In vitro study of the cytotoxic effects of low- and high-molecular-weight fucoidan extracted from New Zealand seaweed *Undaria pinnatifida* in MCF-7 and MDA-MB-231 breast cancer cell lines

Keyu Shi

A thesis submitted to

Auckland University of Technology

in partial fulfilment of the requirements for the degree of

Master of Applied Science (MAppSc)

2018

School of Applied Sciences

In vitro study of the cytotoxic effects of low- and high-molecular-weight fucoidan extracted from New Zealand seaweed *Undaria pinnatifida* in MCF-7 and MDA-MB-231 breast cancer cell lines

Approved by:

Primary Supervisor: A/Prof. Jun Lu

Signature: _____ _____

Second Supervisor: A/Prof. Fabrice Merien

Signature: _____ _____

Table of Contents

List of Figures

List of Tables

Attestation of Authorship

I hereby declare that this submission is my own work and that, to be the best of my knowledge and belief, 'In vitro study of the cytotoxic effects of low and high-molecular-weight fucoidan extracted from New Zealand seaweed *Undaria pinnatifida* in MCF-7 and MDA-MB-231 breast cancer cell lines.', contains no material previously published or written by another person (except where explicitly defined in the acknowledgements) nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Name: ……Keyu Shi……

Signed: …

Date: ……06-08-2018……..

Acknowledgements

First, I would like to thank my primary supervisor Associate Professor Jun Lu and second supervisor Associate Professor Fabrice Merien for giving me this great opportunity to work on this subject. I've always wanted to learn more about biomedical science, especially on cancers, and this is in a way dream comes true for me. I feel so lucky that I have someone so respectable and so knowledgeable as my supervisors and I am really grateful to them for all the guidance and help I received and for them being so kind to me. Additionally, I want to thank Professor Jun for always giving me support and suggestions when I met problems in experiments and for teaching me the linear mixed effect model for data analysis. I would like to thank Professor Fabrice for giving me the training on the operation of Muse cell analyser and for being so supportive when I met the experimental material delay. I know I would not be able to finish this study if there weren't supports and encouragement from you. I greatly appreciate it.

Special thanks to Kelvin Wang for guiding me through all the methods of cell culture, the MTT assay and cell cycle assay. I really appreciate his patience for teaching me all the things that I need to know in the lab and his professional knowledge on operating the flow cytometry. Special thanks to Sonya Popoff and Brid Lorigan for helping me with material orders and a lot other lab supply.

Many thanks to all staff members in the laboratories of the Faculty of Health and Environmental Sciences in Auckland University of Technology. Thank you for being so kind and friendly to me and for willing to help me when I was new at the lab. Additionally, I would like to thank Cindy for sharing some tips on lab techniques with me and thank Yutao for the help on R Studio.

Last but not least, I would like to thank my parents and my grandfather for their unconditional supports, both financially and spiritually, for me on my study. Thank you for supporting me to learn the things I love and not complaining about me being so far away from home. You are the best family I could ever have in the world.

Abbreviations

AA TN: African American triple-negative breast cancer

7-AAD: 7-Aminoactinomycin D

AIs: Aromatase inhibitors

ANOVA: Analysis of Variance

ATCC: American type culture collection

BRCA1 & 2: Breast cancer susceptibility gene 1 & 2

BSA: Breast Screen Aotearoa (programme)

CATN: Caucasian triple-negative breast cancer

Cn: Concentration

CTRL: (Negative) Control

DDW: Distilled water

DISC: Death-inducing signalling complex

DMSO: Dimethyl sulfoxide

DNA: Deoxyriboncleic acid

EGFR: Epidermal growth factor receptor

ER: Oestrogen receptor

ERK: Extracellular signal-regulated kinase

FAS: Fatty acid synthase

FBS: Fetal bovine serum

HDFa: Human dermal fibroblasts, adult

HER-2: The oncogene human epidermal growth factor receptor 2

HMF: High-molecular weight fucoidan

HP: Hydrogen peroxide

HR: Hormone receptor

IC_{50} values: Inhibitory voltage inhibiting cell growth by 50% (used as a parameter of cytotoxicity)

LMEM: Linear mixed effects model

LMF: Low-molecular weight fucoidan

Log: Logarithm

LSGS: Low serum growth supplement

MAP: Mitogen activated protein

MBC: Metastatic breast cancer

MCF-7: Michigan Cancer Foundation-7 (Breast cancer cells)

MDA-MB-231: Invasive ductal breast carcinoma metastasis (pleural effusion)

mM: Millimolar (10^{-3} mol/L)

MMF: Medium-molecular weight fucoidan

MMP: Mitochondria membrane potential

MMPs: Matrix metalloproteinases

MOMP: Mitochondrial outer membrane permeabilization

MRI: Magnetic resonance imaging

MTT: 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide

NO: Nitric oxide

NOS: Nitric oxide synthases

eNOS: Endothelial nitric oxide synthase

iNOS: Inducible nitric oxide synthase

nNOS: Neuronal nitric oxide synthase

NSCLC: Non-small-cell lung cancer

OD value: The average absorbance value

PARP: Poly (ADPribose) polymerase

PARPi: Poly (ADPribose) polymerase inhibitors

PBS: Phosphate Buffered Saline

PI: Propidium iodide

PR: progesterone receptor

PS: Phosphatidylserine

p53: Tumour-suppressor phosphoprotein

RNAse A: Ribonuclease A

ROS: Reactive oxygen species

RPM: Revolutions per minute

S.D: Stand deviation

SERDs: Selective oestrogen receptor down-regulators

SERMs: Selective oestrogen receptor modulators

TNBC: Triple-negative breast cancer

Abstract

Breast cancer is known as the top cancer for women worldwide. It is estimated that every year over one million new cases of breast cancer are diagnosed and contribute largely to cancer related deaths. Chemotherapy, including neoadjuvant therapy and adjuvant therapy, is a critical part in treatment for breast cancer that impact on survival and life quality for patients. However, chemo-resistance and adverse effects occur frequently when patients receive chemotherapy or the improved target therapies. New strategies have been proposed to enhance the effects of anticancer drugs as combing them with natural dietary compounds, decreasing drug dose administered and reducing the toxicity to normal cells.

Fucoidan is noticed for its anti-cancer potential in treating breast cancer as well as in many other cancers. It is a natural bioactive compound derived from brown algae that has low toxicity and multiple anti-cancer pathways, the potential of which makes it a candidate for therapeutic agent using alone or in combination with other cytotoxic drugs. Base on the molecular weight, fucoidan can be categorised into three ranges: high-molecular weight fucoidan (HMF, >300k), medium-molecular weight fucoidan (MMF, 300-10k) and low-molecular weight fucoidan (LMF, <10k). In this study, the inhibitory effects of HMF and LMF from New Zealand *Undaria Pinnatifida* have been studied against breast cancer. Two breast cancer cell lines, MCF-7 and MDA-MB-231, have been used in this study representing ER-positive type and triple-negative type of breast cancer. A fibroblast (HDFa) cell line has also been used in this study, representing non-cancer cells, to examine toxicity of fucoidan.

By conducting MTT assays, apoptosis assay and other related mechanism assays on cancer cells, the findings in this study indicate that LMF exhibited much better inhibition on proliferation of breast cancer cells than HMF. Dose-dependent inhibition by LMF was observed in both MCF-7 and MDA-MB-231 after incubated for 48, 72 and 96 hours. MCF-7 cells are more sensitive to LMF than MDA-MB-231 by a distinction of about 20% inhibition at the highest concentration of LMF (56.6% inhibition at 200 µg/ml and 39.2% inhibition at 300µg/ml,72hrs, respectively) and time-dependent manner of inhibition was only observed in MCF-7. The IC_{50} of LMF to MCF-7 cells over 72 hours was determined to be about 19 µg/ml and dropped to 10.5 µg/ml after 96 hours. Induction of caspase-dependent apoptosis was observed in MDA-MB-231 cells

through intrinsic apoptosis pathway alone or with the extrinsic pathway. An activation of NOS stimulated by LMF was observed in MDA-MB-231 cells at a dose-dependent manner. No obvious cytotoxicity of LMF to HDFa cells was observed by 72 hours incubation in a cell cycle assay. To conclude, LMF from New Zealand *Undaria Pinnatifida* showed great anti-cancer effects against these two types of breast cancer, therefore, it has great potential to be used as a therapeutic agent or a supplement to combine with other chemo-agents for treating breast cancer, even though it may not be potent enough to treat this type of cancer alone.

Chapter 1 Introduction

1.1 Background

Breast cancer is one of the leading cancers in both developed and developing countries. It is the top cancer among women and a major cause of cancer-related death. In 2012 alone, there were nearly 1.7 million new breast cancer cases diagnosed worldwide, representing 25% of all cancers. High incidence rates and mortality rates of breast cancer have made it a non-negligible threat and burden to public health in many countries.

The incidence of breast cancer is currently highest in more developed regions including New Zealand (4^{th} highest in the world), while increasing rapidly in developing countries such as Asian countries. High risk population are identified among women who are mutated stability gene BRCA1and/or BRCA2 carriers, mostly those with positive family histories, compared to others with negative family histories. Besides inherited factor, other risk factors such as age, obesity, hormones and life style all have impacts on the incidence of breast cancer. As many other cancers, survival for breast cancer patients largely depends on the stage at diagnosis. Early stages of breast cancer are considered curable in most cases, whereas treatment is extremely difficult if stage IV metastasis is developed. In the past two decades, the introduction of breast screening programme in many countries and the improvement on therapy options (targeted therapies) have decreased the mortality of breast cancer to some extent. Nevertheless, the complex protein status and molecular basis of malignant breast tumours make the treatment of breast cancer challenging.

Except for the stages of tumour growth and the health condition of patients, therapy options for breast cancer patients is dependent on the clinical classification of the tumour(s). Based on the receptor status on the cancer cell membrane, breast cancer can be categoried into three groups: oestrogen receptor positive (ER^+) and/or progesterone receptor positive (PR^+) type, the oncogene human epidermal growth factor receptor 2 positive ($HER\text{-}2^+$) type and all receptors negative type. For receptor-positive groups, patients typically receive targeted therapies with or without chemotherapy as standard treatment, of which has less adverse effects than tranditional chemotherapy. For all receptor negative breast cancer patients or patients who have developed metastasis, tranditional chemotherapy is the primary therapy option. In both targeted therapies and

chemotherapy, adverse effects and intrinsic resistance/drug resistance are the main obstacles in improving overall survival and life quality for patients. Recent efforts by researchers have carried out a more sophisticated molecular classification of breast cancer, trying to provide better prognosis for patients and look for therapy targets but not yet indicated in improving therapies. Therefore, new anti-cancer agents with lower toxicity, mainly natural products, are demanded in breast cancer treatment to either apply alone or combined with other therapeutic agents.

Fucoidan is one the natural compounds that has been extensively studied in the past two decades. It consists of a group of sulphated carbohydrates derived from marine brown algae (eg, *Fucus vesiculosus*, *Cladosiphon okamuranus*, *Laminaria japonica*, and *Undaria pinnatifida*) and has a long history been used as dietary supplements in Asia for medicinal purposes (Tocaciu et al., 2018). The anti-cancer activities induced by fucoidan as well as many other biofunctions such as anticoagulant and anti-inflammatory activities indicate its great potential to be utilised in the medical field.

It is reported that fucoidan has been found in all the brown seaweeds examined so far, while absent in green algae and red algae (Gupta & Abu-Ghannam, 2011). Fucoidans derived from various brown seaweed species have been tested for their inhibitory effects on various types of cancer and their anti-cancer pathways. The cytotoxicity of fucoidan from distinctive seaweed species seems to be variable, mostly due to the difference in their chemical composition involving changes in molecular structure, molecular size, sulphate contents, etc. Even fucoidan extracted from the same seaweed species has a composition which is influenced by geographic sites and harvesting time. Generally, fucoidan can induce cell apoptosis (one of the programmed cell deaths), cause cell cycle arrest and regulate several signalling pathways in various cancer cells, as well as its ability to inhibit metastasis and enhance immune response (Atashrazm, Lowenthal, Woods, Holloway, & Dickinson, 2015). Due to its action in plural anti-cancer pathways as well as its selective cytotoxic effects, fucoidan shows promising therapeutic value when applying it alone or in combination with other cytotoxic drugs in treating a heterogeneous and diverse disease such as breast cancer.

In previous in vitro studies, breast cancer cell lines including MCF-7, MDA-MB-231, 4T1 and T-47D have been reported to respond to the inhibitory effect of fucoidan (from various seaweed species), and partial aspects of the mechanisms have been explored.

New Zealand has a rich source of brown seaweed *Undaria pinnatifida,* a fucoidan-enriched seaweed, along the coast, which is not yet fully exploited and utilised. Considering that fucoidan extracted from *Undaria pinnatifida* in east Asia has been reported for its anti-cancer properties in previous research, examining the anti-cancer potential in fucoidan extracted from New Zealand *Undaria pinnatifida* seems to be a meaningful new direction for the seaweed's utilisation. In this study, inhibitory effects of high molecular weight and low molecular weight fucoidan will be examined on two breast cancer cell lines and mechanisms will be explored. Findings in this study could at least partially fill in the gaps of knowledge in this area and offer some ideas for further research.

1.2 Objectives of Study

The main purpose of this study is to examine the inhibitory effects of fucoidan from two different ranges of molecular weight (< 10k and >300k) on cancer cells. The inhibitory effects of HMF and LMF on cancer cell lines will be measured and compared to see if the molecular weight impacts the cytotoxicity of fucoidan. Two breast cancer cell lines were used in this study. The sensitivity of each of them to HMF and LMF will be tested and compared. If the cytotoxic effect of HMF and LMF to breast cancer are significant and considered has certain therapeutic value, then subsequent mechanism studies will be conducted exploring anti-cancer pathways in fucoidan-induced inhibition on breast cancer. The cytotoxicity of HMF and LMF to a noncancerous cell line will also be examined if they are proven cytotoxic to cancer cell lines. The specific goals in this study are summarized as the following points:

1: Testing whether HMF and LMF can supress the growth of breast cancer cell lines MCF-7 and MDA-MB-231. If so, try to find the optimal concentration range and action time as well as determine the IC_{50} value of HMF and LMF.

2: Conducting a cell cycle assay on the fibroblast cell line HDFa and examine if the cytotoxic effects of HMF and LMF are selective to cancer cells.

3. If the inhibition of HMF and LMF on breast cancer cell proliferation is confirmed significant, the study will continue forward with mechanism assays on the anti-cancer pathways of fucoidan using selective kits and a cell analyser.

1.3 Overview

This thesis consists of six chapters, of which chapter 1 is the introduction.

Chapter 2 is the literature review section, which comprehensively introduces the aetiology of cancer, a review of breast cancer and fucoidan literatures. The review of breast cancer includes its incidence and mortality worldwide, the risk factors for this disease and mainly on the development of treatment for this cancer in relation to its clinical classifications. The review on fucoidan includes the value of natural products, the general composition and structure of fucoidan and its anti-cancer potential for therapies in cancer treatment. Its main anti-cancer pathways related to this study has been reviewed and explained in this chapter as well. Factors that have an impact on biofunctions of fucoidan are then listed and discussed. The achieved research output so far on fucoidan against breast cancer is also stated in this chapter, discussing its therapeutic potential for treating breast cancer.

Chapter 3 is the methodology section. All materials, equipment and assay protocols involved in this study are described, as well as experimental design, data analysis and statistical data analysis. Major assays in this study include cytotoxicity assay (MTT assay), cell cycle assay, cell counting, cell apoptosis assay, multi-caspase assay, mitochondria membrane potential assay and a nitric oxide assay.

Chapter 4 presents the findings, which are all the results obtained from the study. This section includes the data and graphics for cytotoxicity assays, cell cycle assay and all the anti-cancer mechanism assays

Chapter 5 discusses the results shown in Chapter 4. By comparing and combining all the findings together, some assumptions will be made about the mechanisms based on statistics from this study and previous research.

Chapter 6 is the overall conclusion. It summarises the findings obtained from this study as well as discusses some limitations. Future research will be suggested based on the conclusion and other related literature sources.

Chapter 2 Literature Review

2.1 Aetiology of cancer

Cancer is one of the leading chronic diseases responsible for causing huge public health burden in both developed and developing countries. It is a disease that is characterised by uncontrolled abnormal cell division and its potential or trend to spread to other organs of the body, and in essence are genetic mutations in the genome of somatic cells (DeVita, Lawrence, & Rosenberg, 2016). In terms of cancer, there are over 200 various cancer types so far characterized. The most common types of cancers such as lung, stomach, liver, colon, and breast cancers, belong to the category of carcinomas, also known as malignant neoplasms. Other categories include sarcoma, leukemia, lymphoma and myeloma and central nervous system cancers. Becoming metastatic is the main reason for cancers ending with death, accounting for high cancer mortality rates each year.

2.11 Risk factors for cancer

Cancer is a multifactorial disease. Age, for one, is a major factor. Normally, elderly populations are at more risk than young populations for cancer incidence due to the time genetic mutations require to develop. However, this cannot explain certain types of cancers happening at childhood such as luekemia, in which the aetiology is not quite fully understood yet. Of great concern, there is a trend around the world that cancer incidence is increasing in younger groups of people. Aside from non-modifiable intrinsic factors, including inherited genetic mutations (family history), hormones and immune conditions, some extrinsic factors from individual lifestyle and behaviour are also closely related to cancer incidence. These external factors include, but are not limited to tobacco (or other toxic chemical exposures), alcohol, radiation, excess body weight, poor nutrition and physical inactivity. In some cancer cases, infectious agents may be the cause (American Cancer Society, 2018). For instance, human papillomavirus (HPV) is widely known as a risk factor in cervical cancer.

2.12 Cancer genes and their pathways

Although cancer is essentially a genetic disease, a single mutation in cancer genes is not powerful enough to break all safeguards of mammalian cells and lead to cancer. Therefore, cancer is a disease contributed to by multiple defective genes (Vogelstein &

Kinzler, 2004). There are generally three types of cancer genes involved in cancer development.

Oncogenes and tumour suppressor genes are the two types that directly take part in cancer progression. Alterations in oncogenes afford a selective growth advantage on cells by stimulating cell birth. Their activations result in the gene constitutively active even where the wild-type gene is not. They typically mutate at specific hotsports, affecting the same codon or clustered at neighboring codons in neoplasms. Their mutations are missense in most cases and affect only one allele (DeVita, Lawrence, & Rosenberg, 2016). Tumour suppressor genes, on the contrary, promote tumourigenesis by inhibting cell apoptosis or cell-cycle arrest. When mutations occur, they reduce the activity of gene products. Generally, their missense alterations are required in both maternal and paternal alleles in order to provide a selective advantage on cells.

Stability genes are another critical type of cancer genes that function in cancer progression through different pathways. The main responsibility of stability genes in cells is to repair subtle mistakes formed when DNA replicates or is exposed to mutagens. They also control certain processes where numerous chromosomes are involved. If stability genes work properly, genetic mutations in cells should be always kept to a minimum. However, once the specialised genes become inactivated, the possibility of genetic alterations happening in other genes will increase (Friedberg, 2003), including alterations in cancer genes. For some hereditary cancers that run in families, such as breast cancer, the most common inherited genetic mutations are in stability genes rather than oncogenens or tumour supressor genes (Vogelstein & Kinzler, 2004). The representative BRCA1 and BRCA2 genes in hereditary breast cancer are both under the category of stability genes.

In conclusion, stability genes act as protectors to minimise genetic alterations in cells. For other cancer genes, especially tumour suppressor genes, only after the protecters lose their efficacy can the alternative suppressors act physiologically. What is worth noticing here is that the rate of mutation is increased in all genes and may affect people in various ways, but only when oncogenes and tumour suppressor genes are affected do they become cancerogenic. Then, as a result of a somatic mutation, an advantaged clonal expansion will develop and continue towards the neoplastic process (Nowell, 2002).

2.2 Breast cancer

2.2.1 Epidemiology and risk factors

Breast cancer is one of the top cancers among women in both developed and developing countries, with over 1,300,000 cases and 450,000 deaths estimated each year. In 2012 alone, there were approximately 1.7 million newly diagnosed breast cancer cases worldwide (Maisonneuve, 2017), which amounts to 25% of all female cancers that year and was surpassed by only lung cancer. The incidence of breast cancer is highest in more developed regions such as Europe, Northern America and Oceania, and relatively lower in less developed regions of the world including Asia and Africa (Fig. 1) (GLOBOCAN, 2012). Nevertheless, the incidence rates of breast cancer in developing regions are continuously increasing due to further urbanisation and adoption of western lifestyles. Surprisingly, the incidence rates in developed countries have been observed to stabilise or even drop slightly since the early 1980s. This is viewed as a result of increased social awareness on breast cancer and benefits from policies supporting mammographic screening programmes. Additionally, it is believed to be partially contributed to by the decreasing use of hormone replacement therapy (since 2005) which has proven to be related to hormone-sensitive breast cancer (Lakhani, 2012).

The mortality rates of breast cancer have been reported to have similar trend in timeline and in geography relative to incidence. That is, the mortality declined in high-risk developed regions after the 1980s. Population screening and improved adjuvant therapies contributed equally to this decline in breast cancer mortality. When diagnosed at an early stage, the prognosis for breast cancer patients is often considered optimistic and curable. Despite all the progress on the detection and treatment of breast cancer, it is still a leading cause of cancer death, mostly due to the recurrence and metastasis in breast cancer patients (as cited in Gao, Zhong, Xie, Peng, & Han, 2016). Breast cancer now ranks as the second cause of cancer death after lung cancer in developed countries (198,000 deaths, 15.4% of all cancer deaths in 2012), and it remains the most frequent cause of cancer death in less developed regions (324,000 deaths, 14.3% of all cancer deaths in 2012) (Maisonneuve, 2017).

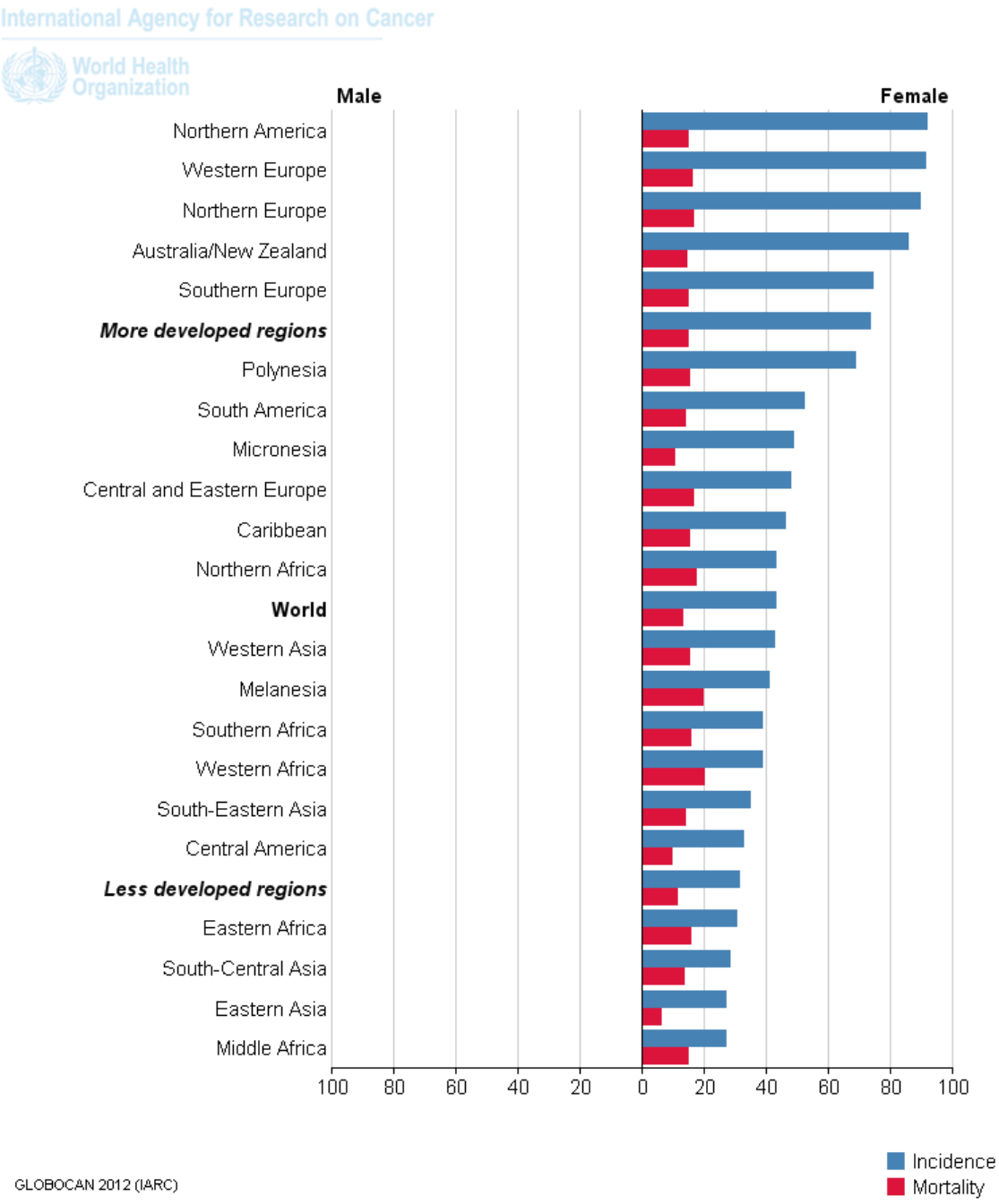


Figure 1. Estimated age-standardised rates of breast cancer (World) per 100,000 population (GLOBOCAN 2012, IARC)

Chart retrieved from http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx

There are multiple risk factors for breast cancer. The very first distinction is one's gender. Although breast cancer is not a cancer limited to only females, male breast cancer is extremely rare in patients (less than 1%) (Losurdo et al., 2017). Therefore, most research of breast cancer focuses on the aetiology and treatment for female

patients. For women with a positive family history, their chance of having breast cancer in their lifetime will be much higher than others because they are likely to carry inherited BRCA1 and/or BRCA2 gene mutations. Even without genetic factors, the risk of developing breast cancer increases with age. It is estimated that approximately 19% of breast cancer patients are diagnosed between the ages of 30 and 49, 37% of them are diagnosed between 50 and 64, and 44 % of women who are at least 65 years old (Bonilla, Tabanera, & Mendoza, 2017). Hormones are also known for having a great impact on the risks of breast cancer. Both oestrogen and progesterone are capable of increasing breast cancer risk. Other risk factors include breast density, menstrual and menopausal history, radiation exposure and individual life style (e.g. smoking and drinking) (Bak, Gupta, Wahler, & Suh, 2016).

Table 1. Breast cancer risk factors (Carlson, 2008)

Intrinsic risk factors	Extrinsic risk factors
Gender	Smoking
Age	Alcohol (Drinking)
Positive Family History	Obesity
Oestrogen	Older age at first birth
BRCA1/2 mutation	Prior thoracic radiation
Proliferative breast disease	
Early first period and/or Late menopause	

2.2.2 Breast cancer in New Zealand

New Zealand has the 4th highest age-standardised incidence rate of breast cancer in the world, which is 89.4 per 100,000 population (as cited in McKenzie et al., 2014). So far, breast cancer remains the top cancer for female patients in New Zealand with an incidence far higher than other types of cancer (Fig. 2). The mortality of breast cancer experienced an extensive decrease (as shown in Fig. 3) since 1998 when the New Zealand government started the Breast Screen Aotearoa (BSA) programme-- a free national breast screening programme for women aged between 45 and 69. This programme allows females to have a free examination of their breasts by mammogram every two years and largely improves the survival of patients by detecting breast cancer at an early stage.

An ethnic disparity in breast cancer mortality in New Zealand has been observed and studied by several researchers. As a country full of immigrants, the NZ population consist of approximately 74% European, 14.9% indigenous Māori, 11.8% Asian, 7.4%

Pacific people and 1.2% Middle Eastern/Latin American/African (NZ Census 2013). It has been reported that the mortality of breast cancer is higher in Māori women and Pacific Island women (5-year survival rate 74% and 72%) compared to the mortality rates in non-Māori and non-Pacific women (5-year survival rate 83%, mainly refer to Europeans) (as cited in Muthukaruppan, 2011). Evidence shows that this survival difference is largely due to socio-economic inequalities instead of ethnicity (Jeffreys et al., 2005), although Māori women tend to present more aggressive breast cancer. The attendance rates in BSA programme in NZ were 39% for Māori women and 58% for non- Māori women (Curtis, Wright, & Wall, 2005), which lead to the delays in diagnosis and makes treatment more challenging for Māori patients.

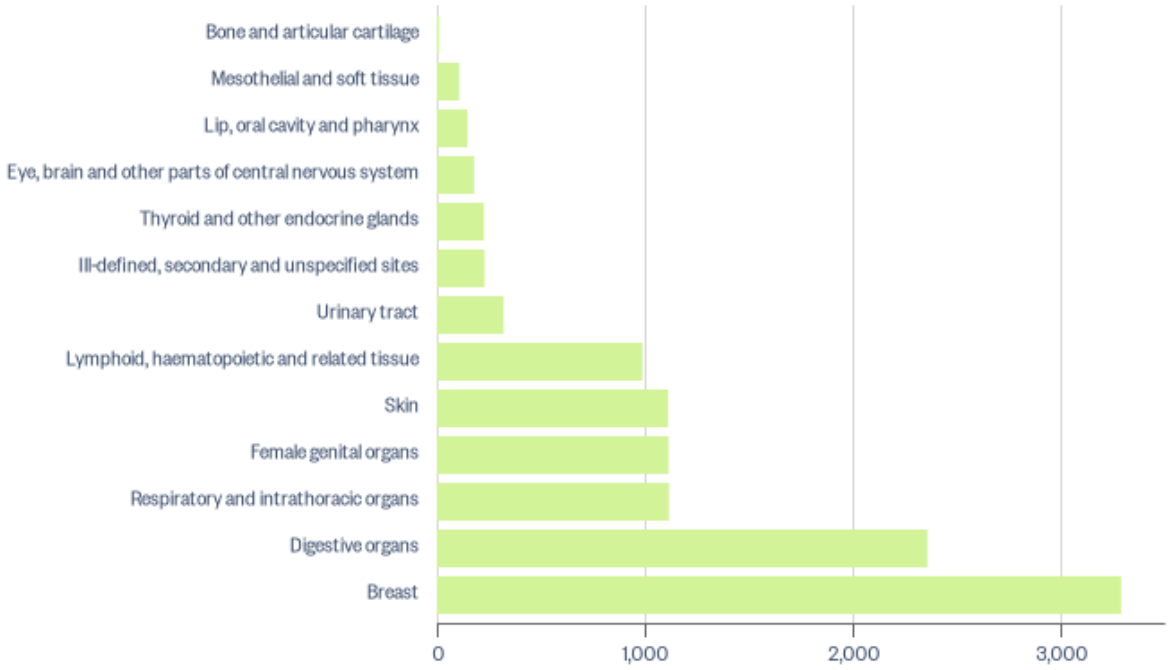


Figure 2. New cancer registrations in the New Zealand female population. By cancer site or type, 2015. (Ministry of Health, 2015)

Chart retrieved from https://figure.nz/chart/sqJArKCVJfUNi4iC-b6NKns5ZlKOXu622

Data available from http://www.health.govt.nz/publication/new-cancer-registrations-2015

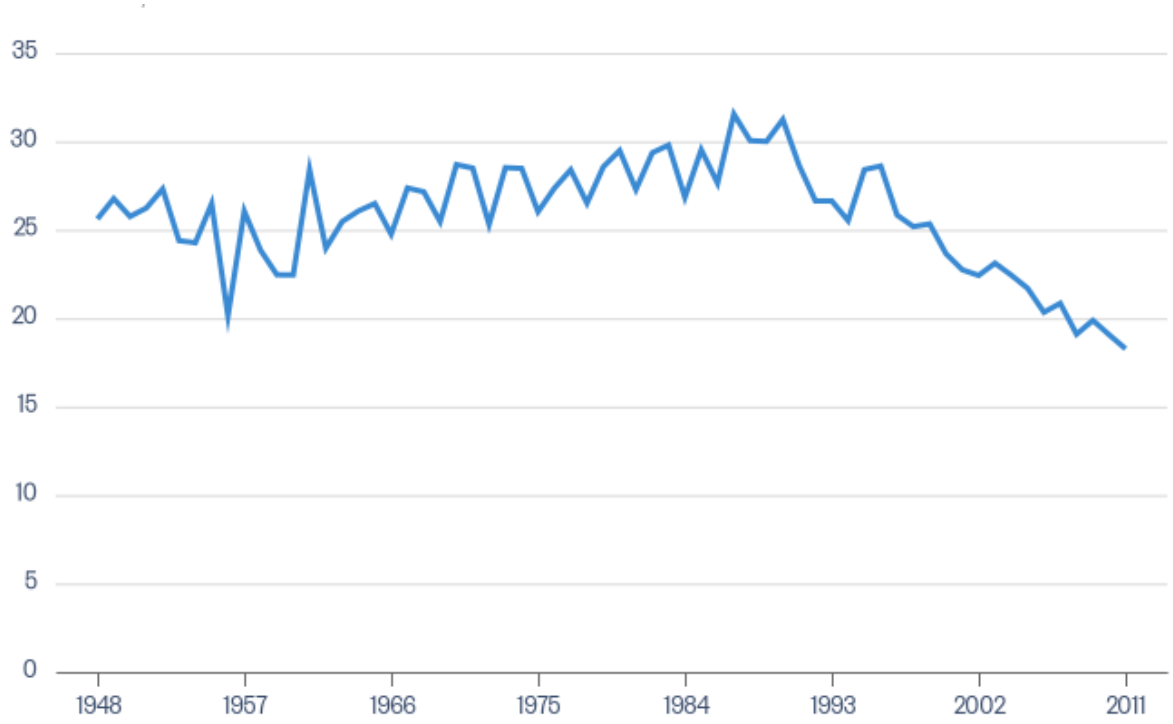


Figure 3. Death rate for breast cancer in New Zealand. Females, 1948-2011, age-standardised rate per 100,000 population. (Ministry of Health, 2014)

Data available from https://www.health.govt.nz/publication/cancer-historical-summary-1948-2011

2.2.3 Classification of breast cancer in relation to therapies

As a very heterogeneous disease, breast cancer can be classified into multiple categories serving different purposes based on the status of the nodules, tumour size, histopathology, grade, stage and some intrinsic expressions of proteins (Uscanga-Perales, Santuario-Facio, & Ortiz-López, 2016). The most basic classification of this disease is based on its pathology, which consists of ductal carcinoma, lobular carcinoma (*in-situ* or invasive) and medullary carcinoma. Under this category, invasive ductal carcinoma and invasive lobular carcinoma are the two most common morphological subtypes, representing approximately 80% and 10% of all invasive breast cancers, respectively (Rivenbark, O'Connor, & Coleman, 2013). It has been reported in 2012 that breast cancer comprises over 100 morphologically distinct tumour types and subtypes recognized by the World Health Organisation (Lakhani, 2012).

For better guiding therapies, all morphological subtypes of breast cancer will be further divided into clinical classifications based on their molecular diversity. There are two main clinical classifications: One is based on the protein expression results of

immunohistochemical staining, the other is based on the gene expression profiling using DNA microarrays.

2.2.3.1 Immunohistochemical classification of breast cancer

Traditionally, breast cancers are classified into three subgroups according to the presence or absence of three immunohistochemical biomarkers on the cells, namely hormone receptor (HR) positive breast cancer, HER-2 amplified breast cancer and triple-negative breast cancer (TNBC).

The HR positive group is also known as ER-positive breast cancer, with the majority of them also expressing PR. This is the most numerous and diverse group, accounting for 70-80% of all breast cancer cases (Dixon, 2014). Due to its sensitivity to oestrogen, patients often receive endocrine therapy as a standard treatment and have better prognosis than ER negative cancer patients.

HER-2 (sometimes also called c-ErbB-2 or Neu) is amplified in approximately 15-20% of breast cancer tumours (Lakhani, 2012). It has been defined that "a tumour is determined as HER2-positive if the number of tumour cells displaying strong overexpression (3 + cells) exceeds 10% of the total tumour population; equivocal if the number of tumour cells displaying moderate HER2 overexpression (2 + cells) exceeds 10% of the total tumour population and negative otherwise" (as cited in Escrivá-de-Romaní, Arumí, Bellet, & Saura, 2018, p 80). With the overexpression of the gene, this type of breast cancer has an advantage of treatment using HER-2 targeted therapies.

When the expression of all ER, PR and HER-2 receptors are negative in the tumour, it is commonly known as triple-negative breast cancer. TNBC accounts for about 12-17% of breast cancers (Merino Bonilla, Torres Tabanera, & Ros Mendoza, 2017). It is more aggressive than HR-positive and HER-2 overexpressed breast cancers and unfortunately lack effective biomarkers, which makes cytotoxic chemotherapy currently the only option for TNBC patients (Tinoco, Warsch, Glück, Avancha, & Montero, 2013), thus has a much worse expectation of survival. Compared to HR positive cancer patients, TNBC patients are typically younger, with an age below 50 years old. Within this younger age group, statics shows that the incidence of TNBC is especially higher in those who have African ancestry and/or carry BRCA1/2 gene mutations. On the contrary, most HR-positive breast cancer patients are post-menopausal and BRCA1/2 mutations are less common.

Immunohistochemical classification of breast cancer has great therapeutic values, and it has been set as a routine management in the clinic. However, there are a few limitations on applying this classification. The most critical aspect is that it only allows physicians to identify candidate patients to certain therapies, but it does not guarantee all patients will respond to the therapy and actually benefit from it. In other words, immunohistochemical ER-positive breast cancer patients may receive endocrine therapy as standard treatment, or anti-HER-2 therapy to those who have HER-2 positive cancer, but the reality is only a limited proportion of patients will actually benefit from these treatments (Viale, 2015). Chances are there are false-positive (ER^+ /PR^-) or false-negative (ER^- /PR^+) results that cannot be corrected by immunohistochemical staining (Patani, Martin, & Dowsett, 2013), consequently lead to poor clinical implications in prognostication and patient stratification for treatment (Li et al., 2010). Similarly in HER-2 breast cancer cases, false-negative HER2 status lead to negligence of anti-HER2 directed therapy while false-positive HER2 results lead to unnecessary administration of costive, prolonged and non-beneficial treatment (Escrivá-de-Romaní, Arumí, Bellet, & Saura, 2018). Patients being responsive or resistant to given therapies cannot be distinguished until they receive treatment. Therefore, researchers have kept looking for new biomarkers (for treatment or the resistance to the treatment), and other ways to more precisely predict individual therapeutic efficiency (Viale, 2015).

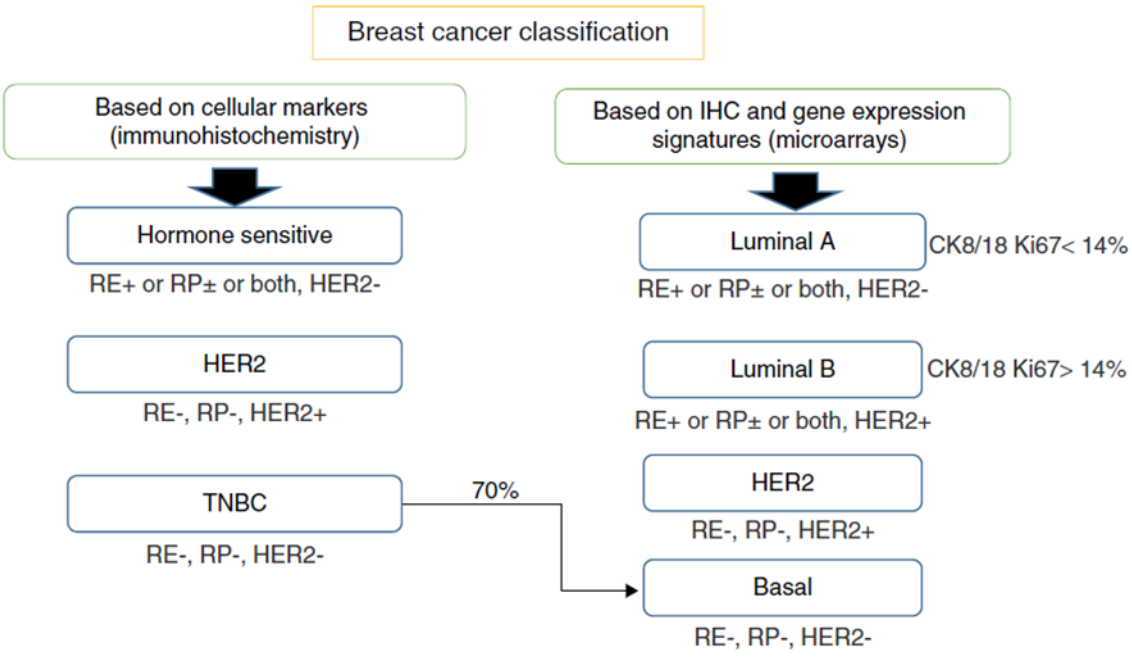


Figure 4 Two clinical classifications of breast cancer (Uscanga-Perales, Santuario-Facio, & Ortiz-López, 2016, p. 106)

2.2.3.2 Molecular classification of breast cancer

During the last 15 years, the development of gene expression profiling allows researcher to provide a more sophisticated classification of breast cancer. According to unique gene expression profiles of breast cancers from DNA microarrays, they can be re-classified into 4 main intrinsic molecular subtypes: LuminalA, Luminal B, HER2-enriched, basal-like and normal-like breast cancer. This is a classification that is officially adopted by the panelists of the St. Gallen Breast Cancer Conference in 2011 (Goldhirsch et al., 2011).

Luminal A and B breast cancers are both ER-positive and/or PR-positive. They are differentiated by status of HER-2 and a proliferative biomarker Ki-67 (also known as MKI67). Luminal A type is typically HER-2 negative with low levels of the protein Ki-67. Because of the relatively slower growth rate of cells and advantage of endocrine therapy, it has a best prognosis of 5-year survival of 80% (Sørlie et al., 2001), with low histological grade and low recurrence rates. Luminal B includes both HER-2 positive and negative proportions. The Ki-67 protein level in luminal B type tumours are slightly higher than in luminal A type, which makes it slightly more aggressive. The standard cut-offs for Ki-67 to distinguish luminal A and luminal B has been first defined in 2001 in the St. Gallen Conference to be 14%, but later in 2013 it was challenged and suggested to be raised to 20% for a better separation of luminal tumours, and then raised to 20-29% in 2015. So far, the front two Ki67 labelling indexes are both commonly applied (Gallardo et al., 2018).

HER2-enriched breast cancer is HR negative and HER-2 positive. Unlike the HER-2 positive group defined by immunohistochemical staining, this type doesn't have approximately half of tumours also express ER and/or PR as in the former (as cited in Vici et al., 2015). It is clinically more aggressive than luminal tumours. Most successful treatments are with HER-2 targeted therapies. What worth mentioning here is that one minor constituent of clinically triple-negative breast tumours is actually genetically expressed as the HER-2–enriched subtype. This is suspected to be the explanation for

why some patients who are clinically HER-2 negative gain a benefit from HER-2–targeted agents (Perou, 2011).

Basal-like breast cancer is a major subtype of TNBC, with a proportion of approximately 70%. Although it is a 'triple-negative' immunophenotype, it has its own distinctive clinical performance, histological features, chemotherapeutic response, sites of distant relapse and outcome (Badve et al., 2011). Basal-like type can be distinguished from other TNBCs based on its unique expression of high-molecular-weight cytokeratin 5/6 ($CK5/6^{+}$) and/or epidermal growth factor receptor (EGFR/HER1) (Palazzo & Colleoni, 2017). A high expression of p53 or mutations of the TP53 gene has also been observed in up to 85% of basal-like tumours (Badve et al., 2011), which is commonly associated with drug resistance.

Besides basal-like phenotype, there are some other subsets of TNBC sometimes also categorised into main intrinsic molecular subtypes. The most known subsets include a claudin-low subtype, which is named after its low expression of many of the claudin genes (i.e. claudin3, 4, and 7) (Perou, 2011). Even within the basal-like subtype, it can be further subdivided into BL1 and BL2 types to better identify molecular characteristic for therapeutic purpose (Lehmann et al., 2011). Thus, TNBC is a highly diverse group of breast cancers. Despite all the effort looking for new biomarkers and molecular analysis, TNBC subtypes currently still have poor prognosis in absence of therapies. Chemotherapy remains the only treatment that patients can benefit from.

Molecular classification of breast cancer provides a better and more precise prognosis for patients (i.e. risk of cancer recurrence, resistance, benefit from chemotherapy, etc.), and it is considered a future direction for precise treatment. However, it has not yet been used in guiding therapies in the clinic. Several new targeted therapies for breast cancer are currently ongoing clinical trials with more still trying to find effective antibodies.

2.2.4 Breast cancer metastasis

Regardless of the progress in treatment, metastasis often develops in breast cancer patients. Metastasis is defined as "the migration of tumour cells from the primary tumour, followed by intravasation, survival, extravasation of the circulatory system, and progressive colonization of a distant site" or in genetic level "tumour cells being genomic instable, promoting selection for characteristics that enable invasion and

distant organ colonization" (Marino et al., 2013, p. 1085). Most metastatic breast cancer (MBC) develops after endocrine therapy, target therapy and chemotherapy, and it can develop months or even years after a patient has been treated for breast cancer. Only 5% of breast cancer patients are initially diagnosed as stage IV disease (metastasis) (Marino et al., 2013). Common metastatic sites for MBC include the bone, brain, liver, and lungs (National Cancer Institute, 2017). If not bilateral breast cancer, then the most common site for metastasis is from the breast of initial tumour(s) to the other side (Peart, 2017). So far, there is no proven cure for MBC patients. Therapy is considered only palliative in MBC treatment, accompanying with mounting adverse effects. Therefore, MBC remains a leading cause of death of breast cancer patients. The 5-year survival rate of MBC patients is approximately 26% (as cited in Peart, 2017).

The main obstacles in MBC treatment involve the complexity, heterogeneity and genomic instability of MBC tumours. As mentioned in the previous section, prognosis and therapeutic options of breast cancers are based the expression of biomarkers and predictive molecular signatures. However, in MBC, information from the primary tumour in a patient may not accurately reflect the information of metastatic site(s). It is worse when there are plural metastatic sites in patient, because one metastasis may vary from another (Marino et al., 2013), and makes the application of therapy challenging. The redundancy of mechanistic pathways is another hallmark of MBC. Genes that promote breast cancer metastasis abound, and include ERBB2, CTNNB1, KRAS, PI3KCA (alias PI3K), EGFR, MYC, TWIST1, SNAI1 (alias SNAIL), SNAI2, MET, and ID1 (as cited in Marino et al., 2013). Cancer stem cells and dormant cells also contribute to the complexity of the presence of tumour subpopulations.

2.2.5 Management of breast cancer

The management of breast cancer typically involves screening, biopsy, surgery, radiotherapy, neoadjuvant therapies and adjuvant therapies. Sometimes it also includes the chemo-prevention and surgical-prevention of breast cancer for high-risk patients but will not be further discussed here in this thesis due to its relevance.

The detection and diagnosis of breast cancer routinely start with the imaging of both breasts. There are three imaging tools commonly applied in the clinic. The baseline imaging method for most patients (age > 40 years) is mammography whereas in younger group (age < 40 years) is ultrasound. Sometimes imaging of breast tissue from

both tools can be equivocal due to a dense breast or a large tumour, magnetic resonance imaging (MRI) is then applied to improve the pre-treatment staging of the breast. MRI is known for its advantage of high sensitivity and precise imaging. It is also routinely requested for patients at very high risk and those with invasive lobular carcinoma (Lakhani, 2012). After screening, the stage of the breast tumour will be assessed combined with the results of biopsies and general blood test, providing information about tumour size, evidence of lymph node spread and metastasis (New Zealand Guidelines Group, 2009).

Depending on the staging, breast conserving surgeries or mastectomies will be arranged to patients unless the cancer is inoperable. In some cases, but not always, a neoadjuvant therapy will be given to patients with large tumours prior to surgery to shrink tumour size, so later they could have breast conserving surgeries instead of the initial decision of mastectomies (as cited in Cain et al., 2017). After surgery, patients usually receive adjuvant treatment in the form of radiotherapy and/or adjuvant therapies to kill the remaining cancer cells and prevent the recurrence of cancer.

2.2.6 Neoadjuvant and adjuvant systemic therapies in breast cancer treatment

Chemotherapy is a systemic treatment for cancer that can be used as both neoadjuvant therapy and adjuvant therapy for patients. Neoadjuvant therapies refer to those pre-operative chemotherapies that given to patients before surgery, and adjuvant therapies are the ones given to patients after surgery in addition to other treatments (Peart, 2017). In current breast cancer treatment, they mainly consist of three kinds of therapies depending on the clinical classification of the removed tumour.

Endocrine therapies are effective treatment recommended for ER-positive breast cancer patients, using selective oestrogen receptor modulators (SERMs), selective oestrogen receptor down-regulators (SERDs) and aromatase inhibitors (AIs) as major agents. These endocrine agents can block oestrogen stimulation at the receptor level (anti-oestrogens) or get the oestrogen source away from tumour cells (Lønning, 2000), therefore extensively inhibit growth of ER-positive tumour cells. SERMs are the most widely applied in endocrine therapy, with tamoxifen (first-generation SERM) and raloxifene (second-generation SERM) being the standard adjuvant agents.

HER-2 targeted therapy is a great success in breast cancer treatment. HER2-positive breast cancer patients used to face worse prognosis of shorter time to disease relapse,

increased incidence of metastases and higher mortality than those with HER2-negative cancer (as cited in Perez et al., 2014). With the emergence of anti-HER2 agents, such as trastuzumab (also known as Herceptin) and sometimes combined with lapatinib, they stop cells from growing rapidly.

For TNBC patients, there is currently no other standardised therapy approved in the clinic, but only cytotoxic chemotherapy given as neoadjuvant and adjuvant therapies. There has been a focus on PARP inhibitors (PARPi) that can possibly be utilised for TNBC treatment. As stated before, there is a large proportion of TNBC patients who are mutated-BRCA1/2 carriers. Poly (ADPribose) polymerase (PARP) is an important enzyme in repairing DNA errors (Garber, 2017). In breast cancer, PARPi, which target the PARP enzymes, is selectively cytotoxic to BRCA1/2 mutated or DNA-damage repair deficient patients, implicating its potential value in TNBC treatment (Wang, Shi, Huang, & Guan, 2018). The clinical trial of this PARP targeted therapy is yet ongoing.

Although with all these selective therapeutic options in the clinic, traditional chemotherapy yet plays a critical role in breast cancer treatment. For one, its significance in TNBC treatment has been fully stated. Second, it is required where endocrine therapy and other targeted therapies have limitations. Normally targeted therapies present less adverse effects than traditional chemotherapy, but it is considered that endocrine agents or anti-HER2 agents should be applied in combination with chemotherapy when patient's response to targeted therapy is not high enough. Also, chemotherapy is an alternative for patients when they unfortunately have endocrine resistance. Approximately 1 in 4 patients expressing both the ERα and PgR proteins in their tumours will not respond to first-line endocrine therapy despite being ER-positive (intrinsic resistance) (Lønning, 2000), and the mechanism is rather complex. Third, for breast cancer patients who have developed metastasis, chemotherapy is often required due to the molecular heterogeneity and instability of MBC tumours.

2.3 Demand of natural products in cancer treatment

Toxicity to normal cells and drug resistance are the main reason for the adverse effects of chemotherapy and its failure. To improve survival and life quality of patients, there has been efforts looking for new bioactive compounds and their possible combinations with current anti-cancer agents, especially natural compounds. Natural products are typically low toxicity or non-toxic to normal cells, with multiple

biofunctions. A substantial number of natural products exhibit anti-cancer properties, and some of them can activate or mediate plural pathways to directly or indirect inhibit cancer development. Recent reports have indicated that natural dietary compounds can successful increase the therapeutic effects of anti-cancer drugs when used in combination, reducing the toxicity to normal cells by allowing lower doses of the drug administered (as cited in Pádua, Rocha, Gargiulo, & Ramos, 2015). In recent decades, the focus of source of natural products has turned from plants to marine organism, mainly due to their high biodiversity and rich resources.

In breast cancer treatment, the difficulty to accomplish effective therapies due to the molecular heterogeneity and diversity in breast cancer has drawn extensive attention. Side effects and drug resistance have been observed in both chemotherapy and targeted therapies in breast cancer treatment. There has been some research studying natural compounds derived from sea product applying alone or combined with endocrine agents or chemo-agents in therapies, especially those marine products with multiple anti-cancer pathways.

2.4 Fucoidan

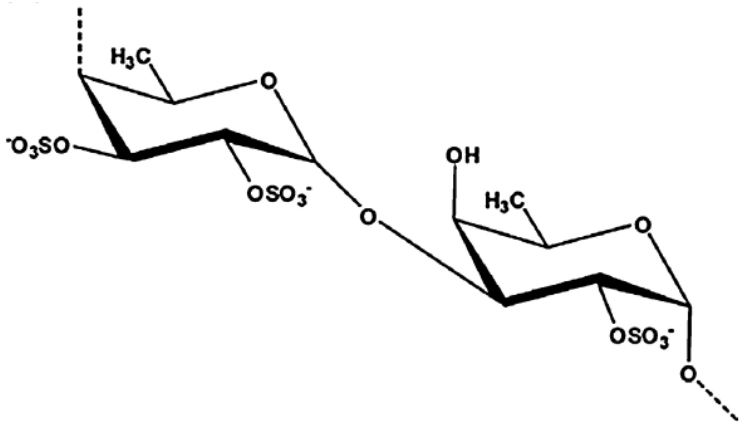


Figure 5. Chemical structure of fucoidan (Pádua, Rocha, Gargiulo, & Ramos, 2015, p. 93)

Polysaccharides are the most abundant among the natural products produced by plants and they widely exist in plants, animals, microorganisms and algae (Wijesinghe & Jeon, 2012). Fucoidans (Fig. 5) is a type of complex sulphated polysaccharides derived mainly from various brown algae species but also exist in other marine animal species such as sea cucumber and sea urchin (as cited in Vo & Kim, 2013). It shows great therapeutic value due to its ability to possesses numerous biological activities

including antiproliferative, antioxidant, anti-inflammatory, antiviral, anticoagulant, anti-tumour, anti-metastatic, anti-angiogenesis, anti-allergic and immunomodulatory activities (as cited in Chizhov et al., 1999; Vo & Kim, 2013). It was first named as "fucoidin" when it was discovered and isolated from marine brown algae by Kylin in 1913 (as cited in Senthilkumar, Manivasagan, Venkatesan, & Kim, 2013). Its current name "fucoidan" is according to IUPAC rules, and sometimes also called as fucan, fucosan or sulfated fucan (Berteau & Mulloy, 2003).

2.4.1 Composition and structure of fucoidan

The main components of fucoidan are L-fucose and sulphates, along with small proportions of monosaccharides (mannose, galactose, rhamnose, glucose, xylose, etc.), uronic acids, acetyl groups and proteins (as cited in Wu et al., 2016). Fucose is the fundamental sub unit of the fucoidan polysaccharide, which is a hexose deoxy sugar with the chemical formula $C_6H_{12}O_5$ (Wijesinghe & Jeon, 2012). Generally, the molecular structure of the fucose backbone is repeated (1–3)-α-L-fucopyranose or alternating and repeated (1–3)- and (1–4)-α-L-fucopyranose (as cited in Wu et al., 2016).

The chemical composition and molecular structure of extracted fucoidan may vary based on multiple determining factors. First, the component of fucoidan extracted from various brown seaweed species is variable. In a few brown seaweed species, such as Fucus vesiculosus, their chemical composition of fucoidan are relatively simple, just mainly being L-fuocse and sulphates, while most fucoidans from brown seaweeds have rather complex chemical compositions (Li, Lu, Wei, & Zhao, 2008). Despite the distinctions dependent on seaweed species, environmental factors such as geography and seasons that have impacts on the growth of brown seaweeds will also have influence on the composition of fucoidan. Other than these factors interacting with marine organisms, methods of extraction and purification of fucoidan have direct impacts on their composition and molecule structures. Normally, fucoidans are isolated from brown seaweeds either using acidic solutions or hot water. The use of acids or not, what kinds of acids, extraction time and temperature and purification procedures can all generated distinctive alternations on composition and structures of fucoidan (Ale, Mikkelsen, & Meyer, 2011). For instance, fucoidan extracted or analysed by H_2SO_4 solution might lead to a higher level of sulphate content due to this specific acid hydrolysis (Ale, Mikkelsen, & Meyer, 2011). The composition of fucoidan can be

variable to some extent depending on brown seaweed species, environment, extraction conditions of fucoidan as well as purification conditions of fucoidan, thereby influencing its performance in biofunctions.

2.4.2 Anti-cancer properties of fucoidan

Fucoidan-mediated anti-cancer activities have been demonstrated in vivo and in vitro in different types of cancers (Atashrazm, Lowenthal, Woods, Holloway, & Dickinson, 2015). Directly, fucoidan interferes with several signaling pathways such as PI3K/AKT pathway and the pathways of cell apoptosis and the cell cycle, inhibiting cancerous cell growth. Other biofunctions of fucoidan such as immune system activation and anti-angiogenesis change the in vivo environment and make tumour cells lose the advantage to expand, therefore killing cancer cells indirectly.

2.4.2.1 Effects of fucoidan on cell apoptosis

Induction of apoptosis in cancer cells is one of the best known anti-cancer pathways of fucoidan. Apoptosis is a morphologically distinct form of cell death. It is characterized by cytoplasmic shrinkage and chromatin condensation that facilitates the removal of cells without inducing inflammation (Elmore, 2007), commonly known as programmed cell death. In cancerous cells, this apoptosis process is suppressed, resulting in cells which then start to grow out of control.

There are two pathways to mediate the process of cell apoptosis, namely the mitochondrial pathway and the death receptor pathway. Both pathways suppress the growth of cancer cells through activating caspases, which then induce cell death by degrading proteins (Kim, Park, Lee, & Park, 2010).

The mitochondrial pathway is the intrinsic pathway, activated by several cellular stress inducers that cause the release of apoptogenic factors such as cytochrome C from the mitochondrial intermembrane space into the cytosol. Cytochrome C together with the adaptor protein Apaf-1 then lead to the activation of caspase-9 in the cytosol, of which can cleave and activate effector caspases (i.e. caspase-3 and caspase-7) (Hengartner, 2000). During the process of the intrinsic pathway, the mitochondrial membrane potential (MMP) in cells is changed. Mitochondrial outer membrane permeabilization (MOMP) is regulated by the interaction effect between the Bcl-2 family of proteins and requires BAX and/or BAK activation (Schug, Gonzalvez, Houtkooper, Vaz, & Gottlieb, 2011). The ability of these Bcl-2 family members to form

ion channels has suggested that these proteins open pores in the outer mitochondrial membrane, allowing the exit of cytochrome C (as cited in Banafa et al., 2013).

The death receptor pathway as an extrinsic pathway of apoptosis is relatively more complex. There are two subtypes of extrinsic pathways. Generally, extrinsic apoptosis is initiated at the plasma membrane by stimulation of death receptors such as fatty acid synthase (FAS) and tumour necrosis factor receptor-1. Binding of these death receptors leads to receptor oligomerization and induces the binding of adapter molecules including the adaptor protein Fas-associated death domain (FADD) and initiator caspase-8. These adapter molecules recruit caspase-8 to the receptor complex to form the death-inducing signalling complex (DISC). Upon DISC formation, caspase-8 undergoes dimerization. Caspase-8 initially cleaves itself forming a heterodimer of which is necessary for recognition of other substrates, such as effector caspases and the proapoptotic Bcl-2 family member BID (Schug, Gonzalvez, Houtkooper, Vaz, & Gottlieb, 2011; Banafa et al., 2013).

The difference between type I and type II extrinsic pathways depends on the level of caspase-8 activation upon DISC formation. In the type II pathway, caspase-8 must engage the intrinsic (mitochondrial) pathway to amplify the death signal and execute apoptosis due to the slow DISC assembly and low production of active caspase-8. This pathway transition is achieved through caspase-8 processing of BID. Cleaved/truncated BID interacts with other BCL-2 family members on the surface of the mitochondria, resulting in MOMP. Alternatively, the type I pathway occurs where large amounts of DISC and active caspase-8 are formed, leading to a direct cleavage of effector caspases in the cytosol (Schug, Gonzalvez, Houtkooper, Vaz, & Gottlieb, 2011).

It has been reported that the mitochondrial pathway is the primary pathway of most fucoidan-mediated apoptosis in various cancers (Senthilkumar, Manivasagan, Venkatesan, & Kim, 2013), although fucoidan can induce cell apoptosis through both pathways. In 2010, Kim et al. revealed that low concentrations of fucoidan (5–20 μg/mL) extracted from *F. vesiculosus* induced apoptosis of HT-29 and HCT-116 colon cancer cell lines in a dose- and time-dependent manner. The cytotoxic effect of fucoidan is better on HT-29 cells than on HTC116 cells, and activations of caspase-3,7,8,9 were observed in the experiment. They also reported later in 2011 that fucoidan extracted from *Undaria pinnatifida* can induce apoptosis in A549 human lung carcinoma cells,

with caspase-9 activities observed. There are, however, a few exceptions where fucoidan mediates cell apoptosis caspase-independently. In 2011 and 2013, Zhang et al. observed that fucoidan obtained from *Cladosiphon navae-caledoniae* Kylin can induce apoptosis in human breast cancer cell lines MCF-7 and MDA-MB-231 through mitochondrial-medicated pathway. While the apoptosis in MDA-MB-231 cell line is caspase-dependent, a caspase-independent apoptotic pathway was activated in MCF-7 cancer cells through activation of ROS-mediated MAP kinases and regulation of the Bcl-2 family protein-mediated mitochondrial pathway.

2.4.2.2 Effects of fucoidan on cell cycle

The cell cycle is the mechanism of cell division. A complete cell cycle consists of G0 phase (quiescence), G1, S, G2 and M phases (proliferation) and then back to quiescence. As with many other anti-cancer agents, fucoidan intervenes the processes of the cell cycle in various cancerous cells to inhibit cancer growth. Typical fucoidan-induced cell cycle arrest is in sub G0/G1 cell accumulation (suggestive of dead cells/apoptotic cells), while cell cycle arrest in other phases has also been observed. Significant down regulation of cyclin D1, cyclin D2 and CDK4 are demonstrated in this process, which is considered to be the mechanism of fucoidan-induced cell cycle arrest (Atashrazm, Lowenthal, Woods, Holloway, & Dickinson, 2015).

2.4.2.3 Effects of fucoidan on metastasis

Anti-metastatic effects of fucoidan has been observed in several studies. In a study of A549 human lung cancer cells, anti-metastatic behaviour of fucoidan is owing to its inactivation of ERK1/2 pathway, which is a pathway involved in the invasive or migratory behaviour of numerous malignancies (Suthiphongchai, Promyart, Virochrut, Tohtong, & Wilairat, 2003). Fucoidan also suppressed MMP-2 activity and protein expression in a dose-dependent manner in A549 human lung cancer cells. Matrix metalloproteinases (MMPs) such as MMP-2 and MMP-9 play critical roles in tumour metastasis. The degradation of extracellular matrix (ECM) is crucial for cellular invasion, indicating the inevitable involvement of matrix degrading proteinases for the process (Lee, Kim, & Kim, 2012).

In addition to ERK1/2, the PI3K–Akt signal pathway has been shown to regulate the invasion and metastasis of non-small-cell lung cancer (NSCLC) as well as the development and progress of various other tumours. PI3K overexpression is highly

correlated with the development, invasion, and metastasis of NSCLC. Fucoidan inhibits the phosphorylation of PI3K–Akt in time and concentration-dependent manners (Liao, Wang, Zhang, & Liu, 2006).

2.4.2.4 Other indirect anti-cancer pathways of fucoidan

Besides targeting pathways in cell proliferation, fucoidan also regulates other activities in vivo that against cancer growth.

It has been reported that fucoidan can enhance the immunity system to eliminate cancer cells. Maruyama, Tamauchib, Iizuka and Nakano reported in 2006 that fucoidan mediates tumour destruction through type 1 T-helper (Th1) cell and NK cell responses (Vo & Kim, 2013, p.22), and the quantity of macrophages increased as well. Both NK cells and macrophages can be activated to kill cancer cells (Wu et al., 2016).

Anti-angiogenesis activity is another way of fucoidan suppressing tumour growth. Tumour requires vessels to supply nutrients and oxygen along with the blood to support its growth. Fucoidan has been found to inhibit the binding of a key angiogenesis promoting molecule VEGF to its cell membrane receptor (Koyanagi, Tanigawa, Nakagawa, Soeda, & Shimeno, 2003). The inhibition of the formation of new vessels consequently inhibits the expansion of tumour cells.

Prevention of cancer development has also been mentioned in fucoidan studies. The antioxidant characteristic of fucoidan effectively eliminates oxygen free radicals in vivo before they attack DNA and proteins in somatic cells, reducing cancer incidence.

2.4.3 Therapeutic value of fucoidan in breast cancer treatment

Anti-cancer properties of fucoidan in breast cancer have been reported both in vitro and in vivo studies, involving various cell signalling pathways. Fucoidan isolated from *Cladosiphon okamuranus* inhibited the proliferation of MCF-7 cells in a time- and dose-dependent manner, and induced apoptosis through the extrinsic pathway. Meanwhile, it showed no cytotoxic effect on normal human mammary epithelial cells (Zhang, Teruya, Eto, & Shirahata, 2011). Fucoidans from *Saccharina japonica* and *Undaria pinnatifida* (derived from East Asia) inhibited both cell proliferation and colony formation in T-47D breast cancer cells. Besides cytotoxic effects, fucoidan was proven to block MDA-MB-231 breast carcinoma cells' adhesion to platelets, which implied its potential for tumour metastasis suppression (as cited in Pádua, Rocha, Gargiulo, & Ramos, 2015). In animal model, fucoidan extracted from *Fucus vesiculosus* inhibited 4T1 mouse breast

cancer cell growth in vivo and in vitro via downregulation of the Wnt/β-catenin signalling pathway without causing cytotoxic effects in normal cells. A decrease of vascular endothelial growth factor (VEGF) expression was also observed in 4T1 cells, indicating the antiangiogenic activity of fucoidan (Xue et al., 2013).

As a non-toxic anti-cancer agent, fucoidan can be used in combination with chemotherapy agents (including endocrine/targeted therapies) to lower the toxicity of therapy to patients as well as generate synergistic inhibitory effects on breast cancer. A recent study has reported a combination treatment of fucoidan (obtained from Japan) and three chemotherapeutic agents (cisplatin, tamoxifen and paclitaxel) on two breast cancer cell lines (MCF-7 and MDA-MB-231). Compared to using treatments of fucoidan or drugs alone, this combination treatment exhibit highly synergistic inhibitory effects on the growth of breast cancer cells. It is stated that fucoidan enhanced the downregulation of the anti-apoptotic proteins Bcl-xL and Mcl-1 by these chemotherapeutic drugs and intracellular ROS levels, and reduced glutathione (GSH) levels in breast cancer cells. A protective effect of normal human fibroblast TIG-1 cells by fucoidan to prevent apoptosis from cisplatin and tamoxifen has also been observed, indicating a decrease on side effects of therapy (Zhang, Teruya, Yoshida, Eto, & Shirahata, 2013). The anti-metastatic property of fucoidan is also a promising quality to improve overall survival for patients, especially for MBC patients. Taken together, these outcomes suggest a favourable characteristic of fucoidan for its application in breast cancer treatment.

2.4.4 Determining factors for fucoidan anti-cancer activities

2.4.4.1 Sulphate content

Sulphate groups in fucoidan are considered to be associated with its anti-cancer performance. Normally, the increase of sulphate content in fucoidan enhance its anti-cancer effects. A recent research in 2010 has compared the anti-cancer activities of purchased native fucoidan and its over-sulphated product. They found that cancer growth was more effectively inhibited under the treatment of over-sulphated fucoidan than unprocessed ones, especially in the low-molecular weight group (F5-30 kDa) (Cho, Lee, & You, 2010). It is believed that the loose construction of low-molecular-weight fucoidan molecules allows them to have higher extent of sulphate substitution (Ale, Mikkelsen, & Meyer, 2011). As a result, over-sulphated LMF showed better inhibition on cancer proliferation than over-sulphated native fucoidan and large-molecular-weight

(F>30 kDa) fucoidan. Apart from the sulphate percentage of fucoidan, the sulphated position on the polysaccharide molecule backbones are related to its anti-cancer activity as well.

The reason for sulphate content correlation to anti-cancer properties of fucoidan is possibly the negative charge it gives to fucoidan molecules, which helps in the recognition of biological targets (e.g. growth factors receptors) (as cited in Wu et al., 2016). However, being sulphated is not a compulsory condition for fucoidan molecules to be bioactive. Evidence has been found that the fucoidan extracted from brown seaweed *S. hornery* can exert anti-cancer effects on human skin melanoma cancer cell line SK-MEL-28 and human colon cancer cell line DLD-1 in vitro experiments while no sulphate group has been detected in it (as cited in Wu et al., 2016).

2.4.4.2 Molecular weight

Molecular weight is another critical factor that has impact on anti-cancer performance of fucoidan. The molecular weight of fucoidan polymers ranged from 21 to 1600 kDa, exhibiting significant variations (as cited in Yang, Chung, & You, 2008). Low-molecular-weight fucoidan (LMF) has been reported to have higher anti-cancer properties than native fucoidan and high-molecular-weight fucoidan (HMF). In polysaccharides, lower molecular weight usually means smaller molecular size, which enables LMF to enter cancer cells much easier than HMF (Wu et al., 2016). The solubility of LMF and HMF is another related factor. Although sometimes LMF solubility in water or medium might not be quite ideal due to its hydrophobicity, HMF as large molecules are more commonly known for its relatively low solubility. Hence, lower molecular weight is conductive to enhance the anti-cancer activity of fucoidan. A recent research in 2013 confirmed the significance of fucoidan molecular weight. Choi and Kim (2013) found out that if choosing proper method to degrade polysaccharides in fucoidan without reducing sulphate content (γ-irradiation was applied in that research), the anti-cancer activity of fucoidan can be enhanced by simply decreasing molecular weight.

Attention needs to be paid to the facts that molecular weight and sulphate content are not always dominating factors in the anti-cancer effects of fucoidan. The molecular structure of fucoidan remains critical. In 2010, Cho et al. produced three fucoidan fractions with molecular weights of <5, 5–30 and >30 kDa and reported that the F5-30K

showed the most tumor growth inhibitory effect despite the sulfate amount in F<5K being greater than in the two other fractions. Nevertheless, sulphate content and molecular weight of fucoidan should be taken into consideration when testing its anti-cancer properties.

2.4.5 Fucoidan from *Undaria pinnatifida*

Brown seaweeds including wakame (*Undaria pinnatifida*) are popular food in East Asia. *Undaria pinnatifida* (Harvey) Suringar is native to the cold temperate seas of the Northern Hemisphere. It is endemic to Japan, Korea and China, expanding along some regions of the coastlines of Australia and New Zealand. In New Zealand, it is an unwanted organism due to its growth in a diverse range of habitats, with harvest allowed (with permission) in places such as mussel farms and highly infested coastlines (as cited in Mak, Hamid, Liu, Lu, & White, 2013).

Undaria pinnatifida is a great source of fucoidan. It contains the richest fucoidan content when mature, which is a fucoidan yield of approximately 57.3–69.9% in September in New Zealand. *Undaria pinnatifida* in the sea in Japan (Peter the Great Bay) showed similar properties. When it matures in June-July, the amount of crude fucoidan rose markedly from 3.2% (in April) to 16.0% of dry weight (Skriptsova, Shevchenko, Zvyagintseva, & Imbs, 2010). It has been reported that fucoidan derived from this type of seaweed has a higher sulphate and L-fucose content (Kim, Lee, & Lee, 2010). Compared to common fucoidan, fucoidan extracted from *Undaria pinnatifida* contains more sulphated galactofucans than sulphated fucose (as cited in Vishchuk, Ermakova, & Zvyagintseva, 2011), which enable them to exhibit a broader range of bioactivities. Synytsya et al. (2010) examined the cytotoxic effect of fucoidan derived from Korea brown seaweed *Undaria pinnatifida* in five distinctive cancer cell lines (PC-3, HeLa, A549, and HepG2). Cell viability shows that the inhibition is in a similar pattern to the commercial fucoidan.

Fucoidan from *Undaria pinnatifida* is also confirmed to be non-toxic. In 2010, Kim and Lee investigated genotoxicity of fucoidan from Sporophyll of *U. pinnatifida* in three genotoxicity tests and confirmed that fucoidan presents no significant genotoxic concern under the anticipated conditions of use. In the same year, Chung et al. reported that up to 1000 mg/kg body weight per day of the consumption of fucoidan from

Undaria pinnatifida showed no sign of toxicity in rodents (after up to 28 days of daily administration).

Chapter 3 Methodology

3.1 Cell lines involved in this study

Table 2. Cell line information

Cell Line Designation	Catalogue Number	Cell Line Description	Supplier
MCF-7	HTB-22	Human mammary gland, breast; derived from metastatic site: pleural effusion; (ER^+, $PR^{+/-}$, $HER\text{-}2^-$)	ATCC
MAD-MB-231	HTB-26	Human mammary gland/breast; derived from metastatic site: pleural effusion; (ER^-, PR^-, $HER\text{-}2^-$)	ATCC
HDFa	PCS-201-012	Human Fibroblast	ATCC

The two breast cancer cell lines used in this study are differential on the immunohistochemical classification of breast cancer. MCF-7 cells are obtained from ER-positive breast cancer, with PR either positive or negative and HER-2 receptor negative, clinically more sensitive to endocrine agents such as tamoxifen. MDA-MB-231 is a highly metastatic cancer cell line where all three receptors (ER, PR, & HER-2) in the cells are negative, thus has fewer chemotherapeutic agent options. Both of two cell lines are widely utilized for in vitro studies of breast cancer and are considered as powerful experimental tools that, at least partially, reflect breast cancer heterogeneity in vitro (Lewinska, Adamczyk-Grochala, Kwasniewicz, Deregowska, & Wnuk, 2017). HDFa is a normal cell line used to examine toxicity of fucoidan in this study.

All three cell lines (Table 2) were stored at -80 °C or in liquid nitrogen. After thawing the cell lines, they were maintained in 25 or 75 cm2 tissue culture flasks containing 5 ml or 12 ml of completed growth culture medium, in 37 °C incubator with 5% CO2 humidified air.

3.2 Cell proliferation assay

To study the cytotoxic effects of high- and low-molecular weight fucoidan on cancer cell proliferation, cell viability of MCF-7 and MDA-MB-231 incubated in a range of various concentrations for various time periods was determined by using the methylthiazol-diphenyl-tetrazolium (MTT) assay. The MTT assay is a kind of colorimetric assays. This method enables measuring the decrease of yellow MTT compound by mitochondrial succinate dehydrogenase. After MTT entering the cells, it reaches to mitochondria and react with mitochondrial succinate dehydrogenase, turning into an insoluble and dark purple formazan product. Organic solvents such as Dimethyl sulfoxide (DMSO) are then used to solubilise the formazan product, causing a change in the colour of solvent which can be measured spectrophotometrically. The consumption of MTT only occurs in metabolically active cells, therefore this assay is a reliable way to indirectly reflect the number of viable cells (Hansen, Nielsen, & Berg, 1989).

Table 3. Major materials in cell culture and cell proliferation assay

Number	Material	Supplier
1	Cell culture medium (RPMI 1640)	Life technologies
2	Cell culture medium (106)	Life technologies
3	L-glutamine (200 mM; 100 ml)	Life technologies
4	Penicillin-Streptomycin (10,000 U/ml; 100 ml)	Life technologies
5	Trypan Blue Solution, 0.4%	Life technologies
6	TrypLE™ Express Enzyme (1X), no phenol red	Life technologies
7	PBS (Phosphate buffered saline), pH 7.2, no calcium magnesium and phenol red	Life technologies
8	Sterile filtered fetal bovine serum (FBS)	Life technologies
9	Low Serum Growth Supplement (LSGS)	Life technologies
10	MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (Cat No. M2128-1G)	Sigma- Aldrich (St Louis, USA)
11	DMSO (Dimethyl sulfoxide) (Cat No. 102952)	Merck-Chemicals
12	25 and 75 cm^2 cell culture flasks, 5, 10 and 25 ml sterile disposable pipette tips, 15 and 50 ml centrifuge tubes, 96-well tissue culture plates, 6-well tissue culture plates, etc.	BD (Becton Dickinson) Bioscience (Auckland, NZ)

3.2.1 Preparation of complete cell culture medium

Cells of MCF-7 and MDA-MB-231 were cultured in RPMI 1640 base medium, additionally with 1% Penicillin-Streptomycin, 1% L-glutamine and 10% fetal bovine serum.

Cells of HDFa was cultured in Medium 106, a sterile, liquid medium for the culture of human dermal fibroblasts, with 1.96% LSGS.

3.2.2 Preparation of MTT stock solution

12 mM (5 mg/ml) MTT solution was prepared by dissolving 50 mg MTT powder in 10ml sterile phosphate buffered saline (PBS). In order to fully dissolve the powder, the mixture was vortexed and filtered through a sterile Millex GV 0.22 μm syringe filter to remove any pathogens and spontaneously formed formazan crystals. The prepared MTT solution was stored at 4°C for short term storage (within two weeks) or at -20°C for long term storage, protected from light until use.

3.2.3 Preparation of HMF and LMF stock solution

Table 4. General description of fucoidan used in this study

Type of Fucoidan	Molecular Weight	Supplier
High-molecular-weight fucoidan (HMF) from *Undaria pinnatifida*	>300k	Extracted at Auckland University of Technology (AUT), New Zealand
Low-molecular-weight fucoidan (LMF) from *Undaria pinnatifida*	<10k	Extracted at Auckland University of Technology (AUT), New Zealand

Fucoidan from two ranges of molecular-weight were used in this study. Both HMF and LMF from *Undaria pinnatifida* were extracted at Auckland University of Technology (AUT), New Zealand, and each dissolved in complete cell culture medium (RPMI 1640 base medium with 1% Penicillin-Streptomycin, 1% L-glutamine and 10% fetal bovine serum) and medium 106 (with 1.96% LSGS) respectively. HMF was dissolved to a final concentration of 300 μg/ml as stock solution. LMF was dissolved to a final concentration of 600 μg/ml as stock solution.

Prepared fucoidan stock solution were divided and stored in centrifuge tubes at -80℃, protected from light until use.

3.2.4 Cell culture protocols

3.2.4.1 Thawing frozen cells

The cryovial containing frozen cells was carefully taken out from liquid nitrogen and placed in a 37°C water bath immediately. The vial was gently swirled in water bath to complete a quick thaw (within a minute) with only a minor amount of ice left in the vial,

then dried and wiped with 70% ethanol on the surface of the vial. The thawed cells were transferred into a centrifuge tube containing 3 ml pre-warmed complete cell culture medium. After a centrifugation of 5-6 minutes, the clear supernatant was carefully discarded without disturbing the cell pellet at the bottom of tube. Lastly, cells were re-suspended in 1-2 ml complete cell culture medium and transferred into a 25 cm^2 tissue culture flask, incubated at 37°C with 5% CO_2.

3.2.4.2 Medium replacement

After cells cultured in good condition for a few days but are not yet confluent, they require a medium change to replenish nutrients and maintain the correct pH. The deficient cell culture medium was carefully removed and discarded from the culture flask, followed by a genlte wash for adherent cells using 1X sterile PBS without calcium and magnesium (approximately 5 ml for each 25 cm^2 tissue culture flask and 10-11 ml for each 75 cm^2 tissue culture flask). Fresh pre-warmed complete cell culture medium was then slowly added into the flask. The flask was returned to the 37°C incubator right after the medium change.

3.2.4.3 Passaging adherent cells

When cells reached ~70-80% confluent, they were passaged to a new tissue culture flask. Before passaging cells, 1X sterile PBS, the dissociation reagent TrypLE™ Express Enzyme and complete cell culture medium were pre-warmed in 37°C water bath. PBS was first used to wash cells after old medium discarded. Then, 1-2 ml of the TrypLE™ Express Enzyme was added to the cell culture flask (approximately 0.5 ml per 10 cm^2) and incubated at 37°C for approximately 2-5 minutes (specific incubation time varies among cell lines). The detachment was stopped when completed over 90% by adding complete cell culture medium twice the volume of the used TrypLE™ Express Enzyme. The mixture was then transferred to a 15 ml centrifuge tube and centrifuged for 5-7 minutes (specific centrifuge speed and time vary among cell lines). After the centrifugation, the supernatant was carefully discarded without disturbing the cell pellet at the bottom of tube. Cells were re-suspended in 1-2 ml complete cell culture medium and 10 µl of cell suspension was taken for counting. Lastly, an appropriate amount of cell suspension was passaged into a new tissue culture flask containing fresh complete cell culture medium, incubated at 37°C with 5% CO_2.

For the three cell lines used in this study, they were not split less than 1:10 to ensure the cell density be appropriate for the cells to grow. Normally, MDA-MB-231 cells (from a confluent flask) was passaged to a new flask at 10-15% of collected cells and MCF-7 was at 15-20%. HDFa was passaged to a new flask at a percentage about 20%. Cells passaged at these percentages could reach 70~80% confluent in 2 to 4 days and be ready for experiments or for sub-culturing.

3.2.4.4 Freezing cells

Freezing medium was stored at -20°C and was thawed and kept at 4°C before use (the appropriate freezing medium is dependent on cell line types), containing a cryoprotective agent such as DMSO or glycerol. The procedure of harvesting cells from the cell culture flask was the same as used during the sub-culturing. After centrifugation, cells were resuspended in 1-2 ml growth medium and counted for an approximate total cell number. Based on the recommended cell density for storage using freezing medium and the total cell number, cells were re-centrifuged to remove growth medium and resuspended in proper amount of cold freezing medium. Aliquots of the cell suspension were dispensed into cryogenic storage vials. The cryovials containing the cells were first stored at -80°C overnight then transferred to liquid nitrogen and stored in the gas phase above the liquid nitrogen.

3.2.5 MTT cell proliferation assay protocol

MTT assays in this study were performed following the protocol first described by Mosmann in 1983.

3.2.5.1 Major equipment used in assays

Table 5. Major equipment and materials applied in MTT assay

Number	Equipment
1	Multiple pipette
2	Inverted microscope
3	Centrifuge
4	Haemocytometer
5	Multi-functional orbital shaker
6	Microtiter plate reader (FLUOstar Omega, Alphatech) with 540 and 680 nm filters

3.2.5.2 Procedure for MTT cell proliferation assay

Table 6. Procedure for MTT assay

Step	Action
1	Harvesting cells from culture flask
2	Conducting a cell count and adjusting cell density
3	Seeding cells on 96-well plates, with 100 µl in each well
4	Adding treatments, with 100 µl into each well
5	Adding MTT solution
6	Adding DMSO and measure absorbance

Step 1: Cell preparation

Sterile PBS, the TrypLE™ Express Enzyme and complete cell culture medium were pre-warmed in 37°C water bath before cell preparation. The procedure of harvesting cells from the cell culture flask was the same as used during the sub-culturing. After the centrifugation, the supernatant in tube was carefully discarded. Cells were gently re-suspended in 1-2 ml of fresh complete cell culture medium.

Step 2: Cell counting

A minor amount of the cell suspension was taken to perform a cell count. 10 µl of cell suspension was removed onto a piece of parafilm and mixed with equal amount of Trypan Blue. 10 µl of this mixture was then injected to one side of the haemocytometer, by which the number of cells could be counted under the microscope. If cells were too concentrated, the cell suspension was then diluted to a relatively lower concentration to present a clear view for cell count on the haemocytometer. The concentration of cell suspension in culture medium was calculated using the following equation:

Cell density in suspension (cells/ml) = (Counted cell number / Number of squares) ×dilution factor ×10,000 cells/ml

The dilution factor is 2 for the sample diluting in 1:1 with Trypan blue.

Step 3: Seeding cells

The seeding density for both breast cancer cell lines in this study was 50,000 cells/ml. Required amount of cell suspension from step 2 was diluted to the seeding density with complete cell culture medium to make a cell stock suspension. After dilution, cell stock suspension was seeded into each well in 96-well plates at 100µl/well. For one column of six wells, complete cell culture medium was added in each well

instead of cell stock suspension to set as a blank control. After seeding, plates were placed and kept in the incubator, incubated at 37°C with 5% CO_2.

Step 4: Adding treatment

Treatment was added to cells after about 18 hours incubation when cells were attached to the bottom/wall in each well. A range of various concentrations of treatment were freshly prepared with pre-warmed complete cell culture medium on the day of the experiment. 100 µl of each concentration of treatment was gently added to corresponding wells in 96-well plates. For one column of six wells containing cells, complete cell culture medium was added instead of treatment to set as a negative control. Treatment was not required for the Day 0 plate. All plates were placed back to the incubator and kept incubated.

Step 5: Adding MTT solution

Because fucoidan does not interact with MTT or influence colorimetry, there was no requirement of changing medium before adding MTT solution. After incubation for a set of time (48, 72 and 96 hours in the case of this study), 10 µl of MTT reagent was added to each well in the 96-well plate and gently shaken the plate to ensure the approach of MTT to cells. The plate was then placed back for incubation for about 4 hours, after which dark purple formazan was visible in wells.

Step 6: Quantification

170 µl of the supernatant in each well was gently removed away with only minor amount of liquid left in each well to prevent formazan loss. DMSO was then added to each well (100 µl/well) to fully dissolve purple precipitate, using an orbital plate shaker to make sure all precipitates are dissolved completely. After another incubation of 20 to 30 minutes at 37°C, the plate was set for 5 minutes automatic shaking in the plate reader and absorbance was measured at a wavelength of 540 nm. The average absorbance value (OD value) was calculated from readings of the six replicates in each column. The linear relationship between absorbance value and cell number was exhibited using plots of absorbance on the Y-axis against cell number on the X-axis.

3.2.6 Determination of MTT assay linearity range

Appropriate cell density and culturing time are critical for a good linear relationship that is presented between the MTT assay results and the number of viable cells. The

number of cells vs. absorbance standard curve is used to determine the linearity of the MTT assays. The number of cells in cell viability studies is suggested to fall within the linear portion of the curve.

The highest cell concentration set in this study for MCF-7 cell line and MDA-MB-231 cell line was 400,000 cells/ml. Cell suspensions were kept in 1.5ml micro-tubes and using a series of one in two (1:2) dilutions (by complete cell culture medium) to prepare micro-tubes of final cell suspension. An aliquot of 100µl cell suspension each concentration was seeded into corresponding wells of the 96-well plate. Each concentration has a set of six replicate wells. Step 6 (in section 3.2.6.2) was conducted approximately 18 hours after seeding.

Table 7. (1:2) Dilution plan for making cell linearity standard curve

Number	Cell concentration (cells/ml)
1	400,000
2	200,000
3	100,000
4	50,000
5	25,000
6	12,500
7	6,250
8	3,125

3.2.7 Determination of cell doubling time

Cell doubling time is the length of time required for cell population to double when they are in logarithmic growth period. The doubling times of two breast cancer cell lines in this study were calculated from the cell growth curve.

100 µl of cell suspension (density 50,000 cells/ml) was seeded into each well in four 96-well plates. After cultured for a set of time (0, 24, 48 and 72 hrs) in the incubator, the absorbance reflecting cell number was measured daily using MTT assay. Based on the absorbance values, the relationship between absorbance and time required for cell growth was presented. According to the equation of cell number vs. absorbance standard curve, the absorbance for doubled cells (100,000 cells/ml) can be calculated and then looked up for the time in the cell growth curve. Therefore, the cell doubling time was obtained by the equation of cell growth curve. According to the two curves, cells doubling time for MCF-7 is 40.617 hours and for MDA-MB-231 is 32.023 hours.

3.2.8 Determination of the inhibitory effects of HMF and LMF

Step 1：Cell seeding

The determined cell density for both MCF-7 and MDA-MB-231 cell lines was 5, 000 cells per well, with each well containing 100 μl of cell suspension.

Step 2: Treatment preparation

HMF solution of various concentrations for both cell lines was prepared using the 300μg/ml HMF stock solution and complete cell culture medium RPMI 1640, diluted into the following concentrations (as shown in Table 8):

Table 8. HMF dilution plan for MCF-7 and MDA-MB-231

Number	Treatment concentrations of HMF	Final concentrations of HMF in plates (1:2 dilution)
1	240 μg/ml	120 μg/ml
2	200 μg/ml	100 μg/ml
3	160 μg/ml	80 μg/ml
4	120 μg/ml	60 μg/ml
5	80 μg/ml	40 μg/ml
6	40 μg/ml	20 μg/ml
7	20 μg/ml	10 μg/ml
8	10 μg/ml	5 μg/ml

LMF solutions of various concentrations for MCF-7 cell line treatments were prepared using the 600 μg/ml LMF stock solution and complete cell culture medium RPMI 1640, diluting into the following concentrations (as shown in Tables 9 & 10):

Table 9. LMF dilution plan for MCF-7

Number	Treatment concentrations of LMF	Final concentrations of LMF in plates (1:2 dilution)
1	400 μg/ml	200 μg/ml
2	200 μg/ml	100 μg/ml
3	100 μg/ml	50 μg/ml
4	50 μg/ml	25 μg/ml
5	20 μg/ml	10 μg/ml
6	10 μg/ml	5 μg/ml
7	4 μg/ml	2 μg/ml
8	2 μg/ml	1 μg/ml
9	1 μg/ml	0.5 μg/ml

LMF solutions of various concentrations for MDA-MB-231 cell line treatments were prepared using the 600μg/ml LMF stock solution and complete cell culture medium RPMI 1640, diluting into the following concentrations:

Table 10. LMF dilution plan for MDA-MB-231

Number	Treatment concentrations of LMF	Final concentrations of LMF in plates (1:2 dilution)
1	600 μg/ml	300 μg/ml
2	500 μg/ml	250 μg/ml
3	400 μg/ml	200 μg/ml
4	300 μg/ml	150 μg/ml
5	200 μg/ml	100 μg/ml
6	100 μg/ml	50 μg/ml
7	50 μg/ml	25 μg/ml
8	10 μg/ml	5 μg/ml
9	2 μg/ml	1 μg/ml

Step 3: Adding treatment

Treatments were added into each plate approximately 18 hours after seeding cells, which ensures all the cells were attached to the bottom/wall in each well. When adding treatment, 100 μl of each concentration of treatment was slowly adding into the corresponding wells, and each treatment concentration has a column of six replicate wells. One column of six wells received only medium instead of treatment to set them as negative controls. The plate designs of HMF and LMF to MCF-7 and MDA-MB-231 cell lines are displayed as shown (Fig. 6 & 7):

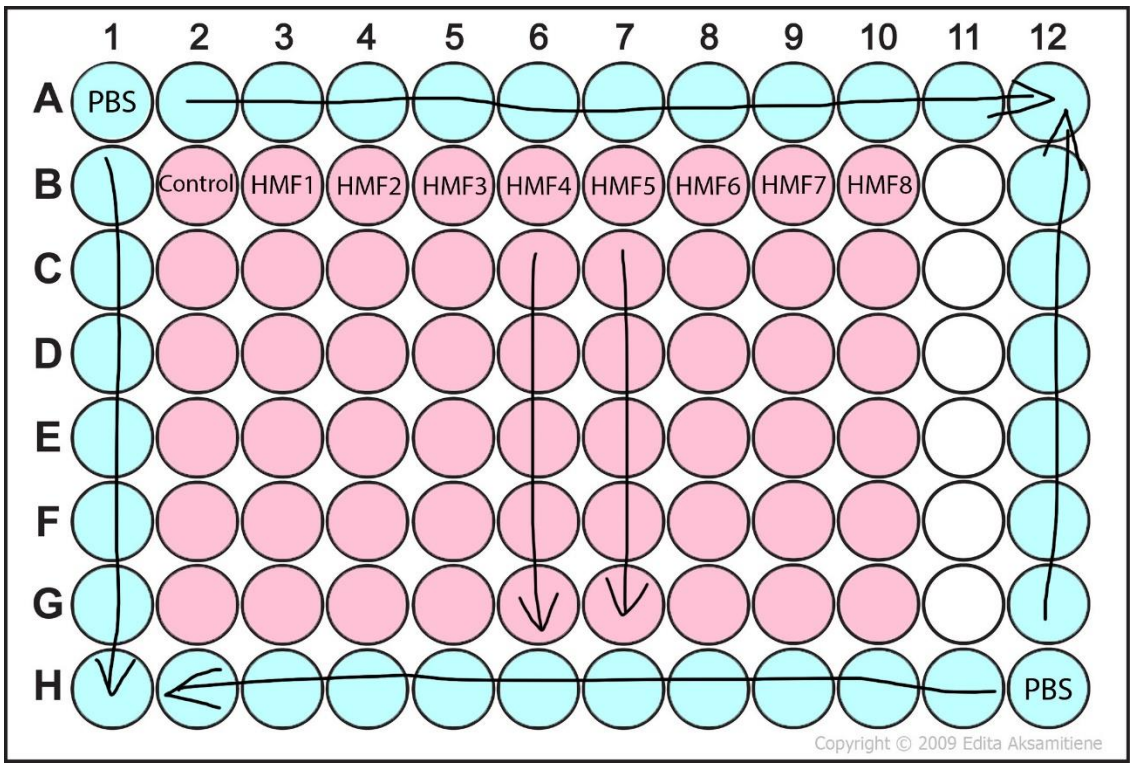


Figure 6. 96-well plate design for both cell lines testing HMF

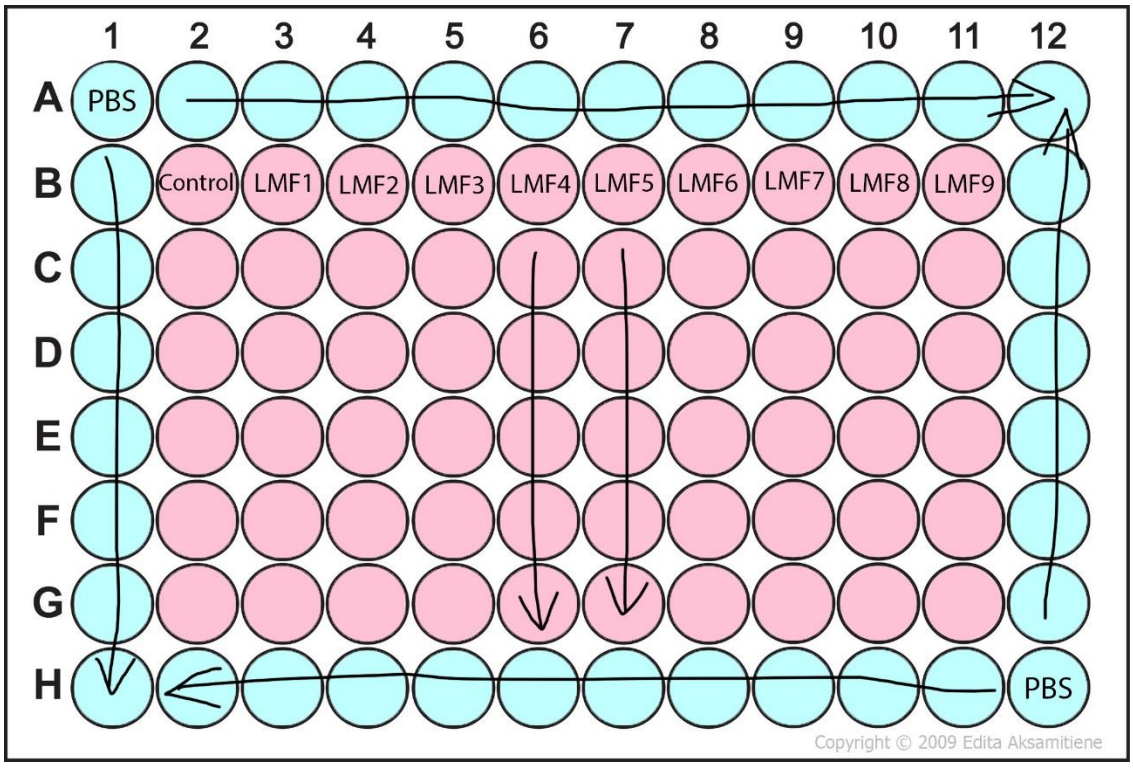


Figure 7. 96-well plate design for both cell lines testing LMF

3.3 Cell cycle assay

The cell cycle assay in this study was only performed on HDFa (fibroblast) cell line to examine the toxicity of fucoidan. Based on the previous results of the inhibitory effects of HMF and LMF, only LMF showed cytotoxicity to two breast cancer cell lines, therefore it required examination in this assay. Fucoidan typically induce cell cycle arrest in cancerous cells thereby inhibiting cancer growth and is selectively not cytotoxic to normal cells.

Flow cytometry is a critical biophysical technology applied extensively in biomedical and clinical field for multiple purposes (i.e. cell counting, cell sorting, biomarker detection and protein engineering). For cell cycle assay, a single parameter cellular DNA content is measured using flow cytometry. There is a linear relationship between cellular fluorescence intensity and amount of DNA that can be measured by using fluorescent dyes specifically binding to DNA. The emitted fluorescence from the DNA-specific dyes reflects DNA content in different phases of the cell cycle proportionally. One of the most frequently used dyes to quantitatively assess DNA content is PI, which binds to DNA by intercalating between the bases and has little or no sequence preference. Due to the fact that PI also binds to RNA, nucleases such as Ribonuclease (RNAse) are necessary to eliminate the interference (Hansen, Nielsen, & Berg, 1989).

3.3.1 Major equipment and materials used in cell cycle assay

Table 11. Major equipment and materials used in cell apoptosis assay

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

3.3.2 Protocols for cell cycle analysis

3.3.2.1 80% Ethanol preparation

80% Ethanol was prepared by distilled water and absolute ethanol. The solution was then stored at -20℃ until use.

3.3.2.2 RNAse A (Ribonuclease A) solution preparation

RNAse A solution was prepared by dissolving the RNAse A powder (100 mg) in manufacturer's vial in distilled water (DDW) and made a final concentration of 1mg/ml as a stock solution. The stock solution was divided into microtubes and stored at -20℃.

3.3.2.3 PI stock solution preparation

10 mg of PI powder was contained in the vial. The PI stock solution was prepared by dissolving PI powder in 10ml distilled water to prepare a 1mg/ml solution. The prepared PI stock solution was stored at 4℃, protected from light until use.

3.3.2.4 Cell preparation and treatment preparation

Step 1: Cell seeding

Cells in the culture flask were collected the same way as in passaging adherent cells. The seeding density of cells was at 50,000 cells/ml in 6-well plates, with 2 ml cell suspensions contained in each well. A parallel group (plate) was set in the experiment. The two plates were kept in the incubator for about 18 hours to allow all cells attached to the bottom/wall of the wells.

Step 2: Adding treatment

Treatment was prepared using the 600 μg/ml LMF stock (of medium 106) and complete culture medium 106 for HDFa, diluted into the concentrations of 300 μg/ml, 150 μg/ml and 75 μg/ml (Table 12). Prepared LMF solution of all concentrations were kept in 15 ml centrifuge tubes with at least 4 ml in each tube. Old medium in plates was carefully removed prior to steadily adding 2 ml LMF of each concentration into corresponding wells (as shown in plate design Fig. 8). 2 ml fresh complete medium was added instead of LMF in the control wells. Both plates were kept incubated with treatment for 72 hours.

Table 12. LMF dilution plan for HDFa

Number	Treatment concentrations of LMF (1:2 dilution of the stock)	Final concentrations of LMF in plates (replace)

1	300 μg/ml (3.5ml stock +3.5ml medium)	300 μg/ml 2ml/well
2	150 μg/ml (3ml No.1 +3ml medium)	150 μg/ml 2ml/well
3	75 μg/ml (2ml No.2 +2ml medium)	75 μg/ml 2ml/well

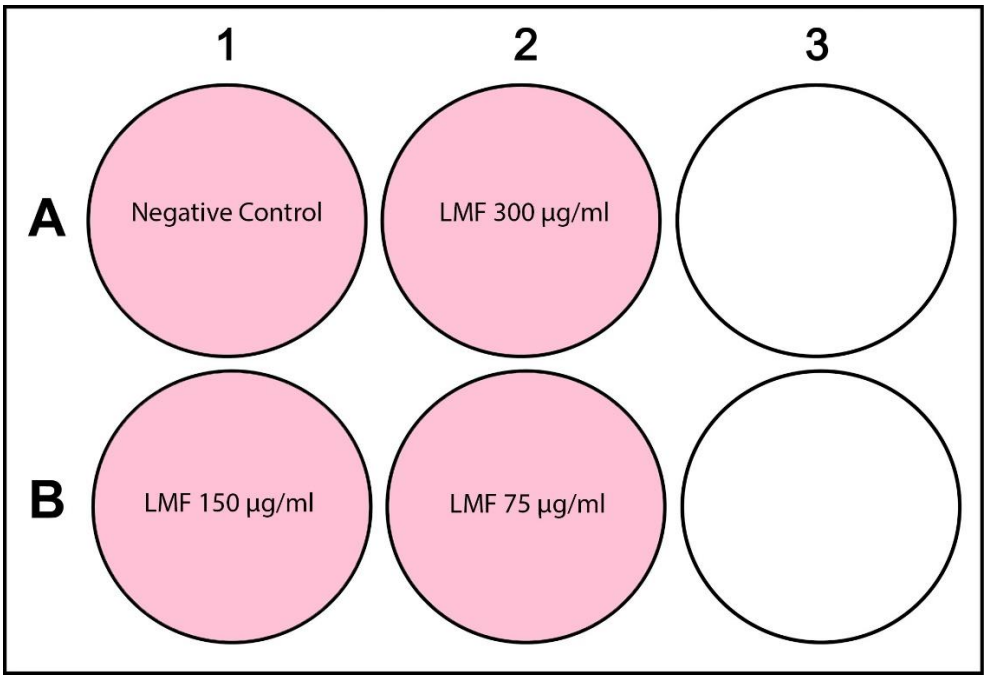


Figure 8. 6-well plate design for cell cycle assay

3.3.2.5 Cell harvesting and sample preparation

The whole process of collecting and preparing cell samples was conducted on ice.

Step 1: Cell collecting

Supernatant in each well was collected to labelled 15 ml centrifuge tubes accordingly. Cells in each well were washed by 500 μl PBS and the PBS was also collected into relevant tubes. After wash, 500 μl TrypLE™ Express Enzyme was added to detach cells, during which the plate was placed back in the incubator for no more than 5 minutes. Then, 1 ml cell culture medium was added into each well to stop detaching and the mixture was transferred into corresponding tubes. Each well was washed by PBS one more time and collected.

Step 2: Centrifugation

All tubes containing cells were centrifuged at 1200 RPM at 4°C for 5 minutes. Supernatant in each tube was carefully removed after centrifugation and 1 ml PBS was added into each tube following another centrifugation of 5 minutes.

Step 3: Cell resuspension

After the second centrifugation, supernatant in each tube was discarded but left only a minor amount of PBS in tubes. Centrifuge tubes were then gently swiped left and right 3 times on a tube rack for cell re-suspension.

Step 4: Cell fixing and storage

80% ethanol was taken out of -20℃ freezer and use immediately. 1 ml ice cold 80% ethanol was slowly added into each tube while placed on the vortex and set at low speed. In order to avoid formation of aggregates in cells, tubes were slightly tilted when adding ethanol to assure ethanol dropping to the side of the tube instead of directly onto the cells. After that, tubes were sealed with parafilm and stored at -20℃ for more than two days but less than 10 days before cells were taken to analysis.

3.3.2.6 Cell cycle analysis

Step 1: Permeabilizing solution preparation

The permeabilizing solution consists of 0.1% Triton-X100, RNAse A and PBS. In the study, 10 µl Triton-X100 + 500 µl RNAse A + 9,490 µl PBS was mixed and prepared for 1 ml permeabilizing solution for each test tube.

Step 2: Ethanol removal

Tubes containing cells were first centrifuged at 1200RPM for 2-3 minutes before the removal of the parafilm and the discard of the ethanol. Cells were then washed by 3 ml ice cold PBS which was later discarded after a second centrifugation. This wash and centrifuge process was repeated twice in total to completely remove the ethanol.

Step 3: PI staining

After the ethanol in each tube was removed, 1ml permeabilizing solution was added to each tube to resuspend cells and fully mixed. Cell suspension in tubes was then transferred to test tubes and sent for incubation at 37℃ for about 45 minutes. After permeabilization, 5 µl of PI stock solution was added to each test tube and fully mixed

with cell suspension. Cells in test tubes were incubated for another 5 minutes in dark at room temperature before they were loaded to the flow cytometer for measurement.

3.4 Cell mechanism assays

In relation to the inhibitory effect of LMF on breast cancer cell lines, a few more assays were conducted in this study to explore its anti-cancer mechanisms. Five kits purchased from Merck Millipore were used in mechanism studies. Mechanism assays were conducted in MDA-MB-231 cell line.

3.4.1 Major equipment and materials used in assays

Table 13. Major equipment and materials used in cell mechanism assays

Number	Equipment and Materials	Supplier
1	6-well cell culture plates	BD (Becton Dickinson) Bioscience (Auckland, NZ)
2	1.5 ml microcentrifuge tubes	Sigma- Aldrich
3	Deionized water	
4	LMF stock solution (with complete PRMI 1640 medium)	
5	30% Hydrogen peroxide (HP)	Sigma- Aldrich
6	Muse™ Count & Viability Kit Catalog No. MCH100102	Merck Millipore
7	Muse™ Annexin V & Dead Cell Kit Catalog No. MCH100105	Merck Millipore
8	Muse™ MultiCaspase Kit Catalog No. MCH100109	Merck Millipore
9	Muse™ MitoPotential Kit Catalog No. MCH100110	Merck Millipore
10	Muse™ Nitric Oxide Kit Catalog No. MCH100112	Merck Millipore
11	Muse™ Cell Analyzer	Merck Millipore

3.4.1.1 Muse™ cell analyzer

The Muse™ cell analyzer purchased from Merck Millipore is a smaller scale of flow cytometer. It uses patent-pending fluorescent detection and micro-capillary technology to deliver rapid, accurate and quantitative cell analysis of both suspension and adherent cells (in a size between 2 to 60 μm) in diameter. Cell samples loaded into this device

should be suspended into culture medium/specified buffer and in an appropriate concentration depending on the requests in assay protocols.

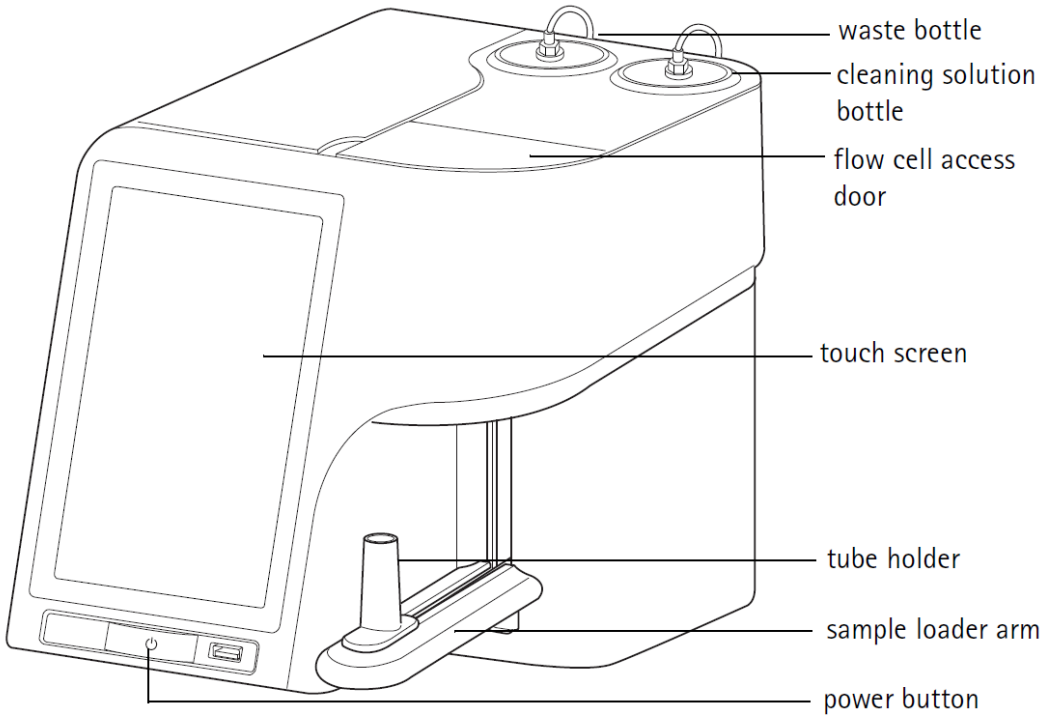


Figure 9. The Muse™ cell analyzer

3.4.2 Preparation of hydrogen peroxide stock solution

10 mM hydrogen peroxide (HP) was used in this study to prepare the positive control for multicaspase assay (Lewinska, Adamczyk-Grochala, Kwasniewicz, Deregowska, & Wnuk, 2017). A solution of 0.5 ml 30% HP was diluted in a 50ml centrifuge tube containing 49.5 ml sterile deionized water to make a 0.3% HP stock solution and kept at 4℃. When preparing the positive control, 0.5 ml of HP stock solution was diluted into 4.5 ml complete cell culture medium to make 10 mM HP medium solution.

3.4.3 Cell samples preparation

Step 1: Cell seeding

MDA-MB-231 cells were seeded at a density of 45,000 cells/ml in 6-well plates, with 2ml cell suspension in each well. All plates were kept in a 37 ℃ incubator for about 18 hours to ensure all the cells were attached to the bottom/wall of the wells.

Step 2: Adding treatment

Treatment was prepared using the 600 μg/ml LMF stock (with RPMI 1640 medium) and complete culture medium, diluted into final concentrations of 300 μg/ml, 150

μg/ml, 50 μg/ml and 5 μg/ml (as shown in Table 14). All treatments were kept in 15 ml centrifuge tubes with at least 6 ml in each tube. Old medium in plates was carefully removed and 2 ml LMF solution of each concentration was steadily added into corresponding wells (as the plate design shown in Fig. 10). 2 ml fresh complete medium was added instead of LMF solution in the wells of negative control and positive control (for multicaspase assay). Plates were kept incubated with treatment for 48, 72 and 96 hours respectively, with one plate for one time point.

Half an hour before harvesting cells, old medium in the positive control well was removed and changed into 2 ml 10 mM HP medium solution. Cells were then incubated for 30 minutes until collection.

Table 14. LMF dilution plan for Muse assays

Number	Treatment concentrations of LMF (dilution of the stock)	Final concentrations of LMF in plates (replace)
1	300 μg/ml (5.5ml stock +5.5ml medium)	300 μg/ml 2ml/well
2	150 μg/ml (4.5ml No.1 +4.5ml medium)	150 μg/ml 2ml/well
3	50 μg/ml (3ml No.2 +6ml medium)	50 μg/ml 2ml/well
4	5 μg/ml (1ml No.3 +9ml medium)	5 μg/ml 2ml/well

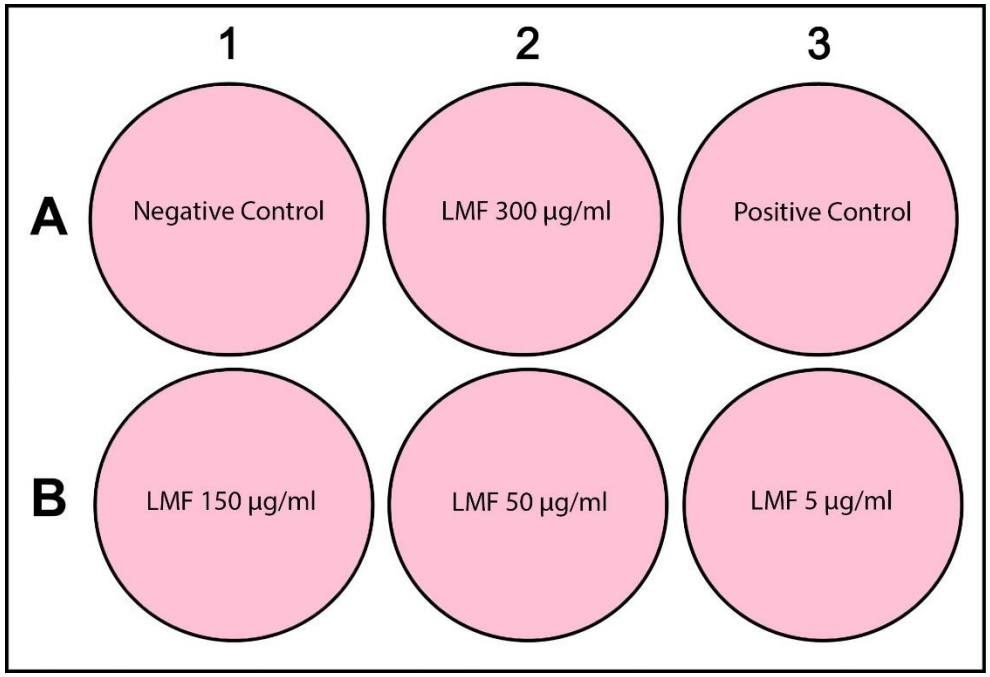


Figure 10. 6-well plate design for mechanism assays

Step 3: Cell harvesting

After being incubated for the required time, old medium/treatment in each well was carefully removed and discarded. 1 ml of PBS was added into each well. After a gentle rinse, PBS in each well was collected into corresponding labelled 15 ml centrifuge tubes. Then, 0.5 ml TrypLE™ Express Enzyme was added into each well to detach cells. During detaching, the plate was placed back in the incubator for no more than 5 minutes until most cells were detached from the wall/bottom. 1 ml complete cell culture medium was added to terminate detachment, and the mixture in each well was collected. Another 1 ml PBS was added to each well, rinsed, and transferred into tubes to ensure all cells were collected.

After collection, centrifuge tubes were centrifuged at 1200 RPM at room temperature for 5 minutes. Supernatant in each tube was discarded. Appropriate amount of complete culture medium was added into each tube to re-suspend cells. A cell counting step is required here for cell suspension in each tube, using the haemocytometer, and adjusting cell concentrations of each sample to be in the range of 1×10^5- 5×10^5 cells/ml (by dilution or re-centrifugation).

3.4.4 Cell viability assay

In this assay, viability of harvest cells was measured. Cell concentrations in each sample were re-checked and adjusted into 1×10^5- 5×10^5 cells/ml for the following assays.

3.4.4.1 Test principle

The Muse™ Count & Viability Reagent differentially stains viable and non-viable cells based on their permeability to two DNA binding dyes present in the reagent. The DNA-binding dye in the reagent stains cells that have lost their membrane integrity, allowing the dye to stain the nucleus of dead and dying cells. The membrane-permeant DNA staining dye stains all cells alive or dead with a nucleus.

The Muse™ Count & Viability Software Module then performs calculations automatically and displays data in two dot plots.

3.4.4.2 Kit components

Muse™ Count & Viability Reagent

3.4.4.3 Assay protocol

Step 1: The Muse™ Count & Viability Reagent was stored at 4℃. Proper amount of this reagent was added into each microcentrifuge tube. The amount of reagent used is depending on concentration of cell sample and can be referred from Table 15.

Table 15. Cell suspension dilution table

Concentration of Original Cell Suspension	Dilution Factor	Cell Suspension Volume	Count & Viability Reagent Volume	Concentration of Diluted Cells
1 x 10^5 to 1 x 10^6 cells/mL	10	50 µL	450 µL	<1 x 10^5 cells/mL
1 x 10^6 to 1 x 10^7 cells/mL	20	20 µL	380 µL	<5 x 10^5 cells/mL
1 x 10^7 to 2 x 10^7 cells/mL	40	20 µL	780 µL	<5 x 10^5 cells/mL

Step 2: Cell suspension in each sample was gently remixed by pipetting 3-5 times. Required amount of cell suspension was then removed into test tubes and labelled. All tubes were kept incubated for 5 minutes at room temperature to ensure a complete staining.

In the case of this study, 50 µl cell suspension was added into 450 µl reagent in each test tube and incubated for 5 minutes.

Step 3: The mixture in each test tube was gently remixed again, then loaded on Muse™ Cell Analyser for measuring.

3.4.5 Cell apoptosis assay

Cell apoptosis is a complex process involving multiple changes in cells. The ability of apoptosis to modulate the death of a cell is recognized for its great therapeutic potential, thereby emphasis has been placed on signalling pathways that control cell apoptosis.

Caspases play a central role in propagating the process of programmed cell death (apoptosis) in response to proapoptotic signals. In most cases of cell apoptosis, caspase activation has been observed and they were differentially activated in intrinsic and extrinsic pathways of cell apoptosis.

Changes in mitochondria is another feature related to cell apoptosis. Loss of the mitochondrial inner transmembrane potential is often, but not always, observed to be associated with the early stages of apoptosis (through the intrinsic pathway). Mitochondrial membrane potential changes have been implicated in caspase-dependent apoptosis as well as caspase-independent cell death processes. Depolarization of the inner mitochondrial membrane potential is thus a reliable indicator of mitochondrial dysfunction and cellular health, which has become increasingly important in the study of apoptosis and drug toxicity.

To study the fucoidan-induced cell apoptosis in MDA-MB-231cell line, three assays were applied namely Annexin V & Dead Cell Assay, MultiCaspase Assay and MitoPotential Assay.

3.4.5.1 Annexin V & dead cell assay

3.4.5.1.1 Test principle

Phosphatidylserine (PS) is a membrane component normally localized to the internal side of healthy cell membrane. During the apoptosis process, PS will be translocated to the outer surface of the cell membrane. Annexin V utilized in this assay is a calcium-dependent phospholipid-binding protein that has high affinity for PS, thus can binding to the PS on the external membrane of apoptotic cells. Therefore, apoptotic cells can be distinguished from non-apoptotic cells.

A dead cell marker 7-AAD is also used in this assay as an indicator of cell membrane structural integrity. It is excluded from live, healthy cells, as well as early apoptotic cells. Four populations of cell can be distinguished in this assay (as listed in Table 16).

Table 16. Four populations of cells in annexin V & dead cell assay

Four populations of cells in annexin V & dead cell assay
non-apoptotic cells: Annexin V (-) and 7-AAD (-)
early apoptotic cells: Annexin V (+) and 7-AAD (-)
late stage apoptotic and dead cells: Annexin V (+) and 7-AAD (+)
mostly nuclear debris: Annexin V (-) and 7-AAD (+)

3.4.5.1.2 Kit components

Muse™ Annexin V & Dead Cell Reagent (Part No. 4700-1485, 100 tests/bottle)

3.4.5.1.3 Assay protocol

Step 1: The Muse™ Annexin V & Dead Cell Reagent was stored at 4℃ and pre-warmed to room temperature before use. 100 µl of this reagent was added into each test tube.

Step2: Cell suspension in each sample was gently remixed by pipetting 3-5 times. 100 µl of cell suspension was then removed into the reagent in test tubes and labelled. Due to the reagent is light-sensitive, all tubes were kept incubated in the dark for 20 minutes (at room temperature) to ensure a complete staining.

Step 3: The mixture in each test tube was gently remixed again, then loaded on Muse™ Cell Analyser for measuring.

3.4.5.2 Multi-Caspase assay

3.4.5.2.1 Test principle

This is a Pan Caspase assay that can detect the presence of multiple caspases including caspase-1, 3,4, 5, 6, 7, 8, and 9. It utilizes a derivatized VAD-peptide, which is membrane permeable, that can detect the activity of multiple caspases by binding to them with resulting fluorescent signal proportional to the number of active caspases in the cell, appearing as increased signal in the Caspase axis. The dead cell dye 7-AAD is also used in this assay to provide information on membrane integrity or cell death. Four populations of cell can be distinguished in this assay (as listed in Table 17).

Table 17. Four populations of cells in multi-caspase assay

Four populations of cells in multi-caspase assay
(LL) Live cells: caspase (-) and 7-AAD (-)
(LR) Caspase (+) cells exhibiting pan caspase activity: caspase (+) and 7-AAD (-)
(UR) Late stage of caspase activity cells: caspase (+) and 7-AAD (+)
(UL) Necrotic cells: caspase (-) and 7-AAD (+)

3.4.5.2.2 Kit components

The kits used are commercially available and their components are listed in the below table.

Table 18. Kit components in multi-caspase assay

Kit components in multi-caspase assay
Muse™ Multi-Caspase Reagent (Part No. 4700-1530, 100 tests/bottle)
Muse™ Caspase 7-AAD (Part No. 4700-1510, 100 tests/bottle)
Muse™ 10× Caspase Buffer (Part No. 4700-1535, 100 tests/bottle)
1X PBS (Part No. 4700-1515, 100 tests/bottle)
Anhydrous DMSO (Part No. 4300-0160, 100 tests/bottle)

3.4.5.2.3 Reagent preparation

3.4.5.2.3.1 Preparation of multi-caspase reagent stock solution

Step 1: 50 µl Anhydrous DMSO was added to the Muse™ multi-caspase reagent vial.

Step 2: There was a small amount of powder in each vial. Thoroughly mix the vial by repeatedly inverting until the reagent was completely dissolved.

Step 3: The Muse™ multi-caspase reagent stock solution was divided with 3-4 µl/tube in microcentrifuge tubes and store at –15 to –25°C, desiccated and protected from light. Stock solution in each microcentrifuge tube can be thawed twice, since more than two freeze/thaw cycles of the reconstituted Muse™ multi-caspase reagent stock solution will take up moisture and cause deterioration of the reagent.

3.4.5.2.3.2 Preparation of assay buffer

Assay buffer in this assay is 1× caspase buffer, prepared from 10× caspase buffer.

Step 1: The Muse™ 10× caspase buffer was pre-warmed to room temperature to completely dissolve any crystals that may have formed during storage.

Step 2: For each experiment, 150 µl of 10× caspase buffer was diluted in 1,350 µl deionized water, fully mixed and stored in a 1.5 ml centrifuge tube.

3.4.5.2.3.3 Preparation of multi-caspase reagent working solution

Step 1: The multi-caspase reagent stock solution was thawed and diluted with 1× PBS as suggested in the following table (19):

Table 19. Preparation of multi-caspase reagent working solution

Component	1 to 10 Tests	10 to 40 Tests
Stock solution	1 µl	2 µl
1× PBS	159 µl	318 µl

Step 2: The prepared multi-caspase reagent working solution was stored at 2 to 8°C, protected from light until use and must be used the same day it is prepared.

3.4.5.2.3.4 Preparation of caspase 7-AAD working solution

Step 1: The Muse™ caspase 7-AAD was diluted with 1× caspase buffer as suggested in the following table (20):

Table 20. Preparation of caspase 7-AAD working solution

Component	1 Test	6 Tests
Muse™ caspase 7-AAD	2 µl	12 µl
1× caspase Buffer	148 µl	888 µl

Step 2: The prepared caspase 7-AAD working solution was stored at 2 to 8°C, protected from light until use and must be used the same day it is prepared.

3.4.5.2.4 Preparation of cell suspension

According to the assay protocol, cells should be resuspended in 1× caspase buffer to proceed the staining.

Step 1: 50 µl of cell suspension with medium from each sample was transferred into labelled microcentrifuge tubes.

Step 2: Labelled microtubes were centrifuged for 30 seconds, then supernatant in each tube was carefully removed.

Step 3: 50 µl of 1× caspase buffer was added into each tube with cells resuspended.

3.4.5.2.5 Assay protocol

Step 1: 5 µl of Muse™ multi-caspase reagent working solution was added to each labelled tube.

Step 2: Mix thoroughly by pipetting up and down 3-5 times.

Step 3. Tubes were then loosely caped and incubated for 30 minutes in the 37°C incubator with 5% CO_2.

Step 4: After incubation, 150 µl of Muse™ caspase 7-AAD working solution was added to each tube.

Step 5: Mix thoroughly by pipetting up and down 3-5 times.

Step 6: Samples were incubated at room temperature for another 5 minutes, protected from light.

Step 7: Samples were remixed thoroughly and run on Muse™ cell analyser.

3.4.5.3 MitoPotential assay

3.4.5.3.1 Test principle

The Muse™ Mitopotential Assay utilizes the Mitopotential Dye, a cationic, lipophilic dye to detect changes in the mitochondrial membrane potential and 7-AAD as an indicator of cell death. High membrane potential drives accumulation of Mitopotential dye within inner membrane of intact mitochondria, resulting in high fluorescence. Cells with depolarized mitochondria demonstrate a decrease in fluorescence and a downward shift. Four populations of cell can be distinguished in this assay (as listed in Table 21).

Table 21. Four populations of cells in mitopotential assay

Four populations of cells in mitopotential assay
(LL) Live cells with depolarized mitochondrial membrane: MitoPotential (-) and 7-AAD (-)
(LR) Live cells with intact mitochondrial membrane: MitoPotential (+) and 7-AAD (-)
(UR) Dead cells with depolarized mitochondrial membrane: MitoPotential (+) and 7-AAD (+)
(UL) Dead cells with intact mitochondrial membrane: MitoPotential (-) and 7-AAD (+)

3.4.5.3.2 Kit components

Table 22. Kit components in mitopotential assay

Kit components in mitopotential assay
Muse™ MitoPotential Dye (Part No.4700-1580, 100 tests/vial)

Muse™ MitoPotential 7-AAD (Part No. 4700-1585, 100 tests/vial)
1X Assay Buffer (Part No. 4700-1330, 100 tests/bottle)

3.4.5.3.3 Preparation of Muse™ MitoPotential working solution

Step 1: The Muse™ MitoPotential Dye was previously stored at –20°C. After thaw, it was divided with 3-4 µl/tube in microcentrifuge tubes to make stocks, desiccated and protected from light. Stock in each microcentrifuge tube can be thawed twice and more than two freeze/thaw cycles of the Muse™ MitoPotential Dye should be avoided.

Step 2: The Muse™ MitoPotential Dye stock in one microtube was thawed and diluted with 1× Assay Buffer in 1:1000 (i.e. Table 23):

Table 23. Preparation of mitopotential dye working solution

Component	10 Test	20 Tests
Muse™ MitoPotential Dye	1 µl	2 µl
1× Assay Buffer	999 µl	1998 µl

Step 3: The prepared mitopotential working solution was stored at room temperature, protected from light until use and must be used the same day it is prepared.

3.4.5.3.4 Preparation of cell suspension

According to the assay protocol, cells should be resuspended in 1× assay buffer to proceed the staining.

Step 1: 100 µl of cell suspension with medium from each cell sample was transferred into labelled new microcentrifuge tubes.

Step 2: Labelled microtubes were centrifuged for 30 seconds, then supernatant in each tube was carefully removed.

Step 3: 100 µl of 1× assay buffer was added into each tube with cells resuspended.

3.4.5.3.5 Assay Protocol

Step 1: 95 µl of mitoootential working solution was added to each labelled tube and mixed thoroughly by pipetting up and down 3-5 timess.

Step 2: Cells were incubated for 20 minutes in a 37°C CO_2 incubator.

Step 3: 5 µl of Muse MitoPotential 7-AAD reagent was added to each well and mixed thoroughly by pipetting up and down 3-5 times.

Step 4: Samples were incubated for another 5 minutes at room temperature.

Step 5: Samples were remixed thoroughly and run on Muse™ cell analyser.

3.4.6 Nitrosative stress measurement

Nitric oxide (NO) is a unique diffusible molecular messenger that occupies central roles in mammalian pathophysiology (Brüne, Sandau, & Von Knethen, 1998), produced from conversion of L-arginine into L-citrulline by the enzyme NO synthases (NOS). It is known to be involved in a variety of biological functions and activities ranging from vasodilation, neurotransmission, and anti-microbial and anti-tumour activities. In breast cancer, both endothelial nitric oxide synthase (eNOS) as well as inducible nitric oxide synthase (iNOS) have been detected in a substantial amount of human breast tumours, where their expression patterns correlate with tumour grades (Martinez et al., 2016). Depending on the amount, duration, and the site of NO production, NO has been implicated involved with both anti-cancer pathways and pro-cancer pathways (Ying and Hofseth, 2007).

In this study, fucoidan-related changes in the levels of nitric oxide were evaluated using Muse™ nitric oxide Kit.

3.4.6.1 Test principle

The Muse™ nitric oxide kit utilised a reagent called DAX-J2 Orange, which is a membrane permeable novel reagent and generates a highly fluorescent product upon NO oxidation inside the cell, thus can simultaneously monitor the count and percentage of cells exhibiting nitric oxide activity. 7-AAD is also included in this kit as an indicator for dead cells. Four populations of cells can be distinguished in the assay (as listed in Table 24):

Table 24. Four populations of cells in nitric oxide assay

Four populations of cells in nitric oxide assay
Live Cells with no NO activity (negative): NO (-) and 7-AAD (-)
Live Cells with NO activity: NO (+) and 7-AAD (-)
Dead Cells with NO activity: NO (+) and 7-AAD (+)

Dead Cells with no NO activity: NO (-) and 7-AAD (+)

3.4.6.2 Kit Components

Table 25. Kit components in nitric oxide assay

Kit components in nitric oxide assay
Muse® Nitric Oxide Reagent (Part No. 4700-1666, 100 tests/bottle)
Muse® 7-AAD (Part No. 4700-1673, 100 tests/bottle)
1× Assay Buffer (Part No. 4700-1330, 100 mL)

3.4.6.3 Preparation of cell suspension

According to the assay protocol, cells should be resuspended in 1× assay buffer at 1× 10^6 to 1×10^7 cells/ml to proceed the staining.

Step 1: 100 µl of cell suspension with medium from each sample was transferred into labelled microcentrifuge tubes.

Step 2: Labelled microtubes were centrifuged for 30 seconds, then supernatant in each tube was carefully removed.

Step 3: 50 µl of 1× assay buffer was added into each tube with cells resuspended.

3.4.6.4 Preparation of Muse™ nitric oxide reagent working solution

Step 1: The Muse™ nitric oxide reagent was previously stored at–15°C to –20°C. After thaw, it was divided with 3-4 µl/tube in microcentrifuge tubes to make stocks, desiccated and protected from light for long term storage. In this way, the freeze-thaw cycles of reagent can be minimized.

Step 2: One microtube of the Muse™ nitric oxide reagent, 7-AAD and 1× assay buffer were prewarmed to room temperature, protected from light. Right before use, the nitric oxide reagent working solution was freshly prepared by diluting the stock solution 1:1000 in 1× assay buffer (i.e. Table 26).

Table 26. Preparation of nitric oxide reagent working solution

Component	10 Test	50 Tests

Muse™ nitric oxide reagent	1 µl	5 µl
1× assay buffer	999 µl	4995 µl

3.4.6.5 Preparation of Muse™ 7-AAD working solution

Step 1: The Muse™ 7-AAD was diluted with 1× assay buffer as suggested in the following table (Table27) to make a working solution.

Table 27. Preparation of 7-AAD working solution

Component	1 Test	6 Tests
Muse™ 7-AAD	2 µl	12 µl
1× Assay Buffer	88 µl	528 µl

3.4.6.6 Assay protocol

Step 1: 10 µl of prepared cell suspension was added into each test tube and labelled.

Step 2: 100 µl of Nitric Oxide reagent working solution was added to each tube.

Step 3: Mix thoroughly by pipetting up and down 3 to 5 times and loosely capping the tube.

Step 4: Samples were incubated for 30 minutes in the 37°C incubator with 5% CO_2.

Step 5: 90 µl of 7-AAD working solution was added into each tube after incubation.

Step 6: Samples were incubated for another 5 minutes at room temperature, protected from light.

Step 7: Samples were remixed thoroughly and run on Muse™ Cell Analyser.

3.5 Data analysis

3.5.1 Analysis of MTT assay results

IC_{50} commonly refers to the concentration of a drug (inhibitor) where the response is reduced by half. It is an important reference to evaluate the inhibitory effects of HMF and LMF in this study. IC_{50} values were calculated by PRISM® software (Graphpad, Version 7.0) and were obtained by selecting the model of dose-response inhibition, nonlinear regression (curve fit): Log (inhibitor) vs. Response-Variable slope (four parameters).

3.5.2 Analysis of cell cycle assay results

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

3.6 Statistical analysis

All experiments in this study were performed at least two times. MTT assays were performed more than three times. Statistical differences in single group of data and among multiple groups were determined by one-way ANOVA (Analysis of Variance) and Linear Mixed Effects Model (LMEM) with R-Studio® software (version 3.5.0). For determining significance, p-value < 0.05 were considered significant and p-value < 0.01 were considered very significant.

Chapter 4 Results

4.1 Inhibitory effects of HMF and LMF on MCF-7 and MDA-MB-231 breast cancer cell lines

The inhibitory effects of fucoidan from two different ranges of molecular weight from New Zealand *Undaria pinnatifida* were examined on MCF-7 and MDA-MB-231 cell lines using the MTT assays. The linearity was determined first for each cell line, then the inhibitory effects of HMF and LMF against breast cancer was evaluated in this study.

4.1.1 Linearity determined by MTT assays for MCF-7 and MDA-MB-231 cell lines

The linearity in cell number vs. absorbance standard curve is a critical reference for establishing the initial beginning value in the MTT assays of the study. Good linearity assures cells were growing in good condition. Cell seeding density used in studies should fall within the linear portion of the curve and was neither too high nor too low for cells to grow normally.

4.1.1.1 MCF-7 cell line

The linearity between cell quantity of MCF-7 and absorbance is presented in Fig. 11. The linear relationship is best at cell densities ranging from 200,000 cells/ml to 3125 cells/ml ($R^2 = 0.9744$) between the absorbance, determined at 540nm wavelength.

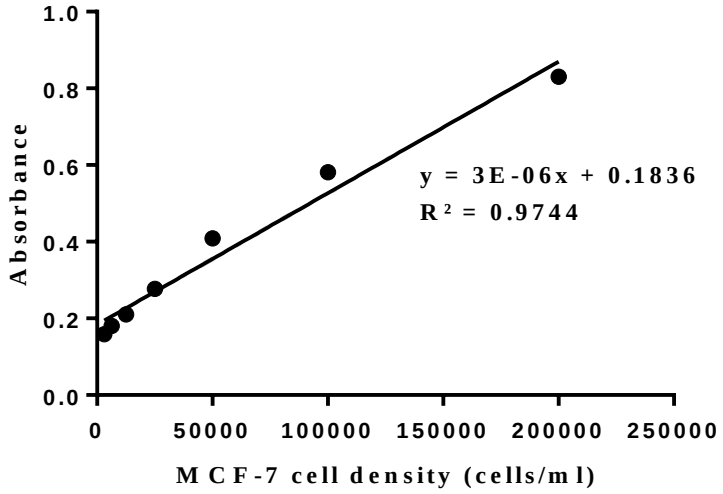


Figure 11. Linearity between MCF-7 cell quantity and absorbance values

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

4.1.1.2 MDA-MB-231 cell line

The linearity between cell quantity of MDA-MB-231 and absorbance is presented in Fig. 12. The linear relationship is best at cell densities ranging from 200,000 cells/ml to 6250 cells/ml (R^2 = 0.9813) between the absorbance, determined at 540nm wavelength.

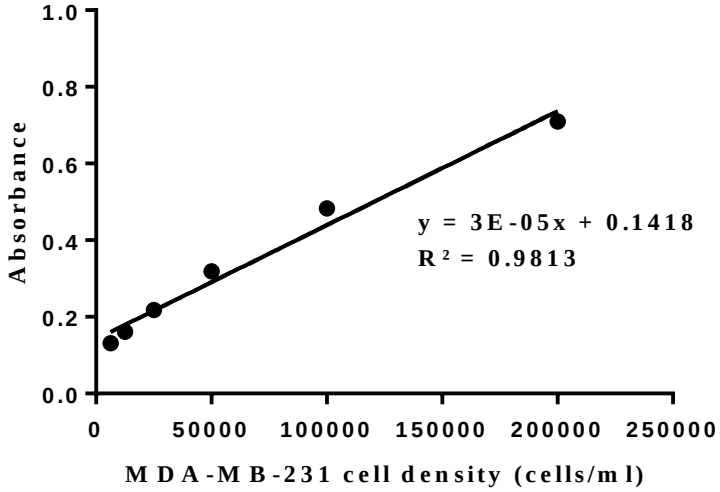


Figure 12. Linearity between MDA-MB-231 cell numbers and absorbance values

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

According to linearity curves of both cell lines, a cell density at about 45,000 to 50,000 cells/ml is considered a suitable cell seeding density, which is neither too high nor too low, for both cell lines in MTT assay and mechanism assays.

4.1.2 Inhibitory effect of HMF on MCF-7 and MDA-MB-231 cell lines

HMF is less studied in literature regarding fucoidan use against cancer. It is extensively believed that its large molecular size, which is hard to be absorbed and metabolized in vivo, limited its anti-cancer potential. In this study, HMF ranging from 120 µg/ml to 5 µg/ml have been tested for its inhibitory effect on MCF-7 and MDA-MB-231 cell lines. In both cell lines, HMF exhibited very limited inhibition on cell growth.

In MCF-7, no obvious dose-dependent inhibition was observed after incubation of 48 and 72 hours ($p = 0.1818$ & $p= 0.2738$, One-way Anova). Cell viabilities were only dropped less than 8.5% after treated with HMF in various concentrations for 48 hours and 17.5% for 72 hours. After 96 hours, cell viability was decreased in a slightly wider range from 5 µg/ml to 120 µg/ml groups ($p = 0.01281$, One-way Anova).

Overall effects of HMF on MCF-7 cells were analysed by ANOVA in a linear mixed effects model using LMF concentration (Cn) and incubation time (Time) as fixed effects and individual well in 96-plate as random effect. The results indicate that, in general, HMF did not cause much difference on the inhibition of cells treated with various concentrations (P_{Cn} = 0.1868).

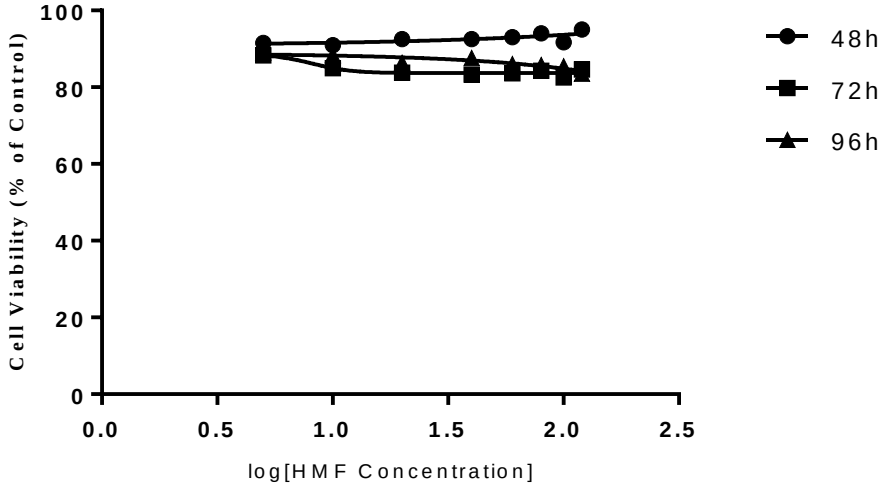


Figure 13. Effect of HMF on MCF-7 cell line

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

In MDA-MB-231, the inhibitory effect of HMF cells was similar to the inhibition on MCF-7 cells. A slight dose-dependent decrease (86.39% to 77.91% with HMF from 5 µg/ml to 120 µg/ml) was observed after incubation of 48 hours and remained nearly unchanged to 72 hours till 96 hours (as shown in Fig. 13). However, the most significant inhibition with HMF treatment is still around 20%, far from enough to become a potential therapeutic agent for cancer treatment.

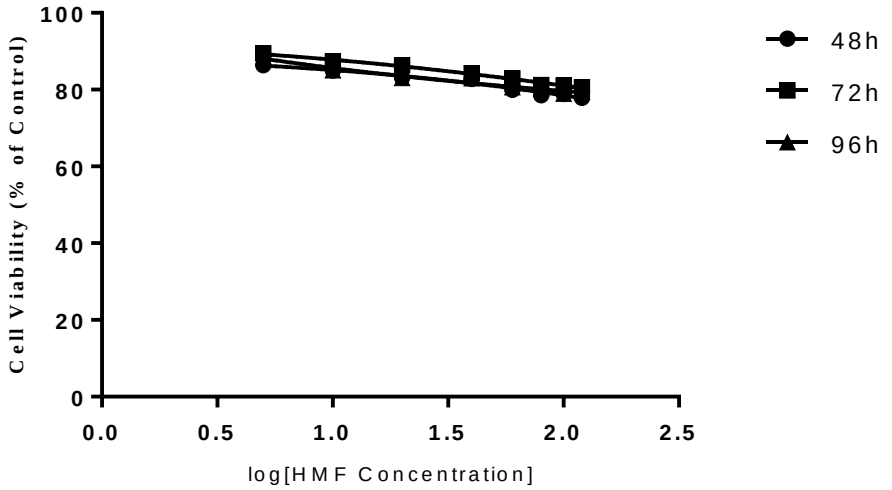


Figure 14. Effect of HMF on MDA-MB-231 cell line

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

4.1.3 Inhibitory effect of LMF on MCF-7 and MDA-MB-231 cell lines

After treatments with various concentrations of LMF and incubated for 48, 72 and 96 hours, LMF showed significant inhibition on cell proliferation in both MCF-7 and MDA-MB-231 cell lines. As can be seen from the inhibition curve presented in figures 14 and 15, in both studies LMF inhibits cell growth in a dose-dependent manner.

In the MCF-7 cell line, cells were treated with LMF from a range of highest 200 µg/ml to lowest 0.5 µg/ml. The inhibitory effect of LMF was most significant at LMF concentration of 200 µg/ml after incubation of 96 hours (cell viability dropped to 42.42% compared to negative controls) and was at a very close value after 72 hours (43.38%). The IC_{50} of 96 and 72 hours was 10.540µg/ml and 19.087 µg/ml of LMF, respectively. The best inhibition rate at 48 hours was 55.23% at 200 µg/ml of LMF, therefore IC_{50} for cells incubated for 48 hours would be over 200 µg/ml. MCF-7 showed a good sensitivity to LMF, with 0.5% µg/ml LMF inhibiting cell growth by 18% at 72 hours and 22% by 96 hours.

Effects of LMF concentrations and incubation time were analysed by ANOVA in a linear mixed effects model using LMF concentration (Cn) and incubation time (Time) as fixed effects and individual well in 96-plate as random effect. Data shows that the effect of LMF concentrations on inhibition in MCF-7 cells is significant (P_{Cn}= 0.0283) and was in a very significant time-dependent manner (P_{Time} < 0.0001). There was no interaction of the effect of LMF concentrations and incubation time ($P_{Time:Cn}$= 0.3624).

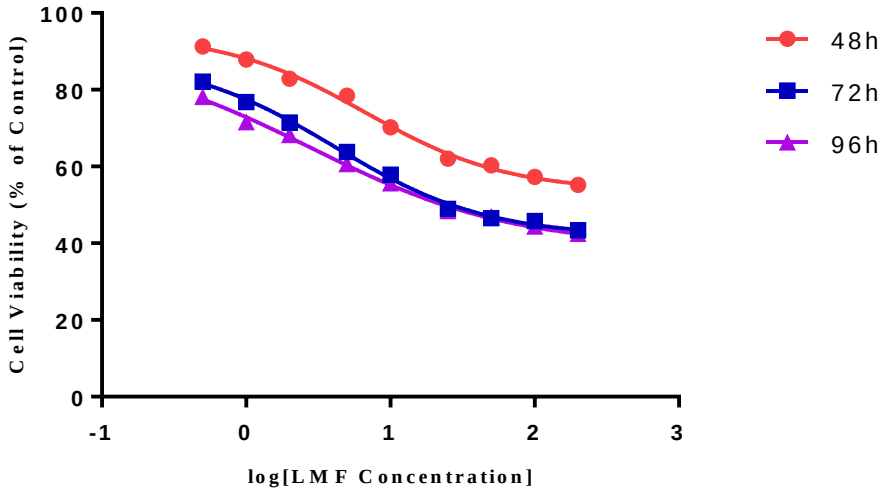


Figure 15. Effect of LMF on MCF-7 cell line

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

In the MDA-MB-231 cell line, cells were treated with LMF from a range of highest 300 µg/ml to lowest 1 µg/ml. The inhibitory effect of LMF was most significant at LMF concentration of 300 µg/ml after incubation of 72 hours (cell viability dropped to 60.76% compared to negative controls), although the inhibition rates at 48 and 96 hours were very close to this value (62.01% and 64.41%, respectively). The IC_{50} of LMF for MDA-MB-231 cells would be above 300 µg/ml for all incubation times. Although the dose-dependent inhibition exhibited by LMF was observed after each incubation, there was a reverse in the data which showed up on cell viability in LMF lower concentrations (< 150 µg/ml) and was observed for all repeated independent tests. From 72 hours to 96 hours, an increased cell viability of approximately 6-10% was observed with the decreasing concentrations of LMF, indicating that a higher concentration of treatment is required for a better inhibition on MDA-MB-231 cells.

Effects of LMF concentrations and incubation time were analysed by ANOVA in a linear mixed effects model using LMF concentration (Cn) and incubation time (Time) as fixed effects and individual well in 96-plate as random effect. Data shows that the effect of LMF concentrations on inhibition in MDA-MB-231 cells is significant (P_{Cn} = 0.0169) and the effect of time was also very significant (P_{Time} = 0.0046). Due to the reverse of cell viability in LMF lower concentration groups, the inhibitory effect of LMF on MDA-MB-231 is not considered time-dependent. There was an interaction of the effect of LMF concentrations and incubation time ($P_{Time:Cn}$ < 0.0001).

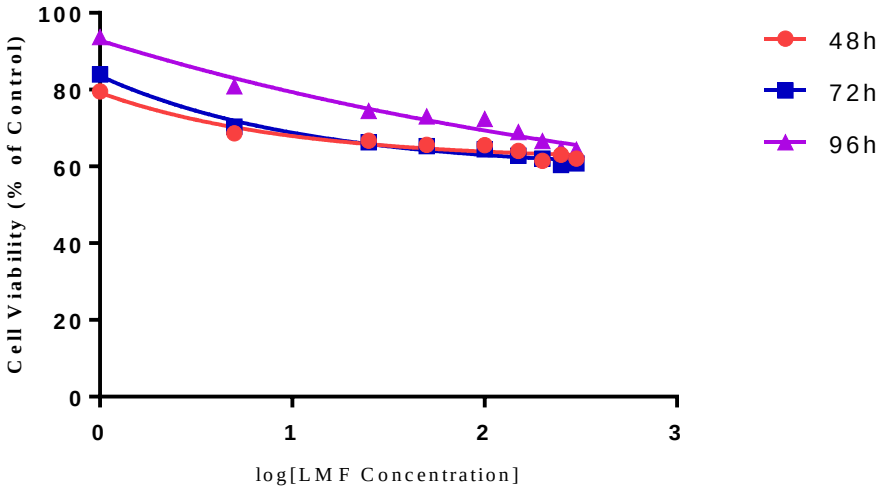
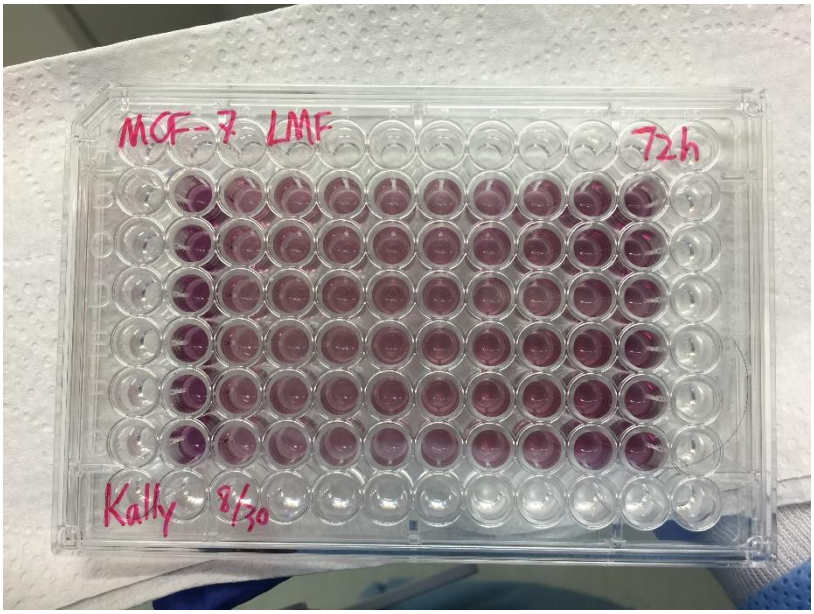


Figure 16. Effect of LMF on MDA-MB-231 cell line

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



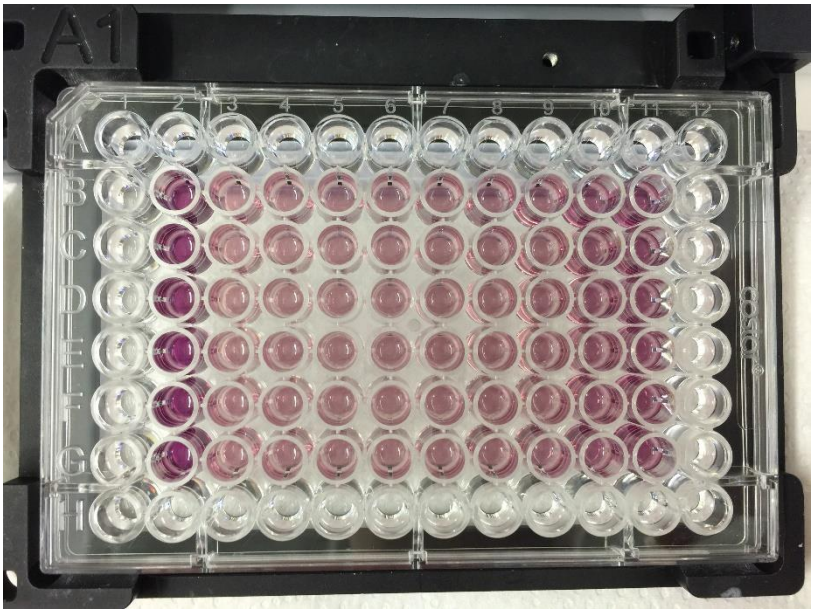
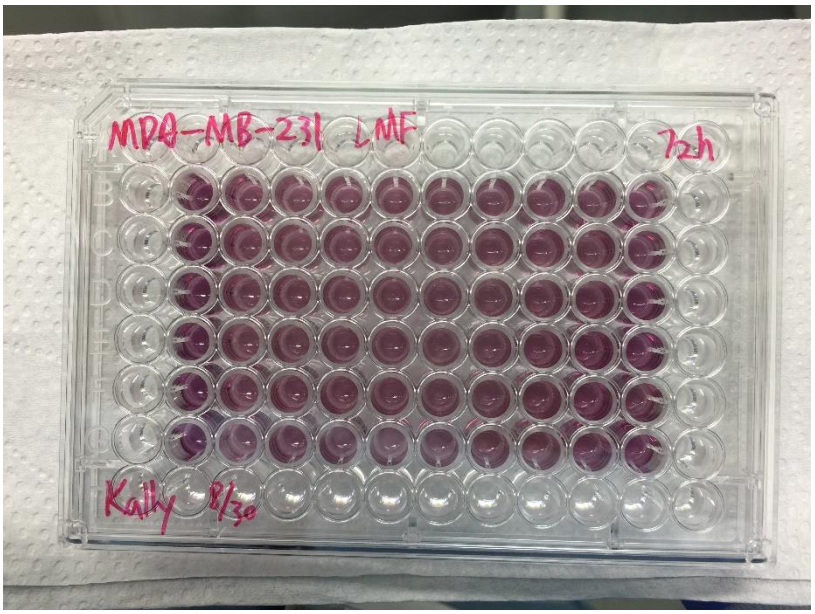


Figure 17. MTT test on MCF-7 cell line treated with LMF in various concentrations (incubation of 72 hrs)



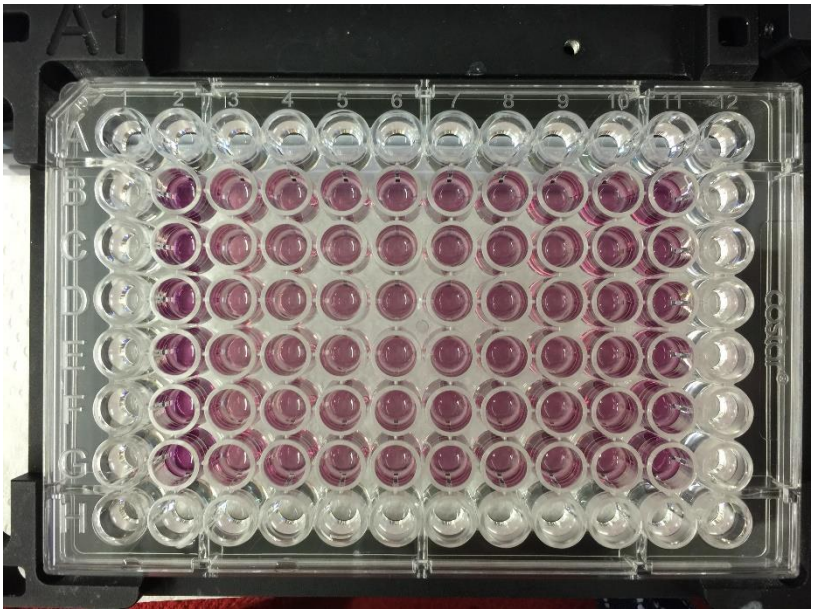
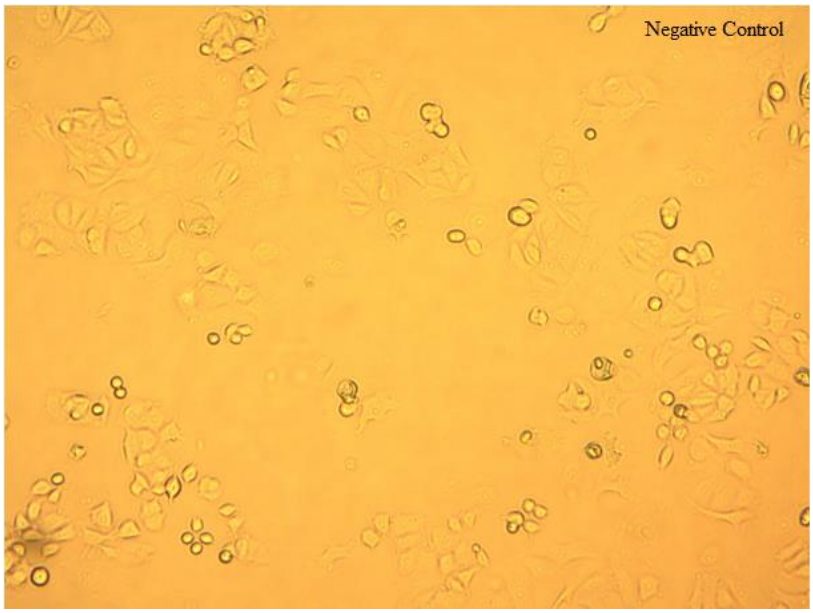


Figure 18. MTT test on MDA-MB-231 cell line treated with LMF in various concentrations (incubation of 72 hrs)

Figures 17 and 18 show the 96-well plates after absorbance measurement in MTT assays. The treatment designs in plates have been shown in the methodology section of this thesis. The No.2 column is the negative control group and the highest concentration of LMF group is No.3 column. The dose-dependent inhibitions by LMF were clearly reflected in colour from dark purple to light purple in both cell lines.



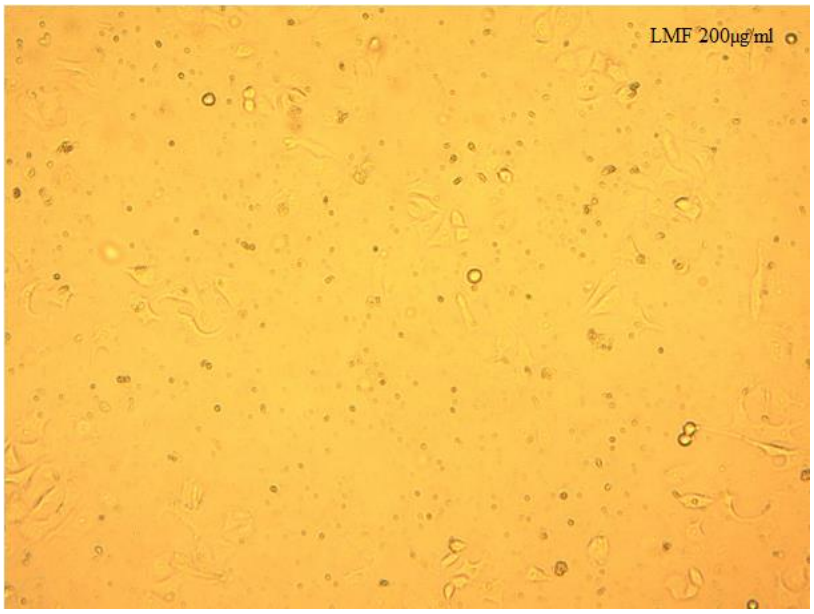
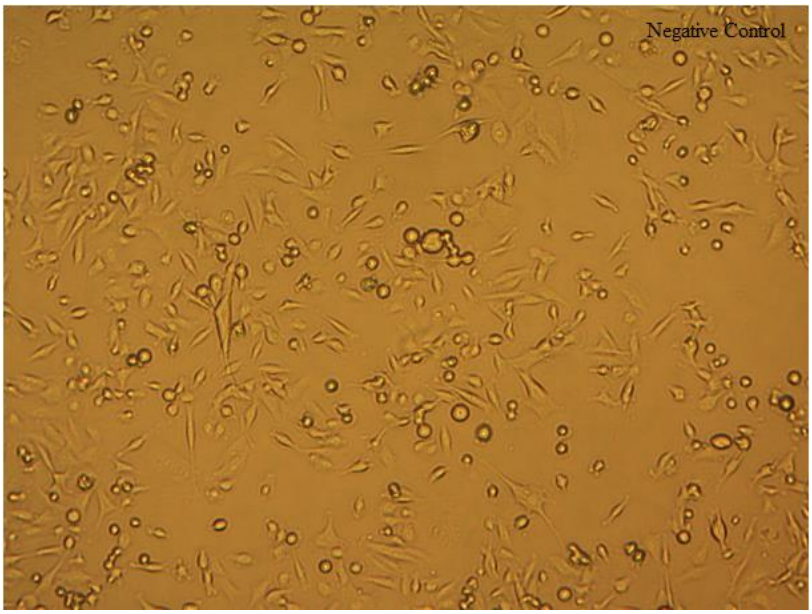


Figure 19. Comparison of MCF-7 treated with/without 200 µg/ml LMF after 48 hrs



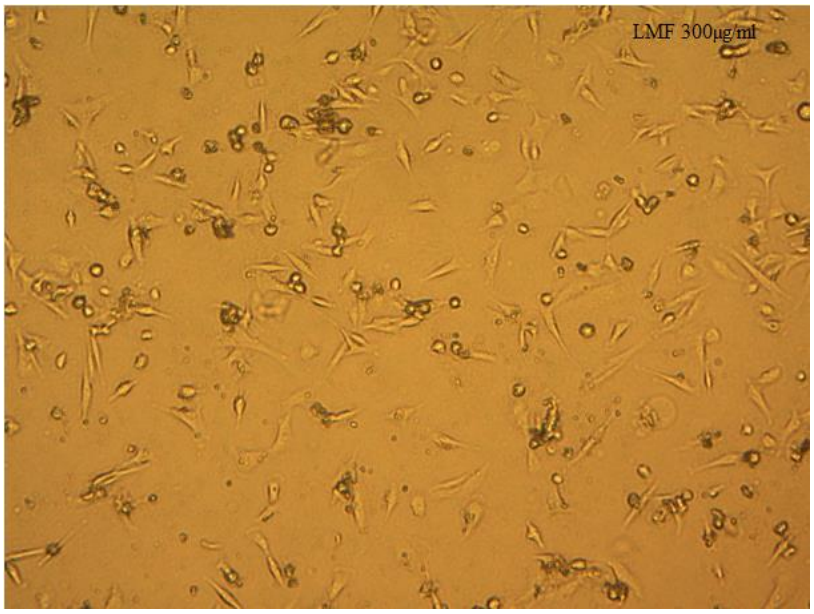


Figure 20. Comparison of MDA-MB-231 treated with/without 300 µg/ml LMF after 72 hrs

Figures 19 and 20 show the decreases in population of cells in both cell lines after incubation with LMF, which were observed under microscope. The decreases were most obvious at LMF concentration of 200 µg/ml and 300 µg/ml, respectively.

4.2 Effect of LMF on noncancerous cell line in cell cycle

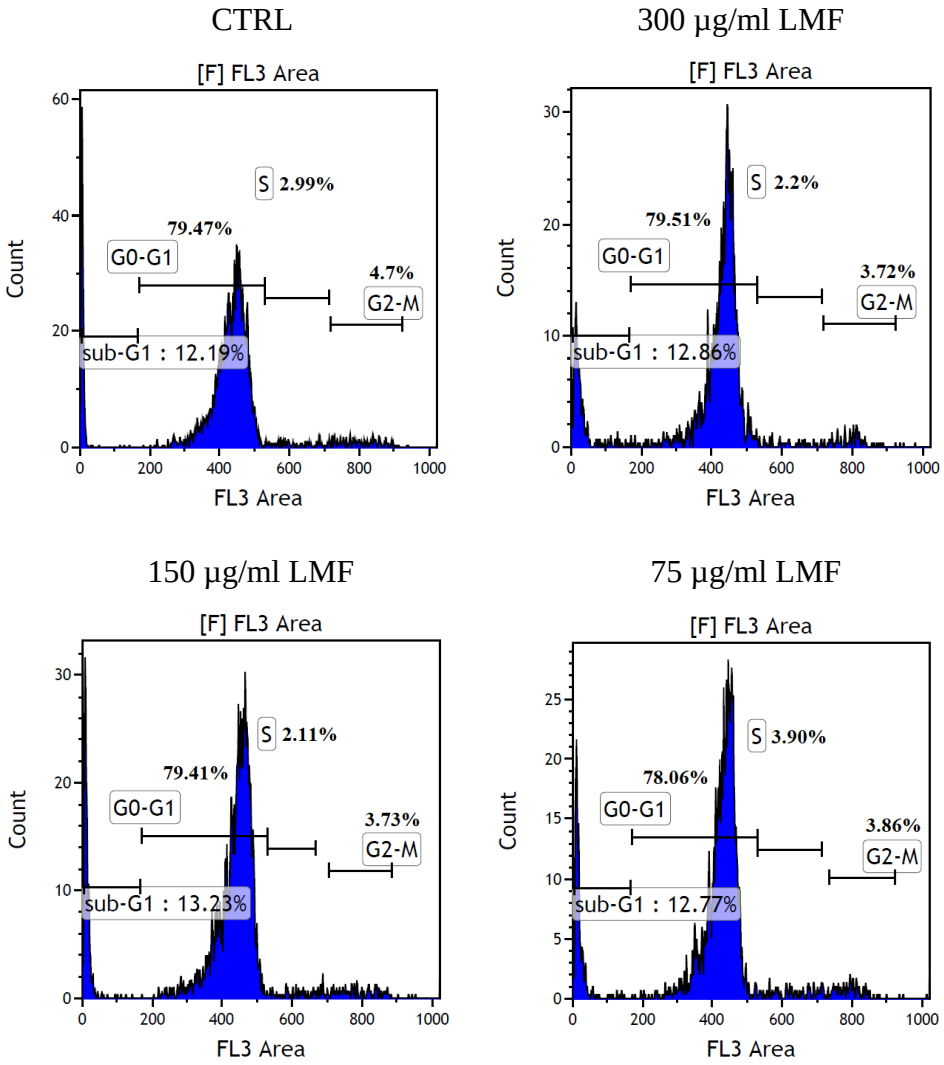


Figure 21. Cell cycle distribution of HDFa cells treated with/without LMF after 72 hrs

HDFa cells were seeded at the same density as two breast cancer cell lines in 6-well plates, which is 50,000 cells/ml, and treated with LMF after all cells were attached to the plate.

In previous MTT assays, the cytotoxic effect of LMF on breast cancer cells has been indicated. In this assay, three different concentrations of LMF were used to test whether LMF will inhibit cell growth of noncancerous cells.

It has been reported in multiple literatures that fucoidan typically induces sub G0/G1 cell accumulation in a variety of cancer cell types. As can be seen from Fig. 21, no obvious percentage changes (< 1%) have been observed in G0-G1 phase and sub-G1 phase in cells treated with LMF compared to negative controls. Cell cycle arrest was

also not observed in S phase and G2-M phase, indicating that LMF is not cytotoxic to HDFa cells up to a concentration at 300 µg/ml.

4.3 Mechanism assays

4.3.1 Cell viability in prepared samples

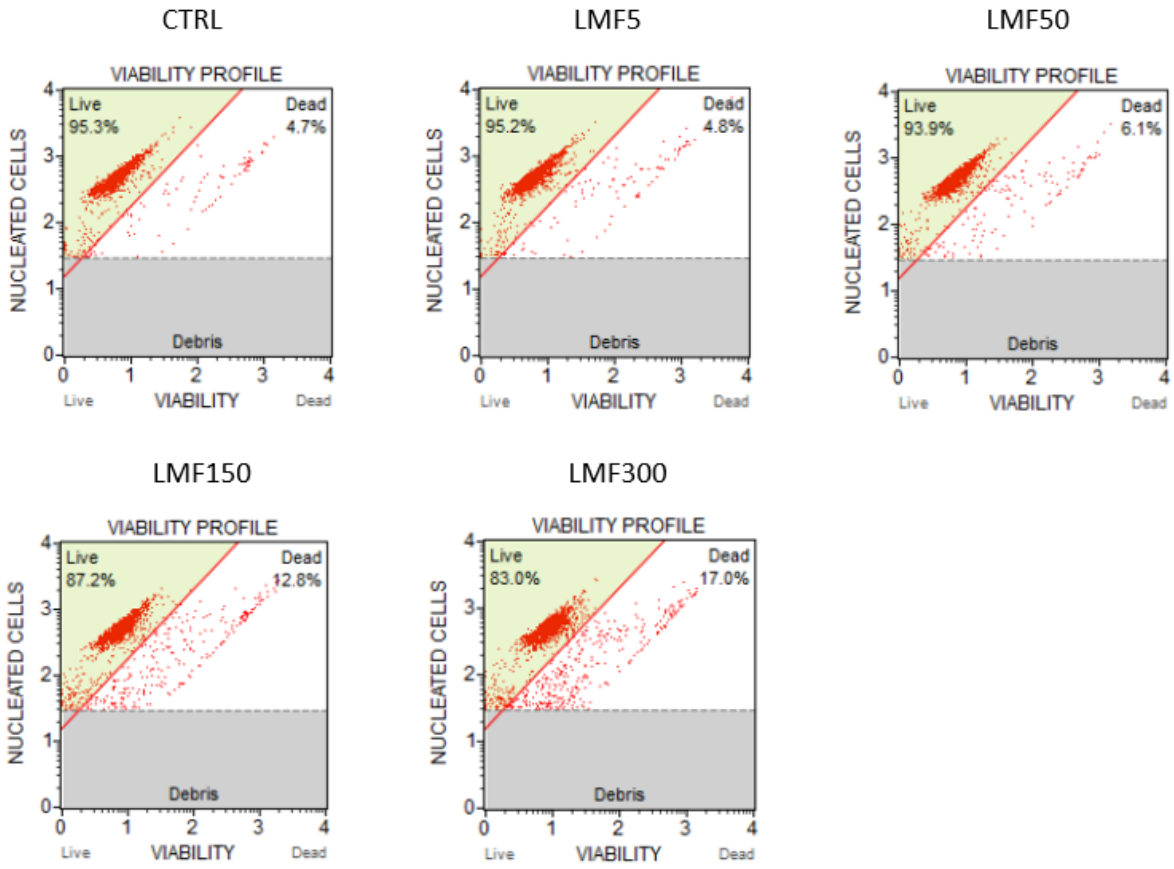


Figure 22. MDA-MB-231 cell viability after being treated with LMF (µg/ml) for 48 hrs and harvest (supernatant discarded)

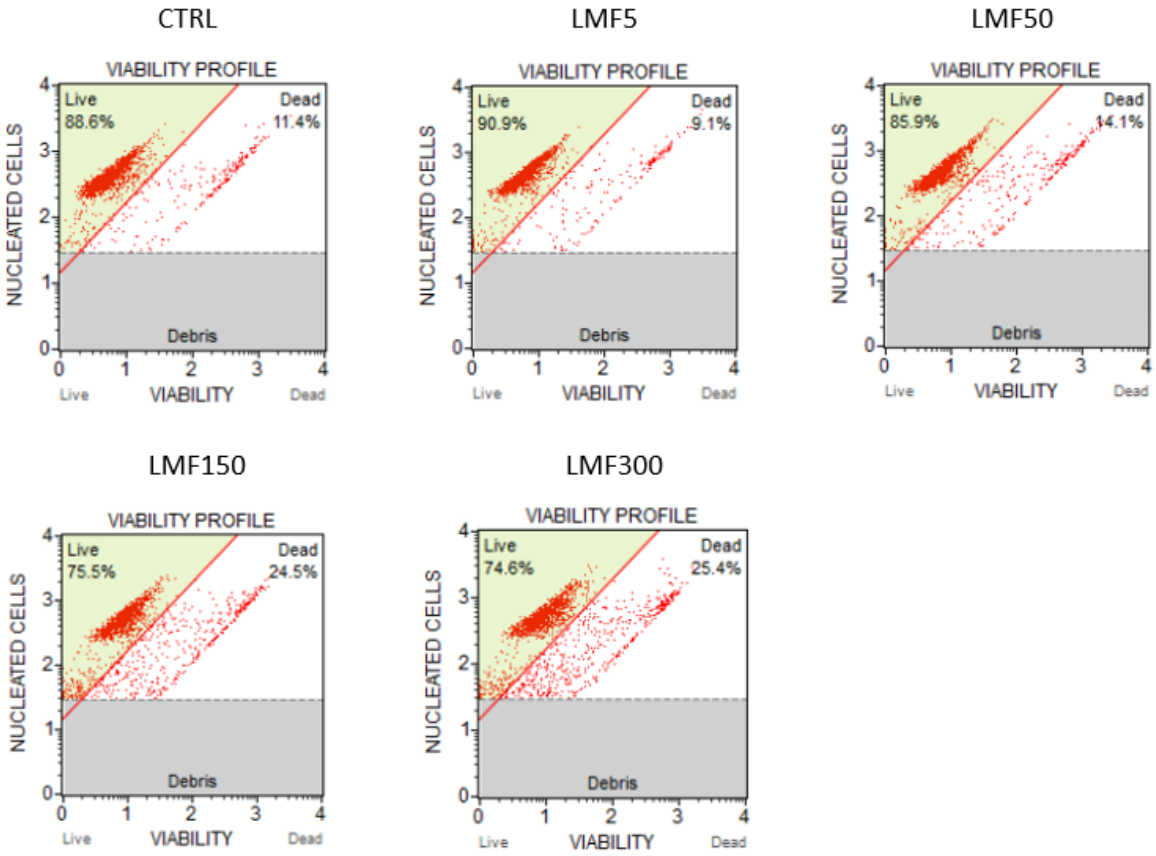


Figure 23. MDA-MB-231 cell viability after being treated with LMF (µg/ml) for 72 hrs and harvest (supernatant discarded)

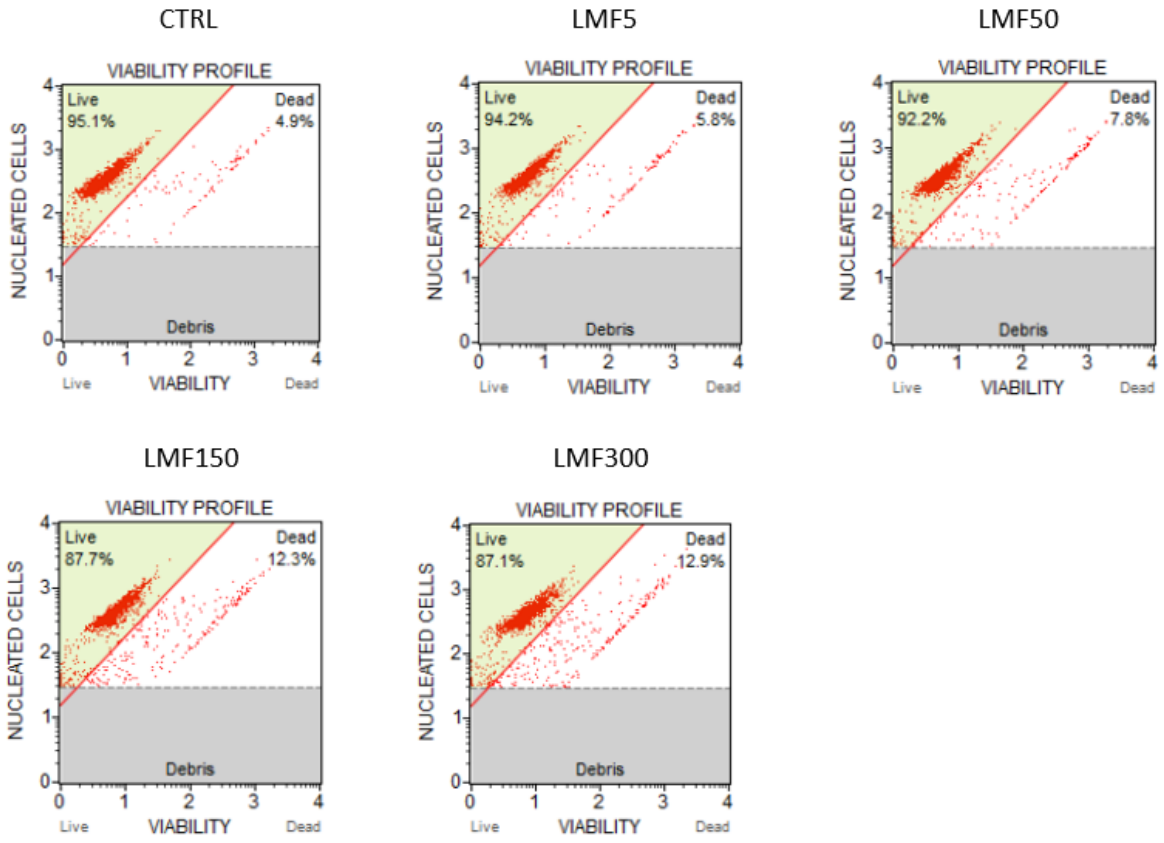


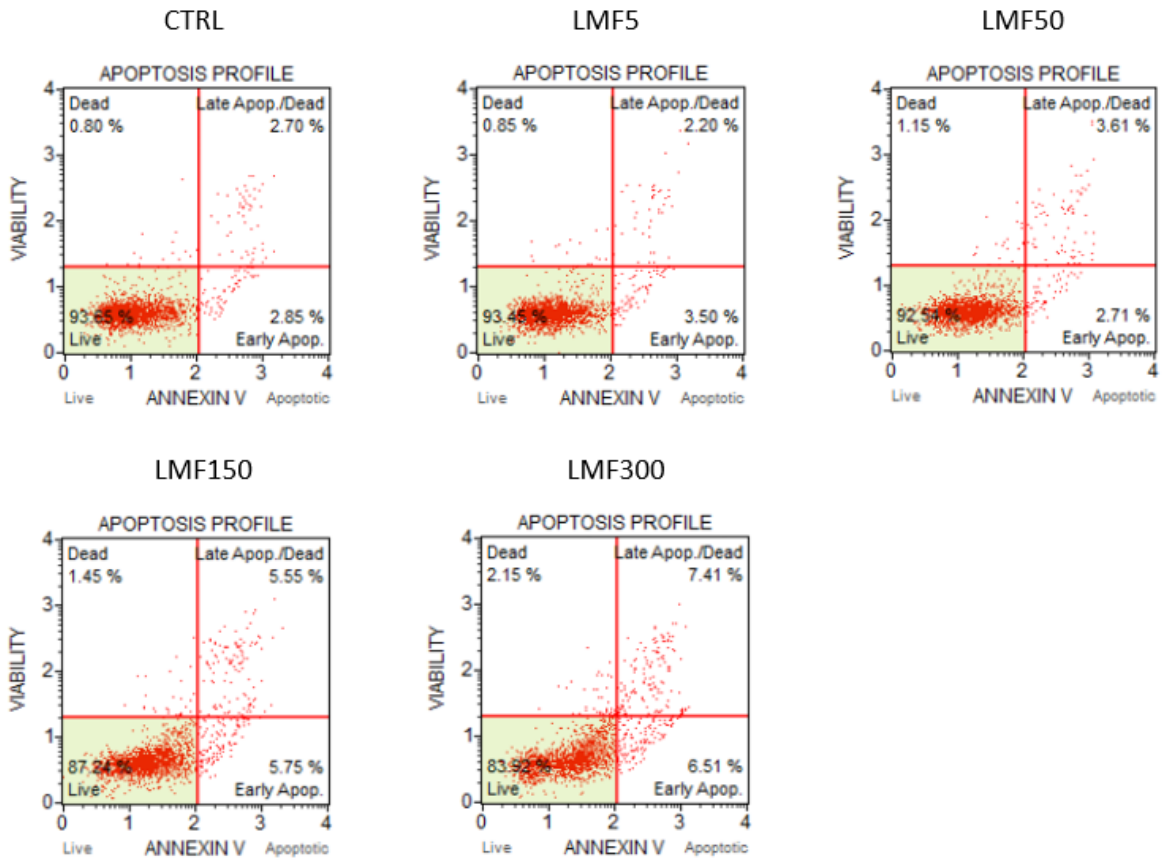
Figure 24. MDA-MB-231 cell viability after being treated with LMF (µg/ml) for 96 hrs and harvest (supernatant discarded)

To ensure the loading of cell samples and minimize the case of tiny particles from LMF treatment causing clots in the (Muse) flow cytometry system, treatment in each well of 6-well plates were discarded as was the old culture medium in negative controls. Different from previous MTT assays, cell viabilities of harvested cells were measured in this assay. Therefore, cell viability in each cell sample is generally good, but a slight dose-dependent trend can still be observed in each time point.

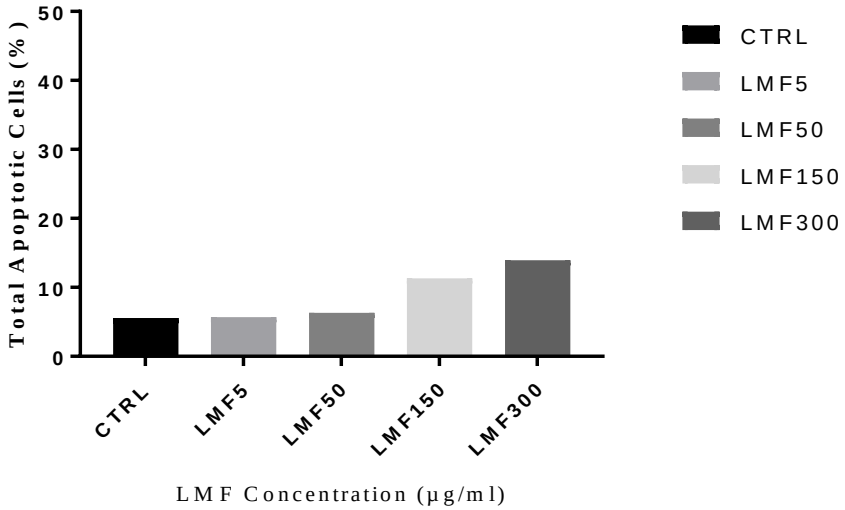
After cells were treated with LMF for 48 hours and harvested from each well in plate, the viability of cells treated with 300µg/ml were dropped from 95.3% to 83.0%; After cells were treated with LMF for 72 hours and harvested, the viability of cells treated with 300µg/ml were dropped from 88.6% to 74.6%; After cells were treated with LMF for 96 hours and harvested, the viability of cells treated with 300µg/ml were dropped from 95.1% to 87.1%. Overall, an average of roughly 10% viability decrease was observed in cells treated with the highest concentration of LMF as the most significant decrease. The difference between viabilities of harvest cells indicates that LMF treated cells are more likely to experience demise during harvesting than cells not

treated with LMF, probably due to different apoptotic cell percentage between cell samples, as was examined in the next assay. No obvious time-dependent manner was observed in this assay.

4.3.2 Cell apoptosis

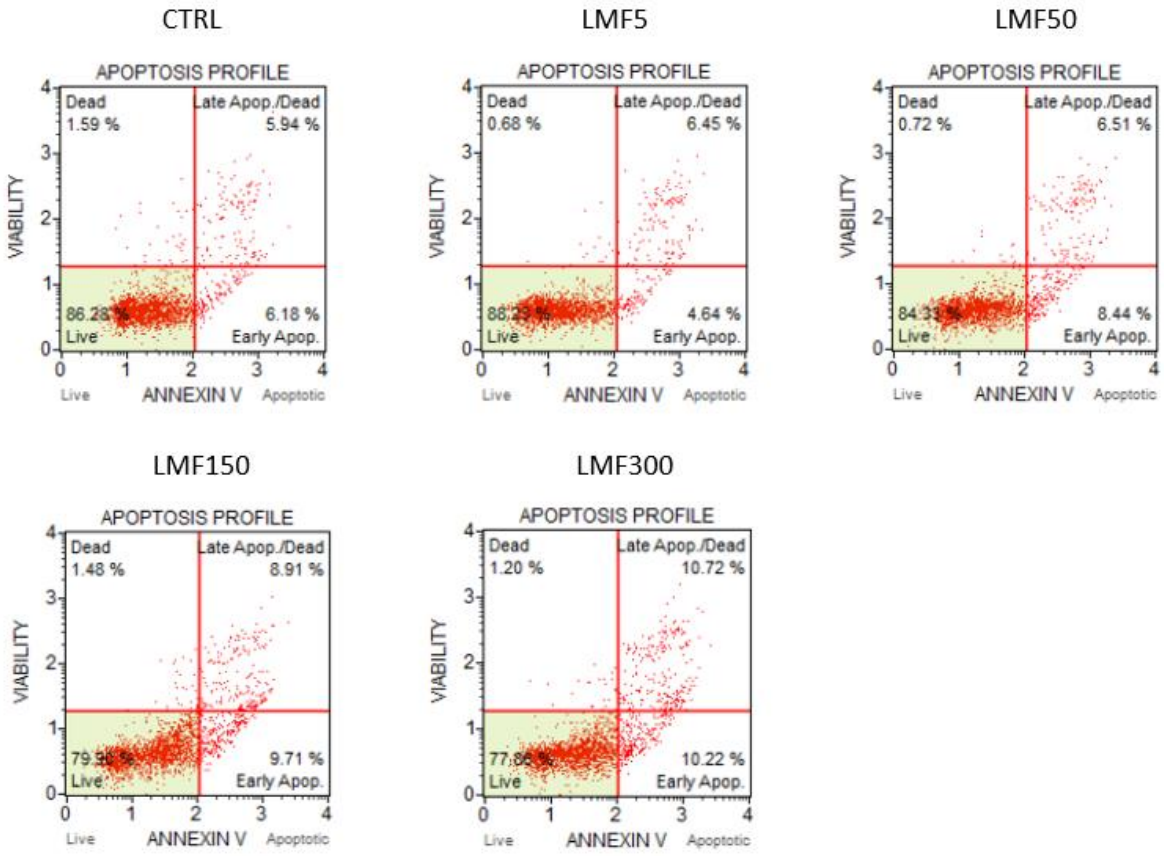


(A)

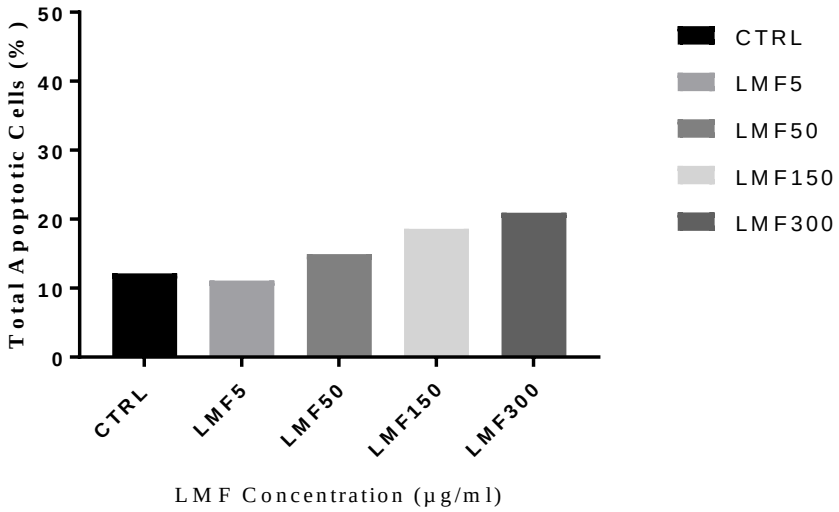


(B)

Figure 25. The apoptotic cells distribution in MDA-MB-231 cells after being treated with LMF (µg/ml) for 48 hrs and harvest

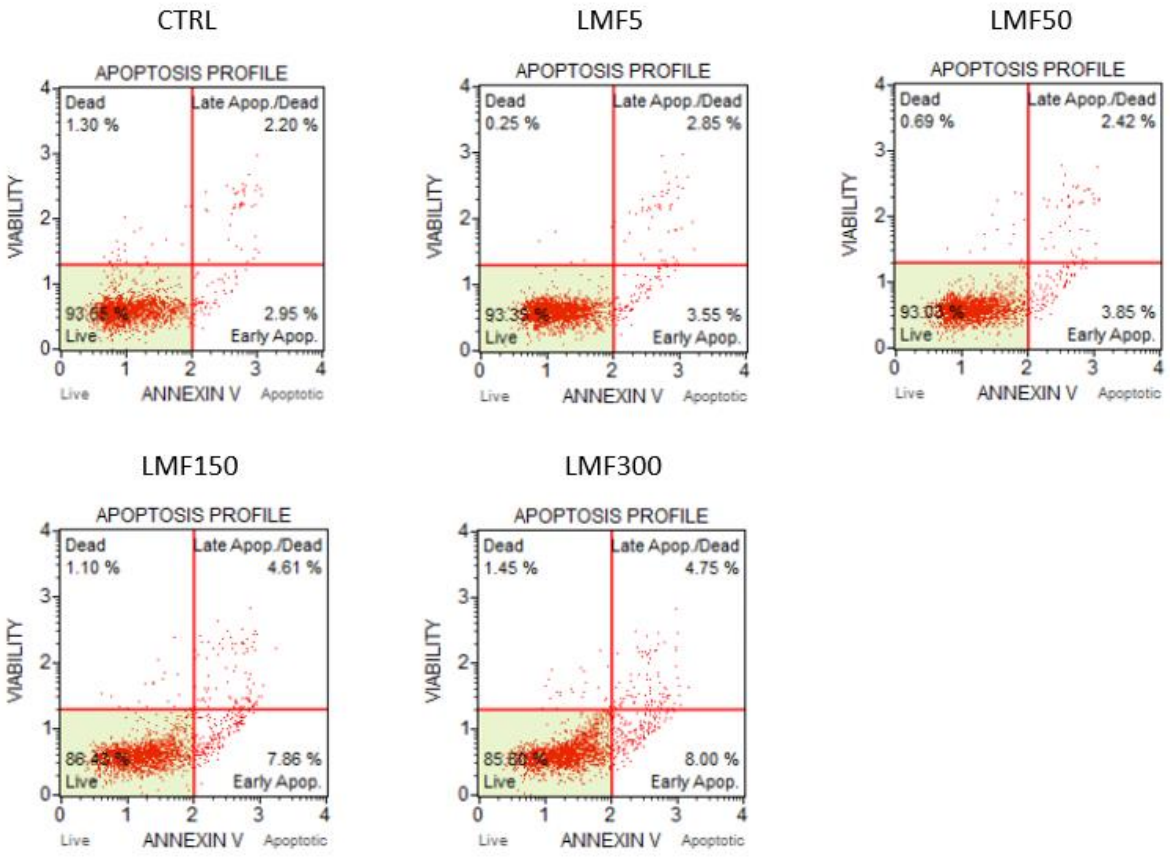


(A)

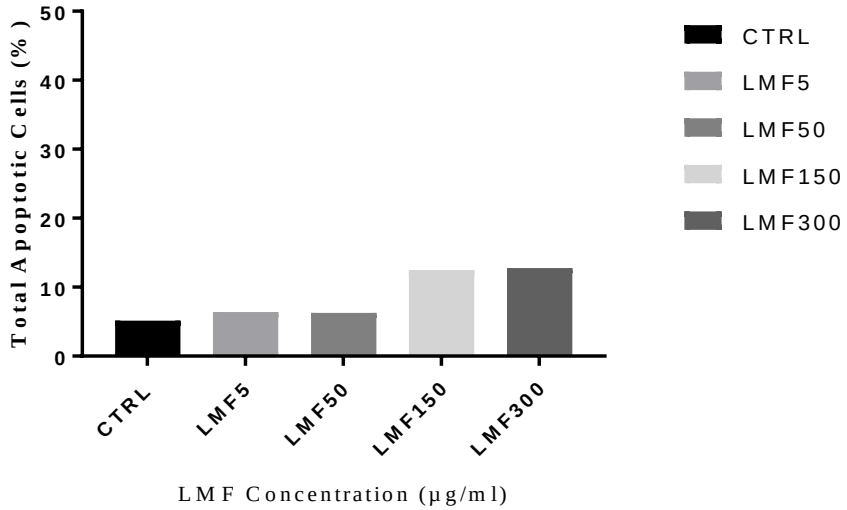


(B)

Figure 26. The apoptotic cells distribution in MDA-MB-231 cells after being treated with LMF (µg/ml) for 72 hrs and harvest



(A)



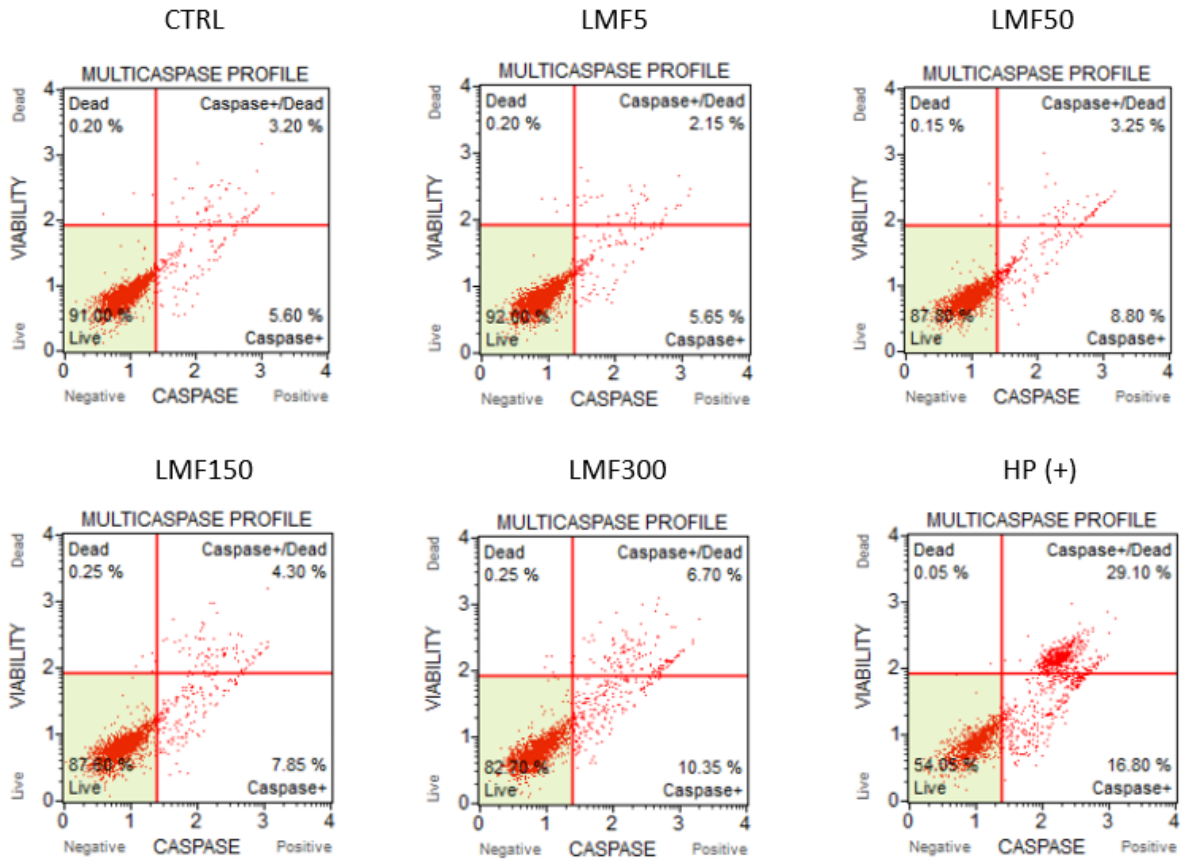
(B)

Figure 27. The apoptotic cells distribution in MDA-MB-231 cells after being treated with LMF (µg/ml) for 96 hrs and harvest

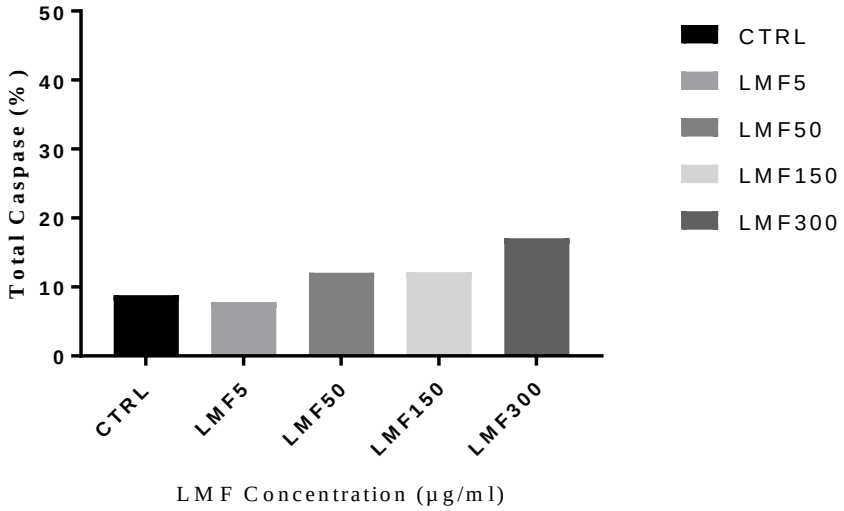
LMF-induced apoptosis was observed in MDA-MB-231 cells after 48, 72 and 96 hours (figures 25-27). The increase of apoptotic cells was most significant in cells treated with 300 µg/ml LMF (total apoptotic cells were 13.93% after 48 hrs, 20.94% after 72 hrs and 12.75% after 96 hrs). The average of this increase was about 8% (8.38%, 8.81% and 7.6% after 48, 72 and 96 hrs, respectively) compared to negative controls in all three points, thus no obvious time-dependent manner was observed in this assay.

LMF-induced apoptosis was also observed in cells treated with 150 µg/ml LMF, and the increase is slightly lower than in cells treated with 300 µg/ml LMF (about 2%) after 48 and 72 hours and almost the same after 96 hours. The changes in cells treated with lower concentrations (50 µg/ml and 5 µg/ml) of LMF were not obvious compared to negative control. It seems that LMF-induced apoptosis in MDA-MB-231 cells was evenly distributed in early stage and late stage. The discard of treatment solution may have some impact on the percentage of late apoptotic/dead cells, but generally LMF is confirmed to induce apoptosis in MDA-MB-231 cells in a dose-dependent manner. The results in this assay aligns with the trends in MTT assays relative to the inhibitory effect of LMF on the MDA-MB-231 cell line.

4.3.3 Caspase activity

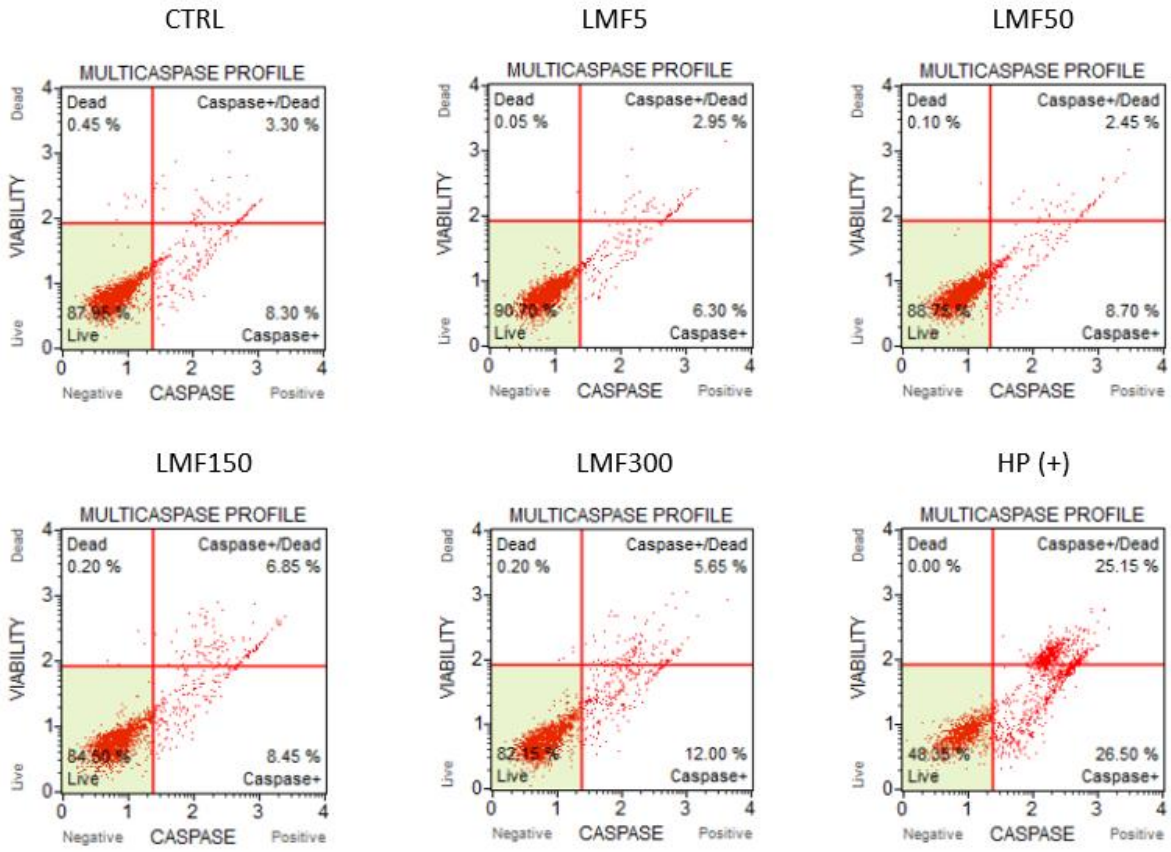


(A)

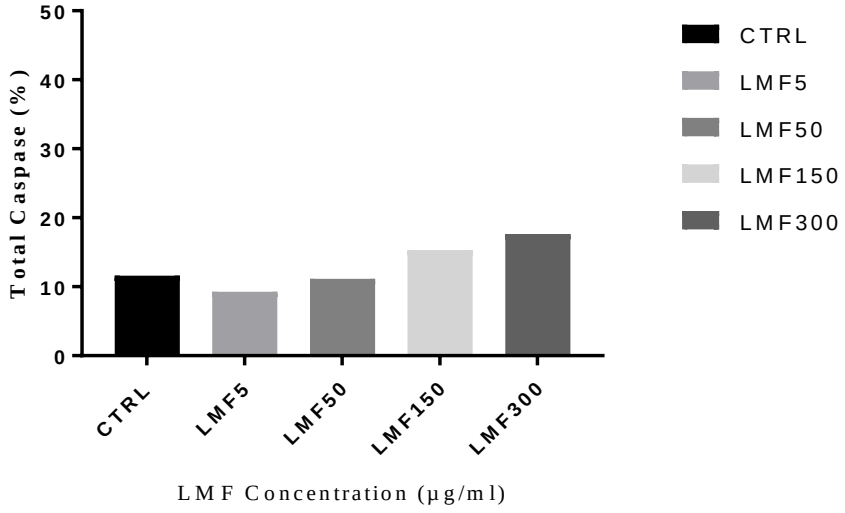


(B)

Figure 28. The caspase activation in MDA-MB-231 cells after being treated with LMF (µg/ml) for 48 hrs and harvest

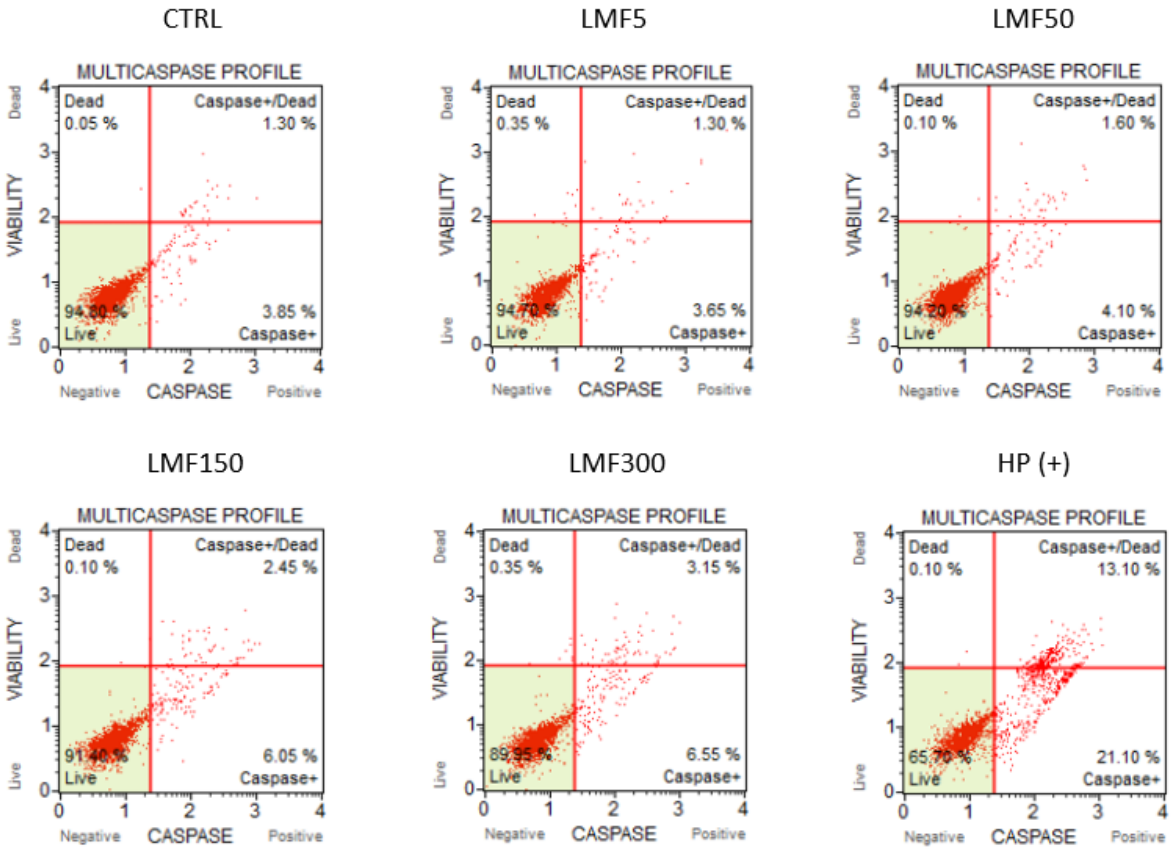


(A)

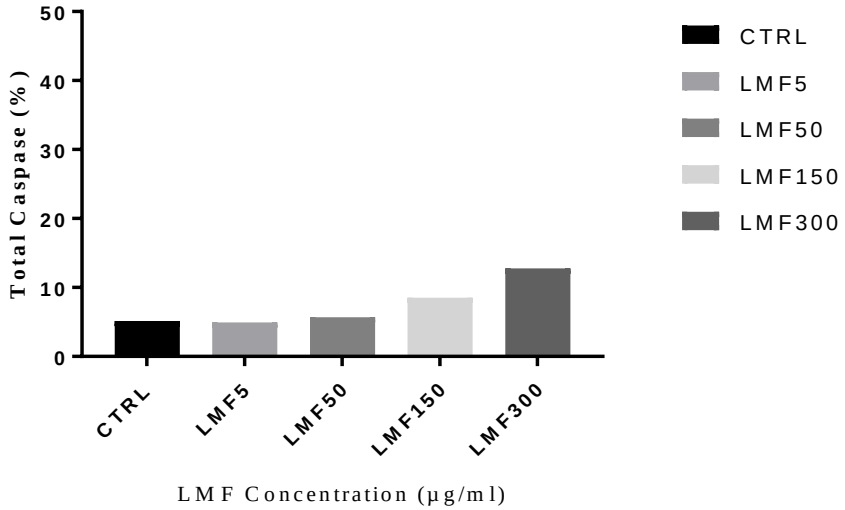


(B)

Figure 29. The caspase activation in MDA-MB-231 cells after being treated with LMF (µg/ml) for 72 hrs and harvest



(A)



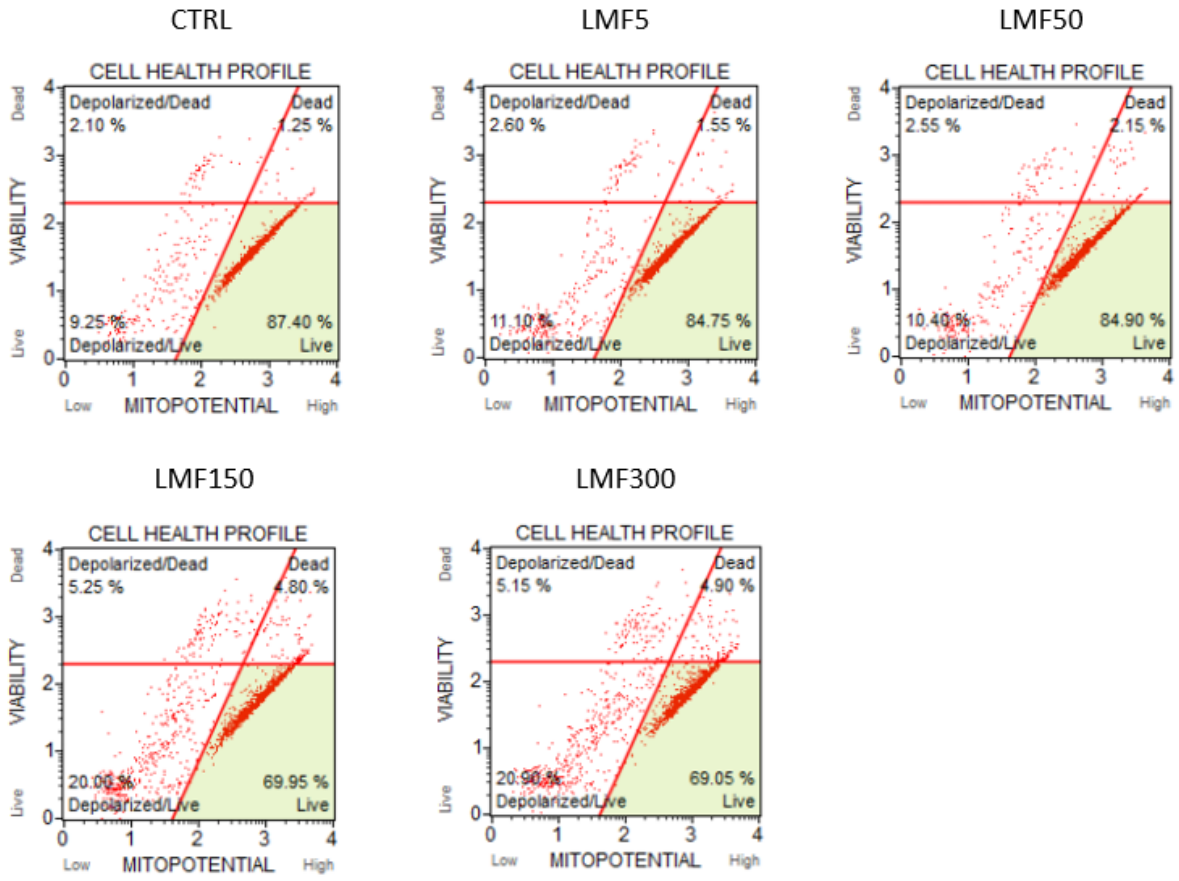
(B)

Figure 30. The caspase activation in MDA-MB-231 cells after being treated with LMF (µg/ml) for 96 hrs and harvest

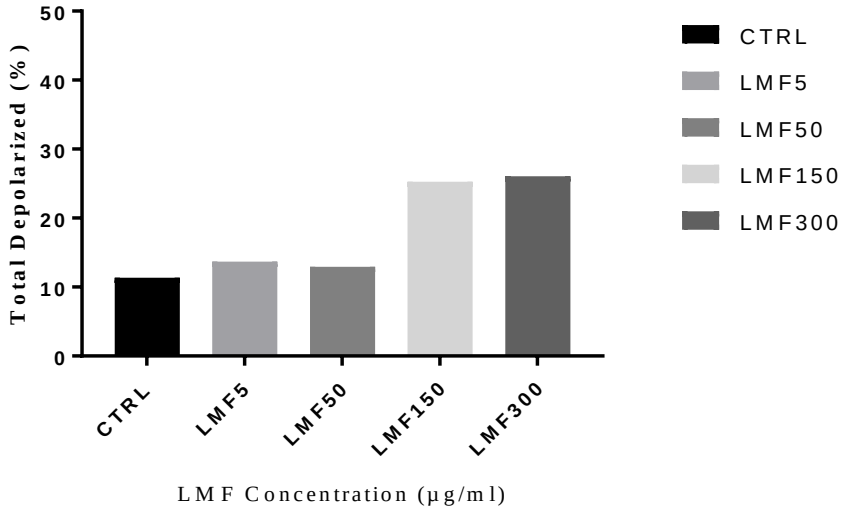
As shown in figures 28 to 30, caspase activation was observed during LMF-induced apoptosis in MDA-MB-231 cells. A 10 mM hydrogen peroxide (HP) medium dilution was used in this assay to prepare a positive control, and the MDA-MB-231 cell line responded to it as a very significant increase of total caspase was observed.

Although not as high as in the positive control, the most obvious increase of total caspase was also observed in cells treated with 300 µg/ml LMF in all three time points (17.05%, 17.65% and 12.75% after 48, 72 and 96 hours, respectively), and a slightly lower increase in cells treated with 150 µg/ml LMF (12.15%, 15.30% and 8.50% after 48, 72 and 96hrs, respectively). Compared to negative controls (8.80%, 11.6% and 5.15% after 48, 72 and 96hrs, respectively), the increase of total caspase in cells treated with lower concentrations (50 µg/ml and 5 µg/ml) of LMF were generally not obvious. This trend of caspase activation in cells corresponds to the trend in cell apoptosis assay. No obvious time-dependent manner was observed on caspase activity in this assay.

4.3.4 Mitopotential measurement

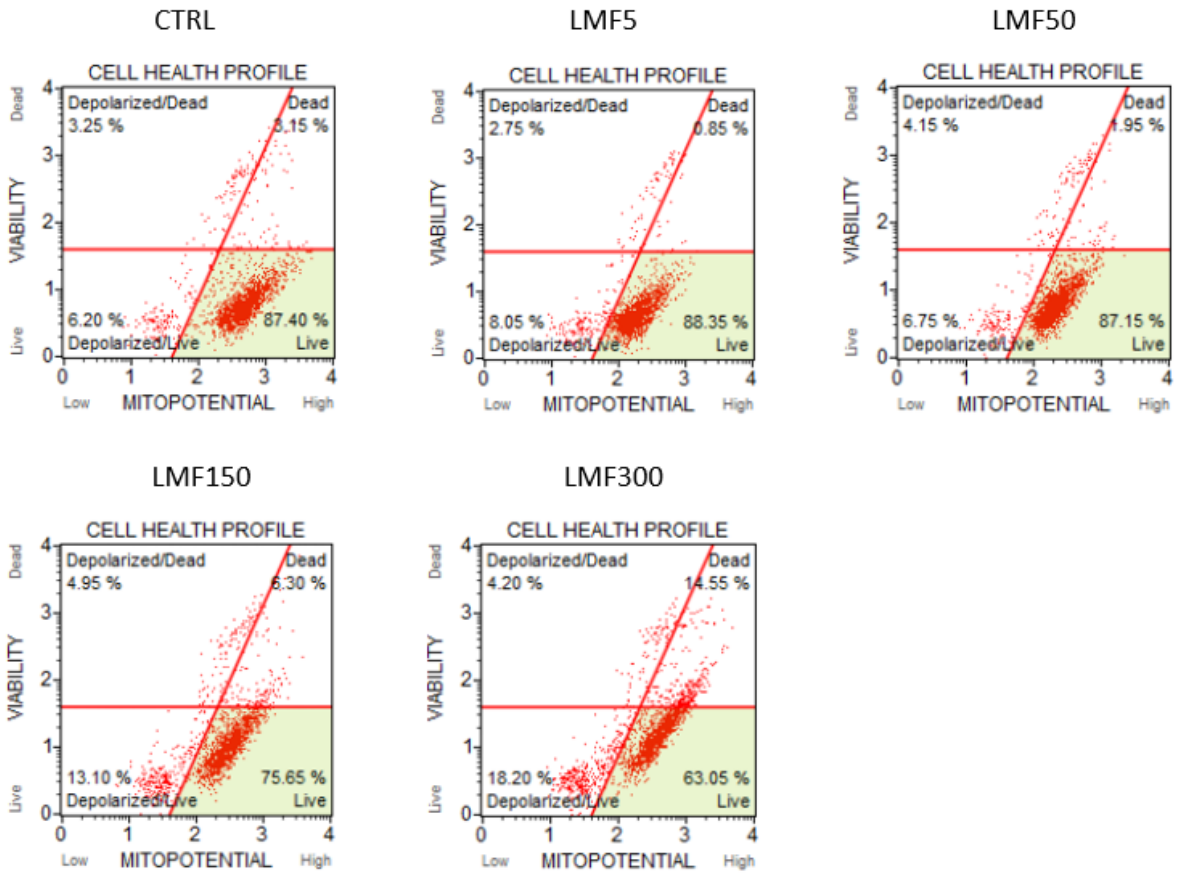


(A)

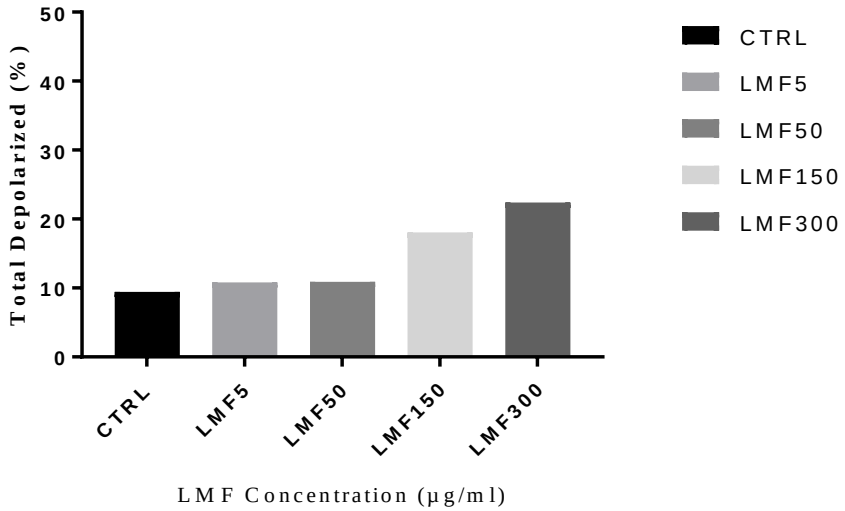


(B)

Figure 31. The status of mitochondria in MDA-MB-231 cells after being treated with LMF (µg/ml) for 48 hrs and harvest

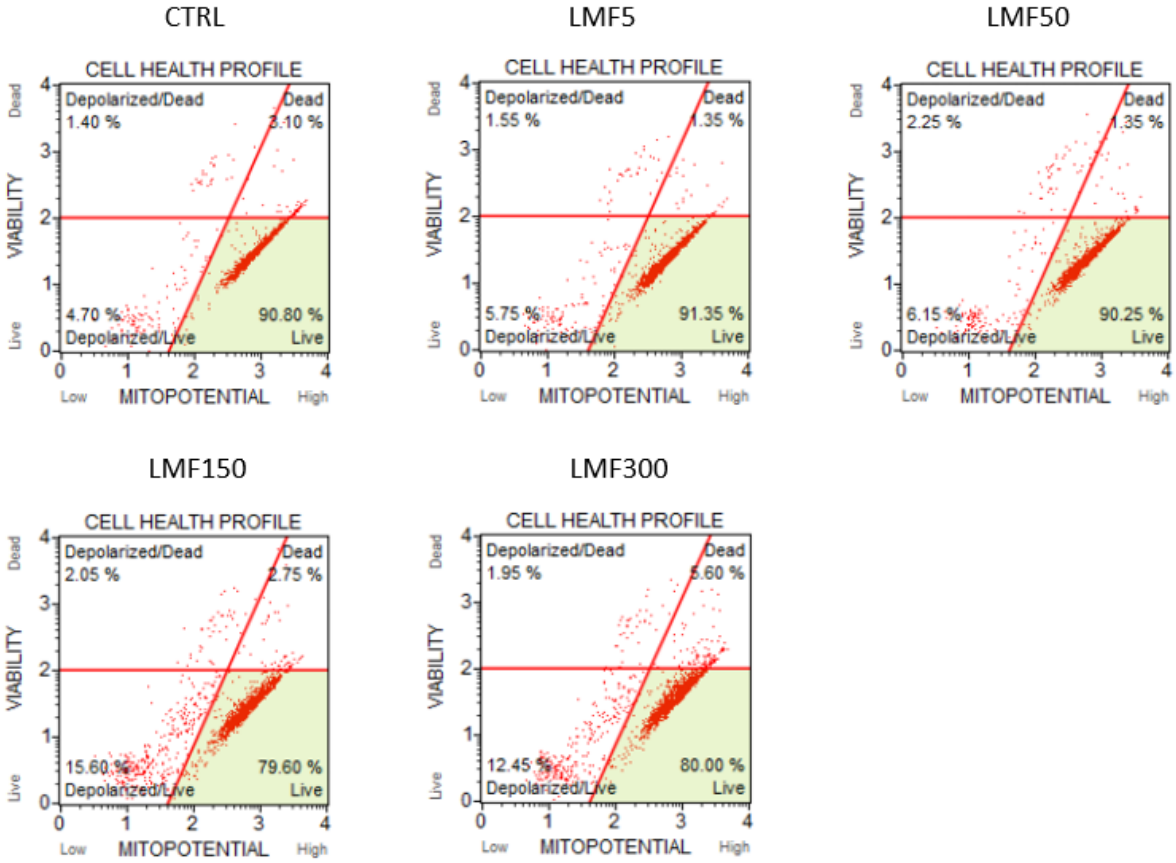


(A)

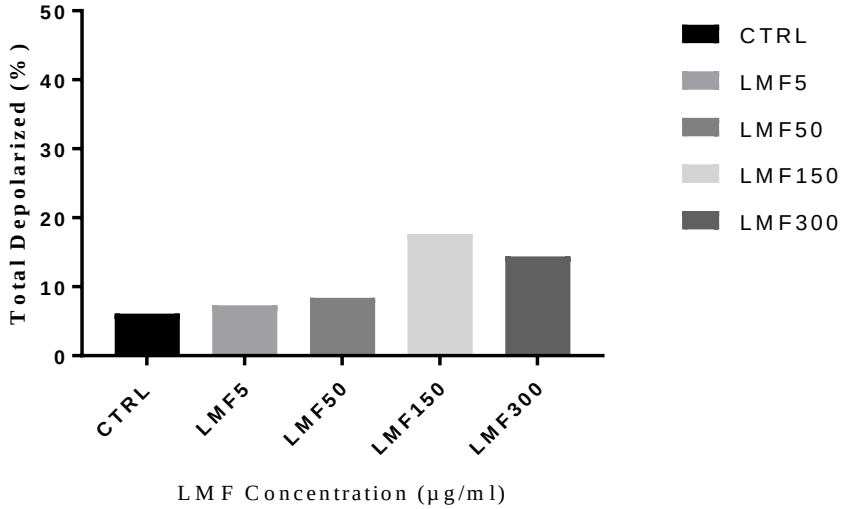


(B)

Figure 32. The status of mitochondria in MDA-MB-231 cells after being treated with LMF (µg/ml) for 72 hrs and harvest



(A)



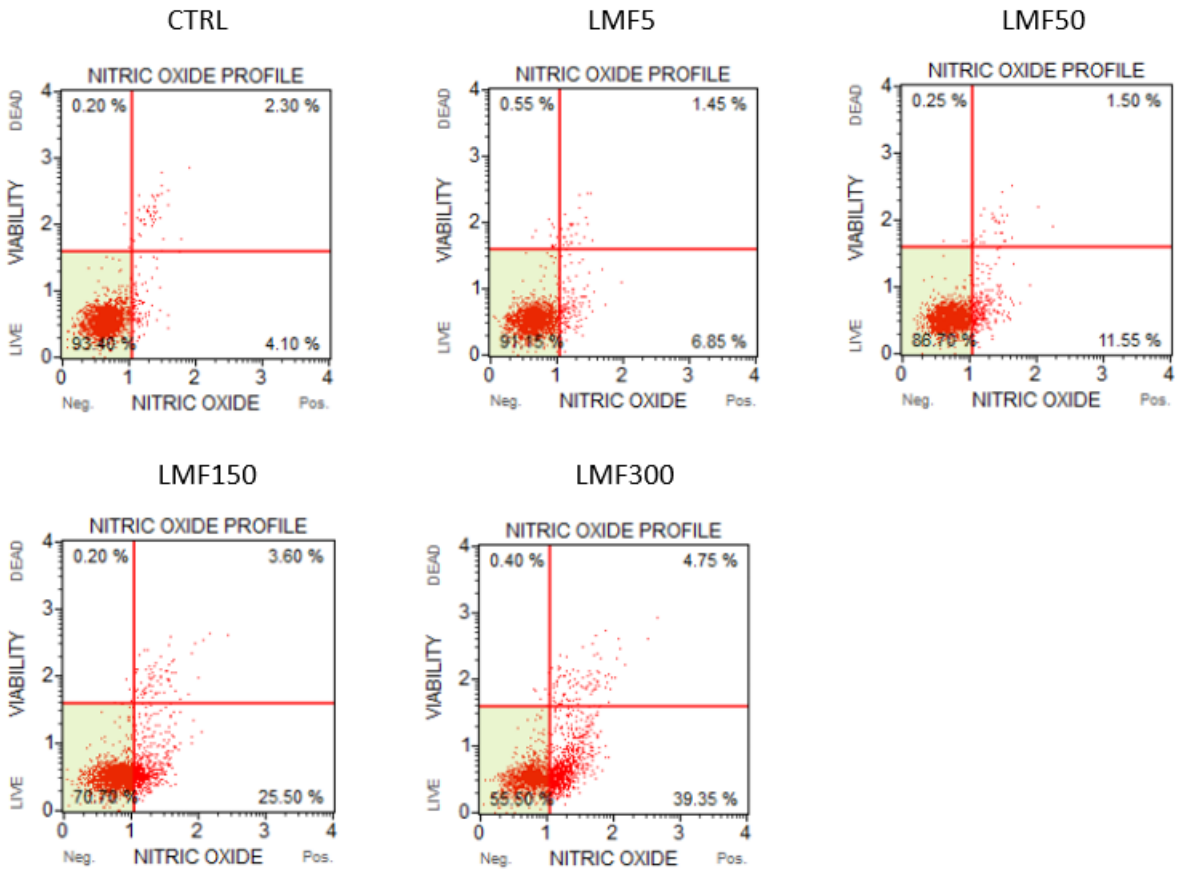
(B)

Figure 33. The status of mitochondria in MDA-MB-231 cells after being treated with LMF (µg/ml) for 96 hrs and harvest

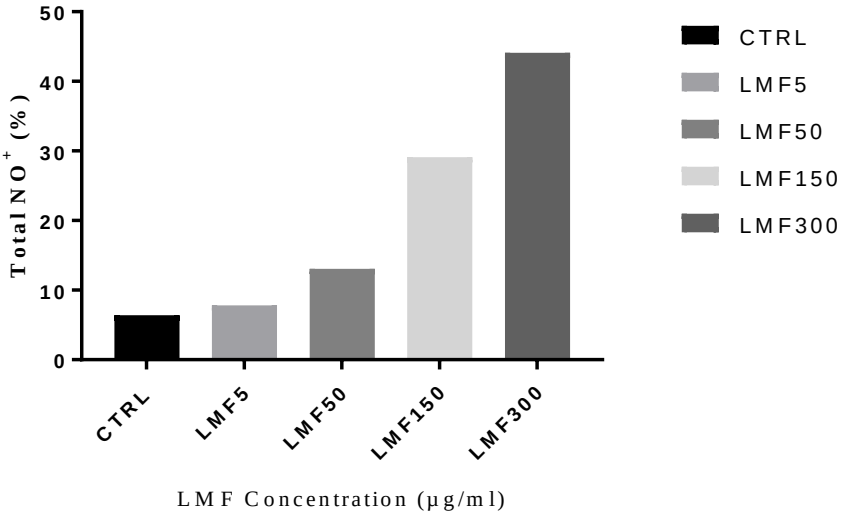
The depolarization of mitochondria membrane potential is closely associated with cell apoptosis pathways and caspases activated in apoptosis. A similar trend in loss of MMP has been observed in cells treated with various concentrations of LMF as in apoptosis and caspase assays.

MMP loss was most significant in cells treated with 300 µg/ml and 150 µg/ml LMF, with 26.05% and 25.25%, 22.40% and 18.05%, 14.40% and 17.65% in cells after 48, 72 and 96 hours respectively. The number of cells with depolarized mitochondria in these two samples was more than doubled when compared to negative controls, while only a minor increase (1-2%) was observed in the percentage of cells treated with 50 µg/ml and 5 µg/ml LMF. No obvious time-dependent manner was observed on loss of MMP in this assay.

4.3.5 Nitrosative stress in cells

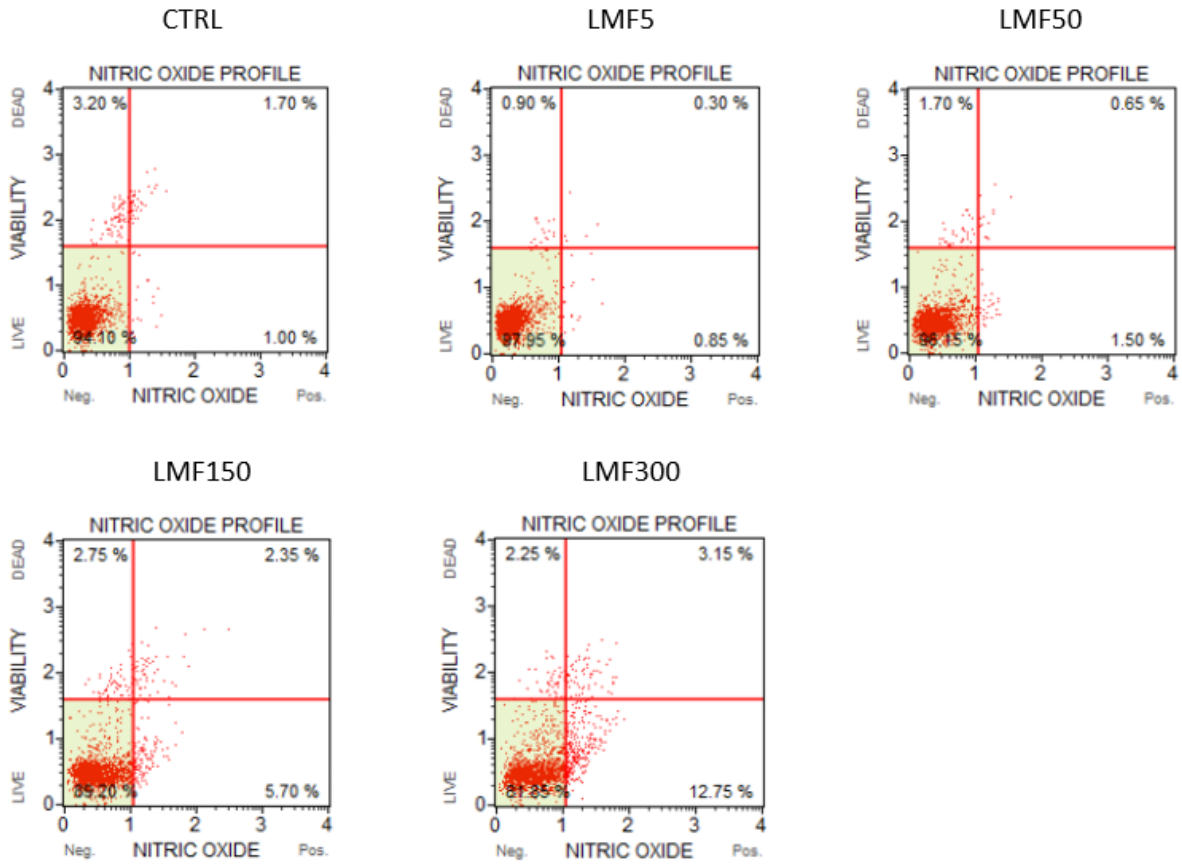


(A)

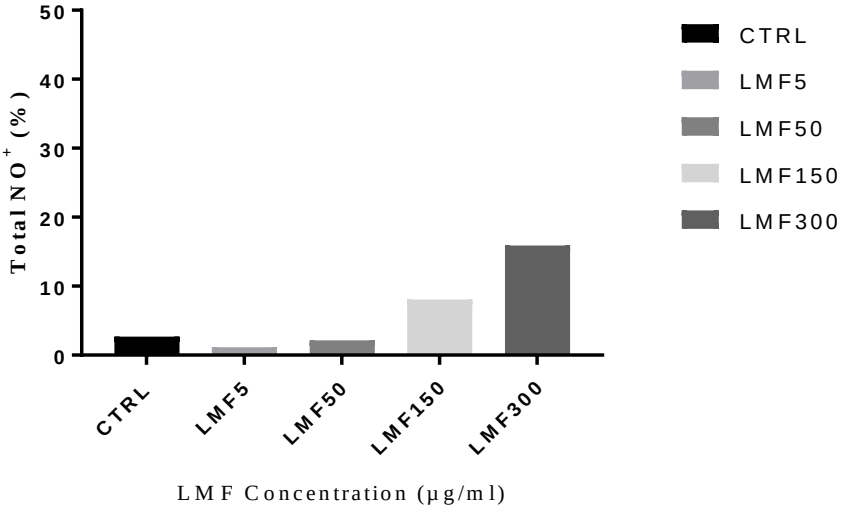


(B)

Figure 34. The nitrosative stress parameters in MDA-MB-231 cells after being treated with LMF (µg/ml) for 48 hrs and harvest

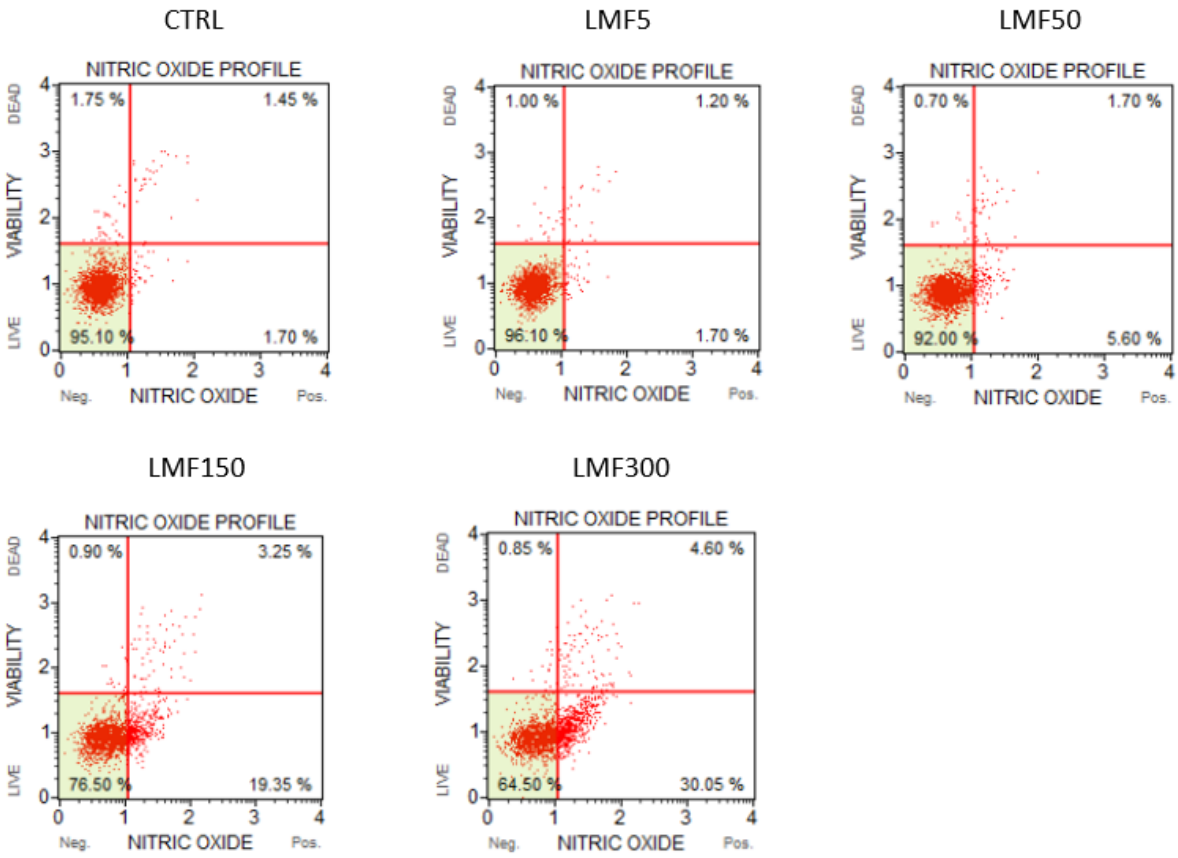


(A)

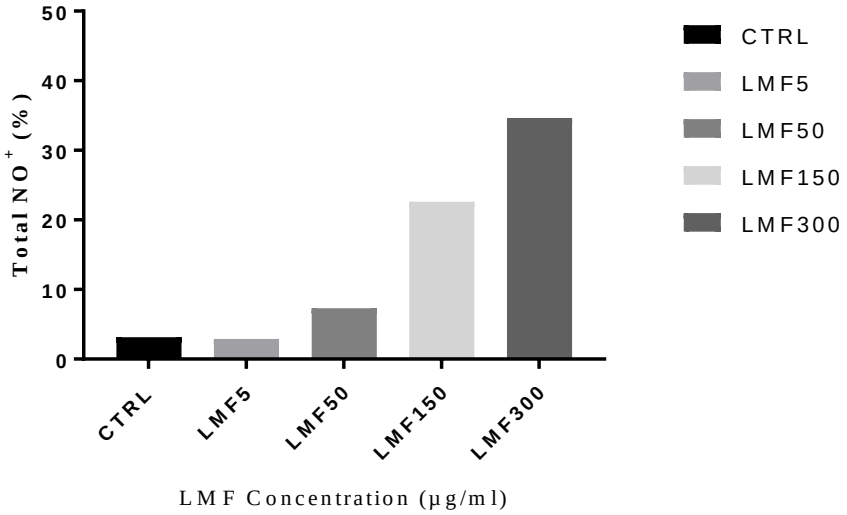


(B)

Figure 35. The nitrosative stress parameters in MDA-MB-231 cells after being treated with LMF (µg/ml) for 72 hrs and harvest



(A)



(B)

Figure 36. The nitrosative stress parameters in MDA-MB-231 cells after being treated with LMF (µg/ml) for 96 hrs and harvest

There was a 1 mM MAHMA NONOate (one of the NO donors) medium solution freshly prepared and used on cells in this assay, trying to make a positive control. However, the MDA-MB-231 cell line somehow did not respond to it, therefore data of a positive control is not presented here.

Nevertheless, LMF significantly induced NO production in MDA-MB-231 cells. The increase of nitrosative stress in cells is in a clear dose-dependent manner in all three time points. Possibly due to the reactive chemistry characteristic of NO, the increase of NO in cells incubated for different periods of time seems to be variable but not time-dependent. The highest level of NO was increased in cells treated with 300 μg/ml at about 37.70% after an incubation of 48 hours, 13.20% after 72 hours and 31.50% after 96 hours, compared to negative controls. In contrast to former assays, the increase of NO production was largely concentrated in viable cells than dead cells, with only a minor percentage of NO (less than 5%) detected in cells that lost viability. NO was also detected in negative controls in a minor amount, indicating its existence in MDA-MB-231 cells without any treatment.

Chapter 5. Discussion

Although fucoidan has been extensively studied due to its promising anti-cancer properties and pathways, the fucoidan usage in clinical practice is rather complicated. The complexity of fucoidan usage is largely contributed by endogenous (i.e. structure, sulphate content, etc.) and exogenous (i.e. dose, route of administration) factors that sometimes leading to variable and contradictory research output. Therefore, fucoidan from various brown seaweed species and various geographic distribution sites has been examined with in vitro and in vivo studies. In this study, research is focused on the cytotoxicity of different molecular-weight fucoidan extracted from *Undaria pinnatifida*, which is native to east Asia but now widely distributed along coastlines in New Zealand. HMF and LMF have been tested in this study in vitro against breast cancer. So far, there are a few reports of fucoidan extracted from *Undaria pinnatifida* in Japan and Korea against breast cancer, but few data are available on the effect of fucoidan from New Zealand *Undaria pinnatifida* on breast cancer cell lines.

5.1 Cytotoxicity of fucoidan in relation to their molecular weight

The cytotoxic effects of HMF and LMF were significantly different in both MCF-7 and MDA-MB-231 cell lines. It appears that HMF has very limited effect on slowing down growth of cancer cells but cannot induce obvious cell death either by dose-dependent manner or time-dependent manner. Its large molecular size may contribute mostly to this performance that makes HMF having difficulties entering cancer cell membrane. Compared to the cell viability in negative control groups, the existence of HMF in cell culture medium seems to exert minor disadvantages on cancer proliferation (cell viability in treatment groups decreased from 100% to around 80-90%), however the inhibitory effect of HMF did not show enough therapeutic value.

LMF exhibited great suppression on cell viability in both MCF-7 and MDA-MB-231 cell lines. Dose-dependent inhibitions of cell proliferation have been observed in each time point of 48, 72 and 96 hours in both types of cells. In the MCF-7 group, decrease of the cell population has been observed in cells treated with all LMF concentrations (from 200 μg/ml to 0.5 μg/ml) from 48 hours to 72 hours. While cell viability continued to decrease slightly at 96 hours in lower concentration groups (< 10 μg/ml of LMF), the cell viability in higher concentration groups (≥ 10 μg/ml of LMF) remains relatively stable (decreased about 1-2%). In general, LMF showed a dose-dependent manner and a

time-dependent manner in inhibitory effects on MCF-7 cells, with the best inhibitory effect at 200 µg/ml of LMF at 96 hours where cell viability decreased to around 40% compared to negative control.

In MDA-MB-231 cells, no time-dependent manner has been observed in the decrease of cells treated with LMF from 48 hours to 96 hours. In contrast, the inhibition on cell viability experienced a retrogress in cell groups treated with LMF concentrations lower than 200 µg/ml at 96 hours compared to 48 hours. The inhibition rates on MDA-MB-231 in cell groups treated with highest LMF concentrations (300, 250 and 200 µg/ml) were similar and relatively stable from 48 hours to 72 hours, at a decrease to cell viability of around 60% compare to negative controls. Although not time-dependent, the dose-dependent manner of the inhibitory effect of LMF was exhibited in all time points.

MCF-7 cells appear to be much more sensitive to LMF than MD-MB-231 cells, with the best inhibitory effect nearly 20% higher in the MCF-7 group than in the MDA-MB-231 group and the IC_{50} value at only 10.54 µg/ml after 96 hours (IC_{50} was above 300 µg/ml for MDA-MB-231 cells by 96 hours). The reverse of cell viability over time only appeared in MDA-MB-231cells groups, not in MCF-7. A possibility supported by literature is suggested here, namely that the difference between the p53 status in two cell lines may contribute to their distinction of LMF-sensitivity. Tumour protein p53, also known as TP53, is a protein coded by the TP53 gene and is able to suppress cancer growth by regulating cell apoptosis, cell cycle progress and activation of DNA repair proteins. The p53 in the MCF-7 cell line is the wild type, whereas in MDA-MB-231 it harbours mutated p53. Tumour cells which harbour mutated p53 are typically more resistant to certain anti-cancer drugs because mutated p53 no longer renders the tumour suppressing abilities of the wild type p53, which also makes breast cancer of this type clinically more challenging to treat.

Findings from Zhang et al. (2011) have reported a similar trend in growth inhibition in MCF-7 and MDA-MB-231 cell lines. They used LMF (72%, <500 MW; less than 28%, 800 kDa peak MW) derived from seaweed Mozuku of *Cladosiphon novae-caledoniae* Kylin from the Kingdom of Tonga and have tested the effect of LMF on the growth of several cancer cell lines, including these two breast cancer cell lines. Their data shows that LMF exerted 60% growth inhibition in MCF-7 cells and 41% growth inhibition in MDA-MB-231 cells after LMF treatment of 820µg/ml for 96 h, which is a

very similar result as found in this study. Differences in the highest concentrations of LMF in two different studies indicates, in a way, that LMF from New Zealand *Undaria pinnatifida* exhibits better efficacy than LMF from *Cladosiphon novae-caledoniae* Kylin, considering that LMF in this study can reach very close to the inhibitory effect on the same cell lines using one third to half of the concentration used in Zhang's treatment (200 µg/ml & 300 µg/ml of LMF compared to 820 µg/ml). In a following study, they raised a hypothesis that the distinct caspase-3 expressions may contribute to the different effects of LMF on two cell lines (Zhang, Teruya, Eto, & Shirahata, 2013). They argued that MCF-7 cells do not express full-length caspase-3 to induce a positive feedback loop and does not undergo formation of apoptosomes in vivo, while caspase-3 is expressed in MDA-MB-231 cells. Overall, the fact that MCF-7 cells have a higher sensitivity to LMF than MDA-MB-231 cells has been proven.

5.2 Examination of LMF-induced apoptosis and mechanism

Inducing cell apoptosis is one of the main anti-cancer mechanisms of fucoidan. The percentage of apoptotic cells in LMF treated MDA-MB-231 cells and related hall markers were examined in this study.

As can be seen from the figures, apoptosis was significantly induced at LMF concentrations of 150 µg/ml and 300 µg/ml at 48 hours, 72 hours and 96 hours. Increase has been observed in both early apoptotic cell percentage and late apoptotic cell percentage, with an average increase of approximately 8% in total apoptotic cell percentages with the highest concentration comparing to the negative control in all three time points. No obvious time-dependent manner has been observed in this assay, while the total apoptotic cell percentage appears to be correlated to LMF in a dose-dependent manner.

Caspase activity has been observed in fucoidan-mediated apoptosis in this study. From 48 hours to 96 hours, an increase in total caspase in cells has been observed in each time point with an increasing dose of LMF, indicating that the LMF-induced cell apoptosis in MDA-MB-231 is a caspase-dependent apoptosis. There are multiple caspases involved in cell apoptosis process and they are expressed as inactive zymogens that become activated upon cleavage by other caspases in a so-called caspase activation cascade. Among those caspases, caspase-2, -8, -9 and -10 function as initiators in apoptosis pathway whereas caspase-3, -6 and -7 are effectors (Jänicke, 2009). Caspases

detected in this study were not identified by the multi-caspase assay and will require other assays to better explore which pathway(s) of cell apoptosis was induced by LMF in MDA-MB-231. Nevertheless, the LMF-mediated apoptosis in this study is confirmed as caspase-dependent apoptosis.

Mitochondria status is also closely related to cell apoptosis. Loss of mitochondria membrane potential (MMP) is often considered as a sign that is associated with the early stages of cell apoptosis. In this study, the increase of cells with depolarized mitochondrial membrane have been observed in all three time points with the increasing dose of LMF. Compared to negative controls, the highest concentrations of LMF (150 µg/ml & 300 µg/ml) significantly doubled the percentage of cells with mitochondria depolarization, indicating that MMP was changed during LMF-induced cell apoptosis. This finding implies the pathway of fucoidan-induced apoptosis, which is a mitochondria-mediated apoptosis. As has been stated in the literature review section, loss of MMP can occur either in the intrinsic pathway of apoptosis or in type II extrinsic pathway (which will later engage with the intrinsic pathway), whereas type I extrinsic pathway does not involve changes in MMP. Therefore, at least activation of the intrinsic apoptosis pathway is confirmed in LMF-induced apoptosis in MDA-MB-231, while the involvement of the extrinsic pathway of cell apoptosis needs to be further examined by caspase-8 detection. By confirming the mitochondria-mediated pathway of apoptosis, the activation of caspase-9 is expected in LMF treated MDA-MB-231 cells as well as caspase-3 and caspase-7. This result is consistent with literature that the intrinsic pathway is the primary pathway in fucoidan-induced apoptosis in cancer cell lines.

To conclude, LMF induced caspase-dependent apoptosis in MDA-MB-231 cells and it was activated through the mitochondria-mediated (intrinsic) pathway. The apoptosis was significantly induced by higher doses of LMF while LMF lower than/including 50 µg/ml presents only minor effects in apoptosis and apoptosis related activities, which is consistent with MTT results. Whether the extrinsic pathways participated in LMF-induced apoptosis will require further research of identifying caspases that have been observed to be activated in the apoptosis process.

5.3 Examination of nitrosative stress parameters in LMF treated MDA-MB-231 cells

NO is a short-lived and colourless gas which can diffuse between cells easily due to its lipophilic nature. It is essential in many physiological functions and has rather complex biological effects. In relation to cancer, NO is known to be expressed in a wide range of mammalian cells including breast cancer cell lines, and its role in cancer progresses is variable between inhibition and promotion. NO is generated from the terminal guanido nitrogen atom of L-arginine by various NADPH-dependent NOS, with neuronal (n) NOS, iNOS and eNOS being the three main isoforms of NOS and more recently a mitochondria NOS has been identified (Lala, & Chakraborty, 2001).

According to the output of the Nitric Oxide assay conducted in this study, LMF seems to stimulate NO production in MDA-MB-231 cells. Elevation of nitrosative stress in cells has been observed with the treatment of LMF in each time point and appears to be positively correlated to the dosage of LMF, which means dose-dependent. The increase of total nitric oxide positive cells was most remarkable with the 300 µg/ml LMF that was roughly 7 times higher than in negative controls at 48 and 72 hours, and 11 times higher than in negative controls at 96 hours, secondly at 150 µg/ml, while minor increases were observed with a lower LMF concentration 50 µg/ml.

The generation of NO under certain conditions is cytotoxic to various pathogens and tumour cells (Umansky & Schirrmacher, 2001), and the similar trends in LMF-induced cell apoptosis and LMF-stimulated NO production seems to imply the correlation. However, this hypothesis is not supported by other research findings. It is stated that NO-mediated apoptosis in tumour cells involves upregulation of the tumour suppressor protein p53, activation of caspases, chromatin condensation and DNA laddering. As mentioned before, the p 53 in MDA-MB-231 cell line is mutated, which is linked to NO-resistance, thus is unlikely to perform cell apoptosis through regulating this pathway. Research from Martinez et al. (2016) comparing the cytotoxic effect of NO on two different types of TNBC cells indicated that other mechanism pathways of NO-mediated apoptosis are also ineffective in MDA-MB-231 cells. In this research, breast cancer cells were treated with various concentrations of long-acting NO donor and found that TNBC cells derived from African American (AA) and Caucasian (CA) female breast cancer patients, respectively, respond differently to nitrosative stress. All AA TN cells demonstrated NO-induced mitochondria-mediated apoptosis, whereas

MDA-MB-231 as a CA TN cell line was unable to interact with NO and induce apoptosis. Therefore, it is considered that NO-mediated cell apoptosis is not contributing to LMF-induced apoptosis in MDA-MB-231 cells.

Contrary to the cytotoxic effect of NO, it is reported that low concentrations of NO may prevent cells from apoptosis induced by other apoptotic stimuli (Choi, Pae, Jang, Kim, & Chung, 2002). This may explain why cell viability of MDA-MB-231 cells inhibited by LMF in lower concentrations (<150 µg/ml) was slowly reversed in the MTT assays of this study. However, the protective role of NO as observed with in vitro experiments does not necessarily mean similar promotion of TNBC tumours in vivo. Fucoidan is also known for its ability to stimulate iNOS in macrophages and increase NO production. Normally, this over-production of NO in macrophages will be released to tumour cells and induce NO-mediated apoptosis to suppress tumour growth (Takeda et al., 2012). For CA TN tumours, their resistance to NO will not enable the apoptosis, where the over-production of NO will also inactivate the protective effect upon tumour cells. Actually, it has been stated that iNOS expression by macrophages, stromal cell and tumour cells accounted for most of the NOS activity in breast cancer (Lala & Chakraborty, 2001). Since fucoidan is able to stimulate iNOS in macrophages, it is speculated that LMF stimulates iNOS in MDA-MB-231 cells in the same way and leads to the increase of NO production.

Due to some limitations of time and materials, mechanism assays were not yet successfully performed on the MCF-7 cell line, thus whether LMF can increase nitrodasive stress in MCF-7 cells or not currently remains unknown. There is, however, another previous research tested a natural compound called diosmin (a citrus fruit flavonoid) on MCF-7 cells using the same nitric oxide kit as in this study (Lewinska, Adamczyk-Grochala, Kwasniewicz, Deregowska, & Wnuk, 2017). Their findings indicate that diosmin induced programmed cell death in the MCF-7 accompanied by nitrosative stress, which at least indicates the existence of NOS in the MCF-7 cells. Considering that MCF-7 cells are with the wild type of p53, they are theoretically not resistant to NO if fucoidan stimulates NO generation in the MCF-7 cells as well. In this hypothesis, NO-mediated apoptosis is likely to contribute to the inhibitory effect of LMF on MCF-7. This hypothesis could be another theory of why MCF-7 is more sensitive to LMF than MDA-MB-231 cells. A future mechanism study to analyse the cytotoxic effect of LMF in MCF-7 cells is suggested.

Based on previous research on the effect of fucoidan on MDA-MB-231 cells, there are some other properties of LMF/fucoidan which are suspected that may have a link to the role of NO. For one, fucoidan has an anti-metastatic function on MDA-MB-231 cells. Cumashi et al. (2007) stated a decreased by 80% of cells attached to the platelet-coated plate in the presence of fucoidan, of which the interaction is associated with early steps of tumour cell migration and induces platelet aggregation allowing the tumour cell cluster to survive in the micro-vascular system. Fucoidan was found to inhibit P-selectin residing on the platelet surface and led to reduced numbers of attached tumour cells, and most circulating tumour cells do not survive attack from immune cells or the shear forces of the blood stream. This is one of the anti-metastasis mechanisms of fucoidan. NO is also one of the anti-adhesive substances that can lead to cancer metastasis inefficiency. It is reported that NO can inhibit cell adhesion, interfere with the assembly of focal adhesion complexes and disrupt the cell–extracellular matrix interactions (Monteiro, Silva, & Stern, 2004). In the interaction between tumour and host cells (macrophages), local production of NO by the iNOS influences adhesive processes, which causes tumour cells lose their capacity to colonize elsewhere (Monteiro, Silva, & Stern, 2004). In some types of tumour cells, NO mediate adhesion-related apoptosis (anoikis), consequently prevents metastasis. For MDA-MB-231, combining the facts that LMF induced cell apoptosis as well as stimulated NO production in tumour cells, may be worth some further mechanism studies.

Another role of NO is suggested to be its synergy with cytotoxic drugs. In several previous studies of combined treatments on breast cancer, the ability of fucoidan to synergize with standard anti-cancer agents (i.e. cisplatin, tamoxifen and paclitaxel) and reduce toxicity has been reported. NO, as another molecule critical in sensitization of tumour cells to chemotherapeutic agents, has also been found able to synergize with certain chemotherapeutic agents, mostly with doxorubicin and cisplatin (Jeannin et al., 2008). It has been examined that NO donor agents effectively reduced the resistance in MDA-MB-231 cells to doxorubicin by 33% to 50%, and enhanced the inhibitory effect of the treatment (Muir, Adams, & Graham, 2006). Therefore, apart from the synergistic ability of fucoidan itself, whether levels of NO increased by LMF contribute partially to the synergy in the combined therapy of fucoidan and cytotoxic drugs (i.e. cisplatin) or not, this again may be worth some further mechanism studies.

Taken together, it is confirmed by this Nitric Oxide assay that LMF increased NO production in MDA-MB-231 cells. Although the increase in NO is consistent with the dose-dependent inhibitory effect by LMF on MDA-MB-231 cells, MDA-MB-231 cells are functionally unable to carry out NO-mediated apoptosis. The NO resistance in MDA-MB-231 cells and the protective role of low concentration NO in cells are considered possible explanations for relatively lower sensitivity to LMF in MDA-MB-231 compared to MCF-7 cells. To the best of my knowledge, few research projects have studied fucoidan/LMF stimulation of NOS in breast cancer cells, while more of the studies focus on the anti-cancer pathway of fucoidan-activated macrophages and stimulation of iNOS in macrophages. Considering the fact that iNOS expression is also the main NOS activity in breast cancer, it is suspected that LMF may stimulate iNOS in MDA-MB-231 the same way as in macrophages. Despite no cytotoxicity of NO to MDA-MB-231 cells, there are some other functions of NO that may have a link with previously reported anti-cancer properties of fucoidan on MDA-MB-231 cells, including metastasis inefficiency and the synergy with cytotoxic chemotherapeutic agents. Further mechanism studies are suggested to better examine the relationship between increased NO in breast cancer cells and fucoidan anti-cancer properties.

Chapter 6 Conclusion

6.1 Overall conclusion

By using MCF-7 and MDA-MB-231 cell lines as experimental models, the inhibitory effects of HMF (>300k) and LMF (<10k) against ER^+ and triple-negative breast cancers have been examined in this study. Molecular weight is shown to be a critical factor for fucoidan from *Undaria pinnatifida* in its anti-cancer potential. HMF in this study failed to effectively suppress cancer cell proliferation, causing only 20% inhibition by 96 hours. A suppression at this percentage is normally not considered to be characteristic of a therapeutic value. Compared to HMF, LMF exhibited much better inhibition on both cell lines, decreaseing approximately 60% cell growth in MCF-7 and about 40% in MDA-MB-231 at the highest concentration. Dose-dependent inhibition by LMF was observed in both cell lines but only in the MCF-7 was a time-dependent correlation apparent, with an IC_{50} of 19.087 µg/ml at 72 hours and 10.540µg/ml at 96 hours. For LMF treatment, MCF-7 and MDA-MB-231 showed distinctive sensitivities to the treatment. MCF-7 is more sensitive to LMF than the ER^- cancer cells and this similar trend was also observed by another LMF study testing LMF from *Cladosiphon novae-caledoniae* Kylin on these two breast cancer cell lines (Zhang et al., 2011). In their study, the assumption was made that the expression of caspase-3 in MDA-MB-231 but not MCF-7 may be the reason for the distinction of sensitivity. Due to the detection of LMF-stimulated NO production in breast cancer cells in this study, and combing literature, the mutated p53 status and NO-resistance in MDA-MB-231 but not MCF-7 (wild type p53) is suspected to contribute to this sensitivity distinction as well. This may become a hypothesis for further research.

As for the cytotoxicity of LMF to normal cells, no obvious suppression was observed on HDFa cell line, one of the typical noncancerous cell lines, by LMF at a dosage which is suppressive on breast cancer cells (up to 300 µg/ml) in this study. Although data of LMF on more noncancerous cell lines are required to fully study its toxicity, the selective cytotoxicity of LMF to cancer cells is in line with previous literatures.

LMF-induced apoptosis was observed in this study in MDA-MB-231 cells at higher LMF concentrations (150 µg/ml and 300 µg/ml) along with the caspase activation and depolarized mitochondrial membrane. This indicates that LMF induced caspase-dependent apoptosis in this cell line and the mitochondria-mediated (intrinsic) pathway

of apoptosis was involved. In this way, caspase-9 activation was expected in the apoptosis process and the status of caspase-8 (corresponding to the extrinsic apoptosis pathway) needs to be confirmed by further study.

LMF-stimulated NOS activity was observed in MDA-MB-231 cells. It is reported in literature that iNOS expression by macrophages, stromal cells and tumour cells accounts for most of the NOS activity in breast cancer (Lala & Chakraborty, 2001). Based on the ability of fucoidan to activate iNOS in macrophages, it is suspected that LMF may stimulates iNOS in breast cancer cells in a similar way. For certain cancer types, including MCF-7, the increase of NO could lead to NO-mediated cell apoptosis in the same way as the immune response in vivo. Unfortunately, due to the mutated p53, MDA-MB-231 is resistant to this apoptosis pathway.

6.2 Limitations

It is unfortunates that mechanism assays were not able to be performed in theMCF-7 cell line due to time and material limits, but it can always be conducted later by other researchers when possible. The comparison of data from MCF-7 and MDA-MB-231 can better indicating the anti-cancer pathways of LMF, and the hypothesis on sensitivity distinction between MCF-7 and MDA-MB-231 can be examined once this is accomplished.

Another improvement required for this study is the S.D value for data from cell cycle assays and mechanism assays. Although all results are repeatable, and the effects/trends related to LMF were confirmed, the third independent experiment (repeat) was not able to be performed due to the extensive time spent on MTT assays. It is also considered better if one more non-cancerous cell lines can be added and tested to prove the selective cytotoxicity of LMF to cancer cells rather than normal cells.

6.3 Future research

The anti-cancer potential of LMF from New Zealand *Undaria pinnatifida* has been confirmed in this study and the inhibitory effects on two breast cancer cell lines were considerable but not enough to treat breast cancer alone. A combined study for LMF and other breast cancer therapeutic agents is suggested to test the synergy effects. There have been a few recent studies reported that fucoidan (obtained from Japan) enhanced the anti-cancer properties of chemo-agents (i.e. tamoxifen, cisplatin, paclitaxel) on breast cancer cells through multiple mechanisms, but more data are required to fill in

the knowledge gap of this field. Although endocrine therapies as standard treatment for ER^+ breast cancer are considered less toxic than traditional chemotherapies, combined therapy is still critical to allow better suppression of tumour growth and reduce the risk for drug resistance. Both the non-toxic property of LMF and the potential of LMF-stimulated NO production in tumour cells on sensitization of tumour cells to chemotherapeutic agents are promising qualities for LMF to become a candidate in combined chemotherapy treating breast cancer.

Additionally, as mentioned in the discussion section, determination of whether the LMF-induced NO increase contributes to anti-cancer properties of fucoidan on MDA-MB-231 cells, may warrant further mechanism studies in areas such as anti-metastasis and synergy effects.

LMF tested in this study was extracted from New Zealand *Undaria pinnatifida* using ethanol. With other extraction and purification methods or with a different molecular weight range, the composition of LMFs may be variable therefore perform distinctively. There are some studies that already have been done on LMFs from brown algae species using certain extraction/purification methods about their compositions and bioactivities. To better understand and compare the anti-cancer potentials of all LMFs, further studies on LMFs are recommended.

Reference

Ale, M. T., Mikkelsen, J. D., & Meyer, A. S. (2011). Important determinants for fucoidan bioactivity: A critical review of structure-function relations and extraction methods for fucose-containing sulfated polysaccharides from brown seaweeds. *Marine drugs, 9*(10), 2106-2130.

American Cancer Society: Cancer Facts and Figures 2018. Atlanta, Ga: American Cancer Society, 2018.

Atashrazm, F., Lowenthal, R. M., Woods, G. M., Holloway, A. F., & Dickinson, J. L. (2015). Fucoidan and cancer: a multifunctional molecule with anti-tumor potential. *Marine drugs, 13*(4), 2327-2346.

Badve, S., Dabbs, D. J., Schnitt, S. J., Baehner, F. L., Decker, T., Eusebi, V., ... & Palacios, J. (2011). Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. *Modern Pathology, 24*(2), 157.

Bak, M. J., Gupta, S. D., Wahler, J., & Suh, N. (2016). Role of dietary bioactive natural products in estrogen receptor-positive breast cancer. *In Seminars in cancer biology (Vol. 40, pp. 170-191)*. Academic Press.

Banafa, A. M., Roshan, S., Liu, Y. Y., Chen, H. J., Chen, M. J., Yang, G. X., & He, G. Y. (2013). Fucoidan induces G 1 phase arrest and apoptosis through caspases-dependent pathway and ROS induction in human breast cancer MCF-7 cells. *Journal of Huazhong University of Science and Technology [Medical Sciences], 33*(5), 717-724.

Berteau, O., & Mulloy, B. (2003). Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology, 13*(6), 29R-40R.

Bonilla, J. M., Tabanera, M. T., & Mendoza, L. R. (2017). Breast cancer in the 21st century: from early detection to new therapies. *Radiología (English Edition), 59*(5), 368-379.

Brüne, B., Sandau, K., & Von Knethen, A. (1998). Apoptotic cell death and nitric oxide: activating and antagonistic transducing pathways. *BIOCHEMISTRY C/C OF BIOKHIMIIA*, *63*, 817-825.

Cain, H., Macpherson, I. R., Beresford, M., Pinder, S. E., Pong, J., & Dixon, J. M. (2017). Neoadjuvant Therapy in Early Breast Cancer: Treatment considerations and common debates in practice. *Clinical Oncology, 29*(10), 642-652.

Cancer Genome Atlas Network. (2012). Comprehensive molecular portraits of human breast tumours. *Nature, 490*(7418), 61.

Carlson, R. W., (2008, February 9). *Breast Cancer: Risks, Prevention, Diagnosis and*

Treatments. [Video file].
Retrieved from https://www.youtube.com/watch?v=oJUVlTm39AM

Chizhov, A. O., Dell, A., Morris, H. R., Haslam, S. M., McDowell, R. A., Shashkov, A. S., ... & Usov, A. I. (1999). A study of fucoidan from the brown seaweed *Chorda filum*. *Carbohydrate Research, 320*(1)*,* 108-119.

Cho, M. L., Lee, B. Y., & You, S. G. (2010). Relationship between oversulfation and conformation of low and high molecular weight fucoidans and evaluation of their in vitro anticancer activity. *Molecules*, *16*(1), 291-297.

Choi, B. M., Pae, H. O., Jang, S. I., Kim, Y. M., & Chung, H. T. (2002). Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator. *BMB reports*, *35*(1), 116-126.

Choi, J. I., & Kim, H. J. (2013). Preparation of low molecular weight fucoidan by gamma-irradiation and its anticancer activity. *Carbohydrate polymers*, *97*(2), 358-362.

Chung, H. J., Jeun, J., Houng, S. J., Jun, H. J., Kweon, D. K., & Lee, S. J. (2010). Toxicological evaluation of fucoidan from *Undaria pinnatifida* in vitro and in vivo. *Phytotherapy research, 24*(7)*,* 1078-1083.

Curtis, E., Wright, C., & Wall, M. (2005). The epidemiology of breast cancer in Maori women in Aotearoa New Zealand: implications for screening and treatment. *NZ Med. J, 118,* 1-10.

DeVita, V. T., & Lawrence, T. S. & Rosenberg, S. A. (Eds.). (2016). *Cancer: Principles & Practice of Oncology Review* (10th ed., pp. 16-56). Philadelphia, USA: Wolters Kluwer.

Dixon, J. M. (2014). Endocrine resistance in breast cancer. *New Journal of Science, 2014.*

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic pathology, 35*(4)*,* 495-516.

Escrivá-de-Romaní, S., Arumí, M., Bellet, M., & Saura, C. (2018). HER2-positive breast cancer: Current and new therapeutic strategies. *The Breast, 39,* 80-88.

Friedberg, E. C. (2003). DNA damage and repair. *Nature, 421*(6921), 436.

Gallardo, A., Garcia-Valdecasas, B., Murata, P., Teran, R., Lopez, L., Barnadas, A., & Lerma, E. (2018). Inverse relationship between Ki67 and survival in early luminal breast cancer: confirmation in a multivariate analysis. *Breast cancer research and treatment, 167*(1)*,* 31-37.

Gao, H., Zhong, F., Xie, J., Peng, J., & Han, Z. (2016). PTTG promotes invasion in human breast cancer cell line by upregulating EMMPRIN via FAK/Akt/mTOR signaling. *American journal of cancer research, 6*(2)*,* 425.

Garber, J. (2017). Judy Garber on PARP Inhibitors for Triple-Negative Breast Cancer.

Oncology (Williston Park, NY), 31(11)*,* 814.

GLOBOCAN. (2012). Breast Cancer Estimated Incidence, Mortality and Prevalence Worldwide in 2012.
Retrieved from http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx

Goldhirsch, A. 2., Wood, W. C., Coates, A. S., Gelber, R. D., Thürlimann, B., Senn, H. J., & Panel Members. (2011). Strategies for subtypes—dealing with the diversity of breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. *Annals of oncology, 22*(8)*,* 1736-1747.

Gupta, S., & Abu-Ghannam, N. (2011). Bioactive potential and possible health effects of edible brown seaweeds. *Trends in Food Science & Technology*, *22*(6), 315-326.

Hansen, M. B., Nielsen, S. E., & Berg, K. (1989). Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *Journal of immunological methods, 119*(2)*,* 203-210.

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature, 407*(6805)*,* 770.

Jeannin, J. F., Leon, L., Cortier, M., Sassi, N., Paul, C., & Bettaieb, A. (2008). Nitric oxide-induced resistance or sensitization to death in tumor cells. *Nitric Oxide*, *19*(2), 158-163.

Jeffreys, M., Stevanovic, V., Tobias, M., Lewis, C., Ellison-Loschmann, L., Pearce, N., & Blakely, T. (2005). Ethnic inequalities in cancer survival in New Zealand: linkage study. *American Journal of Public Health, 95*(5)*,* 834-837.

Jänicke, R. U. (2009). MCF-7 breast carcinoma cells do not express caspase-3. *Breast cancer research and treatment*, *117*(1), 219-221.

Kim, K. J., Lee, O. H., & Lee, B. Y. (2010). Genotoxicity studies on fucoidan from Sporophyll of *Undaria pinnatifida*. *Food and chemical toxicology, 48*(4)*,* 1101-1104.

Kim, E. J., Park, S. Y., Lee, J. Y., & Park, J. H. (2010). Fucoidan present in brown algae induces apoptosis of human colon cancer cells. *BMC gastroenterology, 10*(1)*,* 96.

Koyanagi, S., Tanigawa, N., Nakagawa, H., Soeda, S., & Shimeno, H. (2003). Oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities. *Biochemical pharmacology, 65*(2)*,* 173-179.

Lakhani, S. R. (Ed.). (2012). WHO Classification of Tumours of the Breast (4th ed., pp. 23). International Agency for Research on Cancer.

Lala, P. K., & Chakraborty, C. (2001). Role of nitric oxide in carcinogenesis and tumour progression. *The lancet oncology*, *2*(3), 149-156.

Lee, H., Kim, J. S., & Kim, E. (2012). Fucoidan from seaweed *Fucus vesiculosus*

inhibits migration and invasion of human lung cancer cell via PI3K-Akt-mTOR pathways. *PloS one, 7*(11), e50624.

Lehmann, B. D., Bauer, J. A., Chen, X., Sanders, M. E., Chakravarthy, A. B., Shyr, Y., & Pietenpol, J. A. (2011). Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *The Journal of clinical investigation, 121*(7), 2750-2767.

Lewinska, A., Adamczyk-Grochala, J., Kwasniewicz, E., Deregowska, A., & Wnuk, M. (2017). Diosmin-induced senescence, apoptosis and autophagy in breast cancer cells of different p53 status and ERK activity. *Toxicology letters*, *265*, 117-130.

Li, Q., Eklund, A. C., Juul, N., Haibe-Kains, B., Workman, C. T., Richardson, A. L., ... & Swanton, C. (2010). Minimising immunohistochemical false negative ER classification using a complementary 23 gene expression signature of ER status. *PloS one, 5*(12), e15031.

Liao, D. W., Wang, L., Zhang, X. G., & Liu, M. Q. (2006). Expression and significance of PTEN/PI3K signal transduction-related proteins in non-small cell lung cancer. *Chinese journal of cancer, 25*(10), 1238-1242.

Losurdo, A., Rota, S., Gullo, G., Masci, G., Torrisi, R., Bottai, G., ... & Santoro, A. (2017). Controversies in clinicopathological characteristics and treatment strategies of male breast cancer: A review of the literature. *Critical reviews in oncology/hematology, 113,* 283-291.

Lønning, P. E. (2000). Is there a growing role for endocrine therapy in the treatment of breast cancer? *Drugs, 60*(1), 11-21.

Maisonneuve, P. (2017). Epidemiology, Lifestyle, and Environmental Factors. *In Breast Cancer (pp. 63-72)*. Springer, Cham.

Mak, W., Hamid, N., Liu, T., Lu, J., & White, W. L. (2013). Fucoidan from New Zealand *Undaria pinnatifida*: Monthly variations and determination of antioxidant activities. *Carbohydrate polymers, 95*(1), 606-614.

Marino, N., Woditschka, S., Reed, L. T., Nakayama, J., Mayer, M., Wetzel, M., & Steeg, P. S. (2013). Breast cancer metastasis: issues for the personalization of its prevention and treatment. *The American journal of pathology, 183*(4), 1084-1095.

Martinez, L., Thames, E., Kim, J., Chaudhuri, G., Singh, R., & Pervin, S. (2016). Increased sensitivity of African American triple negative breast cancer cells to nitric oxide-induced mitochondria-mediated apoptosis. *BMC cancer*, *16*(1), 559.

Maruyama, H., Tamauchi, H., Iizuka, M., & Nakano, T. (2006). The role of NK cells in antitumor activity of dietary fucoidan from *Undaria pinnatifida* Sporophylls (Mekabu). *Planta Med 72*(15): 1415-1417.

McKenzie, F., Ellison-Loschmann, L., Jeffreys, M., Firestone, R., Pearce, N., & Romieu, I. (2014). Healthy lifestyle and risk of breast cancer for indigenous and

non-indigenous women in New Zealand: a case control study. *BMC cancer, 14*(1), 12.

Ministry of Health. (2014). Cancer: Historical summary 1948–2011. Wellington: Ministry of Health.

Monteiro, H. P., Silva, E. F., & Stern, A. (2004). Nitric oxide: a potential inducer of adhesion-related apoptosis—anoikis. *Nitric Oxide*, *10*(1), 1-10.

Muir, C. P., Adams, M. A., & Graham, C. H. (2006). Nitric oxide attenuates resistance to doxorubicin in three-dimensional aggregates of human breast carcinoma cells. *Breast cancer research and treatment*, *96*(2), 169-176.

Muthukaruppan, A. (2011). Gene Expression Analysis in Breast Cancer (Doctoral thesis, Auckland University of Technology, Auckland, New Zealand). Retrieved from https://researchspace.auckland.ac.nz/handle/2292/6997

National Cancer Institute. (2017) *Metastatic cancer.* Retrieved July 10, 2018, from https://www.cancer.gov/types/metastatic-cancer. Accessed

New Zealand Guidelines Group, (2009). Management of early breast cancer. *Wellington: New Zealand Guidelines Group*

Nowell, P. C. (2002). Tumor progression: a brief historical perspective. *In Seminars in cancer biology (Vol. 12, No. 4,* pp. 261-266). Academic Press.

Palazzo, A., & Colleoni, M. (2017). Adjuvant Systemic Therapies by Subtypes: Guidelines. *In Breast Cancer (pp. 535-539)*. Springer, Cham.

Patani, N., Martin, L. A., & Dowsett, M. (2013). Biomarkers for the clinical management of breast cancer: international perspective. *International journal of cancer, 133*(1), 1-13.

Pádua, D., Rocha, E., Gargiulo, D., & Ramos, A. A. (2015). Bioactive compounds from brown seaweeds: phloroglucinol, fucoxanthin and fucoidan as promising therapeutic agents against breast cancer. *Phytochemistry Letters, 14,* 91-98.

Peart, O. (2017). Metastatic Breast Cancer. *Radiologic technology, 88(5),* 519M-539M.

Perez, E. A., Romond, E. H., Suman, V. J., Jeong, J. H., Sledge, G., Geyer Jr, C. E., ... & Winer, E. P. (2014). Trastuzumab plus adjuvant chemotherapy for human epidermal growth factor receptor 2–positive breast cancer: planned joint analysis of overall survival from NSABP B-31 and NCCTG N9831. *Journal of clinical oncology, 32*(33), 3744.

Perou, C. M. (2011). Molecular stratification of triple-negative breast cancers. *The oncologist, 16*(Supplement 1), 61-70.

Rivenbark, A. G., O'Connor, S. M., & Coleman, W. B. (2013). Molecular and cellular heterogeneity in breast cancer: challenges for personalized medicine. *The*

American journal of pathology, 183(4)*,* 1113-1124.

Schug, Z. T., Gonzalvez, F., Houtkooper, R. H., Vaz, F. M., & Gottlieb, E. (2011). BID is cleaved by caspase-8 within a native complex on the mitochondrial membrane. *Cell death and differentiation, 18*(3)*,* 538.

Senthilkumar, K., Manivasagan, P., Venkatesan, J., & Kim, S. K. (2013). Brown seaweed fucoidan: biological activity and apoptosis, growth signaling mechanism in cancer. *International journal of biological macromolecules, 60,* 366-374.

Skolnick, M. H., Frank, T., Shattuck-Eidens, D., & Tavtigian, S. (1997). Genetic susceptibility to breast and ovarian cancer. *Pathologie-biologie, 45*(3), 245-249.

Skriptsova, A. V., Shevchenko, N. M., Zvyagintseva, T. N., & Imbs, T. I. (2010). Monthly changes in the content and monosaccharide composition of fucoidan from *Undaria pinnatifida (Laminariales, Phaeophyta)*. *Journal of Applied Phycology, 22*(1)*,* 79-86.

Suthiphongchai, T., Promyart, P., Virochrut, S., Tohtong, R., & Wilairat, P. (2003). Involvement of ERK1/2 in invasiveness and metastatic development of rat prostatic adenocarcinoma. *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics*, *13*(5), 253-259.

Synytsya, A., Kim, W. J., Kim, S. M., Pohl, R., Synytsya, A., Kvasnička, F., ... & Park, Y. I. (2010). Structure and antitumour activity of fucoidan isolated from sporophyll of Korean brown seaweed *Undaria pinnatifida*. *Carbohydrate Polymers, 81*(1)*,* 41-48.

Sørlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., ... & Thorsen, T. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proceedings of the National Academy of Sciences, 98*(19)*,* 10869-10874.

Takeda, K., Tomimori, K., Kimura, R., Ishikawa, C., Nowling, T. K., & Mori, N. (2012). Anti-tumor activity of fucoidan is mediated by nitric oxide released from macrophages. *International journal of oncology*, *40*(1), 251-260.

Thomsen, L. L., Miles, D. W., Happerfield, L., Bobrow, L. G., Knowles, R. G., & Moncada, S. (1995). Nitric oxide synthase activity in human breast cancer. *British Journal of Cancer*, *72*(1), 41.

Tinoco, G., Warsch, S., Glück, S., Avancha, K., & Montero, A. J. (2013). Treating breast cancer in the 21st century: emerging biological therapies. *Journal of Cancer, 4*(2)*,* 117.

Tocaciu, S., Oliver, L. J., Lowenthal, R. M., Peterson, G. M., Patel, R., Shastri, M., ... & Fitton, J. H. (2018). The effect of *Undaria pinnatifida* fucoidan on the pharmacokinetics of letrozole and tamoxifen in patients with breast cancer. *Integrative cancer therapies*, *17*(1), 99-105.

Umansky, V., & Schirrmacher, V. (2001). Nitric oxide-induced apoptosis in tumor cells. *Advances in cancer research*, *82*, 108-133.

Uscanga-Perales, G. I., Santuario-Facio, S. K., & Ortiz-López, R. (2016). Triple negative breast cancer: Deciphering the biology and heterogeneity. *Medicina universitaria, 18*(71), 105-114.

Viale, G. (2015). A bad tumor biomarker is as bad as a bad drug: the gap between genomics data and phenotype to predict response. *The Breast, 24,* S23-S25.

Vici, P., Pizzuti, L., Natoli, C., Gamucci, T., Di Lauro, L., Barba, M., ... & Mariani, L. (2015). Triple positive breast cancer: a distinct subtype?. *Cancer treatment reviews, 41*(2)*,* 69-76.

Vishchuk, O. S., Ermakova, S. P., & Zvyagintseva, T. N. (2011). Sulfated polysaccharides from brown seaweeds *Saccharina japonica* and *Undaria pinnatifida*: isolation, structural characteristics, and antitumor activity. *Carbohydrate research, 346*(17)*,* 2769-2776.

Vo, T. S., & Kim, S. K. (2013). Fucoidans as a natural bioactive ingredient for functional foods. *Journal of Functional Foods, 5*(1)*,* 16-27.

Vogelstein, B., & Kinzler, K. W. (2004). Cancer genes and the pathways they control. *Nature medicine, 10*(8), 789.

Vriend, J., & Reiter, R. J. (2015). Breast cancer cells: modulation by melatonin and the ubiquitin-proteasome system–a review. *Molecular and cellular endocrinology, 417,* 1-9.

Wang, X., Shi, Y., Huang, D., & Guan, X. (2018). Emerging Therapeutic Modalities of PARP inhibitors in Breast Cancer. *Cancer treatment reviews.*

Wijesinghe, W. A. J. P., & Jeon, Y. J. (2012). Biological activities and potential industrial applications of fucose rich sulfated polysaccharides and fucoidans isolated from brown seaweeds: A review. *Carbohydrate Polymers, 88*(1)*,* 13-20.

Wu, L., Sun, J., Su, X., Yu, Q., Yu, Q., & Zhang, P. (2016). A review about the development of fucoidan in antitumor activity: Progress and challenges. *Carbohydrate Polymers, 154,* 96-111.

Xue, M., Ge, Y., Zhang, J., Liu, Y., Wang, Q., Hou, L., & Zheng, Z. (2013). Fucoidan inhibited 4T1 mouse breast cancer cell growth in vivo and in vitro via downregulation of Wnt/β-catenin signaling. *Nutrition and cancer*, *65*(3), 460-468.

Yang, C., Chung, D., & You, S. (2008). Determination of physicochemical properties of sulphated fucans from sporophyll of *Undaria pinnatifida* using light scattering technique. *Food Chemistry, 111*(2)*,* 503-507.

Ying, L., & Hofseth, L. J. (2007). An emerging role for endothelial nitric oxide synthase in chronic inflammation and cancer. *Cancer research*, *67*(4), 1407-1410.

Zhang, Z., Teruya, K., Eto, H., & Shirahata, S. (2011). Fucoidan extract induces apoptosis in MCF-7 cells via a mechanism involving the ROS-dependent JNK activation and mitochondria-mediated pathways. *PloS one, 6*(11), e27441.

Zhang, Z., Teruya, K., Eto, H., & Shirahata, S. (2013). Induction of apoptosis by low-molecular-weight fucoidan through calcium-and caspase-dependent mitochondrial pathways in MDA-MB-231 breast cancer cells. *Bioscience, biotechnology, and biochemistry, 77*(2), 235-242.

Zhang, Z., Teruya, K., Yoshida, T., Eto, H., & Shirahata, S. (2013). Fucoidan extract enhances the anti-cancer activity of chemotherapeutic agents in MDA-MB-231 and MCF-7 breast cancer cells. *Marine drugs*, *11*(1), 81-98.

Appendix

A1: Cell viability of MCF-7 cells after treated with HMF for 48, 72 and 96 hours.

Table S1. Cell viability of MCF-7 cells after treated with HMF for 48, 72 and 96 hours. Data are presented as means ± S.D, n=6.

HMF (µg/ml)	MCF-7 48 hrs	MCF-7 72 hrs	MCF-7 96 hrs
120	95.06±0.03	84.64±0.01	83.56±0.03
100	91.69±0.02	82.52±0.02	85.44±0.03
80	94.02±0.01	84.31±0.01	85.78±0.03
60	93.04±0.01	83.63±0.01	86.08±0.03
40	92.51±0.02	83.27±0.03	87.62±0.03
20	92.50±0.01	83.75±0.01	86.40±0.03
10	90.96±0.01	84.99±0.03	88.34±0.03
5	91.56±0.01	88.27±0.02	89.03±0.03
0	100	100	100

A2: Cell viability of MDA-MB-231 cells after treated with HMF for 48, 72 and 96 hours.

Table S2. Cell viability of MDA-MB-231 cells after treated with HMF for 48, 72 and 96 hours. Data are presented as means ± S.D, n=6.

HMF (µg/ml)	MCF-7 48 hrs	MCF-7 72 hrs	MCF-7 96 hrs
120	77.91±0.01	80.66±0.01	79.38±0.01
100	78.87±0.02	81.14±0.02	78.99±0.01
80	78.58±0.01	81.22±0.01	80.35±0.01
60	79.97±0.01	82.92±0.01	80.65±0.01
40	82.80±0.01	84.28±0.01	82.96±0.01
20	83.16±0.01	86.37±0.01	83.04±0.01
10	84.98±0.01	87.35±0.01	85.06±0.01
5	86.39±0.03	89.42±0.01	88.44±0.03
0	100	100	100

A3: Cell viability of MCF-7 cells after treated with LMF for 48, 72 and 96 hours.

Table S3. Cell viability of MCF-7 cells after treated with LMF for 48, 72 and 96 hours. Data are presented as means ± S.D, n=6.

HMF (µg/ml)	MCF-7 48 hrs	MCF-7 72 hrs	MCF-7 96 hrs
200	55.23±0.01	43.38±0.01	42.42±0.01
100	57.30±0.01	45.81±0.00	44.41±0.01
50	60.30±0.01	46.58±0.01	46.89±0.01
25	62.00±0.01	48.92±0.01	48.37±0.01
10	70.24±0.01	57.84±0.01	55.67±0.01

5	78.43±0.01	63.80±0.01	60.60±0.01
2	82.86±0.02	71.49±0.01	68.21±0.02
1	87.89±0.02	76.82±0.02	71.50±0.01
0.5	91.33±0.01	82.12±0.02	78.10±0.02
0	100	100	100

A4: Cell viability of MDA-MB-231 cells after treated with LMF for 48, 72 and 96 hours.

Table S4. Cell viability of MDA-MB-231 cells after treated with LMF for 48, 72 and 96 hours. Data are presented as means ± S.D, n=6.

HMF (μg/ml)	MCF-7 48 hrs	MCF-7 72 hrs	MCF-7 96 hrs
300	62.01±0.02	60.76±0.02	64.41±0.01
250	63.05±0.01	60.36±0.01	64.48±0.01
200	61.49±0.01	62.06±0.01	66.62±0.01
150	64.03±0.01	62.77±0.01	68.95±0.01
100	65.56±0.01	64.49±0.01	72.37±0.01
50	65.59±0.01	65.29±0.01	73.03±0.01
25	66.69±0.01	66.31±0.02	74.45±0.01
5	68.65±0.01	70.41±0.01	80.85±0.01
1	79.58±0.02	83.97±0.01	93.71±0.02
0	100	100	100

A5: Effects of HMF concentrations (Cn) and incubation time analysed by ANOVA in a LMEM

Table S5. Effects of HMF concentrations and time in the MCF-7 analysed by ANOVA in a LMEM

MCF-7 with HMF	numDF	denDF	F-value	p-value
Time	1	134	43.10	<.0001
Cn	1	6	2.22	0.1868
Time:Cn	1	134	14.89	0.0002

Table S6. Effects of HMF concentrations and time in the MDA-MB-231 analysed by ANOVA in a LMEM

MDA-MB-231 with HMF	numDF	denDF	F-value	p-value
Time	1	134	5.10	0.0255
Cn	1	6	46.13	0.0005
Time:Cn	1	134	0.12	0.7295

A6: Effects of LMF concentrations (Cn) and incubation time analysed by ANOVA in a LMEM

Table S7. Effects of LMF concentrations and time in the MCF-7 analysed by ANOVA in a LMEM

MCF-7 with LMF	numDF	denDF	F-value	p-value
Time	1	151	263.7279	<.0001
Cn	1	7	7.5877	0.0283
Time:Cn	1	151	0.8346	0.3624

Table S8. Effects of LMF concentrations and time in the MDA-MB-231 analysed by ANOVA in a LMEM

MDA-MB-231 with LMF	numDF	denDF	F-value	p-value
Time	1	151	8.2676	0.0046
Cn	1	7	9.7169	0.0169
Time:Cn	1	151	73.1301	<.0001