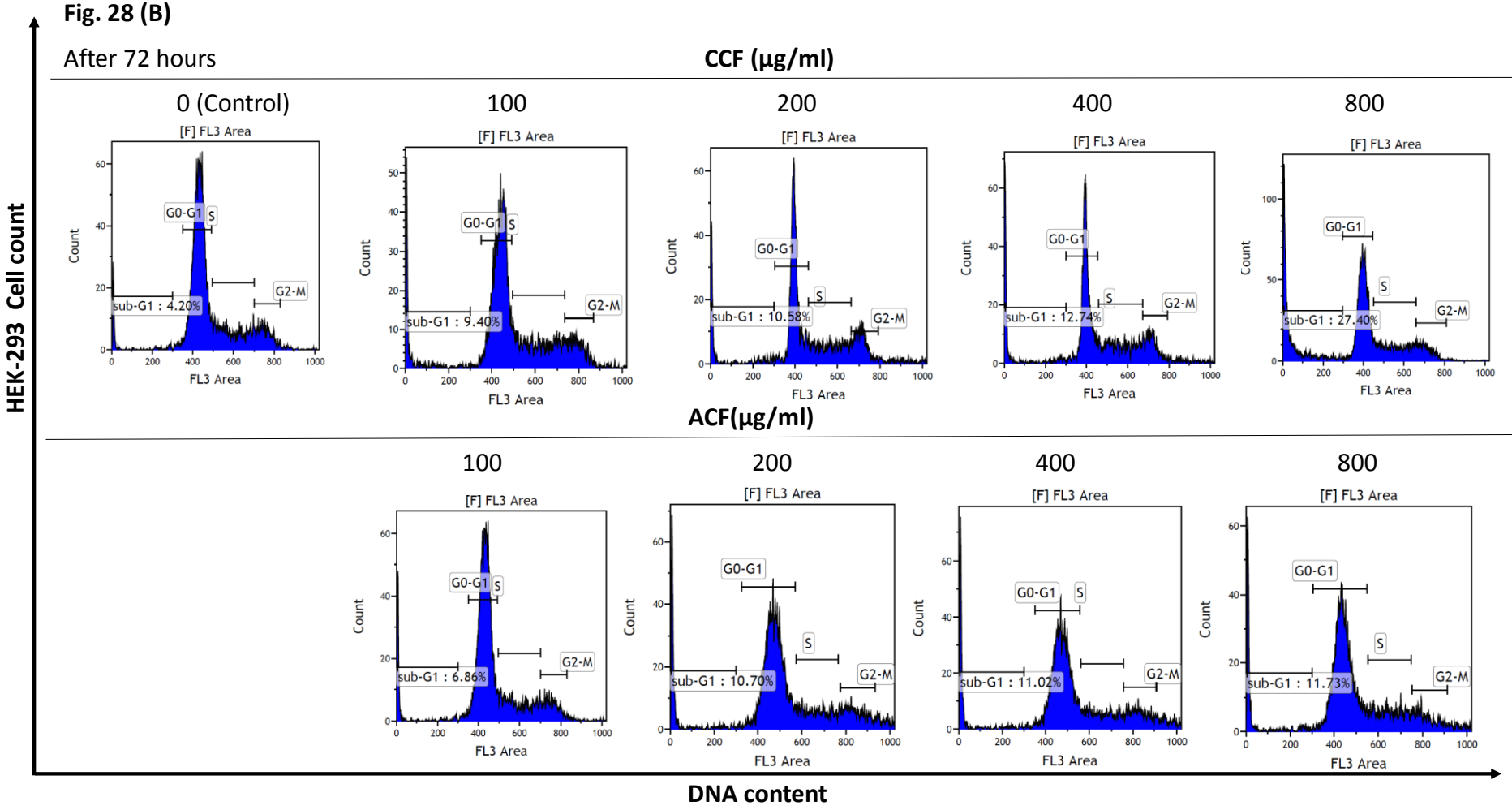
ACF, UPF and CPF fucoidan had very similar intensity of inducing apoptosis in HEK-293 cells. At 800 µg/ml of ACF, UPF and CPF, apoptosis only increased by 7.03%, 8.70% and 6.94% respectively when compared to control. Hence, on the basis of percentage of sub-G1, the decreasing order of apoptosis inducing effect of different type of fucoidan tested in this study against HEK-293 cells are CCF > UPF > ACF > CPF. It would be worth mentioning, however, that UPF, ACF and CPF induces the apoptosis in HEK-293 cells only by a low margin of around 7% and CPF at 100 µg/ml even increased the cell growth by 1.94%.

Fig. 28(A)



	Before Starvation	After starvation
Sub-G1 :	2.97%	2.19%
G0-G1 :	60.69%	74.48%
S :	19.82%	11.86%
G2-M :	14.55%	5.45%

Fig. 28 (B)


After 72 hours
CCF (µg/ml)
0 (Control)
100
200
400
800
ACF(µg/ml)
100
200
400
800
[F] FL3 Area
Count
FL3 Area
G0-G1
S
G2-M
sub-G1 : 4.20%
sub-G1 : 9.40%
sub-G1 : 10.58%
sub-G1 : 12.74%
sub-G1 : 27.40%
sub-G1 : 6.86%
sub-G1 : 10.70%
sub-G1 : 11.02%
sub-G1 : 11.73%
HEK-293 Cell count
DNA content

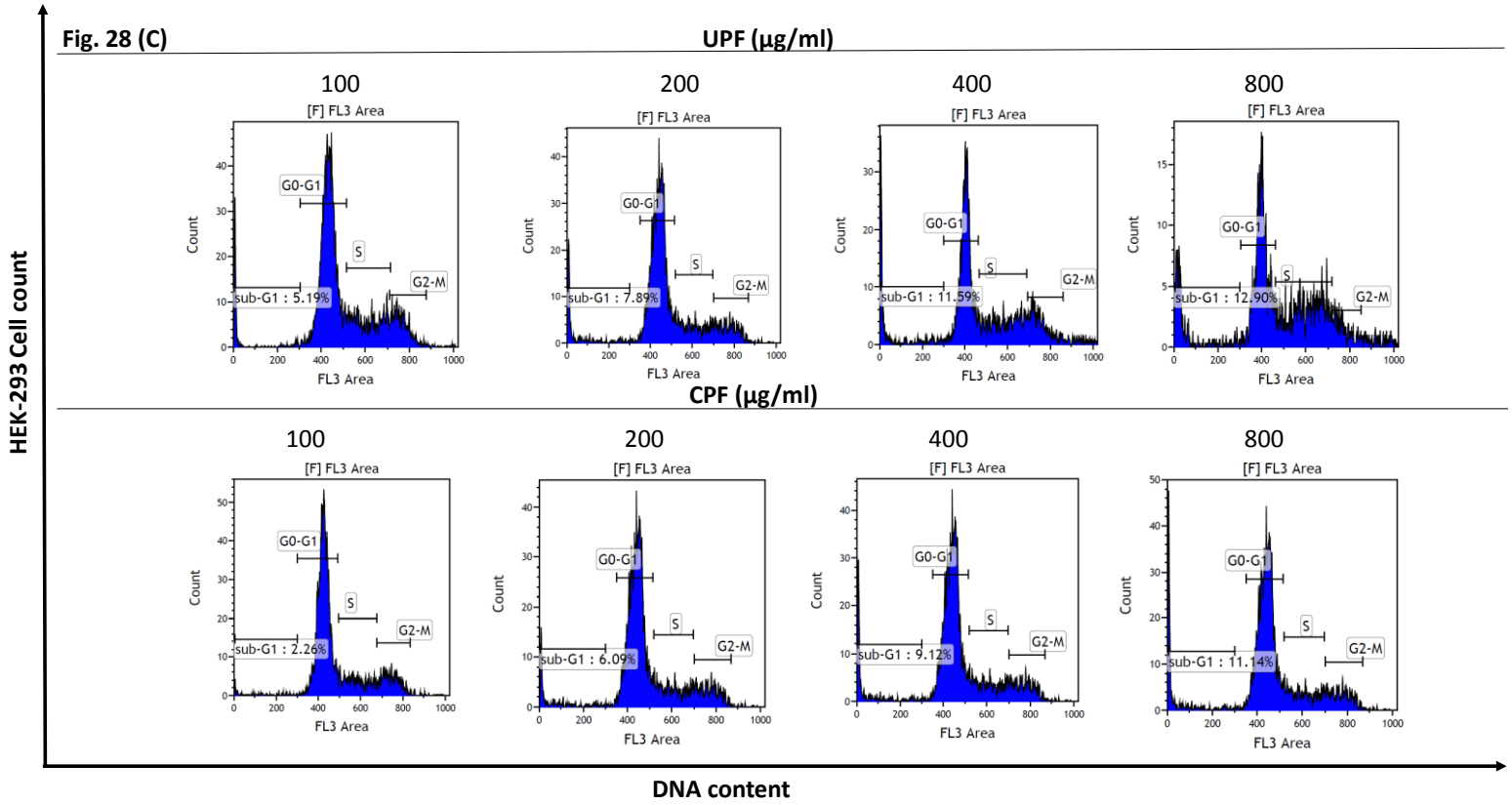


Figure 28: Apoptosis inducing effect of CCF, ACF, UPF and CPF represented as DNA histogram. (A) Represents the cell percentage of HEK-293 cells before and after starvation. HEK-293 cells were treated for 72 hours with CCF, ACF, UPF and CPF (100, 200, 400 and 800 µg/ml). The cells were then harvested and analyzed for their DNA content by flow cytometry. Figure 28 (B) and (C) represents sub-G1 fraction, of HEK-293 cells after treatment with CCF, ACF, UPF and CPF.

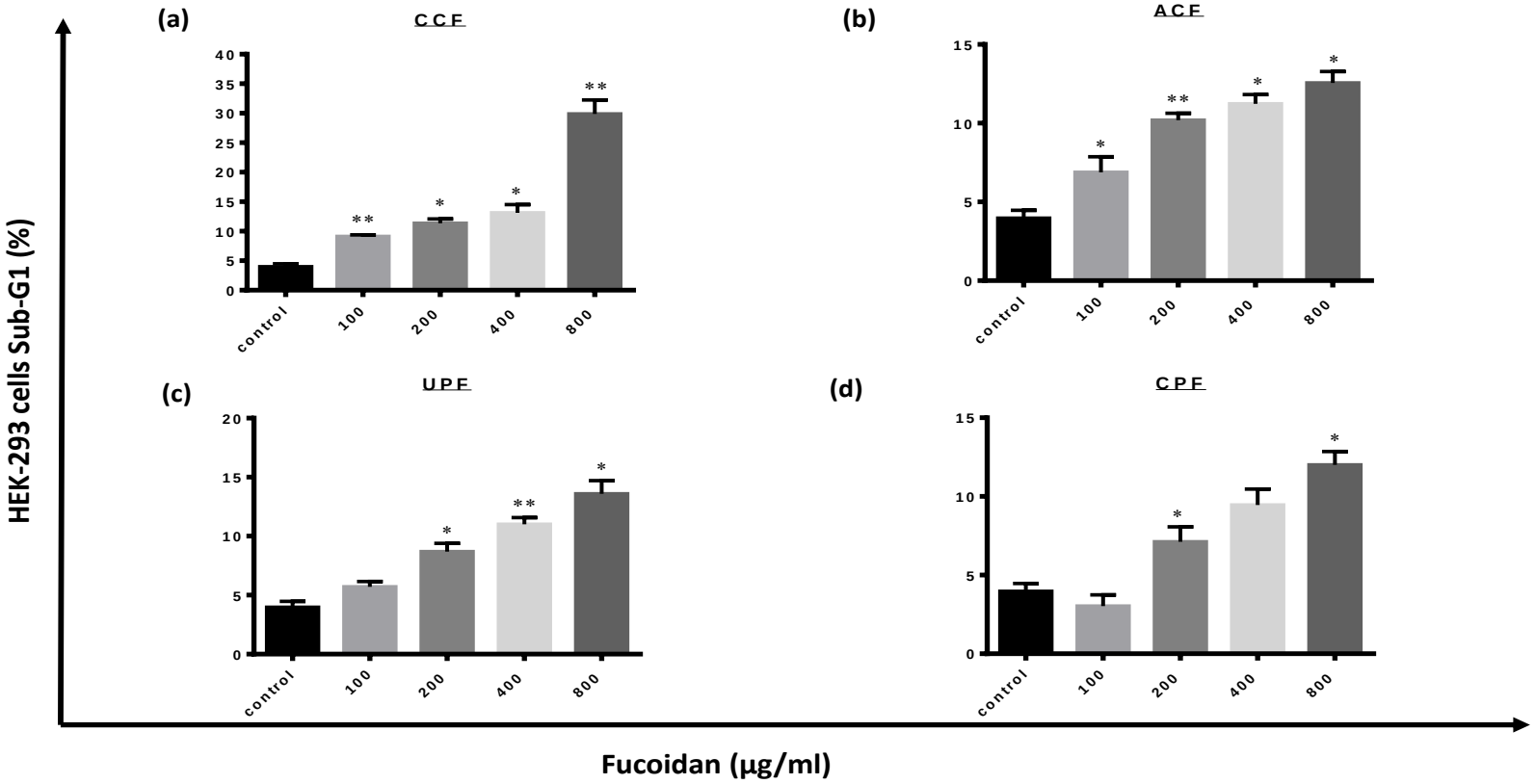


Figure 29: The HEK-293 cell percentage of sub-G1 fraction in the cell cycle after 72 hours of treatment with fucoidan (CCF, ACF, UPF and CPF). Data are presented as mean ± SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the control (control; without fucoidan).

CHAPTER FOUR

4. DISCUSSION

In spite of advancement in treatment options available for prostate cancer, mortality rates for advanced prostate cancer (i.e. metastatic and androgen independent metastatic stage), remains alarmingly high (prostate cancer is the second leading cause of cancer death in males in industrialized countries) (Society, 2013; Stewart & Wild, 2014). The present chemotherapy regime available for treating prostate cancer is accompanied with low effectiveness and high side effects, leading to deterioration of patient's condition after treatment (Jemal, Siegel, Xu, & Ward, 2010). Hence there is an urgent need to identify novel chemotherapeutic agents with high activity and low unwanted secondary effects.

Brown algae (seaweed) contain large amounts of cell-wall polysaccharides (Mohsen, Mohamed, Ali, & El-Sayed, 2007), most of which are sulfated polysaccharide fucoidans. Due to its abundance, and multiple biological activities (like anticancer, anticoagulant, antimitogenic, anticompliment, antiviral, anti-inflammatory, and antioxidant activities), fucoidan has been proposed as an important chemotherapeutic agent and is actively investigated in life sciences research (Cumashi et al., 2007).

In the present study, crude and pure fucoidan from *F. vesiculosus* (CCF, CPF from Sigma), crude fucoidan extracted at AUT from New Zealand *U. pinnatifida* (ACF) and pure fucoidan from *U. pinnatifida* (UPF from sigma) were investigated for their antiproliferative and apoptosis inducing activities on human prostate cancer cell lines. Two human prostate cancer cell lines namely PC-3 and DU-145 were utilized as a study model to examine the anticancer potential of CCF, ACF, UPF and CPF on prostate cancer cells. It is worth mentioning here that among prostate cancer cell lines available, PC-3 (Tilley, Wilson, Marcelli, & McPhaul, 1990) and DU-145 (Nakayama et al., 2000) are established to be comparable to androgen-independent cancer cells (androgen-independent stage of prostate cancer is considered to be metastatic advanced prostate cancer) and therefore to study drugs against these cell lines can be an indication of how a potential agent would

manifestate its action against advance prostate cancer. Thereafter, to test the toxicity and efficacy of fucoidan on normal or noncancerous cells, HEK-293 cells were also analysed.

To the best of my knowledge, the anticancer potential of crude and pure fucoidan from *Fucus Vesiculosus* (CCF, CPF) and crude fucoidan extracted from New Zealand *U. pinnatifida* (ACF) on human prostate cancer cells were carried out for the very first time.

4.1 Evaluation of Antiproliferative activity of fucoidan

Antiproliferative activity of fucoidans on various cell lines, was estimated by MTT Cell Proliferation Assay. All the three cell lines were treated with various concentration (5, 25, 50, 100, 200, 400, 800 and 1000 µg/ml) of each type of fucoidan and the cell viability was estimated as a time (day 1, 2, and 3) and dose dependent manner. Here cell viability was calculated as the ratio of absorbance in treated cultures to that in untreated control cultures. At day 3 (i.e. at 72 hours after treatment) the results were found to be significantly different and much easier to comprehend.

Comparison and ranking of different types of fucoidan examined in this study for their anticancer activity is done on the basis of IC_{50} values, which were obtained by GraphPad Prism software, version 6. IC_{50} value is the measure of effectiveness of a drug and it indicates the concentration of a drug which is needed to reduce a biological process by half (i.e. 50%). The lower IC_{50} values (corresponding to the most cytotoxic substance) suggest higher drug sensitivity and hence higher anticancer potential (Gibson, 2009; Krippendorff, Lienau, Reichel, & Huisinga, 2006).

4.1.1 Inhibitory effect of fucoidan (CCF, CPF, ACF and UPF) on prostate cancer cells (PC-3, DU-145 cells)

The four types of fucoidan tested in this study exhibited an inhibitory effect against tumour cell growth, with varying efficiencies and selectivities. In the current study, CCF (available commercial crude fucoidan obtained from *F. vesiculosus*) inhibited the proliferation of PC-3 and DU-145 cells in a time and dose dependent manner. The proliferation of PC-3 and DU-145 (except at 5 µg/ml) cells decreased consistently at various concentrations of CCF, with

increasing days (i.e. day 1, 2, and 3) (Figure 11 and 17). CCF was found to be more effective against PC-3 cells with an IC_{50} value of 74.88 µg/ml than on DU-145 cells with an IC_{50} value of 331.9 µg/ml.

The estimated IC_{50} values for ACF, UPF and CPF on PC-3 cell line was more than 1000 µg/ml. On the basis of tentative IC_{50} values, calculated using the Prism software, ACF (IC_{50} 903 µg/ml) has better antiproliferative activity than UPF (IC_{50} 1247 µg/ml) and CPF (IC_{50} 1964 µg/ml). At 1000 µg/ml (highest concentration tested in this study), the cell viability decreased by 48.1%, 35.94% and 26.66% for ACF, UPF and CPF respectively. Both above mentioned findings support the fact that CCF exhibited the lowest IC_{50} value, corresponding to highest cytotoxic effect against PC-3 cells. This was followed by ACF, UPF and CPF (Figure 13 and Table 14).

As mentioned before (Section 3.1.4) we do not have any results for ACF activity on DU-145. The probable reason behind this finding is discussed in section 4.4. Proceeding with antiproliferative activity of other fucoidans tested against DU-145 cells, UPF (IC_{50} 995 µg/ml) was shown to have high antiproliferative effect than CPF (IC_{50} 1587 µg/ml). Hence, the trend of inhibition exhibited by CCF, UPF and CPF against DU-145 cells was CCF > UPF > CPF.

From the above discussion it is clear that of all the four fucoidans tested in this study, CCF was the most effective and promising agent against both PC-3 cells and DU-145 cells, but with varying margins. The possible reasons behind CCF being more sensitive towards PC-3 than DU-145 cells could be due to phenotypic or genetic differences between the cell lines (Mitchell, Abel, Ware, Stamp, & Lalani, 2000). It has been documented that DU-145 and PC-3 cells are androgen-receptor negative and harbour non-functional p53. But for displaying a high sensitivity to *in vitro* inhibitory activity it is important that the cell line displays an active p38 (as found in PC-3), and thus the androgen receptor nor the p53 status is of any primary importance for the differences observed (Skjøth & Issinger, 2006).

Prior to this study, there were no reports examining the anticancer activity of fucoidan from *Fucus vesisculus* against human prostate cancer. Thus it was difficult to have a comparative study. However, some marine algal species have been investigated and reported for their anticancer activity on different cancer types, which are discussed below.

Previous studies have indicated that fucoidan from *F. vesiculosus* exhibited strong cytotoxic activity against human lymphoma HS-sultan cells (Aisa et al., 2005), human acute leukemia NB4 and HL-60 cells (Jin et al., 2010), human colon cancer HT-29, HCT116 cells (E. J. Kim, Park, Lee, & Park, 2010), human breast cancer MCF-7 cells (Banafa & Roshan, 2013) and human bladder carcinoma 5637, T-24 (Cho et al., 2014).

In most of the above mentioned studies (i.e. for human bladder carcinoma 5637, T-24, human lymphoma HS-sultan cells, human acute leukemia NB4 and HL-60 cells), the IC_{50} value was near to 100 µg/ml. This dose of 100 µg/ml of fucoidan is analogues to IC_{50} value of 74.88 µg/ml obtained against PC-3 cells, hence, the previous mentioned studies were in agreement to our study.

After comprehensive literature review, only one article was located which evaluates the anticancer effect of fucoidan from *U. pinnatifida* on human prostate cancer (H.-J. Boo et al., 2013). In this study, fucoidan induced cell death in a dose dependent manner and at 200 µg/ml, 45.1% of prostate cancer cells were killed. In this study, at 200 µg/ml 45.08% prostate cancer cells were killed. Hence, when compared, our results are in agreement with previous studies.

During comparative analysis of the antiproliferative effect of fucoidans (crude and pure form of fucoidan from *Fucus* and *Undaria*) it was inferred that the crude fucoidan of either of the two species tested in this study (*Fucus* and *Undaria*) was found to exhibit more antiproliferative effect than the pure fucoidan against prostate cancer cell lines; i.e. crude forms were more effective in inhibiting the growth of cancerous cells than the pure forms. Fucoidans bioactivities have been said to be related to their structural make-up, monosaccharide composition, sulfate content, position of sulfate ester groups, and molecular weight (B. Li et al., 2008). Fucoidan structure and monosaccharide composition itself is dependent upon various aspects like, the source of fucoidan, the part of seaweed from which it is extracted, the time and location of harvesting and the extraction methods.

Out of the four types of fucoidan evaluated in this study, three were commercially available fucoidan from sigma (i.e. crude and pure fucoidan from *F. vesiculosus* and pure fucoidan from *U. pinnatifida)*. The forth fucoidan tested was a crude form of fucoidan provided by

AUT university and it was extracted from New Zealand *U. pinnatifida*. When the specification provided by sigma for each type of fucoidan was studied, it was enlisted that crude fucoidan from *Fucus* had sulphur content in range from 12 to 30 %, and its molecular weight ranged from 2 X 10^3 to 2 X 10^4 Da. The crude fucoidan from *Undaria* also had considerable high sulphate content and lower molecular weight (chemical composition discussed in section 4.3). For the pure forms, from either of the species, other than their % of purity (which was ≥ 95%), their chemical composition is discussed in detail in section 4.3. As this discussion (section 4.3) and other literature proposes, higher degree of sulphation and lower molecular weight are linked with greater *in vitro* activity (Ale et al., 2011) and thus one can propose that higher inhibitory effects of crude fucoidan was as a result of these factors.

Other probable reasons responsible for the pure form being less effective than the crude form might be, that during the purification process, the sulphate content (which has been documented to be associated with anticancer property) was decreased by a process called desulphation (as it may have elucidated out with various solvent used during the multistep purification process), and also due to harsh conditions employed during the extraction and purification processes. Our assumptions are supported by one of the studies which mentions that native form of fucoidan has more anti-cancer activity than hydrolysed fucoidan (B. Li et al., 2008).

4.1.2 Inhibitory effect of fucoidan (CCF, CPF, ACF and UPF) on noncancerous cell line (HEK-293)

After determining the degree of antiproliferative effect by all the four fucoidans against prostate cancer cells, fucoidans were tested for their cytotoxicity on normal human embryonic kidney cells (HEK-293). Thus by comparing the degree of toxicity of CCF, ACF, UPF and CPF on normal cell lines verses the cancer cell line, a treatment option for prostate cancer with higher efficacy and lower or nil side effect can be proposed.

The IC_{50} values obtained for CCF, ACF, UPF and CPF after 72 hours of treatment on HEK-293 cells were 268.2, 958.4, > 2000, > 5000 µg/ml respectively. From the mentioned data, CPF seems to be the least cytotoxic on proliferation of HEK-293. The probable reasons behind

the pure form of fucoidan being less cytotoxic against normal cells could be because of decrease in sulphate content, as during the purification process, sulphate is eluded out. This in turn decreases the negative charge of fucoidan, hence decreasing the formation of fucoidan–protein complex involved in apoptosis and finally it's *in vitro* activity (Haroun-Bouhedja, Ellouali, Sinquin, & Boisson-Vidal, 2000).

When the cell inhibition % of HEK-293 cells at various dose of each fucoidan was compared with that of prostate cancer cells (PC-3 and not DU-145 cells as there was no result obtained for ACF inhibition action on DU-145 cells) it was inferred ACF was selective in antiproliferation activity between prostate cancer and normal cell line. Furthermore, there is only one study available, which evaluated the anticancer effect of *Undaria* fucoidan on PC-3 cells (H.-J. Boo et al., 2013). Herein, it was mentioned that *undaria* fucoidan had minimum cytotoxic effect on normal cells. Previous studies evaluating the in vitro activity of *undaria* fucoidan on different cancer cells showed the same result as concluded in this study (H. J. Boo et al., 2011; Synytsya et al., 2010)

Another point to be noted here is that CPF increased HEK-293 cells proliferation at 5, 25, 50 100 and 200 µg/ml. The maximum % increase of cells were at 5 and 25 µg/ml of CPF. Thus it can be rightly said that CPF along with being less toxic toward normal cells, were also helping the normal cells to grow at low doses. Probably, the low dose might decrease the bioavailability of drug and hence, decrease the interaction between the drug and cells, leading to the growth of cells. There have been similar reports mentioning low doses of chemotherapeutic drugs ("metronomic" dosing) (Bocci, Nicolaou, & Kerbel, 2002), enhancing noncancerous cell growth, however this effect (obtained in our study) has not been cited prior for fucoidan treatment.

Results from antiproliferative assay reveals that CCF was the most effective against prostate cancer cells, but on the same time had similar cytotoxic effect on normal cells as well. Though, pure fucoidan from *Undaria* and *Fucus* were less cytotoxic to normal cells they were not significantly inhibiting the prostate cancer cells. Furthermore, ACF was found to be second most effective fucoidan against the carcinoma cells along with lesser cytotoxicity towards the normal cells, especially at lower doses.

4.2 Evaluation of Apoptosis inducing activity

Many researchers have proposed various mechanisms, for antiproliferative activity of a possible agent against cancer cells, like induction of apoptosis, cell cycle arrest, inhibition of angiogenesis, inhibition of various growth factors and inhibition of metastasis (Cragg, Grothaus, & Newman, 2009). Out of these mechanisms, apoptosis is one of the most pervasive pathways through which chemo-preventive or chemotherapeutic agents can inhibit the overall growth of cancer cells.

The apoptosis inducing activity of CCF, ACF, UPF and CPF was evaluated by Flow cytometric analysis of apoptosis using PI DNA staining. Here the proportion of sub-G1 hypodiploid cells (which is proportional to cell death) was estimated after 72 hours of drugs addition for each cell lines (Riccardi & Nicoletti, 2006). By comparing the % of sub-G1 fraction, obtained after cells were treated with different concentrations (100, 200, 400 and 800 µg/ml) of fucoidan, the effectiveness of cell death for different types of fucoidan was estimated. Along with determining the index of the apoptotic DNA fragmentation (i.e. % of sub-G1) this procedure helps with simultaneous analysis of cell-cycle parameters of surviving cells (i.e. cell cycle distribution). Thus any arrest or blockage of cells at any particular cell cycle phase was also determined. Before we start evaluating apoptosis inducing activity by different fucoidan evaluated in this study, it is important to analyse the effect of serum starvation on each cell line.

4.2.1 Cell Synchronization by Serum Starvation

Cell synchronization is a process in which cells representing different stages of the cell cycle are selected and brought to the same phase. It offers a unique strategy to study the molecular and structural events taking place as cells travel through the cell cycle. It allows the exact study of individual phases of cell cycle, the regulatory mechanisms which determine cell cycle regulation at the level of gene expression and posttranscriptional protein modification, and contributes to drug discovery (Baserga, 1985).

It could be done either by “Physical Fractionation" or "Chemical Blockade." The latter is most widely accepted and practised method of cell synchronization, as it is easier to

perform and results obtained are very reproducible. Cells synchronization by chemical blockade is either performed by inhibition of DNA synthesis or nutritional deprivation (Pardee, 1974). As nutritional deprivation involved only serum free medium (as reagent), which was much easier to prepare and handle, in this study various cells used for analysis were synchronized by serum starvation method.

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 G0-G1 phase. G1 phase plays an important part in the cell cycle and determines whether a cell commits to division or to leave the cell cycle (COOPER, 2003). Moreover, serum elimination helps reduce the analytical interference and offers more reproducible experimental conditions (Pirkmajer & Chibalin, 2011).

In this study various cells used for analysis were synchronized by serum starvation which involves incubation of cell culture in FBS (fetal bovine serum) free culture medium for 24 hours. For PC-3 and HEK-293 cells synchronisation the serum free medium used was RPMI 1640 and for DU-145 cells EMEM serum free medium was used. PC-3 and HEK-293 cells resulted in good synchronization after 24 hours of serum starvation.

However, for DU-145 cells, the effectiveness of serum starvation was less compared to PC-3 cells (as compared to 12.94% and 13.79% of synchronization of PC-3 and HEK-293 cells respectively only 6% of DU-145 cells got synchronized). The probable reason might be, DU-145 cells being more aggressive phenotype, was less dependent on growth factors in the medium and thus its deprivation did not make any differences to its growth while the cells were serum starved to attain synchronization.

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. Previous study reported that certain concentration of serum which could result in a slight cell leakage under the period of serum starvation would be the most appropriate serum (Jackman & O'Connor, 2001). Moreover, the time period for which cultured cells are starved is also an 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 cells are not starved for optimum time, it could not help the leakage cells complete the second cycle and may then block them from further division.

Other probable reasons for comparatively less synchronization of DU-145 cells, would be complete serum starvation, which might have been too harsh on DU-145 cells and short duration of serum starvation time (which was 24 hours). As all the three cell lines were synchronized to G0-G1 phase, it was decided to keep the synchronization condition (of complete serum starvation for 24 hours) constant for all the cell lines as it would be much logical to compare the apoptosis inducing effect of fucoidan on cell lines which have undergone the same conditions of synchronization.

4.2.2 Effect of fucoidan (CCF, CPF, ACF and UPF) on induction of apoptosis in prostate cancer cells

Fucoidan anticancer property has been attributed to various mechanisms like inducing accumulation of sub-G1 population, chromatin condensation, and blockage of cells in different phases of cell cycle and internucleosomal fragmentation of DNA (Yamasaki-Miyamoto, Yamasaki, Tachibana, & Yamada, 2009). These different mechanisms responsible for the anticancer nature of fucoidan are because of differences in structural make-up, monosaccharide composition, sulfate content, position of sulfate ester groups, and molecular weight of fucoidan from different sources and also upon time and location of harvesting (Patankar et al., 1993). For example, Fucoidan from *Fucus vesiculosus* has been shown to induce apoptosis in human lymphoma HS-Sultan cells (Aisa et al., 2005), human leukemia U937 cells (Teruya, Konishi, Uechi, Tamaki, & Tako, 2007), and MCF-7 human breast cancer cells (Yamasaki-Miyamoto et al., 2009). However, fucoidan from *C. okamurans* and *U.pinnatifida* have also been shown to arrest the HTLV-1 infected T-cell HUT-102-cells and human prostate cancer cells (PC-3) in G2-M and G0-G1 phase respectively (H.-J. Boo et al., 2013).

In the present study, CCF, ACF, UPF and CPF increased the % of sub-G1fraction in a dose dependent fashion but with different efficacy at 72 hours. Out of all the four fucoidans

tested in this study against PC-3 cells, CCF was the most effective in inducing apoptosis as CCF treatment (800 µg/mL) increased the sub-G1 fraction by 28.55% at 72 hours (Figure 24 B) compared to that of control. Additionally ACF, UPF and CPF treatment (800 µg/mL) increased the sub-G1 fraction by 14.01%, 13.92% and 10.30% respectively compared to control (Figure 24 C). As listed in appendix A1, A2 and A3 there was nonsignificant arrest of PC-3 cells at any other phase of cell cycle.

For DU-145 cells, also all the three fucoidan (except ACF) increased the sub-G1 fraction in a dose dependent manner. From the results it was proposed that CCF was the most effective (at 800 µg/mL, 31.08% of sub-G1 fraction) and CPF was the least effective (at 800 µg/mL, 10.64% of sub-G1 fraction) in inducing apoptosis in DU-145 cells (Figures 26, B and C). Hence, both prostate cancer cells had similar response toward different fucoidan tested in this study fucoidan tested in this study.

Apoptosis is mediated through either, the extrinsic (cytoplasmic) pathway whereby death receptors trigger the apoptosis from outside the cell, or intrinsic (mitochondrial) pathway in which changes in mitochondrial membrane potential (MMP) leads to cytochrome C release and death signal activation from inside the cell (Fulda & Debatin, 2006). These pathways are said to activate caspases which are a family of cysteine proteases that play essential roles in apoptosis (programmed cell death), necrosis, and inflammation (Nicholson & Thornberry, 1997).

Fucoidan from different sources have been shown to activate both pathways which mediate apoptosis. According to previous studies, caspase 8 and 9, which are best characterized molecules for extrinsic and intrinsic pathways respectively are documented to be activated by fucoidan (E. J. Kim et al., 2010). For example, Fucoidan from *Fucus vesiculosus* (very low dose of 20 µg/ml) have shown to inhibit the growth of human colon and breast cancer cells by apoptosis induction which was mediated through intrinsic pathway. It has also been mentioned that, fucoidan activated caspase 3 and 7 in these cancer cell line. However, it induced apoptosis in T leukemia cells at much higher concentration of 3 mg/ml (Haneji et al., 2005).

After extensive literature review, only one article was found which studied the anticancer effect of commercial pure fucoidan from *U.pinnatifida* on PC-3 cells. It was documented that at 1000 µg/ml, fucoidan induced apoptosis in 34.7% of PC-3 cells. On the other hand in our studies, treatment with crude and pure fucoidan (800 µg/ml) from *Undaria* sp. increased the sub-G1 fraction by 13.92% and 14.01% respectively compared to control. The differences in both the studies could be attributed to the fact that even though fucoidan is obtained from same seaweed it was harvested from different locations, and as already mentioned the activity of fucoidan varies with different source and location of harvesting.

In the same study, in order to elucidate the mechanism of induction of apoptosis in PC-3 cells, various effects of fucoidan on different pathways were examined. It was shown that *Undaria* fucoidan treatment led to activation of caspase-8 and caspase-9, i.e. activated both intrinsic and extrinsic pathways. Both these pathways induce apoptosis via interaction of MAPK/ERK and PI3/Akt signalling pathways, hence these pathways affect each other. The MAPK/ERK pathway (also known as the Ras-Raf-MEK-ERK pathway) is a chain of proteins in the cell that communicates a signal from a receptor on the surface of the cell to the DNA in the nucleus of the cell. It regulates apoptosis. In mammals, there are more than a dozen MAPK genes. The best known are the extracellular signal-regulated kinases 1 and 2 (ERK1/2). They are said to regulate apoptosis (Raman, Chen, & Cobb, 2007). On the other hand, the PI3K/Akt pathway inhibits apoptosis (Cregan et al., 2004). The results of this study proposed that, fucoidan from *Undaria pinnatifida* induced apoptosis in PC-3 cells through activation of the ERK1/2-MAPK pathway and inactivation of the PI3K/Akt pathway.

Another major finding of this study was that along with inducing apoptosis, fucoidan also down-regulated Wnt/β-catenin signalling pathway, which is reported to be up-regulated in a large portion of prostate cancer patients pathway (as activation of the Wnt/β-catenin pathway has effects on prostate cell proliferation, differentiation and the epithelial-mesenchymal transition, which is thought to regulate the invasive behaviour of tumour cells) (Zi et al., 2005). Hence fucoidan has an additional advantage of being more specific for prostate cancer as it has been reported to directly down-regulate Wnt/β-catenin signalling, which is very critical in the manifestation of the invasive nature of prostate cancer.

Thus, a drug which could block the activity of PI3K /Akt and activates ERK1/2 would ideally lead to the inhibition of proliferation and induction of apoptosis. Moreover as we have not estimated the effect of different fucoidans used in our study on various pathways, which are critical for cell survival and death (as it was out of the scope of this research); it can be proposed that CCF and ACF (two of most effective fucoidans in this study) impose their apoptosis inducing effect by differentially regulating PI3K/Akt, ERK1/2 signalling pathways.

All of these inferences taken together suggest that fucoidan has the capability to inhibit (the proliferation of prostate cancer cells) and interact with several components of the apoptosis pathway and it might have therapeutic potential for prostate cancer treatment.

4.2.3 Effect of fucoidan (CCF, CPF, ACF and UPF) on induction of apoptosis in noncancerous cells (HEK-293)

One of the most important criteria for a chemo preventive agent is said to be an agent with minimal side effect. An anticancer drug should not only lengthen lifespan of a patient suffering from cancer, but should also improve the quality of life. Thus after determining the degree of apoptosis inducing effect by all the four fucoidans against prostate cancer cells, they were tested for their cell death activity on normal human embryonic kidney cells (HEK-293).

The experiments results (Figure, 28B, 28C and 29) show that CCF had the maximum apoptosis inducing effect (at 800 µg/ml, 23.2%) on HEK-293 cells. Additionally, UPF, ACF and CPF had similar apoptosis inducing effect (at 800 µg/ml, 8.7%, 7.03% and 6.94% respectively) on HEK-293 cells compared to control. Most of the above discussed research work evaluating the anticancer nature of fucoidan on different types of cancer cells have shown to have no inverse effect on the proliferation of normal cell lines. However, Hee et al. showed that fucoidan from *Fucus vesiculosus* inhibited the growth and induced apoptosis in normal lung fibroblast cells (HEL-299).

4.3 Comparison between types of Fucoidan analysed in this study

Like many polymers, fucoidans are also polydisperse, which do not have a discrete molecular weight, but have a mixture or range of molecular weights. Fucoidans are also highly branched molecules, hence, even though a particular fucoidan consists of similar molecular weight mixture, the fractions that would be obtained after extraction (by hydrolysing the original polysaccharides) would be large in number with different molecular configuration and activity. Moreover, fucoidan extracted from a same species have been documented to differ in their structure, depending upon the time, location of harvesting and they even differ in their chemical composition if extracted from different part of the same seaweed. Hence, differences in structure attributes to the differences in bioactivity of a fucoidan from same source.

Fucoidan from different species are distinctly different in their structural motifs. In this thesis, four different types of fucoidan were analysed and tested for their anticancer potential on human prostate cancer cells. Out of all the fucoidans tested CCF (commercial crude fucoidan from *F. vesiculosus*) was found to be most effective and had maximum antiproliferative and apoptosis inducing effect against cancer cells. ACF (crude fucoidan from New Zealand U. pinnatifida, extracted at Auckland University of Technology) was the second most effective agent. The other two fucoidans UPF and CPF (commercial pure fucoidan from *Undaria* and *Fucus* respectively) were shown to exert more or less similar *in vitro* activity (IC_{50} values too high, thus less anticancer potential).

Inferences drawn from extensive literature review (Fitton, Stringer, & Karpiniec, 2015; B. Li et al., 2008), AUT studies (Wilfred Mak et al., 2014) and from information available at Sigma database, we put forward the probable chemical composition and molecular weight profile of fucoidans tested in this study and their implications on respective activity of different fucoidan.

The commercial crude fucoidan from *F. vesiculosus*, was reported to be composed majorly of fucose (45.9%) and sulphate (32.1%) with traces of uronic acid (8.7%), xylose (3.6%) and

galactose (3.2%). The commercial pure fucoidan from *F. vesiculosus* consist of around 43.1 % fucose, 17.96% sulfate, 2.2% galactose, 3.1% uronic acid and 8.8% xylose. When the molecular weight of both crude and pure forms of fucoidan from *Fucus* sp were compared, it can be inferred that crude and pure fucoidan peak average MW (kDa) were around 54.7 and 32.9 respectively. Higher sulphate content regulates the antiproliferative potential of fucoidan. It has even been shown that over-sulphation enhances the anticancer activity of fucoidan by increasing the negative charge and thus facilitating the interaction of fucoidan–protein complexes in various pathways linked to cell proliferation (and ultimately inhibiting cell growth) (Soeda, Ishida, Shimeno, & Nagamatsu, 1994). Hence, it can be proposed that the higher activity of crude fucoidan (CCF) is attributed to its higher sulphate content. Additionally, another important point mentioned in above cited reviews regarding high activity of crude fucoidan was, it might contain contaminants (like polyphenols) which may have chemical moieties having antiproliferative effect against cancer cells. Thus these chemical moieties might further add up to the *in vitro* activity of crude form.

The structure of fucoidan obtained from U. pinnatifida is documented to be divergent from that of *Fucus* species (K.-J. Kim, O.-H. Lee, & B.-Y. Lee, 2010) as it contains significant amount of galactose. According to information available from study on crude fucoidan isolated from New Zealand *U.pinnatifida* (Wilfred Mak et al., 2014) at AUT (ACF), crude fucoidan was documented to be composed of 39.24% fucose, 25.19% sulphate, 26.48% galactose, 3.43% uronic acid, along with minor amounts of protein. Commercial pure fucoidan from *Undaria sp*. (UPF) was used as a standard in the same study (for comparing the chemical composition of fucoidan extracted at AUT from New Zealand *Undaria pinnatifida*), and it was documented to be composed of 33.74% fucose and 13.96% sulphate, 22.98% galactose and 0.67% uronic acid. The peak average molecular weight of ACF and UPF were estimated to be 81 and 51.2 kDa respectively. The higher activity of ACF could be attributed to higher sulfate and uronic acid content in comparison to UPF. And due to which the negative charges on fucoidan molecule was enhanced and thus leading to an increased interaction of the molecule with the protein involved in cell proliferation and finally resulting in greater suppression of cell growth.

Moreover, it has been documented that lower molecular weight and milder extraction methods directly regulates the inhibitory effect of fucoidan. However in this study the crude fucoidan were shown to possess higher molecular weight than the pure forms. The concept that lower molecular weight fucoidan having enhanced *in vitro* activity appears to be very complex and not clearly understood. Furthermore, it has been reported that lower molecular weight alone does not facilitate higher activity. Different factors are interrelated and possibly they manifestate the antiproliferative activity together rather than alone (B. Li et al., 2008). In chemistry, conformation of molecule (that is whether it is in compact or loose form) also plays a critical role in deciding the activity of a compound (L. Zhang, Li, Xu, & Zeng, 2005). Thus, possibly, loose conformation of fucoidan (having high molecular weight) have more hydroxyl groups exposed and are available for interaction with receptors on cancer cells, whereas in compact conformation of fucoidan (low molecular weight) hydroxyl groups are more involved in intramolecular interaction than with receptors on cancer cells. This might be possible because high molecular weight compounds are heavily branched structures which might result in the loose conformation of the compound, and vice versa. Thus in this study, possibly, the loosely or high molecular weight fucoidan have more antiproliferative activity than relatively compact or low molecular weight fucoidan.

Another fact to consider would be that in order to obtain a purer form of fucoidan, the native molecule has to undergo various chemical procedure like hydrolysis, which could be mild (like acid and enzymatic hydrolysis) or severe (radical depolymerisation and heating at high temperature). Milder hydrolysis condition has been reported to confer higher antitumour activity than under harsh condition by less desulphation (Le Gal, Ulber, & Antranikian, 2005). Thus, in this study the pure forms of fucoidan analysed might have been obtained after severe hydrolysis, and in turn lead to desulphation or debranching and possibly decreasing or modifying the *in vitro* activity.

Thus it can be proposed that fucoidan is a very complex entity, with its anticancer activity being linked to its structural composition, which is in turn governed by various factors like, species, time and location of harvesting, maturity of the plant and extraction methods. Hence to fully establish the *in vitro* activity of fucoidan and its reproducibility, it is

imperative to have a clear idea of its structure and to standardise the protocols which facilitates and enhances the activity.

4.4 Probable justification for the inconsistent characteristics of ACF with DU-145 cells

During the course of the study, we came across an unexpected situation. When ACF was being evaluated for its antiproliferative and apoptosis-inducing effect against human prostate cancer cell lines, PC-3 and DU-145, results were obtained for ACF against PC-3 cells but not against Du-145 cells. For MTT cell proliferative assay, the OD readings were too high to be analysed in the prism software to obtain IC_{50} value. Apoptosis assay by Beckman Coulter MoFlo™ XDP flow cytometer also could not attain any results in the dot plot graph.

After careful deliberation, the 96 well plate design was modified to identify the wells with high OD readings. It needs to be noted here that very high absorbance reading was not obtained when ACF was tested for its anticancer potential on PC-3 cells growth and for cytotoxic effect on HEK-293 normal cells, thus ACF did not seemed to have any issue with MTT reagent used in the cell proliferation assay.

Here the culture medium used for DU-145 cells was EMEM, which was different from RPMI-1640 culture medium used for PC-3 cells and HEK-293. Hence the 96 well plate was designed to find out the interaction and corresponding OD reading between MTT and

- EMEM medium;
- DU-145 cells with EMEM medium;
- DU-145 cells with EMEM medium along with different concertation's of ACF to be analysed (each in separate columns);
- EMEM medium along with different concertation's of ACF to be analysed (each in separate columns)

The visual development in the plate is depicted in Figures 15 and 16, which were obtained before and after MTT assay.

After MTT assay was performed, it was observed that the OD reading for first two columns (EMEM medium alone i.e. blank and DU-145 cells along with medium i.e. negative control) were accepted and in range (i.e. neither too low nor too high). But in the other two sets of columns (containing DU-145 cells with various concentration of ACF and EMEM medium with various concentration of ACF) the OD reading was way too high.

It can be inferred from the above observation that OD values were in normal range when EMEM medium and DU-145 cells alone interacted with MTT reagent. High OD readings were observed when different concentrations of ACF along with EMEM medium interacted with MTT reagent. Hence, as high OD values were only observed when MTT reagent interacted with ACF along with EMEM medium and not with DU-145 cells or EMEM medium alone, it could be that chemical interactions between EMEM medium and ACF fucoidan were interfering with MTT reagent contributing to very high OD readings values and dark blue appearance.

Crude fucoidan from New Zealand *U.pinnatifida* (ACF) have been tested for its antioxidant activity via DPPH and CUPRAC assays in earlier studies. The results showed that ACF had strong antioxidant activity in both assays. Moreover Vitamin C, a proven potent antioxidant was used as a positive control in the study, and antioxidant potential of ACF was found to be very similar to vitamin C (Wilfred Mak, 2012). Now as we know that, in MTT cell proliferation assay the cell viability is directly linked to the metabolic activity of cells. The NAD-(P)-H-dependent cellular oxidoreductase enzymes, present in the mitochondria of viable cells reduces the MTT reagent (a tetrazolium dye) to insoluble purple formazan, which are further solubilized using DMSO and quantified by spectrophotometric means.

It should be emphasized here that if MTT reagent is being reduced to formazan by anything else other than dehydrogenase enzymes (i.e. non-enzymatically), then it would give false positive results. Additionally, reducing agents like vitamin C (which has very high antioxidant activity) have been shown to interfere with the MTT assay and gives high absorbance or O. D. values.

Furthermore, according to ATCC database, EMEM medium used for culturing DU-145 cells, is documented to a have higher pH range and higher % of vitamins and glucose in

comparison to RPMI 1640 medium used for culturing PC-3 and HEK-293 cells. Culture medium at elevated pH has also been documented to accelerate spontaneous reduction of tetrazolium salts (i.e. non enzymatic reduction) and result in increased background absorbance values (T. L. Riss et al., 2004).

Hence, the probable reasons behind obtaining very high O. D. value for MTT proliferation assay when ACF fucoidan (crude New Zealand U. pinnatifida fucoidan extracted at AUT) was tested on DU-145 human prostate cancer cells could be owing to the strong reducing nature of ACF which was further assisted by EMEM medium as it has a higher working pH along with comparatively higher content of vitamin which have been shown to interfere with MTT reagent and resulting in high absorbance value.

Throughout the apoptosis assay experiments, 20,000 events per sample were analysed, but when different concentrations of ACF treated DU-145 cells were analysed, there were very few cells (nearly hundred) which could be the gated in dot plot graph compared to the control, which is DU-145 cells without treatment. (Figure 30, 31, 32, 33 and 34 shown in appendix A4). Thus, the DNA histogram could not gate any cells in any phases of cell cycle (as is clear from Figure 26, B). One of the possibility behind very few cells being gated or shown to be present in the dot plot graph, could be that the DU-145 cells were actually dying after treatment with various concentration of ACF, thereby resulting in less or negligible cell density compared to the control cells (untreated cells). May be the doses of ACF which have been tested in this study are potent inhibitor and being cytotoxic at the concentration tested for DU-145 cells in particular and thus lower doses of ACF should also be checked for its antiproliferative activity.

Furthermore, there is a need for an elaborate study utilizing various other assays to quantify cell viability and apoptosis inducing activity of ACF fucoidan against DU-145 cells.

CHAPTER FIVE

5. CONCLUSION

5.1 Conclusion

Universally, the goal of cancer treatment has been eradication of tumour cells, with minimal damage to healthy cells. Prostate cancer is one of the cancer which is very difficult to treat as it progresses to more invasive and advance stage. As the current treatment options available to patients suffering from this life threatening disease, have severe side effects, natural substances are being extensively explored for their anticancer activity as they have low toxicity. Out of all the natural resources available, the marine environment offers maximum diversity as it covers more than 70% of the earth's surface and represent an enormous resource for the discovery of potential chemotherapeutic agents.

The present study, demonstrates that crude and pure fucoidan from *F. vesiculosus* and *U. pinnatifida* (CCF, CPF, ACF and UPF) have antiproliferative and apoptosis-inducing effect against human prostate cancer cells (PC-3 and DU-145). All the four fucoidans exhibited inhibitory effect against prostate cancer cells in comparison to the control (without treatment) in both time and dose dependent manners but with varying efficiencies and selectivities. The crude forms of fucoidan from either of the species were found to exhibit more antiproliferative effect than pure forms of fucoidan, As CCF (commercial crude fucoidan from F. vesiculosus) was most effective in inhibiting the growth of human prostate cancer cell line, which was followed by ACF (Crude fucoidan from New Zealand U. pinnatifida extracted at AUT).

Once the antiproliferative effect of fucoidans were evaluated, the mechanism of action through which inhibitory effect was tested by flow cytometric analysis of apoptosis. In the present study, CCF, ACF, UPF and CPF increased the % of sub-G1fraction (i.e. degree of apoptosis) in a dose dependent fashion but with different efficacy. During apoptosis assay also the crude forms exhibited more cell death than pure form.

Cytotoxic effect of various fucoidan tested in this study were analysed on normal cells as well. It was demonstrated that CCF was the most toxic and inhibited the growth of normal cells the most, whereas ACF was comparatively less cytotoxic than CCF and exhibited lower apoptosis inducing activity on normal cells.

Even though ACF was less capable of inhibiting the growth of prostate cancer cells in comparison to CCF (the most effective), owing to its lesser toxicity towards normal cells, ACF would be a more apt option, which needs to be further investigated and established employing other bioassays against prostate cancer. Thus this study highlights that fucoidan form New Zealand *U.pinnatifida* might have therapeutic potential for advanced prostate cancer treatment with milder side effect.

5.2 Future Research directions

In this study, results were indicative of the fact that fucoidan exhibited anticancer potential against human prostate cancer cells. Further experiments were carried out to elucidate the mechanism behind this *in vitro* activity. Results revealed that fucoidan mediates its anticancer activity by inducing apoptosis. Many anticancer drugs in chemotherapy and natural products induce tumour cell death via apoptosis or programme cell death mechanism. As documented, apoptosis occurs via two principal pathways; the mitochondria-mediated (intrinsic) and death receptor-mediated (extrinsic) pathways. These pathways activate executive caspases- enzymes that degrade protein.

Fucoidan has been shown to induce these pathways by regulating different caspase activities. Thus to further identify the relevant mechanism responsible for fucoidan-induced apoptosis, it is imperative to determine whether or not fucoidan activates caspases (by studying the up and downregulation of various proteins and its consecutive receptors), via Western blotting using antibodies that detects the cleaved forms of the enzymes.

Wnt/β-catenin signalling pathway, which leads to the prostate cell proliferation and differentiation, amongst others, has been reported to be up-regulated and to play a critical role in the development and invasiveness of prostate cancer (Yokoyama, Shao, Hoang, Mercola, & Zi, 2014). Furthermore, the cell lines used in this study are highly invasive androgen-independent prostate cancer cell lines (PC-3 and DU-145) and according to

previous studies, demonstrate high level of Wnt/β-catenin signalling (Chen et al., 2004). Thus it would be advantageous to study the after effect of fucoidan treatment, on the expression of Wnt/β-catenin signalling-related protein. This would provide a new insight into the treatment option for advanced prostate cancer treatment, and directly relate fucoidan action with metastatic androgen-independent (advanced) prostate cancer.

During the course of the study, ACF was found to be interfering with EMEM medium, which was used for culturing DU-145 cells. Thus no results could be obtained for inhibition effects of ACF against DU-145 cells. In order to elucidate the reasons behind these anomalies, further research should be carried out to better understand this interference effect of ACF.

An emerging approach of combination therapy, in which an established chemotherapeutic drug is combined with a naturally occurring novel agent, has been gaining rapid popularity owing to its promising results of higher efficacy against different cancer cells. It should also be noted here that the rationale behind combination therapy is to combine drugs which have different mechanism of action as it decreases the formation of resistant cancer cells (Xiong, Ma, Lai, & Lavasanifar, 2010). In advanced prostate cancer patients, the options available for treatment are very limited. Thus it would be beneficial to study the combinational effects of chemotherapeutic drug used against prostate cancer (like Docetaxel) with a naturally occurring polysaccharide like fucoidan.

In this study, four different types of fucoidan were evaluated for their antiproliferative effect against prostate cancer cells. Among all, ACF (crude fucoidan from New Zealand *U. pinnatifida*) was found to be selectively active against prostate cancer cells (than the normal cells). Earlier, Undaria species was considered to be an unwanted pest in New Zealand. However, now it has been shown to possess high antioxidant and antiproliferative activity (W Mak, Hamid, Liu, Lu, & White, 2013). Thus in order to establish the chemotherapeutic nature of New Zealand *Undaria* fucoidan it would be important to elucidate and establish its clearer structure, bioavailability mechanism, and pharmacokinetic.

Furthermore, an extensive *in vivo* study of fucoidan on mouse models would be very essential in reinforcing the significant anticancer activities of fucoidans used in this study,

especially the ACF (crude fucoidan from New Zealand *U. pinnatifida* extracted at AUT), considering the fact that it's cytotoxicity was minor on normal cells.

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APPENDIX

A1: Cell cycle distribution of PC-3 cells after treatment with CCF, ACF, UPF and CPF

Treatment(µg/ml)	Sub-G1	G0-G1	S	G2-M
Control	2.80± 1.02	65.74±0.24	14.73±0.92	16.73±0.1.08
CCF 100	10.86±1.43	62.59±0.85	7.09±0.45	19.46±0.63
CCF 200	12.82±0.23	60.78±0.30	6.32±0.14	20.08±0.34
CCF 400	20.37±0.74	56.18±0.55	4.57±0.26	18.88±1.09
CCF 800	28.55±1.34	50.52±0.51	3.52±0.20	17.41±0.35
ACF 100	9.53±0.0.45	72.15±0.61	8.56±0.82	9.76±0.33
ACF 200	10.68±0.93	70.09± 0.21	7.25±0.51	11.98±0.16
ACF 400	12.69±0.28	62.82±0.35	8.92±0.14	15.57±0.98
ACF 800	14.01±0.76	59.74±0.11	9.25±0.26	14.12±0.67
UPF 100	5.32±0.70	50.52± 1.98	18.54±0.32	22.62±0.52
UPF 200	11.44±0.94	48.52±0.93	19.00±0.45	18.04±0.67
UPF 400	12.12±0.61	45.61±1.48	20.49±0.21	21.78±0.32
UPF 800	13.92±0.74	42.25±0.43	19.69±0.67	22.14±0.99
CPF 100	1.35±0.38	53.61±2.89	20.64±0.11	22.42±0.87
CPF200	2.23±1.19	53.95±0.91	21.62±0.56	22.20±0.21
CPF 400	8.17±0.60	47.62±0.32	19.91±0.98	21.30±0.62
CPF 800	10.30±1.12	48.28±0.78	20.65±0.23	19.77±0.67

A2: Cell cycle distribution of DU-145 cells after treatment with CCF, UPF and CPF

Treatment(µg/ml)	Sub-G1	G0-G1	S	G2-M
Control	5.56±0.76	74.63±1.77	11.49±0.37	8.21±0.98
CCF 100	9.37±0.49	73.62±1.09	6.95±0.63	10.06±0.59
CCF 200	13.19±0.63	65.12±0.82	7.02±0.85	14.67±1.25
CCF 400	14.95±1.04	60.19±1.09	9.25±2.09	12.02±0.98
CCF 800	31.08±1.46	50.12±0.40	6.75±0.77	12.05±1.09
UPF 100	5.86±0.23	70.91±1.09	6.21±0.88	17.02±1.86
UPF 200	8.09±0.50	72.65±0.72	7.95±2.88	11.31±0.98
UPF 400	8.95±0.89	71.25±0.15	8.02±1.38	11.78±0.21
UPF 800	11.30±1.35	69.09±1.86	8.95±0.68	10.66±0.14
CPF 100	5.56±0.72	76.83±0.15	7.65±1.82	9.96±0.14
CPF200	6.27±0.77	78.25±0.51	8.21±1.86	7.27±1.11
CPF 400	10.20±0.61	69.08±0.61	8.00±0.36	12.72±0.21
CPF 800	10.64±1.06	68.56±0.31	7.25±1.11	12.95±0.87

A3: Cell cycle distribution of HEK-293 cells after treatment with CCF, ACF, UPF and CPF

Treatment(µg/ml)	Sub-G1	G0-G1	S	G2-M
Control	4.20±0.50	70.26±0.49	14.65±0.99	10.89±0.87
CCF 100	9.40±0.23	65.92±1.44	13.92±0.07	10.76±0.98
CCF 200	10.58±0.75	60.10±0.94	12.56±0.22	16.76±1.11
CCF 400	12.74±1.38	58.25±0.49	12.03±0.22	14.50±0.62
CCF 800	27.40±2.35	43.65±0.18	14.09±0.49	14.86±0.66
ACF 100	6.86±0.99	65.59±0.25	12.75±0.32	14.8±0.93
ACF 200	10.70±0.44	59.65±1.78	13.62±1.11	16.03±0.21
ACF 400	11.02±0.59	53.25±0.53	14.29±0.32	21.44±0.61
ACF 800	11.73±0.72	56.62±1.01	15.81±0.58	15.84±0.62
UPF 100	5.91±0.46	62.32±0.22	15.24±0.07	17.25±0.66
UPF 200	7.89±0.70	60.97±0.44	14.57±0.23	16.57±0.95
UPF 400	11.59±0.57	56.05±0.43	16.51±0.55	15.85±1.09
UPF 800	12.90±1.10	49.75±0.29	18.83±0.87	18.52±0.54
CPF 100	2.26±0.71	67.25±0.93	16.29±1.34	14.2±0.27
CPF200	6.09±0.94	60.92±1.13	15.86±0.66	17.13±0.07
CPF 400	9.12±1.02	58.61±0.01	14.39±0.09	17.88±1.22
CPF 800	11.14±0.85	54.26±0.09	15.65±0.98	18.95±0.32

A4: Dot plot representation for DU-145 cells before and after treatment with ACF

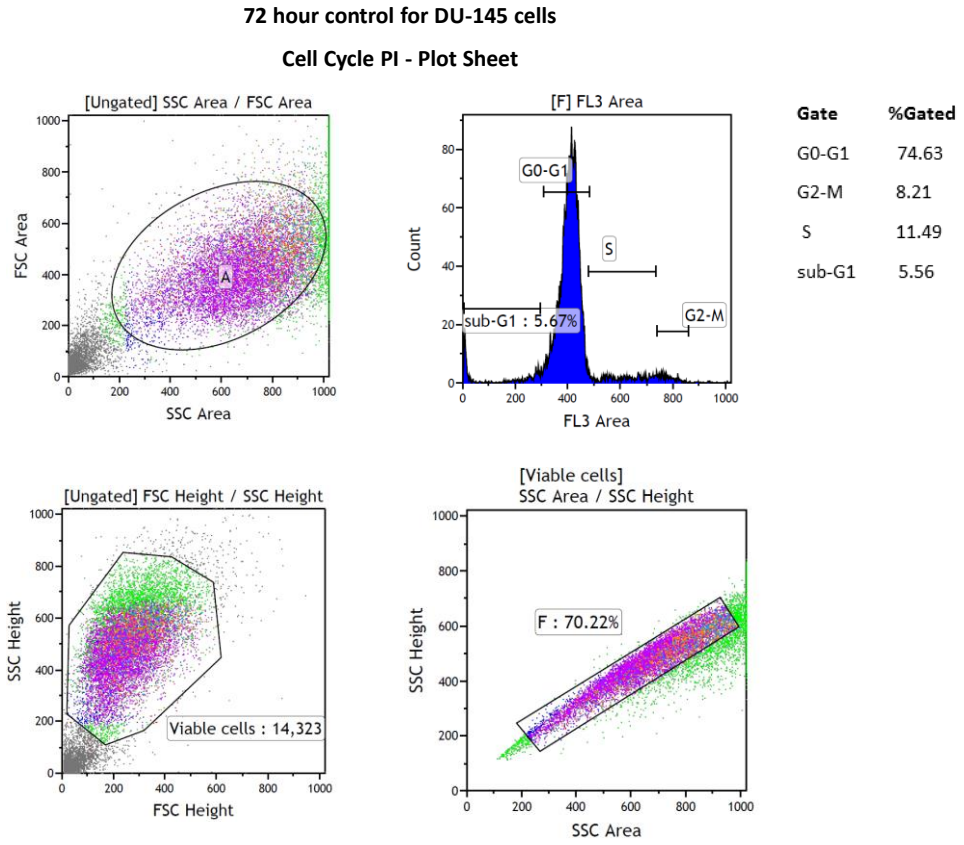

Figure 30: Dot plot representation for control DU-145 cells (cells without any treatment) at 72 hours.

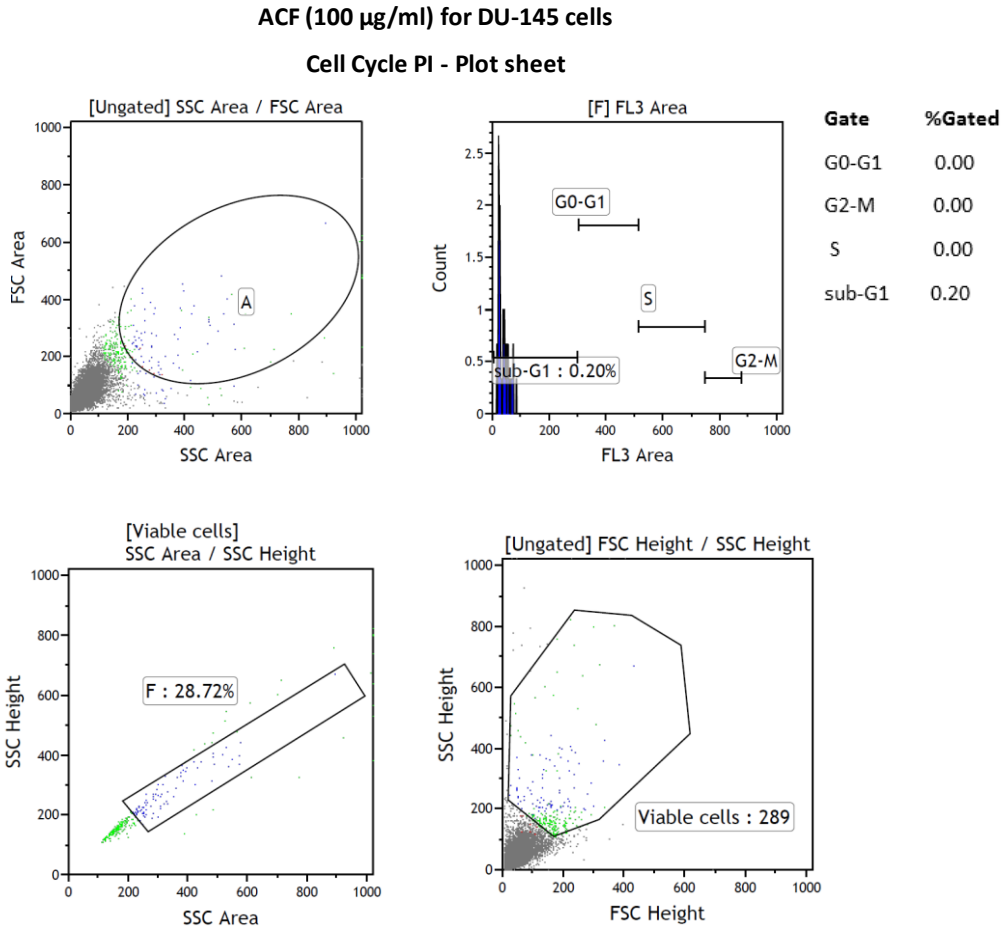

Figure 31: Dot plot representation for DU-145 cells after treatment with ACF (100 µg/ml) at 72 hours.

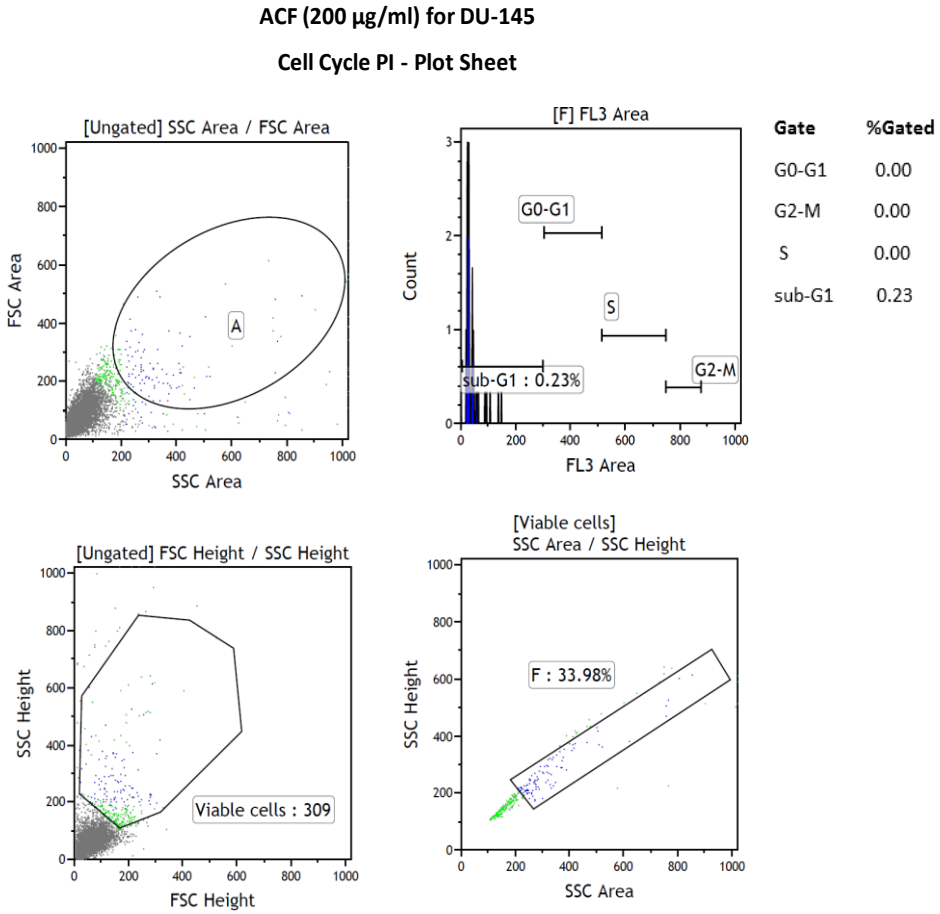

Figure 32: Dot plot representation for DU-145 cells after treatment with ACF (200 µg/ml) at 72 hours.

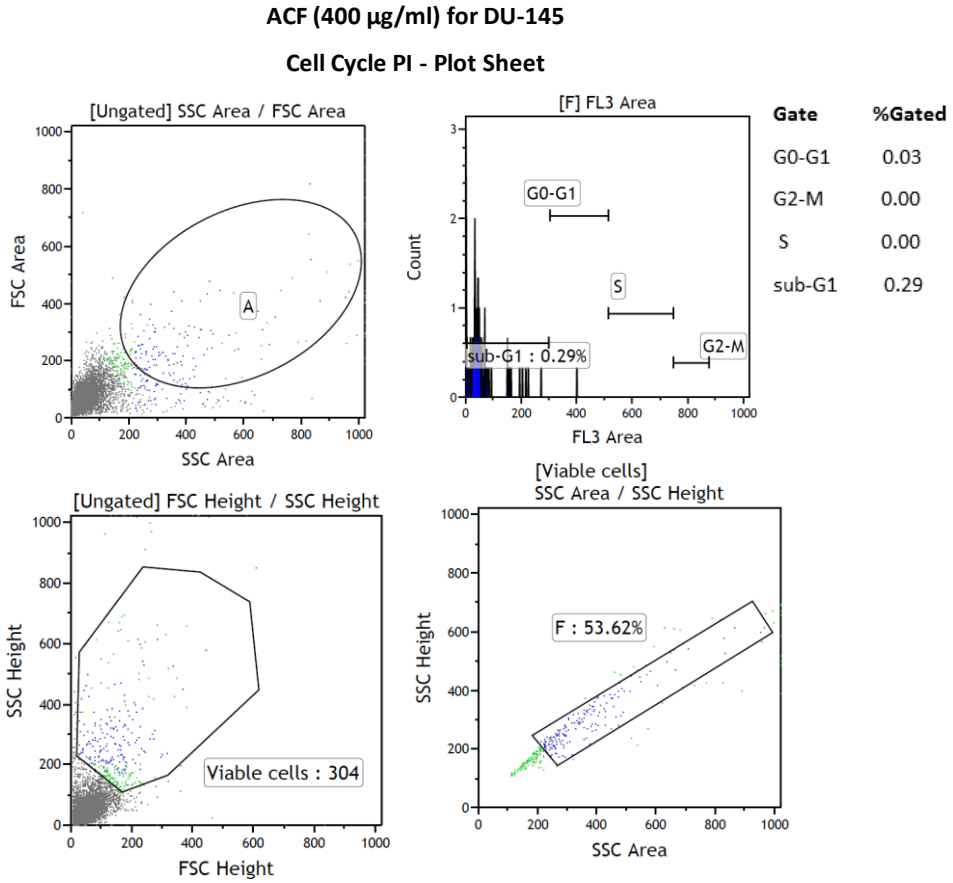

Figure 33: Dot plot representation for DU-145 cells after treatment with ACF (400 µg/ml) at 72 hours.

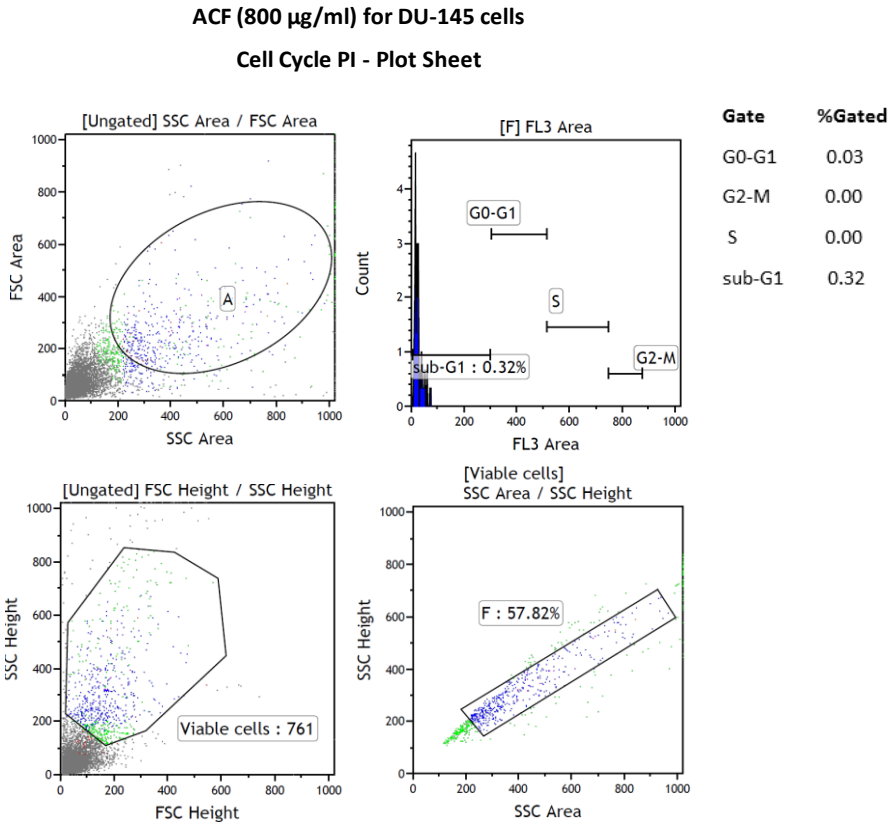

Figure 34: Dot plot representation for DU-145 cells after treatment with ACF (800 µg/ml) at 72 hours.

A 5: Voltage used for running the samples during Flow cytometric analysis

Table 18: The FL3 voltage set for each cell line

	Voltage (volts)	
Cell line	**Starvation**	**42 hours**
PC-3	464	470
Du-145	474	480
HEK-293	485	500