



UNIVERSITY of the
WESTERN CAPE

**EFFECTS OF FUCOIDAN AND CHEMOTHERAPEUTIC AGENT
COMBINATIONS ON MALIGNANT AND NON-MALIGNANT
BREAST CELL LINES**

by

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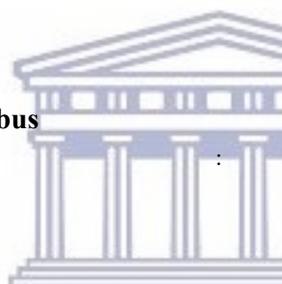
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DECLARATION

I, **Aisha Ibrahim Abudabbus**, declare that “**Effects of Fucoidan and Chemotherapeutic Agent Combinations on Malignant and Non-Malignant Breast Cell Lines**” is my original work and that all the sources that I have used or cited have been indicated and acknowledged by means of complete references, and that this document has not been submitted for degree purposes at any other academic institution.

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A handwritten signature in blue ink, appearing to read 'Aisha Ibrahim Abudabbus'.

Date Signed

UNIVERSITY of the
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DEDICATION

*This dissertation is dedicated to my family, for being the
light, fuel, and greatest source of joy in my life.*

With all my love,

Aisha Abudabbus



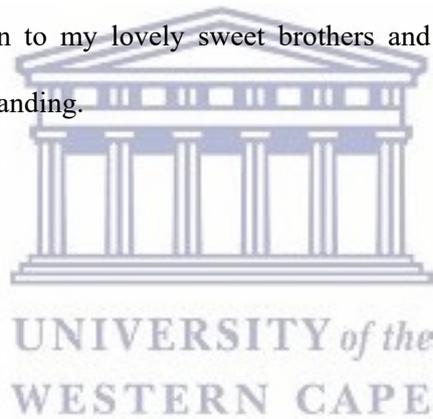
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ABSTRACT

Background

Breast cancer is currently one of the most common malignancies in women. Fucoïdan (FUC) is a natural polysaccharide with anticancer properties. Despite a number of *in vitro* and *in vivo* studies reporting the efficacy of fucoïdan in treating various cancers, few studies have measured the efficacy of fucoïdan in combination with cancer drugs. Drugs like cisplatin, doxorubicin and taxol are important in breast cancer treatment. However, in recent years, supplements have gained importance in its treatment. Fucoïdan, a sulfated polysaccharide mainly found in brown algae and seaweed, is a new candidate for breast cancer therapy because of its antitumour activity. This study was aimed at determining the cytotoxic, apoptotic and cell cycle distribution effects of fucoïdan and its synergistic and/or antagonistic effects in combination with cisplatin, doxorubicin and taxol in the breast cancer cell line, MCF-7, relative to the normal MCF-12A non-malignant breast epithelial cell line.

Methods

The IC₅₀ value of each agent was obtained against MCF-7 and MCF-12A cells using the MTT-cytotoxicity assay. Apoptosis was determined with the Annexin VFITC/PI assay, Active Caspase-3/-7, and -9 and cell cycle assays, followed with Hoechst-33342 staining. MCF-12A non-cancerous epithelial breast cells was used as the control.

Results

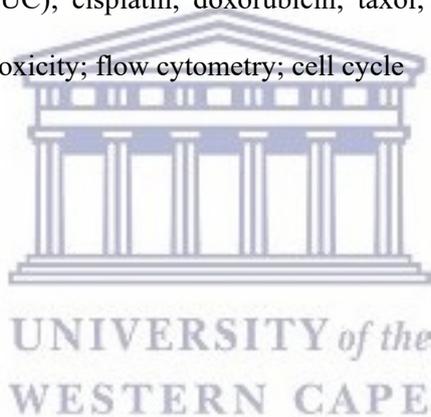
Overall, fucoïdan significantly increased the cytotoxic effect of the chemotherapeutic agents. Consistently, costimulation of MCF-7 cells with any chemotherapeutic agent in the presence of fucoïdan further increased apoptosis induction, caspase-3/-7 and caspase-9 activation, particularly, in cisplatin- and taxol-challenged cells more than fucoïdan-doxorubicin compared to untreated controls. Furthermore, fucoïdan treatment resulted in G1 phase cell cycle arrest of MCF-7 cells and accumulation of the sub-G1 population as revealed by flow cytometry.

Fucoidan-drug combinations strongly induced the accumulation of MCF-7 cells in the G2/M and sub-G1 phase. In contrast, no significant differences for cytotoxicity and apoptosis or cell cycle profile were found between fucoidan treated and untreated MCF-12A cells.

Conclusions

Fucoidan is an effective antitumour agent either alone or in combination with cisplatin, doxorubicin and taxol in MCF-7 breast cancer cells. These findings suggest that fucoidan is a candidate natural product for breast cancer combination therapies. Further studies are required to evaluate cancer-specific and fraction-specific mechanisms of fucoidan for translation into *in vivo* tumour models.

Keywords: fucoidan (FUC); cisplatin; doxorubicin; taxol; MCF-7 cells; MCF-12A cells; apoptosis; caspase; cytotoxicity; flow cytometry; cell cycle



LIST OF ABBREVIATIONS

AIF	Apoptosis inducing factor
AIs	Aromatase inhibitors
AJCC	American Joint Committee on Cancer
Annexin-V-FITC/PI	Annexin-V-fluorescein isothiocyanate/propidium iodide
ANOVA	Analysis of variance
APAF-1	Apoptosis protease activating factor 1
APAF-1	Apoptotic protease activating factor-1
ATM	Ataxia-telangiectasia-mutated
Bax	Bcl-2 associated x-protein
Bcl-2	B-cell lymphoma-2
Bcl-2	B-cell lymphoma 2
BMI	Body mass index
CARD	Caspase recruitment domain
Caspase	Cysteine aspartic acid-specific proteases
CDDP	Cisplatin or Cis-dichlorodiammineplatinum (II) complex
CDK	Cyclin-dependent kinase
CDKi	Cyclin-dependent kinase inhibitor
c-IAP1	Cellular IAP1
CMF	Cyclophosphamide, methotrexate, 5-fluorouracil
DED	Death effector domain
DFS	Disease-free survival
DIABLO	Direct IAP binding protein with low PI
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulphoxide
DOX	Doxorubicin
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
EGFR	Epidermal growth factor receptor
EGFR	Kinases, epidermal growth factor receptor
ER	Oestrogen receptor
ERK	Extracellular-regulated kinase

ERα	Oestrogen receptor alpha
ERβ	Oestrogen receptor beta
FACS	Florescence activated cell sorter
FADD	Fas-associated death domain
FBS	Foetal bovine serum
FUC	Fucoidan
IAPs	Inhibitor of apoptosis proteins(s)
IARC	International Agency for Research on Cancer
IBC	Inflammatory breast cancer
IBM	IAP-binding motif
MAP	Mitogenic activated protein
MAP	Mitogenic activated protein
MCF-7	Human breast adenocarcinoma cell line
NSCLCs	Non-small-cell lung carcinomas
OS	Overall survival
p53	Phosphoprotein 53 (tumour protein/tp53)
PARP-1	Poly (ADP) ribose polymerase-1
PBS	Phosphate buffered saline
PCD	Programmed cell death
PI3K	Phosphatidylinositol-3-kinase
PR	Progesterone receptor
Rb	Retinoblastoma
RFS	Recurrence-free survival
SERM	Selective oestrogen receptor modulators
Smac	Second mitochondria-derived activator of caspase
TAX	Paclitaxel or taxol
TRADD	TNF receptor-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
TSGs	Tumour suppressor genes
VEGF	Vascular endothelial growth factor
WHO	World Health Organization ()
XIAP	X-linked inhibitor of apoptosis protein

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CHAPTER 1

BACKGROUND TO THE STUDY

1.1 Introduction

Breast cancer is a serious global health problem, being the second most common of all cancers and by far the most frequent cancer among women [1]. In 2012, breast cancer was estimated at 12% of all cancers diagnosed, i.e., affecting 1.7 million people [2,3]. The goal of cancer treatment is eradication of tumour cells, ideally with minimal damage to healthy tissue. Because of the side effects of many current treatments, the use of natural substances of low toxicity is of great cancer therapeutic interest.

Life began in the sea and oceans with a particularly rich biodiversity, covering over 70% of the earth's surface. The marine environment contains several numbers of plants, animals and microorganisms which produce a wide diversity of natural products [4]. A polysaccharide known as fucoidan is extracted from marine brown algae and is known to consist largely of L-fucose and sulphate [5-7].

The structure of fucoidan is highly complex and may differ substantially in composition and chemical structure between species, depending also on regional and seasonal influences, and even on the method of extraction [8-10]. They were identified in the first half of the last century [11,12]. Fucoidan has been reported to possess antioxidant, antiviral, antibacterial, anti-inflammatory and anticoagulant activities which have attracted global interest [5,6,13]. Accumulating evidence support the notion that the use of fucoidan as a supplement provides protection against various cancers. Several studies have reported that fucoidan has antiangiogenic and antiproliferative activity in cancer cells *in vitro*, as well as inhibitory activity of tumour growth in mice [14,15].

Fucoidan has been shown to display antimetastatic activity by blocking the interactions between cancer cells and the basement membrane [16]. Fucoidan inhibits tumour cell proliferation and tumour cell adhesion to various substrates, but its exact mechanisms of action are not yet completely understood. A number of *in vitro* and *in vivo* studies have indicated that fucoidan contains strong anticancer bioactivity [17]. Since fucoidan also possesses immunomodulatory effects [18,19], it is postulated that it may have protective effects against the development of side effects when co-administered with chemotherapeutic agents and radiation [20,21].

1.2 Problem Statement

Cancer is a global public health problem and the burden of this disease continues to increase despite enormous efforts to lessen the impact on the individual, family and society [22]. According to GLOBOCAN, roughly 12.7 million cancer cases and 7.6 million cancer deaths have occurred in 2008; of these, 56% of the cases and 64% of the deaths occurred in the economically developing world. Breast cancer has the highest incidence and continues to be the main cause of cancer deaths among females, contributing to 23% of the total cancer cases and 14% of the cancer deaths [3,23]. Breast cancer incidence and mortality estimates reported by GLOBOCAN in 2012 (globocan.iarc.fr) and data from 39 countries from the International Agency for Research on Cancer (IARC) and mortality data from 57 countries from the World Health Organization (WHO), indicate that breast cancer mortality rates are falling in most high-income countries, in the face of increasing or stable incidence rates.

However, of major concern are the increasing incidence and mortality rates in developing countries coupled with variations in breast cancer rates and trends that indicate disparities in patterns of risk factors and access to and availability of early diagnosis and treatment public health services [24]. The development of resistance to chemotherapy is considered a major hindrance to treatment of various cancers. Globally, decreased disease-free or overall survival (DFS/OS) is due mainly to recurrence and metastases of breast cancers in patients who do not

respond to targeted therapies from the start (*de novo drug resistance*) and even those who do respond to targeted therapies such as tamoxifen and trastuzumab and show signs of remission initially, only to turn refractory (relapse) to these treatments with time (*acquired drug resistance*) [25-32]. Moreover, most chemotherapeutic drugs presently being used exhibit adverse side effects on the human body, namely, bleeding, hair loss, diarrhoea, and immunosuppression [33-35] which have negative impacts on the lives of affected patients [36-42].

An improved understanding of the long-term side effects of adjuvant chemotherapy and targeted therapy is therefore crucial to integrate our growing knowledge of breast cancer biology with standard high-quality histopathologic measures to better identify patients most likely to benefit from the various options for combined multimodality therapy [43-48]. In recent years, an ever-growing literature suggests that nature is a rich source of multi-targeting phytochemicals (nutraceuticals) that have the potential to overcome drug resistance in cancer cells [49-53]. Nonetheless, many of these phytochemicals like curcumin or resveratrol have not fared as expected in translational studies due to their limited water solubility, consequent poor bioavailability and metabolic instability [52].

Hence, there is a direct need to explore new natural products and metabolites isolated from microorganisms, animals and plants possessing high selectivity and efficacy alone and in combination with conventional chemotherapeutics against drug-resistant tumour cells without toxicity to normal cells [54-58]. Apoptosis as a highly regulated programmed cell death has become a matter of great interest in cancer therapy and oncology because of the high potential of various anticancer agents in inducing apoptosis in a variety of cancer cells [59,60]. Thus, screening for natural products capable of inducing apoptosis in cancer cells that can be used collaterally with other cytotoxic drugs is of interest in order to elevate the therapeutic efficacy and reduce the side effects in cancer therapy. In this study, fucoidan (FUC), a natural polysaccharide composed of sulphated L-fucose residues extracted from marine brown algae

(*Phaeophycophyta*) and some echinoderms (sea urchin and sea cucumber) [7,10,13,17,20,21,61,62] was tested for its potential anticancer effects alone or in combination with cisplatin, doxorubicin and taxol against non-malignant MCF-12A breast epithelial cells and MCF-7 cancer cells.

1.3 Research Hypothesis

Combinations of fucoidan (FUC) with conventional antineoplastic agents such as cisplatin (CDDP), doxorubicin (DOX) and taxol (TAX) act synergistically to yield enhanced anticancer effects than those predicted by their individual activities against MCF-7 breast cancer cells and MCF-12A normal breast epithelial cells *in vitro*.

1.4 Aim of the Study

The aim of the study was to investigate the combinatorial effects of FUC, CDDP, DOX and TAX on the survival (cell proliferation, cell cycle transition and apoptosis) of MCF-7 breast cancer cells and MCF-12A normal breast epithelial cells *in vitro*.

1.5 Objectives of the Study

The objectives of this study are summarized below:

- ❧ Determine the effects of fucoidan in combination with chemotherapeutic drugs (cisplatin, doxorubicin and taxol) on the human breast carcinoma cell line MCF-7 and MCF-12A normal breast epithelial cell line.
- ❧ Examine the cytotoxicity of each compound against MCF-7 and MCF-12A cells.
- ❧ Evaluate the morphology of MCF-7 and MCF-12A cells following the fucoidan-drug combination treatments.
- ❧ Determine if the cytotoxic effects of fucoidan-drug treatments occur via apoptotic or

necrotic cell death.

- ❧ Confirm the involvement of caspases in fucoidan-induced apoptosis.
- ❧ Study the effects of fucoidan-drug treatments on the cell cycle profile of MCF-7 and MCF-12A cells.



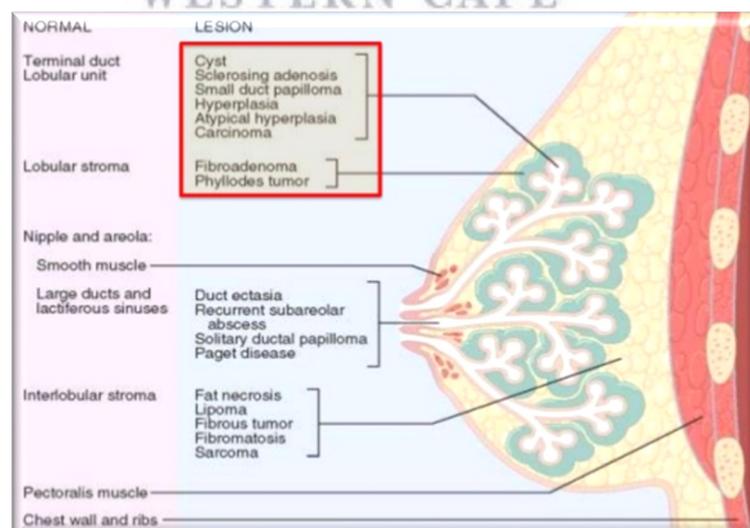
CHAPTER 2

LITERATURE REVIEW

2.1 The Normal and Cancerous Mammary Gland

Breasts present as two skin appendages with their underneath structures and glands [63]. They constitute human mammary glands which are located in front of the anterior chest wall. In women, the normal breast is composed of epithelial and mesenchymal elements. The epithelial element is made up of lobules and ducts (luminal cells) that form and deliver milk [64] and the myoepithelial cells (basal cells).

Ducts and lobular glands are surrounded by fibrovascular mesenchymal tissues formed from fibroblasts, adipocytes and blood vessels (Figure 2.1). Mammary glands undergo crucial changes in pregnancy and lactation. The breast is affected by many types of neoplastic and non-neoplastic lesions [65-67] as shown in Figure 2.1.



Source:[68] Lester SC. The breast. In: Kumar V, Abbas AK, Fausto N, Aster JC, eds. *Robbins and Cotran Pathologic Basis of Disease*. Philadelphia: Elsevier Health Sciences; 2014.

Figure 2.1: Normal structure of breast and its abnormalities

Cancer is a genetic and multifactorial disease. It evolves when single cells develop multiple mutations driving cells from a normal to a malignant state [69,70]. Each cancer is different from other types in its own genomic and proteomic constitutions. However, for cancer to evolve it should acquire the so called *Must Hallmarks*, including: self-efficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless potential for replication, genomic instability, sustained angiogenesis, tissue invasion and metastasis [71-73]. Other hallmarks that contribute to tumorigenesis and cancer progression include avoiding immune destruction, enabling replicative immortality, tumour-promoting inflammation, genome instability and mutation and deregulating cellular energetics [72]. Likewise, breast cancers have characteristically different patterns of gene expression [74,75].

2.2 Epidemiology of Breast Cancer

Breast cancer is a significant cause of morbidity and mortality in women. It represents a heavy health burden with a high incidence of recurrence and treatment failure. In women, breast cancer is the most common cancer and the second most common type of cancer associated with death after lung cancer [76-80]. Despite the fact that breast cancer is sporadic, there is a small proportion occurring in a hereditary or familial form [65,81,82]. In many developed countries like Canada and USA, 1 out of 8 women above 45 years of age develop a form of breast cancer in their life [83]. Breast cancer is more common in Western and well-developed countries, such as northern European countries, USA, Canada, Australia and New Zealand [24,84,85].

However, it is less common in the Middle East, Africa and even much less common in some Asian countries such as Japan [77,86,87]. According to GLOBOCAN 2012, Southern Africa had a breast cancer incidence and mortality rate of 39 and 16 per 100,000, respectively [84,88]. The incidence of breast cancer is 50% higher in urban compared to rural provinces. In the US, it accounts for approximately 1700 new cases of breast cancer every year. The rate of breast cancer in immigrant women tends to become similar to the rate in native-born women from

the same area, indicating contribution of environmental factors [89,90]. In 2008, GLOBOCAN reported an estimated 12.7 million cancer cases and 7.6 million cancer deaths worldwide [22,23,91]. More recent figures approximated 14 million new cases and 8.2 million deaths in 2012, which demonstrates an increase of roughly 1 million and 600,000, respectively [88]. Worldwide statistics covering the period 2008-2012 demonstrated a consistent trend in higher incidence in breast cancer rates in developed countries compared to developing countries (692,200 and 794,000 versus 691,300 and 883,000, respectively) but also interestingly a higher mortality rate in developing countries compared to those in developed countries (268,900 and 324,000 versus 189,500 and 198,000, respectively [23,88]. The reason for the high variation in incidence rates could be attributed to hormonal and reproductive factors. This involves the use of oral contraceptives and postmenopausal hormone therapy [92,93].

Higher death rates in developing countries are attributed to lack of access to early screening methods such as mammography which are costly and lack of education and awareness of breast cancer [23,88,94]. Genetic risk factors, race and ethnicity account for about 5-10% of all breast cancer occurrences. According to the American Cancer Society (cancer.org), black females showed a consistent increase (0.3% per year) in breast cancer incidence over a 10 year period (2002-2013) compared to white American females [94]. The mortality rate of breast cancer in black females were 42% higher than those of white females during 2008-2012 period. The overall 5 year survival rate in black females were 80% in comparison to 91% in white females for breast cancer diagnosed between 2005 and 2011 [94].

2.3 Risk Factors for Breast Cancer

Scientists have discovered many risk factors for the development of breast cancer, including lifestyle and reproductive factors [95,96], early menarche and late menopause, having the first child after age 35, fewer children or nulliparity [97,98] and high stress levels [99,100]. Increased consumption of animal fat, lack of exercise, high body mass index (BMI) and obesity [101], alcohol intake [102] and cigarette smoking are also common risk factors [103].

Other widespread factors include exogenous oestrogen intake [102] after menopause like hormone replacement therapy or oral contraceptive pills [104-109]. Another risk factor is exposure to nuclear radiation especially for younger women. A good example of this is what happened in Hiroshima and Nagasaki in 1945 [110]. Genetic factors also play a vital role in breast cancer genesis [75,111,112]. Mutations in certain genes like oncogenes or tumour suppressor genes and mutations in the tumour suppressor genes BRCA1 and BRCA2 [95,113-115] increase the risk of early or bilateral breast cancer [116-119]. In fact, 5-10% of new breast cancer cases are attributed to these mutations [87,120,121].

Recently, it has been reported that regular antibiotic intake [122-126] may be another risk factor due to suppression of the immune system and increased expression of prostaglandins [127]. However, it has been asserted that there is little evidence that would implicate the use of antibiotics, antidepressants, statins, and antihypertensives in the aetiology of breast cancer [128]. Recently, both aspirin and acetaminophen use were inversely associated with breast cancer incidence [129], but that oral contraceptive use and regular alcohol consumption may be associated with inflammatory breast cancer (IBC) [102]. On the other hand, factors that decrease the incidence of breast cancer are first pregnancy before the age of 20 with a relative risk of 0.5 compared to 1 in nulliparous women [97,98,130,131]. Other protective factors of breast cancer are normal BMI, regular exercise, early menopause, multiple pregnancies, and breast feeding and living in Asia [101,132,133].

2.4 Classification and Staging of Breast Cancer

The taxonomy used for staging breast cancer is based on the following criteria:

- ❧ Tumour size (T)
- ❧ Lymph node spread (N) and
- ❧ Presence or absence of distant metastasis (M)

This taxonomy is known as the TNM classification as shown in Tables 2.1 and 2.2 [134,135].

Table 2.1: Taxonomy used for staging breast cancer

<i>The classification of tumor size (T), lymph node spread (N) and presence or absence of distant metastasis (M) system of breast cancer according to American Joint Committee on Cancer (AJCC).</i>	
Tumor size	
TX	Primary tumour cannot be assessed
T0 N0	Evidence of primary tumour (clinically occult)
Tis Carcinoma <i>in situ</i>	Includes both ductal and lobular carcinoma <i>in situ</i> and Paget's disease of the nipple without invasion and without tumor
T1	Tumour less than 2.0 cm in greatest dimension
T1mic	Microinvasion present, 0.1 cm or less in greatest dimension
T1a	Tumour more than 0.1 cm but not more than 0.5 cm in greatest dimension
T1b	Tumour more than 0.5 cm but not more than 1.0 cm in greatest dimension
T1c	Tumour more than 1.0 cm but not more than 2.0 cm in greatest dimension
T2	Tumour more than 2 cm but not more than 5 cm in greatest dimension
T3	Tumour more than 5 cm in greatest dimension
T4	Tumour of any size with direct extension to chest wall or skin as described below
T4a	Extension to chest wall
T4b	Oedema or ulceration of the skin of the breast or the presence of satellite nodules confined to the same breast
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma
Regional lymph nodes (N)	
NX	Regional nodes cannot be assessed (or previously removed)
N0	No regional lymph node metastasis
N2	Metastasis to ipsilateral axillary nodes fixed to one another or to other
N3	Metastasis to ipsilateral internal mammary lymph nodes
Distant metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No evidence of distant metastasis
M1	Distant metastasis (includes metastasis to ipsilateral supraclavicular lymph nodes)
Sources: [134,135]	

Table 2.2: TNM staging in breast cancer

TNM clinical stage classification			
Stage I	T1	N0	M0
	T0	N1	M0
Stage IIA	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
Stage IIIB	T3	N2	M0
	T4	N0	M0
	T4	N1	M0
Stage IIIC	T4	N2	M0
	Any T	N3	M0
Stage IV	Any T	Any N	M1

2.5 Treatment of Breast Cancer

Despite the increase in incidence of breast cancer over the last decades, the cure rate has also increased and key points for curing of breast cancer is early detection and better understanding of the molecular mechanisms behind breast cancer progression. The main goal of treatment is to block growth of the primary tumour and prevent loco-regional and distant metastasis to the liver, lungs, bone and brain.

2.5.1 Surgery

The main and standard current treatment of breast cancer is surgery in the form of lumpectomy/quadrantectomy or modified sub-partial mastectomy, including axillary lymph node clearance; this is usually referred to breast conserving surgery [136,137]. Sentinel lymph node biopsy is a useful diagnostic procedure with or without any clinical evidence of early tumour spread to the axilla [138]; sentinel node is the first node in breast lymphatic drainage [138,139]. After surgery, patients receive chemo (adjuvant) and radiotherapy [140]. Sometimes it is necessary to shrink the tumour size and decrease blood supply to the tumour before surgery and then neo-adjuvant chemo/radiotherapy is recommended prior to surgery [141]. This usually improves overall survival (OS) and recurrence-free survival (RFS) [142].

2.5.2 Chemotherapy

Chemotherapy is used as neoadjuvant and adjuvant treatment of breast cancer before and/or after surgery to prevent relapse and to decrease the likelihood of recurrence of micro-metastasis [143,144]. Usually more than one drug is given (combination chemotherapy) to overcome single drug toxicity [145-151]. The likelihood of success of chemotherapy depends on many factors such as tumour grade, clinical stage, age of patient, lymph node spread, oestrogen receptor (ER) status [152] and many others. These drugs given at a high dose make them toxic to other normal cells.

Drugs recommended to breast cancer patients after evaluations in clinical trials and act in different ways, for example, by inducing DNA damage [153] or by targeting signalling pathways like epidermal growth factor receptor (EGFR) [29,154-156]. Breast cancer cells in the S phase of the cell cycle respond more to chemotherapy than other tumour cells with low S phase. In one study, S-phase fractions of the primary tumour was tested in premenopausal breast cancer patients who participated in a randomized trial [157]. These studies concluded that the benefit from adjuvant chemotherapy compared with radiotherapy is largely confined to patients with highly proliferative tumours.

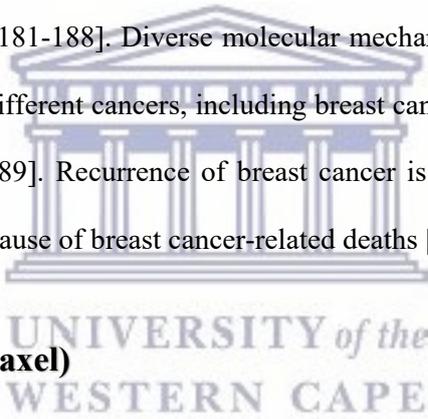
2.5.2.1 Cisplatin

The anticancer activity of cisplatin was discovered in the mid-1960s [158-160]. Cisplatin and its analogue carboplatin, [*cis*-diammine-1,1-cyclobutanedicarboxylate platinum (II)] are among the most commonly used antitumour drugs in current oncology settings [161]. Other platinum compounds include carboplatin, iproplatin, tetraplatin (ormaplatin) and oxaliplatin [160,162], used alone and in combination with other drugs to treat various types of cancer, including breast cancer [144,163-167]. It is generally accepted that the binding of cisplatin to genomic DNA (gDNA) in the cell nucleus is largely responsible for its antitumour properties [162], and the damage so induced can interfere with normal transcription and/or DNA replication mechanisms of cells [168]. Eventually, this disruption in DNA processing triggers cytotoxic processes that lead to the death of the cancer cell. Failure to repair cisplatin-induced DNA damage may finally result in the triggering of apoptosis or programmed cell death or cell suicide [159]. Despite being one of the most effective broad-spectrum anticancer drugs, various resistance mechanisms to cisplatin have been described, including decreased accumulation of the platinum compound into cells, enhanced repair of the platinum- DNA damage, increased efflux of the agent and raised cellular glutathione levels [160,169,170]. Such resistance mechanisms present a major obstacle to effective breast cancer therapy [162].

2.5.2.2 Doxorubicin

Doxorubicin (DOX) is an anthracycline antibiotic. Its intermediate precursor, daunorubicin, was first isolated from *Streptomyces peucetius* in 1969 [171,172]. DOX was then formed by a C-14 hydroxylation of daunorubicin. DOX possesses powerful antitumour activity against a range of cancer cell lines [173]. DOX is therefore used as an anticancer drug because of its ability to inhibit growth and proliferation of tumours and to induce apoptosis [174]. However, the clinical use of DOX is restricted due to a dose-related cardiotoxicity and cancer cell resistance to treatment [175,176]. Studies are underway to alleviate the cardiotoxicity without interference with DOX's killing efficacy [177]. The ability of tumour cells to evade

mechanisms leading to cell death is one of the hallmarks of cancer [71-73,178]. The success of DOX as an antitumour drug is thought to be multifactorial. One of the mechanisms employed by DOX is intercalation into the DNA of cells and another involving the inhibition of enzymes essential in DNA replication. This ultimately leads to apoptosis and cell cycle arrest [179]. DOX becomes cytotoxic to human cells after metabolic conversion to doxorubicinol, resulting from cytoplasmic NADPH-dependent aldose, aldehyde, and carbonyl reductases, through reduction of a carbonyl group. Doxorubicinol interacts with iron and produces ROS, which affects biomacromolecules [175]. This may induce free-radical injury to DNA and intracellular accumulation of ROS. Excess ROS can result in the activation of the mitochondrial death pathway resulting in apoptosis [180]. Several clinical trials on metastatic and triple negative breast cancer have been conducted using DOX in combination with other chemotherapeutic drugs [148-150,156,163,181-188]. Diverse molecular mechanisms promote the development of DOX resistance and different cancers, including breast cancer, can express a wide array of drug-resistance genes [189]. Recurrence of breast cancer is a key clinical impediment and represents the principal cause of breast cancer-related deaths [25,190,191].



2.5.2.3 Taxol (Paclitaxel)

Taxol (paclitaxel) is a widely used chemotherapy drug in the treatment of breast cancer [184,192]. It was first discovered in 1967 [193], entered into clinical trials in 1984 [194,195] and has been a leading chemotherapeutic agent ever since [196,197]). The mechanism of action of taxol involves its interference with microtubule assembly [198-200]. Taxol prevents the disassembly of microtubules during mitosis [201]. When taxol binds to tubulin, the microtubules become locked in the polymerized state, and thus cells are restricted from G2 to M phase transition [202,203]. The end result is that the cells are not able to replicate [204]. Another effect of taxol is that it inhibits the anti-apoptosis protein Bcl-2, and induces apoptosis in breast cancer cells [205]. Recently, pathologic complete response rates in triple-negative, HER2-positive, and hormone receptor-positive breast cancers following anthracycline-free neoadjuvant chemotherapy with carboplatin and paclitaxel in the presence or absence of

trastuzumab have been documented [144]. Various other clinical trials describe the efficacy of taxol in combination regimens [151,154,186,206-208]. However, taxol, like most other chemotherapy drugs, has a high level of toxicity as well as a multitude of side effects. The consequence of the toxicity of taxol at a higher dosage is neuropathy which limits its use in patients. Furthermore, cancer cells develop resistance to taxol after prolonged exposure [209,210].

2.5.3 Hormonal Therapy

Cancers of the ovaries and breasts have hormone receptors in the cells (e.g. oestrogen receptor, ER-positive or progesterone receptor, PR-positive) are called hormone receptor-positive [211,212]. In these cancers, the female hormone oestrogen promotes the growth of the cancer. Hormone therapy for breast cancer works by blocking the effects of oestrogen or lowering oestrogen levels. In hormonal therapy, ER-positive tumours are targeted by selective oestrogen receptor modulators (SERMs) like tamoxifen and raloxifen [152,213]. They work as oestrogen agonists and antagonists and they are given for up to 5 years, reducing recurrence and mortality by more than 60% in the first 10-15 years (Early Breast Cancer Trialists' Collaborative Group /EBCTCG) [191]. They also reduce incidence of contralateral breast cancer. Aromatase inhibitors (AIs) inhibit peripheral conversion of androgens to oestrogen and thereby reduce the primary source of oestrogen in postmenopausal women [214]. This family of drugs also includes non-steroidal inhibitors like anastrozole and letrozole [152,215-220], steroidal inhibitors like exemestane and formestane [152,154]. Some results showed that a switch to AIs after two to three years of tamoxifen treatment might be more effective for the remaining 5 years and improve disease-free survival (DFS) [221,222].

2.5.4 Radiotherapy

Radiotherapy in breast cancer aims to decrease loco-regional spread of the disease [157,223-228]. The therapy can be external beam radiation and internal beam radiation (also called brachytherapy) [229]. External radiotherapy is more commonly used in breast cancer.

Radiotherapy can be neoadjuvant to shrink tumour size or decrease vascularization and can be given after surgery along with adjuvant therapy like cyclophosphamide, methotrexate, 5-fluorouracil (CMF) [230-232].

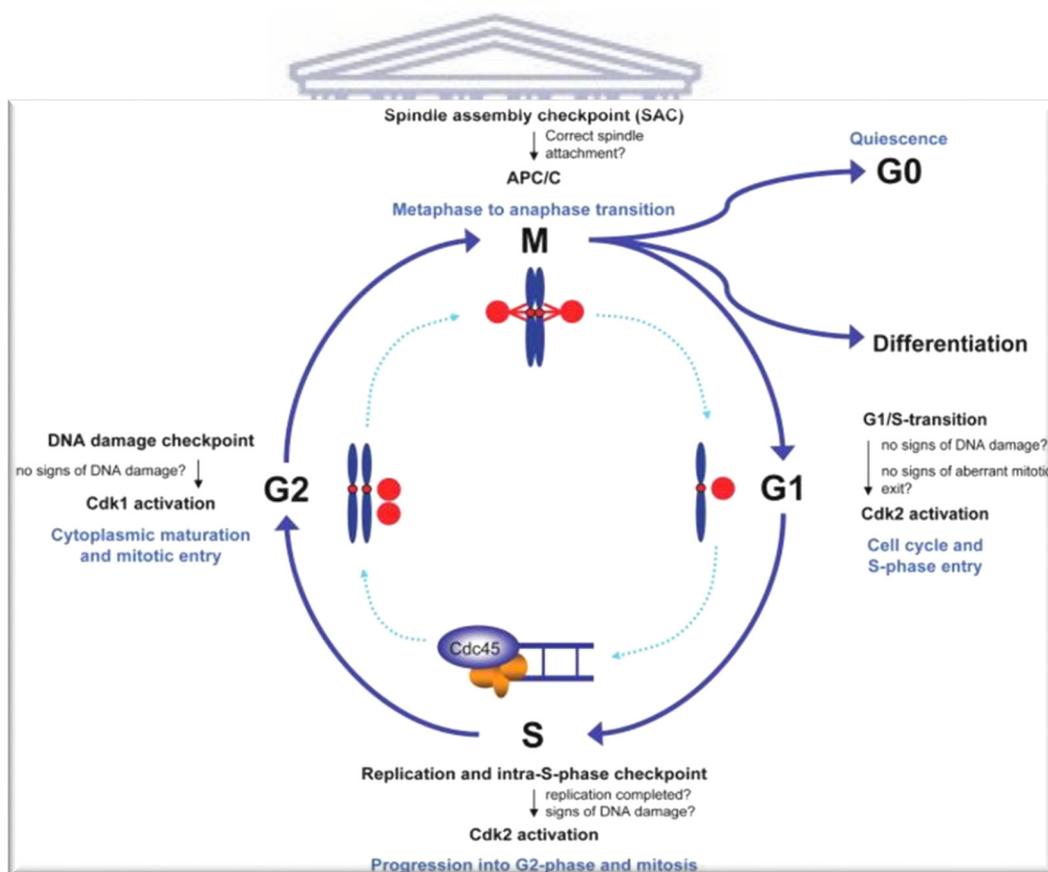
2.5.5 Immunotherapy

According to the expression pattern of oestrogen receptor alpha ($ER\alpha$), oestrogen receptor beta ($ER\beta$) and HER2, breast cancer has been reclassified using these molecular criteria into normal like, luminal A, luminal B, ERBB2 and basal [233-237]. HER2/neu is a tyrosine kinase growth factor receptor expressed in 25-30% of primary breast cancers [156,238-240]. Another upcoming therapeutic target is vascular endothelial growth factor (VEGF), which stimulates new blood vessel formation (angiogenesis) in breast cancer [154,241]. New antibody therapeutic modalities are to target HER2/neu and VEGF using monoclonal antibodies (trastuzumab and bevacizumab) [144,154,156,183,206,242-244]. Microarray studies of gene expression patterns in breast cancer have recognized cancer fingerprints that can distinguish invasive gene signatures, and they are very useful predictive and prognostic tools [245,246]. Palliative chemotherapy is given to terminal patients in stage IIIB or stage IV with disseminated disease and distant metastases. It improves patient survival up to 12-24 months in 10-70% of terminal patients [247].

2.6 The Cell Cycle

The process of faithfully replicating the genetic information of a cell and its subsequent division into two daughter cells is known as the cell cycle [26,71,72,248]. The cell cycle can be envisioned as alternating cycles of interphase where the cell grows in size, synthesizes RNA and proteins, and replicates its DNA, and mitosis (M) where the newly replicated cell divides. At times, cells may exit the cell cycle after M and remain quiescent in a resting phase (G₀). Once the cell receives a mitogenic stimulus, cells re-enter the cell cycle at gap phase 1 (G₁), a growth phase prior to replication, followed by entry into synthesis (S), where the DNA of the cell is replicated, succeeded by the gap phase 2 (G₂), a growth phase preceding cell division

in M (Figure 2.2) [249]. Progression through the four stages of the cell cycle is ordered and tightly regulated to ensure that only one round of DNA replication occurs per cell cycle and that cell division only occurs after replication is complete. To combat stressors that cells may encounter as they progress through the cell cycle, such as misaligned chromosomes or damaged DNA, cells elicit checkpoints that halt cell cycle progression and allow the cell to try correct the problem [73]. However, if the damage is too severe or if repair is untimely, cells will either undergo apoptosis, which is a programmed cell death, or become senescent, which is a state of permanent arrest. Cell cycle arrest and elimination of damaged cells by apoptosis are extremely important processes to an organism in preventing the transmission of damaged or incompletely replicated DNA to new daughter cells and thereby acts as a preventative measure against diseases like breast cancer [250,251].



The human cell cycle can be divided into four phases: G1-phase, S-phase, G2-phase and M-phase (mitosis). Cells must proceed through the cell cycle in a unidirectional manner and cell cycle progression is restricted to cells that have fulfilled specific requirements to enter the next phase of the cell cycle. Whether requirements for cell cycle progression are met is supervised by checkpoints which hold back cells at cell cycle transitions [249].

Figure 2.2: The cell cycle and its checkpoints

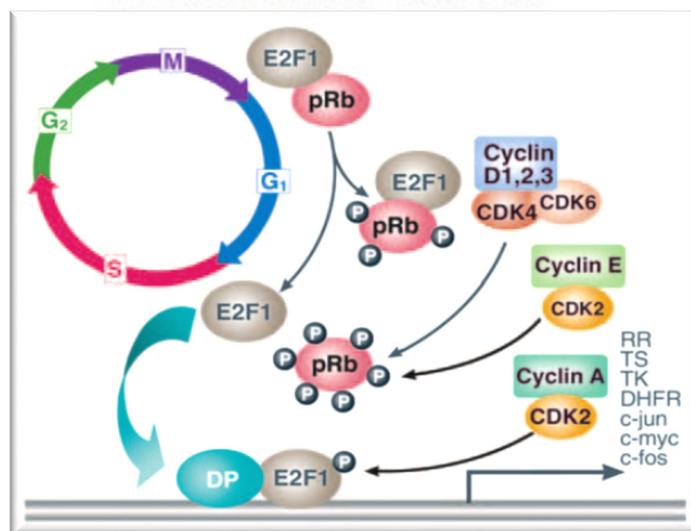
2.6.1 Cell Cycle Regulation

Normal cells need to decide when to divide (i.e., enter the cell cycle) and when to stay in G₀ [252]. This is a tightly regulated and carefully balanced process. The entry into the cell cycle (G₁) is generally governed by the restriction point or transition point beyond which cell progression through the cell cycle is independent of external stimuli such as exposure to nutrients or mitogen activation. This point of determination is thought to divide the early and late G₁ phase of the cell cycle. Progression of a cell through the cell cycle is promoted by a number of cyclin dependent kinases (CDKs). CDKs are a family of serine-threonine protein kinases that control progression of the cell cycle by phosphorylating target proteins at specific times [253-255]. CDK protein levels remain constant during the cell cycle, so their activity is regulated by expression of the cyclin family of regulatory proteins [254,256,257]. Timed expression and rapid ubiquitin mediated proteolysis limits cyclin expression to specific intervals of the cell cycle, limiting the activity of CDK to certain phases of the cell cycle [257-261].

During early G₁, the cyclin D family (consisting of cyclin D1, D2 and D3) forms a complex with CDK4 or CDK6 [262]. The cyclin D-CDK4/6 complex initiates entry of cells into the S phase, primarily by phosphorylation of the retinoblastoma (Rb) family of proteins [263,264]. CyclinE-CDK2 and continued Rb phosphorylation push cells from G₁ into S phase [265,266]. The complex of cyclin A and CDK2 is required for cells to progress through S phase as seen in Figure 2.3 [267,268].

In the G₂ and M phases of the cell cycle, cyclin A forms a complex with Cdk1, and this complex is required for transition from G₂ to M [269]. Regulation of these cyclin/CDK complexes is accomplished by a variety of mechanisms [254,270-272]. For example, cyclin/CDK complexes are regulated by a class of proteins known as cyclin-dependent kinase inhibitors (CDKi) which play important roles in coordinating proliferation during normal development and differentiation, as well as during cellular stress [270,273].

There are two broad classes of CDKi proteins, the INK4 and CIP families [254,274,275]). The INK4 family, including p16INK4a, target CDK4 and CDK6 and prevent their association with cyclin D. Without cyclin D association, CDK4/6 remains inactive and the cell cycle is unable to progress through G1. The Cip/Kip family of cyclin dependent kinase inhibitors, including p21 and p27, is able to inactivate multiple cyclin-CDK complexes. They are able to block cell cycle progression in G1 and, to a lesser extent, S and M phases of the cell cycle and thereby help elicit a checkpoint [276,277]. The integrity of the cell's genome is monitored by the transcription factor p53 which halts progression of the cell cycle to allow time for DNA repair. This is accomplished by p53 inhibition of Rb phosphorylation [278,279]. The level of p53 is maintained low in normal proliferating cells. In the presence of DNA damage, p53 binds to its sequence-specific DNA site – gene induction results in increased p53 protein [279]. Thus, p53 controls cell cycle through upregulation of p21 (CKI), an active inhibitor of CDKs 2, 4 and 6. The inhibition of kinase activity prevents phosphorylation of Rb and the cell is arrested at G1 to allow time for DNA repair. The p53 induced un-redeemable damaged cell enters into apoptosis [280] by inducing the expression of the pro-apoptotic protein Bax [279]. Mutant p53 mediates survival of breast cancer cells [281].



Hypophosphorylated Rb complexes with the transcription factor E2F. CDK2, CDK4, and CDK6 phosphorylate Rb. E2F is released; binds to DNA with DP, resulting in E2F-dependent transcription for the G1 to S transition [250].

Figure 2.3: Schematic of G1-S phase regulation of the eukaryotic cell cycle

2.6.2 The Cancer Cell Cycle

The delicate balance between the rate of cell cycle progression (cell division) and cell growth (increased cell mass) on the one hand and programmed cell death (apoptosis) on the other is important to cancer development [261,282]. Tumour heterogeneity and diversity do not stand as barriers to share some prominent properties such as unrestricted proliferation [283]. Aberrations in proteins that are key to cell proliferation control and survival are important to all types of tumour formation [284]. The connection between altered cell cycle regulatory proteins and malignant transformation had been described by several scientific reports which led to the notion of cancer as a disease of the cell cycle [285].

Overexpression of CDKs and cyclins and loss of CKI and pRb expression are altered modifications linked to tumorigenesis that result from chromosome alterations such as amplifications and translocations of oncogenes and deletions of tumour suppressors or epigenetic inactivation such as methylation of tumour suppressor promoters [286,287]. Dysregulation of CDKs is known to induce constitutive mitogenic signalling and defective responses to anti-mitogenic signals cause aberrant proliferation, genomic and chromosomal instability [288,289]. CDKs (CDK4, 6 and 2) involved in G1 to S transition control and regulations have been shown to be altered in 80-90% of tumours [290,291].

ATM (ataxia-telangiectasia-mutated)-CHK2-p53, a DNA damage checkpoint pathway, reportedly leads to CDK hyperactivity, unregulated cell cycle progression, genomic instability, and cancer when they are dysregulated [288,292]. Such genes involved in cancer transformation are termed oncogenes (growth promoting) and tumour suppressor (growth inhibiting) genes [293-297]. More than 100 oncogenes and 30 tumour suppressor genes (TSGs) are known. Oncogenes and TSG are a major focus of human cancer studies and additions to both classes of cancer genes that have no cognates among the tumour viruses have been identified. Oncogenes in their normal proto-oncogene state drive the cell cycle forward, allowing cells to proceed from one stage to the next [298]. This highly regulated process

becomes dysregulated due to the activation of genetic alterations that lead to cellular transformation. Tumour suppressor genes, on the other hand, restrict cell cycle progression. Their control over cell division is lost with genetic alterations leading to their inactivation [298]. Epigenetic changes in these genes are contributing factors to the unrestricted proliferative potential of cancer cells [299,300]. These changes show the negative impact of mutagens (exogenous and endogenous), germ line mutations and various types of genomic instabilities acquired during tumour development. Both the oncogenes and TSG are known to code for diverse functions such as growth factors (cytokines), growth factor receptors, adapter molecules, protein kinases, G-proteins, nuclear transcription factors, molecules that repair DNA, apoptosis, metastasis and invasion. Several essential manifestations of alterations in these genes lead to self-sufficiency in growth signals: insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis [71-73].

2.7 Apoptosis

2.7.1 Overview of Apoptosis

There are several types of programmed cell death (PCD) mechanisms that have been identified, including mitotic catastrophe, oncosis, necrosis / necroptosis, pyroptosis, entosis and apoptosis [301-308]. Cells that are damaged by external injury undergo necrosis, while cells that are induced to commit programmed suicide because of internal or external stimuli undergo apoptosis [309]. This dissertation will look attentively at apoptosis since it has been shown to be closely related to anticancer therapy [310,311].

The term “apoptosis” is derived from the Greek words “dropping off” and refers to the falling of leaves from trees in autumn. In 1972, apoptosis was described as a morphologically distinct form of cell death [301]. However, certain components of the apoptosis concept had been explicitly described many years previously [312]. Apoptosis or programmed cell death (PCD) is an evolutionarily and genetically conserved biological process that is indispensable during

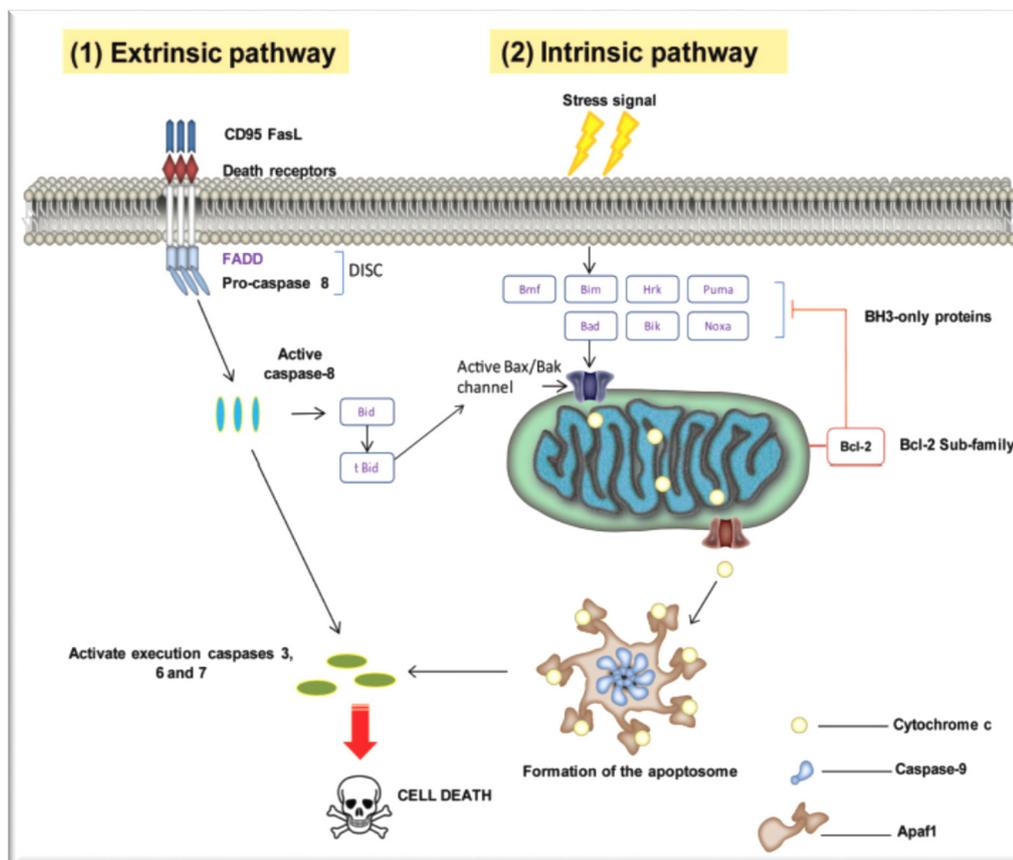
normal development, tissue homeostasis, regulation of the immune system and development of the nervous system [313-315]. During development, structures that are no longer needed are removed by apoptosis. Throughout life, apoptosis eliminates cells that are useless or potentially dangerous such as aged, infected, injured or mutated cells. Deregulation of apoptosis can lead to cancer [305]. PCD is characterized by morphologic changes such as chromatin condensation, nuclear fragmentation and pyknosis (i.e., the reduction of cell volume) [308], as well as biochemical changes that include caspase activation, breakdown of DNA and protein and membrane surface modifications that allow the apoptotic cell to be recognized and engulfed by phagocytic cells [305].

Light and electron microscopy are used to identify the various morphological changes that occur during apoptosis [312]. Early markers of apoptosis such as cell shrinkage and reduction of cell volume are visible by light microscopy [301], while electron microscopy can better define subcellular changes [59]. With cell shrinkage, the cells are smaller in size, the cytoplasm is dense and the organelles are more tightly packed. Pyknosis is the result of chromatin condensation and this is the most characteristic feature of apoptosis [316].

Extensive plasma membrane blebbing occurs followed by karyorrhexis (i.e., the destructive fragmentation of the nucleus of a dying cell) and separation of cell fragments into apoptotic bodies during a process called “budding”. Apoptotic bodies consist of the cytoplasm with tightly packed organelles with or without a nuclear fragment. The organelle integrity is still maintained and all of this is enclosed within an intact plasma membrane. These bodies are subsequently phagocytosed by macrophages, parenchymal cells, or neoplastic cells and degraded within phagolysosomes. There is essentially no inflammatory reaction associated with the process of apoptosis or with the removal of apoptotic cells. Apoptotic cells exhibit several biochemical features such as protein cleavage, protein cross-linking, DNA breakdown, and phagocytic recognition that together result in the distinctive structural pathology described previously [317].

2.7.2 Mechanisms of Apoptosis

An understanding of the underlying mechanism of apoptosis is important as it plays a pivotal role in the pathogenesis of many diseases [59,301,306,307,318-323]. To date, research indicates that there are two major pathways, the death receptor pathway (extrinsic) and the mitochondrial pathway (intrinsic) [306,324]. Both pathways eventually lead to a common pathway or the execution phase of apoptosis as demonstrated in Figure 2.4 [325]. However, there is now evidence that the two pathways are linked and that molecules in one pathway can influence the other [326,327]. A third less well-known initiation pathway is the intrinsic endoplasmic reticulum pathway [328].



(1) The extrinsic pathway is mediated by the ligation of TNF/CD95/Fas ligands to the membrane. This triggers the formation of the death-inducing signaling complex (DISC) composed of FADD and pro-caspase 8. Caspase 8 activation occurs due to the induced proximity of pro-enzyme molecules. Caspase-8 also activates the pro-apoptotic protein Bid which feeds into the intrinsic pathway. (2) The intrinsic pathway is mediated primarily by the Bcl-2 super family. BH3 pro-apoptotic proteins inactivate Bcl-2 pro-survival partners releasing Bax and Bak. Homo-oligomerization of Bax and Bak at the OMM results in the release of cytochrome c and the downstream activation of caspase-9 via a conformational change. Both pathways converge to activate the executioner caspases leading to cell death [325].

Figure 2.4: Schematic of the extrinsic and intrinsic apoptosis pathways

2.7.2.1 The Extrinsic Death Receptor Pathway

The extrinsic death receptor pathway begins when death ligands bind to a death receptor [317]. The best known death receptors are the type 1 TNF receptor (TNFR1) and a related protein called Fas (CD95). Their ligands are called TNF and Fas ligand (FasL), respectively. These death receptors have an intracellular death domain that recruits adapter proteins such as TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD), as well as cysteine proteases like caspase-8. Binding of the death ligand to the death receptor results in the formation of a binding site for an adaptor protein. This ligand-receptor-adaptor protein complex is known as the death-inducing signalling complex (DISC). This leads to the initiation of the assembly and activation of procaspase-8. The activated form of the enzyme, caspase-8, is an initiator caspase, which initiates apoptosis by cleaving other downstream or executioner caspases, typically caspase-3. The active caspase-3 is responsible for the cleavage of a number of so-called death substrates that lead to the well-known characteristic hallmarks of an apoptotic cell [329].

2.7.2.2 The Intrinsic Mitochondrial Pathway

The cell autonomous or intrinsic pathway is initiated within the cell and is largely centred around and/or regulated by the mitochondria [330]. The mitochondrial pathway can be induced by extra- or intracellular stress (hypoxia, DNA-damage, insufficient amount of growth factors, high concentration of cytosolic calcium), or due to oxidative stress. This pathway causes increased permeability of the outer mitochondrial membrane leading to the release of pro-apoptotic molecules such as cytochrome C into the cytoplasm [331]. Membrane permeability is controlled by members of the Bcl-2 family which are further classified as pro-apoptotic and anti-apoptotic [318,331]. Cytoplasmic release of cytochrome C activates caspase-3 and forms a complex apoptosome with ATP, APAF-1 (apoptosis protease activating factor 1) and caspase-9 [318,332]. Family members of IAP (inhibitors of apoptosis proteins) can bind directly to caspases and inhibit their activity [327,333]. IAPs are negatively regulated by

proteins from the mitochondrial intermembrane such as second mitochondria-derived activator of caspase (Smac), Direct IAP Binding Protein with Low pI (DIABLO) and Omi/high temperature requirement protein A (HtrA2) [334-336]. In addition to the caspase activator protein, some other molecules such as AIF (Apoptosis Inducing Factor) and endonuclease G have also been found to cause apoptosis by chromatin condensation and high molecular weight DNA fragmentation [337].

2.7.3 Major Players in Apoptosis and Carcinogenesis

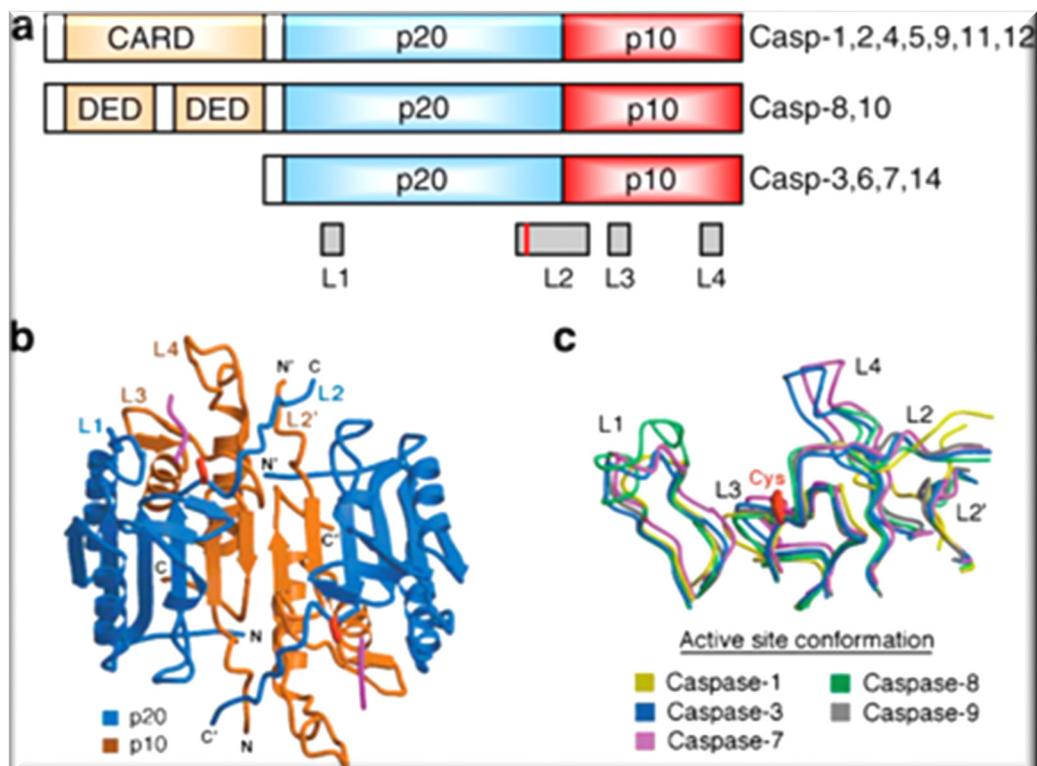
2.7.3.1 Apoptosis and Carcinogenesis

Normal cellular homeostasis is maintained by a balance between the processes of growth and cell death [328,338]. Imbalances in either can lead to uncontrolled cell growth and the development of cancer and decreased apoptosis [323,339-341]. As early as the 1970s, apoptosis has been linked to the elimination of potentially malignant cells, hyperplasia and tumour progression [301]. Thus dysregulation of apoptosis or its resistance may increase many mitogenic proteins or growth promoting pathways such as mitogenic activated protein (MAP) kinases, epidermal growth factor receptor (EGFR) and phosphatidylinositol-3-kinase (PI3K), often leading to uncontrolled growth that serves to promote oncogenesis [320,342]. In general, the mechanisms by which evasion of apoptosis occurs can be broadly divided into (1) reduced caspase function, (2) disrupted balance of pro-apoptotic and anti-apoptotic proteins, and (3) impaired death receptor signalling.

2.7.3.1.1 Caspases

Apoptosis is mediated by intracellular cysteine proteases called caspases which share the ability to cleave their substrates after aspartate residues [323]. To date, 14 mammalian caspases have been identified of which caspases-2, -3, -6, -7, -8, -9 and -10 have been shown to be involved in apoptosis [343]. Caspases are produced as inactive zymogens containing a prodomain, a p20 large subunit and a p10 small subunit. Most of the caspases are activated by

proteolytic cleavage. Active caspase is a heterotetramer, containing two small and two large subunits. Caspases are divided into initiator caspases (caspase-2, -8, -9, -10) and effector caspases (caspase-3, -6, and 7) [343]. Initiator caspases have long prodomains that contain the protein-protein interaction motifs, death effector domain (DED) or caspase recruitment domain (CARD) (Figure 2.5). DED and CARD are involved in interacting with the upstream adaptor proteins. The short prodomain containing effector caspases are typically cleaved and activated by upstream initiator caspases. Active effector caspases are responsible for downstream execution steps of apoptosis by cleaving multiple cellular substrates. To date over 400 caspase substrates have been identified [344].



(a) Domain organization of caspases and the location of catalytic center loops (L1–L4). Initiator caspases have long prodomains, CARD or DED, whereas executioner caspases have short prodomains. Loops are shown in gray. The active site Cys is shown by a red line. Processing that separates p20 and p10 subunits occurs in L2. The resulting large subunit portion of the L2 loop of one monomer and small subunit portion of the L2 loop of the neighboring monomer (L2') are involved in loop bundle formation (b and c). (b) Ribbon representation of the active caspase-3 structure showing the positions of the active center loops (L1–L4, L2') based on the crystal structure of the complex of caspase-3 with peptide inhibitor (in pink). (c) The active site conformations of the caspases with known structures. Loops L1 and L3 are highly conserved, whereas L2 and L4 are responsible for the differences in substrate binding specificity. CARD, caspase recruitment domain; DED, death effector domain [345].

Figure 2.5: Structure and domain organization of mammalian caspases

In some cell types, the caspase-9 enzyme is found in the mitochondrial intermembrane space and is released into the cytosol together with cytochrome C after rupture of the outer mitochondrial membrane [346-348]. Once released, caspase-9 interacts with and is activated by the apoptosis-activating factor Apaf-1 in a cytochrome C- and dATP-regulated manner [349-352]. Subsequently, procaspase-3 is recruited to the Apaf-1/caspase-9 complex and undergoes proteolysis and activation [352,353]. Interestingly, caspase-9 contains a caspase-3 cleavage site at position 330, and it has been shown that procaspase-9 is also a substrate of caspase-3 during apoptosis [354]. Indeed, in a cell-free system of apoptosis, caspase-9 processing can be enhanced by caspase-3 [355]. Furthermore, these authors showed that cytochrome C mediates a hierarchical activation of numerous caspases in addition to caspase-9 and caspase-3, such as caspase-2, -6, -7, -8 and -10, in a complex proteolytic cascade. Once activated, caspase-9 translocates to the nucleus, where it may participate in nuclear dismantling during apoptosis [346]. Studies investigating Apaf-1 and caspase-9-deficient animals have shown that the release of mitochondrial cytochrome C and the subsequent Apaf-1-dependent activation of caspase-9 and caspase-3 represent a common pathway used by many apoptosis-inducing stimuli that is important for tumour suppression by p53 [356-358].

Recent evidence suggests that caspase-3 plays an important role in several key events during apoptosis such as nuclear fragmentation, DNA fragmentation, and membrane blebbing in a cell type-specific and stimulus-specific manner [359]. Furthermore, caspase-3 has been reported to play a role as an amplifier of the apoptotic signals, i.e., by the cleavage of Bcl-2 [348,360,361]. The role of caspase-3 was studied extensively in caspase-3-deficient animals [357,362]. These animals exhibit massively impaired developmental apoptosis in the brain, whereas programmed cell death in other organs occurs normally. Caspase-3-deficient embryonic stem cells are resistant to UV- and sorbitol-induced cell death, whereas γ -irradiation-induced cell death occurs normally. Similar results were obtained in the breast cancer MCF-7 cells that harbor a spontaneous deletion of 47 bp within exon 3 of the CASP-3 gene. This mutation introduces a premature stop codon and leads to a complete absence of

caspase-3 protein and activity. A comparison of MCF-7 cells and CASP-3-transfected MCF-7 cells revealed that DNA fragmentation and membrane blebbing were severely affected after TNF3 or staurosporine treatment [363]. Caspases remain one of the important players in the initiation and execution of apoptosis. It is therefore reasonable to believe that low levels of caspases or impairment in caspase function may lead to a decreased in apoptosis and carcinogenesis. Downregulation of caspase-9 was found to be a frequent event in patients with stage II colorectal cancer and correlated with poor clinical outcome [364]. Moreover, loss of caspases-3 expression and function could contribute to breast cancer cell drug resistance and survival [365].

2.7.3.1.2 Inhibitor of Apoptosis Proteins (IAPs)

Enzymatic activity of caspases can be directly regulated by inhibitors of apoptosis proteins (IAPs) [366]. The IAP family of proteins consists of eight human analogues, including cellular IAP1 (c-IAP1). All IAP proteins contain one or more BIR (baculoviral IAP repeat) domains which are thought to be responsible for caspase inhibition. All IAPs, except survivin, contain one or more other functional domains, for example, RING domain, which possesses E3-ubiquitin ligase activity, and the CARD domain [367,368]. X-linked inhibitor of apoptosis protein (XIAP) is the best-described IAP and possibly the most potent suppressor of apoptosis. The linker region that precedes the BIR2-domain binds to an active site of caspase-3 or -7 and prevents substrate binding. The BIR2 domain interacts with the N-terminus of the caspase small subunit.

The BIR3 domain of XIAP binds to the IAP-binding motif (IBM) on the N-terminus of the small subunit of caspase-9, which is exposed upon proteolytic processing of caspase-9. A distinct part of the BIR3 domain heterodimerizes with an interface of caspase-9, which is required for homodimerization of caspase-9. XIAP can also inhibit apoptosis through the E3-ubiquitin ligase activity of its RING domain that mediates proteosomal degradation of proteins, including caspases [369]. In contrast to XIAP, the anti-apoptotic activity of c-IAP1 and c-IAP2

is thought not to be primarily related to direct inhibition of caspases [319].

Rather, they interfere with caspase activation by targeting caspases or Smac/DIABLO to proteosomal degradation through their RING domain or binding to Smac/DIABLO sequestering it from XIAP, thus facilitating XIAP-mediated inhibition of caspases. Abnormal IAP expression has been reported in pancreatic cancer cells and was found to be responsible for resistance to chemotherapy. In addition, Livin was observed to be expressed in melanoma and lymphoma and A pollon was upregulated in gliomas and was responsible for cisplatin and camptothecin resistance [370,371]. Survivin was noted to be overexpressed in haematological malignancies and also in non-small-cell lung carcinomas (NSCLCs) [372].

It has been suggested that overexpression of survivin in the majority of NSCLCs together with abundant or upregulated expression of XIAP may indicate that these tumours are endowed with resistance against a variety of apoptosis-inducing conditions [373,374]. Accumulating evidence suggests that survivin expression can identify the lesions at highest risk for malignant transformation and invasion. Its presence in body fluids might be an important biological marker and predictive sign of treatment outcome [375]. Abnormal expression of the survivin gene in different tumours and its role in tumorigenesis are related to function during mitosis, rather than inhibition of apoptosis [376]. IAPs are the most important regulators of apoptosis because they synchronize both the intrinsic and extrinsic pathways of apoptosis. Eight human IAP proteins are known to date with survivin and XIAP being the most extensively characterized.

2.7.3.1.3 The Bcl-2 Family of Proteins

B-cell lymphoma 2 (Bcl-2) was the first protein of this family to be identified more than 30 years ago and it is encoded by the *BCL2* gene [377,378]. The second member of a range of proteins is found in human B-cell lymphomas with the t(14;18) chromosomal translocation. All the Bcl-2 members are located on the outer mitochondrial membrane. All family members

contain at least one of the BCL-2-homologous domains (BH1-BH4). BH3 is responsible for the anti- or pro-apoptotic behaviour, and certain pro-apoptotic members contain only the BH3 domain. There are two main groups of the Bcl-2 proteins: (1) the pro-apoptotic proteins (e.g. Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk) made up of the BH-3 only proteins, so named because at the time of cellular stress they get activated and initiate apoptosis, and (2) the anti-apoptotic proteins (e.g. Bcl-2, Bcl-X L , Bcl-W, Bfl-1 and Mcl-1) that contain all four BH domains and protect the cell from apoptotic stimuli by blocking the mitochondrial release of cytochrome C [335,379-382]. Disruption of the balance between pro- and anti-apoptotic members can lead to dysregulation of apoptosis.

In many human tumours, decreased production of pro-apoptotic molecules like Bax and overexpression of the anti-apoptotic proteins cause drug resistance and the survival of tumour cells [383]. Subsequently, the ratio of pro-apoptotic to anti-apoptotic Bcl-2 family members is critical in determining whether the cell will undergo apoptosis. Increased expression of Bcl-2 causes resistance to chemotherapeutic drugs and radiation therapy, while downregulation of Bcl-2 expression may promote apoptotic responses to anticancer drugs. In addition, overexpression of Bcl-2 may result in accumulation of cells in the G0 phase of the cell cycle and contribute to chemoresistance [384].

2.7.3.1.4 Tumour Protein 53 (p53)

The p53 protein, also called tumour protein 53 (or TP53), is one of the best known tumour suppressor proteins [278,385-396], encoded by the tumour suppressor gene *TP53* located at the short arm of chromosome 17 (17p13.1) [397]. P53 is named after its molecular weight, i.e. 53 kDa [398]. The protein controls a multitude of important cellular responses that may vary from protecting the integrity of the genome, inducing apoptosis, regulating glycolysis and autophagy, to even promoting cell differentiation [307,399]. Cells committed to die via p53-dependent apoptosis typically follow the mitochondrial pathway, although p53 can also modulate cell death through death receptors. Furthermore, most evidence suggests that the key

contribution of p53 to apoptosis is primarily dependent on transcriptional activity. P53 has the ability to activate transcription of various pro-apoptotic genes including those encoding members of the Bcl-2 family, such as the BH-3 only proteins Bax, Noxa, and Puma [400]. In addition, it has been found that when a p53 mutant was silenced, it resulted in reduced cellular colony growth in human cancer cells, which was found to be due to the induction of apoptosis. Recent studies have shown that finely tuned, complex control of p53 by Mdm-2 (mouse double minute-2, an oncoprotein) is a key step in ursodeoxycholic Acid (UDCA) modulation of p53-triggered apoptosis [401].

2.8 Overview of Marine Algae

Human beings have depended on natural products as a resource of drugs for thousands of years. Plant-based drugs have formed the basis of traditional medicine systems that have been used for centuries in many countries such as Egypt, China and India [402-404]. Today, plant-based drugs continue to play an essential role in health care. The WHO estimated that 80% of the populations in developing countries rely on the use of plants for treatment of different ailments [405,406]. According to a study published in 1993, at least 119 chemicals, derived from 90 plant species, can be considered as important drugs in one or more countries [407]. Plant-derived drugs were shown to represent about 25% of the American prescription drug market, and over 50% of the most prescribed drugs in the US had a natural product either as the drug or as the starting point in the synthesis or design of the agent [408].

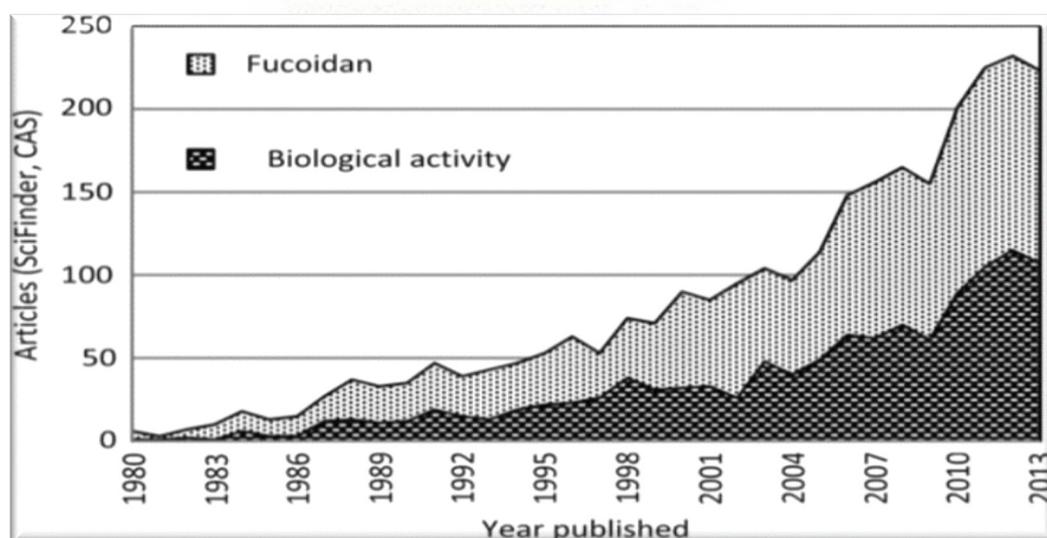
In the past twenty years, there has been a lot of progress in the fight against cancer. Advances in cellular and molecular biology have helped our understanding of the different mechanisms of this disease. Natural products have contributed significantly to the development of anticancer drugs. Seaweeds are macroalgae known for their richness in minerals and certain vitamins, but also for bioactive substances like polysaccharides, proteins, lipids and polyphenols used in the development of new pharmaceutical agents [409]. Currently, the *Phaeophyceae* or brown algae attract much attention since they are a rich source of structurally

different polysaccharides such as alginates, laminarans and fucoidans, exhibiting various biological activities including anticancer properties [409,410].

2.9 Fucoidan

2.9.1 Statistical Data for Scientific Publications Related to Fucoidan

Fucoidan is a natural polysaccharide made essentially of sulphated L-fucose residues extracted from marine brown algae (*Phaeophycophyta*) and some echinoderms (sea urchin and sea cucumber). Named “fucoidan” according to IUPAC rules, it has also been called fucan, fucosan or sulphated fucan [411]. Over the last decade, the number of published articles on fucoidan-related topics has increased dramatically (Figure 2.6) since the first publication by Kylin in 1913 [11,12]. As more scientists continue to explore this unique polysaccharide, more biological health benefits are being discovered [13]. Recent interest in fucoidan had focused primarily on the antitumour, anticoagulant and antioxidant activities, as well as activities against liver and urinary system failures [412,413].

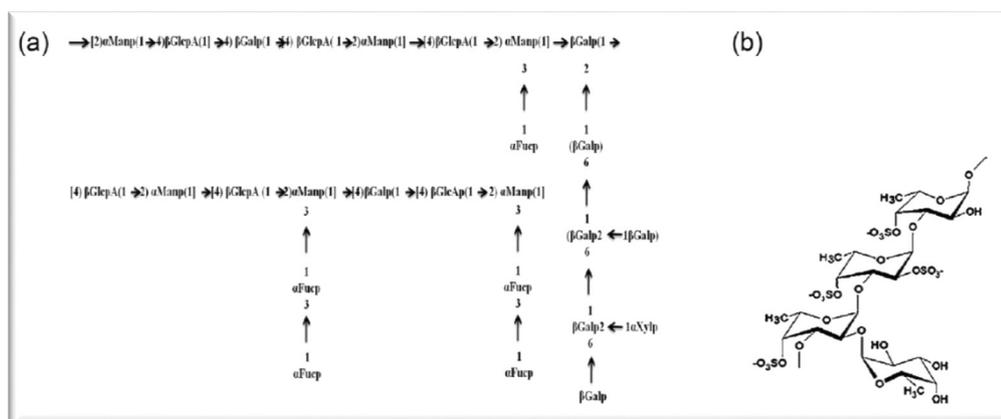


Publications related to fucoidan studies and their biological activities (ca 20% of such papers are dedicated to angiogenesis investigation and antitumor effect). Searched in April, 2014 with SciFinder (CAS) database (Ustyuzhanina et al., 2014).

Figure 2.6: Statistical data for scientific publications related to fucoidan

2.9.2 Characterization of Fucooidan

In 1950, Percival and Ross [414] were the first to allude to a fucooidan structure extracted from the cell walls of brown algae, *Fucus vesiculosus*. Subsequent reports appeared of a polysaccharide structure consisting mainly of fucose and sulphate [415,416]. The chemical composition and structure of fucooidan are very diverse and fluctuates significantly depending on the algae species, place of cultivation or habitat, harvesting time and even the method of extraction [417,418]. The main skeleton of fucooidan consist of α -1,3-linked sulphated l-fucose; a repeating sequence of alternating α (1 \rightarrow 3) and α (1 \rightarrow 4) glycosidic bonds is also possible [8,411]. The main chains may contain carbohydrate (L-fucopyranose and D-glucuronic acid) and non-carbohydrate (sulfate and acetyl groups) components. Fucooidan also contains minor components such as galactose, glucose, mannose, and xylose. Due to structural heterogeneity and complex branching patterns, the structure of fucooidan has not yet been fully elucidated [419,420]. Despite complexities, the structural backbone of numerous fucooidan extracts from brown seaweeds *Fucus vesiculosus* [421], *Ascophyllum nodosum* [422], *Sargassum kjellmanianum* [423], *Sargassum thunbergii* [424] and *Cladosiphono kamuranus tokida* [425] have been clarified. The content of L-fucose ranged from 12.6 to 36.0% and sulfate ranged from 8 to 25%. The structure of fucooidan is depicted in Figure 2.7.



(a) Basic model structure of fucooidan polysaccharide. (b) A chemical structure of brown seaweed sulfated polysaccharide [426].

Figure 2.7: Structure of fucooidan

2.9.3 Health Benefits and Biological Activities of Fucoidan

For many years, research has essentially focused on plants and terrestrial microorganisms, mainly because of these specimens are easily available and folk traditions have described beneficial effects from their use. Research on fucoidan has thus far been carried out in Japan, Korea, France, Australia, China and the United States. Studies have indicated that fucoidan is non-toxic, non-allergenic and has no negative effects on the human body once consumed [427]. Nutraceutical and food supplements containing fucoidan have been marketed for a number of years with no known adverse effects [428]. No toxicological changes were observed when rats were orally administered with up to 1000 mg/kg body weight per day of fucoidan for 28 days [429].

It is clear that activity is related to structural features of fucoidan: degree of sulfation [430], molecular weight [431,432] and linkage pattern [433]. Biological activities of fucoidan may also vary depending on the source of seaweed, compositional and structural traits, charge density, distribution, bonding of the sulfate substitutions [434,435] and purity of the fucoidan extract [411,436]. Pharmacologically, fucoidan affects many pathophysiological processes and possible therapeutic properties include antitumour and immunomodulatory [437,438], antiviral [439], antithrombotic and anticoagulant [440], anti-inflammatory [441,442] and antioxidant effects [443]. Various renal [444] and uropathic disorders [445] have also responded positively to fucoidan treatment. Fucoidan isolated from brown seaweed such as *Undaria* and *laminaria* have been reported to display anticoagulant, antiviral and anticancer properties [13,446,447].

Fucoidan possesses excellent natural antioxidant activities [448]. Fucoidan stimulates the immune system in several ways, and the numerous important biological effects are related to its ability to modify cell surface properties [449]. Oral intake of fucoidan present in dietary brown seaweed might confer protective effects through direct inhibition of viral replication and stimulation of the immune system (innate and adaptive) [13]. Fucoidan has been reported to be effective *in vivo* upon oral, intraperitoneal, or intravenous administration [450-452].

2.9.4 Anticancer Activity of Fucoidan

Several different therapeutic strategies such as chemotherapy, radiation therapy, surgery or combinations have been used to treat different types of cancer. Unfortunately, several of these treatments provide only minimal benefits and long term side effects [453-455]. The therapeutic potential of natural bioactive compounds such as the polysaccharide fucoidan is now well documented and may allow for the development of a new generation of therapeutic measures against cancer [456]. Although the mode of action of fucoidan is not well understood, it has been shown to induce cytotoxicity and apoptosis in cancer cells [17,21,61,62,426,457-459]. Fucoidan can also affect cancer cells indirectly e.g. as an antiangiogenic agent by cutting off the nutrient supply to cancer cells [62].

Furthermore, fucoidan has been shown to display immune-stimulating effects on dendritic (DC) cells [460-463] and natural killer (NK) cells [412,464]. Thus, fucoidan can enhance anticancer immunity through immune cell activation and influx and stimulation of the production of anticancer cytokines [10,421]. The main mechanisms by which fucoidanis have been postulated to inhibit cancer include scavenger receptor modulation, immune activation, anti-angiogenesis, blockade of metastasis, mobilization of stem cells and interference with SDF1/CXCR4 axis, anti-oxidant and pro-oxidant effects [17]. The anticancer activity of fucoidan is indeed likely to be via more than one single pathway.

Fucoidan was found to inhibit proliferation and to induce apoptosis in human lymphoma HS-Sultan cell lines [421]. Fucoidan extracted from brown seaweeds *Eclonia cava*, *Sargassumhornery* and *Costariacostata* displayed anticancer effects on human melanoma and colon cancer cells [465]. Human malignant melanoma cancer cell (SK-MEL-28 and SK-MEL-5) growth was inhibited by fucoidan isolated from *Fucus evanescens* [433]. Fucoidan from *L. saccharina*, *L. digitata*, *F. serratus*, *F. distichus* and *F. vesiculosus* strongly blocked MDA-MB-231 breast carcinoma cell adhesion, proliferation and induced apoptosis [20,459] and has been implicated in tumour metastasis [5,466]. Sulphated polysaccharides from brown

seaweeds *S. japonica* and *U. pinnatifida* possess high antitumour activity and inhibited proliferation and colony formation of breast cancer and melanoma cell lines [467]. Fucooidan administered to Raji lymphoblastoid cancer cells eradicated cells within 72 hours of incubation while not attacking normal cells [468,469]. Numerous more recent studies reported on the anti-carcinogenic properties of fucooidan, while displaying little or no effect on normal cells. Fucooidan exhibits cytotoxic effects against CCL39 cancer cells [435,470]. Intraperitoneally injected crude extracts of *Fucus vesiculosus* induced apoptosis of 4T1 breast cancer cells in tumour-bearing mice, but did not cause apoptosis of some other cancer cells *in vitro* [471].

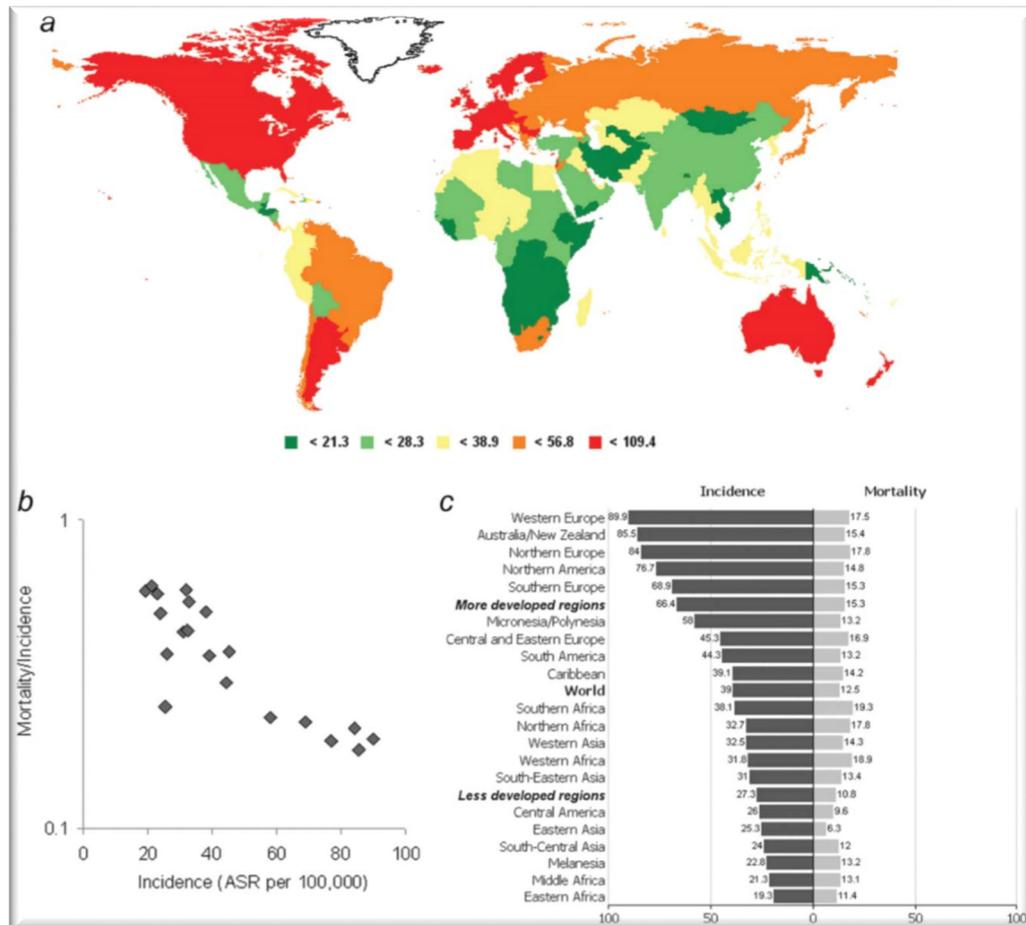
Highly purified fucooidan derived from the brown algae *Sargassum mclurei* was less cytotoxic, but inhibited colony formation in DLD-1 colon cancer cells when used at up to 200 µg/ml for 48h [472,473]. An earlier study showed that fucooidan derived from *Fucus vesiculosus* failed to induce apoptosis in K562 erythroleukemic [474], but did so in human HT-29 and HCT116 colon cancer cells [455]. These results collectively suggest that apoptotic activity of fucooidan on cancer cells may be cell type specific.

Fucooidan's anticancer properties were supported by a study that compared the breast cancer rates in Japan with other developed nations [475]. Women who consumed miso soup more than six times a week were reported to have half the risk of breast cancer compared to women who did not. They concluded that the brown seaweed components such as fucooidan may inhibit cancer cell formation. Figure 2.8 shows the breast cancer rates across the world in 2008. Cancer rates were excessively high throughout the United States, Australasia and many parts of Europe, but relatively low in most parts of Asia [1] where seaweed consumption is high [476].

2.9.5 Effects of Fucooidan on Cell Cycle and Apoptosis

Both cell cycle and apoptosis control major regulatory functions of growth and development in all living organisms [477,478]. Cell cycle deregulation and apoptosis resulting in uncontrolled cell proliferation are the most frequent alterations that occur during the

development and progression of cancer [72,307,311,318,340,479]. For this reason, a blockade of the cell cycle and apoptosis induction are regarded as effective strategies for eliminating cancer.



Source: [1] (a) Estimated age-standardized incidence rates per 100,000 (World): breast cancer. (b) Breast cancer: relationship between incidence and Mortality: Incidence ratio (20 regions). (c) Estimated age-standardized incidence and mortality rates for breast cancer.

Figure 2.8: Breast cancer rates across the world in 2008

Fucoïdan has been shown to moderately arrest the cell cycle at the G0/G1 phase in Huh7 cells at a concentration of 1.0 mg/ml [480]. Cell proliferation was inhibited and the G2/M ratio was increased in hepatocellular cells lines at 22.5 $\mu\text{g/ml}$ [481]. In HUT-102 T-cell lymphoma cells, fucoïdan induced the accumulation of cells in the G1/S phase [425]. In the breast cancer cell line, MCF-7, 820 $\mu\text{g/ml}$ fucoïdan was shown to increase the accumulation of cells in the sub-

G1 phase [482]. The mechanism of the action of fucoïdan against cancerous cells remains to be fully elucidated. Significant downregulation of cyclin D1, cyclin D2 and CDK4 in cancer cells has been demonstrated [425,483]. Fucoïdan extracted from *Fucus vesiculosus* increased the level of p21/WAF1/CIP1 in PC3 cells and downregulated E2F, a transcription factor that controls progression of cells from G1 to S phase [483]. In human bladder cancer cells, fucoïdan have been reported to downregulate cyclin E, CDK2 and CDK4, resulting in G0/G1 arrest [484]. Furthermore, immunoprecipitation assays revealed a significant increase in the binding of p21/WAF1/CIP1 to CDK2 and CDK4, suggesting that the induced G0/G1 arrest is due to suppression of CDK activity following direct binding of this CDK inhibitor to CDKs 2 and 4.

Induction of apoptosis is a critical factor in cancer therapy. Previous work showed that fucoïdan, extracted from *Cladosiphon okamuranus*, have strong antiproliferative and apoptotic effects on MCF-7 cells in a dose-dependent manner, while not affecting proliferation of normal human mammalian epithelial (HMEC) cells [485]. Fucoïdan exhibited the characteristics of apoptotic cell death such as induction of chromatin condensation, fragmentation of nuclei and DNA, and cleavage of specific proteins. Moreover, fucoïdan was shown to stimulate accumulation of sub-G1 populations, chromatin condensation, and internucleosomal fragmentation of DNA. Because these are representative features of apoptosis, it may be concluded that fucoïdan induce apoptotic cell death in MCF-7 cells.

Effector caspases, such as caspase-3 or -7, have been shown to activate DNase, resulting in fragmentation of DNA in response to various apoptotic stimuli [485]. Activation of caspase-7 and PARP cleavage are hallmarks of apoptosis in MCF-7 cells [486]. Cleavage of PARP and activation of caspase-7 were induced after treatment of MCF-7 cells with fucoïdan and the caspase-7 inhibitor, z-DEVD-fmk, terminated fucoïdan-induced apoptosis. Caspase-3 is known to be activated and plays a pivotal role in fucoïdan-induced cell death [421,430]. These results suggest that caspase-7 is required, while activation of caspase-3 is not a prerequisite for fucoïdan-induced apoptosis in MCF-7 cells [485]. MCF-7 cells show a defect in caspase-

3, but express caspase-7, an executioner caspase capable of cleaving poly (ADP-ribose) polymerase (PARP) [487,488]. Caspase-3 was found to be activated in HS-Sultan (malignant lymphoma) cells treated with fucoidan [421]. Fucoidan was also found to upregulate caspases-3 and -9 and Bax (proapoptotic proteins) in HCT-15 (human, colon, colorectal adenocarcinoma) cells, whilst Bcl-2 and Akt (anti-apoptotic proteins) were reduced [489]. In a study of the effects of fucoidan on HT-29 and HCT116 human colon cancer cells, fucoidan was found to cause apoptosis in a dose-dependent manner, while having no effect on normal FHC colonocytes. Molecular analysis of the HT29 cells showed that fucoidan increased levels of a number of pro-apoptotic proteins involved in the mitochondrial-mediated pathway of apoptosis [455]. These included cleaved caspase-3, -7, -8 and -9, cleaved PARP, Bak and truncated Bid. A number of anti-apoptotic effectors were reduced including XIAP, survivin and Mcl-1. Enhancement of mitochondrial membrane permeability, as well as release of cytochrome c and Smac/Diablo from the mitochondria, was also observed.

Molecular changes, including increased levels of Fas-L, TNF-related apoptosis-inducing ligand (TRAIL) and DR5 implicated the activation of the death receptor-mediated apoptotic pathway. In contrast, no changes in caspases, ERK, p38, p53 or pAKT levels were found to accompany the apoptosis-induced by fucoidan in HeLa cells [490]. However, apoptosis inducing factor (AIF) was found in the cytosol, as well as upregulation of Bax and downregulation of Bcl-2. In U937 cells, apoptosis was induced by a sulphated polysaccharide from *Ecklonia cava* [491]. This was shown to be correlated with an upregulation of Bax and PARP, activation of caspases 7 and 8, and downregulation of Bcl-2.

2.9.6 Fucoidan as a Synergistic Anticancer Agent

Fucoidan could be useful as an adjunct oral therapy during or after conventional chemotherapy or radiotherapy [61]. Several studies, using *in vitro* models, have noted potential synergies with chemotherapy. The ability of fucoidan to work in synergy with standard anticancer agents has recently been investigated [20]. The combinatorial effect of fucoidan from

Cladosiphonnavae caledoniae and three commonly used anticancer agents cisplatin (CDDP), tamoxifen (TAM) and paclitaxel (Taxol) on signal transduction pathways were investigated. Fucoïdan plus anticancer agents reduced the ERK phosphorylation in MDA-MB-231 breast cancer cells compared to untreated control or fucoïdan alone. Another study examined the synergistic effect of a high molecular weight (HMW) fucoïdan with colorectal cancer chemotherapy agents oxaliplatin plus 5-fluorouracil/leucovorin (FOLFOX) and irinotecan plus 5-fluorouracil/leucovorin (FOLFIRI) [492]—test patients received 4.05 g fucoïdan dissolved in 150 ml of liquid per day for 6 months. From the commencement of chemotherapy, toxicities and chemotherapy efficiency were compared. Fucoïdan caused no side effects such as allergic dermatitis. Diarrhoea, neurotoxicity and myelosuppression were not suppressed by fucoïdan, whereas general fatigue was significantly decreased from 60% to 10%. The patients were followed for approximately 15 months. The survival rate of patients who received fucoïdan was, however, not significantly different from control participants.

Fucoïdan has shown to affect the migration and invasion of multiple myeloma (MM) cells treated with the chemotherapy drug cytarabine [493]. Human myeloma cell lines RPMI8226 and U266 were treated with crude fucoïdan from *F. vesiculosus* for 72h followed by cytarabine for 6 h. Fucoïdan reduced cell migration through a Boyden chamber and downregulated expression of CXCR4 and MMP-9. Fucoïdan from *Saccharinacichorioides* has been reported to act synergistically with a low dose of resveratrol (a natural polyphenol extracted from foods and beverages) on an invasive and highly motile HCT-116 colon cancer cell line [494]. In the colony formation assay, fucoïdan plus resveratrol reduced the colony number by 60% compared to 34% and 27%, respectively, when treated with resveratrol or fucoïdan alone. Dietary fucoïdan synergistically reduced cell growth in the OE33 cell line when it was combined with lapatinib, a targeted therapy that acts as a tyrosine kinase inhibitor in advanced HER2-positive breast cancer cells [21]. In a xenograft transplantation study, the effect of fucoïdan alone or in combination with cyclophosphamide has been examined on tumour growth [437]. Nine days after the injection of Lewis lung carcinoma cells into mice, fucoïdan

from *Fucus evanescens* was administered to animals alone or combined with cyclophosphamide. Fucoïdan treatment showed marked antitumour (33% tumour growth inhibition) and anti-metastatic (29% reduction of the number of metastases) activities. However, fucoïdan did not exhibit a synergistic effect with cyclophosphamide on tumour growth.



CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

This chapter focuses on the description of the research methodology and design that has been chosen for the study. It summarizes the materials and methods used such as chemicals required, drugs tested and the maintenance of breast cell cultures, MCF-7 and MCF-12A. MCF-7 is a human breast cancer cell line that was first isolated in 1970 from a malignant adenocarcinoma breast tissue of a 69-year old woman [495,496]. MCF-7 is the acronym of Michigan Cancer Foundation - 7, referring to the institute in Detroit where the cell line was established. MCF-7 cells are useful for *in vitro* breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium.

MCF-12A is a human breast immortalized cell line derived from the non-malignant epithelial breast tissue of a 60-year old woman [496]. MCF-12A is the acronym of Michigan Cancer Foundation – 12A. Analyses of MCF-7 breast carcinoma cells and MCF-12A exposed to fucoidan (FUC), cisplatin (CDDP), doxorubicin (DOX) and taxol (TAX) included dose response curves (cytotoxicity assays) and determination of apoptosis by means of Annexin-VFITC/PI assays, caspase-3/7 and -9 assays. Additionally, analysis of the cell cycle distribution and morphological staining of apoptotic cells using Hoeschet-33324 dye were performed. The statistical methods used for data analysis are also described.

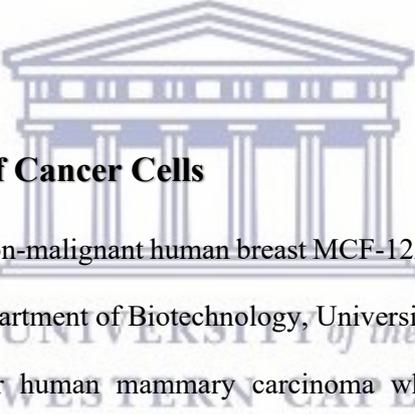
3.2 Materials

All reagents used in this study were of analytical grade. Fucoidan, bisbenzimidazole, Hoechst-33342 tri-hydrochloride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trypan blue, dimethyl sulfoxide (DMSO) and N,N-dimethylformamide (DMF) were

purchased from Sigma-Aldrich (St. Louis, MO, USA). Cisplatin (CDDP), doxorubicin (DOX) and taxol (TAX) were obtained from Tocris Bioscience, R & D Systems Company (Minneapolis, MN). FITC-AnnexinV/DEAD cell apoptosis kit, caspase-9 colorimetric kit and FxCycle PI/Rnase staining solution were purchased from Thermo Fisher Scientific (Waltham, MA). Heat-inactivated foetal bovine serum (FBS), vibrant fam caspase-3 and -7 kit, phosphate buffered saline (PBS), F-12 glutamax (DMEM F-12 glutamax) and penicillin/streptomycin were obtained from Gibco, Life Technologies (Waltham, MA). Fucoidan stock solution (10 mg/ml) was prepared in distilled water, filtered through a 4.5 µm pore filter and stored at -20°C. Stock solutions (1 mM) of cisplatin was prepared by dissolving the powder in DMF, doxorubicin and taxol were dissolved in DMSO. For each experiment, the working solutions for all drugs were prepared freshly and protected from light.

3.3 Methods

3.3.1 Maintenance of Cancer Cells



The MCF-7 cancer and non-malignant human breast MCF-12A cell lines were donated by Prof Mervin Meyer of the Department of Biotechnology, University of The Western Cape. MCF-7 is a model cell line for human mammary carcinoma which exhibits some features of differentiated mammary epithelium [497]. MCF-7 cells were maintained as an adherent monolayer culture in commercially defined Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (100 U/ml penicillin and 100 µg/ml streptomycin).

Incubation conditions were 37°C in a 95% relative humidity (RH) in an atmosphere of 5% CO₂. The solutions used were cell culture grade quality and the laboratory ware used in cell culture were commercially presterilized and disposable. All tissue culture operations were carried out in a Heraeus incubator (Hanau, Germany) or NU-5510E NuAire DHD autoflow automatic CO₂ air-jacketed incubator and an AireGard NU-201-430E horizontal laminar airflow tabletop workstation that provides a HEPA filtered clean work area (NuAire).

3.3.2 Cell Harvesting (Passaging)

Cells were grown in either 25-cm² or 75-cm² attached types, filter cap culture flasks (Greiner Bio-One Germany). When the attached cell density on the flask surface exceeded 80% confluency, the cells were detached with trypsin-EDTA. Medium was aspirated and cells attached to flask surface were washed out with FBS-free growth medium. Trypsin-EDTA was added (1 ml for 25-cm² and 3 ml for 75-cm² flasks) and discarded immediately leaving small amount of trypsin-EDTA (~0.5 ml in 25-cm² and ~1 ml in 75-cm² flasks). The flasks were then incubated at 37°C until detachment of cells was observed. Growth medium was added and detached cells were resuspended and passaged into new culture flasks or separated for further experiments or cryopreserved.

3.3.3 Freezing and Thawing of Cells

For freezing, trypsinated and detached cells (concentration no more than 5×10^6 cells/ml) were centrifuged at 800 rpm for 5 min in 15-ml falcon conical tubes (Greiner). The supernatant was discarded and the cell pellet resuspended in 1 ml of cold cryopreservation medium (90% v/v FBS and 10% v/v DMSO (Sigma-Aldrich, USA)). The cell suspension was transferred to cryovials (Greiner) and maintained on ice for 30 min. The cryovials were then kept in a -20°C freezer for 24h and transferred to a -80°C freezer. For thawing, cryovials were removed from -80°C freezer and immediately transferred to 37°C. When they were completely thawed, aliquots were transferred into the 15-ml falcon tubes and cells pelleted at 800 rpm for 5 min. The supernatant was discarded and pellet was resuspended in complete medium. Cells were maintained in defined growth conditions.

3.3.4 Cell Counting and Viability Testing

Cell counts were carried out using TC20TM automated cell counter (Bio-Rad). The automated cell counter uses an optical method to count the cells in fluid samples enclosed in a cell counting slide or chamber. Cell viability was assessed by mixing cell suspension with 0.4%

trypan blue stain in a 1:1 (v/v) ratio and 10 μ l trypan blue stained cell suspensions was placed in the counting slide chamber and inserted into the cell counter, with the total number of cells counted together with the number of viable cells displayed on the screen of the cell counter in 30 sec. Thereafter, the required number of cells needed for a particular experiment was calculated in relation to the number of viable cells displayed on the cell counter.

3.3.5 MTT Cell Proliferation Assay

Growth inhibitory or inducing effects of various substrates on cell lines was determined by the MTT-proliferation assay. The MTT-[3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] colorimetric assay is based on a membrane permeable dye which is metabolized to dark-blue crystals of formazan by mitochondrial dehydrogenases of living cells which can be measured at wavelengths between 500 and 600 nm by spectrophotometry [498]. Cells were grown to confluency in 75-cm² flasks and after trypsinization and addition of medium, the cell number was corrected to 4.5×10^4 cells per well.

Cell suspension (100 μ l) was seeded into 96-well plates and the plates incubated at 37°C overnight. After one day, culture media was removed and cells were incubated with 100 μ l of medium supplemented with varying concentrations of fucoidan (400, 600, 800, 1000 and 1200 μ g/ml) and chemotherapeutic agents at a log range of concentrations: cisplatin (0.02 – 200 μ M) (0.006 – 60 μ g/ml), doxorubicin (0.002 – 20 μ M) (0.0012 – 11.6 μ g/ml) and taxol (0.02 – 200 nM) (0.000017 – 0.17 μ g/ml). A background of 100 μ l of medium and a control of 100 μ l of medium and cells were also included.

In combination experiments, the cells were treated with both of fucoidan (50 μ l) and one of the chemotherapeutic agents (50 μ l). After indicated times of incubation 10 μ l of MTT (5 mg/ml) was added to each of the wells and incubated for 4 h at 37°C. The medium was aspirated and replaced with 150 μ l/well of DMSO, gently shaken for 15 min to dissolve the formazan salt. The colour intensity of the formazan solution was measured at 595 nm using a

microplate reader (Glomax Multi Detection System, Promega, USA). The percentage inhibition of proliferation was calculated using the formula below and the half maximal inhibitory concentration of a drug (IC₅₀) values calculated from log dose-response curves using Graph Pad Prism version 6.05 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Only the inner rows of the microtitre plate were used for these studies to minimize cell growth variations due to different medium evaporation rates at the periphery. Readings were automatically generated from the machine and recorded based on the formula below:

$$\text{Cell Viability (\%)} = \frac{\text{Extract Concentration Reading} - \text{Blank Reading}}{\text{Control Reading} - \text{Blank Reading}} \times 100\%$$

3.3.6 Cell Cycle Analysis

Samples were prepared for flow cytometry as described previously [499]. Briefly, cells were seeded at a density of 2×10^5 cells/well in 6-well plates and incubated for 24 hours at 37°C in a CO₂ incubator to form a monolayer. The cells were treated with fucoidan and IC₅₀ concentrations of CDDP, DOX and TAX for 24 h. After treatment, the cells were washed with 2 ml cold PBS and trypsinized, combined with any floating cells, pelleted, washed twice with PBS. Slowly, 70% of ice-cold ethanol was added to the cell while vortexing to reduce cell clumping.

The cells were stored at -20°C for 48 h, after which they were pelleted at 6000 rpm for 10 min. Cells were washed in 2X PBS and 0.5 ml of propidium iodide (PI) master mix containing 100 µg/ml RNase and 40 µg/ml in PBS. Cell cycle phase distribution was determined by fluorescence activated cell sorting (FACS) using a FACS Calibur Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA). DNA content of 50,000 events was determined by

FlowJo[®] software (Tree Star, Inc., Ashland, USA), which provided histograms to evaluate the cell cycle distribution. Cells with sub-G1 DNA content were considered apoptotic cells [500].

3.3.7 Annexin V FITC/PI Assay

The flow cytometer is highly sophisticated equipment used to analyze individual cells in heterogeneous populations. It allows thousands of cells to pass through a light beam every second [321], and then it can distinguish these cells based on size and health amongst other parameters. This technique was employed to investigate whether the fucoidan was inducing cytotoxicity to the cells by either necrosis or apoptosis. Annexin-V (Ambion Annexin V-FITC Apoptosis Detection Kit) was used to quantitate the percentage of cells undergoing apoptosis, necrosis and viable cells. Annexin-V has a high calcium dependent affinity for phosphatidylserine (PS) residues; these residues are normally embedded in the cytoplasmic plasma membrane in healthy cells but are translocated to the surface of the cells during apoptosis. Therefore, Annexin-V can bind to these residues acting as a probe to detect and measure apoptosis [501].

MCF-7 and MCF-12A cells were seeded into 35-mm diameter petri dishes (surface area 962 mm²) at 3x10⁵ cells per dish in 2 ml culture medium and allowed to attach for 24 h. The cells were treated for the indicated times with desired concentrations of FUC alone or in combination with IC₅₀ concentrations of DOX, TAX or CDDP. Hydrogen peroxide (H₂O₂) 25 µM was used as positive control for necrosis. After the treatment, cells were harvested and centrifuged at 3000 rpm for 5 min. The pellets were washed in 1% PBS and resuspended in Annexin-V binding buffer. The cells were centrifuged at 3500 rpm for 5 min and supernatants discarded. Cell extracts were suspended in 100 µl Annexin-V binding buffer and 5 µl FITC-Annexin added and allowed to incubate in the dark for 15 min at room temperature. PI (5 µl) diluted in 1 x Annexin-V binding buffer (1:10 v/v) was added and allowed to incubate for 15 min in the dark at room temperature. Annexin-V binding buffer (400 µl) was added to wash the Annexin/PI stained cells. Then the cells were mixed gently and kept on ice.

Immediately, all samples were analyzed on a Becton Dickinson FACS Calibur instrument (BD Biosciences Pharmingen, San Diego, CA, USA) measuring the fluorescence emission at 530 nm and 575 nm. A minimum of 10,000 cells per sample were acquired and analyzed using FloJo[®] flow cytometry analysis software. Cells in the early stages of apoptosis were positively stained with Annexin-V, whereas cells in late apoptosis were positively stained with both Annexin-V and PI.

3.3.8 Hoechst-33342 Staining

Hoechst stains are part of a family of blue fluorescent dyes used to stain DNA [502]. These bis-benzimidides were originally developed by Hoechst-AG, which numbered all their compounds so that the dye Hoechst-33342 is the 33342nd compound made by the company. There are three related Hoechst stains: Hoechst-33258, Hoechst-33342, and Hoechst-34580. The dyes Hoechst-33258 and Hoechst-33342 are the ones most commonly used and they have similar excitation/emission spectra. Hoechst-33342 was soluble in organic solvents such as DMF or DMSO. Concentrations can be achieved of up to 10 mg/ml. Aqueous solutions were store at 2–6°C and protected from light (Molecular Probes, 2005).

Cells were seeded (3×10^5 cells) on sterile coverslip (2 x 22 mm) and grown in 35-mm diameter petri dishes (surface area 962 mm²), allowed to adhere for 24 h, and treated with (400, 800, or 1000) µg/ml fucoidan or/and IC₅₀ of CDDP, DOX and TAX individually or in combination for 24 h, and appropriate controls were included.. The used medium was discarded, washed three times with PBS and stained using 1 ml of 2 µg/ml in PBS Hoechst-33342 for 10-30 min at room temperature. Then the labeling solution was discarded and the cells washed three times in PBS. Subsequently, the coverslips were mounted as described previously [503]. Apoptotic cells were observed by a DMI-4000B inverted fluorescence microscopy (Leica, Germany) with excitation ~353 nm and emission ~483 nm. Each image shown is representative of 20 randomly observed fields.

3.3.9 Caspase-3/7 Assay

Caspase 3 and 7 are called effector caspases which are responsible for initiating the hallmarks of the degradation phase of apoptosis [345,362]. To confirm the dependence of apoptosis on caspase activity, the test was done with the specific caspase inhibitor, FLICA. The activities of caspase-3/7 were measured using the Vybrant® FAM Caspase-3/7 kit (Invitrogen, Molecular Probes, Cat no. V35118). Briefly, cells were grown in 6-well plates until the semiconfluency stage and then treated with indicated concentrations of desired different concentrations of FUC alone or in combination with IC₅₀ of CDDP, DOX, and TAX for 24h in culture medium. After trypsination, the medium was removed and were combined with any floating cells, pelleted, and re-suspended in fresh culture medium.

The untreated and treated cells were stained with 10 µl of 30X FLICA working solution (prepared by adding 1 part of FLICA reagent stock solution to 4 parts PBS). The cells were mixed by flicking tubes and incubated for one hour at 37 C° and 5% CO₂ in the dark, with mixing the tubes twice during incubation to minimize cell setting. Cells were washed with wash buffer solution (one part of 10X wash buffer to 9 parts distilled water) and pelleted twice, supernant discarded and the cell pellet resuspended in 400 µl of 1X wash buffer solution, then analyzed on a Becton Dickinson FACS Calbur instrument fitted with a 488 nm excitation and green emission for the FLICA-stained cells. A minimum of 10,000 cells per sample were acquired and analyzed using FloJo® flow cytometry analysis software.

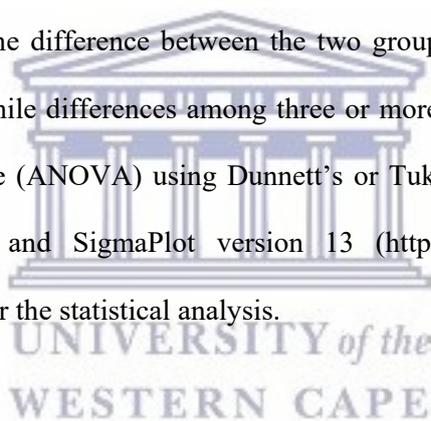
3.3.10 Caspase-9 Assay

Caspase-9 is an initiator caspase that plays a crucial role in many forms of drug-induced cell death triggered by release of cytochrome C from the mitochondria into the cytosol [356,357,504]. Upon the release of cytochrome C into the cytosol, procaspase-9 is activated through the formation of a multi-protein complex, termed an *apoptosome*, by the binding of cytochrome C to the activating factor Apaf-1 [505]. Once the apoptosome is formed and caspase-9 activated, downstream effector caspases, such as caspase-3, can then be activated

to promote the cell death programme. After the indicated treatments, cells were collected and lysed on ice for 10 min. Caspase-9 activity was measured using a colorimetric protease assay kit according to manufacturer's specifications (Invitrogen) and LEDH-pNA as substrate. The amount of free pNA, which absorbed the light, was determined by measuring absorbance at 405 nm using Glomax Multi-Detection System (Promega, USA).

3.4 Statistical Analysis

Each experiment was performed at least in triplicate and repeated three times. The results are presented as the means \pm standard error of the mean (SEM). IC_{50} values were estimated from nonlinear regression analysis using the log inhibitor vs normalized response module in GraphPad Prism (version 7.03 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). The difference between the two groups was analyzed using the two-tailed Student's t-test while differences among three or more groups were analyzed by one-way analysis of variance (ANOVA) using Dunnett's or Tukey's multiple comparisons test. GraphPad Prism 7.03 and SigmaPlot version 13 (<https://systatsoftware.com/products/sigmaplot/>) were used for the statistical analysis.



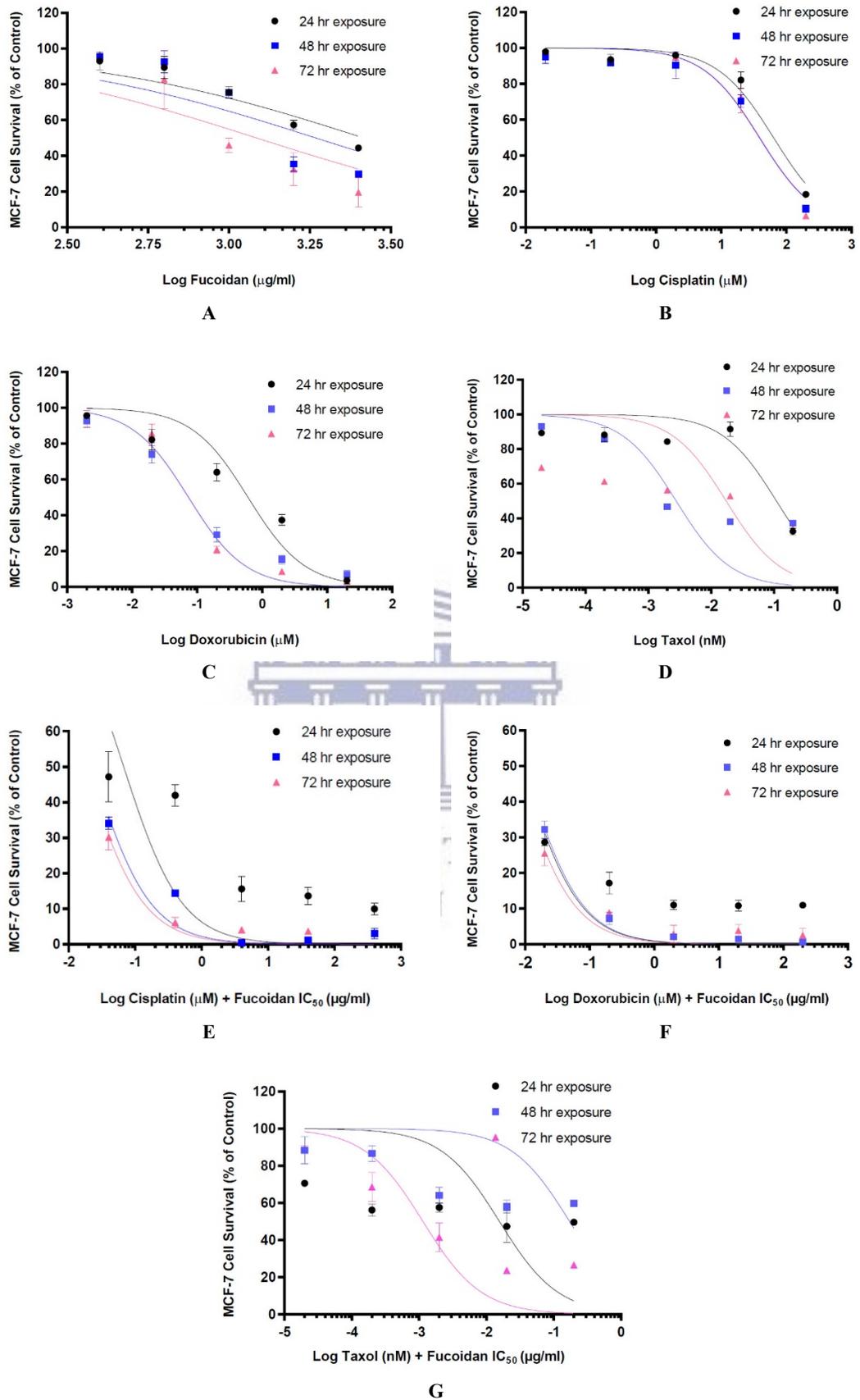
CHAPTER 4

RESULTS AND DISCUSSION

4.1 Cytotoxicity of Fucoidan and Anticancer Drugs in MCF-7 and MCF-12A Cells

Several studies have shown that fucoidan has anticancer activity against breast cancer cells *in vitro* and *in vivo* [456,506]. The primary aim of this investigation was to determine whether a combination of fucoidan (FUC) with cisplatin (CDDP), doxorubicin (DOX) and taxol (TAX) would exert enhanced potency in combination than their individual effects against MCF-7 breast cancer and non-malignant human breast MCF-12A epithelial cell lines. As a first step toward accomplishing this aim, we examined the cytotoxicity of each compound alone against human breast carcinoma cell line MCF-7 cells and a non-malignant cell line MCF1-2A as a control. The MTT cell viability assay was employed for this purpose. MCF-7 and MCF-12A cells were treated with fucoidan (400-1200 $\mu\text{g/ml}$), cisplatin (0.2-200 μM), doxorubicin (0.002-20 μM) and taxol (0.2-200 nM) for 24, 48 and 72 hours as described in the Materials and Methods section (Chapter 3).

Fucoidan reduced the viability of the MCF-7 in a dose- and time-dependent manner. The IC_{50} (concentration at which 50% of proliferating cells are inhibited) values of fucoidan against MCF-7 cells were lower than those obtained for non-cancerous MCF-12A cells, which implies that the cancerous MCF-7 cells were more sensitive to fucoidan cytotoxicity (Tables 4.1 to 4.3 and Figures 4.1 and 4.2). Cisplatin (CDDP) is a widely used chemotherapeutic agent for breast cancer [167,170,507]. In this study, CDDP suppressed both MCF-7 and MCF-12A cell growth in a time- and concentration-dependent manner (Figures 4.1 and 4.2, respectively). As presented in Tables 4.1 and 4.2, the IC_{50} values of CDDP against MCF-7 and MCF-12A cells decreased in a time-dependent manner.



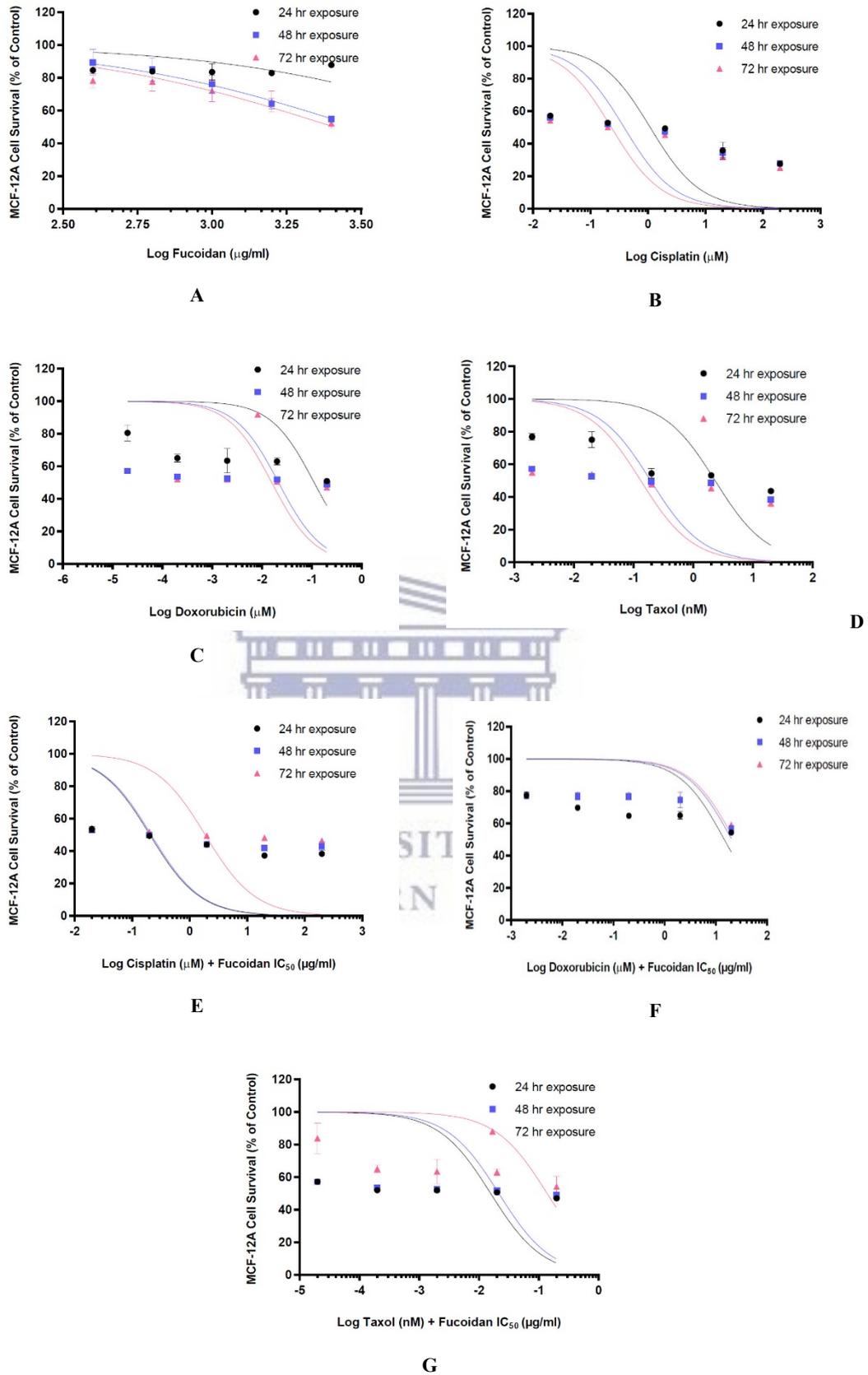
Graphs were drawn with GraphPad Prism version 7.03 for Windows, www.graphpad.com.

Figure 4.1: Effects of drugs alone and in combination with fucoïdan on MCF-7 cells

Table 4.1: Potency of drugs alone and in combination with fucoïdan in MCF-7 cells

Drugs	Time (h)	Drugs Alone				Drugs with Fucoïdan				Fucoïdan with Drugs				Potency Ratio for Drug	Potency Ratio for Fucoïdan
		IC ₅₀	95% CI		R ²	IC ₅₀	95% CI		R ²	IC ₅₀	95% CI		R ²	Drug Alone/Drug+Fuc	Fucoïdan Alone/Fuc+Drug
			Lower	Upper			Lower	Upper			Lower	Upper			
Fucoïdan (µg/ml)	24	2610	2238	3043	0.9										
	48	1854	1374	2502	0.7										
	72	1217	885.5	1672	0.7										
Cisplatin (µM)	24	63	52.52	76.88	0.9697	0.06	0.0359	0.1251	-0.2	326	280.2	378.7	0.8	1058	8.0
	48	38	30.31	48.81	0.9556	0.02	0.01879	0.02687	0.9	125	96.35	162.8	0.7	1923	14.8
	72	39	30.22	50.67	0.9546	0.02	0.01523	0.02019	0.9	103	79.27	133.4	0.6	1957	11.8
Doxorubicin (µM)	24	0.61	0.4067	0.9006	0.8886	0.01	0.0058	0.0142	-1.14	153	137.6	169.8	0.8	61	17.1
	48	0.07	0.05593	0.09612	0.9504	0.01	0.0089	0.0106	0.9	104	77.33	140.9	0.6	7	17.8
	72	0.07	0.05977	0.09185	0.9767	0.01	0.0059	0.0087	0.8	93	75.56	115	0.7	7	13.1
Taxol (nM)	24	0.11	0.07562	0.1576	0.7932	0.02	0.0049	0.0487	-12.68	1343	1118	1614	-0.2	7	1.9
	48	0.002	0.001333	0.005753	0.2524	0.17	0.07436	0.3962	-1.874	2706	2303	3180	0.7	0.012	0.7
	72	0.02	0.006394	0.04683	-4.713	0.00	0.0006	0.002	0.5	971	772.1	1220	0.8	18.2	1.3

IC₅₀ values were estimated from nonlinear regression analysis using the log inhibitor vs. normalized response module in GraphPad Prism version 7.03 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.



Graphs were drawn with GraphPad Prism version 7.03 for Windows, www.graphpad.com.

Figure 4.2: Effects of drugs alone and in combination with fucoïdan on normal MCF-12A cells

Table 4.2: Potency of drugs alone and in combination with fucoïdan in MCF-12A cells

Drugs	Time (h)	Drugs Alone				Drugs with Fucoïdan				Fucoïdan with Drugs				Potency Ratio for Drug Alone/Drug+Fuc	Potency Ratio for Fucoïdan Alone/Fuc+Drug
		IC ₅₀	95% CI		R ²	IC ₅₀	95% CI		R ²	IC ₅₀	95% CI		R ²		
			Lower	Upper			Lower	Upper			Lower	Upper			
Fucoïdan (µg/ml)	24	8624	5525	13461	-9										
	48	3078	2665	3555	0.8803										
	72	2565	2148	3063	0.6614										
Cisplatin (µM)	24	1.1	0.2587	4.648	-6.178	0.2	0.04436	0.9378	-25.8	758.1	598.5	960.4	-1.13	5.50	11.4
	48	0.39	0.09123	1.63	-6.725	0.22	0.04193	1.105	-64.23	826.2	617.6	1105	-5.91	1.77	3.7
	72	0.23	0.05964	0.8994	-5.917	1.87	0.3244	10.77	-171.7	1003	729.1	1381	-18.42	0.12	2.6
Doxorubicin (µM)	24	0.11	0.03055	0.4076	-6.03	14.65	4.292	49.98	-10.09	2243	1846	2725	0.2	0.0075	3.8
	48	0.02	0.003814	0.1215	-181.2	20.48	7.947	52.76	-4.741	3192	2549	3998	0.2	0.0010	1.0
	72	0.02	0.00275	0.08979	-124.8	22.92	9.146	57.42	-5.417	3433	2759	4272	0.1	0.0009	0.7
Taxol (nM)	24	2.33	0.6604	8.206	-124.8	0.015	0.00275	0.08979	-124.8	1095	801.4	1497	-12.78	155	7.8758
	48	0.19	0.03797	0.9896	-181.2	0.02	0.003814	0.1215	-181.2	1164	841.4	1610	-20.55	10	2.6443
	72	0.13	0.02652	0.6624	-4.992	0.14	0.03792	0.504	-4.992	2161	1689	2766	0.3349	1	1.1870

IC₅₀ values were estimated from nonlinear regression analysis using the log inhibitor vs. normalized response module in GraphPad Prism version 7.03 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

Table 4.3: IC₅₀ values of fucoïdan cisplatin, doxorubicin and taxol for MCF-7 and MCF-12A cell lines

Individual Treatment			
Drug	Time (h)	IC ₅₀ Values*	
		MCF-7	MCF-12A
Fucoïdan mg/ml	24	2610	8624
	48	1854	3078
	72	1217	2565
Cisplatin µM	24	63.54	1.10
	48	38.46	0.39
	72	39.13	0.23
Doxorubicin µM	24	0.61	0.11
	48	0.07	0.02
	72	0.07	0.02
Taxol nM	24	0.11	2.33
	48	0.002	0.19
	72	0.02	0.13
Combination Treatment			
Drug	Time (h)	IC ₅₀ Values*	
		MCF-7	MCF-12A
Fucoïdan/Cisplatin	24	0.06	0.20
	48	0.02	0.22
	72	0.02	1.87
Fucoïdan/Doxorubicin	24	0.01	14.65
	48	0.01	20.48
	72	0.01	22.92
Fucoïdan/Taxol	24	0.015	0.015
	48	0.17	0.02
	72	0.13	0.14

*See Tables 4.1 and 4.2 for associated 95% confidence intervals. IC₅₀ values were estimated from nonlinear regression analysis using the log inhibitor vs. normalized response module in GraphPad Prism version 7.03 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

The IC_{50} s for single exposure of both MCF-7 and MCF-12A to DOX and TAX was lower than CDDP (Tables 4.1 to 4.3), implying that DOX and TAX were more potent than CDDP in these cells. However, when MCF-7 cells were exposed to a combination of FUC and CDDP, the IC_{50} for FUC decreased 8-, 14.8 and 11.8-fold for the 24, 48 and 72h exposure times, respectively (Table 4.1). More remarkably, in MCF-7 cells, the combination of FUC and CDDP increased the potency of CDDP in the combination 1058-, 1923- and 1957-fold, respectively for the 24, 48 and 72h exposures (Table 4.1). A decrease in IC_{50} of a drug signifies an increase in its potency. The combination of FUC and DOX also increased the potency of FUC in MCF-7 cells, i.e., 17-, 17.8- and 13-fold for the 24, 48 and 72h time points whereas the potencies for DOX in the combination increased 61-fold after 24h, but only 7-fold for the 48 and 72h exposures (Table 4.1).

The increased potencies for FUC in combination with CDDP and DOX, respectively, were almost identical, but the potency for CDDP was increased more significantly. In the case of the combination of FUC with TAX, increases in potency were noted for both TAX and FUC after 24 and 48h while the potency for TAX was increased 18-fold after 72h (Table 4.1). For MCF-12A cells, the combination of FUC with CDDP increased their respective potencies by 11.4- and 5.5-fold after 24h, but moderately for FUC (3.7- and 2.6-fold for the 48 and 72h exposure times, whereas neither FUC nor DOX exhibited increased potencies for all the times, except for FUC at 7.8-fold after 24h (Figure 4.2).

In fact, in combination with FUC, the potency of DOX was decreased almost a 1000-fold for all the times of exposure (e.g., at 24h, the IC_{50} of 0.11 μ M for DOX in the combination with FUC was increased to 14.65 μ M). In the case of TAX combined with FUC, only TAX exhibited increased toxicity (155- and 10-fold after 24 and 48h) while FUC show improved potencies of 7.8-, and 2.6-fold after 24 and 48h. As presented in Table 4.3, FUC enhanced the anti-proliferative influence of CDDP against MCF-7 cells while the cytotoxicity effect of CDDP was ameliorated in MCF-12A cells. Treatment of MCF-7 cells with DOX alone showed IC_{50}

values of 0.61, 0.07 and 0.07 μM at 24, 28 and 72 h, respectively, while against MCF-12A cells the values were 0.11, 0.02 and 0.02 μM . This implies that MCF-12A cells were slightly more sensitive to DOX alone than MCF-7 cells. Fucoidan, however, increased the sensitivity of MCF-7 to DOX while it significantly ($p < 0.0001$) exacerbated the sensitivity of MCF-12A cells to DOX. This finding is consistent with recent reports that prospective studies of anthracycline-free platinum-containing neoadjuvant chemotherapy are warranted in locally advanced breast cancer patients with HER2⁺ and triple-negative breast cancer [144].

Taxol (TAX) is an active microtubule interfering agent for the treatment of advanced breast cancer [198,199]. The cytotoxic effects of TAX against both MCF-7 and MCF-12A cells increase in a time- and dose dependent fashion as presented in Tables 4.1 to 4.3. Judged by the IC₅₀ values in the combined treatment presented in Table 4.3, FUC did not protect MCF-12A cells from the cytotoxicity effect of TAX. This probably implies that FUC might not play a role in the mechanism involved in TAX-induced anti-proliferative activity.

These findings indicated that the chemotherapeutic drugs showed indiscriminate dose and time dependent cytotoxicity to both the cancerous and non-cancerous breast cells as presented in Figures 4.2 and 4.3 and Tables 4.1 to 4.3. However, FUC does not compromise the cytotoxic effects of the chemotherapeutic drugs against MCF-7 cells while the cytotoxic effect of DOX and CDDP were notably reduced in the presence of FUC against MCF-12A cells.

4.2 Morphological Effects of Fucoidan and Anticancer Drugs on MCF-7 and MCF-12A Cells

The images visualized and captured with a Zeiss microscope confirm clearly that varied morphological changes occur in MCF-7 breast cancer cells and MCF-12A normal breast epithelia cells following 24 hours exposure to 400, 600, 800, 1000 and 1200 $\mu\text{g/ml}$ FUC in combination with IC₅₀ concentrations of CDDP, DOX and TAX (Figures 4.3 to 4.6), but this was not investigated further.

Fucoidan induced morphological changes, such as a reduction in cell volume comparing with the control and under the same conditions. The results indicated that single treatment with the mentioned concentrations of FUC decreased the MCF-7 cells viability in a time-dependent manner. After 24 hours of culturing, 1000 $\mu\text{g/ml}$ FUC significantly decreased viable MCF-7 cell number as compared with those of untreated controls. Additionally, the combination treatment on MCF-7 showed that FUC with the chemotherapeutic drugs strongly inhibited MCF-7 viability.

4.3 Apoptotic Effects of Fucoidan and Anticancer Drugs on MCF-7 and MCF-12A Cells

To determine whether FUC-induced cytotoxicity on MCF-7 breast cancer cells occurs via apoptotic or necrotic cell death, the cells were stained with Annexin-V-FITC / PI double staining using flow cytometry. Live cells are negative for both PI and annexin (lower left quadrant), early-stage apoptotic cells are PI-negative and annexin-positive (upper left quadrant), late-stage apoptotic cells are positive for both PI and annexin (upper right quadrant), and necrotic cells are positive for PI and negative for annexin-V (lower right quadrant). The data showed that FUC treatment caused cells to lose their phospholipid membrane asymmetry. The exposure of phosphatidylserine to the outside of the plasma membranes was detected by annexin-V-FITC staining in MCF-7 cells.

The annexin-V staining results (Figures 4.7 and 4.8) indicate that FUC (400, 800, 1000 $\mu\text{g/ml}$) causes a significant ($p < 0.001$) increase in the number of early apoptotic cells (annexin-V-positive) in a dose-dependent manner (19.5%, 21.8%, 31.8%, respectively (Figure 4.8A). The effect of FUC (1000 $\mu\text{g/ml}$) on MCF-7 cancer cells was significantly higher than the 400 and 800 $\mu\text{g/ml}$ treatments. Figure 4.8B shows the results of the same treatments on late-stage apoptosis in MCF-7 cancer cells. FUC at 400 and 800 $\mu\text{g/ml}$ increased late stage apoptosis in MCF-7 cells, but not at 1000 $\mu\text{g/ml}$, all relative to control.

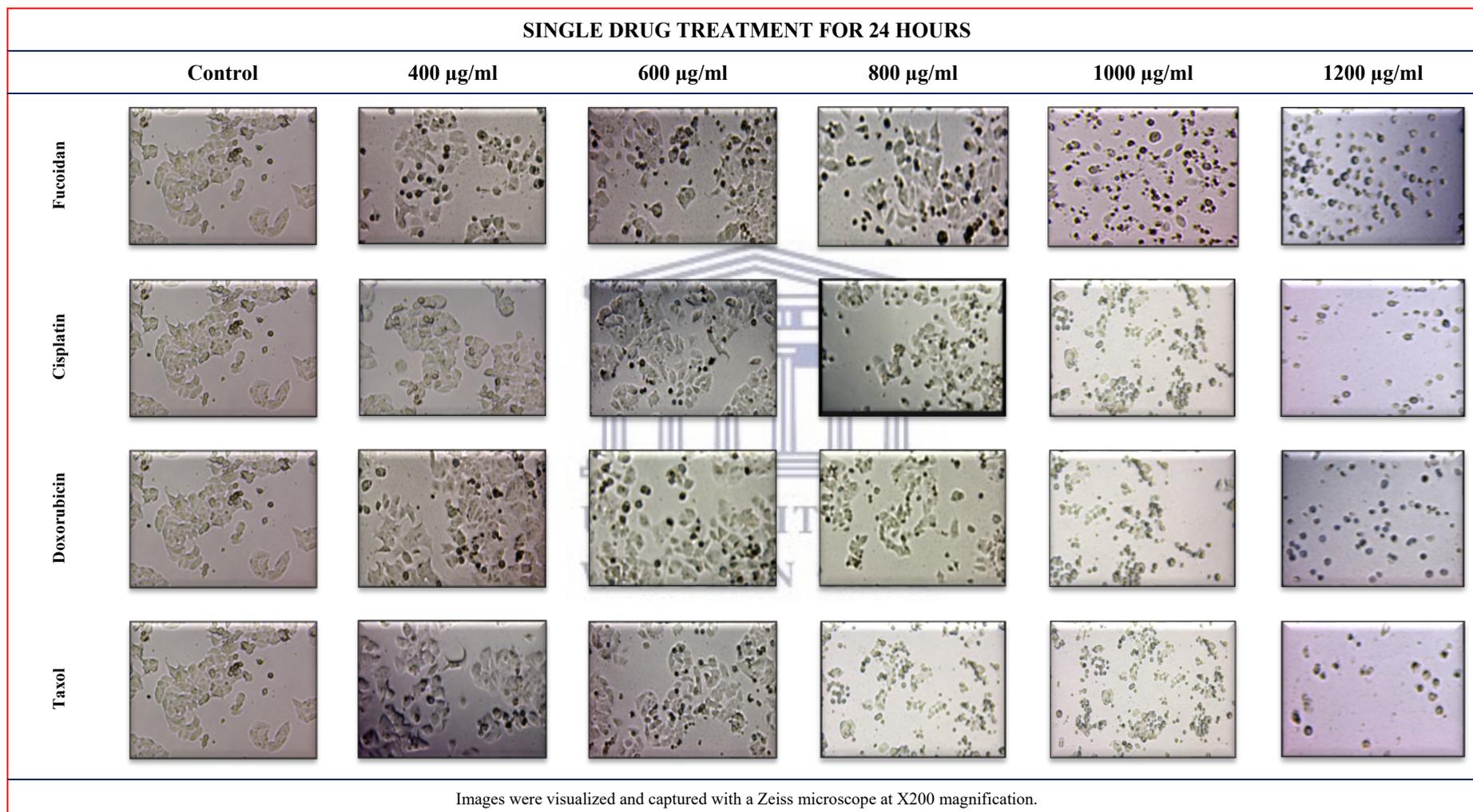


Figure 4.3: Morphological effects of single drug treatment on MCF-7 cells

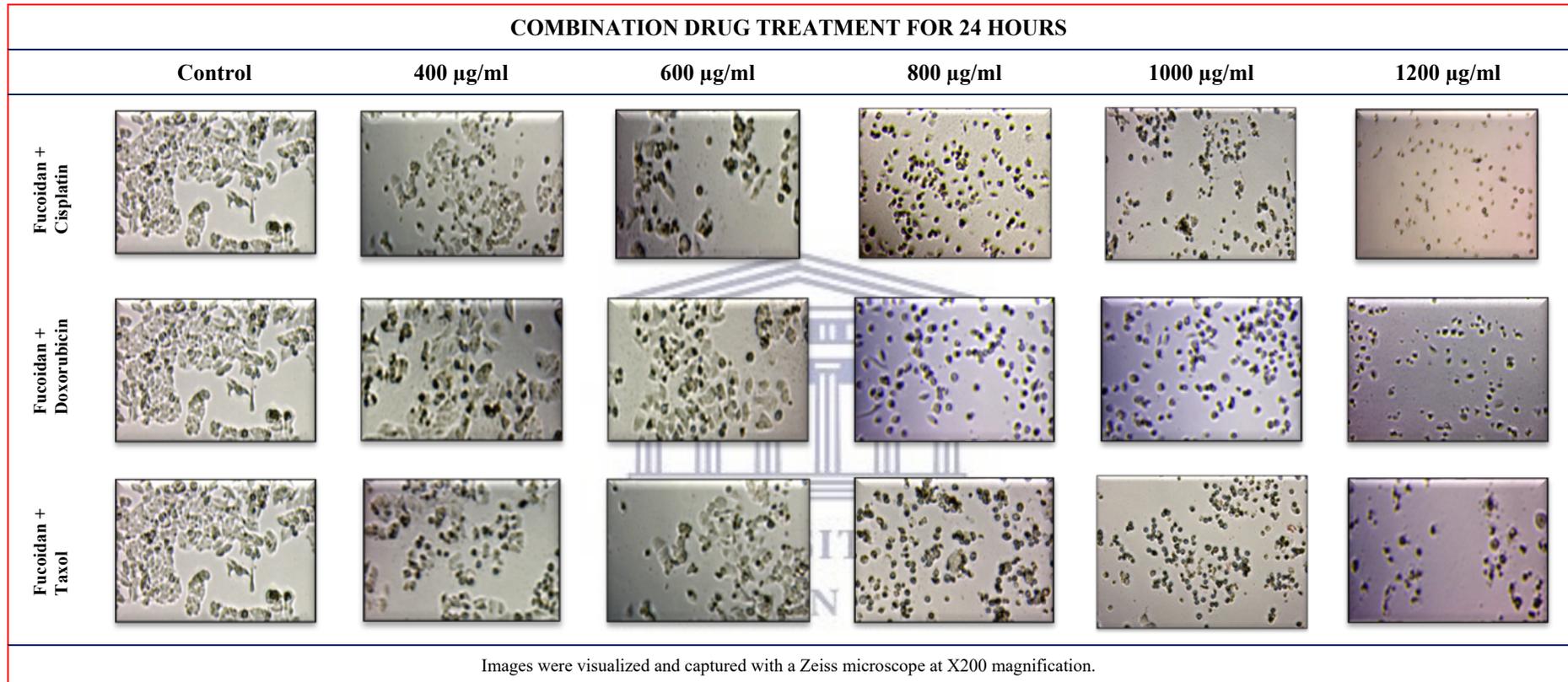


Figure 4.4: Morphological effects of combination drug treatment on MCF-7 cells

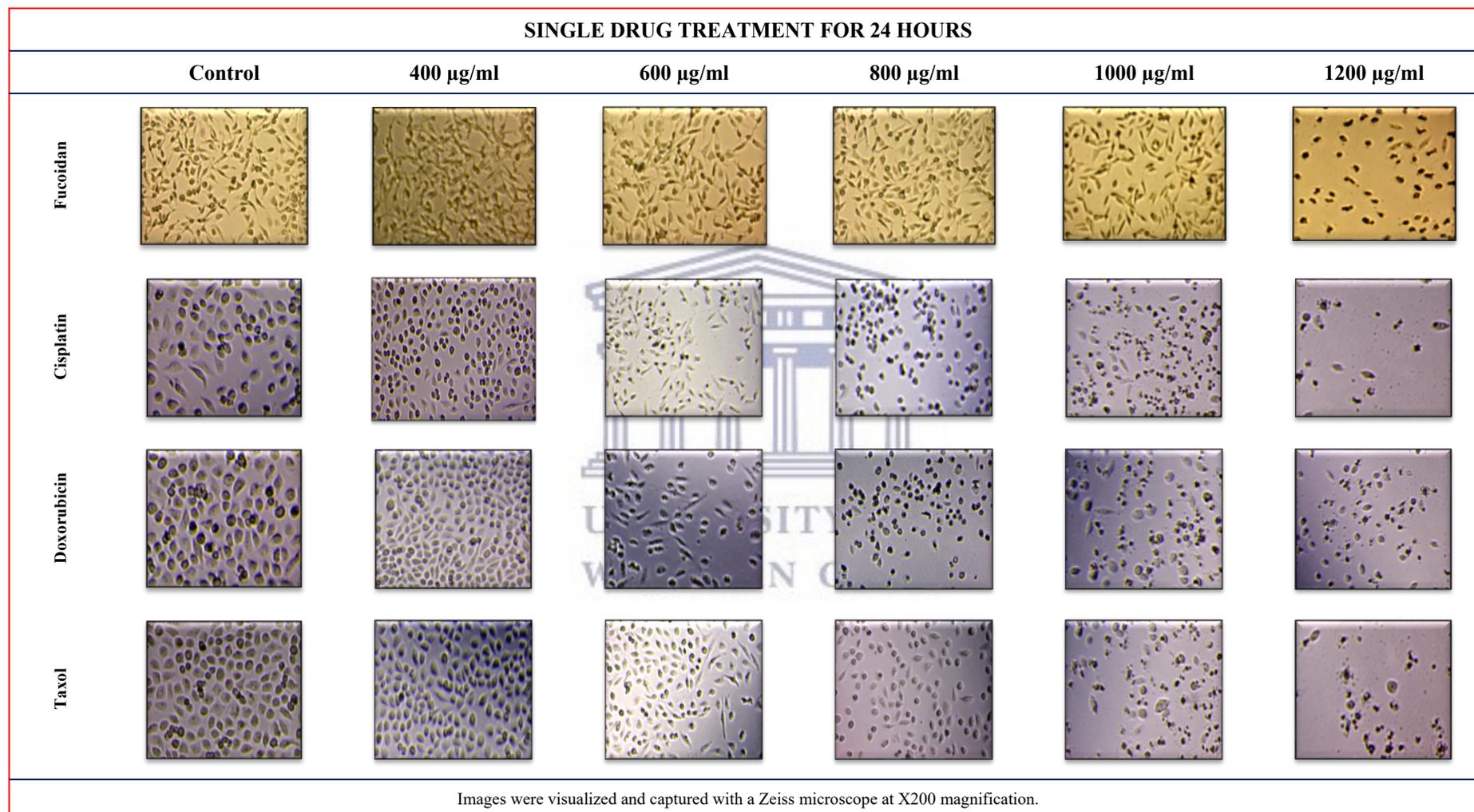


Figure 4.5: Morphological effects of single drug treatment on MCF-12A cells

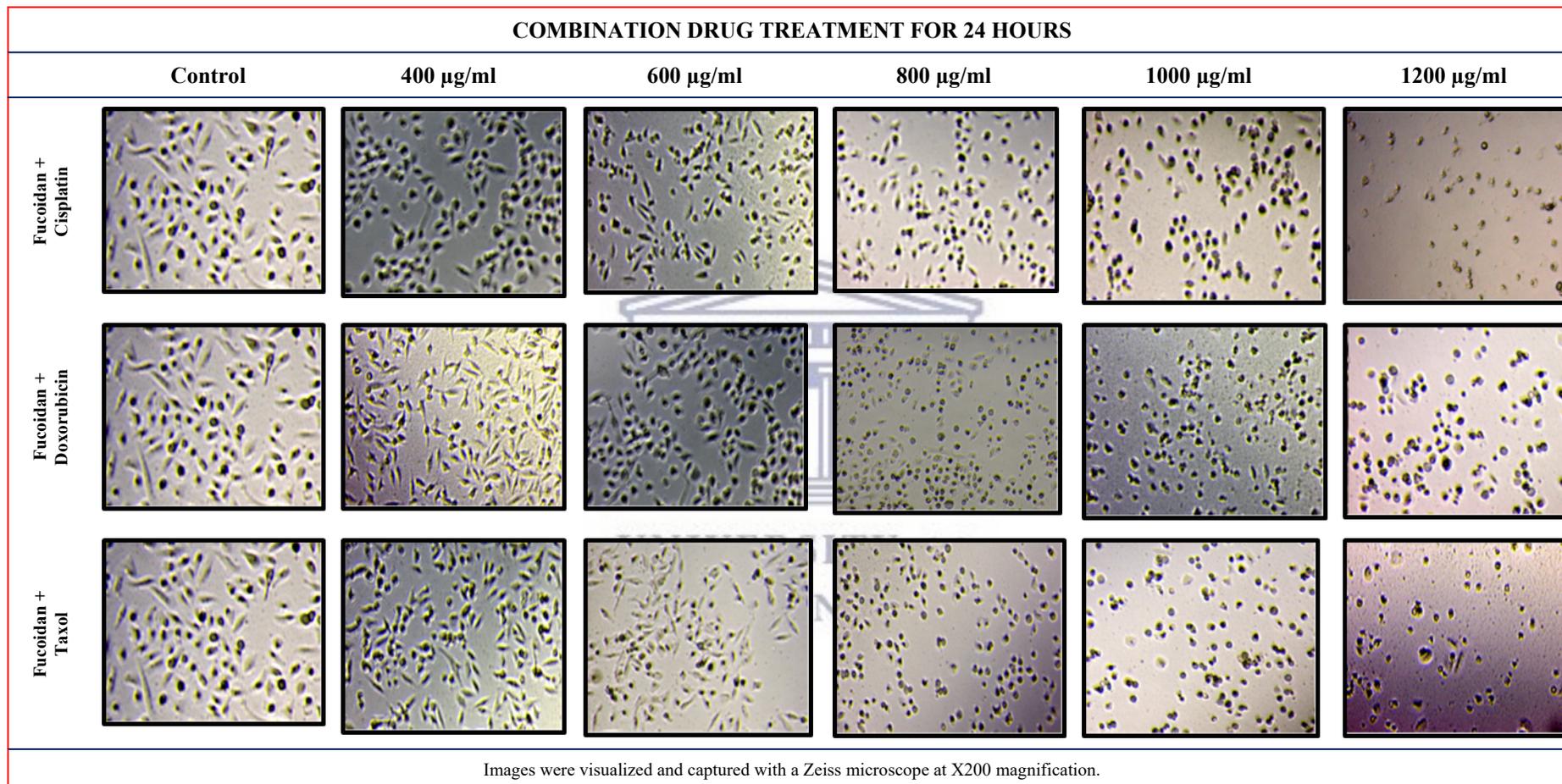


Figure 4.6: Morphological effects of combination drug treatment on MCF-12A cells

Figure 4.8B shows the results of the same treatments on late-stage apoptosis in MCF-7 cancer cells. FUC at 400 and 800 $\mu\text{g/ml}$ increased late stage apoptosis in MCF-7 cells, but not at 1000 $\mu\text{g/ml}$, all relative to control. However, CDDP alone and in combination with FUC significantly ($p < 0.001$) increased late-stage apoptosis in MCF-7 cancer cells as did DOX alone and in combination with FUC at 800 and 1000 $\mu\text{g/ml}$. TAX alone had no effect on late-stage apoptosis, but TAX in combination with FUC 400 and 1000 $\mu\text{g/ml}$ did.

Figures 4.9 and 4.10 A and B summarize similar experiments performed on MCF-12A normal non-malignant breast epithelial cells. FUC, CDDP, DOX and TAX significantly ($p < 0.001$) induced an increase in early apoptosis of MCF-12A cells (Figures 4.9 and 10A). Remarkably, the early apoptosis-inducing effects of these drugs were found to be attenuated when combined with FUC in a concentration-dependent manner. FUC alone at 1000 $\mu\text{g/ml}$ significantly ($p < 0.001$) enhanced late-apoptosis in MCF-12A cells as did CDDP alone and in combination with 400 and 1000 $\mu\text{g/ml}$ FUC (Figure 10B). DOX or TAX alone did not affect late-apoptosis, but did so significantly when administered in combination with 400, 800 and 1000 $\mu\text{g/ml}$ FUC.

4.4 Effects of Fucooidan and Anticancer Drugs on Caspase Activation in MCF-7 and MCF-12A Cells

Initiator caspase-8 and -9 and effector caspase-3 and -7 perform critical roles in the induction of apoptosis. Activated initiator caspases can cleave and activate effector caspases, which, in turn, cleave a variety of cellular substrates, most notably poly-ADP-ribose [508]. Fucooidan-induced cell apoptosis in cancer [421,425,426,455,474,489,509], including MDA-MB-231 [459] and MCF-7 [482,485,510] breast cancer cells. In this study, to confirm the involvement of caspase activation in MCF-7 and MCF-12A cells exposed to CDDP, DOX, TAX and FUC alone and in combination, we evaluated the activation of caspase-3/-7 as well as caspase-9. In MCF-7 cells (Figures 4.11 and 4.13A), a significant ($p < 0.05$) dose-dependent increase in caspase-3 and/or -7 was observed after 24h of treatment with all the single and combinations of drugs relative to control, implying involvement caspases in apoptosis of these cells.

Similar results were also obtained for caspase-9 activity in MCF-7 cells (Figure 4.14A), except FUC (400 µg/ml) treatment. In the case of and MCF-12A cells, FUC (400 µg/ml) treatment did not produce any significant effect on caspase-3 and/or -7 activity, but all other treatments did (Figures 4.12 and 4.13B). Also, all treatments produced significant caspase-9 effects in MCF-12A cells, except FUC alone (400, 800 and 1000 µg/ml), FUC (400, 800 µg/ml) + CDDP, FUC (400, 800 and 1000 µg/ml) + DOX, and FUC (400, 800 and 1000 µg/ml) + TAX (Figure 4.14B). Thus, single exposure of MCF-12A cells to CDDP, DOX and TAX induced caspase-9 activity, but not FUC alone or any of its combinations with CDDP (except in combination with FUC 1000 µg/ml), DOX and TAX.

These results suggested that the fucoïdan from the brown algae *Fucus vesiculosus* could enhance CDDP, DOX and TAX induced apoptosis in human breast cancer cells by activating initiator and effector caspases, which ultimately cause the morphological and biochemical changes observed in apoptotic cells. The findings also point to increased efficacy or synergism of the combinatorial effects between FUC and CDDP, DOX and TAX in breast cancer cell lines.

4.5 Effects of Fucoïdan and Anticancer Drugs on Cell Cycle Arrest in MCF-7 and MCF-12A Cells

Cancer cells exhibit deregulation of cell cycle and apoptosis and activation of oncogenic signal transduction pathway, resulting in abnormal proliferation [26,307,477,478]. The effects of FUC, CDDP, DOX and TAX alone or in combination on cell cycle distribution of MCF-7 and MCF-12A cells was determined by flow cytometry and showed alterations in Sub-G1, G0/G1, S, and G2/M phases of cell cycle under different treatment conditions for 24 hours (Figures 4.15 o 4.18). In untreated MCF-7 cells, 4.20% of control cells were in Sub-G1 58.20% of control cells were in G1 phase, 16.10% in S phase and 21.50% in G2/M phase (Figures 4.15 and 4.16). In untreated MCF-12A cells, 2.20% of control cells were in Sub-G1, 61.80% in G1 phase, 14.60% in S phase and 21.40% in G2/M phase (Figures 4.17 and 4.18).

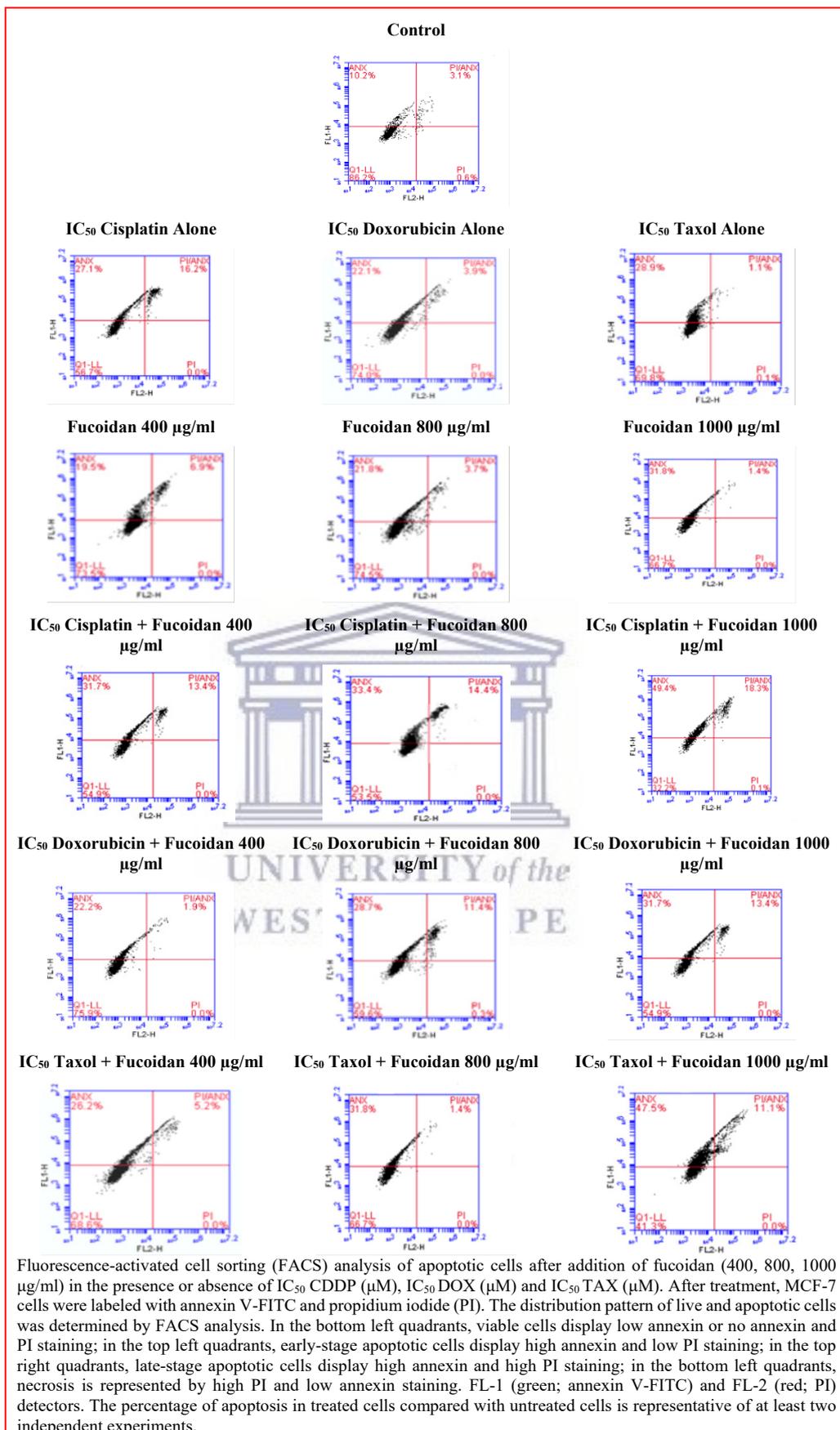


Figure 4.7: Apoptotic effects of drug treatments on MCF-7 cells

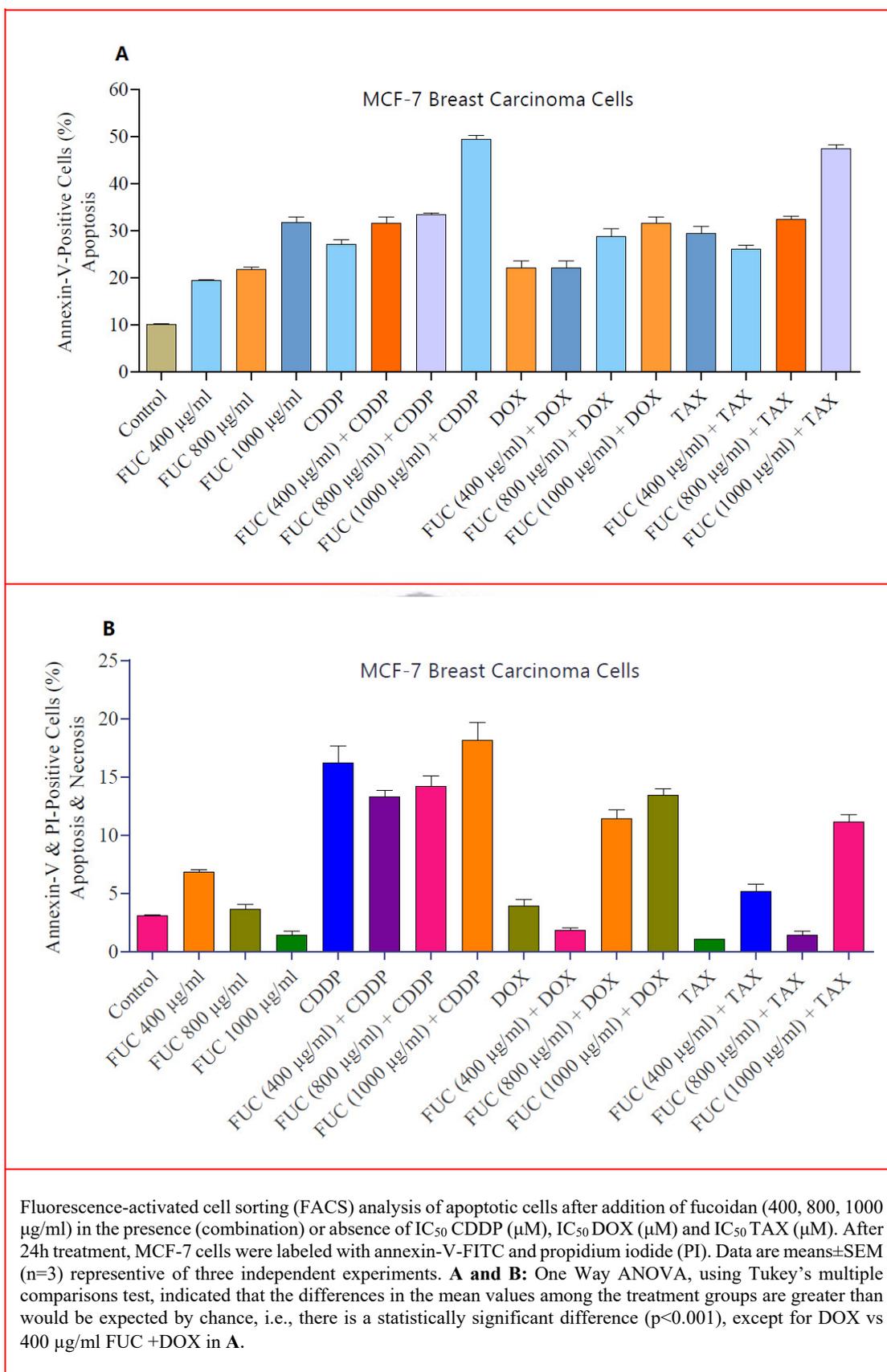


Figure 4.8: Analysis of apoptotic MCF-7 cells by annexin-V-PI staining

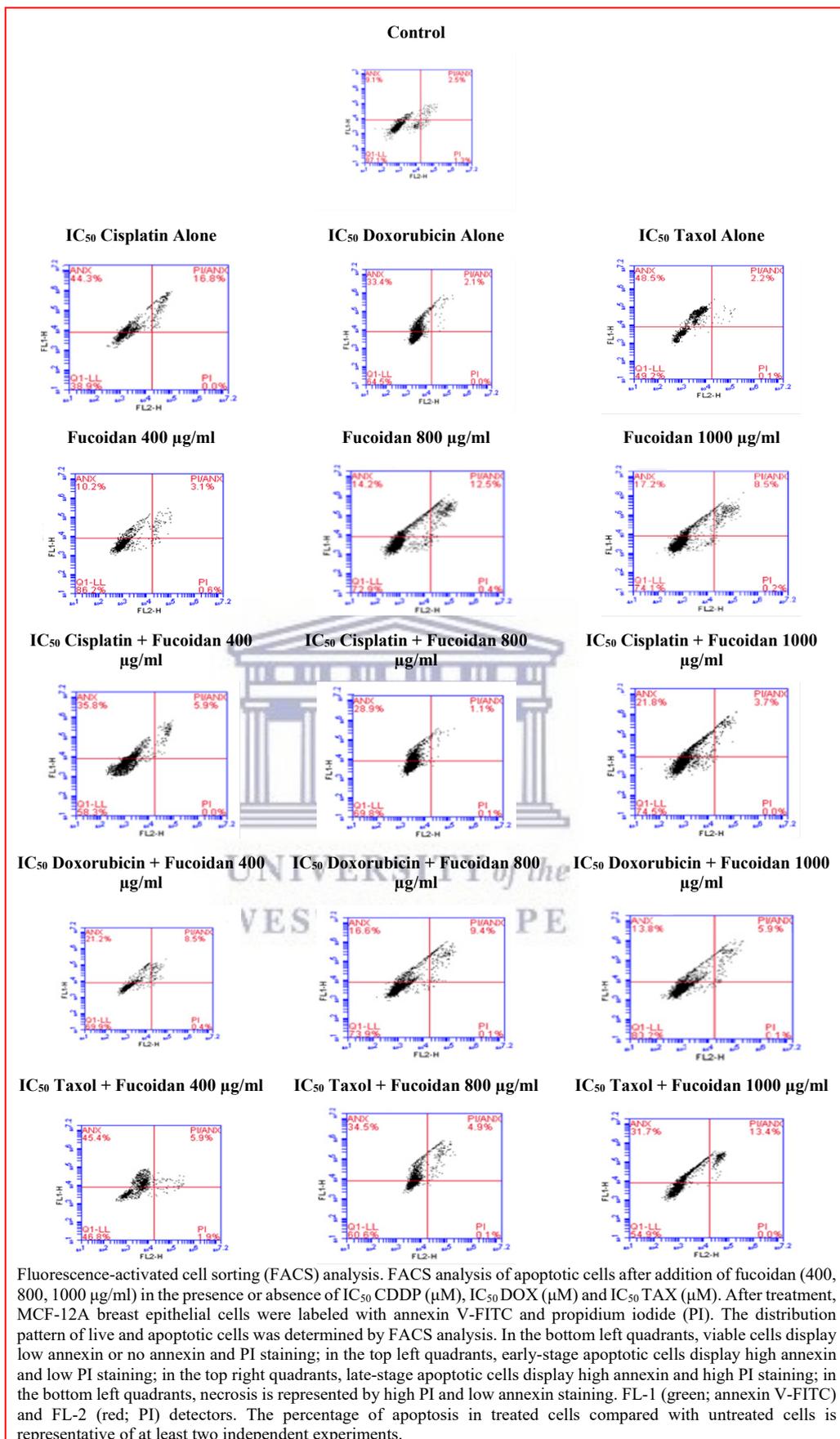


Figure 4.9: Apoptotic effects of drug treatments on MCF-12A cells

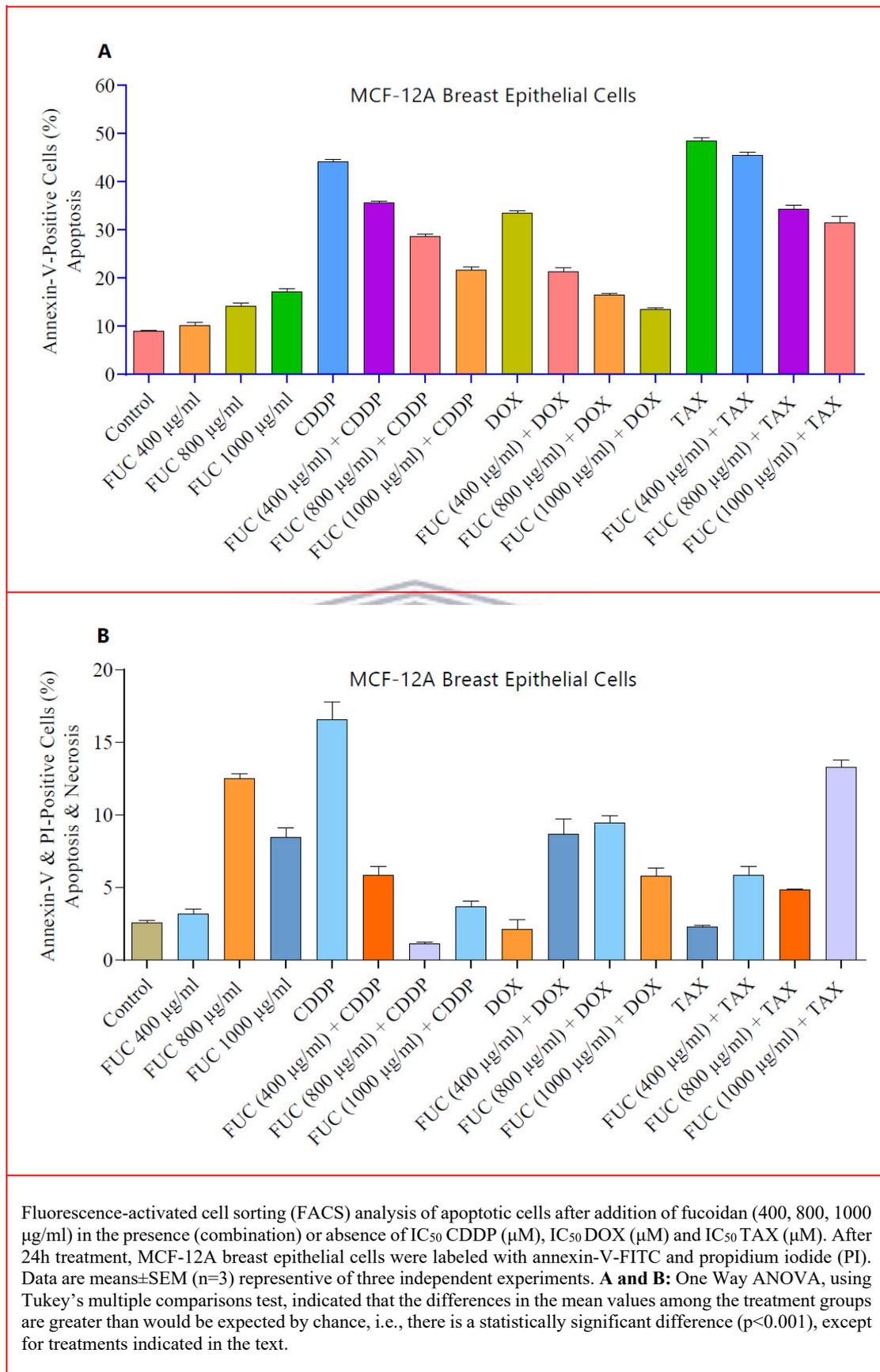


Figure 4.10: Analysis of apoptotic MCF-12A cells by annexin-V-PI staining

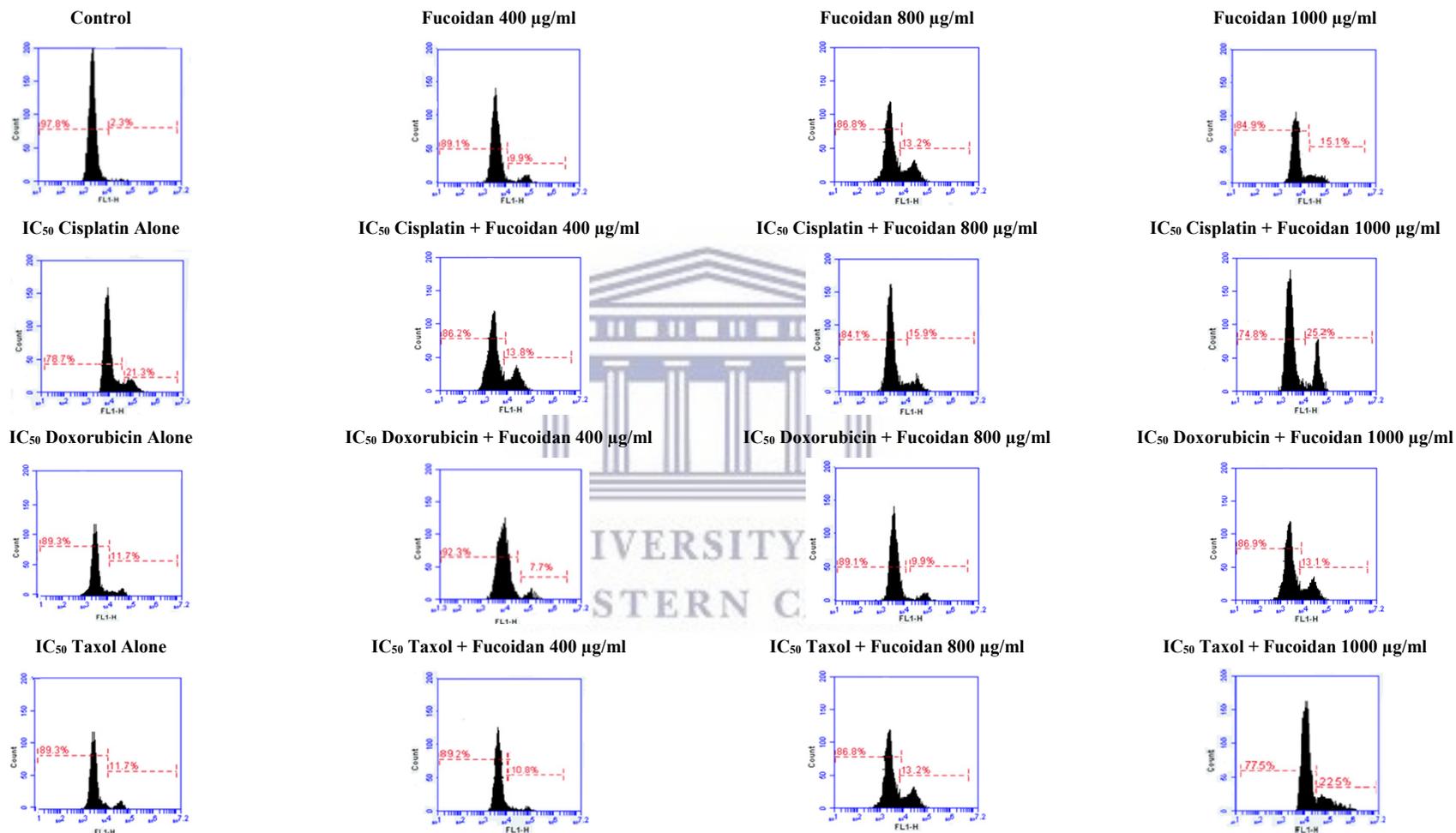


Figure 4.11: Effects of fucoidan and anticancer drugs on caspase activation in MCF-7 cells

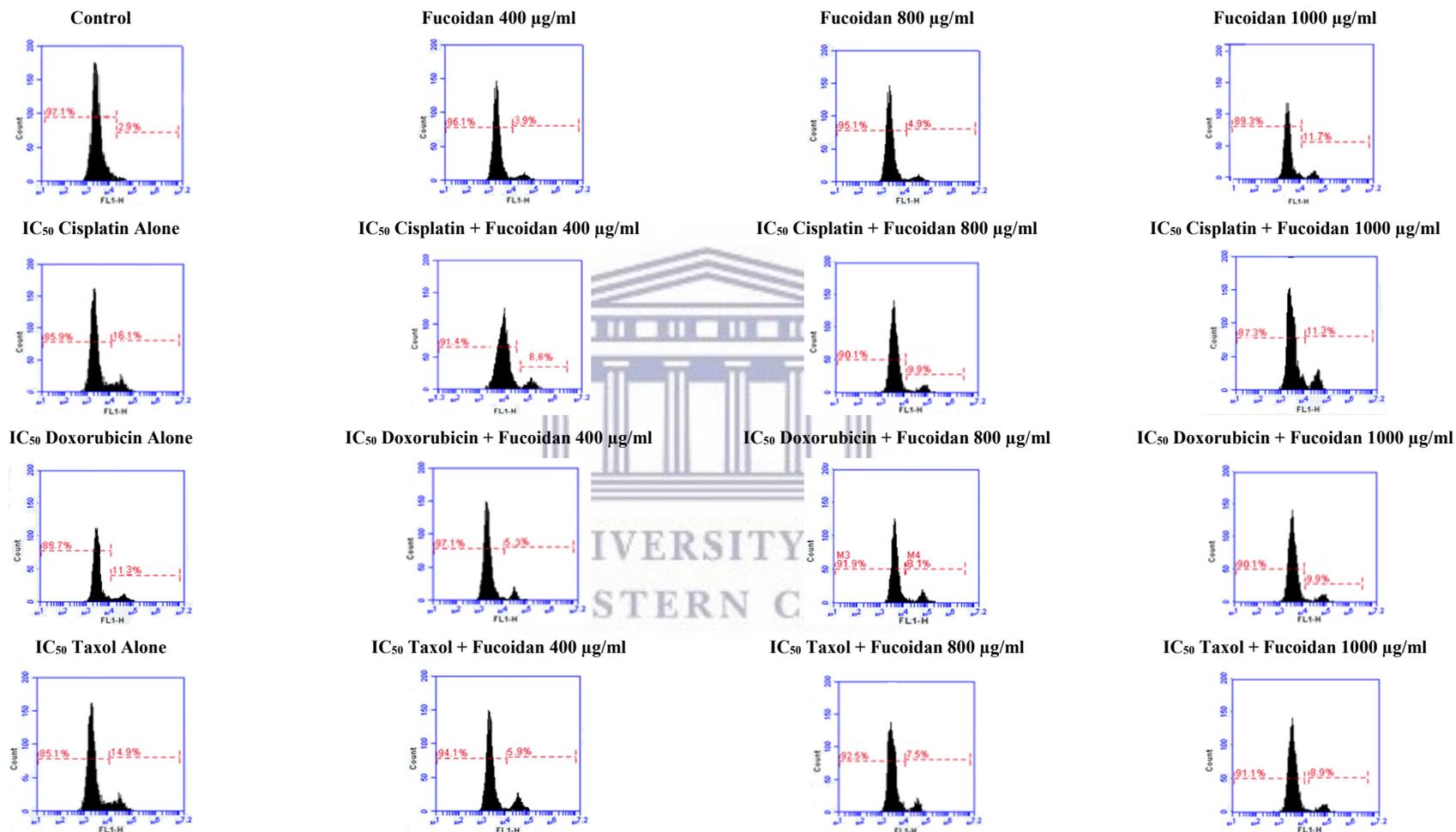


Figure 4.12: Effects of fucoidan and anticancer drugs on caspase activation in MCF-12A cells

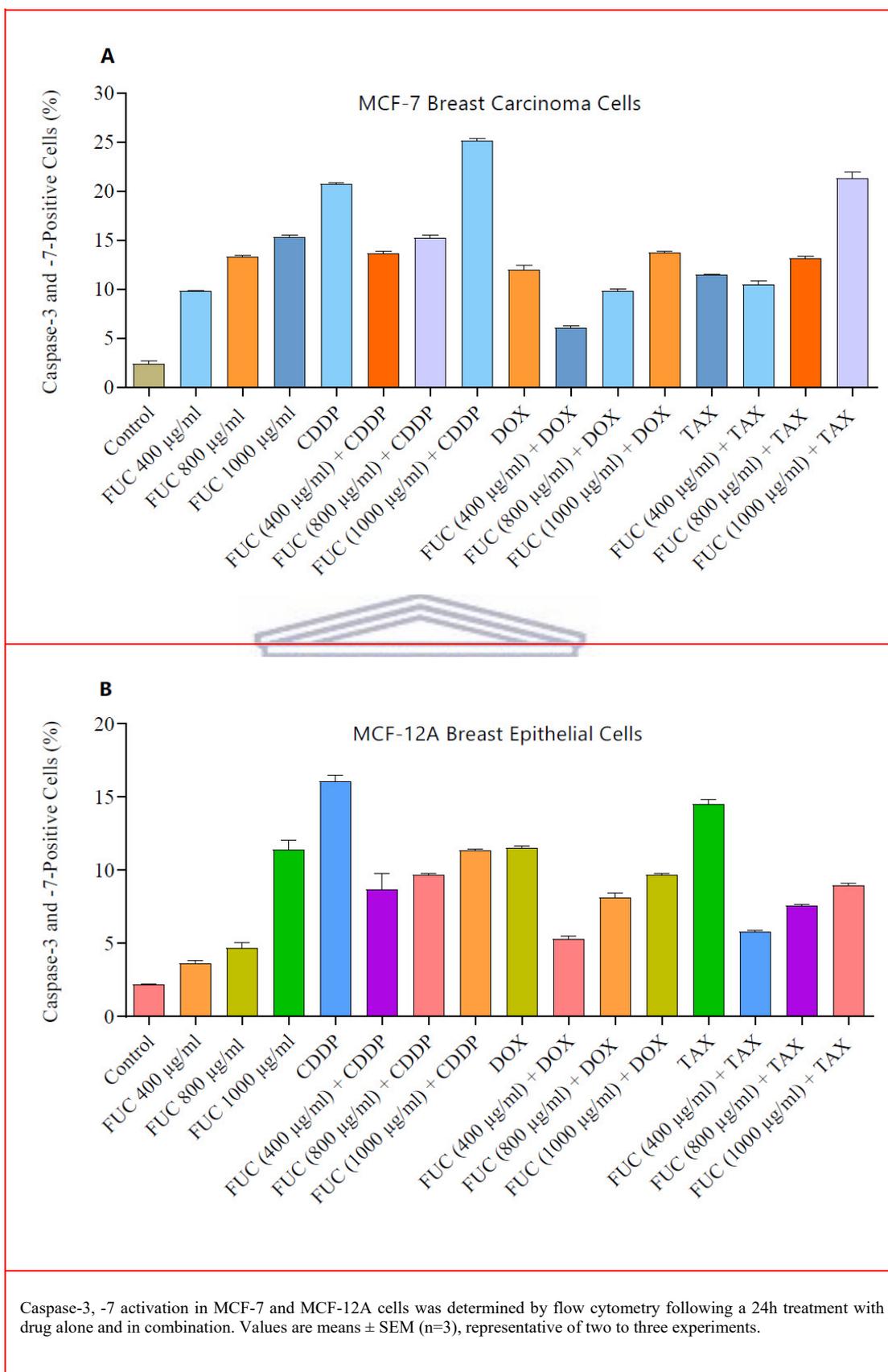


Figure 4.13: Analysis of caspase-3 and -7 activities in the MCF-7 and MCF-12A breast cell lines

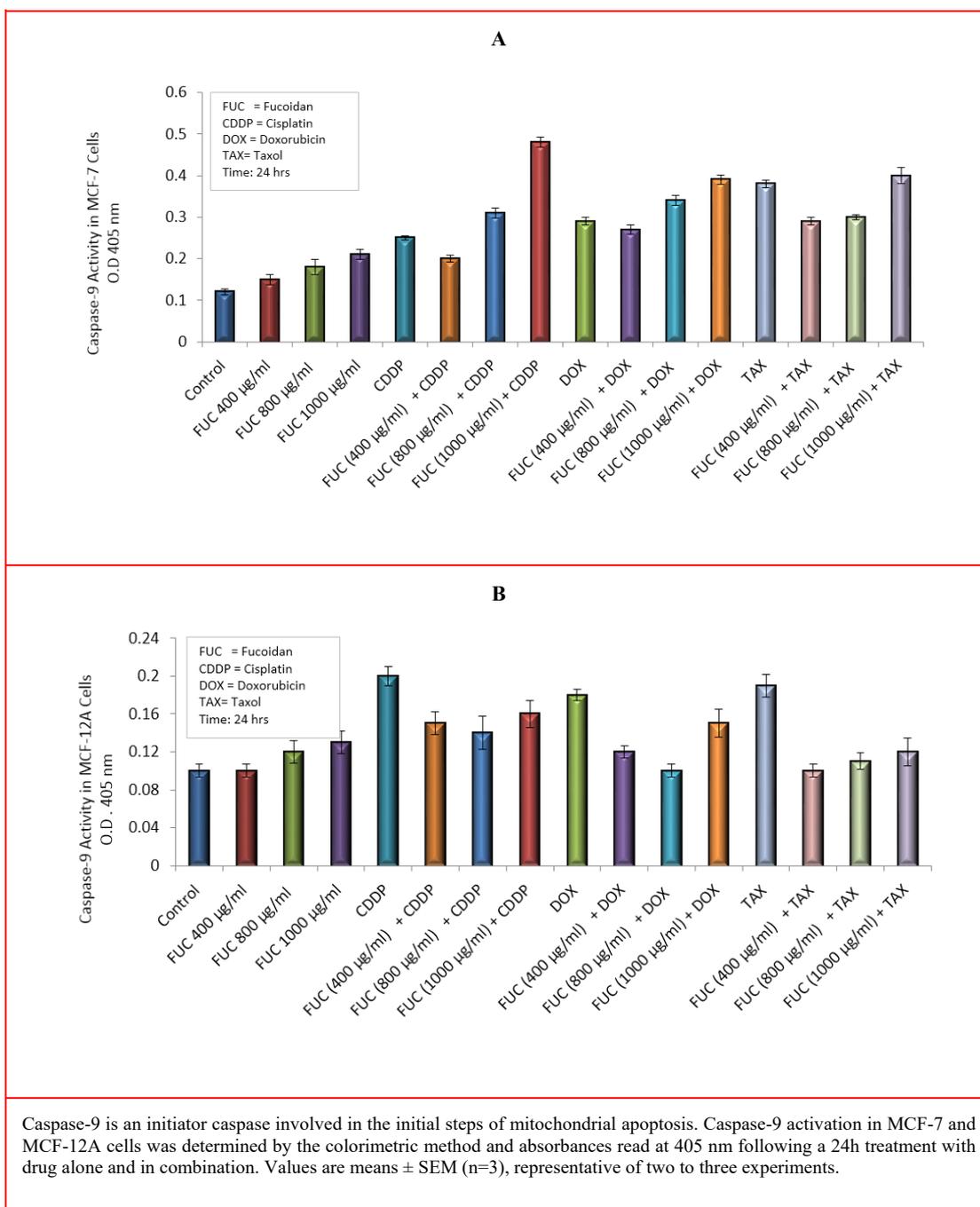


Figure 4.14: Analysis of caspase-9 activity in the MCF-7 and MCF-12A breast cell lines

Fucoïdan interfered with the population of cells in cell cycle phases in a concentration dependent manner. FUC (1000 µg/ml) significantly ($p < 0.0001$) reduced the cell population undergoing DNA synthesis (S-phase) and increased the Sub-G1 phase of MCF-7 cells (Figures 4.15 and 4.16). The treatment reduced the cell population in S-phase from 16% at 400 µg/ml FUC to 4% at 1000 µg/ml FUC when compared with untreated control cells.

Over the same concentration range, populations in the Sub-G1 phase increased from 5% to 24%. G1 and G2/M populations showed no significant changes at 400-800 $\mu\text{g/ml}$ FUC treatments, but at 1000 $\mu\text{g/ml}$, FUC significantly ($p < 0.0001$) reduced G2/M cells population from 21% to 13%. Taken together, these results suggest that the growth inhibitory effect of FUC on MCF-7 cells is the result of it blocking the G1 phase. Individual IC_{50} concentrations of CDDP, DOX and TAX and their combinations with FUC at 400, 800 and 1000 $\mu\text{g/ml}$ were used to treat MCF-7 cells. The three antineoplastic drugs affected the cell cycle of MCF-7 in the same fashion (Figures 4.15 and 4.16). G1- and S- phase populations were reduced, while G2/M- and Sub-G1 phase cell populations increased.

When combined with FUC, these drugs attenuated the effects of FUC on S-phase and Sub-G1 phase cell populations of MCF-7 (Figures 4.15 and 4.16). Conversely, FUC did not have a significant effect on the MCF-12A cell cycle except at a concentration of 400 $\mu\text{g/ml}$ (Figures 4.17 and 4.18). At this concentration, Sub-G1 populations increased non-significantly from 1% to 7% while S-phase cell populations decreased from 11% to 15%. CDDP, DOX and TAX treatment displayed the same pattern on MCF-12A cells as seen by a significantly lowered population of cells in S-phase and G1-phase and an increase in G2/M and Sub-G1 phases (Figures 4.17 and 4.18). The combination of FUC with DOX significantly ($p < 0.0001$) decrease G2/M phase while G1 phase populations were increased non-significantly when compared with cells treated only with DOX. FUC induced the same effects as observed with DOX when combined with TAX to treat MCF-12A (Figures 4.17 and 4.18).

4.6 Confirmation of Apoptosis by Hoechst Assay

The induction of apoptosis by FUC against MCF-7 (Figure 4.19) and MCF-12A cells (Figure 4.20) was confirmed in fluorescence photomicrographs of cells stained with Hoechst-33342. We next evaluated the morphology of each nucleus after treatment with FUC alone or in combination with IC_{50} concentrations of CDDP, DOX and TAX. The images of Hoechst's staining are presented in Figure 4.19.

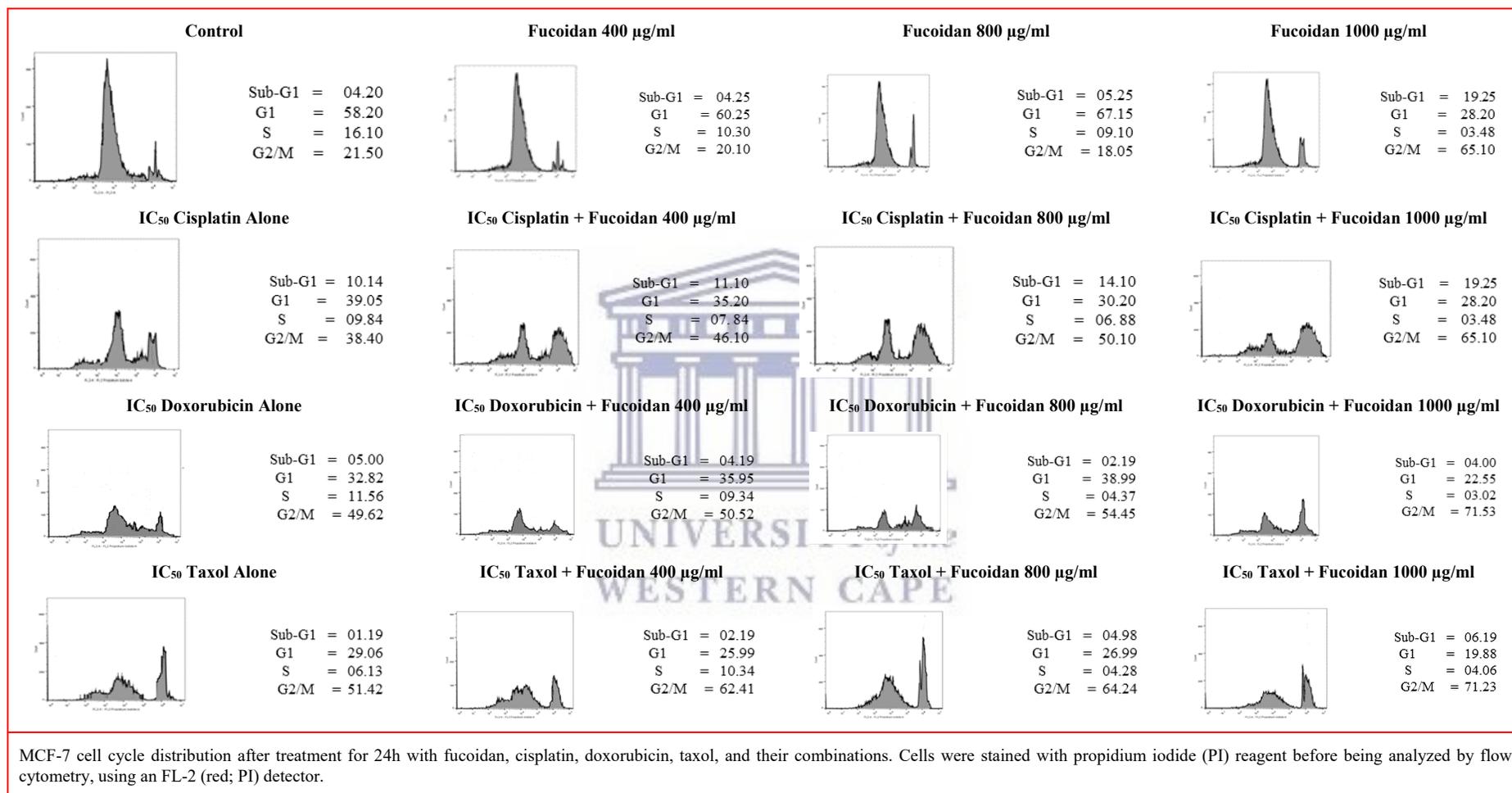


Figure 4.15: Effects of fucoidan and anticancer drugs on cell cycle arrest in MCF-7 cells

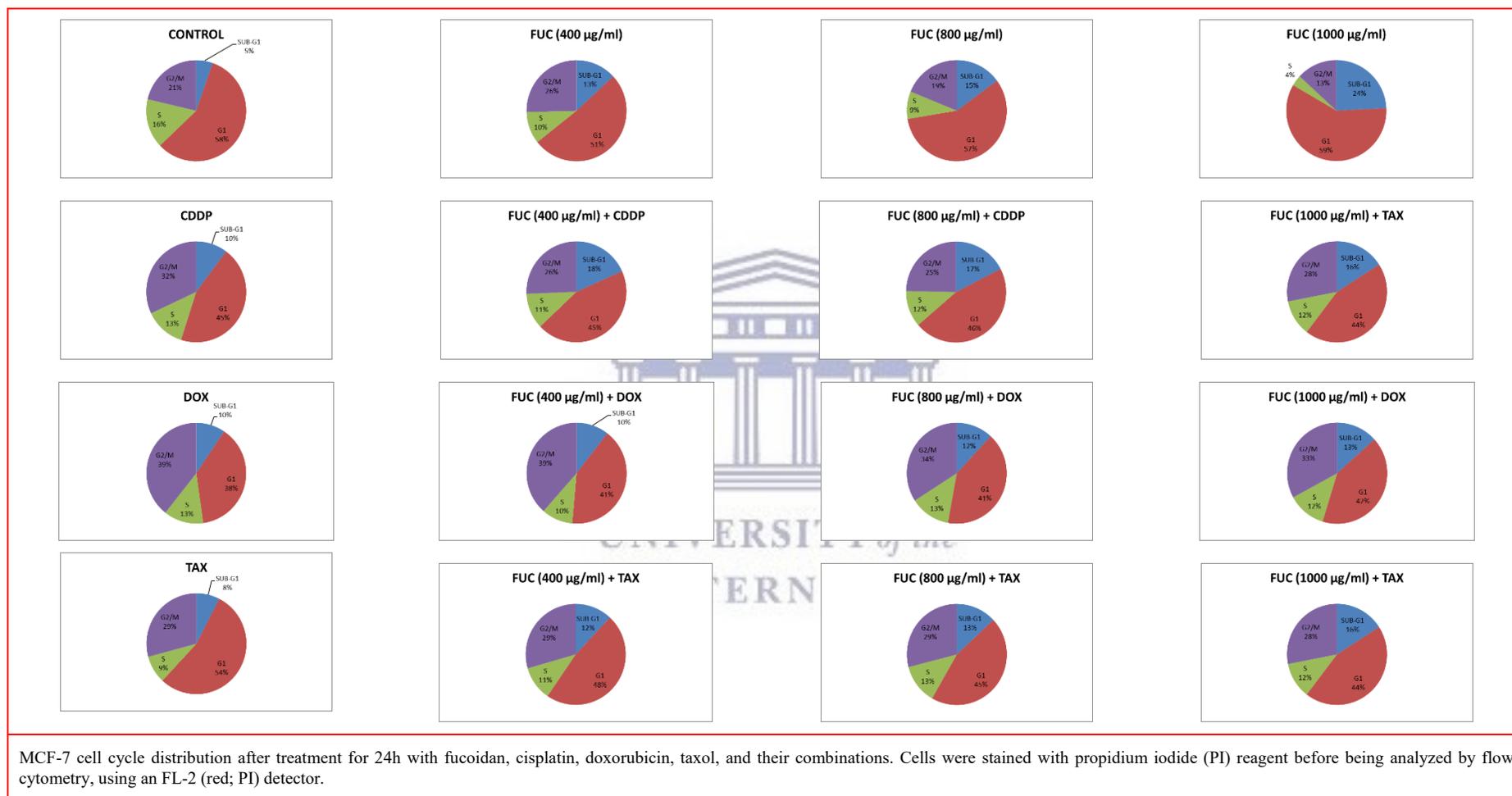


Figure 4.16: Effects of fucoidan and anticancer drugs on cell cycle distribution in MCF-7 cells

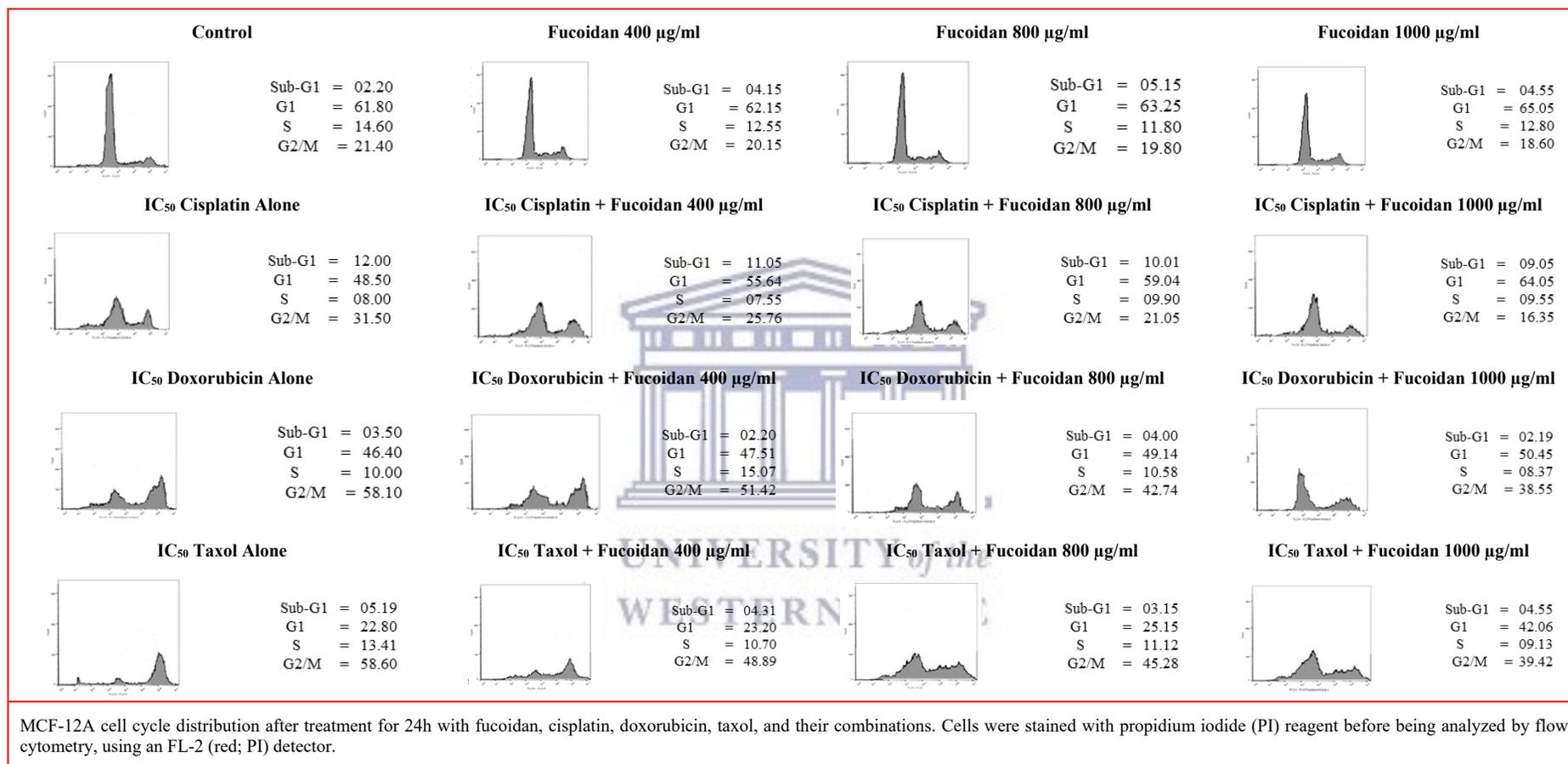


Figure 4.17: Effects of fucoidan and anticancer drugs on cell cycle arrest in MCF-12A cells

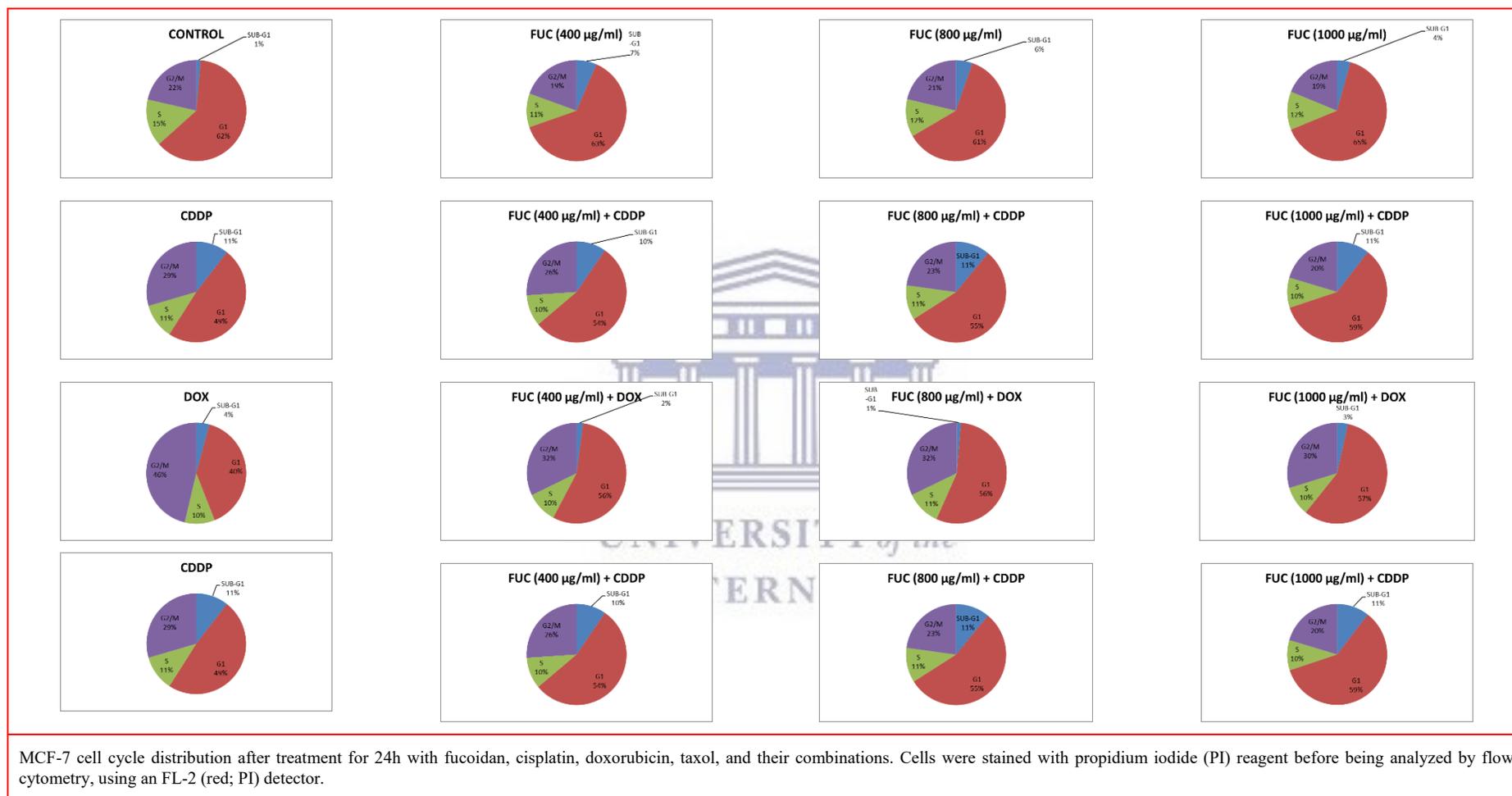


Figure 4.18: Effects of fucoidan and anticancer drugs on cell cycle distribution in MCF-12A cells

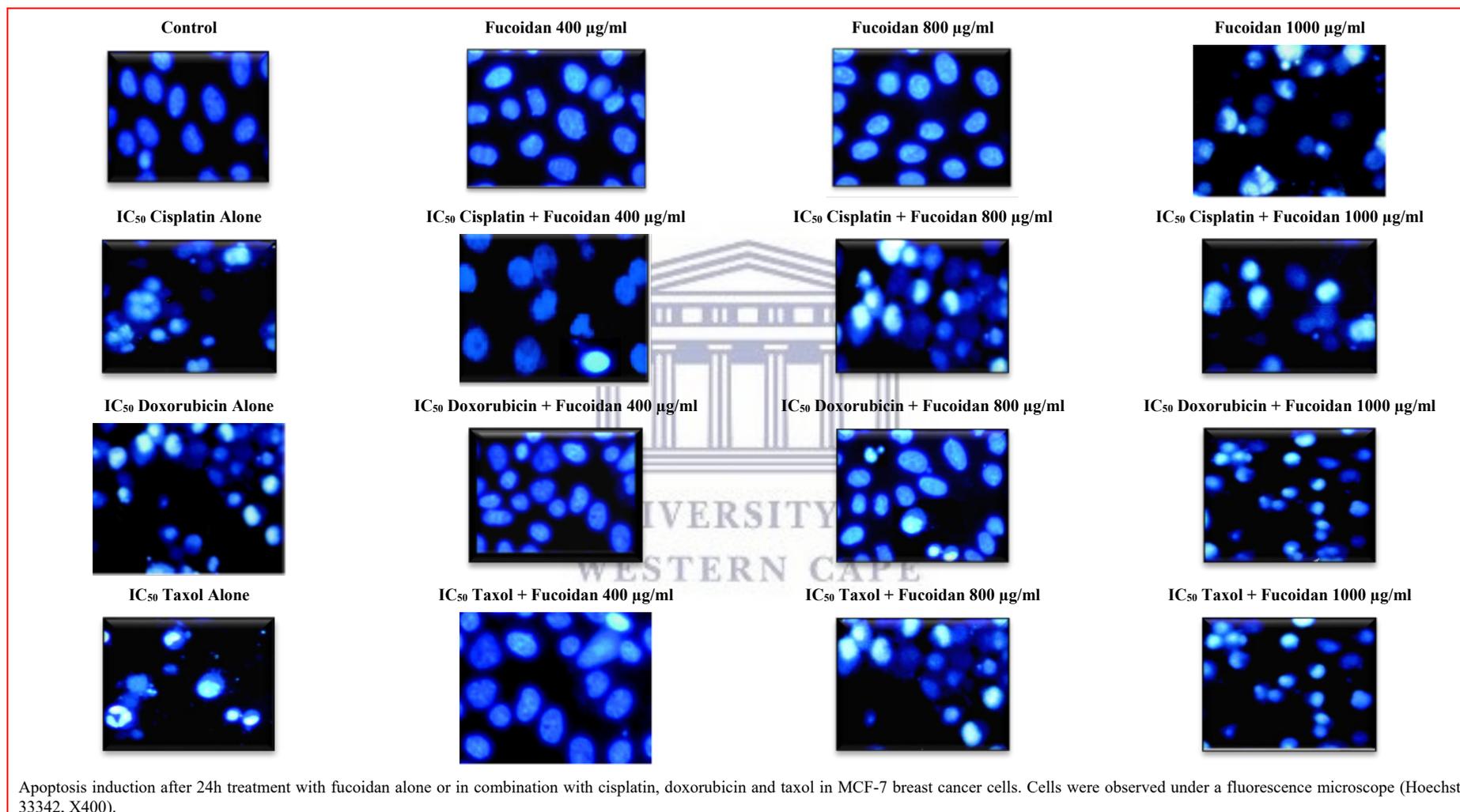


Figure 4.19: Confirmation of apoptosis by Hoechst assay in MCF-7 cells

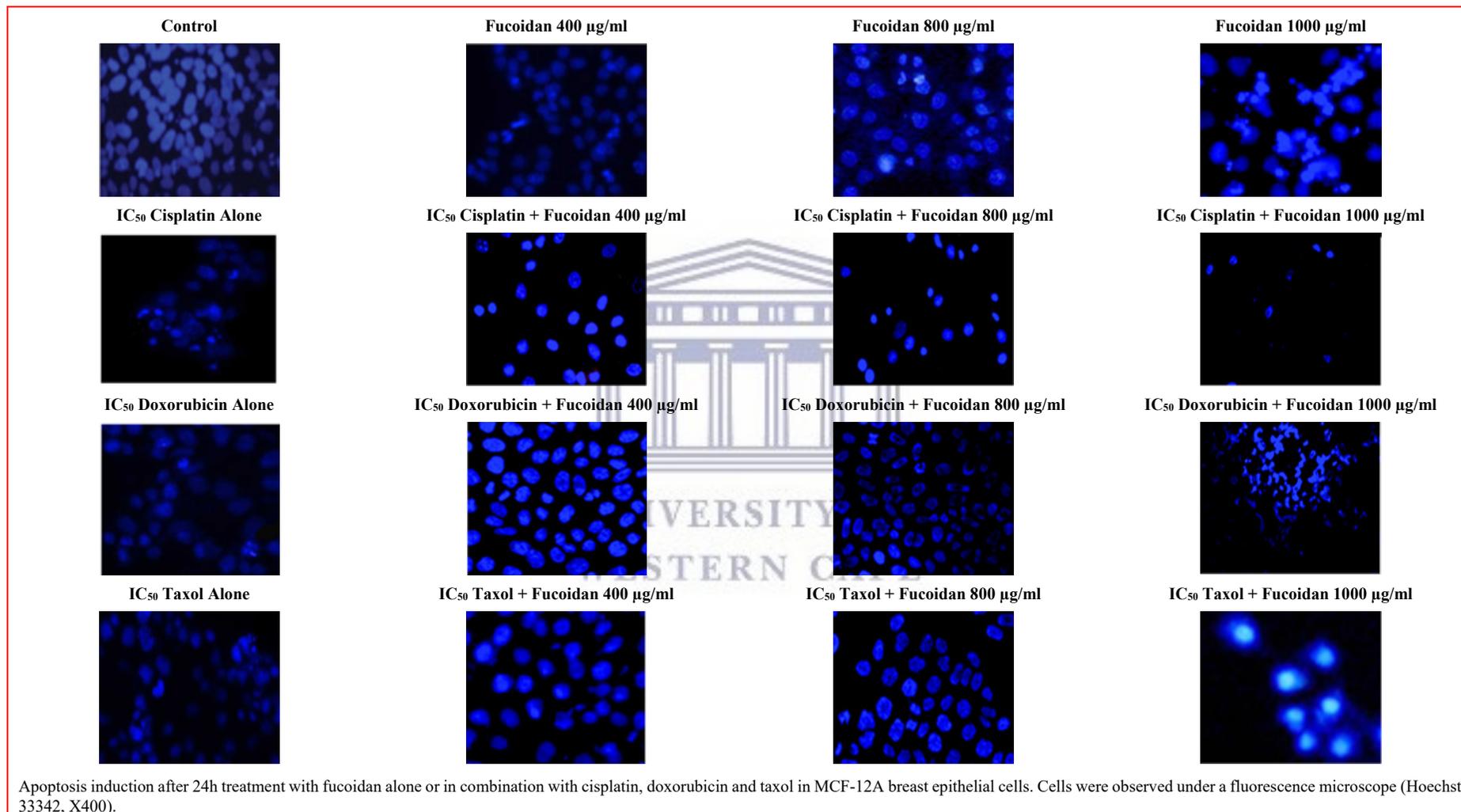


Figure 4.20: Confirmation of apoptosis by Hoechst assay in MCF-12A cells

These results indicate the morphological changes in the cells and also corroborate the results of Annexin V-FITC flow cytometry evaluation. The plates show MCF-7 cell treated with FUC with brightly blue condensed chromatin, typical of cells undergoing apoptosis. The same view was found with MCF-7 cells either treated alone with the drugs or in combination with FUC. This graphic of brightly blue condensed chromatin was found to be absent when FUC was used to treat MCF-12A cells. CDDP, DOX and TAX significantly increased the apoptotic effect of FUC against MCF-7, in contrast to MCF-12A treated cells. This validates the results of Annexin V-FITC flow cytometry evaluation.



CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

5.1 Introduction

Breast cancer is a serious global health problem being the second most common of all cancers and by far the most frequent cancer amongst women [1-3,22]. Natural product-derived compounds isolated from different sources, namely marine organisms, micro-organisms and plants have been a vital source for numerous clinically useful anticancer agents [402,511-513].

Plant- and other naturally-derived compounds with clinically anticancer applications, including vincristine, vinblastine, the two structurally related and naturally occurring polyphenols resveratrol and quercetin, the camptothecin derivative, irinotecan, topotecan and paclitaxel (taxol) have highlighted the role of natural products in the development of new pharmaceutical agents [52,53,55,56,160,402,403,494,514-516]. Fucoïdians, sulfated polysaccharides from brown algae, have recently attracted a lot of attention as a nontoxic compound possessing high anti-tumour, immunomodulating, antioxidant and anticoagulant activities [7,8,10,17,20,21,61,62,426,459,471,472,482,483,494,510,515,517-521].

Therefore, fucoidan has become a focus of great interest because of its anticancer potential and is expected to be a new candidate for low toxic cancer therapy. The synergistic action of the combined drugs is a promising strategy for treating diseases such as cancer because of significant reduction of side effects is attributed to it [145,151,188,466,522-528]

This study sought to evaluate the anticancer effects of fucoidan (*Fucus vesiculosus*) in combination with cisplatin, doxorubicin and taxol in breast cancer cells (MCF-7) and non-malignant breast epithelial cells (MCF-12A).

5.2 Research Hypothesis and Objectives of the Study

In this study, fucoidan (FUC), cisplatin (CDDP), doxorubicin (DOX) and taxol (TAX), alone and in combination were evaluated for their effects on MCF-7 cancer and MCF-12A normal breast epithelial cell proliferation, cell cycle transition, apoptosis and caspase-3, -7, and -9 activation. It was hypothesized that combinations of FUC with conventional antineoplastic agents such as CDDP, DOX and taxol TAX act synergistically to yield greater anticancer effects than those predicted by their individual activities against MCF-7 breast cancer cells and MCF-12A normal breast epithelial cells *in vitro*. Based on the results of this study it can essentially be accepted that, indeed, these fucoidan-drug combinations enhanced their anticancer effects to a greater extent in MCF-7 breast cancer cells than normal MCF-12A breast epithelial cells.

5.3 Context and Significance of the Study

5.3.1 Cytotoxicity

Fucoidan has been reported to exert antitumour activity against PC-3 (prostate cancer) [483], HeLa (cervical cancer), A549 (alveolar carcinoma), HepG2 (hepatocellular carcinoma) cells [529], and MDA-MB-231 and MCF-7 breast cancer cells [20,482]. Fucoidan was shown to inhibit the proliferation of melanoma cells and induce apoptosis by activation of caspase-3 *in vitro* [413]. The sulfate content and molecular weight (MW) of fucoidan polymers have been reported to have a direct relationship to fucoidans' cytotoxic, anticancer and apoptotic activities and because fucoidan is a polymer mixture, fucoidans extracted from different seaweed or locations have different bioactivities [9,412,426,435,436,459,470,472,494,510]. In this study, the IC₅₀ of fucoidan (FUC) for MCF-7 cells was chosen to be 400, 800 and 1000 µg/ml and it was focused on investigating the potential of FUC on induction of apoptosis and its associated mechanisms of action on MCF-7 cells. The MTT assay showed that FUC had cytotoxic activities against the breast cancer cells [485].

It is interesting to note in this study that the non-tumorigenic cell line MCF-12A was not affected markedly by the FUC-mediated anti-proliferative effect [482]. Conversely, inhibition of MCF-7 by FUC showed a dose- and time-dependent relationship at the concentrations (400, 800 and 1000 $\mu\text{g/ml}$). Therefore, FUC may be a good chemopreventive and antitumour candidate without toxic effects on normal cells. Previous studies reported that FUC has cytotoxic effects against different cancer cell lines, including human lymphoma, promyelocytic leukemia, colon carcinoma, breast carcinoma, hepatoma and melanoma [413,421,425,480,482,489].

FUC synergistically enhanced toxicity of the chemotherapeutic agents used against MCF-7 breast carcinoma cells by lowering the IC_{50} values of the drugs. By contrast, in non-cancerous MCF-12A cells, FUC attenuated the toxicity of these drugs in combination by increasing the IC_{50} values except with the TAX combination. Drugs combination that discriminates between cancerous and non-cancerous cells is a plausible and viable strategy of therapeutic efficacy for avoiding possible toxicity and side effects [530].

Cisplatin (cis-diamminedichloroplatinum II) is a widely used chemotherapeutic compound, known to cause DNA damage by alkylating DNA molecules. Cisplatin forms highly reactive, charged, platinum complexes that bind to nucleophilic groups, such as GC-rich sites in DNA. This induces intrastrand and interstrand DNA cross-links as well as DNA-protein cross-links, which inhibit cell growth and result in apoptosis [158-162,165,167-170,531,532]. Apoptosis proceeds, in part, due to the aggregation and multimerization of upstream death effector molecules that concurrently or sequentially activate the cysteinyl aspartate-specific protease (caspase) cascade [324,343,348,508]. Additionally, mitochondria are thought to be a major target of CDDP, and mitochondrial DNA is heavily damaged by CDDP [533,534]. CDDP activates multiple intracellular pathways including those involving cell cycle regulating proteins and caspases [507]. The mechanism of action for the growth-inhibitory effect of cisplatin has been studied in several cell types, including HeLa cells [535], the leukemic cell

line L1210 [536], Ehrlich ascites cells [537], L1210/0 cells [536] and JB1 rat hepatoma cells [538]. In these cells, CDDP was found to arrest the cell cycle in the G2 phase and to induce apoptosis, as indicated by internucleosomal DNA degradation. In contrast, studies demonstrated that cisplatin at 1 μM did not induce the well-defined G2/M-arrest reported for other cell types, but resulted in a marked increase in the rate of cell death. A morphological feature observed, especially with cisplatin-treated MCF-7 cell death, appeared to occur by apoptosis [539].

Doxorubicin is the most widely used drug in the treatment of a variety of human neoplasms including breast cancer [32,172,182]. However, with the increasing use of DOX, acute as well as chronic cumulative dose-dependent cardiomyopathy has been recognized as the major limiting factor for DOX chemotherapy [189,540-544]. Therefore, in this study we investigated the modulatory effect of the natural polysaccharide compound, FUC on DOX cytotoxicity in MCF-7 human breast cancer cell line. In this study, the findings demonstrated that DOX has cytotoxicity towards MCF-7 which was confirmed by the results of induction of apoptosis, where the IC_{50} of DOX induced a 2-fold increase in early apoptosis in comparison with control cells. A previous study showed that DOX exerts strong cytotoxic effects on MCF-7 and T47D breast cancer cell lines with IC_{50} value 400 and 15 nM, respectively [545]. The higher IC_{50} value in MCF-7 cells is due to MCF-7 cell resistance to DOX by overexpressing anti-apoptotic protein Bcl-2, P-glycoprotein, and phosphorylated Akt [546-548]. FUC protects spleen cells from taxol-induced cell death [549]. Likewise, in this study, we found that FUC decrease the cytotoxicity of TAX in MCF-7 breast cancer cells.

5.3.2 Apoptosis

The apoptotic properties of cells are the crucial key approaches used in analyzing the efficacy of chemotherapy and radiation therapy, therefore, apoptosis has apparent diagnostic and therapeutic implications [72,280,306,307,311,320,323,550,551]. Apoptosis consists of various biochemical and morphological changes in the cells, which include chromatin condensation,

cell membrane blebbing, DNA fragmentation, mitochondrial membrane potential changes and activation of the caspase cascade [59]. Programmed cell death has two distinct phases, namely early and late [312]. In healthy and viable cells, phosphatidylserine (PS) is located on the inner surface of the plasma membrane, which will translocate to the outer surface after induction of apoptosis, apparently through an active mechanism [552]. Since PS exposure is a widespread event during apoptosis that occurs earlier than DNA-associated changes and membrane leakage, Annexin-V-FITC is a probe which has been established as an easy method for detection of apoptosis in this phase [553].

In this study, apoptosis was evaluated by Annexin V-FITC/PI and Hoechst staining and caspase-3/7 and caspase-9 activity measurements by flow cytometry and colorimetry. FUC significantly induced the percentage population of cells in early stage apoptosis (Annexin-V only positive cells) without necrosis in MCF-7 cells, while MCF-12A cells in this stage was significantly lower. In contrast to MCF-12A cells, CDDP, DOX and TAX induced MCF-7 cells into early and late stage apoptosis. These results were confirmed with Hoechst staining with brightly blue condensed chromatin, in FUC combined with chemotherapeutic drugs-treated MCF-7 cells, an observation which is absent in MCF-12A cells.

Similar results were reported in a recent study on the combinatorial effects of FUC (an enzymatically digested polysaccharide) and three commonly used anticancer agents, CDDP, tamoxifen (TAM) and paclitaxel (TAX) on MCF-7 [20]. The study exhibited an increased number of Annexin-V-positive cells in a time-dependent manner relative to untreated control after 48 hrs of incubation. The results demonstrated that FUC in combination with CDDP, TAM, or TAX significantly enhanced cell death of MDA-MB-231 and MCF-7 breast cancer cells by regulating the expression of Bcl-2 family proteins, modulating ERK and Akt signaling, and regulating the production of oxidative stress. In an earlier study, FUC induced apoptosis in human lymphoma cells through caspase-3 activation and extracellular-regulated kinase (ERK) pathways [421].

5.3.4 Caspases

The complex cascade of caspases is a hallmark in the process of apoptosis, as it regulates the final demise of the cell [57]. It was reported that commercially available fucoidan (100 µg/ml) from the brown alga *Fucus vesiculosus* induced apoptosis via the activation of caspase-3 and downregulation of the ERK pathway in human lymphoma HS-Sultan cells [421] and via the caspase-8-dependent pathway in MCF-7 breast cancer cells [485]. Commercially available fucoidan (820 µg/ml), activated a caspase-independent apoptotic pathway in MCF-7 breast cancer cells via the activation of ROS-mediated MAP kinases and the regulation of the Bcl-2 family protein-mediated mitochondrial pathway [482].

Galactofucan (200 µg/ml) from *Undaria pinnatifida* was found to induce apoptosis in A549 human lung carcinoma cells through the downregulation of Bcl-2 and the activation of the caspase pathway [509]. Several experiments were performed to investigate the antitumour effect of fucoidan from *F. vesiculosus* on colon cancer. It was reported that the fucoidan (100 µg/ml) induced apoptosis in HCT-15 human colon carcinoma cells via the activation of caspase-3 and -9, accompanied by changes in Bcl-2 and Bax; additionally, there were changes in the phosphorylation of ERK, p38 kinase and Akt [489]. Another study demonstrated that this fucoidan (20 µg/ml) induced apoptosis in HT-29 human colon carcinoma cells and HCT 116 cells via both the death receptor-mediated apoptotic pathway and mitochondria-mediated apoptotic pathway [455].

In this study, the levels of caspase-9 were found to increase when MCF-7 cells were treated with FUC. The activation of caspase-9 after 24h provided evidence that FUC was capable of triggering apoptosis via the mitochondrial pathway [554]. Moreover, the increased activity of caspase-3/7 level upon treatment with FUC illuminated on the possible involvement of extrinsic pathway [555]. The significant increase in the level of caspase-7 upon treatment with FUC revealed the involvement of this executioner caspase in the induction of apoptosis to a greater extent in MCF-7 than in MCF-12A cells.

It is well established that several pathways are usually involved in the apoptosis induced by anticancer compounds, especially from plant-derived natural sources [556-558]. In this study, it was observed that untreated cells showed a decrease in the level of caspase-3,-7 and -9. However, it was significantly and dose-dependently increased upon treatment in MCF-7. Caspase activity is a critical factor in the induction of apoptotic cell death [559]. A previous study demonstrated that FUC induces a caspase-independent apoptotic pathway in MCF-7 cells [482].

5.3.5 Cell Cycle

The regulation of cell death and cell proliferation is tightly controlled by molecules that sometimes have a common role in the death and cell division in multicellular organisms [275,279,322,385]. Indeed, the process of apoptosis is regulated by the proteins that are often involved in cell cycle regulation [284]. Cancer cells in particular have irregular cell cycle progression profiles due to the mutagenic nature and the presence of growth factors [26,248,560]. In this study, this connection was analyzed by cell cycle analysis to confirm the involvement of apoptosis in MCF-7 cells upon 24h treatment with FUC, CDDP, DOX and TAX, alone and in combination. Flow cytometry analysis was carried out to investigate the effect of FUC on the DNA content of MCF-7 cells by cell cycle phase distribution (G0, G1, S, G2 and M) after treatment as indicated above.

The results indicated that there was a significant G1 phase arrest in a dose-dependent manner following the 24h treatment. The number of MCF-7 cells in S and G2/M phases decreased after the treatments. Additionally, the number of MCF-7 cells in sub-G1 phase elevated after treatment by FUC, representing the number of cells undergoing apoptosis, whilst FUC treatment did not have a significant effect on the cell cycle in MCF-12A cells. The significant elevation in the extent of cell death in MCF-7 cells is associated with apoptotic hallmarks. In this regard, it has recently been demonstrated that FUC induced G1 arrest of the cell cycle in EJ human bladder cancer cells via downregulation of pRB phosphorylation [517]. An earlier

report also asserted that FUC stimulated G1 arrest of the cell cycle in cultured human non-small-cell bronchopulmonary carcinoma cells [422]. Another study demonstrated that FUC induced accumulation of cells in G1 phase probably by suppressing the genes related to G1 phase such as cyclin D1 and CDK-4 in a dose-dependent manner on breast cancer cells MCF-7 [510,561].

In the present study, FUC reduced the effects of the drugs on cell populations at G2/M, G1 and S-phases in MCF-12A cells which correlated positively to the amelioration of toxic effects of the drugs against the cells. The influence of FUC on the cell cycle may be due to downregulation of cyclin E, Cdk2 and Cdk4 through binding of p21 as was observed when human bladder cancer cells were treated with FUC [484]. Moreover, synergism has been demonstrated between CDDP and docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) in MKN45 gastric cancer cells as cell cycle analysis showed that the combination treatment increased G0/G1 phase and S phase arrest, and significantly increased the number of apoptotic cells [562].

In MCF-7 cells, DOX induces a G2/M arrest [563,564]. Previous studies have further confirmed the fact that anthracyclines are mostly active on proliferating cells in S and G2/M phases due to the maximal expression of their target enzyme, topoisomerase II at these phases [565,566]. TAX affects tubulin polymerization to promote the stability of microtubules [198], resulting in the accumulation of intracellular microtubules and the breakdown of mitotic spindles to arrest cell cycle progression in the G2/M phase, or cause apoptosis [567,568]. It is known that TAX can suppress cell growth through blocking cell cycle arrest at G2M phases (Horwitz, 1992; Van Amerongen and Berns, 2006). In aforementioned study, MCF-7 cells treated with TAX accumulated significantly in the G2/M phases, and the Sub-G1 apoptotic region. Because TAX induces a p53-independent G2/M arrest (prophase) that triggers the rapid onset of apoptosis [569,570].

The current study demonstrated inhibition of MCF-7 breast cancer cell proliferation by inducing apoptosis and arresting cells in the G1 phase in a dose-dependent manner. FUC strongly enhanced sensitivity to chemotherapeutic drugs CDDP and TAX, while sensitivity to DOX mildly increased. This was seen by a reduction of cell proliferation, apoptotic induction, caspase activation and a notably change in the cell cycle profile. These findings suggest that FUC is a potential candidate for cancer combination therapies. Further studies are necessary to elucidate the protective action of FUC against DOX and CDDP toxicity in non-cancerous MCF-12A cells. *In vivo* and clinical studies are critical to evaluate the safety and utility of these combination treatments in cancer patients.

5.4 Limitations of the Study

This study was limited to MCF-7 and MCF-12A cells. Mechanistic studies of the fucoidan-drug combinations were envisaged, but time and funding constraints hindered such objectives.

5.5 Conclusion and Future Directions

Several studies *in vitro* and *in vivo* have indicated that FUC contains strong anticancer bioactivity. Therefore, FUC retards tumour development and eradicates tumour cells. Moreover, it has been reported that FUC did not induce apoptosis within normal cells at the doses which were toxic for cancer cell lines. Since FUC also possesses immunomodulatory effects, it is postulated that it may have protective effects against development of side effects when it is co-administered with chemotherapeutic agents and radiation. Thus, the chance increased to candidate this compound to synergize with standard anticancer agents and/or reduce toxicity. The present study demonstrates that FUC, isolated from *F. vesiculosus*, combined with the chemotherapeutic agents CDDP, DOX and TAX induce cell growth inhibition through the induction of apoptosis and cell cycle arrest in the human MCF-7 breast cancer cell line. These studies highlight the potential regarding the achievable efficacy of FUC in combination with first line of chemotherapeutic agents in breast cancer treatment.

The FUC used in this study is crude FUC isolated from *F. vesiculosus*, purchased from Sigma-Aldrich Corporation, and it is unclear which parts of the plant they used and what season they harvested the *Fucus*. Therefore, it is necessary to carry out further research to determine the composition of this FUC, and which ingredients play a major role in antitumor activity. Further *in vivo* and clinical studies are needed to evaluate the safety and efficacy of these combination treatments in cancer patients.



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