Anti-Cancerous and Anti-Metastatic Effects of *Corchorus Olitorius* on Breast Cancer Cell Lines

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Abstract

Breast cancer is one of the leading conditions affecting women worldwide, with a high incidence and mortality rate, including Cyprus. Several factors contribute to the development of breast cancer. These include genetic predisposition, reproductive health and modifiable factors such as dietary habits, physical activity, smoking and alcohol consumption.

Given that diet is a significant determinant in the development of breast cancer, the present study focused on *Corchorus olitorius* (commonly known as jute), a locally grown and widely consumed dark green leafy vegetable. The investigation aimed to explore its potential anti-cancer effects on selected breast cancer cell lines: MCF-7, MDA-MB-231 and MDA-MB-468. Acetone, hexane and methanol were used to extract bioactive compounds from *C. olitorius* leaves based on their solubility.

These extracts were subsequently tested to evaluate their potential anti-proliferative, anti-adhesive, anti-migratory and anti-invasive effects *in vitro*. The findings suggest that MDA-MB-468 cells were the most affected in proliferation, adhesion and migration assays, with the hexane and methanol extracts being more potent in possessing anti-cancer effects. These could be attributed to the major compounds identified in GC-MS analysis which include α-linolenic acid and palmitic acid.

Total phenolic and flavonoid content assays in the present study confirmed the presence of polyphenolic compounds in all *C. olitorius* leaf extracts. The DPPH radical scavenging assay has shown that the methanol has the lowest IC_{50} value (51.70 μg/mL), showing its higher antioxidant capacity.

Another objective was to investigate the overall dietary habits of adult women in Northern Cyprus through a survey and to draw potential parallels between the survey findings and the *in vitro* experiments. Even though the survey study was planned as a pilot study with a limited number of participants, certain eating and cooking habits were observable, such as the lower omega 3:omega 6 fatty acid ratio in the intakes, rare use of steaming and the predominant use of olive oil as the staple oil in the region.

The significance of the present study lies in the preliminary evidence it provides regarding the collective anti-cancer effects of *C. olitorius* extracts on breast cancer cell lines. These findings could serve as a foundation for future research.

Articles in Preparation

1. Anti-cancer Effects of *C. olitorius* extracts on Triple Negative Breast Cancer Cell Lines
2. Polyphenolic Content Analysis and Compound Identification of *C. olitorius* Leaf Extracts using TPC, TFC Assays and GC-MS Analysis

Dedications

This study is dedicated to all the women whose lives have been touched by breast cancer - those who are fighting, those who have triumphed, and those we have lost.

Declaration

I hereby declare that during my pursuit of a PhD degree at London Metropolitan University, I have not been registered for any other degree or award at another institution. The work presented in this thesis is my own and has not been submitted elsewhere for any other qualifications. All results and findings are original, except where appropriately cited from other sources.

12.08.2024
Eliz Arter

Abbreviations and Symbols

ACSL4 - Acyl-CoA synthetase long-chain family member 4

AlCl3 - Aluminium chloride

APAF-1 - Apoptotic protease-activating factor-1

ATCC - American type culture collection

BC - Breast cancer

BIA - Bioelectrical impedance analysis

BMI - Body mass index

BRCA - Breast cancer gene

C. olitorius – Corchorus Olitorius

CHO – Carbohydrate

COX - Cyclooxygenases

CO2 – Carbon dioxide

CRP - C - reactive protein

CV - Crystal violet

DCIS - Ductal carcinoma in situ

DHA - Docosahexaenoic acid

DMEM - Dulbecco’s Modified Eagle’s medium

DMSO - Dimethyl sulfoxide

DPPH assay - 11-diphenyl-2-picrylhydrazyl assay

ECM - Extracellular matrix

EGFR - Epidermal growth factor receptor

EMT - Epithelial-mesenchymal transition

EPA - Eicosapentaenoic acid

EPIC - European Prospective Investigation into Cancer and Nutrition

ER - Oestrogen receptor

EV - Extracellular vesicles

FA - Fatty acid

FAK - Focal adhesion kinase

FC - Folin-Ciocalteu’s phenol reagent

FCS – Foetal calf serum

FFQ - Food frequency questionnaire

FFAR4 - Free fatty acid receptor 4

FRSA - Free radical scavenging activity

GA - Gallic acid

GAE - Gallic acid equivalent

GC-MS - Gas chromatography-Mass spectrometry

GF – Growth Factor

HER2 - Human epidermal growth factor 2

HPLC - High pressure liquid chromatography

HepG2 - Human hepatocellular carcinoma cells

HRT - Hormone replacement therapy

HuR - Hu-antigen R protein

IDC - Invasive ductal carcinomas

IDA - Iron deficiency anaemia

IGF-1 - Insulin-like Growth Factor 1

ILC - Invasive lobular carcinomas

IL-6 - Interleukin-6

KEGG - Kyoto Encyclopaedia of Genes and Genomes

LOX - Lipoxygenase

MMP – Matrix Metalloprotease

MS - Mass Spectrometry

MUFA - Monounsaturated fatty acid

MTT Assay - (3-(45- dimethylthiazol-2-yl)-25-diphenyl tetrazolium bromide) assay

NA2CO3 - Sodium carbonate

NaNO2 - Sodium nitrite

NaOH - Sodium hydroxide

NE - No extract

OC - Oral contraceptive

O.D - Optical Density

PARP-1 - Poly(ADP-ribose) polymerase-1

PBS - Phosphate buffered saline

PE - Phycoerythrin

PG - Postgraduate

PR - Progesterone Receptor

PUFA - Polyunsaturated fatty acid

RDA - Recommended dietary allowances

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

RT – Retention time

S.D - Standard deviation

SCFA - Short chain fatty acids

SDS - Sodium dodecyl sulphate

SFA - Saturated fatty acid

TBARS - Thiobarbituric acid reactive substances

TFC - Total flavonoid content

TIMP1 - Tissue inhibitor of metalloproteinase 1

TNBC - Triple negative breast cancer

TNF-α - Tumour necrosis factor-α

TPC - Total phenolic content

TRIM66 - Tripartite motif 66

WHO - World Health Organisation

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Chapter 1 Introduction

1.1 Overview of Breast Cancer

1.1.1 Breast Cancer Statistics

Data collected by the International Agency for Research on Cancer indicate that almost 20 million people were diagnosed with cancer in 2020. Around 10 million deaths were also reported in the same year. Across all ages and both sexes, lung, breast, and colorectal cancers were the three most common types of new cancer cases (Ferlay et al., 2021).

In the same report, breast cancer ranked second in incidence rates, with approximately 2.3 million new cases, and fourth in mortality rates worldwide. In the Republic of Cyprus, in the females and both sexes categories, breast cancer ranked the highest in the new cases across all age groups (Ferlay et al., 2021).

In another report, data from Cyprus Cancer Registry between 2004 and 2017 with follow-up until 2019 suggested that the greatest increase in age-adjusted breast cancer incidence rates was observed in women over 70 years of age. A significant increase in mortality rates was also detected in the same age group. It was concluded that in the older age groups breast cancer incidence rates were still on the rise whereas there was a decrease, although non-significant, in the incidence of advanced stage breast cancers (Quattrocchi et al., 2022).

However, in the northern part of the island, a nation-wide cancer registry does not entirely reflect the actual number of cases as some patients do not apply to state registry. In Northern Cyprus, it is common for patients to apply to private hospitals or choose to receive treatment in Southern Cyprus or in Turkey. This makes it immensely difficult to keep a track of the actual number of cancer patients in Northern Cyprus over time (Gökyiğıt & Demirdamar, 2016).

In an earlier study in which data were collected from North Cyprus Cancer Registry between 1990 and 2004 with 1700 females, breast cancer accounted for 30% of all cancer cases (Hinçal, Taneri, Taneri, & Djamgoz, 2008). A similar result was obtained in another study which involved 1395 females who applied to North Cyprus Cancer Registry between 2007 and 2012. In women, age-adjusted incidence rates were the highest for breast cancer (Pervaiz, Tulay, Faisal, & Serakinci, 2017).

It is apparent that lack of a standardised procedure in Northern Cyprus hinders appropriate data collection and record keeping.

Nevertheless, these findings suggest that both in Southern and Northern Cyprus, breast cancer is the most commonly diagnosed cancer among women.

1.1.2 Aetiology

Data from observational studies suggest that around 90-95% of all chronic diseases including cancer, occur largely due to environmental factors and lifestyle whereas genetic factors account for only 5–10% of all cancers (Anand et al., 2008; Wild, 2014).

A parallel observation is reported for breast cancer. Hereditary factors, such as mutations in breast cancer genes, *BRCA1* and *BRCA2*, account for 5–10% of all breast cancers cases (Fakhri et al., 2022).

Breast and reproductive risk factors also lay the foundation for breast cancer predisposition. These include high circulating hormones, early menarche, first pregnancy at the age of 30 or later, breast size, infertility, nulliparity, no or short breastfeeding, late menopause, oral contraceptives and post-menopausal hormone replacement therapy (HRT). Among other determinants of breast cancer are modifiable risk factors which include physical inactivity, unhealthy dietary habits, overweight and obesity, overconsumption of tobacco and alcohol and exposure to radiation (Houghton & Hankinson, 2021; Howell et al., 2014; Kruk, 2014; Valle, Tramalloni, & Bragazzi, 2015).

Adopting a healthier lifestyle and early detection through cancer screening would provide individuals with long-term benefits and increase the likelihood of cancer prevention and survival rates in patients (Sweeney, 2014).

1.1.2.1 Genetic Predisposition

Although family history increases the risk of developing breast cancer in women by up to 10%, several susceptibility genes have been extensively studied. Most cases of hereditary breast cancers occur as a result of mutations in *BRCA1* and *BRCA2* (Valentini et al., 2024).

These genes code for tumour suppressor proteins which are involved in DNA repair, cell cycle control and cell proliferation. A mutation in one of these genes causes a defect in the encoded proteins (Heisey & Carroll, 2016; Skol, Sasaki, & Onel, 2016; Valentini et al., 2024). It was stated that *BRCA*-associated breast cancer is more aggressive than sporadic cases (Baretta, Mocellin, Goldin, Olopade, & Huo, 2016).

In the general population, lifetime risk of developing breast cancer is 12% (Heisey & Carroll, 2016). However, in earlier meta-analyses, it was shown that the risk of getting breast cancer in *BRCA1*-mutation carriers by age 70 is between 57% and 65% whereas the risk is 45-49% in *BRCA2*-mutation carriers (Antoniou et al., 2003; Sining Chen & Parmigiani, 2007). Ovarian, pancreatic and prostate cancers have also been implicated in *BRCA1* and *BRCA2* mutations (Antoniou et al., 2003; Sining Chen & Parmigiani, 2007; Dhillon, Bajrami, Taniguchi, & Lord, 2016).

One of the other susceptibility genes is *TP53,* which encodes the protein p53 (Skol et al., 2016). P53 is considered to be one of the biomarkers in breast cancer. The encoded protein is a critical transcription factor that acts as a tumour suppressor by binding to specific parts of DNA and regulating the expression of various genes involved in cell cycle, DNA repair, apoptosis as well as angiogenesis and cellular stress response (Flöter, Kaymak, & Schulze, 2017; Sana & Malik, 2015).

It was also suggested that p53 may suppress carcinogenesis through modulation of Reactive Oxygen Species (ROS), temporary cell cycle arrest and cell death programmes (Duffy, Synnott, & Crown, 2017; Flöter et al., 2017).

In most cases mutations occur in the region that codes for the DNA-binding domain of the protein, therefore, causing conformational changes and reducing its binding to the target genes (Duffy et al., 2017; Flöter et al., 2017). It was also reported that BRCA1-positive breast cancers have the highest frequency of *TP53* mutations which demonstrates their strong association (Valentini et al., 2024).

1.1.2.2 Oxidative Stress

Oxidative stress occurs when oxidants, i.e., free radicals like ROS and Reactive nitrogen species (RNS), increase in concentration and exceed the anti-oxidative capacity of the cell which eventually leads to chronic inflammation (Kruk, 2014).

ROS is the major cause of oxidative damage to the structure and function of proteins, lipids and nucleic acids which may lead to tumourigenesis. ROS may arise intrinsically in the mitochondria and immune cells or extrinsically due to radiation, pollution, smoking, alcohol and drugs (Nourazarian, Kangari, & Salmaninejad, 2014).

Growing evidence suggests that increased amounts of ROS are found in a considerable number of cancer cells and that chronic inflammation has a key role in the development of tumourigenesis. Furthermore, many important cellular processes such as metabolism, signalling pathways, cell cycle regulation and cell proliferation are all adversely affected by increased oxidative stress (Kruk, 2014; Nourazarian et al., 2014).

Oxidative stress can occur as a result of obesity in adipocytes triggering a tumourigenic environment and in the breast fat tissue (Rausch, Netzer, Hoegel, & Pramsohler, 2017). It can also occur within a tumour, which is called intratumoural hypoxia, as cells proliferate and increase their oxygen

demand (Gilkes, 2016). It was previously shown that intratumoural hypoxia is associated with a more aggressive and metastasis-prone breast cancer phenotype regardless of the prognostic factors such as the stage and grade of tumour (Z. Liu, Semenza, & Zhang, 2015).

1.1.2.3 Nutrition and Breast Cancer

In a number of clinical and epidemiological studies, the association between diet and development of various cancers, including breast cancer, has been strongly supported (Carruba et al., 2016). Individual dietary habits such as consumption of red meat, processed meat, animal fat and high glycaemic load carbohydrates are considered to be risk factors for breast cancer whereas higher intake of plant-based whole foods, legumes and fruits and vegetables are known to be protective due to their nutrient and non-nutrient phytochemical content (Demetriou et al., 2012; Shapira, 2017; Shin et al., 2023; Jing Wu et al., 2016). Phytochemicals in plant foods have been proposed to exert their anti-cancer effects through various cellular processes and epigenetic modifications (Carruba et al., 2016).

It was also suggested that healthy eating patterns and healthy lifestyle modifications, such as high consumption of fruits and vegetables, moderate physical activity, healthy weight management and avoidance of smoking and alcohol intake, may collectively provide a protective effect against breast cancer in adult women and post-menopausal women (McKenzie et al., 2015; Shin et al., 2023).

In terms of energy intake, it is known that limiting caloric intake is one of the recommendations against cancer. High consumption of energy-dense foods ,i.e., foods with high fat and sugar content, was found to be related to factors, such as increased plasma levels of oestrogen and Insulin-like Growth Factor 1 (IGF-1), which may stimulate cell proliferation (Chandran et al., 2014).

A study demonstrated that overweight/obesity and related dysfunctional metabolic markers, such as fasting blood glucose and plasma triglycerides,

were associated with more aggressive breast cancer types. Specifically, overweight and obesity were found to be significantly associated with triple negative breast cancer (TNBC) in premenopausal women (Agresti et al., 2016).

In order to limit the intake of energy-dense foods, diets rich in water and fibre content, which are characterised by large total weights but lower energy, may be preferred (Jones et al., 2015). This stimulates satiety because of large portion size but lower caloric content. Therefore, diets enriched with fruits and vegetables and those with low fat and refined carbohydrate content, have been suggested as a weight management and breast cancer prevention strategy (Shapira, 2017).

Fibre-dense foods have been demonstrated to be inversely associated with breast cancer. The potential mechanisms of action of fibre in cancer inhibition include combination with and discharge of carcinogens in the gut, promotion of the growth of probiotics which inhibit colonisation of pathogenic bacteria and reduction of circulating oestrogen levels. Furthermore, bacterial fermentation of fibre can promote the synthesis of short chain fatty acids (SCFA). SCFAs inhibit cancer development due to their pro-apoptotic activity. Fibre also prevents a rapid increase in plasma glucose and insulin. This has metabolic benefits which prevent over-eating and obesity but it also reduces the activation of insulin and IGF-dependent signalling pathways thereby inhibiting tumour formation (Sumei Chen et al., 2016; Sonestedt et al., 2008).

In general, dietary guidelines recommend following a healthy eating pattern. This includes consumption of more plant-foods to maximise benefits from nutrients, fibre and a range of phytochemicals, reducing intake of particularly saturated fat, sugars and added salt, managing a health weight and being physically active. A healthy diet pattern supported by healthy lifestyle choices have been shown to reduce the risk of diseases including cancers (*Australian Dietary Guidelines*, 2013; *Dietary Guidelines for Americans*, 2015)

1.1.4 Types of Breast Cancer

Breast carcinomas are heterogeneous and their classification varies depending on certain features (Tsang & Tse, 2020).

Generally, breast malignancies are divided into two on the basis of invasiveness, as either *in situ* or infiltrative. Ductal or lobular carcinoma *in situ* is the name given to non-invasive proliferative epithelial cells confined to the ducts or lobules. Infiltrative or invasive cancers refer to neoplastic cells that have penetrated into stroma which includes the extracellular matrix (ECM) (Cserni, 2020; Makki, 2015).

Broadly, clinical assessment of breast cancer is carried out based on the histological typing and grading of the tumour whose details are given in the World Health Organisation (WHO) tumour classification. Staging of tumours is based on the size of the tumour, nodal status and distant metastasis, which is known as TNM staging (Tsang & Tse, 2020).

Based on the histological classification and pathological growth pattern, the two most common malignancies of breast are invasive ductal carcinomas (IDC) and invasive lobular carcinomas (ILC) (Makki, 2015; McCart Reed, Kalinowski, Simpson, & Lakhani, 2021; Tsang & Tse, 2020; Turashvili, Bouchal, Burkadze, & Kolár, 2005).

ILC are distinguished from IDC based on their absence of E-cadherin which is a critical protein in cell adhesion. Loss of E-cadherin increases the metastatic potential of ILC (McCart Reed et al., 2021; Turashvili et al., 2005). It is also stated that lobular carcinomas grow at a slower rate than ductal carcinomas and tend to be Oestrogen receptor- (ER) and Progesterone receptor (PR)- positive (Korkola et al., 2003).

Ductal carcinoma *in situ* (DCIS) has become a common diagnosis with widespread screening programmes. It is considered to be a possible precursor for the development of IDC and hence women with DCIS are at a higher risk of developing malignant carcinoma in the future than those without DCIS (Makki, 2015).

A widely used histological grading of tumours, which is known as the Nottingham modification of the Scarff- Bloom Richardson grading index, is also used to classify tumours based on how different they are from normal cells. Tumours are then assigned one of the three grades: low grade (Grade I), intermediate grade (Grade II) and high grade (Grade III). Usually the higher the grade; the greater the risk of developing an invasive cancer (Cserni, 2020; Tsang & Tse, 2020).

Another classification is based on hormone receptor availability, which include ER- and PR- receptors, and Human epidermal growth factor 2 (HER2) amplification (Larsen, Thomassen, Gerdes, & Kruse, 2014).

ER-α is a member of a family of ligand-activated transcription factors and is composed of several domains necessary for ligand (oestrogen) binding, DNA binding and initiation of transcription (Dahlman-Wright et al., 2006). It is an important biomarker and as high as 70% of primary breast carcinomas are ERα- positive (Cao & Lu, 2016).

In most cells, ERα and PR are co-expressed hence PR is also considered a target in endocrine therapy. In several sources both can be referred to as 'hormone receptors' (Brisken, 2013; Cao & Lu, 2016). The majority of breast cancers that are ER-positive are also PR-positive (Tsang & Tse, 2020).

PR is similar to ERα in structure with ligand binding, DNA binding and modulating domains. It is also involved in cell-to-cell signalling, activation of transcription and negative regulation of gene expression. PR is expressed in one of the two isoforms: PR-A and PR-B. The two isoforms are expressed at similar levels in a healthy breast but the ratio can change in breast carcinomas in favour of PR-A (Brisken, 2013; Sana & Malik, 2015).

It was previously reported that ER-/PR-positive cancers tend to be of low grade, less aggressive and less metastatic. However, although only a small percentage of breast cancers possess single hormone receptor positivity,

these tend to be more aggressive and less responsive to hormone-dependent therapy (Tsang & Tse, 2020).

HER2 is a gene that encodes a protein in the family of epidermal growth factor receptors (EGFR). There is no ligand binding domain in HER2 and it binds to other EGFRs. After dimerisation, it induces important cell functions such as cell proliferation, differentiation and survival and it prevents apoptosis (Ding, Zhang, Xu, & Zhang, 2017). Expression of *HER2* is amplified in around one fifth of invasive breast cancers (Morsberger et al., 2022). These cancers are generally high grade, hormone receptor negative and with positive lymph node metastasis usually leading to a poor prognosis (J. S. Ross et al., 2009; Tsang & Tse, 2020).

The worst of invasive cancers is TNBC. This category expresses none of the three receptor markers, therefore, named triple negative. TNBC is more aggressive than the other types and is unresponsive to hormone or HER2 treatment leading to a poorer prognosis. TNBC accounts for around 10-15% of invasive cancers. (Larsen et al., 2014; Makki, 2015; Tsang & Tse, 2020).

Ki67 is another molecular marker that is used as a critical diagnostic and prognostic tool in oncology (Gui et al., 2016; Uxa et al., 2021). It is a nuclear protein expressed in proliferating cells but not in resting cells, therefore, representing a key marker in measuring proliferation (Beresford, Wilson, & Makris, 2006). It was previously reported that healthy breast tissue expresses low levels of Ki67 (Yerushalmi, Woods, Ravdin, Hayes, & Gelmon, 2010) and that high expression of Ki67 is significantly associated with poor prognosis (Gui et al., 2016).

It was also stated that the main distinctive factor in determining tumour classification was the presence of ER-positivity (Cao & Lu, 2016). All of the aforementioned markers and receptor availabilities lead to the molecular classification of breast cancer.

Molecular classification comprises of five different sub-types according to their immunohistochemical markers (Boyle, 2012). These are luminal A,

luminal B, HER2-overexpression, basal like or TNBC and normal-like tumours (Boyle, 2012; Tsang & Tse, 2020).

Luminal A cancers, being the most common type, are low grade, ER-/PR-positive, HER2-negative and have the best prognosis compared to the other molecular subtypes (Boyle, 2012; Tsang & Tse, 2020). Following luminal A is luminal B, which is likely to be high grade with a worse prognosis than luminal A. It is ER-/PR-positive, although ER expression is lower, and has varied expression of HER2 (Boyle, 2012; Cao & Lu, 2016; Tsang & Tse, 2020).

The third category is HER2 overexpression. As explained above, this subtype is hormone receptor negative, high grade and usually with lymph node metastasis. However, this subtype is responsive to anti-HER2 therapy which results in improved prognosis (Boyle, 2012; Tsang & Tse, 2020). In previous definitions of this subtype, high ki67 index was also included (Cao & Lu, 2016).

Basal-like or TNBC subtype is high grade with mutations in *TP53* and *BRCA1* (Tsang & Tse, 2020). Normal-like tumours were initially defined but later abolished as their definition was controversial (Boyle, 2012; Tsang & Tse, 2020) leaving four distinct molecular subtypes.

1.1.5 Stages and Metastasis

It is widely accepted that the tumour microenvironment, which is composed of the surrounding vasculature, immune cells and ECM, is an important determinant in the development and spread of tumours (Dumont et al., 2013; A. Huang, Cao, & Tang, 2017).

The stage of tumours have been classified using the TNM staging system developed by the American Joint Committee on Cancer (Amin et al., 2017; Cao & Lu, 2016; Tsang & Tse, 2020). Although there was an update on the staging system to include biomarkers such as hormone receptor positivity and gene expression profiling to obtain a more extensive staging system,

TNM staging is the basis in developing countries where biomarker profiling may not be available (Teichgraeber, Guirguis, & Whitman, 2021).

According to the TNM system, T corresponds to the size of the primary tumour, N represents the absence or presence of cancer in the regional lymph nodes and M shows distant metastases in organs outside the primary tumour area including the regional lymph nodes. In the number staging system, stage 4 is associated with the M category which has generally a low prognosis (Amin et al., 2017; Cao & Lu, 2016).

Metastasis is a complex series of events, known as the metastatic cascade, by which a primary tumour develops into a secondary tumour in a distant site (Thakur, Qiu, Pawar, & Chen, 2024). It is one of the hallmarks of cancer (Hanahan & Weinberg, 2000). It can be divided into five main steps which are local invasion, intravasation, survival in the circulation, extravasation and colonisation (D. X. Nguyen, Bos, & Massagué, 2009).

In the first step, tumour cells detach from the primary tumour site potentially through epithelial-mesenchymal transition (EMT). EMT is characterised by reduced cell-to-cell and cell-to-matrix adhesion and resistance to apoptosis of normal cells that are separated from the ECM. These cancer cells invade the neighbouring tissue locally, enter the blood and lymphatic circulation (known as intravasation) and survive in the circulation, then they exit the circulation (known as extravasation) and colonise a distant site (Melzer, Von Der Ohe, & Hass, 2017; Thakur et al., 2024).

The ‘seed and soil’ hypothesis, which was proposed by Paget in 1889 for metastasis, suggests that disseminated tumour cells (seeds) land into a distant ‘soil’ where they can proliferate and form secondary tumours. It was also suggested that a receptive microenvironment is essential for disseminated cells to adhere and colonise a secondary site (Psaila & Lyden, 2009).

1.2 Potential Therapeutic Uses of *Corchorus Olitorius*

1.2.1 Structure and Medicinal Properties

Corchorus olitorius (Linn.) (*C. olitorius*) belongs to the family Tiliaceae and is commonly known as 'jute mallow', 'tossa jute' or 'bush okra' in English, 'moroheiya' in Japan, 'molehiya' in Cyprus, 'saluyot' in the Philippines and 'ewedu' in Nigeria (Figure 1.2.1.1, photographs taken by the author) (İlhan, Savaroglu, & Colak, 2007; Ujah, Ipav, Ayaebene, & Ujah, 2014). *C. olitorius* is a dark green leafy vegetable native to tropical Africa and Asia and is commonly found in countries including the Philippines, Malaysia, India, Sudan, Japan, South America, the Caribbean and Cyprus (İlhan et al., 2007; Iseri, Yurtcu, Sahin, & Haberal, 2013).

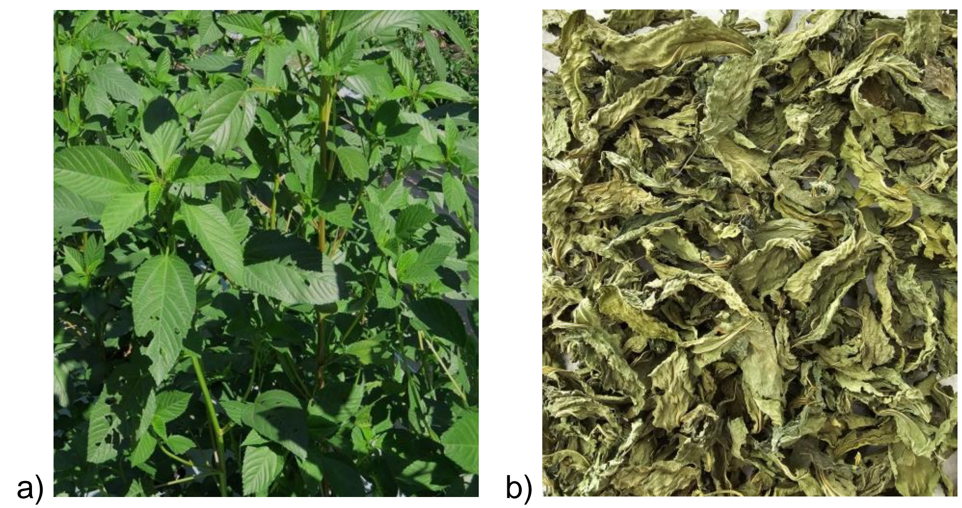


Figure 1.2.1.1: C. olitorius Plant

a) *C.olitorius* in its natural state and b) dried leaves of *C. olitorius*

It has been reported that C. *olitorius* is a good source of vitamins including carotenoids, thiamine (vitamin B1), riboflavin (vitamin B2), nicotinamide (vitamin B3), ascorbic acid (vitamin C), vitamin E and minerals including calcium and iron. It also contains fibre and other bioactive plant compounds (İlhan et al., 2007; Matsufuji et al., 2001; Ujah et al., 2014). It is considered to be the main source of dietary protein in a number of tropical countries because of its high levels of essential amino acids apart from methionine (İlhan et al., 2007).

C. *olitorius* has been used in traditional medicine for the treatment of a wide range of conditions including fever, pain, enteritis, chronic cystitis, dysentery (Iseri et al., 2013; Oboh, Raddatz, & Henle, 2009), gonorrhoea, anaemia and tumours (Ujah et al., 2014). So far the seeds, stems and leaves of C. *olitorius* have been implicated in cardioprotective, antihistaminic, antioxidant, antibacterial, antifungal, anticonvulsant, anti-inflammatory and haematological activities (İlhan et al., 2007; Iseri et al., 2013; Ragasa, Vivar, Tan, & Shen, 2016).

The seeds of C. *olitorius* are particularly rich in cardiac glycosides which are extracted from the plant and used as steroidal pharmaceuticals for heart failure (Matsufuji et al., 2001; Ragasa et al., 2016). Furthermore, terpenes and some phenolic compounds, such as coumarin, have been identified mainly in the seeds. The leaves were found to contain ionones, phenolics, including phenolic acids like chlorogenic acid; flavonoids, mainly quercetin derivatives; tannins; steroids, alkaloids, saponins and terpenoid compounds (Mibei, Ojijo, Karanja, & Kinyua, 2012; Oboh et al., 2009; Ragasa et al., 2016; Ujah et al., 2014).

Six main antioxidant phenolics have been identified in the leaves of *C.olitorius* in a previous study. These were 5-caffeoylquinic acid, also known as chlorogenic acid, 3,5-dicaffeoylquinic acid, quercetin glycosides; quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-(6-malonylglucoside) and quercetin 3-(6-malonylgalactoside), which is the predominant glycoside (Azuma et al., 1999).

The principal phenolic compound with antioxidant properties was chlorogenic acid with a content of 384 mg/100 g of fresh weight of leaves. The total content of quercetin glycosides was found to be 233 mg/100 g fresh weight. In addition to the phenolic compounds in *C. olitorius* leaves, high amounts of ascorbic acid, as high as 258 mg/100 g fresh weight, have been identified whereas vitamin E content, in the form of α-tocopherol, was 14 mg/100 g fresh weight (Azuma et al., 1999).

In another study, the aqueous methanol extract of *C. olitorius* leaves has been shown to possess ~32.6 mg of ascorbic acid per 100 g dry weight (Oboh et al., 2009). Carotenoids as antioxidant vitamins, and other flavonoids, such as isoquercetin, have also been identified in the leaves (Handoussa et al., 2017; Ragasa et al., 2016).

The total polar and non-polar phenol contents were previously shown to be higher than the total ascorbic acid and carotenoid contents, showing that phenols are the predominant antioxidant phytochemicals in *C. olitorius*. In the same study, the aqueous extract was found to have a significantly higher free radical scavenging activity (FRSA) than the hexane extract probably due its higher phenol content (Oboh et al., 2009).

Phenolic compounds have a basic structure of an aromatic ring with one or more hydroxyl groups attached. Flavonoids are one of the major subgroups of polyphenols which have a three-ringed structure. Among the well-established phenolic acids and flavonoids in *C. olitorius* leaves are chlorogenic acid, which is a hydroxycinnamic acid in the phenolic acid subgroup, and quercetin, which is a flavonol in the flavonoid subcategory. Chemical structures of the major flavonoids and phenolic acids are given in Figure 1.2.1.2 (De Araújo et al., 2014; Kopustinskiene, Jakstas, Savickas, & Bernatoniene, 2020; Kundur et al., 2018; Marchiosi et al., 2020; Plazas et al., 2013).

a) b) c) d)

Figure 1.2.1.2: Basic Structure of Major Phenolic Acids and Flavonoids

Chemical structures of a) major flavonoids which include flavonols as a subcategory b) hydroxycinnamic acids as a subgroup of phenolic acids c) chlorogenic acid d) quercetin

1.2.2 Importance of *C. olitorius* in Cancer Research

It has long been known that fruits and vegetables are rich in antioxidants and that higher intakes increase the antioxidant activity in the plasma reducing the risk of some cancers (Katerere, Graziani, Thembo, Nyazema,

& Ritieni, 2012). In a number of studies, *C. olitorius* leaves were found to show antioxidant properties due to their micronutrient and phytochemical content (Morrison & Twumasi, 2010; Morsy, Rayan, & Youssef, 2015; Ragasa et al., 2016). They exhibited total phenol content, total antioxidant capacity, reducing and free radical scavenging activity (Morrison & Twumasi, 2010). The antioxidant activity and FRSA in polar methanol extracts of the leaves have been shown through the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay which was first described by Blois, 1958 and the phenol content was found to be correlated with the scavenging activity of DPPH. Furthermore, phenol content and FRSA were found to be higher in the leaf extract than the seed extract of *C. olitorius* (Iseri et al., 2013; Katerere et al., 2012).

In another study, anti-inflammatory effects of an ethanolic extract of the aerial parts of *C. olitorius*, such as the leaves and stems, were investigated *in vitro* and *in vivo* in rats. Quercetin was reported to be the most predominant constituent of the ethanol extract. A decrease in inflammatory markers, an increase in superoxide dismutase activity and nitric oxide production, restored glutathione levels and potential cytotoxic activity against metastatic melanoma, leukaemia and osteosarcoma cell lines were among the findings in this study. It was suggested that through these mechanisms, phenolics in the ethanol extract of *C. olitorius* exerted their anti-inflammatory effects (Handoussa et al., 2013).

Oleanolic acid, which is a terpene present in *C. olitorius* leaves, was shown to reduce cell viability, increase oxidative stress and stimulate apoptosis in a dose-dependent manner in HCT116 human colon carcinoma cells over 72 hours. It also inhibited tumour growth in HCT116 tumour xenograft mice, supporting the *in vitro* findings of the study (Potočnjak, Šimić, Vukelić, Batičić, & Domitrović, 2022).

Anti-tumour effects of the ethanol extract *C. olitorius* leaves have been investigated on human hepatocellular carcinoma cells (HepG2). The anti-cancer effects were observed at low concentrations, higher than 12.5μg/ml, which were non-toxic to normal hepatic cells within a 24-hour period. The

effects took place in a dose-dependent manner. Nuclear condensation and DNA aggregation as hallmarks of apoptosis were also among the observations of this study. Furthermore, one of the apoptotic proteins, caspase-3, was found to be activated by the ethanol extract *C. olitorius* leaves which mediated caspase-3-dependent apoptotic pathway. Stimulation of this pathway is suggested to be potentially due to the presence of two active compounds; phytol, a diterpene alcohol, and monogalactosyl-diacylglycerol, a lipid, in *C. olitorius* leaves (Li et al., 2012). A similar finding was also documented in a previous study (Furumoto et al., 2002).

A number of studies investigated the synergistic effects of several polyphenols. Healthy eating guidelines across different nations recommend eating a variety of fruits and vegetables, however, many studies concentrate on the efficacy of single compound treatments. Considering that humans consume various foods, these potentially undermine the interactions between different phytochemicals and their synergistic benefits (Ackland, Waarsenburg, & Jones, 2005).

Quercetin, which is a major constituent of secondary plant metabolites, is among the most studied polyphenols. In a study, the combined effects of quercetin and kaempferol on cell proliferation was evaluated using HCT-166 cells. It was demonstrated that the combination of quercetin and kaempferol resulted in a much larger reduction in cell viability than their single treatments in a time- and dose-dependent manner (Jaramillo-Carmona et al., 2014).

Similar effects were observed in a study in which human small intestine (HuTu-80), colon (Caco-2) and metastatic breast cancer cells (PMC42) were used. Combined treatments of quercetin and kaempferol were more effective on suppressing cell proliferation than either of them applied alone. A reduction in the expression of Ki67, was shown to cause cell proliferation inhibition in the same study (Ackland et al., 2005).

Prolonged inflammation is among the underlying causes of cancer and it is crucial to inhibit the production of pro-inflammatory cytokines in the long term. In a study, anti-inflammatory effects of quercetin and catechin were assessed alone and in combination on inflammation-induced macrophage cells, which were isolated from a mouse tumour. In the study, it was shown that a dual treatment resulted in a synergistic, dose-dependent anti-inflammatory response, reducing the expression of pro-inflammatory cytokines without disrupting the normal cell functioning (Li et al., 2019).

Such studies provide *in vitro* evidence that supports the general recommendations about consuming a range of fruits and vegetables of different colours to obtain the most optimal combinations of phytochemicals and gain protection against cancers (Ackland et al., 2005).

1.3 Research Objectives

Literature review suggests that edible plants like *C. olitorius* have long been investigated in cancer therapy due to their phytochemical content and potential anti-tumour effects. The main goal of this study is to investigate the anti-cancer and anti-metastatic effects of *C. olitorius* leaf extracts on various breast cancer cell lines, including non-metastatic, hormone-dependent MCF-7 cells and two metastatic cell lines, MDA-MB-231 and MDA-MB-468 cells.

As mentioned earlier, previous studies have investigated *C. olitorius* leaves more extensively compared with other plant structures due to their high concentration of bioactive compounds. Additionally, the leaves - rather than the stems or flowers - are the part of the plant commonly consumed in local dishes. Therefore, this study focused on evaluating the anti-cancer effects of the leaf extracts.

Acetone, hexane and methanol were used as solvents to extract bioactive plant compounds of varying polarities. The potential synergistic effects and possible mechanisms of action of these phytochemicals as a whole extract,

rather than single compounds, were to be assessed on several cancer-specific behaviours.

The study was intended to examine the proliferation and adhesion of MCF-7, MDA-MB-231 and MDA-MB-468 cells when exposed to acetone, hexane, and methanol extracts of *C. olitorius* leaves.

Additionally, cell migration and invasion, as characteristics of metastatic cells, were assessed using the extracts on MDA-MB-231 and MDA-MB-468 cells. Alongside the invasion assay, levels of CD24 protein were analysed to determine if the extracts would affect its expression.

Another aim of the present study is to examine the eating habits of adult women in Northern Cyprus in a pilot study. An extensive survey was designed to collect information about the demographics, eating and cooking habits and overall breast and reproductive health which may be relevant to breast cancer. The main objective of the survey was to investigate any potential association between the epidemiological data and *in vitro* findings.

Lastly, the present study is intended to provide useful information about the potential synergistic and anti-cancer effects of *C. olitorius* extracts on various breast cancer cells, a topic which needs further research.

Chapter 2 Methodology

2.1 Materials

2.1.1 Plant Material

C. olitorius was the plant material used in the study. Two batches from the 2017 and 2024 harvests were collected from a local grower in Cyprus who used no pesticides. The leaves were separated from the seeds and the stem, and then air-dried at room temperature for a few days without any direct sun exposure.

2.1.2 Chemicals, materials and reagents

Pure methanol, acetone and n-hexane used for extraction were obtained from Fisher Scientific (Loughborough, UK). Narrow neck glass bottles (10-mL) and 75 cm^2 treated, vented cap, sterile tissue culture flasks, 24-well plates and disposable polystyrene weighing papers were also purchased from Fisher Scientific (Loughborough, UK). Cell culture media and supplements including Dulbecco's Modified Eagle's medium (DMEM), L-glutamine (2 mM), foetal calf serum (FCS) and Dulbecco's phosphate buffered saline (PBS) were purchased from Merck. (Dorset, UK). Penicillin (50 U/mL) – Streptomycin (0.05 mg/mL) solution mix used in cell culture and sterile distilled water were obtained from Fisher Scientific (Loughborough, UK). Cell lines used were obtained from American Type Culture Collection, USA. Trypsin-EDTA (0.25%), Dimethyl sulfoxide (DMSO), Crystal violet (CV), Sodium dodecyl sulphate (SDS), Eppendorf tubes, Whatman filter paper and syringes were purchased from Merck (Dorset, UK). Collagen I, rat tail used in migration and invasion assays was obtained from Merck (Dorset, UK). Falcon tubes (50 mL) were purchased from Corning Inc. (Flintshire, UK). Syringe filters were obtained from Silicycle (Quebec, Canada). Disposable hemacytometers were purchased from Cambridge Biosciences (Cambridge, UK). The reagents used in total phenolics and flavonoids assay including Sodium carbonate (Na_2CO_3),

Folin-Ciocalteu's phenol reagent (FC), Gallic Acid (GA), Sodium nitrite ($NaNO_2$), Aluminium chloride ($AlCl_3$), Sodium hydroxide (NaOH) and quercetin were all purchased from Merck (Dorset, UK). Phycoerythrin (PE) Anti-CD24 antibody [SN3] was obtained from Abcam (Cambridge, UK). 1-diphenyl-2-picrylhydrazyl (DPPH) was obtained from Merck & Co. (Kenilworth, USA). Ascorbic acid was purchased from Tekkim Kimya Sanayi (Bursa, Turkey). ELISA kits (Human Caspase 3 Elisa Kit, cat no: E4804Hu, Human Caspase 7 Elisa Kit, cat no: E2257Hu, Human matrix metalloproteinase 9; Gelatinase B Elisa Kit, cat no: E0936Hu) were obtained from BT Lab, China.

2.2 Preparation and Extraction of *C. olitorius*

2.2.1 Solvent Extraction of *C. olitorius*

C. olitorius leaf extracts were prepared as described previously (Assanga et al., 2015). Briefly, 600 g of dried *C. olitorius* leaves were ground using a coffee grinder and three flasks, each containing 200 g of dried leaves and 250 mL of each solvent, were set up. These solvents were methanol, acetone (>98%) and n-hexane in order to extract bioactive plant constituents that are polar, moderately polar and non-polar phytochemicals, respectively. Flasks were covered in aluminium foil to protect against light and placed in a shaker for 5 days at 30°C to encourage phytochemical extraction. Solvents were reconstituted to 250 mL if needed.

The extracts were then filtered to remove the residues by using Whatman No. 1 filter paper. The filtered extracts were separated from the solvent using a rotavapor (Büchi Rotavapor R-200, UK) at 40^{0}C, with rotation speed at 55 rpm and vacuum pressure at 900 mbar. The pressure was decreased gradually to around 200 mbar to evaporate the solvent and concentrate the extracts. The vacuum pressure for evaporation of hexane is known to be 264 mbar, for acetone 370 mbar and for methanol 218 mbar at 30^{0}C. Once the evaporation was complete, extracts were stored at -20^{0}C until next use.

All three solvents used in the extraction process (acetone, hexane and methanol) were evaporated at the end of the procedure. Samples extracted using each of the solvents will be referred to by their solvent names for ease of understanding in the following chapters. In other words, even though the solvents have been evaporated, the solvent names will still be used to easily distinguish and discuss the extracted samples.

2.2.2 Sonication of the Extracts

Further extraction was carried out using a sonicator (Crest Ultrasonics, USA), as described earlier (Y. Yang & Zhang, 2008), to break up any clumps of the plant material left after initial solvent extraction and enable phytochemicals to dissolve more effectively. First, flasks were taken out from -20°C and put on ice to prevent thawing. The powder from each flask was scraped off carefully with a toothbrush in to 10 mL narrow neck glass bottles in the fume cupboard after which their weights were measured. The extracts were resuspended in 10% DMSO (2 mL DMSO + 18 mL sterile distilled water). The sonicator was half-filled with water and each bottle was partially immersed in the water bath for 1 min and then put back on ice to control for the temperature. This was repeated for 10 minutes per extract and it was repeated for all three extracts alternately. Extracts were then stored at -20°C until next use. Final weight of each extract was calculated.

2.2.3 Filtering and Sterilisation of Extracts

Extracts were thawed and diluted in 10% DMSO (5 mL DMSO and 45 mL distilled water). 40 mL of 10% DMSO was prepared in falcon tubes. The dilution ratio of the hexane and methanol extracts was 1:5 such that 10 mL of each extract was diluted in 40 mL of 10% DMSO. The acetone extract was more concentrated and resulted in a slimy thick liquid. Therefore, it was diluted 1:10 with 10 mL of extract and 90 mL 10% DMSO. Leaf extracts were taken up with the help of a syringe and filtered through a sterile syringe filter into sterile falcon tubes. Each extract was then aliquoted into 1mL autoclaved Eppendorf tubes and stored at -80°C. All parts of this procedure was carried out in a tissue cabinet.

Stock concentrations of the extracts were determined by first calculating the initial extract concentrations in 10% DMSO, as given in Table 3.2.2, and entering them into the formula: $C_1V_1=C_2V_2$ where C is the concentration of a solution and V is the volume. Results are given in Chapter 3.

2.2.4 Total Phenolics Assay

Total phenolic content of *C. olitorius* was measured using the Folin-Ciocalteu method as described previously (Chun, Kim, & Lee, 2003) with some modifications. GA was used as the standard to measure the equivalent phenolics. The total final volume in the original assay was made up to 25 mL however, in this study, 12.5 mL was used to save the reagents. All reagents were prepared before carrying out the assay.

Na_2CO_3 (7.5%) was prepared by dissolving 6 g of Na_2CO_3 in 80 mL of distilled water. The working stock concentration of GA was determined to be 5000 µg/mL which was prepared by dissolving 50 mg of GA in 1 mL methanol and 9 mL of distilled water. GA working standards were then prepared from the stock concentration to set up a calibration curve. Diluted GA concentrations were 50 µg/mL, 100 µg/mL, 200 µg/mL, 250 µg/mL, 400 µg/mL and 500 µg/mL. All reagents containing GA were covered in foil since it is light sensitive. Extracts were subsequently prepared at the following concentrations:

Table 2.2.4: Concentration of Extracts used in Total Phenolics Assay

Name of Extract	Concentration of Extract used in the assay (mg/mL)
Acetone – Dilution 1:10	15.0
Acetone – Dilution 1:20	7.5
Acetone – Dilution 1:100	1.5
Hexane – Dilution 1:10	4.0
Hexane – Dilution 1:20	2.0
Hexane – Dilution 1:100	0.4
Methanol – Dilution 1:10	36.0
Methanol – Dilution 1:20	18.0
Methanol – Dilution 1:100	3.6

A total of 16 tubes were prepared for the blank, GA standards and extracts and 4.5 mL of distilled water was added to each tube initially. Into each tube, 0.5 mL of GA standards and extracts were added and after, 0.5 ml of FC phenol reagent was added and mixed. After 5 minutes, 5 mL of 7.5% Na_2CO_3 was added with thorough mixing. Each solution was made up to 12.5 mL with distilled water, mixed and allowed to stand for 90 minutes. The blank contained 0.5 mL distilled water as the sample and the rest of the reagents were the same as the experimental tubes. Absorbance measurements were taken at 750 nm using a VWR UV-VIS Spectrophotometer UV-1600pc (Leicestershire, UK) against the blank. Each sample was analysed in triplicate and the mean value was taken.

Using the absorbance readings of GA standards, a calibration curve was plotted and a linear regression equation was obtained in Microsoft Excel. This equation is:

$$\mathbf{y = mx + c}$$

Where y= absorbance of extract,

m= slope of the calibration curve,

x= concentration of extract and

c= intercept.

Next, concentrations of the extract samples were determined using their absorbance readings and the equation. Extract concentrations were entered into the formula below to calculate Total Phenolic Content (TPC):

$$\mathbf{TPC = cV/m}$$

Where c= concentration from calibration curve

V= volume of the extract used

m= mass of the extract used

TPC of C. olitorius was expressed as mg gallic acid equivalent (GAE)/gram of dry matter of the sample (Bhandari & Rajbhandari, 2014).

2.2.5 Total Flavonoids Assay

Total flavonoids content of *C. olitorius* was determined using the aluminium chloride colorimetric assay as described earlier (Zhishen, Mengcheng, & Jianming, 1999) with some modifications. Quercetin was used as the standard to measure the equivalent flavonoids in this assay. Same as the total phenolics assay, reagent volumes were halved.

Prior to the assay, 5 mL of 5% $NaNO_2$ and 10% $AlCl_3$ and 20 mL of 1M NaOH were prepared. The working stock concentration of quercetin was determined to be 1 mg/mL such that 50 mg of quercetin was dissolved in 50 mL methanol as it is not readily soluble in water. Five working standards were prepared which were 10 µg/mL, 20 µg/mL, 40 µg/mL, 80 µg/mL and 100 µg/mL.

Standard solutions, extracts and the blank were set up. The blank consisted of all the reagents and methanol as the sample. Concentration of the extracts were the same as given in the Total Phenolics Assay (Table 2.2.4). Initially, 2 mL distilled water and 0.5 mL of the samples were added to each tube. At 5 minutes, 0.15 mL of 5% $NaNO_2$ and 0.15 mL of 10% $AlCl_3$ were added. At 6 minutes, 1 mL of 1M NaOH was added and the total volume was made up to 5 mL by topping up with distilled water. All tubes were thoroughly mixed and absorbance measurements were taken in triplicate at 510 nm using a UV-VIS Spectrophotometer.

A calibration curve was plotted with the absorbance readings of quercetin standards and a linear regression equation was obtained in Microsoft Excel. The equation is:

$$\mathbf{y = mx + c}$$

Where y= absorbance of extract,

m= slope of the calibration curve,

x= concentration of extract and

c= intercept.

Concentrations of the extract samples were then determined using their absorbance readings and the equation. Extract concentrations were entered in the formula below to calculate Total Flavonoid Content (TFC):

TFC = cV/m

Where c= concentration from calibration curve

V= volume of the extract used

m= mass of the extract used

TFC of *C. olitorius* was expressed as mg quercetin equivalent (QE)/ g dry matter of the sample (Bhandari & Rajbhandari, 2014).

2.2.6 DPPH Assay

The antioxidant activity of the extracts was measured using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity assay with slight modifications (Kavaz & Faraj, 2023; Khalaf, Shakya, Ashok, Al-Othman, El-Agbar, & Husni, 2008) at Cyprus International University. A stock solution of 10 mg/mL of each extract was prepared in 1% (v/v) DMSO/methanol. The diluted working concentrations (25, 50, 100, 200 and 500 µg/mL) of the extracts were prepared in methanol.

The DPPH solution was freshly prepared in methanol at a concentration of 0.1 mM and it was covered with aluminium foil to protect against light. Ascorbic acid was used as a positive control, with a stock concentration of 1000 µg/mL and serial dilutions of 5, 10, 25, 50 and 100 µg/mL in methanol. A 1 mL volume of each diluted ascorbic acid solution was mixed with 1 mL of DPPH. Methanol with DPPH was used as a negative control. A sample-only control was also prepared using diluted extracts in methanol to measure the background absorbance of the plant extracts without DPPH. For both the negative and sample-only control solutions, a volume of 1 mL was mixed with 1 mL of the DPPH solution.

Similar to the controls, 1 mL of each diluted extract solution was mixed with 1 mL of the DPPH solution. All solutions were incubated in the dark at room

temperature for 30 min before their absorbance was measured at 517 nm using UV-visible spectrophotometer (Shimadzu UV-2600, Kyoto, Japan). A blank consisting of only methanol was used.

Three repeats were taken for each measurement. In order to calculate percent inhibition of the DPPH activity, a corrected absorbance measurement was first calculated using the following equation:

$$\text{Absorbance}_{\text{corrected sample}} = \text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{sample-only control}}$$

Percent inhibition was then calculated using the corrected absorbance reading for each sample with the formula:

$$\text{Percent inhibition (\%)} = [(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{corrected sample}}) / \text{Absorbance}_{\text{control}}] * 100$$

IC_{50} concentration of each extract was calculated on GraphPad Prism which represents the concentration of a sample required to scavenge 50% of the DPPH free radical (Phuyal, Jha, Raturi, & Rajbhandary, 2020). Lower IC_{50} values suggest stronger antioxidant activity showing that a lower amount of the extract is needed to scavenge 50% of DPPH radicals (Biswas et al., 2020). Results are given in Chapter 3.

2.2.7 Gas chromatography-Mass spectrometry (GC-MS) Analysis

Gas chromatography-Mass spectrometry (GC–MS) analysis was carried out at Hacettepe University to determine the chemical composition of *C. olitorius* leaf extracts as described earlier with slight modifications (Kavaz & Faraj, 2023).

The analysis was performed using an AGILENT system (Model: 7890B GC/ 5977A, Series MSD Systems, USA) equipped with a fused-silica HP-5-MS capillary column (30 m in length × 250 µm in width × 0.25 µm in thickness of film). The oven temperature was initially set to 40°C and held for 1 minute, then increased at a rate of 70 °C/min up to 280°C, where it was held for 7 minutes. The inlet temperature was 250°C. Helium was used as the carrier gas at a flow rate of 1 mL/min. The split ratio varied between

10:1 and 500:1 depending on the concentration of the test material, with an injection volume of 1 µL. Mass spectrometric detection was performed in electron ionisation mode at 70 eV. The total run time of the analysis was approximately 42–43 min.

The resulting mass spectra were compared against entries in the GC-MS spectral databases (MassHunter software, USA) for molecular identification of the components present in the extracts.

2.3 Effects of *C. olitorius* extracts on Breast Cancer Cell Lines

2.3.1 Cell Culture Preparation

Cell media used in the experiments was sterile, filtered DMEM with 1000 mg/L glucose and sodium bicarbonate, without L-glutamine. The medium was supplemented with FCS, L-glutamine and pen-strep to provide a sterile, nutrient-dense environment for the cultured cells. Final concentration of the supplements in 500 mL media was 1% L-glutamine, 1% pen-strep and 10% FCS.

Primary breast cancer cell lines obtained from ATCC were stored in liquid nitrogen with 10% DMSO with a low passage number. Cell lines used in this study were MCF-7, MDA-MB-231 and MDA-MB-468 cells. Cell cultures were prepared in 75cm^2 treated, vented cap, sterile tissue culture flasks. For each cell line, 12 mL of cells with media were added to three flasks. Flasks were labelled with the name of the cell line, passage number, initials of the researcher and date. Cultures were kept 37^{0}C 5% CO_2 incubator for 5 days. All steps of media preparation and cell culturing were carried out in a sterilised tissue cabinet. Cell splitting was carried out such that the passage number was kept below 20 in order to prevent any genetic variations from the original strain of cells.

2.3.2 Cell Counting and Splitting

Cell counting and cell splitting protocols were applied as described in the book of Fundamental Techniques in Cell Culture by Sigma-Aldrich and Cell Culture Guidelines published by Abcam (Abcam Protocols, 2018; Sigma-Aldrich, 2016). Cells were removed from the incubator and the culture media was decanted. The flasks were then washed with 2 mL of pre-prepared trypsin and PBS solution, gently shaken and subsequently aspirated. Cells were trypsinised with another 2 mL and put in 37^0C 5% CO_2 incubator for 10 minutes. In order to deactivate trypsin and ensure cell viability, 2 mL of culture media was added after 10 minutes. For non-detached cells, incubation was prolonged for another 10 minutes. The contents of the flask were transferred to a falcon tube using a Gilson pipette and cells were gently mixed ~10 times to get rid of any clumps. The tube was inserted in ice. 10μl of cells were put on the haemocytometer to carry out cell counting. This procedure was repeated for each cell line.

2.3.3 Preparations for the Proliferation Assay

2.3.3.1 Determination of Cell Density for the Proliferation Assay

In order to study the anti-proliferative effects of different *C. olitorius* extracts on cells, the right concentration of cells was determined per cell line. Four dilutions were set up to work out the ideal working cell concentration for each cell line which were:

- 1 x 10^5 cells/mL (1st dilution)
- 0.5 x 10^5 cells/mL (2nd dilution)
- 0.25 x 10^5 cells/mL (3rd dilution)
- 0.125 x 10^5 cells/mL (4th dilution)

These dilutions were prepared in a total of 5 mL. Cell concentrations and absorbance readings for each dilution are given in the tables in Appendix 2.3.3.1.

After preparing a series of 5 mL dilutions for each cell line, 1 mL of each dilution was added to a 24-well plate such that 1 mL of the first dilution was dispensed into all four wells of the first column, 1 mL of the second dilution into the wells of the second column and so forth across the plate. Columns 5 and 6 contained only media, without any cells, as blank. For MCF-7 cells, 3rd dilution was chosen to be used for further experiments since in the first two dilutions maximum cell growth was achieved. For MDA-MB-231 and MDA-MB-468 cells, 2nd dilution was more appropriate.

2.3.3.2 Crystal Violet Staining Assay

In the fume hood, 0.2 g of CV, stored in the cold room, was added to 2 mL methanol and 98 mL distilled water. The mixture was filtered through Whatman filter paper and stored in the cold room in a glass bottle covered in aluminium foil to prevent light exposure. For the preparation of 1% SDS, 1 g of SDS powder was weighed out in a weighing paper, poured into a clean bottle and made up to 100 mL, using approximately 98 mL of distilled water, mixed and stored at room temperature.

Cell plates were taken out of the incubator. Media was removed from all wells carefully. Previously warmed PBS (500 µL) was added to each of the side of the wells carefully to remove any dead cells and impurities which was aspirated later. CV solution (200 µL) was added to each of the wells and left on the bench for 10 minutes. Plates were carefully washed twice in tap water by immersion in a large tray. They were then dried upside down on paper towels in the tissue cabinet for 10 minutes. In order to solubilise the cells and stain, 600 µL of 1% SDS solution was added to all wells. Plates were put on a shaker for ~10 minutes to obtain a uniform colour before taking absorbance readings at 570nm.

2.3.3.3 Proliferation Assay

In order to evaluate the anti-proliferative effects of different *C. olitorius* extract solutions, CV assay was used to stain the adherent cells. Cell proliferation assay using CV staining method was similar to the one described by Feoktistova, Geserick, and Leverkus, 2016. This assay

assumes that dead cells detach from the surface in the washing steps and the absorbance reading obtained is directly proportional to the adherent, active and proliferating cells (PromoKine, 2017). In total, 36 plates were prepared to set up 8 dilutions per extract (3 cell lines x 3 extracts x 4 plates for 8 dilutions). Illustration of the plate is given in Appendix 2.3.3.3.Cells were seeded on to the plates at preferred dilutions. For MCF-7 cells, the working cell concentration was 0.25 x 10^5 cells/mL (116 µL per well) and for MDA-MB-231 and MDA-MB-468 cells, this was 0.5 x 10^5cells/mL which were 223 µL and 202 µL, respectively. A negative control was used containing only cells in 10% DMSO/media without any extract.

All plates were then placed at 37^0C 5% CO_2 incubator for 24 hours. Next day, 100 µL of media was aspirated and 100 µL of diluted extracts were added to the wells with cells. Extract dilution (D) preparations were as follows:

- D1= 1 mL extract + 4 mL 10% DMSO/media
- D2= 1 mL D1 + 2 mL 10% DMSO/media
- D3= 1 mL D2 + 2 mL 10% DMSO/media
- D4= 1 mL D3 + 2 mL 10% DMSO/media
- D5= 1 mL D4 + 2 mL 10% DMSO/media
- D6= 1 mL D5 + 2 mL 10% DMSO/media
- D7= 1 mL D6 + 2 mL 10% DMSO/media
- D8= 1 mL D7 + 2 mL 10% DMSO/media

Concentrations of each extract are given in Table 2.3.3.3 in the Appendix. All plates were incubated at 37^0C 5% CO_2 for 3 days. CV staining assay was performed and absorbance measurements were taken at 570 nm.

These readings were entered into Excel and normalised relative to the control to restrict the data to a certain range and even out differences between different readings. Data normalisation was performed using the mean of control, and data are represented as a percent of the control mean. Statistical analysis was carried out based on the average of the absorbance of the extract-treated replicates per assay and the control

mean. GraphPad Prism 10 (USA) was used to conduct one-way ANOVA test and compare the means of each concentrations relative to the control. All data are presented as a percent of control mean ± S.D of eight wells.

2.3.4 Adhesion Assay

Adhesion assay was carried out as described previously. Collagen was used as the adhesive substrate since it is predominantly widespread in breast cancers (Acerbi et al., 2015). Overall, a total of 13 flasks were seeded for each cell line (4 dilutions x 3 extracts + 1 control).

Firstly, cells were split, counted and seeded on to cell culture flasks. Four of the flasks were labelled as D1, D2, D3 and D4 representing the four dilutions used in the assay and one flask was the negative control. Seeding density was 1 x 10^5 cells/mL. For MDA-MB-231, MCF-7 and MDA-MB-468 cells, the volume of cells equivalent to this cell concentration was 350 µL, 476 µL and 526 µL, respectively.

Cells were incubated at 37^0C and 5% CO_2 overnight. On the same day, six plates were coated with collagen, as described below. Next day, the extracts were diluted in 10% DMSO/media the way described in the proliferation assay (from D1 to D4). Then 1000 µL of media was removed carefully from each flask and 1000 µL of the extract was added to each corresponding flask. As the negative control, 1000 µL of 10% DMSO/media was added to the fifth flask. Flasks were later incubated for 48 hours. After 48 hours, cells were split, transferred to 15 mL centrifuge tubes, made up to 10 mL with media and centrifuged at 1200 rpm for 10 min. They were resuspended in 10 mL of media and counted. The cell seeding density was 1 x 10^5 cells/mL in a total volume of 8 mL. This cell concentration was used to calculate the volume of cells and media needed to be seeded in the 24-well plates.

Cells from each corresponding flask (1000 µL) containing the diluted extracts were added to 8 wells of the 24-well plate and 1000 µL of cells from the control flask was also added to another set of 8 wells. As a blank,

1000 µL of media was placed in the remainder of the wells. All four dilutions of each extract and equivalent volumes of cells are given in Appendix 2.3.4. Plates were then incubated for 2 hours. CV assay was performed and absorbance readings were measured at 570 nm.

2.3.5 Collagen Assay

Collagen coating assay was adapted from a previously described protocol (Manufacturer's Protocols, 2019). In this study, Collagen I (stock solution: 3 mg/mL) was diluted in 50 µg/mL with 20 mM acetic acid (stock solution: 17.4 M) for a total of 100 mL (1.66 mL collagen + 0.115 mL acetic acid + 98.225 mL distilled water used in total). Diluted collagen (200 µL) was added on to each well and plates were left at room temperature overnight. Next day, each well was washed with PBS. Last of liquid was aspirated and the plates were air dried in the tissue cabinet. Dried plates were stored in fridge until use.

2.3.6 Migration Assay

Migration assay was carried out using wound healing assay or 'scratch' assay with the metastatic cell lines MDA-MB-231 and MDA-MB-468 cells. Methodology is as described previously (Justus, Leffler, Ruiz-Echevarria, & Yang, 2014). Briefly, on day 1, when cells were 70-80% confluent, they were detached with trypsin and centrifuged at ~125 g (850-1000 rpm) for 5–7 minutes. They were resuspended and a cell count was performed using a haemocytometer. A working cell solution was prepared at 2×10^5 cells/mL and cells were seeded at 500 µL/well in collagen-coated well plates. Plates were then incubated for 24-48 hours until 90% confluency.

On day 2, when cells were 90% confluent, serum starvation was performed and media was exchanged with 1% serum media and incubated overnight to stimulate migration. On day 3, with medium still in the well, a p200 pipette tip was positioned perpendicularly above the centre of the well and a 'wound scratch' was created swiftly by scraping the monolayer in a straight line. The bottom of plate was marked with a marker pen before scratching the plates. Any remaining medium and cell debris was carefully aspirated

from the edge of the wells and cells were washed with 1% serum media twice. At the end of the washing step, 500 µL of 1% serum containing medium was slowly added to the side of the wells. Wound closure was photographed at 0 hr under Olympus inverted microscope (Southend-on-Sea, UK), magnified 10x with 200 µm scale bar, using the Cell^M Image Analysis and Processing software. For each of the three extracts, three dilutions used were as the following:

- D1= 1000 µL 1:10 diluted extract + 4000 µL 0.1% DMSO/media
- D2= 1000 µL 1:20 diluted extract + 4000 µL 0.1% DMSO/media
- D3= 1000 µL 1:100 diluted extract + 4000 µL 0.1% DMSO/media were prepared in 1% serum and 500 µL was added to each well.

Concentrations of the extracts are given in the table in Appendix 2.3.6. For the negative control, cells were seeded at the same concentration (equivalent to 500 µL) using 0.1% DMSO in 1% serum media.

In order to prevent cell toxication, DMSO concentration was set to 0.1%. Plates were then incubated at 37^0C for 24 hours. Photographs were taken at 24 hr and ImageJ2 software (National Institutes of Health, USA) was used to measure the change in wound width or 'gap closure' in 24 hours. The experiment was repeated three times for each cell line and data were analysed after data normalisation. Percent change in wound width was calculated using the formula: (T_0-T_{24})*100/ control mean.

2.3.7 Invasion Assay

Invasion of the metastatic cell lines, MDA-MB-231 and MDA-MB-468 cells, was examined using the scratch assay as described previously (Justus et al., 2014). On Day 1, when cells have reached 70-80% confluency, they were trypsinised, centrifuged at 125 g for 5-7 minutes and resuspended. A cell count was performed with the help of a haemocytometer and cells were seeded at 500 µL/well in collagen-coated well plates using the working cell concentration at 2×10^5 cells/mL. Plates were incubated for 24-48 hours until 90% confluency has been reached. On day 2, when cells were 90% confluent, media was exchanged with 1% serum media to stop cell

proliferation and encourage their movement. Cells were then incubated at 37^{0}C, 5% CO_2 overnight. On day 3, after wounding and washing twice, collagen top layer was prepared. Prior to the gel preparation, cells in 1% media were put in the fridge for ~20 minutes. All plasticware including pipette tips and 50 mL Falcon tube, were placed in the fridge to inhibit gelling of collagen at room temperature. For the collagen top layer, 6700 µL of 3 mg/mL stock collagen, 100 µL of 10x PBS, 170 µL of 1M NaOH and 2130 µL of sterile distilled water were prepared carefully on ice for 24 wells. After 20 minutes, cells were taken out of the fridge and media was carefully aspirated from wells. Keeping at plate level, 400 µL of collagen gel was carefully loaded on to the wells. Plates were then incubated at 37^{0}C, 5% CO_2 for 30 minutes until a firm gel had formed. Negative control cells contained 1000 µL of 0.1% DMSO in 1% serum media. For the experimental cells, 1000 µL was removed from the following dilutions and added to the wells accordingly:

- 1000 µL 1:20 diluted acetone extract + 4000 µL 0.1% DMSO/media
- 1000 µL 1:20 diluted hexane extract + 4000 µL 0.1% DMSO/media
- 1000 µL 1:100 diluted methanol extract + 4000 µL 0.1% DMSO DMSO/media

All were prepared in 1% serum. The final concentrations of the extracts were 1.50 mg/mL for acetone, 0.40 mg/mL for hexane and 0.72 mg/mL for methanol. Cells were then placed in the incubator for 30 minutes. After 30 minutes, photos of the cells were taken as t=0 hr. All plates were incubated for 48 hours and the control cells were checked for closure of the wound. ImageJ2 was used to measure the change in wound width after 48 hours. The experiment was repeated three times for each cell line and data were analysed after data normalisation. Percent change in wound width was calculated using the formula: $(T_0\text{-}T_{24})*100/$ control mean.

2.3.8 Flow Cytometry and CD24 Antibody Testing

CD24 is a transmembrane protein which is thought to function as an adhesion molecule. It has been previously reported to be overexpressed in

some cancers including breast cancer and promote tumour invasiveness resulting in poor prognosis (Baumann et al., 2005). In this study, the two metastatic cell lines, MDA-MB-231 and MDA-MB-468 cells, were assessed for the expression of CD24.

The protocol is similar to the method described earlier (Calaf, Ponce-Cusi, & Abarca-Quinones, 2018). For each cell line, 4 culture dishes – one for the control, three for each extract - were seeded with cells at a working concentration of 1 x 10^6 cells/100 µL. The extract concentrations were the same as in the invasion assay. In other words, the concentration of extract in acetone was 1.50 mg/mL, in hexane 0.40 mg/mL, and in methanol it was 0.72 mg/mL. In order to obtain the same concentration, after 24 hours of incubation when cells have reached 70-80% confluency, 100 µL of media was replaced with extract in the acetone and hexane extract dishes. The exchange was 20 µL in the methanol extract dish. All plates were incubated at 37^0C, 5% CO_2 for 48 hours.

After 48 hours, cells were harvested with trypsin-EDTA. PBS supplemented with 0.5% FCS was added to inactivate trypsin. Cells were centrifuged to pellet and washed twice to remove dead cells and debris. Detached cells were then resuspended in 0.5% FCS/PBS and a cell count was performed. The appropriate concentrations of cells were set up in 1 mL Eppendorf tubes. Later, 200 µL of cells were seeded on to a 96-well plate. Anti-CD24 antibody (20 µL) was added to treatment cells containing the extract and control cells. They were incubated at 4˚C in the dark for 30-40 minutes. Cells were then analysed in Millipore Guava EasyCite flow cytometer (Sigma-Aldrich, Dorset, UK). Readings of cell concentration (cells/mL) and percent of total cell count for each cell line were taken in triplicate and recorded. Unpaired t-test was conducted to compare means of the control and each extract for statistical significance using GraphPad Prism 10. Cell concentrations of MDA-MB-231 and MDA-MB-468 cells are tabulated in Appendix 2.3.8.

2.3.9 Quantitative Analysis of Caspase 3/7 and MMP9 using ELISA Assay

ELISA assays were carried out at a certified private laboratory for quantitative detection of caspase 3, caspase 7 and MMP9 in MDA-MB-231 and MDA-MB-468 cells following proliferation assay. These assays were performed according to the manufacturer's instructions.

Briefly, media was removed from the wells and cells were washed with PBS twice. At the end of the wash cycle, 0.5 mL of freshly prepared PBS containing protease inhibitors was added to cells. Cells were gently scraped from the plates into Eppendorf tubes and centrifuged at 2000 rpm for 5 mins at 4^0C. The supernatant was discarded and cells were resuspended in 0.5 mL PBS with inhibitors. All steps were performed on ice using prechilled tubes, pipette tips and reagents. Cells per sample were counted and the numbers were recorded. Cell concentration in each sample was adjusted to $1x10^6$ cells/mL and cells were lysed with three freeze-thaw cycles. Each cycle consisted of rapid freezing at -80^0C until the samples were fully frozen followed by rapid thawing in a water bath at 37^0C until fully defrosted. The samples were then centrifuged at 12000 rpm for 20 mins at 4^0C and supernatants were collected to quantitatively determine the levels of caspase 3, caspase 7 and MMP9.

Six standards were prepared using 1:2 serial dilutions to create a standard curve for the ELISA assays. For caspase 3, the final concentrations used were 24, 12, 6, 3, 1.5 and 0.75 ng/mL. For caspase 7 and MMP9, these were 9600, 4800, 2400, 1200, 600 and 300 ng/L. A zero standard, diluent without the standard solution, was used as a blank. After preparation, 50 µL of the standards, including antibody, was added to the standard wells. Then 40 µL of the samples was added to the sample wells followed by 10 µL of detection antibody. This was followed by the addition of 50 µL of streptavidin-HRP to all sample and standard wells. The plate was then tightly sealed and gently tapped to ensure homogeneous mixing of the contents, and incubated for 1 hr at 37 °C.

After incubation, the seal was removed and the plate was washed 5 times with 350 µL 1X wash buffer using an automated plate washer (Mindray, MW-12A, China). The plate was then gently tapped dry to ensure no residual liquid remained. Then, 50 µL of substrate solution A and solution B were added to all wells. The plate was resealed and incubated for a further 10 mins at 37°C in the dark. A blue colour developed in all wells.

Following the final incubation, 50 µL of stop solution was added to all wells, resulting in an immediate colour change from blue to yellow. Absorbance was measured at 450 nm using a microplate reader (Heales, MB-580, China) within 10 mins. The absorbance readings of the standards were used to create a standard curve for each protein on GraphPad Prism. Using 4PL Sigmoidal non-linear regression model, concentrations of enzyme in samples were calculated based on the standard curve generated. The standard curves are given in Appendix 2.3.9. Calculated enzyme concentrations were then normalised, statistically analysed and the results are given as enzyme levels as a percentage of negative control.

2.4 Investigation of the Relationship between Breast Cancer and Eating Habits of Women in Northern Cyprus

2.4.1 Pilot Study Design

Women were recruited from the three main cities in North Cyprus which were Nicosia, Famagusta and Kyrenia. The inclusion criteria for the case group were Turkish Cypriot or Turkish women who have been living in Northern Cyprus for longer than 5 years with a history of breast cancer within the last 10 years, aged 18-65. For the control group women with the same criteria were included with the exception of a history of breast cancer. All participants in both groups had been checked prior to joining the study to confirm their medical condition.

Participants were recruited mainly from the State Hospital in Nicosia and other private health centres. They signed an informed consent (Appendix 2.5) before completing the survey (Appendix 2.4).

The survey consisted of 6 sections: a section about general demographic information, reproductive health and breast health, eating habits, food frequency questionnaire (FFQ) and a section on the anthropometric measurements. Height (m) and waist circumference (cm) measurements were taken with a tape measure. Weight (kg), body mass index (BMI) (kg/m^2) body fat %, body water % and muscle mass (kg) were taken with the measuring device TANITA BC-545N (Holland) using the bioelectrical impedance analysis (BIA) principle. The device sends a very low electrical signal to the body via its metal electrodes. The electrical current passes through water in the muscle tissue but fat tissue applies resistance, also known as impedance. This resistance is then used to calculate body composition measurements via validated Tanita equations (Tanita, 2020).

Measurements were taken three hours after rising or eating without any prior exercise or caffeine, nicotine, alcohol and any diuretic medication intake. Those with an implant or a pacemaker were not measured using BIA; their weight, height and waist circumference measurements were taken only. All data collected from participants were statistically analysed. For the FFQ analysis, a table was created on Excel in which portion sizes of foods were entered. These portion sizes were converted to average daily amounts using multiplication (or conversion) factors assigned to different frequencies. These factors are as follows:

Table 2.5: Conversion Factors used in the FFQ Analysis (Lanigan, 2013)

Frequency of Consumption	Conversion Factor
Never or less than a month	0
Once a month	0.03
Twice a month	0.07
5-6 times a week	0.79
3-4 times a week	0.5
1-2 a week	0.21
Everyday	1
Every meal	3

Daily amounts were then entered in a nutrient analysis software called BeBIS v9, (Turkey) in order to assess participants' nutrient intake.

2.4.2 Statistical Analysis

Descriptive statistics for the study variables were calculated such that frequency (n) and percentage (%) were used for qualitative variables while arithmetic mean, ± standard deviation of the mean and minimum-maximum were measured for quantitative variables. Pearson Chi-Square test or Fisher's Exact test was used to investigate the significance of associations between qualitative variables, depending on the sample sizes in sub-categories. Parametric test assumptions were controlled for quantitative variables. The distributions were tested for normality by using Kolmogorov-Smirnov and Shapiro-Wilk tests, where appropriate. Non-parametric hypothesis testing methods were applied accordingly. Mann-Whitney U test was performed to compare the differences in quantitative variables between the control and case groups. The level of significance was accepted to be 0.05. SPSS programme (Demo Version 26.0 for Mac) was used for statistical calculations.

Chapter 3 – Extraction of *C. olitorius* and Analysis of Its Phytochemical Content

3.1 Introduction

Assessment of the potential health effects of plants or opportunities for an emerging therapy involves extraction or isolation of bioactive compounds (Iseri et al., 2013; Sasidharan, Chen, Saravanan, Sundram, & Latha, 2011).

Extraction is the initial stage in characterising phytochemicals. It is a widely used technique to isolate bioactive compounds of medicinal plants or phytochemicals from the inactive components using a selective solvent. It is preceded by a series of steps which include washing the plant material, drying and grinding. Grinding helps increase the surface area of the plant material which will come in contact with the solvent and eventually give a higher extraction yield (Sasidharan et al., 2011).

Many different solvents can be used in extraction. These include the more polar or hydrophilic solvents such as water, methanol and ethanol; acetone which is of intermediate polarity and the hydrophobic or non-polar organic solvents such as hexane. Choosing the right solvent depends on several factors including the plant material and phytochemicals of interest (Abubakar & Haque, 2020; Pandey & Tripathi, 2014; Sasidharan et al., 2011; Truong et al., 2019).

There are several methods of extraction, such as maceration, ultrasound- or microwave-assisted extraction, and each method has its own advantages and disadvantages. The main goal of extraction is to isolate as much of the bioactive compounds as possible without changing the chemical composition of the plant material being investigated. Therefore, it is very important to choose the right method and obtain an optimal extraction efficiency (Ćujić et al., 2016).

Maceration or solvent extraction is a cheap, simple method which takes place at room temperature and therefore is frequently used for the

extraction of thermolabile compounds. The idea is that the plant material is completely covered and soaked in a particular solvent in a closed unit at room temperature and gently agitated for at least three days to extract the bioactive phytochemicals or secondary plant constituents of similar polarity (Abubakar & Haque, 2020; Pandey & Tripathi, 2014). Compared to other extraction methods, the extraction time can be considered relatively long. Although in some studies it has been shown to have a low extraction yield, other findings suggest that microwave-assisted extraction had a similar extraction yield at lower temperatures (Ćujić et al., 2016; Q. W. Zhang, Lin, & Ye, 2018).

Separation and identification of phytochemicals are more difficult than extraction due to the large number of different phytochemicals found in a combination. Further techniques are needed for isolation of individual phytochemicals including chromatographic methods such as High-Pressure Liquid Chromatography (HPLC) and Gas Chromatography (GC) and non-chromatographic methods such as distillation. Among the most widely used techniques is HPLC in which bioactive compounds, even in minute amounts, can be identified based on the fact that different compounds have different migration rates on the HPLC column. HPLC or GC coupled with mass spectrometry (MS) is a powerful way of identifying and quantifying phytochemical components and yields rapid identification of components especially when a homogenous extract cannot be obtained (Awuchi, 2019; Sasidharan et al., 2011).

In this study, three extracts of *C. olitorius* leaves were prepared by solvent extraction using acetone, hexane and methanol. The temperature was set to 30^0C to help increase the extraction yield without affecting heat-sensitive phytochemicals. Phytochemicals were identified by GC-MS. GC-MS is a powerful technique that can be used in the separation and identification of compounds, particularly volatile phytochemicals, in a plant extract (Orata, 2012).

Since it has been widely used in traditional medicine, the nutritional profile and phytochemical content of the jute leaves were examined in previous

studies. In addition to minerals and antioxidants vitamins, such as B-carotene, Vitamin C and E (Azuma et al., 1999), the leaves of *C. olitorius* have been shown to possess secondary constituents, including polar and non-polar polyphenols and other non-polyphenol phytochemicals, which exhibit antioxidant, free radical scavenging and selective cytotoxic properties (Guzzetti et al., 2021; Handoussa et al., 2013; Morrison & Twumasi, 2010; Nakaziba, Lubega, Ogwal-Okeng, & Alele, 2022; Okunlola, Jimoh, Olatunji, & Olowolaju, 2017).

Polyphenols are a diverse group of phytochemicals which are subdivided based on their chemical structure. Among the subclasses are phenolic acids, which include chlorogenic acid and its isomers; flavonoids, which include quercetin and its derivatives; and tannins, all of which have been identified in the polar extracts of jute leaves (Biswas et al., 2022; Meinhart, Caldeirão, Damin, Filho, & Godoy, 2018; Mitra et al., 2023; Rambaran, 2020)

Flavonoids are one of the largest groups of polyphenols which are also made up of subcategories. These include anthocyanidins, flavonols, flavanols, flavanones, flavones and isoflavone. Water solubility and polarity differ in these subgroups depending on the level of glycosylation – addition of carbohydrates - which makes them more polar (Jakobek, 2015; Kopustinskiene, Jakstas, Savickas, & Bernatoniene, 2020). It has been previously shown that the ethanolic extracts of the leaves had a high flavonoid content and that quercetin derivatives were the major compounds identified (Biswas et al., 2022; Handoussa et al., 2013).

It has been proposed that the number and arrangement of hydroxyl groups, the arrangement of sugar units, the overall 3D structure of the polyphenols and several other factors play key roles in their activity and interactions with the other molecules in the environment (Jakobek, 2015).

Alkaloids and terpenoids (e.g., carotenoids) are non-polar, non-polyphenol phytochemicals which have been previously identified in the leaves of *C. olitorius* (Awuchi, 2019; Azuma et al., 1999). In this study, hexane was

chosen as the organic solvent to extract the non-polar substances including alkaloids, terpenoids and other unidentified phytochemicals from the jute leaves.

Given that in previous studies *C. olitorius* was found to contain a mixture of polar and non-polar polyphenols and considering the critical role of these antioxidant compounds in cancer prevention, free radical scavenging ability and total phenol and flavonoid contents of the jute extracts were investigated in this study. The phytochemical content of each extract was also determined by GC-MS.

3.2 Results

3.2.1 Extraction Yields

The weight of each extract after the initial solvent extraction is given in Table 3.2.1. At this stage, the highest extraction yield was obtained with methanol, followed by hexane and acetone.

Table 3.2.1 Weight of Plant Material after Solvent Extraction

Solvent Extract	Total Weight with flask (g)	Weight of the flask (g)	Weight of plant material extracted (g)
Acetone	38.23	31.40	6.82
Hexane	40.52	31.75	8.77
Methanol	54.70	43.49	11.21

After solvent evaporation and sonication, the final weight of each extract was determined by subtracting the weight of the bottle and DMSO, which were 15.80 g and 20.2 g, respectively. Total final weights of the extracts which were used to prepare stock concentrations are given in Table 3.2.2.

Table 3.2.2: Weight of Each Extract after Solvent Evaporation

Extract	Weight of extract and DMSO with bottle (g)	Weight of extract and DMSO without bottle (g)	Weight of Extract used (g)	Initial Extract Concentrations in DMSO (g/mL)

Acetone	38.97	23.17	2.97	0.15
Hexane	36.80	21.00	0.80	0.04
Methanol	43.21	27.42	7.21	0.36

After evaporation of the liquid, extraction yield of each extract (*x*) was calculated as a percentage of the dry weight of leaves (200 g per extract) using the formula *x**100/200.

The highest extraction yield was obtained with methanol due to its higher weight than the other extracts (3.61%), followed by acetone (1.49%) and finally hexane (0.4%).

Stock concentrations of the acetone, hexane and methanol extracts in 10% DMSO were 15 mg/mL, 8 mg/mL and 72 mg/mL, respectively. In all experiments, further dilutions were prepared based on these concentrations.

3.2.2 Total Phenolics Content

In order to measure the total phenolic content of *C. olitorius*, GA standards were used to plot a calibration curve and determine the GAE. Table 3.2.2a represents the absorbance measurements of GA at 750 nm. These were taken in triplicate and the mean was determined.

Table 3.2.2a: Absorbance Measurements of Gallic Acid Standards at 750 nm

Gallic Acid Concentration (µg/mL)	Mean Absorbance (750 nm)
0	0.000
50	0.285
100	0.511
200	0.756
250	0.934
400	1.564
500	2.017

Using these measurements, a calibration curve was plotted and the following equation was obtained: y = 0.0039x + 0.0415, R^2 = 0.9927 as shown in Figure 3.2.2.

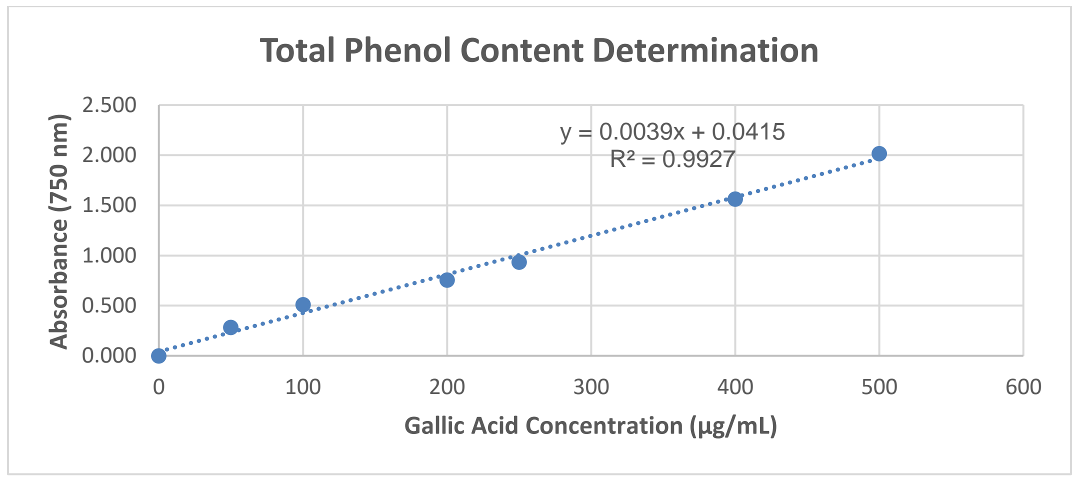


Figure 3.2.2: Calibration Curve for Total Phenol Content in C. olitorius

GA concentrations (x-axis) were plotted against the mean absorbance measurements at 750 nm (y-axis) to determine the linear equation which was y = 0.0039x + 0.0415 (R^2 = 0.9927). This equation was used to calculate the concentrations of extracts using their absorbance values.

Mean absorbance readings of the extracts were entered in the equation to obtain extract concentrations which were then used to calculate TPC. These values are given below, in Table 3.2.2b.

Table 3.2.2b: Absorbance Readings and Concentrations of Extracts

Extracts	Initial Concentrations used in the assay (mg/mL)	Mean Absorbance (750 nm)	Concentrations from equation (mg/mL)
Acetone	15.00	0.103	0.016
	7.50	0.268	0.058
	1.50	0.057	0.004
Hexane	4.00	0.385	0.088
	2.00	0.105	0.016
	0.40	0.019	-0.006
Methanol	36.00	1.800	0.451
	18.00	0.882	0.216
	3.60	0.290	0.064

Since all extracts were further diluted 25 times during assay preparation - 0.5 mL extract in 12.5 mL solution – the final concentrations and the mass of extracts were calculated considering this further dilution. These values were then entered in the formula TPC = Cv/m to obtain total phenol content as mg GAE/g dry matter of the sample. These findings are given in Table 3.2.2c.

Table 3.2.2c: Total Phenol Content of the Extracts

Extracts	Final Concentrations used in the assay (mg/mL)	Mass of Extract (mg)	TPC (mg GAE/g dry matter of sample)
Acetone	0.600	0.30	26.14
	0.300	0.15	193.30
	0.060	0.03	67.66
Hexane	0.160	0.08	549.95
	0.080	0.04	203.53
	0.016	0.01	-365.92
Methanol	1.440	0.72	313.12
	0.720	0.36	299.44
	0.144	0.07	441.89

3.2.3 Total Flavonoids Content

Calculation of the total flavonoids content of *C. olitorius* was similar to that of the total phenol content. As a flavonoid equivalent, quercetin was used in this assay. A standards calibration curve was plotted using the mean absorbance readings of quercetin against predetermined concentrations. These are given in Table 3.2.3a.

Table 3.2.3a: Absorbance Measurements of Quercetin Standards at 510 nm

Quercetin Concentration (µg/mL)	Mean Absorbance (510 nm)
0	0.000
10	0.006
20	0.006
40	0.019
80	0.036
100	0.043

The equation obtained from the curve was y = 0.0004x + 6E-05 (R^2 = 0.9924) as shown in Figure 3.2.3. Mean absorbance readings of the extracts were entered in the equation to find extract concentrations which were used to calculate TFC. These are given in **Error! Reference source not found.**.

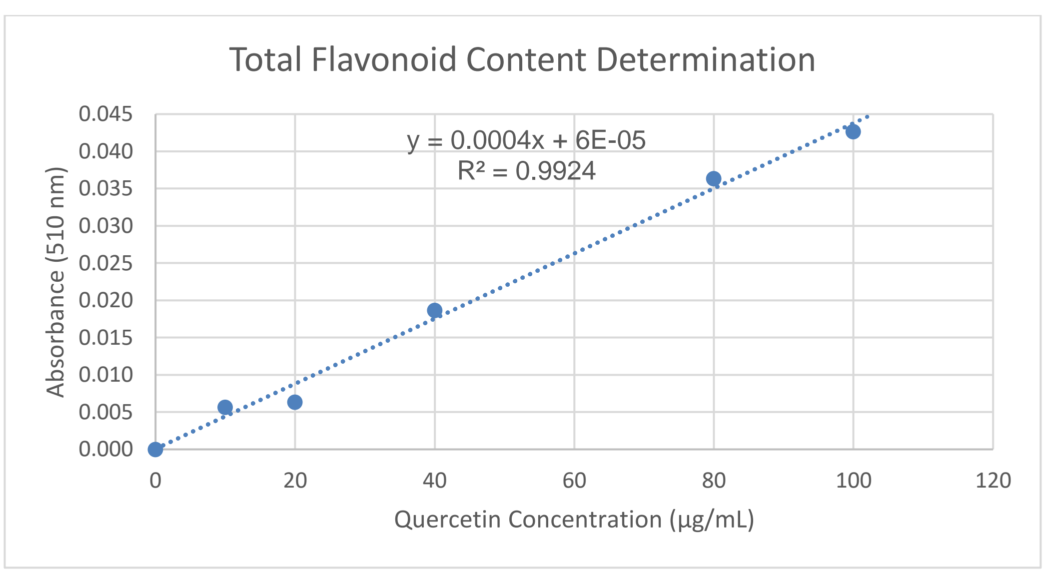


Figure 3.2.3: Calibration Curve for Total Flavonoid Content in C. olitorius

Quercetin concentrations (x-axis) were plotted against the mean absorbance measurements at 510 nm (7-axis) to determine the linear equation which was y = 0.0004x + 6E-05 (R^2 = 0.9924). This equation and the absorbance values of the extracts were used to determine the concentrations of extracts.

Table 3.2.3b: Absorbance Measurements and Concentrations of Extracts

Extracts	Initial Concentrations used in the assay (mg/mL)	Mean Absorbance (510 nm)	Concentrations from equation (mg/mL)
Acetone	15.00	0.039	0.098
	7.50	0.112	0.279
	1.50	0.020	0.050
Hexane	4.00	0.083	0.208
	2.00	0.114	0.286
	0.40	0.003	0.008
Methanol	36.00	0.791	1.977
	18.00	0.458	1.144
	3.60	0.162	0.404

There was a 10-fold further dilution of extracts in the assay solution (0.5 mL in a total of 5 mL). Taking this into consideration, final concentrations and the mass of extracts were determined. Total flavonoid content of *C. olitorius* was then calculated using the formula TFC = Cv/m and expressed as mg QE/gram dry matter of the sample. Results are given in Table 3.2.3c.

Table 3.2.3c: Total Flavonoid Content of the Extracts

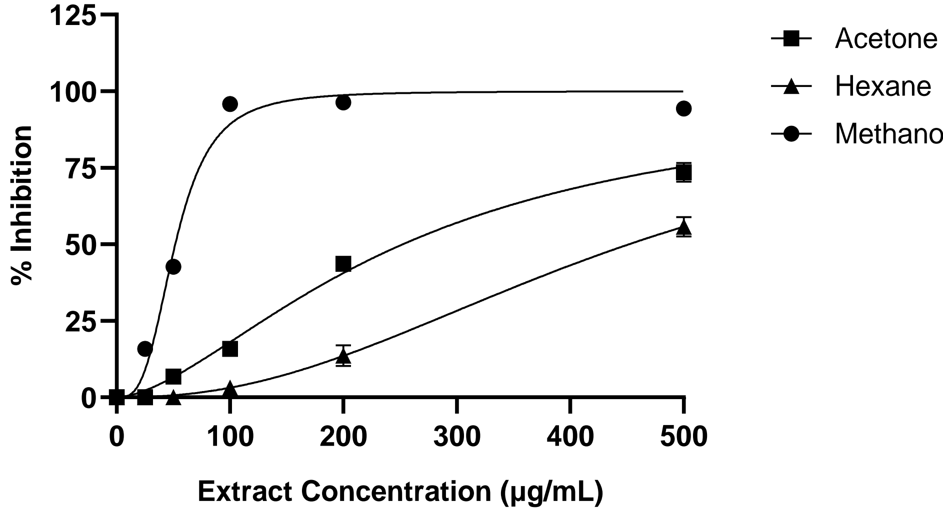
Extracts	Final Concentrations used in the assay (mg/mL)	Mass of Extract (mg)	TFC (mg QE/g dry matter of sample)
Acetone	1.500	0.75	65.46
	0.750	0.38	372.02
	0.150	0.08	332.33
Hexane	0.400	0.20	520.46
	0.200	0.10	1428.42
	0.040	0.02	204.58
Methanol	3.600	1.80	549.26
	1.800	0.90	635.56
	0.360	0.18	1122.27

3.2.4 DPPH Assay

For each extract concentration, absorbance measurements taken at 517 nm were used to measure percent inhibition by chemical reduction of the DPPH free radical. These were then entered into GraphPad Prism to plot an inhibition curve and calculate IC_{50} for each extract using nonlinear regression. This was also repeated for ascorbic acid.

Plots of the percent inhibition against concentration for the extracts and ascorbic acid are given in Figure 3.2.4.

The estimated IC_{50} values of the extracts and ascorbic acid, as the positive control, are given in Table 3.2.4.



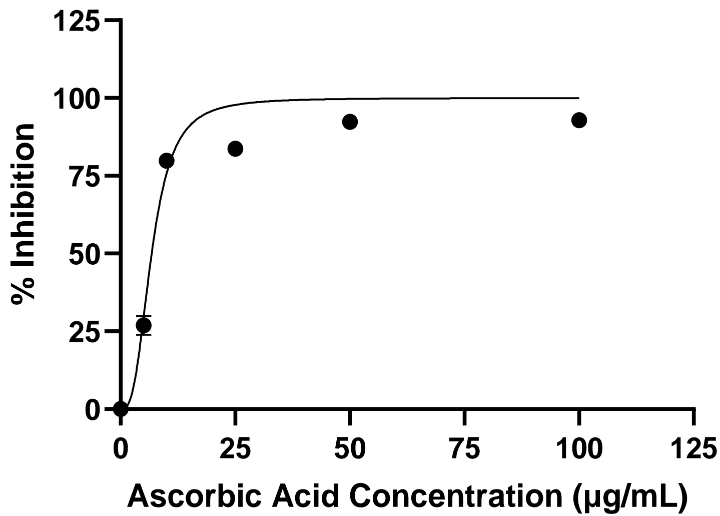


Figure 3.2.4: Percent Inhibition of DPPH Activity by the Extracts and Ascorbic Acid

Percent inhibition of the DPPH activity was calculated using serial dilutions of the extracts (25, 50, 100, 200 and 500 µg/mL), and ascorbic acid (5, 10, 25, 50 and 100 µg/mL) measured in triplicate. Some error bars that are too small and appear hidden by the data points cannot be shown.

Table 3.2.4: Estimated IC_{50} Values of the Extracts and Ascorbic Acid

Sample	IC_{50} (µg/mL)
Acetone Extract	252.40
Hexane Extract	451.70
Methanol Extract	51.70
Ascorbic Acid	6.78

Estimated IC_{50} values were calculated on GraphPad Prism 10 (USA).

3.2.5 GC–MS Analysis of C*. olitorius* Extracts

Phytochemical content of the acetone, hexane and methanol extracts of *C. olitorius* leaves was determined by GC-MS analysis as described in Chapter 2, Section 2.2.7 Chromatographic spectra of the extracts are given below (Figure 3.2.5a, Figure 3.2.5b, Figure 3.2.5c). The phytochemical compositions of each extract are detailed in Table 3.2.5a, Table 3.2.5b and Table 3.2.5c. Molecular structures of the compounds were obtained from the NIST Library (https://webbook.nist.gov/chemistry/) or PubChem (https://pubchem.ncbi.nlm.nih.gov/). Only compounds with ≥80% library matching quality were included. The GC-MS analysis identified

approximately 86% of the acetone extract, 91% of the hexane extract and 19% of the methanol extract.

As seen in Table 3.2.5a, 24 compounds were identified in the acetone extract and it predominantly contained 9,12,15-octadecatrienoic acid, (Z,Z,Z)- (19.341%) - also known as α-linolenic acid, phytol (17.833%), dl-α-tocopherol (10.908%) and n-hexadecanoic acid, also known as palmitic acid (9.496%).

In the hexane extract, a total of 21 compounds were identified and this extract was rich in non-polar fatty acid methyl esters including 9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)- (42.010%), hexadecanoic acid, methyl ester (24.753%), 9,12-octadecadienoic acid (Z,Z)-, methyl ester (10.367%) – also called linoleic acid methyl ester - and phytol (4.505%), as given in Table 3.2.5b.

In the methanol extract, 12 compounds were identified, with 9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)- (6.501%), loliolide (2.126%), n-hexadecanoic acid (2.006%) and 2-methoxy-4-vinylphenol (1.460%) as the major phytochemicals (Table 3.2.5c).

Table 3.2.5a: Phytochemical Composition of the Acetone Extract

#	RT (min)	Extract Peak Area	Compound	Biological Activity	Molecular Formula	Molecular Structure	PubChem ID	Reference
1	10.223	0.234%	Cycloheptasiloxane, tetradecamethyl	-	$C_{14}H_{42}O_7Si_7$		7874	-
2	10.422	0.153%	3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	Anti-cancer	$C_{13}H_{20}O$		638014	(J.-R. Liu et al., 2008)
3	11.126	1.479%	Dihydroactinidiolide	Antioxidant	$C_{11}H_{16}O_2$		6432173	(M. Das et al., 2018)
4	11.733	0.709%	Z-8-Hexadecene	-	$C_{16}H_{32}$		5352971	-
5	13.813	3.352%	Loliolide	Endogenous inducer of plant defense mechanisms	$C_{11}H_{16}O_3$		100332	(Murata et al., 2019)
6	13.913	0.347%	Cyclononasiloxane, octadecamethyl-	-	$C_{18}H_{54}O_9Si_9$		11172	-
7	13.992	1.200%	1-Octadecene	Cytotoxic to gastric cancer cells *in vitro*	$C_{18}H_{36}$		8217	(Y. S. Lee, Kang, Cho, & Jeong, 2007)
	13.992	1.200%	E-15-Heptadecenal	Antibacterial	$C_{17}H_{32}O$		5363097	(Supardy, Ibrahim, Sulaiman, & Zakaria, 2012)
8	14.453	3.173%	Neophytadiene	Anti-inflammatory	$C_{20}H_{38}$		10446	(Bhardwaj, Sali, Mani, & Vasanthi, 2020)

9	14.504	2.565%	2-Pentadecanone, 6,10,14-trimethyl-	Pain alleviation	$C_{18}H_{36}O$	10408	(Avoseh, Mtunzi, Ogunwande, Ascrizzi, & Guido, 2021)
10	14.900	1.453%	Neophytadiene	Anti-inflammatory	$C_{20}H_{38}$	10446	(Bhardwaj et al., 2020)
11	15.352	0.444%	Hexadecanoic acid, methyl ester	Antimicrobial	$C_{17}H_{34}O_2$	8181	(Shaaban, Ghaly, & Fahmi, 2021)
12	15.434	0.981%	Cyclodecasiloxane, eicosamethyl-	-	$C_{20}H_{60}O_{10}Si_{10}$	519601	-
13	15.678	9.496%	n-Hexadecanoic acid	Anti-inflammatory, antimicrobial	$C_{16}H_{32}O_2$	985	(Aparna et al., 2012; Johannes, Litaay, & Syahribulan, 2010)
14	16.049	1.825%	1-Eicosene	-	$C_{20}H_{40}$	18936	-
15	16.519	0.557%	Palmitic Acid, TMS derivative	-	$C_{19}H_{40}O_2Si$	521638	-
16	16.826	0.939%	Dibutyl isophthalate	-	$C_{16}H_{22}O_4$	18405	-
	16.826	0.939%	Terephthalic acid, butyl cycloheptyl ester	-	$C_{19}H_{26}O_4$	91733744	-
17	17.152	17.833%	Phytol	Anti-cancer, anti-inflammatory	$C_{20}H_{40}O$	5280435	(Alencar et al., 2023; Furumoto et al., 2002; Shariare et al., 2021; J. Yu, Jin, Tang, & Huang, 2025)

18	17.335	2.076%	9,12-Octadecadienoic acid (Z,Z)-	Antimicrobial	$C_{18}H_{32}O_2$		5280450	Onoabedje et al., 2024
19	17.391	19.341%	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	Anti-cancer, anti-inflammatory, cardioprotective	$C_{18}H_{30}O_2$		5280934	(Fan, Huang, Guo, Ma, & Yang, 2022; Naghshi et al., 2021)
20	17.593	3.037%	9,12-Octadecadienoic acid (Z,Z)-	Antimicrobial	$C_{18}H_{32}O_2$		5280450	(Onoabedje, Ezugwu, & Abraham, 2024)
21	17.931	0.921%	Cyclohexadecane, 1,2-diethyl-	-	$C_{20}H_{40}$		536940	-
22	19.296	1.198%	4,8,12,16-Tetramethylheptadecan-4-olide	Anti-cancer	$C_{21}H_{40}O_2$		567149	(Swantara, Rita, Suartha, & Agustina, 2019)
24	26.521	10.908%	dl-.alpha.-Tocopherol	Antioxidant, anti-inflammatory, anti-cancer	$C_{29}H_{50}O_2$		2116	(Dada et al., 2023; Galli et al., 2004; Morley et al., 2010)

-: No biological activity has been previously reported. RT: Retention time.

Table 3.2.5b: Phytochemical Composition of the Hexane Extract

#	RT (min)	Extract Peak Area	Compound	Biological Activity	Molecular Formula	Molecular Structure	PubChem ID	Reference
1	8.073	0.198%	Cyclohexasiloxane, dodecamethyl-	-	$C_{12}H_{36}O_6Si_6$		10911	-
2	10.420	0.071%	3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	Anti-cancer	$C_{13}H_{20}O$		638014	(J.-R. Liu et al., 2008)
3	11.125	0.861%	Dihydroactinidiolide	Antioxidant	$C_{11}H_{16}O_2$		6432173	(M. Das et al., 2018)
4	13.222	0.403%	Myristic Acid Methyl Ester	fly-repellent, anti-proliferative, cytotoxic	$C_{15}H_{30}O_2$		31284	(Henderson, Wells, & Jeanne, 1991; Takeara et al., 2008)
5	13.815	0.141%	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one	Anti-inflammatory, antioxidant	$C_{11}H_{16}O_3$		14334	(Han et al., 2021; Jayawardena et al., 2019)
6	14.452	0.663%	Neophytadiene	Anti-inflammatory	$C_{20}H_{38}$		10446	(Bhardwaj et al., 2020)
7	15.145	0.365%	7-Hexadecenoic acid, methyl ester, (Z)-	Anti-inflammatory	$C_{17}H_{32}O_2$		5364431	(Guijas, Meana, Astudillo, Balboa, & Balsinde, 2016)
8	15.145	0.365%	Methyl hexadec-9-enoate	-	$C_{17}H_{32}O_2$		638303	-

9	15.297	1.227%	9-Hexadecenoic acid, methyl ester, (Z)-	-	$C_{17}H_{32}O_2$		643801	-
10	15.355	24.753%	Hexadecanoic acid, methyl ester	Antimicrobial	$C_{17}H_{34}O_2$		8181	(Shaaban et al., 2021)
11	15.441	0.289%	2,2,4,4,6,6,8,8,10,10,12,12,14,14,16,16,18,18,20,20-Icosamethylcyclodecasiloxane	-	$C_{20}H_{60}O_{10}Si_{10}$		519601	-
12	15.668	0.537%	n-Hexadecanoic acid	Anti-inflammatory, antimicrobial	$C_{16}H_{32}O_2$		985	(Aparna et al., 2012; Johannes et al., 2010)
13	16.348	0.158%	Hexadecanoic acid, 14-methyl-, methyl ester	-	$C_{18}H_{36}O_2$		520159	-
14	17.006	10.367%	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	Anti-cancer, cytotoxic, anti-melanogenic	$C_{19}H_{34}O_2$		5284421	(Ko, Shrestha, & Cho, 2018; F. R. Yu et al., 2005)
15	17.063	42.010%	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	Anti-fungal, anti-melanogenic	$C_{19}H_{32}O_2$		5319706	(Johann et al., 2012; Ko et al., 2018)
16	17.154	4.505%	Phytol	Anti-cancer, anti-inflammatory	$C_{20}H_{40}O$		5280435	(Alencar et al., 2023; Furumoto et al., 2002; Shariare et al., 2021; J. Yu et al., 2025)
17	17.301	2.484%	Methyl stearate	Anti-proliferative, cytotoxic	$C_{19}H_{38}O_2$		8201	(Takeara et al., 2008)

18	17.381	1.529%	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	Anti-cancer, anti-inflammatory, cardioprotective	$C_{18}H_{30}O_2$		5280934	(Fan et al., 2022; Naghshi et al., 2021)
19	20.748	0.336%	Docosanoic acid, methyl ester	-	$C_{23}H_{46}O_2$		13584	-
20	26.517	0.948%	6-Methoxy-2,7,8-trimethyl-2-(4,8,12-trimethyltridecyl)chroman	-	$C_{29}H_{50}O_2$		57978470	-
	26.517	0.948%	dl-.alpha.-Tocopherol	Antioxidant, anti-inflammatory, anti-cancer	$C_{29}H_{50}O_2$		2116	(Dada et al., 2023; Galli et al., 2004; Morley et al., 2010)

-: No biological activity has been previously reported. RT: Retention time.

Table 3.2.5c: Phytochemical Composition of the Methanol Extract

#	RT (min)	Extract Peak Area	Compound	Biological Activity	Molecular Formula	Molecular Structure	PubChem ID	Reference
1	8.085	0.881%	Cyclohexasiloxane, dodecamethyl-	-	$C_{12}H_{36}O_6Si_6$		10911	-
2	8.294	1.460%	2-Methoxy-4-vinylphenol	Antioxidant, anti-inflammatory, anti-cancer, anti-metastatic	$C_9H_{10}O_2$		332	(Asami, Kitami, Ida, Kobayashi, & Saeki, 2023; Jeong, Hong, Jeong, & Koo, 2011; Jeong & Jeong, 2010; D. H. Kim et al., 2019; J. Lee, Morshidi, Lee, Sim, & Kim, 2025; Luo et al., 2021)
3	9.382	0.626%	1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopenta[c]pyran-1-yl)ethanone	-	$C_{13}H_{18}O_2$		605654	-
4	11.132	1.217%	Dihydroactinidiolide	Antioxidant	$C_{11}H_{16}O_2$		6432173	(M. Das et al., 2018)
5	12.195	1.051%	Cyclooctasiloxane, hexadecamethyl-	-	$C_{16}H_{48}O_8Si_8$		11170	-

6	12.488	1.622%	Chavicol TMS	-	$C_{12}H_{18}OSi$		21654566	-
7	13.815	2.126%	Loliolide	Endogenous inducer of plant defense mechanisms	$C_{11}H_{16}O_3$		100332	(Murata et al., 2019)
8	15.353	1.216%	Hexadecanoic acid, methyl ester	Antimicrobial	$C_{17}H_{34}O_2$		8181	(Shaaban et al., 2021)
9	15.668	2.006%	n-Hexadecanoic acid	Anti-inflammatory, antimicrobial	$C_{16}H_{32}O_2$		985	(Aparna et al., 2012; Johannes et al., 2010)
10	17.059	1.489%	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	Anti-fungal, anti-melanogenic	$C_{19}H_{32}O_2$		5319706	(Johann et al., 2012; Ko et al., 2018)
11	17.326	0.534%	2(1H)-Naphthalenone, octahydro-4a-methyl-7-(1-methylethyl)-, (4a.alpha.,7.beta.,8a .beta.)-	-	$C_{14}H_{24}O$		41133	-
12	17.383	5.012%	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	Anti-fungal, anti-melanogenic	$C_{19}H_{32}O_2$		5319706	(Johann et al., 2012; Ko et al., 2018)

-: No biological activity has been previously reported. RT: Retention time.

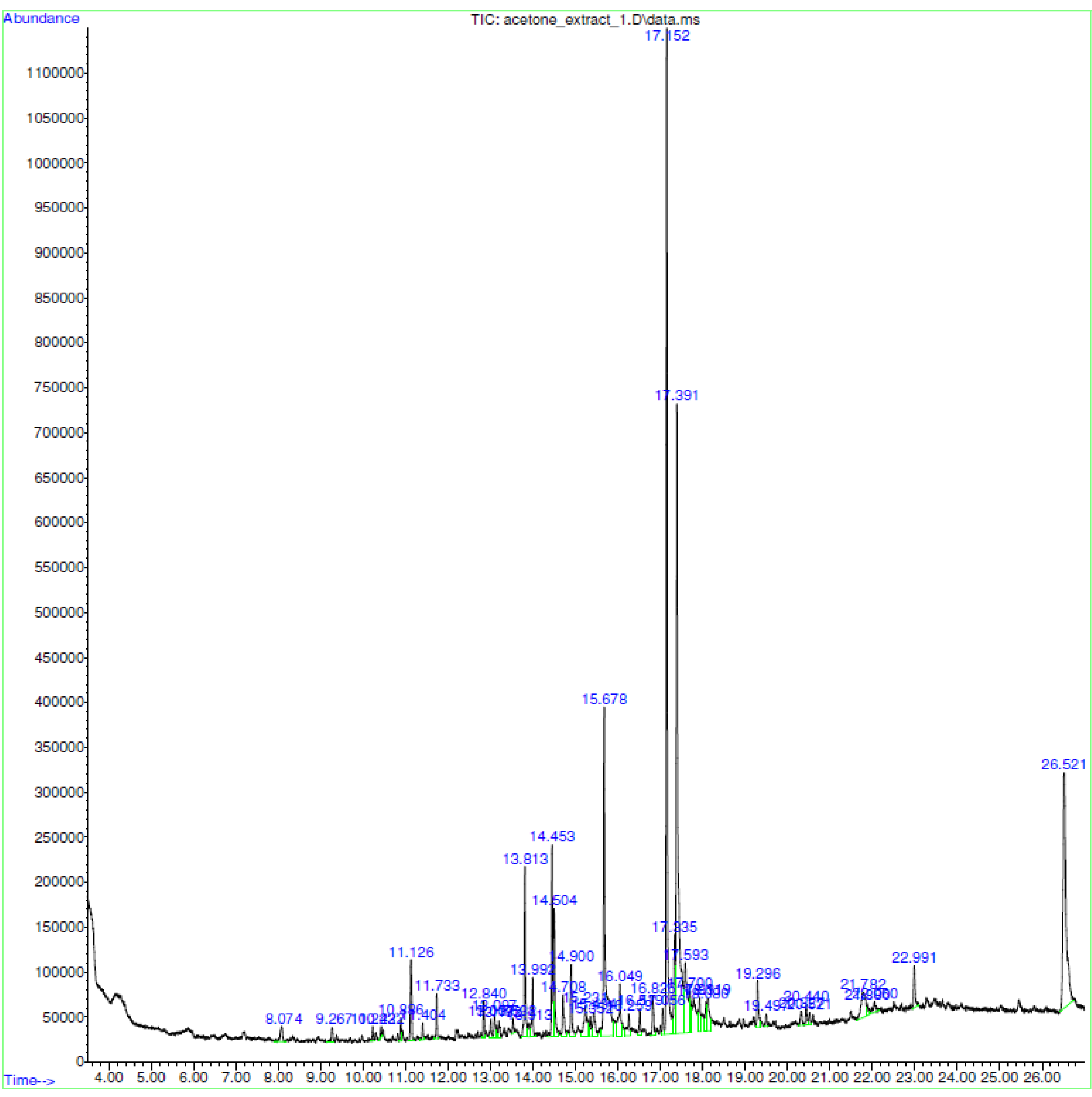


Figure 3.2.5a: Gas Chromatography Spectrum of the Acetone Extract

The spectrum of the acetone extract of *C. olitorius* leaves shows the abundance and retention times of the volatile compounds detected in gas chromatography.

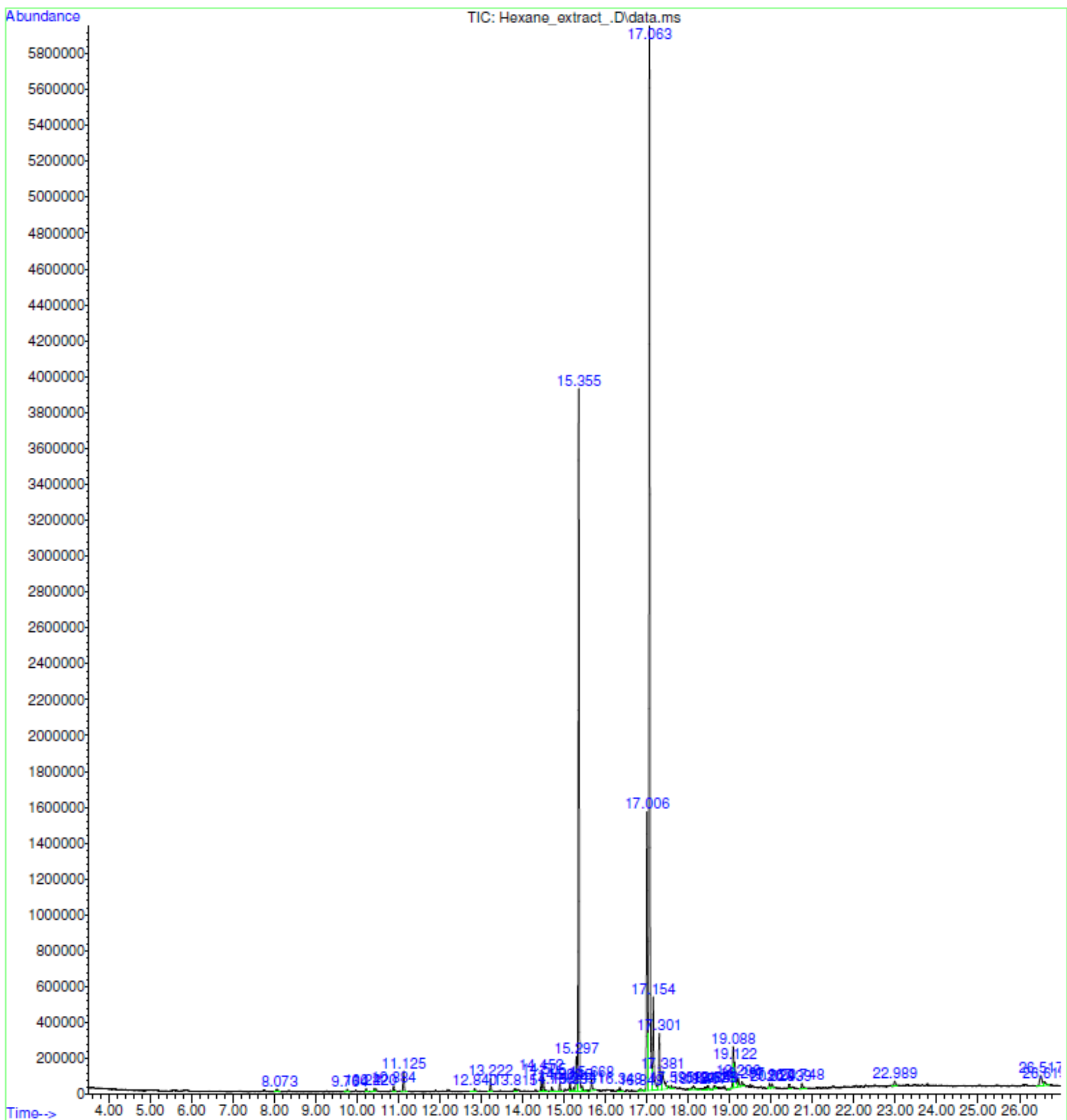


Figure 3.2.5b: Gas Chromatography Spectrum of the Hexane Extract

The spectrum of the hexane extract of *C. olitorius* leaves shows the abundance and retention times of the volatile compounds detected in gas chromatography.

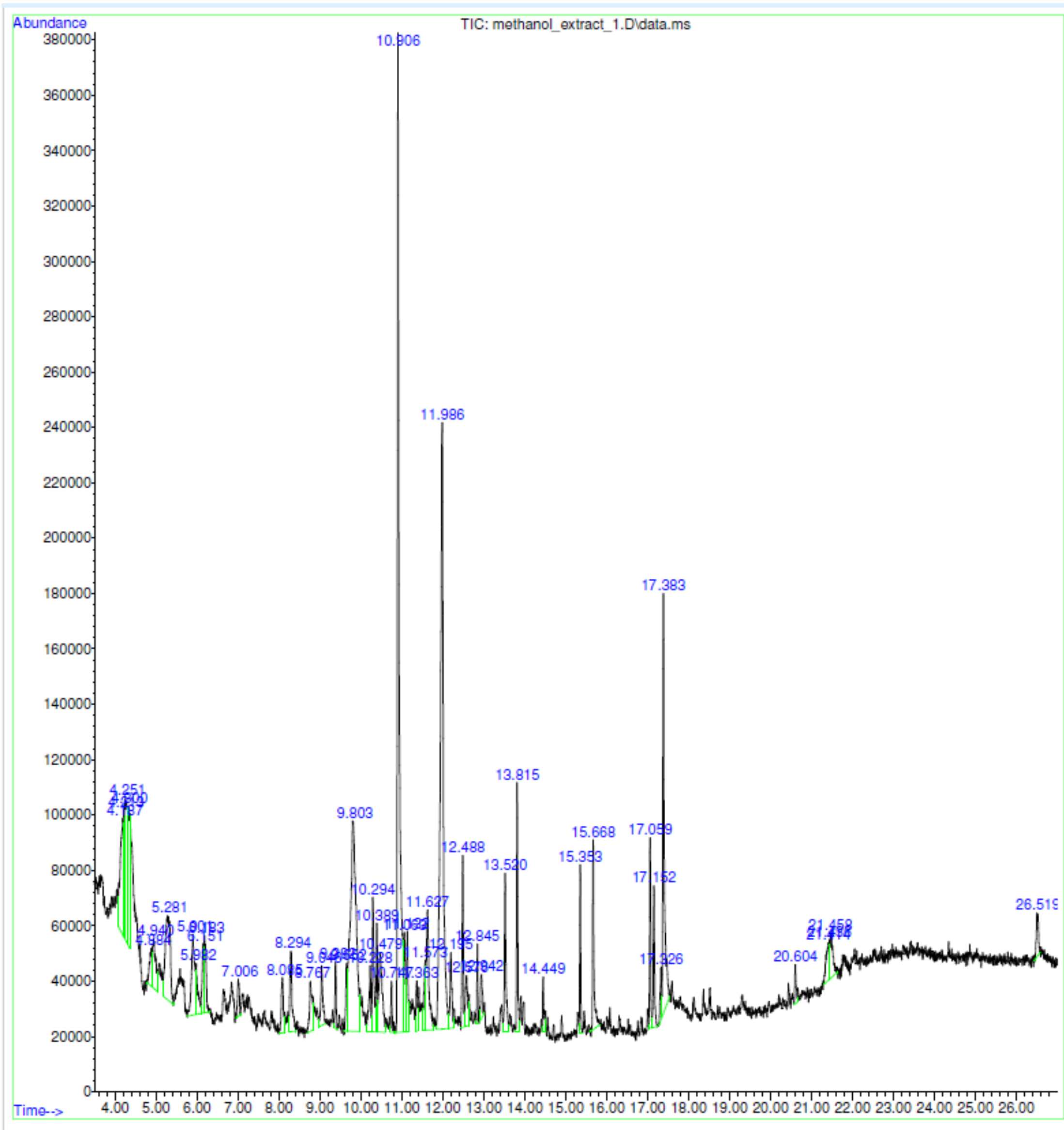


Figure 3.2.5c: Gas Chromatography Spectrum of the Methanol Extract

The spectrum of the methanol extract of *C. olitorius* leaves shows the abundance and retention times of the volatile compounds detected in gas chromatography.

3.3 Discussion

Evaluation of bioactive plant compounds depends on choosing the right extraction method. There are various extraction techniques that can be used and it is important to choose the right extraction method as well as solvents to increase efficiency, selectivity and optimal yield without degrading the desired phytochemicals.

Due to its practical convenience and non-use of high heat, maceration was preferred in this study. Maceration was then followed by sonication to break up and even out clumps of the dissolved plant material. Comparing different extraction methods, such as maceration, ultrasound-assisted extraction etc., was not within the scope of this study. Therefore, a comparison of the efficiency of different techniques was not carried out.

One of the aims of the present study was to use different solvents to extract bioactive plant compounds of various polarities. In this study, methanol extraction resulted in the highest yield (3.61%) compared to hexane and acetone extraction. In a previous study carried out with a citrus plant, *Severinia buxifolia*, methanol also resulted in the highest extraction yield (Truong et al., 2019).

In another study, alcoholic extraction yielded highest whereas hexane extraction was the lowest (Sellami, Ghariani, Louati, Miled, & Gargouri, 2013). In the present study, the lowest yield was also obtained with hexane (0.4%) followed by acetone extraction (1.49%). There could be a number of reasons for this difference in extraction. One of them is the loss of sample during the physical removal of the extracts. Since extracts were concentrated in a glass flask, although great care was taken while collecting the extract, removal with a toothbrush left behind some residual sample. This was particularly observed with the hexane extract. Similarly, due to the sticky and slimy nature of the acetone extract, not all sample was readily scraped off from the flask. The physical loss of sample in both hexane and acetone extracts could be a confounding factor in their overall extraction yield.

In order to compensate for this, the initial extraction step could be performed with three replicates for each extract and the average initial weight of the plant extracts could be measured. Although this may reduce the risk of error, it requires a larger quantity of reagents and plant material.

Another reason for the difference could be the total amount of phytochemicals of varying polarities that was available in the leaves. In

other words, the quantity of polar compounds could be more than that of the non-polar phytochemicals and this may explain the higher extract yield obtained with methanol extraction.

Solvent penetrability, as suggested earlier (Zhang, 2015), could be another explanation for the differences in extraction yield. Methanol may penetrate more readily through the plant cell walls due to its more aqueous nature extracting phytochemicals whereas penetration of the solvent may be more difficult with intermediately polar acetone or non-polar hexane.

3.3.1 TPC and TFC Assays

The findings of the TPC and TFC assays show a relatively higher amount of phenols and flavonoids in the hexane and methanol extracts than in the acetone extract of *C. olitorius (*Table 3.2.2c, Table 3.2.3c).

The higher total phenol and flavonoid content of the hexane and methanol extracts may suggest that phenols and flavonoids in *C. olitorius* leaves are a mixture of highly polar and non-polar compounds rather than of a mid-range polarity as in the case of acetone.

It is also important to bear in mind that since spectrophotometric assays do not isolate or identify specific phenolic compounds, these findings suggest an estimation of their overall content in the plant material. Nevertheless, TPC and TFC assays are relatively inexpensive methods for measuring overall phenolic and flavonoid content of plants.

Furthermore, the use of multiple solvents with different polarities enables the extraction of a wide range of phenolic compounds based on their chemical structure (Sellami et al., 2013).

In the present study, 4 mg/mL of the hexane extract, as the most concentrated of all three dilutions of this extract, had the highest total phenolic content (549.95 mg GAE/g extract). This was followed by the methanol extract. The highest TFC value, 1428.42 mg QE/g extract, was also observed in the hexane extract at 2 mg/mL. TFC values of the

methanol extract were also relatively higher than the acetone extract and other concentrations of the hexane extract.

These findings may indicate that the higher amount of available phenolics and flavonoids in the plant leaves are non-polar and the optimal concentration of the hexane extract to obtain this amount ranges between 2-4 mg/mL. The negative TPC value observed at 0.40 mg/mL of the hexane extract may suggest that this concentration was below the limits determined by the above standards and thus it could not be detected (Steven, 2006).

A similar finding was also observed in a previous study in which hexane extracts of *C. olitorius* leaves resulted in higher TPC and TFC values followed by alcoholic (either methanol or ethanol) extracts. It was suggested that as solvent polarity decreased, flavonoid content of the plant extracts increased (Sellami et al., 2013).

It must also be noted that differences in plant genetics, cultivation methods and practices, geographical conditions and other environmental factors could give rise to variations even in the same plant species (Phuyal et al., 2020).

Following hexane, the methanol extract had the second highest total phenol and flavonoid content (Table 3.2.2c, Table 3.2.3c). In the methanol extract, a dose-dependent pattern was not observed. Although in both TPC and TFC analyses, a reduction was observed from 36 mg/mL to 18 mg/mL, there was an upward trend at the lowest concentration of the methanol extract, which was 3.6 mg/mL.

The higher amount of phenolics and flavonoids in the highly polar methanol environment may be explained by the predominantly polar nature of the bioactive compounds found in the plant leaves. The increase in TPC and TFC seen at the lowest methanol extract concentration may be due to the more specific binding of $AlCl_3$ to different flavonoids in this extract. A higher concentration of the methanol extract may be more toxic and phenols

and/or flavonoids may be neutralising this oxidative toxicity and become partially used up in the process. However, a 10-fold reduction in the concentration (i.e., from 36 mg/mL to 3.6 mg/mL) may not result in such a toxic environment and a higher amount of free, unbound phenols and flavonoids may be available to form complexes with the FC reagent and $AlCl_3$. Alternatively, at higher concentrations, other bioactive compounds may absorb at the same wavelength or there may be non-specific binding with other reducing compounds in the extract resulting in the observed effects.

3.3.2 DPPH Assay

Given the role of oxidative damage in the development of cancer, evaluating the antioxidant capacity of plant extracts is important. The DPPH radical scavenging assay was conducted to measure the antioxidant capacity of the extracts. This is a simple and widely used assay which evaluates the ability of phytochemicals to neutralise free radicals by donating hydrogen or electron. The reaction results in a colour change from purple to yellow in proportion to the potency of the antioxidant compound (Biswas et al., 2020, 2023).

In this study, the methanol extract exhibited the strongest DPPH radical scavenging activity with an IC_{50} value of 51.70 µg/mL. This was followed by the acetone and hexane extracts with IC_{50} values of 252.40 µg/mL and 451.70 µg/mL, respectively. The extracts were compared against ascorbic acid, which had the lowest IC_{50} value of 6.78 µg/mL, possessing the most potent antioxidant activity. This was an expected finding since ascorbic acid is a pure antioxidant whereas the extracts contain a mixture of different phytochemicals of varying antioxidant activities.

Hydro-methanol and methanol extracts of *C. olitorius* leaves were previously reported to have IC_{50} values of 41.1 and 33.1 µg/mL, respectively (Eweka, Eromosele, Eluehike, & Orumwensodia, 2020; Meite, Agbo, Koffi, Djaman, & David N'guessan, 2018), which are similar to the value obtained in the present study. Methanol is an efficient solvent for

extracting strongly polar antioxidants from plant leaves with a higher extraction yield compared to other solvents (Sadat, Hore, Chakraborty, & Roy, 2017).

In previous studies, stronger antioxidant activity was observed in the polar extracts of *C. olitorius* leaves and this correlated with a higher phenol and flavonoid content of the plant extracts (Abuzaid et al., 2020; Fioroni et al., 2023; Oboh et al., 2009). Similarly in this study, the total phenol and flavonoid contents of the methanol extract were also found to be comparable to or higher than the acetone and hexane extracts extract (Table 3.2.2b, Table 3.2.3c).

However, it should be noted that the DPPH assay evaluates only one mechanism of antioxidant activity – free radical scavenging- and therefore, it does not fully represent the more complex antioxidant processes occurring *in vivo*. There is strong evidence showing that the bioactive form of flavonoids found in plants differ from their form *in vivo* and that these phytochemicals undergo extensive metabolism in the small intestine, liver and gut microbiota which alter their antioxidant properties (Williams, Spencer, & Rice-Evans, 2004). Furthermore, not all phenols or flavonoids have the same radical scavenging activity.

It was previously proposed that the antioxidant activity of an extract cannot be predicted solely based on its total phenol or flavonoid content. The antioxidant capacity of polyphenols depends on their overall chemical structure and their interaction with one another and with other phytochemicals present in the extracts. Additionally, the level of glycosylation and hydroxylation of the phenols within the plant may contribute to the overall antioxidant activity observed in this study (Biswas et al., 2020; Katerere et al., 2012).

Additional antioxidant assays, such as oxygen radical absorbance capacity and nitric oxide radical scavenging assays, could be planned as future work to strengthen the findings and provide a broader evaluation of the

antioxidant potential of the extracts, particularly the methanol extract (Fioroni et al., 2023).

3.3.3 GC-MS Analysis

Major lipophilic bioactive compounds in the extracts were identified through GC-MS analysis (Table 3.2.5a, Table 3.2.5b, Table 3.2.5c). In all three extracts, there were a number of siloxane compounds identified as GC-MS artifacts (Table 3.2.5a, Table 3.2.5b, Table 3.2.5c). During the GC-MS analysis, these compounds eluted from the column under the conditions used to elute phytochemicals and are not considered to be a component of *C. olitorius.* Siloxanes are silicon-containing polymers synthetically manufactured for various industrial purposes, including pharmaceutical, medical and cosmetic, and are not naturally found as plant compounds (Mojsiewicz-Pienkowska, Jamrógiewicz, Szymkowska, & Krenczkowska, 2016).

Some compounds were detected at two distinct retention times in the GC-MS analysis. In the methanol extract, 9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)- was identified in two separate peaks at 17.059 and 17.383 mins (Table 3.2.5c). Similarly, in the acetone extract, neophytadiene was detected twice at 14.453 and 14.900 mins (Table 3.2.5a). In the GC-MS analysis, a technique called derivatisation is applied in which compounds of interest are chemically modified to enhance their volatility and detectability (Orata, 2012). The appearance of a compound as two separate peaks may indicate that its derivatisation was incomplete at the first detection and that full conversion to a detectable derivative occurred at the second peak.

Among the other findings of this study is the extraction and identification of certain bioactive compounds in solvents which have different partition coefficient values or logP, which is a measure of lipophilicity. Chemical compounds with a logP value higher than zero, favour lipids over polar solvents and dissolve more efficiently (Atangcho, Navaratna, & Thurber, 2019). Hexane, acetone and methanol have logP values of 3.94, -0.16 and

-0.72, respectively (Royal Society of Chemistry, 2025), reflecting decreasing lipophilicity.

Even though hexane has the highest lipophilicity value and would be expected to dissolve more non-polar compounds, plant compounds with high logP values were also detected in the acetone and methanol extracts in this study. For instance, n-hexadecanoic acid (logP= 7.15) was detected in all three extracts. Likewise, 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-(logP=6.50) was identified in the acetone and hexane extracts (Table 3.2.5a, Table 3.2.5b, Table 3.2.5c).

These findings may suggest that some phytochemicals have partial solubility in solvents with varying lipophilicity and can still be detected, even in small amounts, using a sensitive analytical technique like GC-MS. Additionally, solvents do not exclusively dissolve compounds with identical polarity, but rather those within a similar range of polarity.

Another possible explanation is the co-extraction of plant compounds. Since plant extracts are a complex mixture of various phytochemicals, some components may facilitate the solubilisation and extraction of others, otherwise insoluble in a particular solvent, through molecular interactions.

Extraction and identification of some phytochemicals in solvents having different polarities or logP values is common and has been reported in other studies (Ahmad et al., 2021).

Findings of the GC-MS analysis support those of the DPPH assay. Several compounds identified via the GC-MS analysis have been shown to possess antioxidant activities in previous studies (Table 3.2.5a, Table 3.2.5b and Table 3.2.5c). One such compound is dihydroactinidiolide which was identified in all three extracts. Similarly, α-tocopherol (vitamin E) was identified in the acetone and hexane extracts. As one of the major compounds of the acetone extract, α-tocopherol may be responsible for the moderate free radical scavenging activity of this extract (IC_{50}= 252.40 μg/mL).

In the methanol extract, although it makes up a small percentage of the extract (1.46%), 2-methoxy-4-vinylphenol was also identified as an antioxidant compound. It is a small, single-ring phenolic compound which was shown to have various biological activities, such as anti-inflammatory and anti-cancer in addition to antioxidant, in previous studies (Table 3.2.5c).

Several other chemical compounds accounting for approximately 42% of the extract content with a lower matching quality (<80%), have been identified in the methanol extract which could have partially contributed to the antioxidant and anti-cancer effects observed in this study (data not shown). Among these are two pyrone compounds; 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (76% matching quality) and 2H-thiopyran, tetrahydro-4-methyl- (38% matching quality) eluted at 6.151 min and 10.906 min, respectively; benzofuran, 2,3-dihydro- (64% matching quality) eluted at 7.006 min and phenol, 4-ethenyl-2,6-dimethoxy- (60% matching quality) eluted at 11.363 min.

Benzofuran derivatives were reported to exert anti-proliferative effects on various cancer cell lines and appear to be promising candidates for the synthesis of anti-cancer agents with lower side effects (Abbas & Dawood, 2023; Khodarahmi, Asadi, Hassanzadeh, & Khodarahmi, 2015). Furthermore, pyrone ring-containing compounds are similar to flavonoids in chemical structure since flavonoids also contain a pyrone ring (Liga, Paul, & Peter, 2023).

Although not specifically identified, the methanol extract likely contained another phenolic compound, detected as phenol, TMS derivative (58% matching quality, RT 5.901 min). However, this requires further verification.

Two other phenolic compounds; 4-vinylbenzene-1,2-diol (76% matching quality) eluted at 9.803 min and a quinic acid, 1,3,4,5-tetrahydroxycyclohexanecarboxylic acid (49% matching quality) eluted at 11.986 min were identified. Quinic acid is found in the structure of chlorogenic acid and its isomers (J. G. Xu, Hu, & Liu, 2012) and 4-

vinylbenzene-1,2-diol, also known as 4-vinylcatechol, has been shown to possess antioxidant properties (Terpinc et al., 2011).

Despite having a low match score in GC-MS analysis, these compounds may suggest the potential presence of more phenolic compounds in the methanolic leaf extract of *C. olitorius*. This extract could be therefore further studied using a more suitable method as described below.

These phytochemicals might have also collectively contributed to the free radical scavenging activity observed in the DPPH assay and the high TPC value seen with the methanol extract.

Nevertheless, the relationship between the GC-MS findings and the TPC and TFC assay results should be interpreted with caution. The GC-MS method can detect small compounds that can be derivatised and volatised (Tsermoula, Khakimov, Nielsen, & Engelsen, 2021). Therefore, larger and non-volatile polyphenols or flavonoids with more aromatic rings present in the extracts cannot be readily identified through GC-MS.

Furthermore, thermally unstable, polar compounds extracted with methanol may not be suitable for identification using GC-MS. This may also explain the higher percentage of phytochemicals identified in the hexane (91%) and acetone extracts (86%), as seen in Figure 3.2.5a and Figure 3.2.5b. While the GC-MS method provided high resolution spectral data for compound identification in these extracts, it was less effective for the methanol extract, identifying only 19% of its compounds (Figure 3.2.5c).

Other highly specific analytical methods, such as LC-MS or LC-MS/MS could be a better alternative for the identification of unknown secondary plant metabolites in more polar environments such as ethanol or methanol extracts. However, while these methods could have been more useful for the assessment of methanolic extracts, technical factors such as availability of and access to the laboratories with such instruments, and relevant online libraries which could identify unknown compounds in a mixture were very limited causing a major constraint in this study. Instead

of LC-MS, GC-MS analysis with an extensive online library was accessible and therefore, was used to assess the methanol extract together with the acetone and hexane extracts in the current study. There are also other studies in the literature that have investigated the phytochemical content of methanolic and ethanolic plant extracts using the GC-MS method, supporting its use for analysing more aqueous alcoholic plant extracts when more suitable techniques are not available (Isbilen & Volkan, 2021; Kavaz & Faraj, 2023; Ozbil et al., 2024).

Nevertheless, the GC-MS data show similarities with previous studies investigating the extracts of *C. olitorius* leaves. α-tocopherol was also identified in an earlier study (Azuma et al., 1999). Presence of carotenoids, terpenoids, α-tocopherol and ionones in *C. olitorius* leaves were also reported (Hasan & Kadhim, 2018; Oboh et al., 2009; Ragasa et al., 2016; Ujah et al., 2014). In this study, neophytadiene, 2-Pentadecanone, 6,10,14-trimethyl-, phytol and 4,8,12,16-Tetramethylheptadecan-4-olide were detected as terpenes; dihydroactinidiolide and loliolide as carotenoid metabolites; and 3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)- was identified as β-ionone.

Several fatty acids and fatty acid methyl esters have also been reported in the leaf extracts of *C. olitorius* in previous studies. Among these are hexadecanoic acid and 9,12-Octadecadienoic acid (Z,Z)- (Al-Yousef, Amina, & Ahamad, 2017; Hasan & Kadhim, 2018), hexadecanoic acid methyl ester, methyl stearate, 7-Hexadecenoic acid, methyl ester, (Z)-, 9,12-Octadecadienoic acid (Z,Z)-, methyl ester and 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (T. Nguyen, Aparicio, & Saleh, 2016).

3.4 Conclusion

Different methods can be applied to extract bioactive compounds from plants and they all have advantages and disadvantages. Maceration was preferred in the present study since it does not involve high heat as part of the process and it has a simple, low-cost application.

Evaluation of the extraction yields using methanol, hexane and acetone has shown similarities with previous studies such that hexane extraction resulted in the lowest yield whereas methanol extraction resulted in the highest.

TPC and TFC assays are useful and practical methods to provide basic information about the phenolic and flavonoid content of plant extracts. In this study, hexane and methanol extracts had higher total phenol and total flavonoid content than the acetone extract suggesting that the jute leaves may contain highly polar and non-polar phytochemicals rather than compounds of moderate polarity. One of the main objectives of this study was to investigate the synergistic effect of various phytochemicals, including polar and non-polar compounds. In this study, TPC and TFC assays have provided relevant information about the overall phenol and flavonoid content of the plant leaves.

The DPPH assay has shown the lowest IC_{50} value for the methanol extract, followed by the acetone and hexane extracts, suggesting the presence of highly polar phytochemicals in the methanol extract which possess strong free radical scavenging activity.

The GC-MS analysis has provided more specific information about the phytochemical content of *C. olitorius* leaf extracts and enabled identification of a vast number of bioactive compounds with antioxidant, anti-inflammatory and anti-cancer properties supporting previous evidence in the literature.

Chapter 4 - Effects of *C. olitorius* extracts on Proliferation of Breast Cancer Cells

4.1 Introduction

4.1.1 Cell Proliferation

In normal cells, cell survival and proliferation are tightly regulated processes through different pathways involving growth factors (GF) and hormones. This ensures cells undergo controlled cell-cycle events and cell numbers are maintained in homeostasis via initiation of apoptotic pathways, if necessary (Feitelson et al., 2015).

Apoptosis is a programmed cell death which can be initiated either via the mitochondria-dependent intrinsic pathway or the extrinsic pathway, which involves ligand binding to transmembrane death receptors. Intracellular stress, such as hypoxia, GF withdrawal and DNA damage, can stimulate the intrinsic apoptotic pathway. This induces a series of events in which cytochrome c, a critical protein in the activation of caspases, is released into the cytosol from the inner membrane of mitochondria as a result of mitochondrial outer membrane permeabilisation. Cytochrome c acts as a co-factor for apoptotic protease-activating factor-1 (APAF-1) which subsequently results in the activation of the initiator caspase, caspase 9 and the executioner caspases, caspase 3 and caspase 7. Activation of caspase 3 is a major step in downstream signalling through the apoptotic cascade and therefore, caspase 3 is an important protein in cancer research (Brentnall, Rodriguez-Menocal, De Guevara, Cepero, & Boise, 2013; Chipuk, Bouchier-Hayes, & Green, 2006; Shanmugaraj et al., 2010; Shi, 2002).

Any alteration in signalling pathways allows cells to enter the cell cycle and continue replication. In cancer cells, there may be different factors that cause cell proliferation to continue, including mutations in cancer-critical genes and/or epigenetic factors that act on gene expression. It is relatively

easier to reverse epigenetic modifications with pharmacotherapeutics than target genes involved in cancer development (Feitelson et al., 2015).

Another mechanism in cancer cells that sustains proliferation is the binding of GFs to cell surface receptors which eventually stimulate intracellular pathways to continue cell survival and growth, maintain cellular metabolism and/or escape apoptotic pathways (Feitelson et al., 2015; Hanahan & Weinberg, 2011).

It is therefore important to gain a deeper understanding of these mechanisms in order to look for and design novel anti-cancer therapies.

4.1.2 Breast Cancer Cell Lines

Cell lines used in this study were MDA–MB-231, MDA-MB-468 and MCF-7 cells, which were adherent adenocarcinomas. MCF-7 is an ER- and PR-positive, hormone dependent human cancer cell line which is non-invasive and has low metastatic potential (Comşa, Cîmpean, & Raica, 2015). It represents the most frequently seen breast cancer making up 70% of cases (Milani, Gautam, & Potter, 2009).

MDA-MB-231 and MDA-MB-468 are two highly metastatic breast cancer cell lines evaluated in the current study. Both of them lack ER-, PR-expression and HER2 amplification hence are implicated in the aggressive TNBC (European Collection of Authenticated Cell cultures, 2017; Greenshields, Power Coombs, Fernando, Holbein, & Hoskin, 2019). Since one of the cell lines used in this study represents the majority of breast cancers and the other two are among the most aggressive forms, their proliferative behaviour when treated with *C. olitorius* extracts would be very ideal to investigate.

4.2 Results

In the proliferation assay, 8 different concentrations of each extract were compared against a negative control containing untreated cells in DMSO/media over a treatment period of 72 hrs. All data are presented as

a percent of the control mean ± S.D of eight replicates per each concentration of an extract. Results are given below.

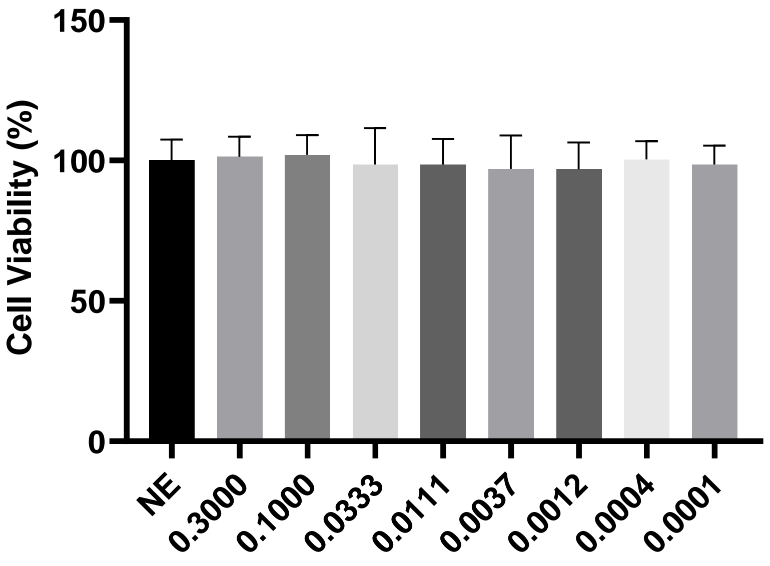
4.2.1 Effects of the Acetone Extract on Breast Cancer Cell Lines

One-way ANOVA test was used to test whether there was a significant difference in the anti-proliferative effects of the acetone extract compared to the control. There was no significant difference between different concentrations of the acetone extract and the control. In other words, bioactive compounds in the acetone extract of *C. olitorius* did not seem to exert any statistically significant anti-proliferative effect relative to the control in any of the three breast cancer cell lines tested.

In MCF-7 cells, there was no statistically significant difference between the means of various concentrations of the acetone extract and the negative control. This suggests that cell viability remained unchanged across both stronger (0.3000 mg/mL) and weaker (0.0001 mg/mL) concentrations (Graph 4.2.1.1).

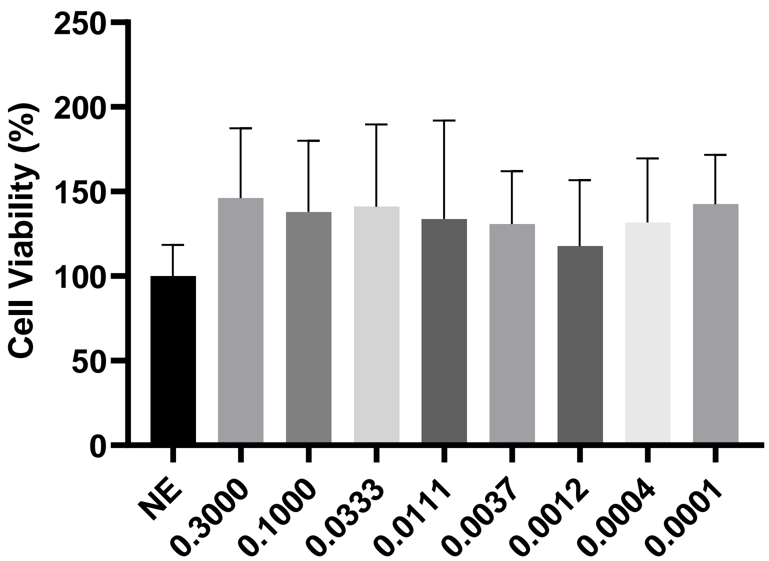
Similarly, the acetone extract had no effect on the proliferation of MDA-MB-231 cells. Although the percent cell viability was higher than the control at all concentrations, the differences were not statistically significant (Graph 4.2.1.2).

On the other hand, percent viability in the extract-treated MDA-MB-468 cells appear to be lower than the control. However, this lower percentage was not statistically significant (Graph 4.2.1.3). Overall, these results suggest that the acetone extract did not inhibit cell proliferation in these cell lines.



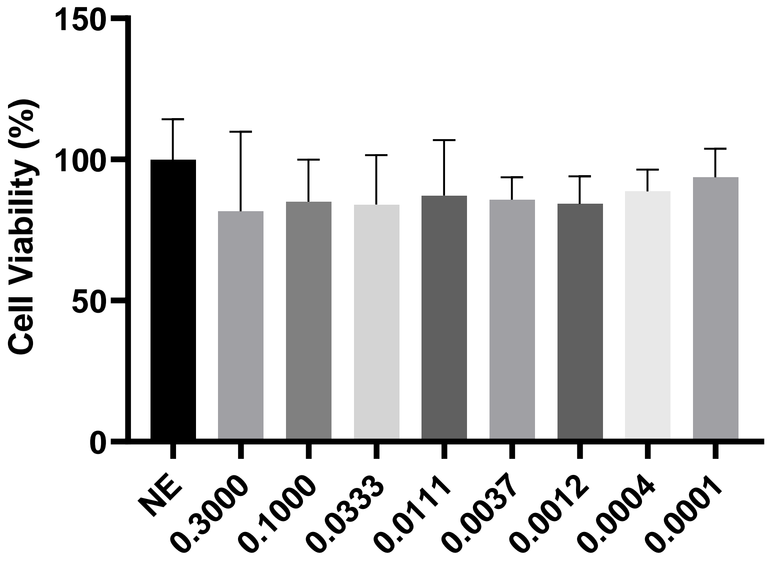
Graph 4.2.1.1: Effect of the Acetone Extract on Proliferation of MCF-7 cells

Anti-proliferative effects of *C. olitorius* extract in acetone of various concentrations were evaluated on MCF-7 cells. The negative control, represented as NE (no extract), consisted of cells in DMSO/media but no extract. Data are presented as a percent of the control mean ± S.D of eight wells. No significant difference was observed when different concentrations were compared to the control.



Graph 4.2.1.2: Effect of the Acetone Extract on Proliferation of MDA-MB-231 cells

Effects of *C. olitorius* extract in acetone of various concentrations were evaluated on MDA-MB-231 cells. The negative control, represented as NE (no extract), consisted of cells in DMSO/media but no extract. Data are presented as a percent of the control mean ± S.D of eight wells. No significant difference was observed when different concentrations of the extract were compared to the control.



Graph 4.2.1.3: Effect of the Acetone Extract on Proliferation of MDA-MB-468 cells

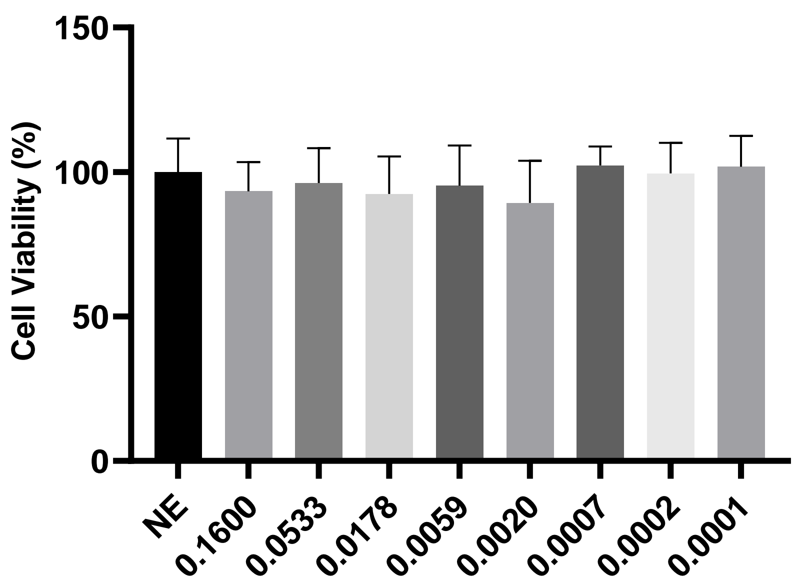
Effects of *C. olitorius* extract in acetone of various concentrations were evaluated on MDA-MB-468 cells. The negative control, represented as NE (no extract), consisted of cells in DMSO/media but no extract. Data are presented as a percent of the control mean ± S.D of eight wells. No significant difference was observed when different concentrations were compared to the control.

4.2.2 Effects of the Hexane Extract on Breast Cancer Cell Lines

Similar to the acetone extract, cell viability as a percent of the control mean was not found to be significantly different in MCF-7 and MDA-MB-231 cells. In MCF-7 cells, the mean viability at each concentration of treatment was almost the same as the control mean. In MDA-MB-231 cells, some concentrations of the extract, such as 0.1600 mg/mL and 0.0178 mg/mL, induced higher viability than the control mean, however, this was not statistically significant (Graph 4.2.2.1, Graph 4.2.2.2).

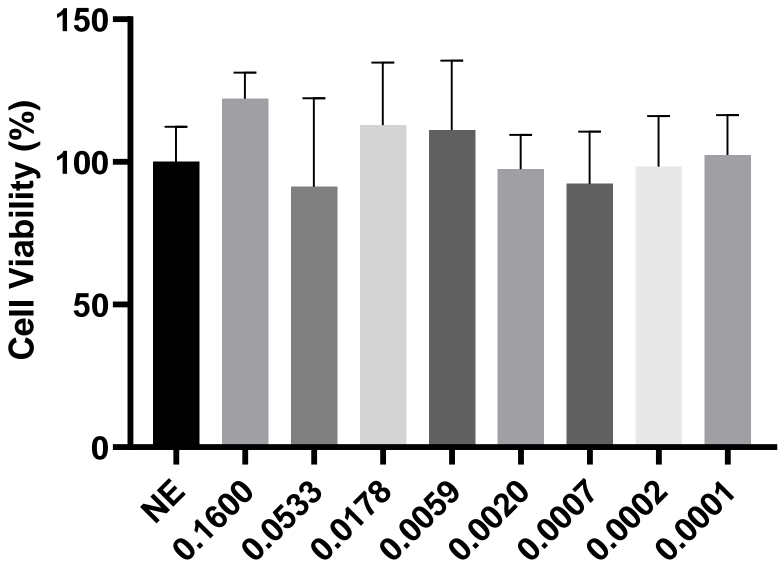
Statistically significant differences between various concentrations of the hexane extract and the control were observed in MDA-MB-468 cells (Graph 4.2.2.3). According to the findings, 0.1600 mg/mL, 0.0533 mg/mL and 0.0001 mg/mL of extract were significantly different from the control at $p<0.05$ level, with means 83.55%, 84.36% and 85.77%, respectively. The

concentrations of 0.0059 mg/mL, percent mean=79.56%, and 0.002 mg/mL, percent mean=79.24%, were also more significantly different than the control ($p<0.001$). In summary, the viability of MDA-MB-468 cells was reduced significantly at these concentrations of the hexane extract compared to the control cells. These findings suggest that the bioactive compounds of *C. olitorius* leaves extracted with hexane exerted their effects inhibiting cell survival and proliferation over 72 hours of incubation. Based on these findings, it can be seen that, of all the concentrations tested, 0.0059 mg/mL and 0.002 mg/mL were found to be the extract concentrations with greatest inhibition.



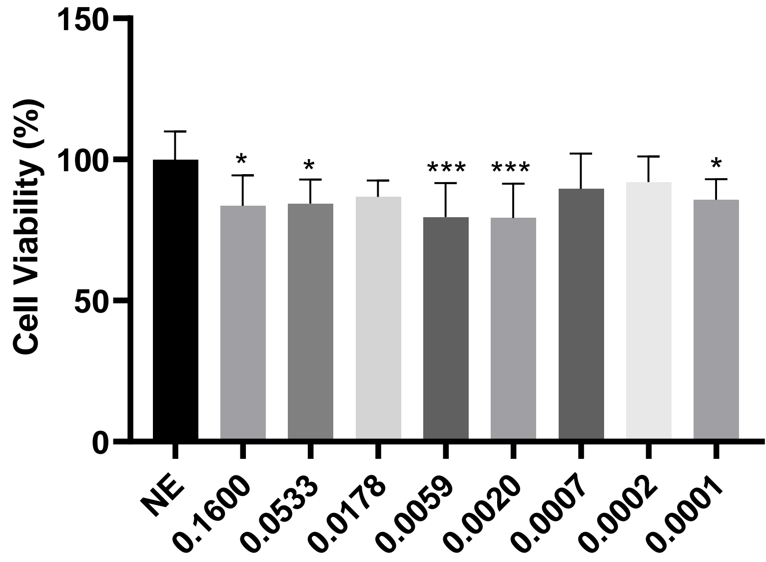
Graph 4.2.2.1: Effect of the Hexane Extract on Proliferation of MCF-7 cells

Anti-proliferative effects of *C. olitorius* extract in hexane were evaluated on MCF-7 cells. The negative control, represented as NE (no extract), consisted of cells in DMSO/media but no extract. Data are presented as a percent of control mean ± S.D of eight wells. None of the eight concentrations were significantly different from the control.



Graph 4.2.2.2: Effect of the Hexane Extract on Proliferation of MDA-MB-231 cells

Effects of C. olitorius extract in hexane were investigated on MDA-MB-231 cells. The negative control, represented as NE (no extract), consisted of cells in DMSO/media but no extract. Data are presented as a percent of control mean ± S.D of eight wells. Different concentrations of the extract were not significantly different from the control.



Graph 4.2.2.3: Effect of the Hexane Extract on Proliferation of MDA-MB-468 cells

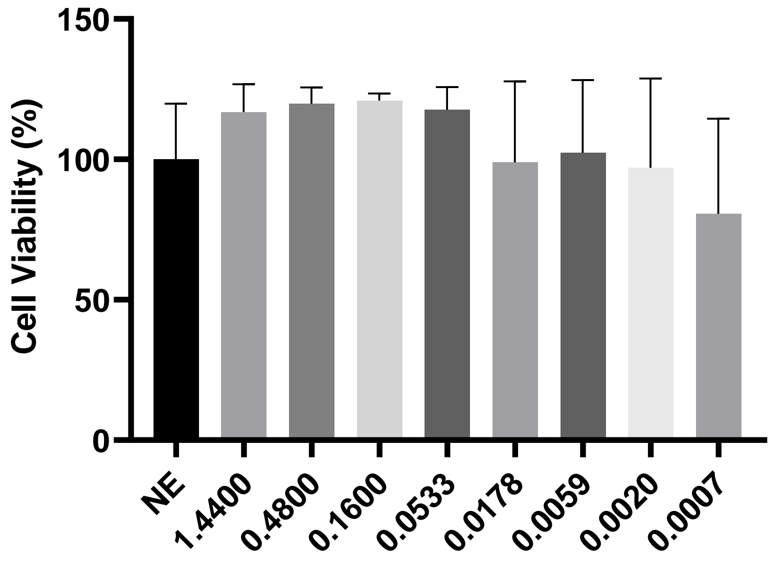
Effects of C. olitorius extract in hexane on MDA-MB-468 cells are given above. A negative control, represented as NE (no extract), was used which consisted of cells in DMSO/media without extract. Data are presented as a percent of control mean ± S.D of eight wells, where * represents statistical significance at p<0.05, and *** at p<0.001 level.

4.2.3 Effects of the Methanol Extract on Breast Cancer Cell Lines

The behaviour of MCF-7 and MDA-MB-231 cells in the presence of the methanol extract was similar to that of the hexane extract. None of the 8 concentrations showed a statistically significant difference from the mean of control (Graph 4.2.3.1, Graph 4.2.3.2). Even though lower concentrations showed reduced cell viability in MCF-7 cells, the difference was not significant.

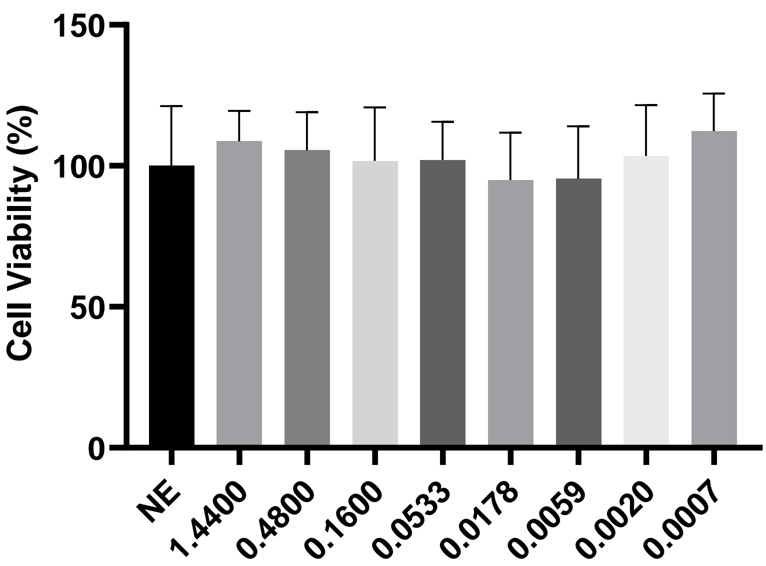
However, in MDA-MB-468 cells, cell viability was significantly different at 0.0533 mg/mL, 0.1600 mg/mL and 0.0178 mg/mL of the methanol extract (Graph 4.2.3.3). The percent mean at 0.0533 mg/mL was 84.99% (p<0.05). Cell viability in the latter two concentrations decreased more significantly than the control with percent means of 83.61% and 82.51%, respectively (p<0.01). According to these findings, of all the concentrations assessed in

this study, 0.0533 mg/mL, 0.1600 mg/mL and 0.0178 mg/mL of the methanol extract seem to inhibit cell proliferation in MDA-MB-468 cells. Since these three concentrations are successive, it appears that the optimal anti-proliferative concentration of the methanol extract is within this range.



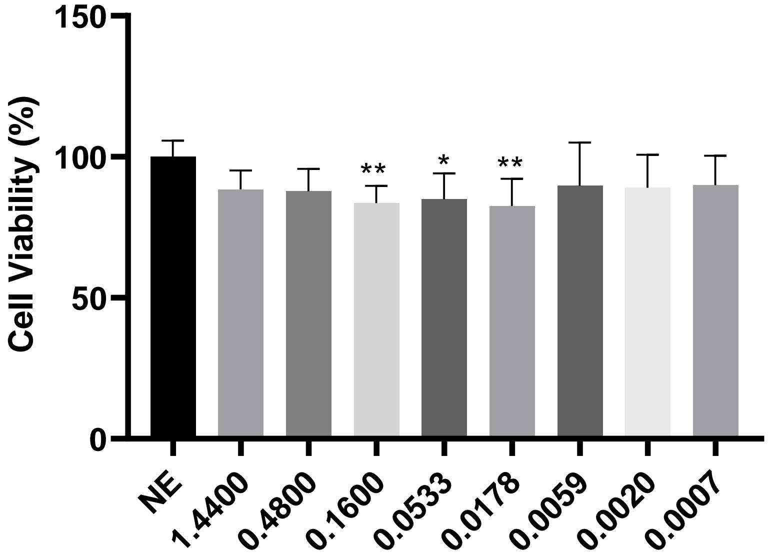
Graph 4.2.3.1: Effect of the Methanol Extract on Proliferation of MCF-7 cells

Effects of *C. olitorius* extract in methanol were evaluated on the proliferation of MCF-7 cells. The negative control, represented as NE (no extract), consisted of cells in DMSO/media but no extract. Data are presented as a percent of control mean ± S.D of eight wells. Different concentrations of the extract were not significantly different from the control.



Graph 4.2.3.2: Effect of the Methanol Extract on Proliferation of MDA-MB-231 cells

Effects of *C. olitorius* extract in methanol were evaluated on MDA-MB-231 cells. The negative control, represented as NE (no extract), consisted of cells in DMSO/media but no extract. Data are presented as a percent of control mean ± S.D of eight wells. No statistically significant difference was observed.



Graph 4.2.3.3: Effect of the Methanol Extract on Proliferation of MDA-MB-468 cells

Different concentrations of *C. olitorius* in methanol were used to examine the effects on MDA-MB-468 cells. The negative control used, represented as NE (no extract), consisted of cells in DMSO/media but no extract. Data are presented as a percent of control mean ± S.D of eight wells in which * represents statistical significance at $p<0.05$ and ** represents significance at $p<0.01$ level.

Overall, results indicate that the acetone extract does not have any anti-proliferative effect on MCF-7, MDA-MB-231 and MDA-MB-468 cells. However, the bioactive compounds released in the hexane and methanol extracts showed significantly inhibited cell proliferation in MDA-MB-468 cells compared to the control.

4.2.4 Assay of Caspase 3 and Caspase 7 Expression

Concentrations of the hexane and methanol extracts, which significantly reduced cell proliferation, were further analysed for potential mechanisms involving caspase-dependent pathways in the metastatic cell lines, MDA-MB-231 and MDA-MB-468. Specifically, 0.16, 0.0533, 0.0059, 0.0020 and 0.0001 mg/mL of the hexane extract, and 0.16, 0.0533 and 0.0178 mg/mL of the methanol extract were further assessed to determine the levels of caspase 3 and caspase 7, which are expected to increase as a trigger for

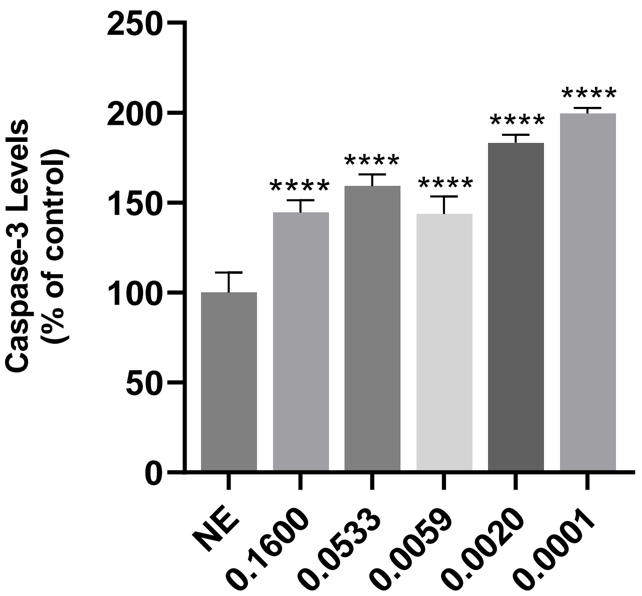
apoptosis. All data are presented as a percentage of the control mean ± S.D, based on statistical analysis of four replicates using one-way ANOVA.

4.2.4.1 Effects of the Hexane Extract on the Expression of Caspase 3 and Caspase 7

In both MDA-MB-231 and MDA-MB-468 cells, the expression of caspase 3 and caspase 7 was significantly higher in cells treated with the hexane extract than untreated controls.

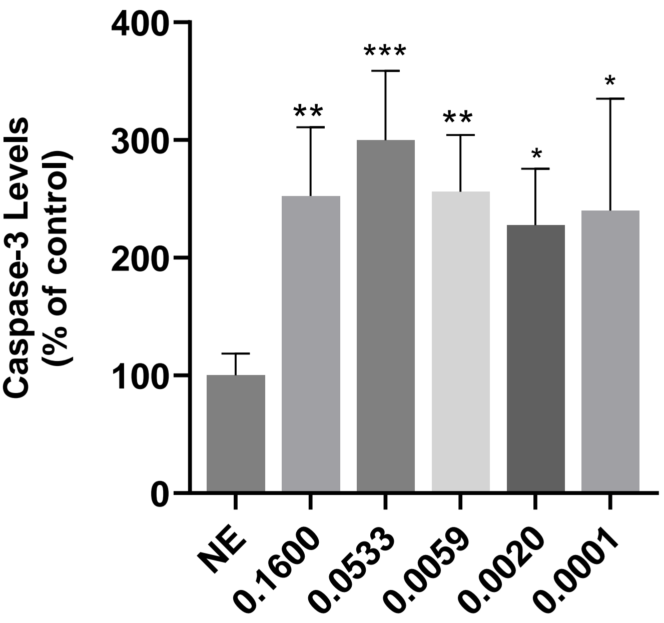
In MDA-MB-231 cells, the percent means of caspase 3 levels that were significantly different from the control were 144.4% for 0.1600 mg/mL, 159.2% at 0.533 mg/mL, 143.6% at 0.0059 mg/mL, 183.2% at 0.0020 mg/mL, and 199.5% at 0.0001 mg/mL of hexane extract ($p<0.0001$). The percent means of caspase 7 levels, which were significantly higher than the control, were 123.2% at 0.1600 mg/mL ($p<0.0001$), 115.0% at 0.533 mg/mL ($p<0.01$), 116.5% at 0.0059 mg/mL ($p<0.01$), 137.1% at 0.0020 mg/mL, and 133.7% at 0.0001 mg/mL of hexane extract ($p<0.0001$).

In MDA-MB-468 cells, the percent means of caspase 3 levels that were significantly different from the control were 252.5% at 0.1600 mg/mL ($p<0.01$), 299.9% at 0.533 mg/mL ($p<0.001$), 256.0% at 0.0059 mg/mL ($p<0.01$), 227.6% at 0.0020 mg/mL, and 240.1% at 0.0001 mg/mL of hexane extract ($p<0.05$). For caspase 7, 0.1600 mg/mL of the hexane extract did not have a percent mean that was significantly different from the control unlike other concentrations. The percent means of those that were significantly higher were 174.1% at 0.533 mg/mL ($p<0.01$), 163.1% at 0.0059 mg/mL ($p<0.05$), 170.1% at 0.0020 mg/mL ($p<0.05$), and 199.4% at 0.0001 mg/mL of hexane extract ($p<0.001$). Results are given below.



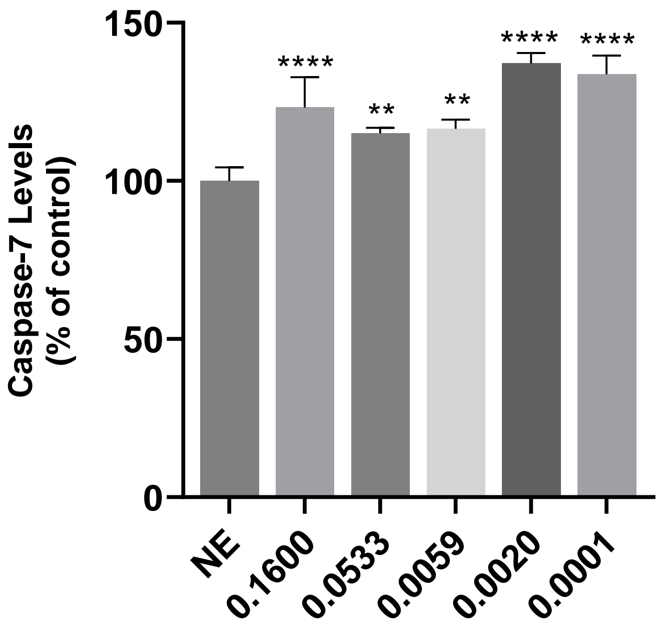
Graph 4.2.4.1a: Effect of Hexane Extract on Caspase 3 Levels in MDA-MB-231 Cells

The levels of Caspase 3 were measured in MDA-MB-231 cells treated with the hexane extract of *C. olitorius*. The negative control, given as NE (no extract), consisted of cells in DMSO/media without extract. Levels of caspase 3 in all concentrations of the hexane extract were significantly higher than the control ($p<0.0001$).



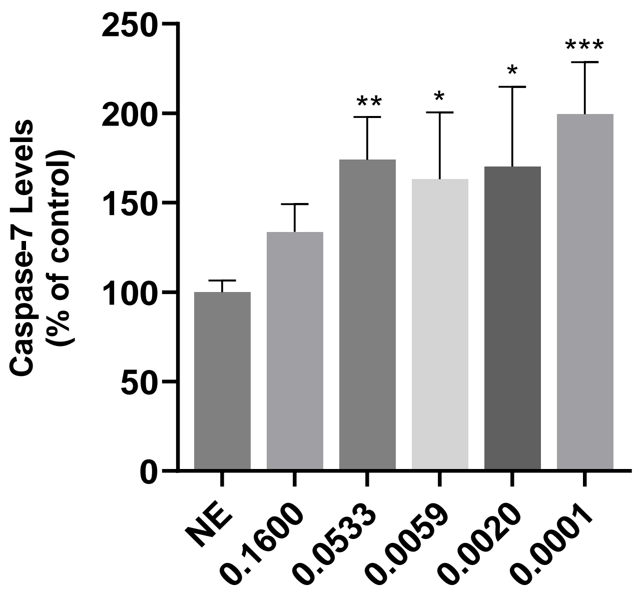
Graph 4.2.4.1b: Effect of Hexane Extract on Caspase 3 Levels in MDA-MB-468 Cells

The levels of Caspase 3 were measured in MDA-MB-468 cells treated with the hexane extract of *C. olitorius*. The negative control, given as NE (no extract), consisted of cells in DMSO/media without extract. Levels of caspase 3 were significantly different from the control in all concentrations of the hexane extract, where * represents statistical significance at $p<0.05$, ** at $p<0.01$ level, and *** at $p<0.001$.



Graph 4.2.4.1c: Effect of Hexane Extract on Caspase 7 Levels in MDA-MB-231 Cells

The levels of Caspase 7 were measured in MDA-MB-231 cells treated with the hexane extract of *C. olitorius*. The negative control, given as NE (no extract), consisted of cells in DMSO/media without extract. Levels of caspase 7 were significantly higher than the control in all concentrations of the hexane extract, where ** represents statistical significance at $p<0.01$ level, and **** at $p<0.0001$.



Graph 4.2.4.1d: Effect of Hexane Extract on Caspase 7 Levels in MDA-MB-468 Cells

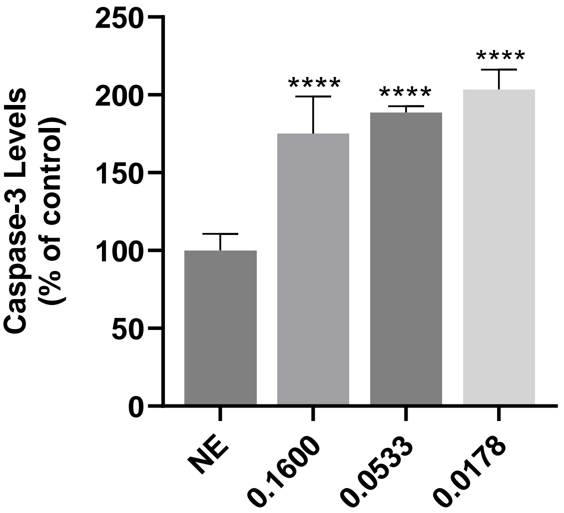
The level of Caspase 7 was measured in MDA-MB-468 cells treated with the hexane extract of *C. olitorius*. The negative control, given as NE (no extract), consisted of cells in DMSO/media without extract. Levels of caspase 7 were significantly higher than the control in all concentrations of the hexane extract, where * represents statistical significance at p<0.05, ** at p<0.01 level, and *** at p<0.001.

4.2.4.2 Effects of the Methanol Extract on the Expression of Caspase 3 and Caspase 7

In MDA-MB-231 cells, the expression of caspase 3 and caspase 7 was significantly higher in cells treated with the methanol extract than their untreated controls. The percent means of caspase 3 levels that were significantly different from the control were 175.1% at 0.1600 mg/mL, 188.8% at 0.533 mg/mL, and 203.4% at 0.0178 mg/mL of methanol extract (p<0.0001). Similarly for caspase 7, The percent means of caspase 7 levels that were significantly higher than the control was 141.0% at 0.1600 mg/mL, 149.8% at 0.533 mg/mL, and 152.3% at 0.0178 mg/mL of methanol extract (p<0.0001).

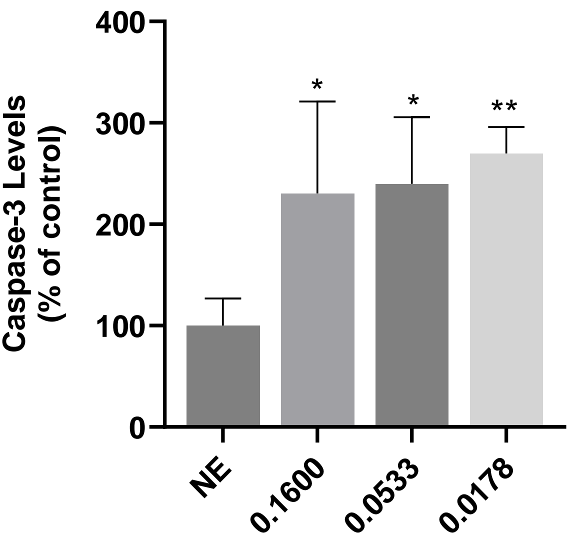
In MDA-MB-468 cells, caspase 3 expression was significantly higher than that of the control. The percent means of caspase 3 levels were 230.6% at 0.1600 mg/mL (p<0.05), 239.9% at 0.533 mg/mL (p<0.05), and 269.8% at

0.0178 mg/mL of methanol extract (p<0.01). However, although the percent mean of caspase 7 was higher in all three extract concentrations, none of them were significantly different from the control. Results are shown below.



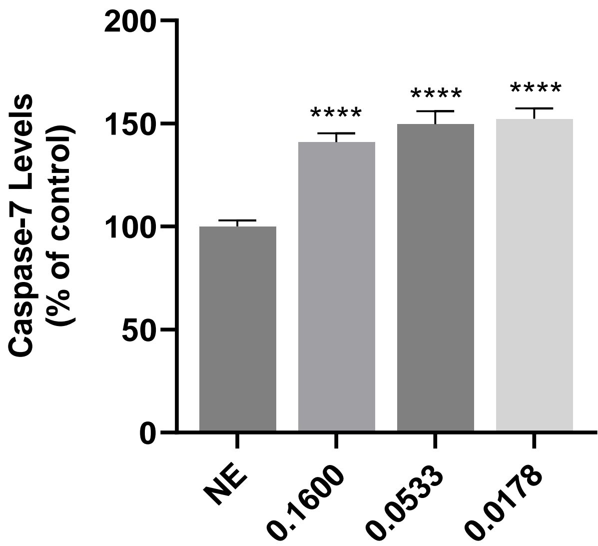
Graph 4.2.4.2a: Effect of Methanol Extract on Caspase 3 Levels in MDA-MB-231 Cells

The level of Caspase 3 was measured in MDA-MB-231 cells treated with the methanol extract of *C. olitorius*. The negative control, given as NE (no extract), consisted of cells in DMSO/media without extract. Levels of caspase 3 in all three concentrations of the methanol extract were significantly higher than the control (p<0.0001).



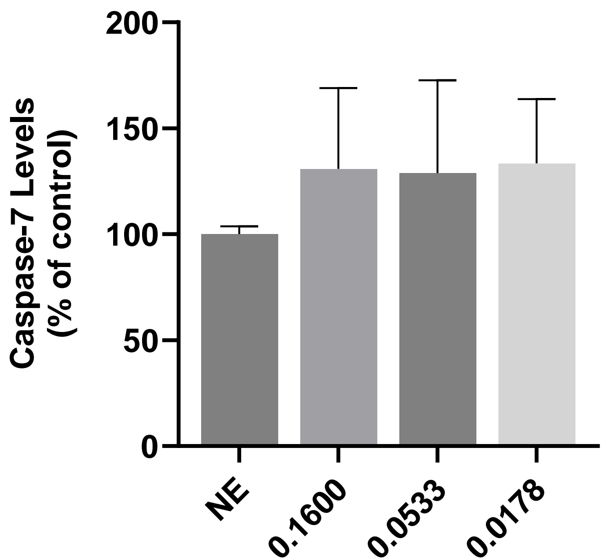
Graph 4.2.4.2b: Effect of Methanol Extract on Caspase 3 Levels in MDA-MB-468 Cells

The levels of Caspase 3 were measured in MDA-MB-468 cells treated with the methanol extract of *C. olitorius*. The negative control, given as NE (no extract), consisted of cells in DMSO/media without extract. Levels of caspase 3 in all three concentrations of the methanol extract were significantly higher than the control, where * represents statistical significance at $p<0.05$, ** at $p<0.01$ level.



Graph 4.2.4.2c: Effect of Methanol Extract on Caspase 7 Levels in MDA-MB-231 Cells

The level of Caspase 7 was measured in MDA-MB-231 cells treated with the methanol extract of *C. olitorius*. The negative control, given as NE (no extract), consisted of cells in DMSO/media without extract. Levels of caspase 7 in all three concentrations of the methanol extract were significantly higher than the control ($p<0.0001$).



Graph 4.2.4.2d: Effect of Methanol Extract on Caspase 7 Levels in MDA-MB-468 Cells

The level of Caspase 7 was measured in MDA-MB-468 cells treated with the methanol extract of *C. olitorius*. The negative control, given as NE (no extract), consisted of cells in DMSO/media without extract. Caspase 7 expression in experimental cells was not statistically different from their untreated controls.

4.3 Discussion

Cell proliferation occurs through the cell cycle and is a tightly regulated series of events which follows replication of DNA, organelles and division of the cell. Disruption of the cell cycle and unbalanced proliferation is among the characteristics of cancer (Loftus, Amend, & Pienta, 2022). The aim of this assay was to investigate the potential anti-proliferative effects of the *C.olitorius* leaf extracts on MCF-7, MDA-MB-231 and MDA-MB-468 cells and further investigate and compare the possible role of caspases, caspase 3 and 7, in inhibition of proliferation in the metastatic cell lines.

CV staining assay is a simple, reproducible, non-enzymatic assay used to study anti-cancer drugs that is based on the principle that cells, which are viable and proliferating, adhere to the surface of the wells whereas those undergoing cell death lose their adherence and are released from the cell population. The cytotoxic dye binds to the proteins and double helix of the

DNA. Therefore, the amount of staining is strongly associated with the amount of DNA in the cell culture. This allows for the estimation of the number of proliferating cells in a population (Feoktistova, Geserick, & Leverkus, 2016; Śliwka et al., 2016).

CV staining is not affected by cellular metabolism and metabolic conditions like the MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, which brings a competitive advantage over this method (Śliwka et al., 2016). However, it may have other limitations.

In some cases, if the cell culture has exceeded its confluence and cells continue proliferating, the absorbance may not truly reflect the number of viable adherent cells (Chiba, Kawakami, & Tohyama, 1998). In this study, various cell concentrations were initially tested to determine the ideal working cell concentration and the absorbance measurements were repeated multiple times to reduce the risk of error.

Another potential limitation of the CV assay is the non-specific binding that could occur in some instances between the dye and dead cells that have not still detached from the surface (Chiba et al., 1998).

In order to eliminate these confounding factors and increase the accuracy of the assay, the experiment was repeated and multiple readings were measured. Furthermore, the washing steps were carried out carefully to remove as many dead cells and debris as possible. Nevertheless, it should be noted that all cell viability assays have their own limitations. However, it was not within the scope of this study to compare their differences.

The solvents used to extract phytochemicals of varying polarity from the leaves of *C. olitorius* were acetone, hexane and methanol. According to the findings of this study, non-metastatic MCF-7 and metastatic MDA-MB-231 cells did not show any significant difference from the control cells in the extracts, suggesting that the phytochemicals did not significantly inhibit proliferative activities of these cell lines. This was also observed with MDA-MB-468 cells in the acetone extract. However, there was a significant

difference between the control cells and extract-treated MDA-MB-468 cells in the hexane and methanol extracts.

In this study, the hexane extract significantly reduced proliferation in MDA-MB-468 cells after a 72-hr treatment, showing the highest inhibitory effect at 0.0059 mg/mL and 0.002 mg/mL with 79.56% and 79.24% inhibition, respectively (Graph 4.2.2.3). Since hexane is an organic solvent which dissolves highly non-polar compounds (Altemimi, Lakhssassi, Baharlouei, Watson, & Lightfoot, 2017), the non-polar bioactive compounds extracted from the leaves and the interaction between them are thought to be responsible for the significant cytotoxic effect.

Unlike hexane, methanol is a highly polar solvent and is used in the extraction of more aqueous substances (Truong et al., 2019). Phytochemicals in the methanol extract also reduced proliferation of MDA-MB-468 cells (Graph 4.2.3.3).

However, in the acetone extract, which is a less polar solvent than methanol but more polar than hexane (Altemimi et al., 2017), bioactive plant chemicals did not have any significant effect on cell proliferation (Graph 4.2.1.3).

Although studies in the literature have largely looked into the anti-cancer effects of individual compounds isolated from plants, several studies investigated how aqueous and alcoholic (ethanol or methanol) extracts affected various cancer cell lines.

In a study, in which A-375 (human melanoma), AGS (gastric cancer) and SUIT-2 (pancreatic cancer) cells were investigated, cells were treated with an aqueous extract of *C.olitorius* and its isolated components, chlorogenic acid and isoquercetin, for 48 hours to evaluate their effects on proliferation. The aqueous extract of *C.olitorius* and its isolates significantly inhibited cell proliferation in a dose-dependent manner. The concentration of the aqueous extract needed to inhibit half of the total cell growth was 2.54, 4.05, and 6.47 mg/mL for AGS, A-375, and SUIT-2 cancer cell lines, respectively

(Tosoc, Nuñeza, Sudha, Darwish, & Mousa, 2021). However, the extract concentrations used for AGS, A-375, and SUIT-2 cells were much higher, about 10-20 times higher than the highest concentration used in the present study. These differences could be due to the use of different cancer cell lines which could affect different molecular pathways within the cell.

In another study, it was reported that HepG2 cells treated with the ethanol extract of the leaves had a significant reduction in cell viability in a dose dependent manner after 24 hours of treatment. It was also observed that the ethanol extract exhibited partial selective cytotoxicity against HepG2 cells even at a concentration of 0.0125 mg/mL with 52.2% inhibition but had no effect on normal hepatocytes (C. Li et al., 2012). The extract concentration of 0.0125 mg/mL is within the range used in this study, which supports the anti-proliferative effects of the methanol extract on MDA-MB-468 cells.

The effects of the methanol extract of *C.olitorius* leaves were also tested on the human colorectal cancer cell line, Caco-2, and similar results were reported. A partial but statistically significant inhibition (39%) was observed on the proliferation of Caco-2 cells without any cytotoxic effects on the healthy human intestinal mucosa cells (Guzzetti et al., 2021).

As mentioned in Chapter 3, the major compounds identified in the acetone extract were α-linolenic acid, phytol, α-tocopherol and palmitic acid (Table 3.2.5a). Previously it was shown that α-linolenic acid supplementation significantly suppressed cell growth in MDA-MB-231, HBL-100, ZR-75 and T-47-D breast cancer cells. However, cell proliferation was restored by 50% when α-tocopherol was given to the cells, showing their opposite effects (Chajès, Sattler, Stranzl, & Kostner, 1995). In the current study, the non-significant anti-proliferative effect of the acetone extract on the experimental cells might be due to the antagonistic effects of α-linolenic acid and α-tocopherol, which were present at a higher amount in this extract. It is also possible that other bioactive compounds had similar antagonistic effects resulting in a final non-significant effect in all the cell

lines tested. These findings highlight the complexity of assessing the collective effects of plant extracts in biological systems.

4.3.1 Evaluation of Caspase 3/7 Expression

In the present study, caspase-dependent apoptosis was further investigated as a possible anti-proliferative mechanism. Since MCF-7 cells are known to be deficient in caspase 3 (S. Yang, Liu, Thor, & Yang, 2007), the two metastatic cell lines, MDA-MB-231 and MDA-MB-468 cells, were analysed and compared for their differences in caspase 3 and caspase 7 expression as the executioner caspases in the apoptotic mechanism.

In both MDA-MB-231 and MDA-MB-468 cells, caspase 3 levels were significantly higher in the hexane extract group than in the controls, with increases of 44.4-99.5% in MDA-MB-231 cells (Graph 4.2.4.1a: and 127.6-200% in MDA-MB-468 cells (Graph 4.2.4.1b). A similar pattern was observed in the experimental cells treated with the methanol extract, with increases ranging from 75.1-103.4% in MDA-MB-231 cells (Graph 4.2.4.2a) and 130.6-169.8% in MDA-MB-468 cells (Graph 4.2.4.2b), suggesting that caspase 3-dependent apoptotic programme might have been activated in the inhibition of proliferation in these cells when treated with the hexane and methanol extracts. However, caspase 7 expression was differentially affected in these cell lines.

In MDA-MB-231 cells, caspase 7 levels were significantly higher in the hexane extract-treated cells than their controls with increases of 16.5-37.1% (Graph 4.2.4.1c) and in the methanol extract-treated cells with 41-52.4% (Graph 4.2.4.2c), whereas in MDA-MB-468 cells, there was no significant difference in caspase 7 levels between the experimental cells and the control in the methanol extract and 0.0016 mg/mL of the hexane extract (Graph 4.2.4.1d, Graph 4.2.4.2d). At other concentrations of the hexane extract, experimental cells showed 63.1–99.4% higher caspase 7 levels than the controls in the MDA-MB-468 cell line.

In a previous study in which the effects of a lipophilic dichloromethane extract and aqueous extract of *C. olitorius* leaves were investigated on

metastatic Colo-741 cells, caspase 3 levels were found to be significantly higher than the control group (Soykut, Becer, Calis, Yucecan, & Vatansever, 2018). Increased caspase 3 activation was also reported in another study in which the effect of ethanolic leaf extracts was evaluated on HepG2 cells (C. Li et al., 2012). These studies support the findings of the present study which suggests that the non-polar (hexane) and polar (methanol) extracts of *C. olitorius* leaves may stimulate caspase 3-dependent pathways in MDA-MB-231 and MDA-MB-468 cells.

Previously, caspase 3 has been shown to be the major executioner of the apoptotic pathway with a broader range of substrates (Lamkanfi & Kanneganti, 2010). It was also reported that caspase 3 processed and cleared caspase 9 more efficiently than caspase 7, suggesting it is a more efficient executioner than caspase 7 in mediating the caspase-dependent cascade (Walsh et al., 2008).

It was shown in animal models that caspase 3 knockout resulted in severe developmental issues and was lethal to mice, whereas caspase 7-deficient mice were viable (Walsh et al., 2008). This suggests that even though caspase 3 and caspase 7 are both effector caspases with overlapping roles, there could be cell type- and stimulus-specific differences in apoptotic regulation between these cell lines (Lamkanfi & Kanneganti, 2010). The type of phytochemicals and their interactions with one other in the methanol extract could lead to a differential effect on the expression of caspase 7 in MDA-MB-231 and MDA-MB-468 cells.

Furthermore, in MDA-MB-468 cells caspase 3 may functionally compensate for caspase 7 and it may be sufficient on its own to execute cell death, completing the breakdown of majority of the substrates during apoptosis. An alternative explanation is that caspase-3 might have been present at higher levels than caspase-7 in this cell line, or that it was activated at an earlier stage of the apoptotic cascade (Walsh et al., 2008), whereas in MDA-MB-231 cells, both caspase 3 and caspase 7 might have been required to initiate the apoptotic cascade. Alternatively, the more

robustly expressed executioner caspase could have been caspase 7 in MDA-MB-231 cells.

Cell line-dependent differences could also explain why the hexane or methanol extracts did not show any significant anti-proliferative effect while having a significantly higher caspase 3 and 7 expression in MDA-MB-231 cells. This could occur due to a number of reasons. Firstly, the amount of both caspase 3 and 7 could have been high enough to be measured in the ELISA assay but not as high to execute the complete cell death. Apoptosis could have been in its early stages in this cell line with a high level of both caspase 3 and 7 but further steps, such as DNA fragmentation and cell shrinkage, have not been completed yet.

Secondly, apoptosis might have occurred in MDA-MB-231 cells but only a certain percentage of cells in the whole population might have undergone apoptosis which subsequently did not create an overall significant anti-proliferative effect in the whole population.

Alternatively, other survival pathways might have compensated for the increase in caspase 3 and 7 overriding apoptotic signals. MDA-MB-231 cells are known to be have mutations in *K-RAS* and *B-RAF* genes which are involved in the regulation of MAPK/ERK signalling pathway (Chavez, Garimella, & Lipkowitz, 2010; Hollestelle et al., 2010; Kenny et al., 2007). This signalling pathway has critical cellular functions including proliferation, cell cycle progression and regulation of apoptosis. Mutations in *K-RAS* and *B-RAF* activate MAPK/ERK signalling pathway, which results in cell survival and metastasis (Y. Guo et al., 2020; O. Li et al., 2023).

MDA-MB-231 cells are also characterised by a mutation in *TP53,* and a deletion in *CDKN2A* which encode the proteins p16INK4a and p14ARF (Chavez et al., 2010; Hollestelle et al., 2010; Kenny et al., 2007). Both proteins are involved in cell cycle regulation, with p14ARF inducing cell cycle arrest through p53 stabilisation (Kresty et al., 2002). It was previously proposed that a cell may use various signalling pathways to activate a final common pathway (Armstrong et al., 1994).

As suggested earlier, caspase 7 might be expressed more abundantly in MDA-MB-231 cells, however, it could have been ineffective in promoting apoptosis. In an earlier study, it was proposed that caspase 7 alone is not sufficient to induce apoptosis and instead of killing cells, caspase 7 appears to remove the apoptotic cells by detaching them from the ECM and clearing the microenvironment (Brentnall et al., 2013).

The loss of function of the regulatory proteins in addition to a destabilised p53 could all collectively promote cell survival and proliferation despite elevated caspase 3 and 7 signals in MDA-MB-231 cells, leading to an overall non-significant difference from the untreated control cells in this assay.

4.3.2 Potential Mechanisms of Action

The specific mechanisms of cytotoxicity which occur in cancer cells but not in healthy cells were identified in previous studies which used either individual bioactive compounds that make up the majority of the plant extracts or food sources that contain a high concentration of these compounds given to mice in *in vivo* studies. In the hexane and methanol extracts, various fatty acid methyl esters were identified as the major compounds, however, these compounds are commonly formed during derivatisation in GC-MS and are used to determine the fatty acid composition in extracts (Ichihara & Fukubayashi, 2010). Therefore, literature on α-linolenic acid (including flaxseeds as its primary source), linoleic acid and palmitic acid, was reviewed to explore potential anti-proliferative mechanisms observed in MDA-MB-468 cells. These were among the major compounds identified in the hexane and methanol extracts.

The cytotoxic mechanisms include activation of caspase-3 (C. Li et al., 2012), compositional changes in the plasma membrane of cancer cells (Wiggins, Kharotia, Mason, & Thompson, 2015), changes in EGFR expression (J. Chen, Mark Stavro, & Thompson, 2002), a sudden increase in the ROS causing oxidative stress within the cancer cell (Guzzetti et al.,

2021; Xi et al., 2022) and formation of lipid peroxidation products (Chajès et al., 1995).

In an earlier study, the anti-proliferative effects of α-linolenic acid were reported on MCF-7, MDA-MB-231 and MDA-MB-468 cells after a 96-hr treatment. It was proposed that this was as a result of α-linolenic acid incorporating into the plasma membrane of cancer cells. This competitive incorporation was thought to lead to alterations and remodelling in lipid rafts, which are normally assemblies for growth signalling receptors in the cell membrane. This subsequently causes growth receptors to move to non-lipid rafts, reducing their activity (Wiggins et al., 2015). The compositional remodelling of lipid rafts was shown to decrease HER2 expression in flaxseed-fed mice (Jiajie Liu et al., 2018) and IGF-1 and EGFR expression in flaxseed-fed mice injected with MDA-MB-435 cells (J. Chen et al., 2002; Chénais & Blanckaert, 2012).

MDA-MB-468 cells are characterised by EGFR amplification (Chavez et al., 2010; Holliday & Speirs, 2011; Kenny et al., 2007). EGFR is an activator of PI3K/Akt pathway which plays an important role in cell growth (Orofiamma, Vural, & Antonescu, 2022). Reduced cell proliferation and the significant increase in pro-apoptotic caspase 3 seen in MDA-MB-468 cells in the hexane and methanol extracts could be due to the competitive incorporation of α-linolenic acid into the cell membrane after a 72-hr incubation, disruption of the lipid composition of the membrane and ultimate inactivation of EGFR downstream signalling including PI3K/Akt pathway.

It was also previously suggested that α-linolenic acid could induce lipid peroxidation, which was indicated by elevated lipid hydroperoxide levels in cellular lipid extracts from breast cancer cells. Increased cellular lipid peroxidation is linked to increased oxidative stress enhancing cell death in cancer cells (Abel, Riedel, & Gelderblom, 2014; Chajès et al., 1995). Incorporation of α-linolenic acid into the lipid bilayer is also thought to alter the oxidative stress response in cells (Chénais & Blanckaert, 2012).

Cancer cells are known to have lower levels of endogenous antioxidant enzymes including glutathione peroxidases. This reduced antioxidative activity makes them more prone to oxidative stress than non-cancerous cells (G. S. Kumar & Das, 1995; Siddiqui, Harvey, & Stillwell, 2008).

As omega-3 fatty acids have more points of unsaturation, the incorporation of omega-3 fatty acids also makes the membrane lipids more susceptible to oxidative stress, which could occur as part of cellular processes including oxidative phosphorylation in the mitochondria. Additionally, the interior lipophilic regions of the lipid bilayer tend to concentrate more oxygen and/or ROS due to their hydrophobic nature. Collectively, these make the membrane lipids primary targets for oxidative damage and result in generation of reactive carbonyl species as lipid hydroperoxides, including malondialdehyde and 4-hydroxyhexenal, in cancer cells (Hardman, Munoz, & Cameron, 2002; Pamplona, 2008; Siddiqui et al., 2008; Virk et al., 2024).

In normal cells, reactive lipid hydroperoxides are detoxified by glutathione peroxidases (Pamplona, 2008; Siddiqui et al., 2008). However, increased lipid peroxidation in the cell membrane due to omega-3 fatty acid insertion overwhelms the already suppressed glutathione-dependent antioxidant systems in cancer cells (Chajès et al., 1995; Kikawa et al., 2010; Virk et al., 2024). Depleted antioxidant systems lead to a further increase in free radical generation, peroxidation of cellular lipids and cytotoxicity in cancer cells (G. S. Kumar & Das, 1995; Siddiqui et al., 2008).

In an earlier study, cytotoxic effects of a well-established cancer drug, doxorubicin, were assessed on MDA-MB-231 cells together with various omega-3 fatty acids, including α-linolenic acid. It was reported that all fatty acids increased the efficacy of doxorubicin, with docosahexaenoic acid (DHA) showing a more pronounced effect than the others and that the fatty acids resulted in generation of lipid hydroperoxides. It was suggested that the enhanced efficacy of doxorubicin could be due to the fatty acids generating lipid peroxides and contributing further to the oxidative stress

and cytotoxicity in the cells (Germain, Chajès, Cognault, Lhcillery, & Bougnoux, 1998).

In another study, in which anti-proliferative effects of various omega-3 and omega-6 fatty acids were examined on SP 2/0 mouse myeloma cells, α-linolenic acid and eicosapentaenoic acid (EPA) were found to have a significant inhibitory effect on proliferation of SP 2/0 cells when compared with other omega-3 and omega-6 fatty acids. It was also reported that the observed inhibitory effects were consistent with a reduction in the levels of glutathione, as the cofactor of glutathione peroxidase. It was suggested that, since cancer cells can develop resistance to cytotoxic drugs by upregulating endogenous antioxidant enzyme activity, coupling the drugs with omega-3 fatty acids may help reduce drug resistance by suppressing the antioxidant systems and inhibiting cell proliferation (U. N. Das, Madhavi, Sravan Kumar, Padma, & Sangeetha, 1998).

Pro-oxidant and cytotoxic effects of α-linolenic acid and EPA on SP 2/0 cells were also shown in another study in which a decrease in antioxidant enzyme activity and an increase in ROS and lipid peroxides were reported. In the same study, the increased oxidative stress through a free radical-dependent process was suggested to have caused an increase in the number of single stranded DNA breaks and damage critical cell membrane proteins, such as Na+/K+-ATPase, rendering them non-functional and leading to apoptosis (G. S. Kumar & Das, 1995).

An increase in lipid peroxidation and caspase 3 expression by α-linolenic acid treatment was observed in another study which used MCF-7 and MDA-MB-231 cells. It was suggested that α-linolenic acid induced lipid peroxidation which might have disrupted the mitochondrial membrane potential, leading to the activation of caspase-dependent apoptosis in these cell lines (Deshpande, Mansara, Suryavanshi, & Kaul-Ghanekar, 2013).

In the present study, formation of lipid peroxides and increased oxidative stress could be another mechanism supporting significantly higher

caspase 3 expression in MDA-MB-231 and MDA-MB-468 cells and significant growth inhibition in MDA-MB-468 cells. The oxidative stress potentially induced by α-linolenic acid in the extracts, could also be enhanced by the presence of pro-oxidants such as iron in *C. olitorius* leaves (U. N. Das, 1999; Njoumi et al., 2018). However, this requires further investigation since the iron content in the extracts may show differences. It is known that processing, such as heating, reduces available iron levels in the leaves (Njoumi et al., 2018). Therefore, processes such as extraction and GC-MS, may result in a decrease in the final iron content of the extracts.

However, intracellular iron levels in MDA-MB-468 cells might have also contributed to the observed anti-cancer effects through an iron-dependent cell death, called ferroptosis. It is a series of events which are initiated by elevated levels of intracellular iron. The excess iron promotes increasing ROS generation, which attack and oxidatively damage highly susceptible polyunsaturated fatty acids in the cell membrane. This lipid peroxidation produces toxic oxidised lipid molecules whose accumulation causes the membrane to lose its structural integrity and rupture, ultimately leading to cell death. This iron-dependent lipid peroxidation resulting in cell death is known as ferroptosis (Tan et al., 2025; F. Yang et al., 2023).

It was previously stated that TNBC cells have an increased pool of bioavailable iron, dysregulated genes involved in iron homeostasis and reduced antioxidant enzyme activity, rendering them more susceptible to ferroptosis-mediated cell death (Greenshields et al., 2019; Tan et al., 2025). In normal cells, ferroptosis is inhibited by glutathione peroxidases however, since these are depleted in cancer cells, lipid peroxides may accumulate and result in ferroptotic cell death (Tan et al., 2025; F. Yang et al., 2023).

Another important regulator associated with ferroptosis is acyl-CoA synthetase long-chain family member 4 (ACSL4). This enzyme is responsible for the incorporation of polyunsaturated fatty acids into cell membranes, enhancing their chances of peroxidation. It was also reported that the expression of ACSL4 is higher in various cancers that undergo

ferroptosis, suggesting its potential significance as a target for future research (Tan et al., 2025).

Previously, it was demonstrated that an iron chelator, DIBI, inhibited proliferation in various breast cancer cell lines including MDA-MB-468 cells and that the MDA-MB-468 cell line was the most sensitive to the drug shown by DNA breaks and cell cycle arrest (Greenshields et al., 2019). This may suggest that the MDA-MB-468 cell line also has high intracellular iron levels.

Taken together, it can by hypothesised that the potential incorporation of α-linolenic acid into the lipid membranes and high levels of free intracellular iron might have sensitised MDA-MB-468 cells to increase lipid peroxidation, overwhelming glutathione peroxidases and eventually leading to ferroptotic cell death. Therefore, in addition to EGFR displacement and inactivation, ferroptosis could also have played a role in reducing proliferation in this cell line compared to the control cells.

Another anti-proliferative effect of α-linolenic acid was shown through the modulation of Akt/NF-κB pathway, which is downstream to PI3K signalling. In healthy non-tumourigenic cells, PTEN is expressed as a tumour suppressor protein that inhibits cell growth and cell cycle progression by suppressing PI3K/Akt pathway (Glaviano et al., 2023; Minami, Nakanishi, Ogura, Kitagishi, & Matsuda, 2014). However, the deletion of *PTEN* in MDA-MB-468 cells results in sustained proliferation in this cell line. Evidence from cell culture assays and MMTV-induced mice suggests that omega 3 fatty acid supplementation, including α-linolenic acid, decreased Akt phosphorylation, reduced the activity of Akt/NF-κB pathway and induced caspase-dependent apoptosis (Jiajie Liu et al., 2018; Schley, Jijon, Robinson, & Field, 2005).

The high concentration of α-linolenic acid in the hexane and methanol extracts might have compensated for the lack of PTEN in MDA-MB-468 cells by reducing the activity of Akt and its downstream signalling pathways.

The apoptotic effects of palmitate were investigated *in vitro* using different breast cancer cell lines and it was shown that palmitate had an anti-proliferative and pro-apoptotic effect on experimental cells compared to their untreated controls. In the same study, it was found to reduce PI3K activity by 60%, suggesting PI3K is implicated in the control of cell growth and survival in breast cancer cells (Hardy, Langelier, & Prentki, 2000).

Palmitic acid was also one of the major compounds identified in all three extracts. The observed effects on MDA-MB-468 cells in the current study may indicate that palmitic acid provides a synergistic support for the anti-proliferative and pro-apoptotic effects of α-linolenic acid in these extracts.

It was previously reported that MCF-7 cells had a low response to omega 3 fatty acid supplementation and that cell viability was not significantly diminished when MCF-7 cells were given omega 3 fatty acids (Chajès et al., 1995). Since α-linolenic acid is also an omega 3 fatty acid and is the major component identified in all three extracts, the non-significant difference between the experimental and untreated cells in the proliferation assay could be explained by the unresponsiveness of MCF-7 cells to this major component of the extracts.

4.3.3 Summary of Findings and Future Research

In summary, findings of this study suggest that the bioactive compounds in the hexane and methanol extracts appear to influence pathways, potentially EGFR, PI3K and Akt signalling pathways involved in cell proliferation in MDA-MB-468 cells. Due to a higher intracellular iron reserve in TNBC cells as suggested in the literature, ferroptosis could have also contributed to the overall effects observed with MDA-MB-468 cells. The non-significant differences in the other cell lines could be attributed to the antagonistic effects of the bioactive compounds in the extracts or cells using various signalling cascades to activate a final common pathway, likely an anti-apoptotic pathway.

From a practical approach, it should also be noted that increased levels of caspase 3 or 7 include both the active and inactive forms as pro-caspases.

In future research, the levels of cleaved caspase 3 as the major executioner could be evaluated.

Additionally, the downstream effects of caspase 3 can be assessed by measuring the levels of its substrates. One such protein is Poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1 is a critical enzyme involved in DNA repair by binding to DNA ends or strand breaks upon DNA damage. In the apoptotic programme, activated caspase 3 cleaves and inactivates PARP-1, which subsequently halts the DNA repair mechanism, leading to cell death (Boulares et al., 1999). Western blotting can be a useful, convenient technique to measure the levels of cleaved caspase 3 and PARP-1. This could provide further confirmation on the anti-proliferative and apoptotic effect of these extracts on TNBC cell lines.

DNA fragmentation, as a further step in apoptosis, could also be assessed using the DAPI staining method. This simple method uses a DNA-binding fluorescent dye that can be visualised using a fluorescence microscope (Khorsandi et al., 2017).

Figure 4.3.3 summarises the various mechanisms potentially involved in reducing proliferation in MDA-MB-468 cells. The diagram was constructed using the ‘05200 N-Pathways in Cancer’ map from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) as a reference framework (KEGG, 2020). It was adapted and modified based on evidence from the literature to highlight the signalling pathways and mechanisms most relevant to the present study.

It should be noted that there could be other compounds in the extracts synergistically activating these mechanisms leading to a final anti-proliferative effect observed in the present study.

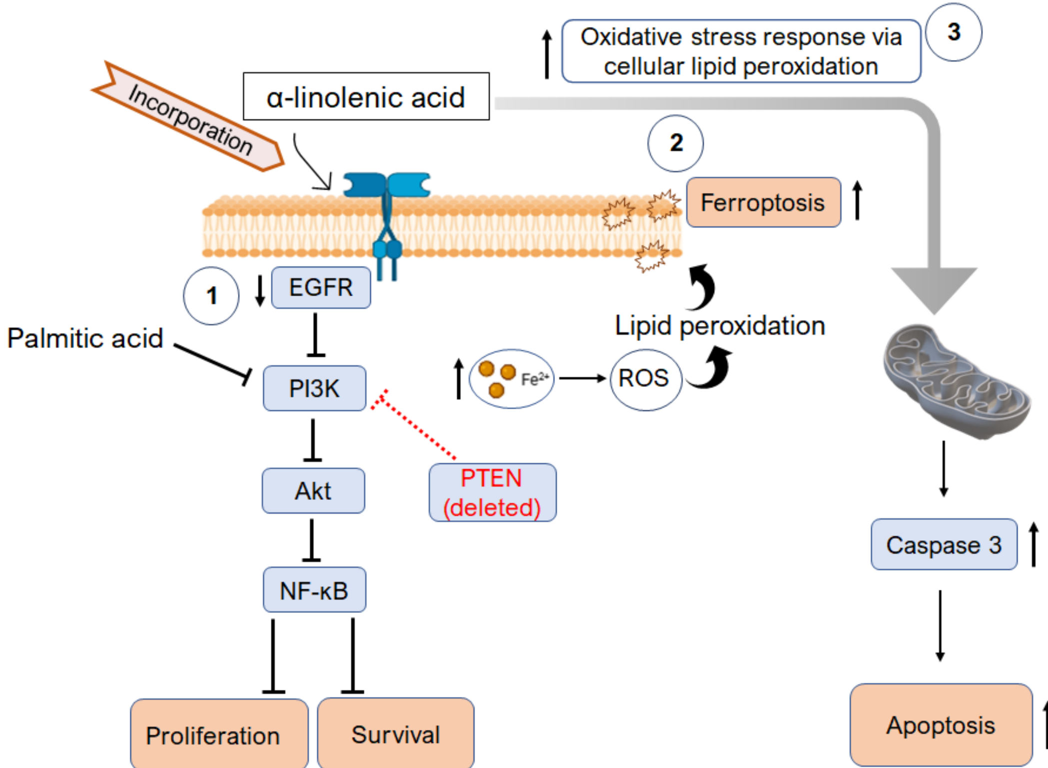


Figure 4.3.3: Overview of Proposed Mechanisms for Proliferation Inhibition in MDA-MB-468 cells

Based on evidence from the literature, proliferation could have been reduced by the major lipophilic components of the hexane and methanol extracts of *C. olitorius*, primarily by α-linolenic acid and palmitic acid, through several mechanisms in MDA-MB-468 cells. Mechanism 1 represents the potential inactivation of EGFR/PI3K/Akt pathway either by the incorporation of α-linolenic acid into the membrane or palmitic acid. Mechanism 2 proposes the potential involvement of membrane rupture and ferroptosis while Mechanism 3 suggests the potential role of lipid peroxides in increased oxidative stress response via cell membrane and DNA damage, leading to apoptosis. PTEN is deleted in this cell line but in non-tumourigenic cells, it inhibits PI3K activation, represented by a dashed line. This diagram was constructed and modified from 05200 N-Pathways in Cancer map from KEGG based on literature findings. Image downloaded from www.biorender.com.

4.4 Conclusion

Preparation of plant extracts in solvents of varying polarities allows for maximum extraction of plant compounds with different properties and helps investigate their effects on cell proliferation and apoptosis.

Until now, no published study appears to have examined the combined anti-proliferative effects of *C. olitorius* leaf extracts on the specific cell lines used in the present study. Previous research has largely focused on assessing individual compounds or limited combinations, rather than

whole extracts. The preliminary findings of this study provide a basis for focusing on whole extracts to gain a deeper understanding of the potential synergistic effects of the bioactive compounds they contain. Furthermore, the use of multiple oestrogen-dependent and -independent cell lines allows for better evaluation and comparison of their molecular differences.

The findings of the proliferation assay suggest that the hexane and methanol extracts exert an anti-proliferative effect on MDA-MB-468 cells, which are also supported by significantly higher expression of caspase 3 in this cell line. Based on literature findings, several mechanisms could be hypothesised including incorporation of α-linolenic acid into the cell membrane, altering its composition and redistribution of receptor proteins, such as EGFR, rendering it non-functional. Other mechanisms could include lipid peroxidation and ferroptotic cell death. These mechanisms require further clarification.

Chapter 5 - Effects of *C. olitorius* extracts on Adhesion of Breast Cancer Cells

5.1 Introduction

Cells are provided with structure and support via a complex, cross-linked matrix of various components including glycoproteins, proteoglycans and polysaccharides in the extracellular space. Thus, ECM plays an essential role in intercellular integrity by forming a stable attachment site for cells. ECM is also involved in the regulation of critical cell activities including cell survival, proliferation and motility through interactions with cell surface receptors. Although it provides architectural structure to cells, ECM is a highly coordinated but dynamic establishment in healthy organisms (Bourgot, Primac, Louis, Noël, & Maquoi, 2020; Janiszewska, Primi, & Izard, 2020). However, during cancer development, changes occur in the ECM which is also used by the growing cancer to create a protective environment to escape cytotoxicity (Baltes et al., 2020; Teng et al., 2014).

Collagen is one of the proteins found extensively within the ECM. It is a proteoglycan, composed of protein and saccharide chains, which can vary in structure and composition in different tissue types. Collagen binds to adhesion molecules on cell surfaces, such as integrins, to provide anchor for the cells. However, although it is a critical part of the ECM, collagen is also associated with cancer development and motility (Bourgot et al., 2020; S. Xu et al., 2019).

For instance, in solid tumours, which can occur in the breast tissue, the ECM is stiffer in structure due to the overexpression and excessive accumulation of fibrillar proteins including different types of collagens. This stiffness sensed by the integrins initiates a signalling cascade promoting cancer cell survival, proliferation and invasion (Bourgot et al., 2020).

In previous studies, it was demonstrated that type I collagen is always expressed in different breast cancers and that its knockdown resulted in

inhibition of metastasis (Jakubzig, Baltes, Henze, Schlesinger, & Bendas, 2018; Jing Liu et al., 2018; Teng et al., 2014). In another study, MCF-7 and MDA-MB-231 cells were found to become more readily attached to type I collagen covering a large spreading area (Teng et al., 2014).

Collagen is a critical research tool widely used in studies to mimic cancer and investigate cell behaviour *in vitro*. Therefore, collagen was used in this study to evaluate the anti-adhesive behaviour of different breast cancer cells.

5.2 Results

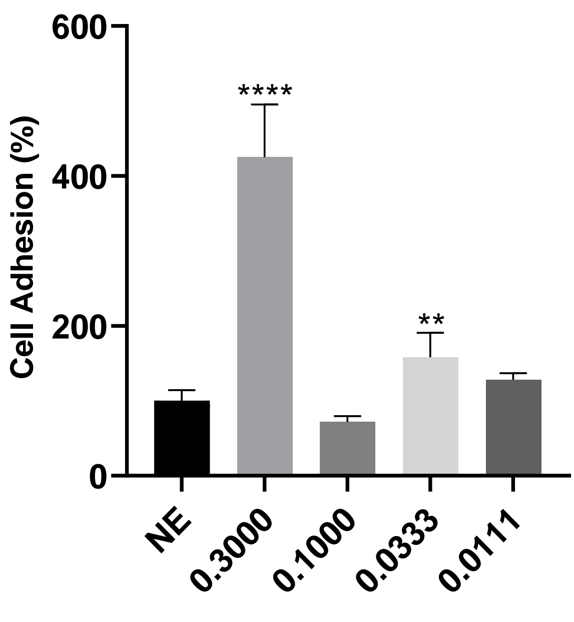
In the adhesion assay, cells were incubated with extracts for 48 hours after which the CV assay was used to evaluate the anti-adhesive effects of the extracts on all three breast cancer cell lines. However, instead of setting up eight dilution series, the first four and more concentrated dilutions were used for each extract to look for more specific effects. Control was a negative control which consisted of cells in 10% DMSO/media with no extract. Results were calculated and statistically analysed as described in Chapter 2. All data are presented as a percent of the mean of control ± S.D of multiple replicates.

5.2.1 Effects of the Acetone Extract on Breast Cancer Cell Lines

According to the findings, cell adhesion was significantly higher at two concentrations of the acetone extract in MCF-7 cells. At 0.300 mg/mL of the extract, adhesion was four times the control (percent mean= 425.1%, $p<0.0001$) and at 0.033 mg/mL it was 1.5 times higher (percent mean= 158%, $p<0.01$). Other concentrations did not differ significantly from the control (Graph 5.2.1.1).

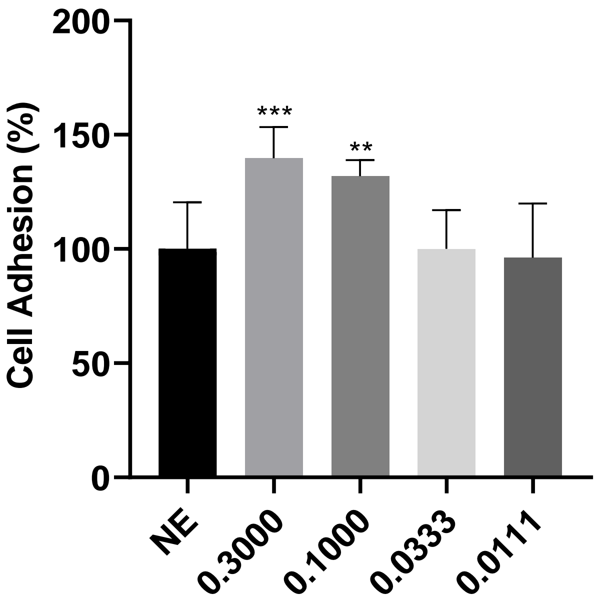
A similar trend was observed in MDA-MB-231 cells. Wells containing 0.300 mg/mL and 0.100 mg/mL of the acetone extract had a significantly higher mean value than the control with percent means 139.8% ($p<0.001$) and 131.9% ($p<0.01$), respectively (Graph 5.2.1.2).

However, cell adhesion was significantly reduced at 0.033 mg/mL of the acetone extract compared to the control in MDA-MB-468 cells (percent mean= 46.75%, p<0.05). Even though at 0.300 mg/mL and 0.011 mg/mL, cell adhesion was lower than the control, none of the other concentrations of the acetone extract were significantly different from the control in this cell line (Graph 5.2.1.3).



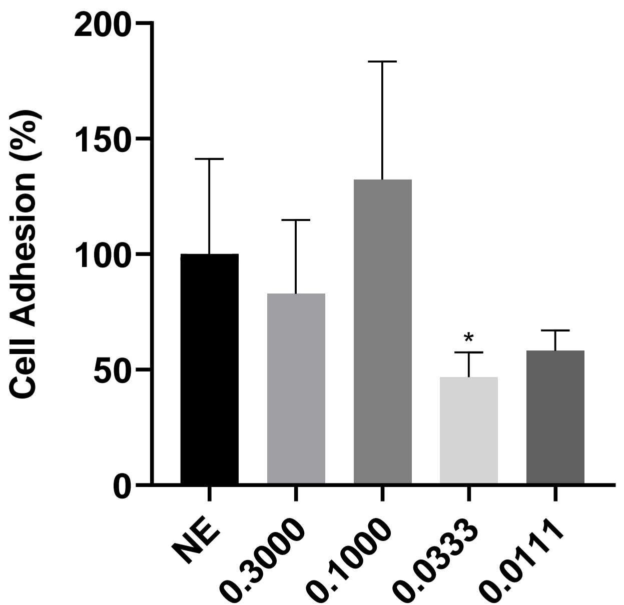
Graph 5.2.1.1: Effect of the Acetone Extract on Adhesion of MCF-7 cells

Anti-adhesive effects of *C. olitorius* extract in acetone of various concentrations were evaluated on MCF-7 cells. The negative control, represented as NE (no extract), consisted of cells in media but no extract. Data are presented as a percent of control mean ± S.D of multiple replicates. ** represents $p<0.01$ level of significance and **** represents $p<0.0001$.



Graph 5.2.1.2: Effect of the Acetone Extract on Adhesion of MDA-MB-231 cells

Effects of *C. olitorius* extract in acetone on the adhesion of MDA-MB-231 cells were evaluated. The negative control, represented as NE (no extract), consisted of cells in media but no extract. Two concentrations, 0.100 mg/mL and 0.300 mg/mL, of the acetone extract were significantly different from the control, at $p<0.01$ and $p<0.001$ level, respectively.



Graph 5.2.1.3: Effect of the Acetone Extract on Adhesion of MDA-MB-468 cells

Effects of *C. olitorius* extract in acetone on the adhesion of MDA-MB-468 cells were evaluated. The negative control, represented as NE (no extract), consisted of cells in media but no extract. Only 0.0333 mg/mL of the extract was significantly different from the control ($p<0.05$).

5.2.2 Effects of the Hexane Extract on Breast Cancer Cell Lines

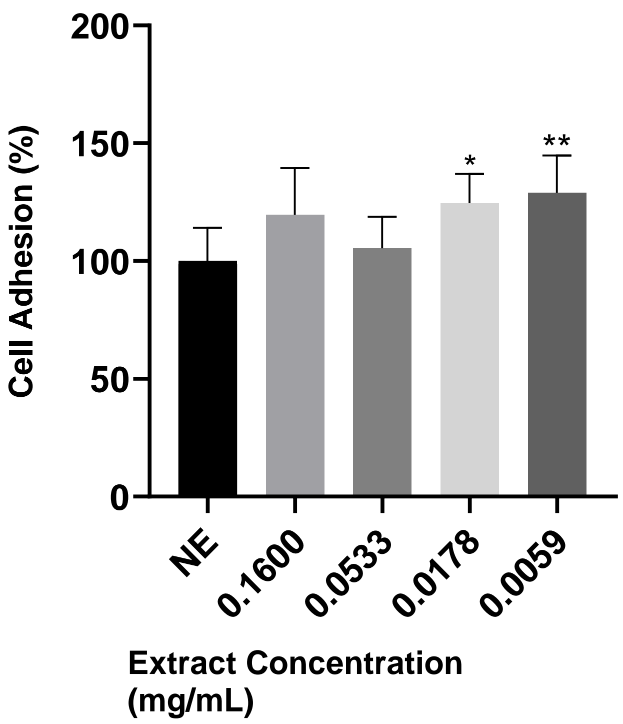
In the hexane extract, both MCF-7 and MDA-MB-231 cells behaved similar to the acetone extract. Some concentrations of the hexane extract showed higher adhesion than the control.

In MCF-7 cells, 0.0178 mg/mL and 0.0059 mg/mL of the extract had significantly higher adhesion than the control with percent means, 124.6% ($p<0.05$) and 129.0% ($p<0.01$), respectively. Although the other two concentrations, 0.1600 mg/mL and 0.0533 mg/mL, were also higher than the control, these differences were not statistically significant (Graph 5.2.2.1).

In MDA-MB-231 cells, cell adhesion was significantly increased at 0.1600 mg/mL, 0.0178 mg/mL and 0.0533 mg/mL of the hexane extract. Compared to the control, percent mean at 0.1600 mg/mL was 124.9%

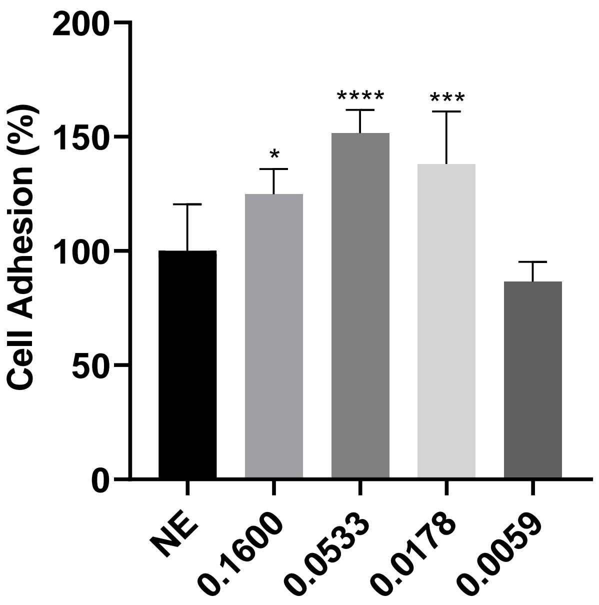
(p<0.05), at 0.0178 mg/mL, 138.1% (p<0.001) and at 0.0533 mg/mL, 151.7% (p<0.0001) (Graph 5.2.2.2).

However, in MDA-MB-468 cells, at all four concentrations cell adhesion was significantly lower than the control. At 0.1600 mg/mL, 0.0533 mg/mL and 0.0178 mg/mL of the extract, cell adhesion was reduced to 62.23%, 63.26% and 64.85%, respectively (p<0.01). Adhesion was decreased to 55.17% (p<0.001) at 0.0059 mg/mL (Graph ***5.2.2.3***).



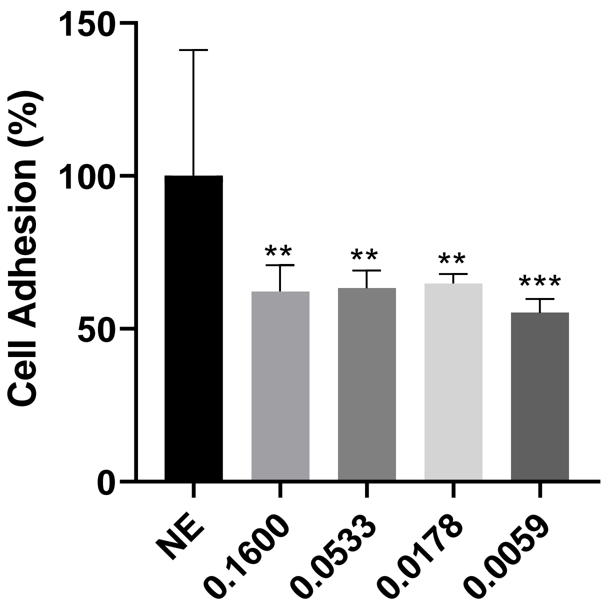
Graph 5.2.2.1: Effect of the Hexane Extract on Adhesion of MCF-7 cells

Effects of *C. olitorius* extract in hexane were evaluated on the adhesive properties of MCF-7 cells. The negative control, represented as NE (no extract), consisted of cells in media but no extract. Data are presented as a percent of control mean ± S.D of multiple replicates. Significant difference from the control was observed at 0.0178 mg/mL and 0.0059 mg/mL of the extract with p<0.05 and p<0.01 significance, respectively.



Graph 5.2.2.2: Effect of the Hexane Extract on Adhesion of MDA-MB-231 cells

Anti-adhesive effects of *C. olitorius* extract in hexane were evaluated on MDA-MB-231 cells. The negative control, represented as NE (no extract), consisted of cells in media but no extract. Of all the concentrations, 0.1600 mg/mL ($p<0.05$), 0.0178 mg/mL ($p<0.001$) and 0.0533 mg/mL ($p<0.0001$) were significantly different from the control.



Graph 5.2.2.3: Effect of the Hexane Extract on Adhesion of MDA-MB-468 cells

Effects of the *C. olitorius* extract in hexane were evaluated on the adhesion of MDA-MB-468 cells. The negative control, represented as NE (no extract), consisted of cells in media but no extract. ** represents significant difference at p<0.01 level and *** at p<0.001.

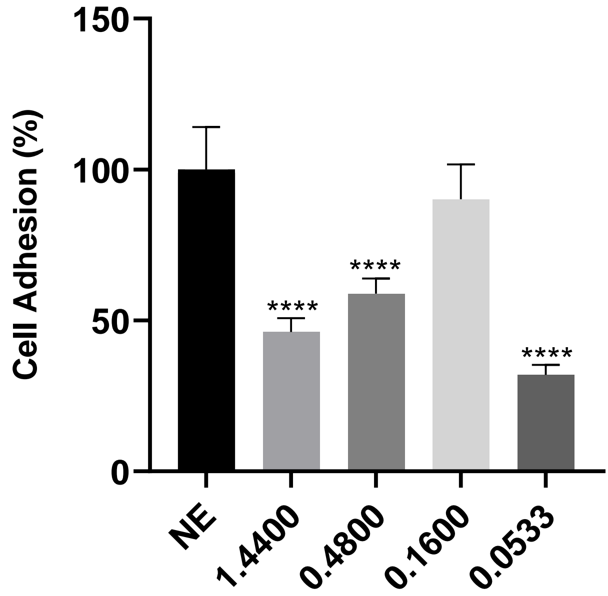
5.2.3 Effects of the Methanol Extract on Breast Cancer Cell Lines

As seen in Graph 5.2.3.1, in MCF-7 cells, three concentrations of the extract reduced adhesion of cells significantly. Percent means of 1.440 mg/mL, 0.480 mg/mL and 0.053 mg/mL were 46.16%, 58.86% and 31.96% of the control mean, respectively (p<0.0001). Overall, the methanol extract appears to inhibit adhesion in MCF-7 cells.

In MDA-MB-231 cells, similar to the acetone and hexane extracts, the methanol extract also increased cell adhesion (Graph 5.2.3.2). Concentrations of 0.480 mg/mL and 0.160 mg/mL significantly enhanced cell adhesion with percent means, 146.9% and 143.9%, respectively (p<0.0001). Other concentrations did not significantly differ from the control.

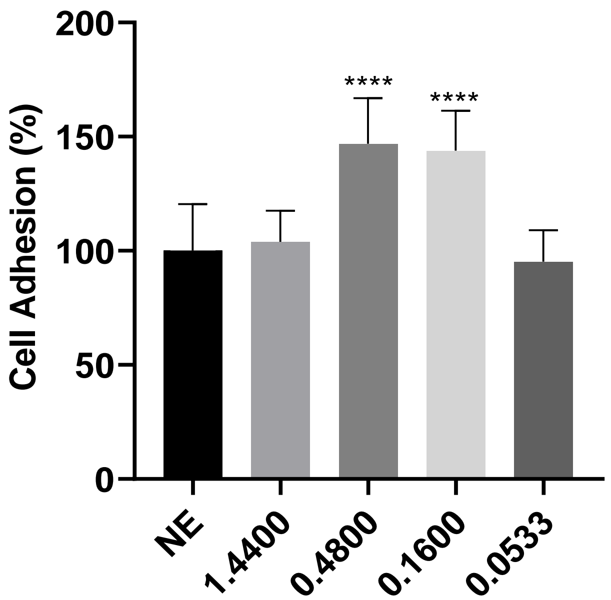
A significant reduction in cell adhesion was observed in MDA-MB-468 cells in the presence of the methanol extract (Graph 5.2.3.3). All four

concentrations had significantly lower percentages than the control. The percent means were 30.58% for 1.440 mg/mL, 30.24% for 0.480 mg/mL, 42.03% for 0.160 mg/mL and 21.42% for 0.053 mg/mL of the extract ($p<0.0001$).



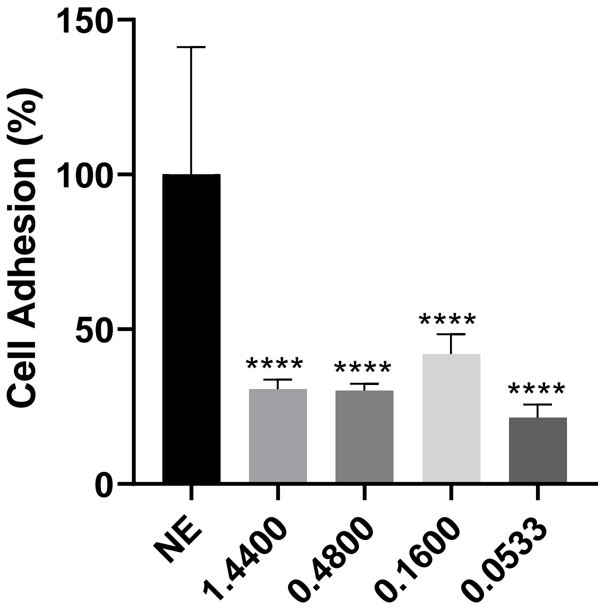
Graph 5.2.3.1: Effect of the Methanol Extract on Adhesion of MCF-7 cells

Anti-adhesive effects of *C. olitorius* in methanol were evaluated on MCF-7 cells. The negative control, represented as NE (no extract), consisted of cells in media but no extract. Data are presented as a percent of control mean ± S.D of multiple replicates. **** shows the concentrations of the extract which are significantly different from the control ($p<0.0001$).



Graph 5.2.3.2: Effect of the Methanol Extract on Adhesion of MDA-MB-231 cells

Anti-adhesive effects of the *C. olitorius* leaf extract in methanol were evaluated on MDA-MB-231 cells. A negative control, represented as NE (no extract), of cells in media was used without any extract. **** shows the statistically significant difference of 0.480 mg/mL and 0.160 mg/mL of the extract from the control cells.



Graph 5.2.3.3: Effect of the Methanol Extract on Adhesion of MDA-MB-468 cells

Effects of *C. olitorius* extract in methanol were evaluated on MDA-MB-468 cells. The negative control, represented as NE (no extract), consisted of cells in media but no extract. All four concentrations of the extract were statistically different from the control ($p<0.0001$).

Unlike the proliferation assay, where only MDA-MB-468 cells were significantly different from the control in the hexane and methanol extracts, all three breast cancer cell lines were significantly different from the control and had either lower or higher cell adhesion in the extracts used.

5.3 Discussion

In this assay, CV staining was used to evaluate the adhesive ability of viable cells on collagen. Since the aim of this analysis was to primarily measure the initial attachment of extract-treated cells to a collagen-rich surface after a 2-hour incubation rather than a long-term stable adhesion, the findings should be interpreted as cellular changes in early adhesion.

Unlike the proliferation assay, all three cell lines investigated in the adhesion assay showed a significant difference from the control in the acetone, hexane and methanol extracts.

Cell adhesion was significantly lower in MDA-MB-468 cells than the control in all three extracts. Although there was a single concentration in the acetone extract which was significantly different from the control, in the hexane and methanol extracts, all concentrations were observed to inhibit cell adhesion. Especially in the methanol extract (Graph 5.2.3.3), all four concentrations appear to inhibit adhesion more significantly ($p<0.0001$).

These observations suggest that various plant compounds, of varying polarities, work synergistically in favour of reducing cell adhesion in this cell line. An inhibitory pattern was also observed with MCF-7 cells in the methanol extract (Graph 5.2.3.1). Neither of these were found to be dose dependent. On the other hand, in MDA-MB-231 cells, 0.480 mg/mL and 0.160 mg/mL of the methanol extract significantly increased adhesion ($p<0.0001$) (Graph 5.2.3.2). This opposite pattern suggests that, even if there is a mixture of different phytochemicals with anti- or pro-adhesive effects in the extracts, the major bioactive compounds in the methanol extract predominantly enhanced adhesion in MDA-MB-231 cells and they exerted their effects optimally at these concentrations.

Behaviour of MDA-MB-231 cells were also similar in the acetone and hexane extracts, with significantly higher adhesion. Altogether, these findings indicate that the bioactive compounds in the extracts stimulate adhesion-related pathways in MDA-MB-231 cells.

In both the hexane and methanol extracts, MDA-MB-231 cells appear to have an inverted U-shaped response (Graph 5.2.2.2, Graph 5.2.3.2). The biphasic dose response seen in biological systems is known as hormesis, which occurs when modest concentrations, around 30-60% greater than the control, cause the maximal response (Calabrese, 2013). The increase observed in the hexane extract was within the range of 25-52%, while this was between 44-47% in the methanol extract. Therefore, it appears that the bioactive compounds in the hexane extract stimulated a hormetic effect in MDA-MB-231 cells and resulted in a final signalling pathway that stimulated adhesion.

Unlike the other two cell lines, there was no particular trend in MCF-7 cells across the extracts. There was an anti-adhesive effect in the methanol extract as mentioned above, whereas adhesion was enhanced in the acetone and hexane extracts. Although availability of data in the literature on the effects of fatty acids on adhesion of MCF-7 cells is limited, it may be possible that the interaction between the fatty acids identified in the methanol extract - mainly α-linolenic and palmitic acid - and polar polyphenolic compounds reduce adhesion in this cell line. Since the acetone and hexane extracts contain more non-polar compounds, this interaction may not be present in these extracts. Alternatively, there could be an opposing effect of various bioactive compounds in the acetone and hexane extracts leading to a final common pathway upregulating adhesion in MCF-7 cells.

Although not identified through the GC-MS analysis in the present study, it was previously shown that treatment of MCF-7 cells with chlorogenic acid (found in the alcoholic fraction of the leaves) increased the expression of E-cadherin while reducing N-cadherin, suggesting a transition from a mesenchymal to an epithelial phenotype. In the same study, it was also proposed that the dosage of individual compounds affected how different cell lines respond to these compounds (Xue et al., 2023).

Furthermore, it was previously suggested that there could be small proportions of individual phenotypes within a population of MCF-7 cells which may show differences in gene and hormone receptor expression as well as signalling pathways (Comşa et al., 2015). The findings with MCF-7 cells in the present study could be because of the influence of the extract compounds on these individual phenotypes which collectively determined whether adhesion would be reduced or increased.

MDA-MB-468 cells are known to express high levels of E-cadherin whereas MDA-MB-231 cells are characterised by lower expression of E-cadherin (Chekhun, Bezdenezhnykh, Shvets, & Lukianova, 2013; Holliday & Speirs, 2011). E-cadherin is involved in cell-to-cell adhesion by holding cells tightly together. It is, therefore, associated with the epithelial state of

cells with weaker cell-to-ECM interactions and represents lower tumourigenicity of this cell line than MDA-MB-231 cells (Chekhun et al., 2013; Dongre & Weinberg, 2019; Majidpoor & Mortezaee, 2021). MDA-MB-231 cells, however, express a mesenchymal state, which is more prone to acquiring EMT and becoming more invasive (Majidpoor & Mortezaee, 2021).

Likewise, both cells have a different claudin expression. Whereas MDA-MB-231 is characterised by low expression of claudins including claudin-3, 4 or 7, MDA-MB-468 does not have a significantly lower expression of the claudin family (Holliday & Speirs, 2011). Claudins are important tight junction proteins and play an essential role in cell adhesion (Murakami-Nishimagi et al., 2023).

Another difference between the two cell lines is the expression of integrins. Integrins are a group of heterodimeric cell surface proteins that bind to collagen in the ECM to mediate intracellular signalling pathways. Their ability to bind to collagen increases the adhesive and invasive potential of cancer cells due to increased interactions and modification of the ECM (Baltes et al., 2020). It was previously reported that collagen-binding integrins predominantly contain the β1 subunit and that MDA-MB-231 cells have a higher expression of β1 integrins than MDA-MB-468 (Klahan et al., 2016; Ziperstein, Guzman, & Kaufman, 2015).

Altogether, the lower cell-to-cell adhering E-cadherin and claudin expression and higher β1 integrin expression contribute to the mesenchymal profile of MDA-MB-231. This EMT mode involves strong interactions with the surrounding ECM via integrins and stimulates proteolytic degradation of the ECM (Friedl, Locker, Sahai, & Segall, 2012; Jia shun Wu et al., 2021), making this cell line more prone to migration and invasion in distant sites (Klahan et al., 2016; Ziperstein et al., 2015).

It was also reported that β1 integrins primarily activate focal adhesion kinase (FAK) which recruits and activates Src family of proteins to stimulate downstream migratory pathways (Baltes et al., 2020). This shows that

MDA-MB-231 cells have a stronger focal adhesion signalling than MDA-MB-468 cells, which are known to heavily rely on amplified EGFR expression and activity (Kenny et al., 2007) and have weaker FAK activation. This was also shown in another study, where the attachment of MDA-MB-231 cells to collagen upregulated FAK activity and its downstream signalling (Ibaragi et al., 2011).

Given the molecular profile of the TNBC cell lines investigated and reduced adhesion observed with all three of the extracts, but especially the hexane and methanol extracts, in MDA-MB-468 cells, one possible mechanism might be further downregulation of integrin-ECM binding and suppression of the already weak integrin-FAK signalling cascade. The anti-adhesive effect of the major phytochemicals might have been reinforced by their interactions with other compounds in the extracts leading to an overall synergistic anti-adhesive effect, potentially preventing successful ‘landing’ and colonisation of cells on the collagen surface.

On the other hand, these bioactive compounds stimulated adhesion in MDA-MB-231 cells, potentially by strengthening integrin activation and its downstream signalling pathway, enabling cells to adhere more strongly to the collagen-dense surface.

As further investigation of the potential pathways associated with adhesion, migration and invasion were also assessed in TNBC cells in the presence of the extracts, as seen in Chapter 6 and Chapter 7. A similar hormetic-like response and enhanced migration in hexane extract-treated MDA-MB-231 cells suggest that similar mechanisms, such as reduced E-cadherin expression, upregulation of FAK-Src coupling and its downstream signalling pathways, could have played an important role in stimulating both the adhesive and migratory capacity in this cell line (Figure 6.3.1). The hexane and methanol extracts reduced migration in MDA-MB-468 cells, also suggesting that the phytochemical composition might have shown their effects through the pathways involved in adhesion and migration.

In order to confirm which proteins are involved in extract-treated TNBC cells, future research could aim to evaluate the expression and activity of integrins and FAK in both cell lines. This would help clarify which pathways are potentially involved in pro- or anti-adhesive behaviour observed in MDA-MB-231 and MDA-MB-468 cells, respectively.

5.4 Conclusion

The early-phase attachment of extract-treated breast cancer cell lines on a collagen surface was measured via the CV assay and the results show increased adhesion through a response similar to hormesis by MDA-MB-231 cells and reduced adhesion without a dose-dependent effect in MDA-MB-468 in all extracts. These results are consistent with the findings of the migration assay discussed in Chapter 6, suggesting related pathways were involved in the observations.

On the contrary, MCF-7 cells exhibited reduced adhesion in the methanol extracts while having increased adhesion in the acetone and hexane extracts. These findings could be attributed to the complex interactions between the bioactive compounds in the extracts and/or subpopulation of cells with different hormone receptor or signalling protein expression.

Chapter 6 - Effects of *C. olitorius* extracts on Migration of Breast Cancer Cells

6.1 Introduction

Metastasis is a complex multi-step process in which a primary tumour grows into a secondary tumour in a separate, distant site (Thakur et al., 2024). Similar to other cellular processes, metastasis is regulated by various signalling pathways and modifications to the ECM. Although not all cancers are metastatic, those that are, usually result in a poor clinical prognosis (Guan, 2015).

Migration is a natural, multistep physiological process which occurs throughout embryonic development, wound healing, tissue repair and as an immunological response to destroy invaders and clear remaining cell debris. However, cell migration is also implicated in disease formation and progression (Ridley et al., 2003). It has been shown that cancer cells also use migratory mechanisms, similar to non-neoplastic cells, to spread within the surroundings and promote metastasis (Friedl & Wolf, 2003).

Migration of tumour cells occurs either as a single cell or multiple cells collectively. Individual cell migration is further classified into mesenchymal and amoeboid migration with distinct features. Amoeboid migration is considered to be the most efficient migration mode as it involves rapid motion of cells and has a highly deformable cell morphology, low cell-ECM attachments without proteolytic degradation of the surrounding matrix and the ability to penetrate through narrow spaces of the ECM. However, mesenchymal mode undergoes EMT and involves strong interactions with the surrounding ECM via integrins and proteolytic degradation of the ECM (Friedl et al., 2012; Jia shun Wu et al., 2021).

On the other hand, collective migration involves multiple cells which retain cell-to-cell interactions and migrate collectively in the ‘leadership’ of certain cancer cells in the leading front of the group. Similar to the mesenchymal

migration mechanism, these multicellular units involve cell-ECM adhesion regulated by integrins and cell-to-cell interactions mediated by cadherins including E-cadherin (Jia shun Wu et al., 2021).

It is known that migrating tumour cells can transition between these modes as an escape mechanism depending on the tumour microenvironment and chemotherapeutic drugs targeting certain signalling pathways (Friedl & Wolf, 2003; Jia shun Wu et al., 2021).

Even though most epithelial cancers, including TNBC, use collective migration mechanisms (Friedl & Wolf, 2003; Ilina et al., 2018; Jia shun Wu et al., 2021), MDA-MB-231 cells demonstrated a mesenchymal type migration in a previous study where three-dimensional collagen matrices were used (Wolf et al., 2003).

As one of the ECM proteins and a stimulator of collective migration (Jia shun Wu et al., 2021), collagen is used in the migration assay to investigate cell migration of the metastatic breast cancer cell lines, MDA-MB-231 and MDA-MB-468.

6.2 Results

Wound healing assay was carried out to investigate the anti-migratory effects of different extracts on the metastatic cell lines, MDA-MB-231 and MDA-MB-468. The negative control used in this assay consisted of cells in 0.1% DMSO/media without extract. Photographs of the control and experimental cells were taken at t=0 hr and 24 hrs after the scratch. The assay was repeated three times. In both cell lines, for the experimental cells only one photo per concentration is shown for clarity but all measurements were included in the calculations.

Data are presented as the percent change in wound width between t=0 hr and t=24 hr compared to the control mean with ± S.D. Data were then entered into GraphPad Prism 10. One-way ANOVA was conducted to compare the means and analyse the statistical significance between the means of the extracts and the control. Figure 6.2a and Figure 6.2b show

the photos of MDA-MB-231 and MDA-MB-468 controls at t=0 hr and t=24 hr, respectively.

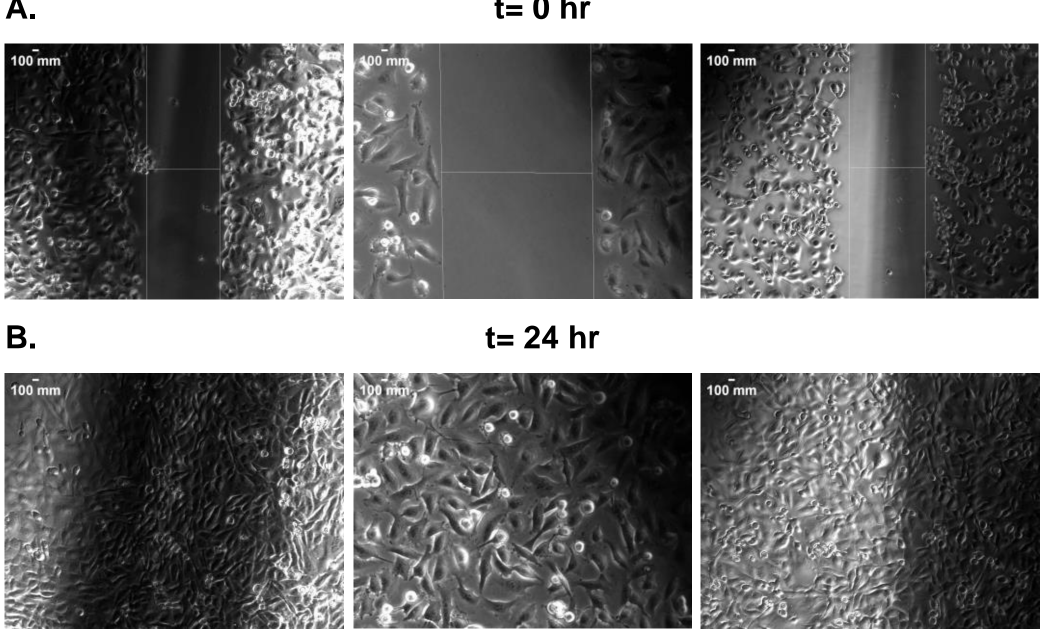


Figure 6.2a: Control cells of MDA-MB-231 at 0 hr and 24 hr of the wound scratch

A. represents the photos of the control cells at t=0 hr with the length of the wound width shown. Gap width was measured as the distance from one end of the wound to the other using NIH Image J2 software. **B.** shows the closure of the wound by the control cells 24 hours after the scratch.

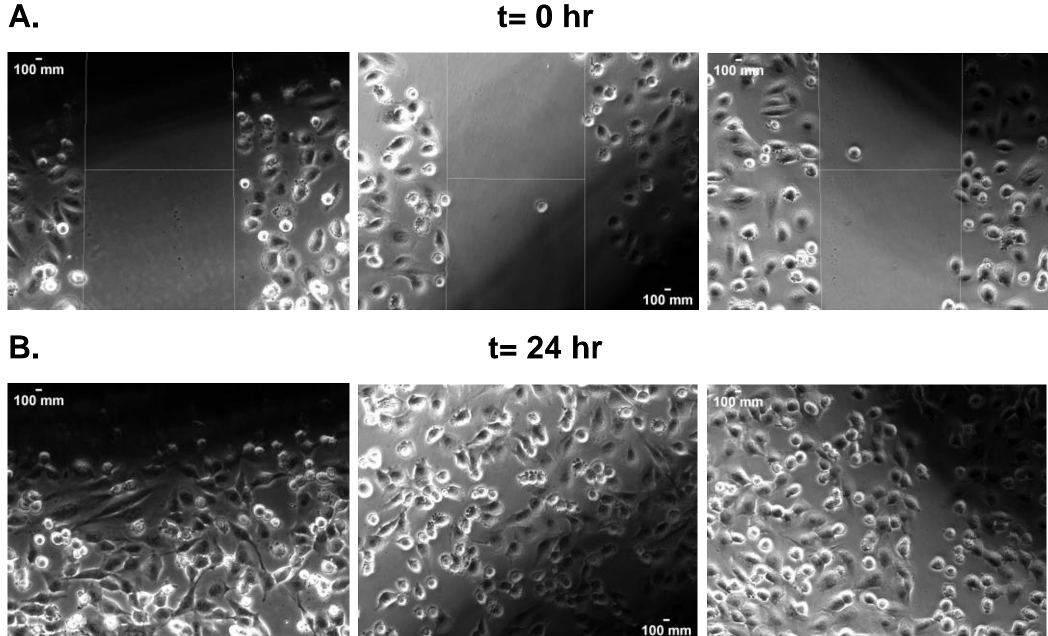


Figure 6.2b: Control cells of MDA-MB-468 at 0 hr and 24 hr of the wound scratch

A. represents the photos of the control cells at t=0 hr with the length of the wound width shown. Gap width was measured as the distance from one end of the wound to the other using NIH Image J2 software. **B.** shows the closure of the wound by the control cells 24 hours after the scratch.

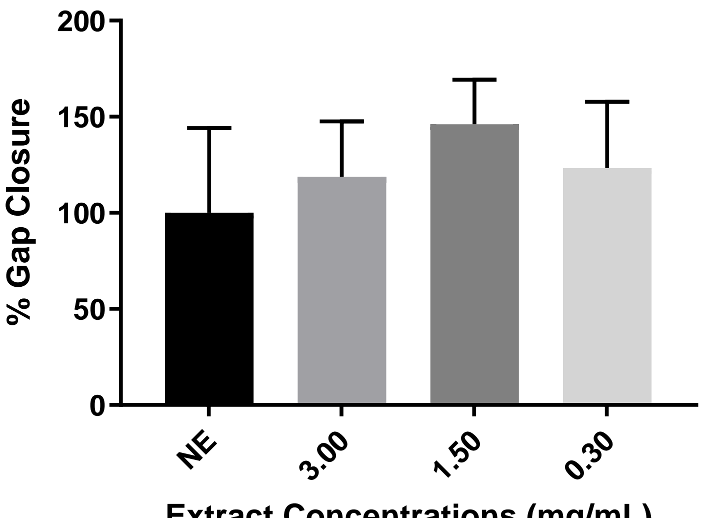
6.2.1 Effects of the Acetone Extract on Breast Cancer Cell Lines

Anti-migratory effects of the acetone extract were evaluated on MDA-MB-231 and MDA-MB-468 cells using collagen as an attachment site.

In MDA-MB-231 cells, at all three concentrations of the extract, wound closure was observed 24 hours after the scratch (Figure 6.2.1.1). Percent change in wound width compared to the control mean was 118.74%, 146.05% and 123.31% at 3.00 mg/mL, 1.5 mg/mL and 0.30 mg/mL of the acetone extract, respectively. Although there seems to be an upward trend up to 1.5 mg/mL, the rate of cell migration declined at 0.30 mg/mL and none of these were significantly different from the control. In other words, no statistically significant inhibitory effect was observed on the migration of MDA-MB-231 in the presence of the acetone extract (Graph 6.2.1.1).

Similarly, in MDA-MB-468 cells, presence of the acetone extract did not have any statistically significant effect on the migration of cells (Figure 6.2.1.2). Percent mean changes in wound width were respectively 85.60%, 78.13% and 68.35% at 3.00 mg/mL, 1.5 mg/mL and 0.30 mg/mL of the extract. Complete wound closure was observed 24 hours after the scratch showing no significant anti-migratory effect of the acetone extract on MDA-MB-468 cells (Graph 6.2.1.2).

In summary, the bioactive molecules in the acetone extract did not have an overall significant anti-migratory effect on the metastatic cells lines tested.



Graph 6.2.1.1: Effect of the Acetone Extract on Migration of MDA-MB-231 cells

Effects of *C. olitorius* extract in acetone were evaluated on the migration of MDA-MB-231 cells. The negative control used, represented as NE (no extract), consisted of cells in media but no extract. Data are presented as a percent of control mean ± S.D. No statistically significant difference was observed between the control and the extract-treated cells.

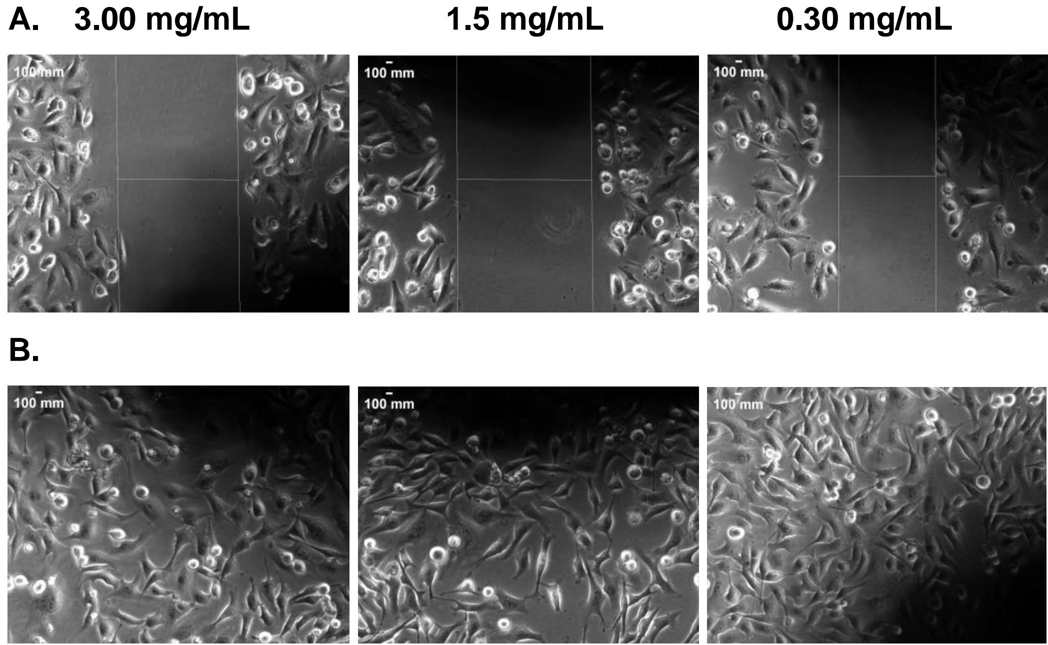
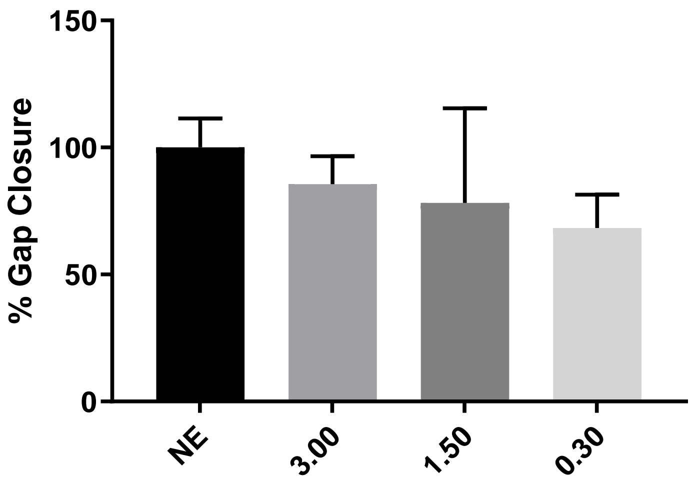


Figure 6.2.1.1: Migration of MDA-MB-231 cells at different concentrations of the acetone extract at 0 hr and 24 hr of the wound scratch

A. represents the three different concentrations of the acetone extract, 3.00 mg/mL, 1.50 mg/mL and 0.30 mg/mL, at **t=0 hr**. The length of the wound width was measured as the distance from one end of the wound to the other using Image J2. **B.** shows the distance travelled by these cells and the closure of the wound 24 hours after the scratch.



Graph 6.2.1.2: Effect of the Acetone Extract on Migration of MDA-MB-468 cells

Effects of *C. olitorius* extract in acetone on MDA-MB-468 cells were statistically analysed. The negative control used, represented as NE (no extract), consisted of cells in media but no extract. No statistically significant difference was observed between the control and the extract-loaded cells.

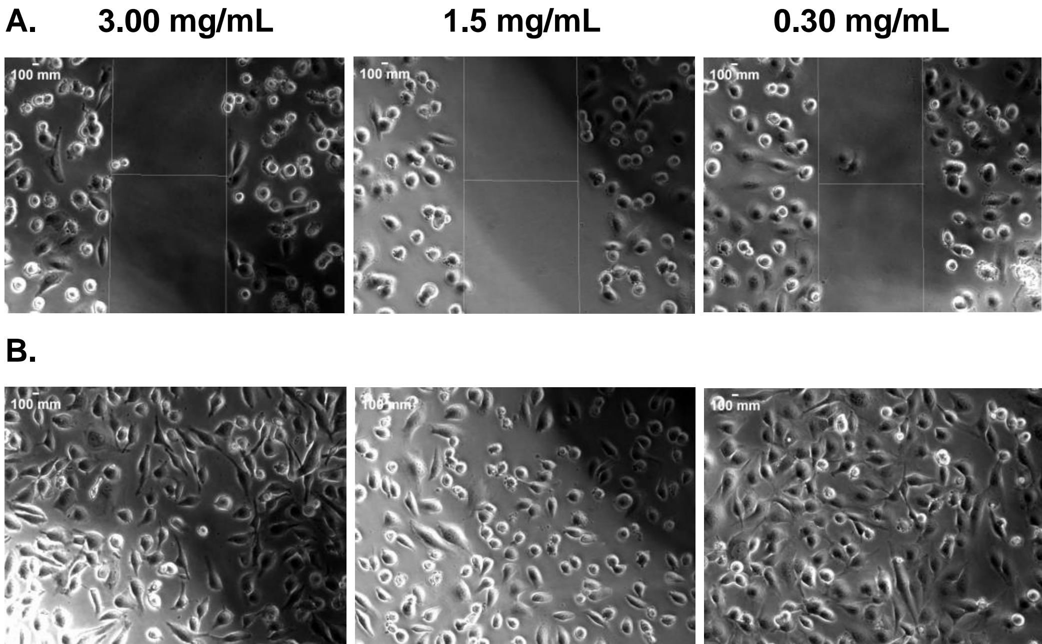


Figure 6.2.1.2: Migration of MDA-MB-468 cells at different concentrations of the acetone extract at 0 hr and 24 hr of the wound scratch

A. represents the three different concentrations of the acetone extract, 3.00 mg/mL, 1.50 mg/mL and 0.30 mg/mL, at **t=0 hr** in MDA-MB-468 cells. The length of the wound width was measured as the distance from one end of the wound to the other using Image J2. **B.** shows the closure of the wound at **t=24 hr**.

6.2.2 Effects of the Hexane Extract on Breast Cancer Cell Lines

The effect of the hexane extract on migration of MDA-MB-231 and MDA-MB-468 cells was also evaluated.

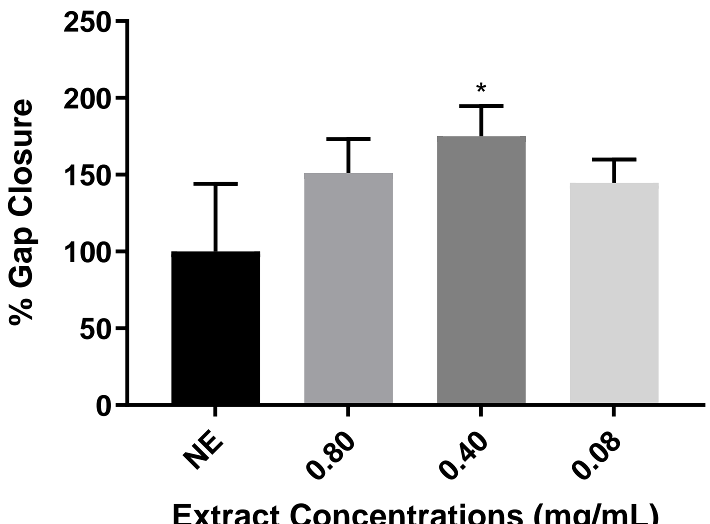
In MDA-MB-231 cells, similar to the acetone extract, there appears to be an incline in cell migration when the extract concentration drops to 0.40 mg/mL after which a reduction is observed in wound closure. However, in the hexane extract, the percent change in wound width at 0.40 mg/mL, which is 175.14%, was statistically higher than the control ($p<0.05$). The other concentrations of the hexane extract were not statistically significant from the control mean even though they were also higher. Percent change at 0.80 mg/mL was 151.18% and 144.66% at 0.08 mg/mL.

Therefore, bioactive compounds of the hexane extract of *C. olitorius* leaves did not have any anti-migratory effect on MDA-MB-231 cells. Rather, a mid-

range concentration of the extract appears to stimulate cell migration in this cell line (Graph 6.2.2.1).

In MDA-MB-468 cells, at 0.40 mg/mL and 0.08 mg/mL of the hexane extract, wounds were mostly closed 24 hours after the scratch (Figure 6.2.2.2). Percent changes observed in wound width at 0.40 mg/mL and 0.08 mg/mL were 119% and 98.48%, respectively. These findings were not significantly different from the control. However, at 0.80 mg/mL, the wound scratch diminished in size but was not completely closed. Percent wound closure at 0.80 mg/mL was 26.37% which was found to be statistically lower than the control (p<0.01) (Graph 6.2.2.2).

Based on these findings 0.80 mg/mL of the hexane extract was found to inhibit migration in MDA-MB-468 cells.



Graph 6.2.2.1: Effect of the Hexane Extract on Migration of MDA-MB-231 cells

Effects of *C. olitorius* extract in hexane on MDA-MB-231 cells were evaluated. The negative control used, represented as NE (no extract), consisted of cells in media but no extract. Data are presented as a percent of control mean ± S.D. * represents significance at p<0.05 level at 0.40 mg/mL.

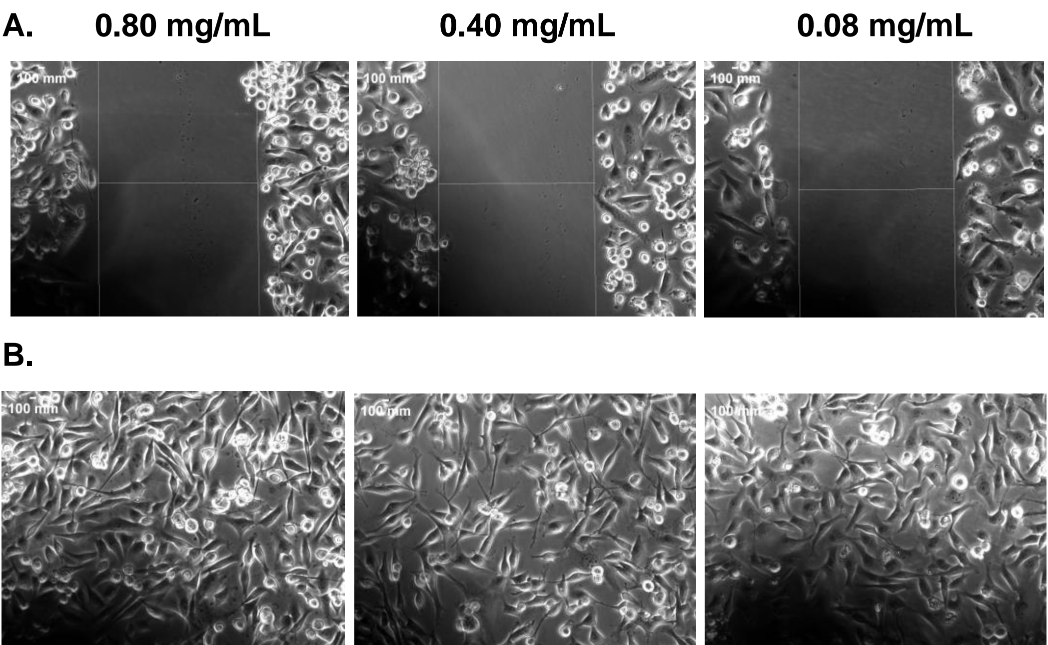
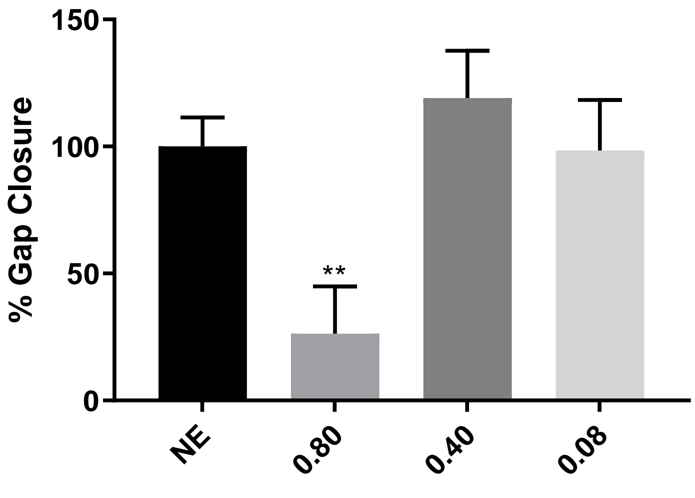


Figure 6.2.2.1: Migration of MDA-MB-231 cells at different concentrations of the hexane extract at 0 hr and 24 hr of the wound scratch

A. represents the three different concentrations of the hexane extract, 0.80 mg/mL, 0.40 mg/mL and 0.08 mg/mL, at **t=0 hr**. The length of the wound width was measured as the distance from one end of the wound to the other using Image J2. **B.** shows the closure of the wound by these cells at **t=24 hr**.



Graph 6.2.2.2: Effect of the Hexane Extract on Migration of MDA-MB-468 cells

Effects of *C. olitorius* extract in hexane on MDA-MB-468 cells were evaluated. A negative control was used, represented as NE (no extract), which consisted of cells in media but no extract. ** shows the statistically significant difference in percent change in wound width at 0.80 mg/mL compared to the control mean ($p<0.01$).

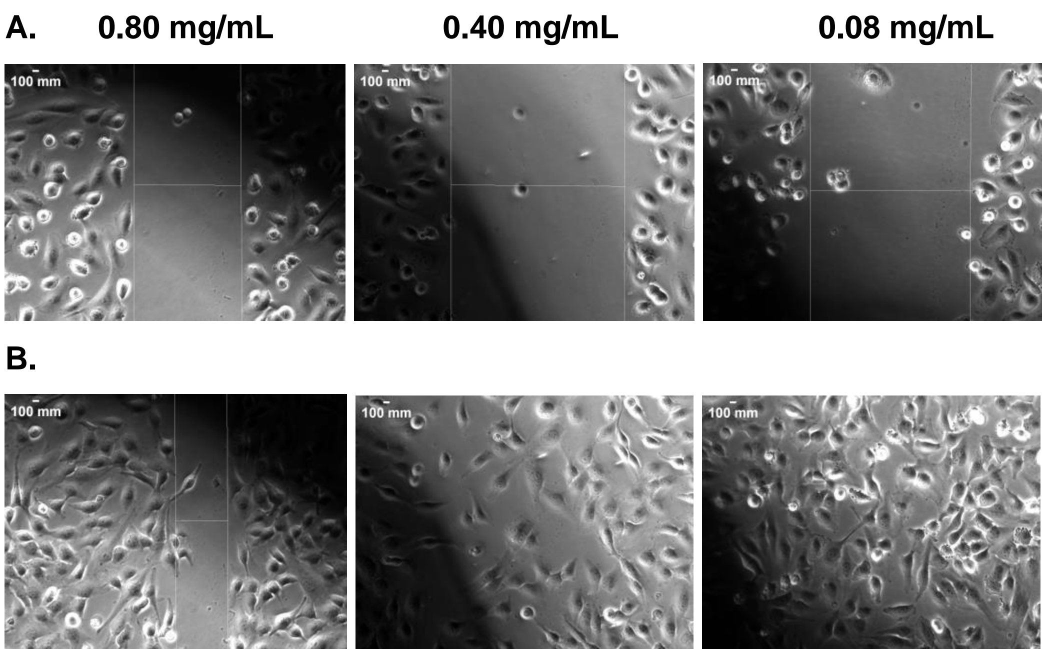


Figure 6.2.2.2: Migration of MDA-MB-468 cells at different concentrations of the hexane extract at 0 hr and 24 hr of the wound scratch

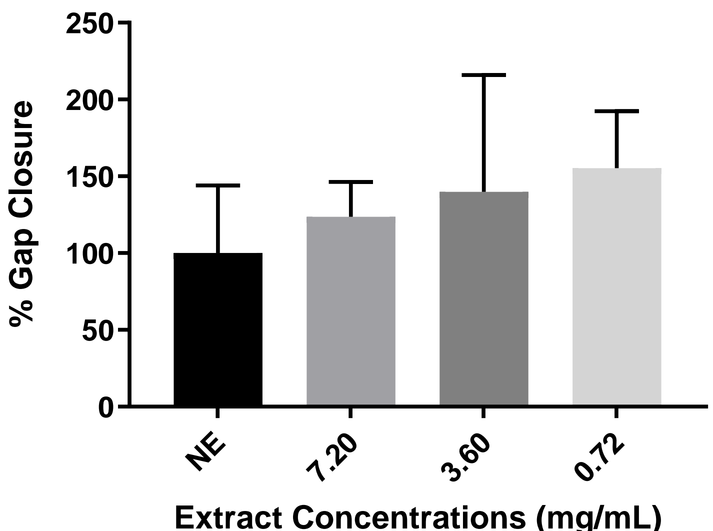
A. represents the three different concentrations of the hexane extract, 0.80 mg/mL, 0.40 mg/mL and 0.08 mg/mL, at **t=0 hr**. The length of the wound width was measured as the distance from one end of the wound to the other using Image J2 software. **B.** shows the distance travelled by the cells at **t=24 hr**. Gap closure was observed at 0.40 mg/mL and 0.08 mg/mL of the extract.

6.2.3 Effects of the Methanol Extract on Breast Cancer Cell Lines

Migration of MDA-MB-231 and MDA-MB-468 cell lines was evaluated in the presence of the methanol extract of *C. olitorius* leaves. As seen in Figure 6.2.3.1, 24 hours after the scratch, the wounds were almost closed in MDA-MB-231 cells at the concentrations tested. Compared to the control mean, percent change in wound width was higher but this was not significantly different at any of the concentrations tested (Graph 6.2.3.1:). Percent changes in wound width were 123.64%, 139.93% and 155.42% at 7.20 mg/mL, 3.60 mg/mL and 0.72 mg/mL of the extract, respectively. Although there appears to be an inverse association between cell migration and extract concentration, phytochemicals in the methanol extract did not have any significant effect on migration in this cell line.

In MDA-MB-468 cells, at all three concentrations, the length of wound width reduced with some wells showing complete wound closure (Figure 6.2.3.2). Means of the percent changes observed were 35.84%, 52.43% and 57.35% at 7.20 mg/mL, 3.60 mg/mL and 0.72 mg/mL, respectively. Although the percent change in wound width compared to the control mean was not statistically significant at 3.60 mg/mL and 0.72 mg/mL of the methanol extract, the partial wound closure observed at 7.20 mg/mL was significantly lower than that of control ($p<0.05$) (Graph 6.2.3.2).

In summary, these findings suggest that 7.20 mg/mL of the methanol extract appears to reduce migration of MDA-MB-468 cells.



Graph 6.2.3.1: Effect of the Methanol Extract on Migration of MDA-MB-231 cells

Anti-migratory effects of *C. olitorius* extract in methanol on MDA-MB-231 cells were evaluated. The negative control used, represented as NE (no extract), consisted of cells in media but no extract. Data are presented as a percent of control mean ± S.D. No significant difference was observed between the control and experimental cells.

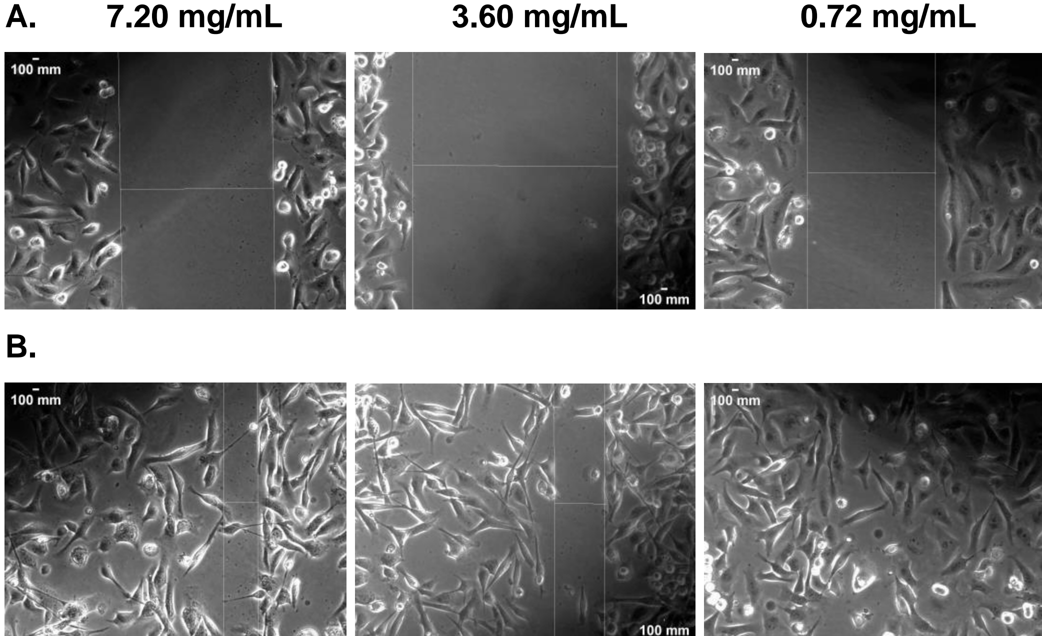
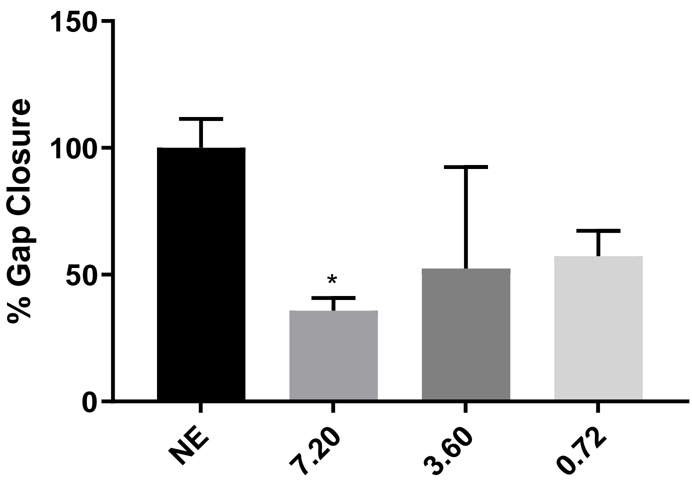


Figure 6.2.3.1: Migration of MDA-MB-231 cells at different concentrations of the methanol extract at 0 hr and 24 hr of the wound scratch

A. represents the three different concentrations of the methanol extract, 7.20 mg/mL, 3.60 mg/mL and 0.72 mg/mL, at **t=0 hr**. The length of the wound width was measured as the distance from one end of the wound to the other using Image J2. **B.** shows the distance travelled by these cells at **t=24 hr**.



Graph 6.2.3.2: Effect of the Methanol Extract on Migration of MDA-MB-468 cells

Effects of *C. olitorius* extract in methanol on the migration of MDA-MB-468 cells were evaluated. The negative control used, represented as NE (no extract), consisted of cells in media but no extract. Percent migration of the cells at 7.20 mg/mL of the extract was significantly lower than the control ($p<0.05$).

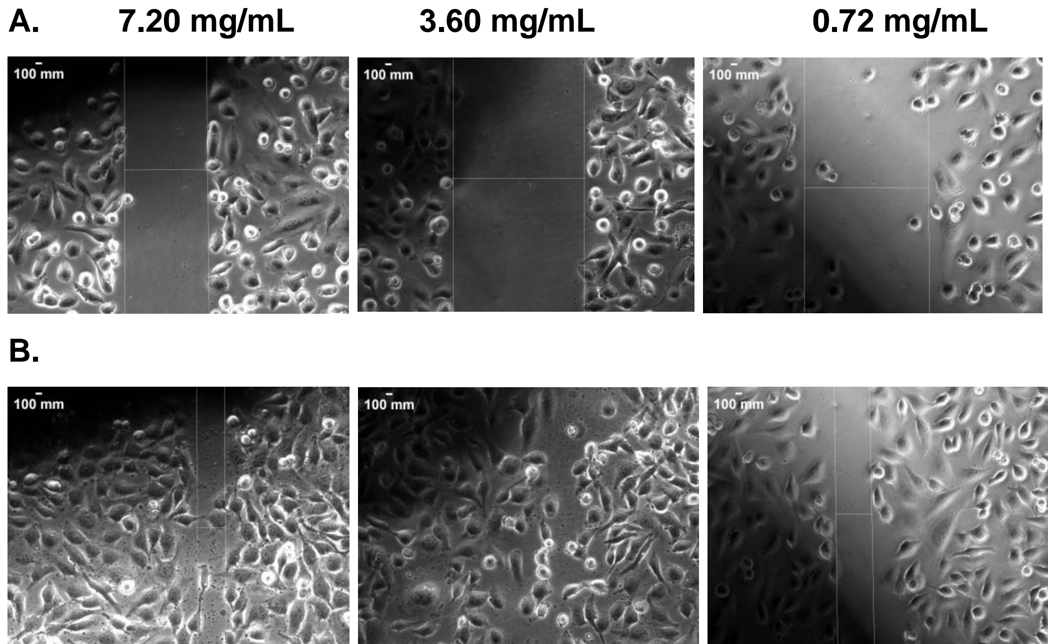


Figure 6.2.3.2: Migration of MDA-MB-468 cells at different concentrations of the methanol extract at 0 hr and 24 hr of the wound scratch

A. represents the three different concentrations of the methanol extract, 7.20 mg/mL, 3.60 mg/mL and 0.72 mg/mL, at **t=0 hr**. The length of the wound width was measured as the distance from one end of the wound to the other using Image J2. **B.** shows the distance travelled by these cells at **t=24 hr**.

6.2.4 Assay of MMP9 Expression

Metalloprotease 9 (MMP9) is among the protein markers upregulated in invasive cancers, degrading the ECM and activating migration (Kundur et al., 2018; Vuoso et al., 2020) therefore, for further exploration of the significantly different effects on migration and possible mechanism of action involving metalloproteases, levels of MMP9 were measured in MDA-MB-231 and MDA-MB-468 cells to compare the effects of the hexane and methanol extracts against their untreated controls. All data are presented as a percentage of the control mean ± S.D, based on statistical analysis of eight replicates using one-way ANOVA.

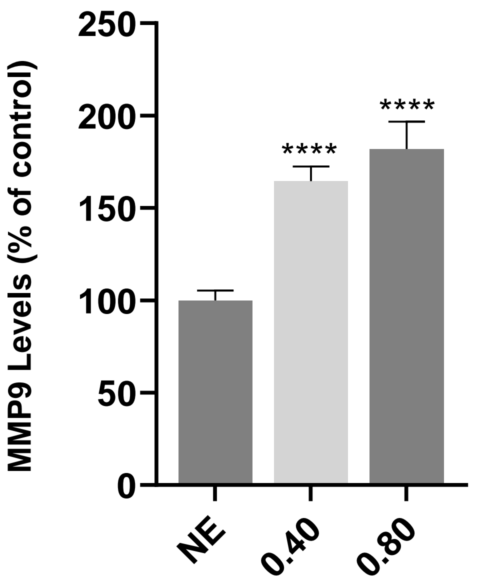
In the hexane extract, concentrations of 0.40 and 0.80 mg/mL were significantly different in the cell lines used. Therefore, the expression level of MMP9 was measured at these two concentrations of the hexane extract.

In the methanol extract, significant difference was observed only at 7.20 mg/mL. Therefore, both TNBC lines were evaluated for their MMP9 expression at this concentration.

6.2.4.1 Effects of the Hexane Extract on the Expression of MMP9

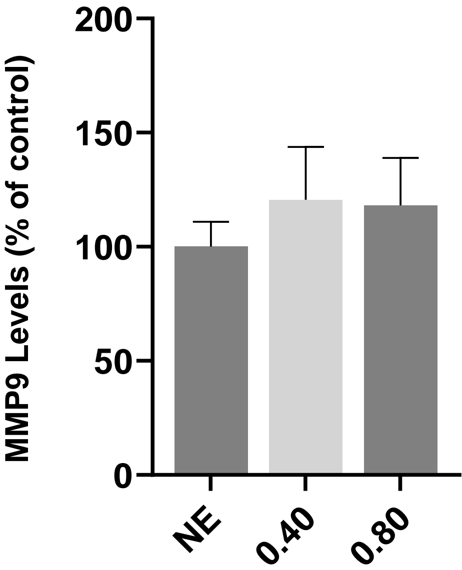
In MDA-MB-231 cells, the expression of MMP9 was significantly higher that of the control in the presence of the hexane extract. The percent means of MMP9 expression in the experimental cells were 164.6% and 182.0% at 0.40 mg/mL and 0.80 mg/mL, respectively ($p<0.0001$).

In MDA-MB-468 cells, the expression was MMP9 was not statistically different from that of control. Results are given below.



Graph 6.2.4.1a: Effect of Hexane Extract on MMP9 Levels in MDA-MB-231 Cells

Levels of MMP9 were measured in MDA-MB-231 cells treated with the hexane extract of *C. olitorius* leaves. The negative control, given as NE (no extract), consisted of cells in DMSO/media without extract. Levels of MMP9 were significantly higher in both concentrations of the hexane extract than the control ($p<0.0001$).

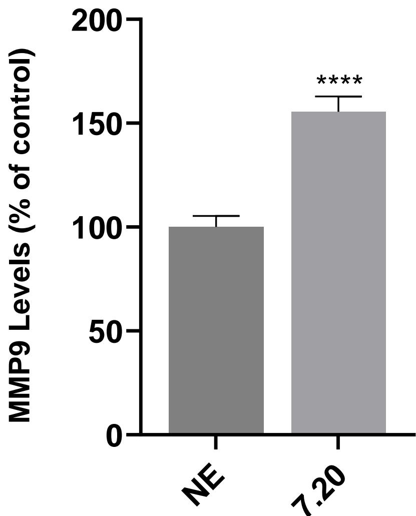


Graph 6.2.4.1b: Effect of Hexane Extract on MMP9 Levels in MDA-MB-468 Cells

Levels of MMP9 were measured in MDA-MB-468 cells treated with the hexane extract of *C. olitorius* leaves. The negative control, given as NE (no extract), consisted of cells in DMSO/media without extract. Levels of MMP9 in experimental cells were not significantly different from the control.

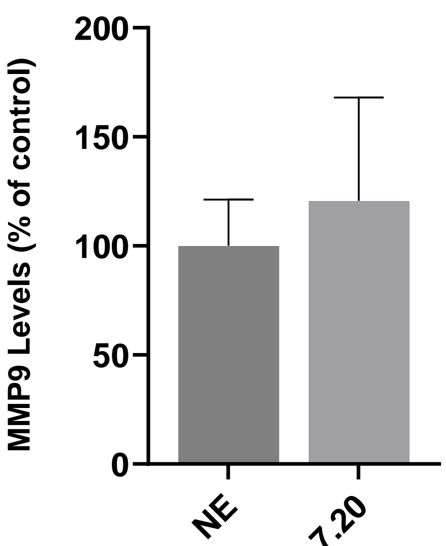
6.2.4.2 Effects of the Methanol Extract on the Expression of MMP9

In MDA-MB-231 cells, the expression of MMP9 was significantly higher that of the control at 7.20 mg/mL of the methanol extract with a percent mean of 155.6% ($p<0.0001$). However, in MDA-MB-468 cells, the expression was MMP9 was not statistically different from that of control. Results are given below.



Graph 6.2.4.2a: Effect of Methanol Extract on MMP9 Levels in MDA-MB-231 Cells

Levels of MMP9 were measured in MDA-MB-231 cells treated with the methanol extract of *C. olitorius* leaves. The negative control, given as NE (no extract), consisted of cells in DMSO/media without extract. Levels of MMP9 were significantly higher than the control ($p<0.0001$).



Graph 6.2.4.2b: Effect of Methanol Extract on MMP9 Levels in MDA-MB-468 Cells

Levels of MMP9 were measured in MDA-MB-468 cells treated with the methanol extract of *C. olitorius* leaves. The negative control, given as NE (no extract), consisted of cells in DMSO/media without extract. MMP9 expression in experimental cells was not significantly different from the control.

6.3 Discussion

A wound healing assay was conducted using collagen to assess the migratory ability of cells incubated with the extracts. Since migration is a characteristic of metastatic cells, only MDA-MB-231 and MDA-MB-468 cell lines were used in this assay.

In MDA-MB-468 cells, a significant difference between the experimental cells and the control group was observed in the hexane and methanol extracts (Graph 6.2.2.2, Graph 6.2.3.2). In both extracts, the strongest concentrations provided an optimum environment for the bioactive compounds to exert their anti-migratory effects in this cell line such that 0.80 mg/mL of the hexane extract decreased migration by 73.6% and 7.20 mg/mL of the methanol extract by 64.2%.

In MDA-MB-231 cells, the only significant difference in cells treated with the hexane extract compared to the control (Graph 6.2.2.1). Unlike MDA-MB-468 cells, 0.40 mg/mL of the hexane extract resulted in a higher migration rate than the control by 75.1% in MDA-MB-231 cells.

Despite having no significant difference from the control cells in the acetone extract, in both the acetone and hexane extracts, MDA-MB-231 cells had an inverted U-shaped response in wound healing assay, which could be indicative of a hormetic response (Graph 6.2.1.1, Graph 6.2.2.1). As discussed in Chapter 5, a hormetic response occurs when modest concentrations, around 30-60% greater than the control values, cause the maximal response (Calabrese, 2013). Although 0.40 mg/mL of the hexane extract is 75.1% greater than the control value, it is possible that the bioactive compounds in the hexane extract stimulated an effect in MDA-MB-231 cells, similar to a hormetic response, and resulted in a final pro-migratory signalling pathway.

6.3.1 Potential Mechanisms of Action in MDA-MB-231 Cells

Linoleic acid, which is one of the major components of the hexane extract, has been previously studied for its migratory effects on various cancer cell

lines, including MDA-MB-231, and several mechanisms have been proposed.

In one such study, the effects of linoleic acid on SKOV-3 cells, which are human ovarian cancer cells, were assessed. It was reported that treatment of cells with a low dose (16 µM) of linoleic acid for 24 hrs increased migration by 20%. In the same study, treated cells were shown to undergo remodelling and altered dynamics in their actin filaments and microtubules, increasing the number of filopodia and proportion of longer filopodia at the leading edge. These protrusions are characteristics of cells elongating on a two-dimensional surface to initiate cell migration. Another finding of the study was the non-significant difference of α-linolenic acid from the control group in the migration rate (Masner, Lujea, Bisbal, Acosta, & Kunda, 2021).

Although a different cell line was used, a lower dose of linoleic acid might have induced similar rearrangements in these cytoskeletal components in MDA-MB-231 cells, thereby enhancing migration speed. Consistent with the non-significance reported in the above study, the effects of α-linolenic acid might have been masked in the present study and therefore did not neutralise the pro-migratory effect of linoleic acid in the hexane extract.

There are a number of studies that investigated the signalling pathways and proteins involved in enhanced cell migration in MDA-MB-231 cells treated with 90 µM of linoleic acid for 48 hrs.

One of the mechanisms proposed in these studies was the cargo content of extracellular vesicles (EV) secreted by linoleic acid-treated MDA-MB-231 cells. When mammary non-tumourigenic epithelial MCF10A cells were given EVs isolated from MDA-MB-231 cells after a 48-hr treatment with 90 µM of linoleic acid, cells changed morphology, grew protrusions and increased migration. However, EVs isolated from untreated MDA-MB-231 cells did not show an increase in migration markers in MCF10A cells, emphasising the key role of vesicle content in cell-to-cell communication and mediating changes in non-tumourigenic cells (Galindo-Hernandez,

Serna-Marquez, Castillo-Sanchez, & Salazar, 2014; Leal-Orta, Ramirez-Ricardo, Cortes-Reynosa, Galindo-Hernandez, & Salazar, 2019).

In both studies, non-metastatic MCF10A cells were found to express higher FAK, vimentin, N-cadherin and lower E-cadherin, which are all markers of increased migration and invasiveness (Galindo-Hernandez et al., 2014; Leal-Orta et al., 2019). It was previously demonstrated that FAK regulates N-cadherin expression in cell adhesion and migration (Mui et al., 2015; Yano et al., 2004).

It was proposed that the metastasis-prone behaviour observed in MCF10A cells was as a result of linoleic acid-induced EVs resulting in a direct activation of PI3K/Akt and ERK-dependent pathways and subsequently increased EMT-like markers (Leal-Orta et al., 2019). PI3K/Akt and ERK-dependent pathways are known to crosstalk and synergise migration through adhesion and EMT modulation. FAK phosphorylation by ERK at the leading edge is thought to regulate adhesion dynamics, enhancing migration (C. Huang, Jacobson, & Schaller, 2004).

EV-stimulated MCF10A cells were also shown to have higher NF-κB-DNA binding activity which stimulated MMP9 expression (Galindo-Hernandez et al., 2014). NF-κB pathway has a critical role in signalling with a tightly regulated transcriptional activity, mediating inflammatory response and cellular invasion (Yu, Lin, Zhang, Zhang, & Hu, 2020).

These studies correlate with the findings of the current study, supporting increased migration and MMP9 expression in MDA-MB-231 cells after incubation with linoleic acid-containing hexane extract for 24 to 48 hours. They also emphasise the importance of EVs in carrying cancer-stimulating signalling molecules and driving non-metastatic cells towards metastasis.

The role of PI3K/Akt signalling pathway and increased FAK in increased migration of MDA-MB-231 cells upon treatment with linoleic acid were reported in other studies.

In one such study, it was demonstrated that 48-hr incubation of MDA-MB-231 cells with 90 μM linoleic acid resulted in eicosanoid secretion which activated FAK and its downstream signalling pathway. One of the downstream metabolites of linoleic acid is arachidonic acid, metabolised by LOX and COX to pro-inflammatory eicosanoids, including prostaglandins and leukotrienes (Serna-Marquez et al., 2013). In the same study, it was suggested that eicosanoids from linoleic acid metabolism by LOXs and COXs mediated FAK as well as Src activation, with Src forming a complex with FAK, and that this coupling promoted a downstream signalling cascade, potentially through reducing E-cadherin expression, that subsequently activated migration (Serna-Marquez et al., 2013; Serrels, Canel, Brunton, & Frame, 2011).

Another pathway promoting cell migration via FAK activation involves one of its downstream proteins, PI3K. Activated PI3K could induce migration through its downstream proteins, Rac, which upregulates the activity of actin and formation of lamellipodia on the leading edge of migrating cells. Activated FAK is also shown to stimulate migration through its interactions with RhoA, which regulates actin assembly and disassembly at the rear end of migrating cells to provide support for lamellipodia formation (X. Zhao & Guan, 2011).

A recent study, in which MDA-MB-231 cells were incubated with 90 μM of linoleic acid for 48 hrs, also reported FAK activation, increased formation of lamellipodia through actin polymerisation and elongation, and increased MMP9 expression (Zaragoza, Castillo-Sanchez, Sanchez-Juarez, Cortes-Reynosa, & Salazar, 2025).

In addition to actin, microtubules and intermediate filaments, including vimentin, make up the major cytoskeletal components of a cell. Activated PI3K/Akt pathway has been known to regulate all three cytoskeletal components, which function collaboratively to enable detachment, formation of protrusions and motility during cell migration (Deng et al., 2022; Zhu et al., 2011).

It was previously shown that linoleic acid enhanced migration in MDA-MB-231 cells through activation of free fatty acid receptor 4 (FFAR4). FFAR4 is activated by free fatty acids, including linoleic acid and its metabolite arachidonic acid, and is involved in the regulation of adipogenesis and inflammation. It was suggested that FFAR4 could act as a tumour-promoting receptor after a 48-hr treatment with linoleic acid, transactivating EGFR, which induces PI3K/Akt cascade to increase migration (Serna-Marquez, Diaz-Aragon, Reyes-Uribe, Cortes-Reynosa, & Salazar, 2017).

One of the characteristics of metastatic breast cancers is higher expression of fascin, a protein involved in the formation of actin-rich cellular protrusions including filopodia (Gonzalez-Reyes, Marcial-Medina, Cervantes-Anaya, Cortes-Reynosa, & Salazar, 2018; Izdebska, Zielińska, Krajewski, & Grzanka, 2023). In a previous study, incubation of MDA-MB-231 cells with 90 µM of linoleic acid for 30 and 60 mins was shown to increase fascin expression in cells. The role of fascin in cell migration was also evaluated through scratch assay after incubation with 90 µM of linoleic acid for 48 hrs. It was reported that cell migration was stimulated through filopodia and lamellipodia formation by fascin overexpression. Furthermore, it was demonstrated that linoleic acid increased MMP9 secretion and that inhibition of fascin expression also inhibited MMP9 expression (Gonzalez-Reyes et al., 2018). A strong relationship between MMP9 and fascin expression with metastasis and poor prognosis was previously shown in breast cancer patients (Youssef & Hakim, 2014).

Given these observations from the literature and the mesenchymal phenotype of MDA-MB-231 cells (Majidpoor & Mortezaee, 2021), it appears that linoleic acid in the hexane extract had a dominating pro-migratory effect on this cell line, potentially affecting several major signalling pathways, such as EGFR/PI3K/Akt and downstream cytoskeletal components, in the present study. This may also account for the significant increase in MMP9 expression observed in MDA-MB-231 cells, as linoleic acid could have stimulated their EMT-prone molecular profile by upregulating MMP9 expression. MDA-MB-231 cells may also have higher

inducible MMP9 reserve due to their mesenchymal type. Evidence from the literature supports increased expression of MMP9 in MDA-MB-231 cells upon incubation with linoleic acid.

However, similar to caspases, increased levels of MMP9 in the ELISA assay after extract treatment represent total enzyme expression, measuring both pro-enzyme and the active form. Therefore, high expression of MMP9 may not directly translate to increased enzyme activity. This potentially explains the non-significant difference between the experimental cells and the control group in invasion assay despite lower gap closure in the treated cells, showing limited proteolytic degradation of the matrix after hexane and methanol extract treatment (Graph 7.2.2.1, Graph 7.2.3.1).

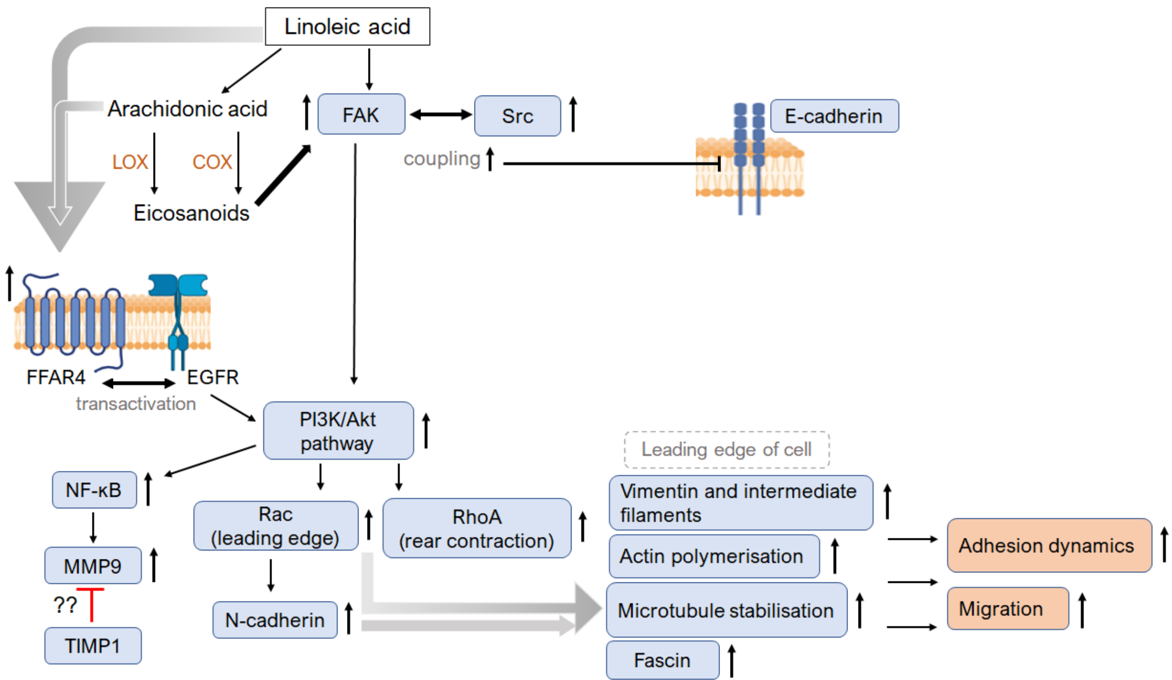
It is possible that upon treatment with the extracts, MDA-MB-231 cells prepared for cytoskeletal remodelling and subsequent migration, shown by elevated metalloprotease expression but MMP9 activity might have been blocked by other factors, such as tissue inhibitor of metalloproteinase 1 (TIMP1). TIMP1 tightly regulates the proteolytic activity of MMP9 in maintaining physiological processes. Any shift in the balance towards increased MMP9 activity causes matrix degradation and increased invasiveness of cells (Ozdemir et al., 2022).

It was previously shown that the most aggressive and metastatic breast cancer cells display highest levels of MMP9 expression and activity, as well as highest MMP9/TIMP1 ratio compared to benign breast tumours (Jinga et al., 2006). Therefore, to gain a deeper understanding of the mechanism behind significantly high expression of MMP9 in MDA-MB-231 without any significant invasive behaviour, future work could be aimed to measure MMP9 activity and TIMP1 expression and activity. This would provide important insight into the potential inhibitory effect of TIMP1 on the highly expressed MMP9 in this cell line and whether the observed effects were due to TIMP1 blocking MMP9 activity.

Lack of a significant difference of MMP9 expression between the treated and untreated group in MDA-MB-468 cells could be explained by their more epithelial-like, less EMT-prone profile displaying higher E-cadherin expression (Chekhun et al., 2013). It was previously reported that MMP9 expression is significantly higher in HER2 overexpressing subtype than basal-like breast cancers, including MDA-MB-468 cells (G. E. Kim et al., 2014). Therefore, their lower baseline MMP9 expression and stronger epithelial-like nature might account for the lack of significant difference between the treated and untreated cells.

Alternatively, even though migration was significantly reduced in MDA-MB-468 cells, lack of a significant difference between the experimental and control cells in MMP9 expression may suggest that the bioactive compounds in the hexane and methanol extracts targeted signalling pathways which did not involve metalloproteases.

Figure 6.3.1 summarises the mechanisms potentially involved in enhancing migration in MDA-MB-231 cells in the present study based on evidence from the literature on linoleic acid, as one of the major lipophilic components of the hexane extract. The diagram was constructed using the '05200 N-Pathways in Cancer' map from KEGG as a reference (KEGG, 2020) and modified based on evidence from the literature as discussed above to highlight the signalling pathways and cellular modifications relevant to the present study.



Linoleic acid
Arachidonic acid
LOX
COX
Eicosanoids
FAK
Src
coupling
E-cadherin
FFAR4
EGFR
transactivation
PI3K/Akt pathway
Leading edge of cell
NF-κB
MMP9
??
TIMP1
Rac (leading edge)
RhoA (rear contraction)
N-cadherin
Vimentin and intermediate filaments
Actin polymerisation
Microtubule stabilisation
Fascin
Adhesion dynamics
Migration

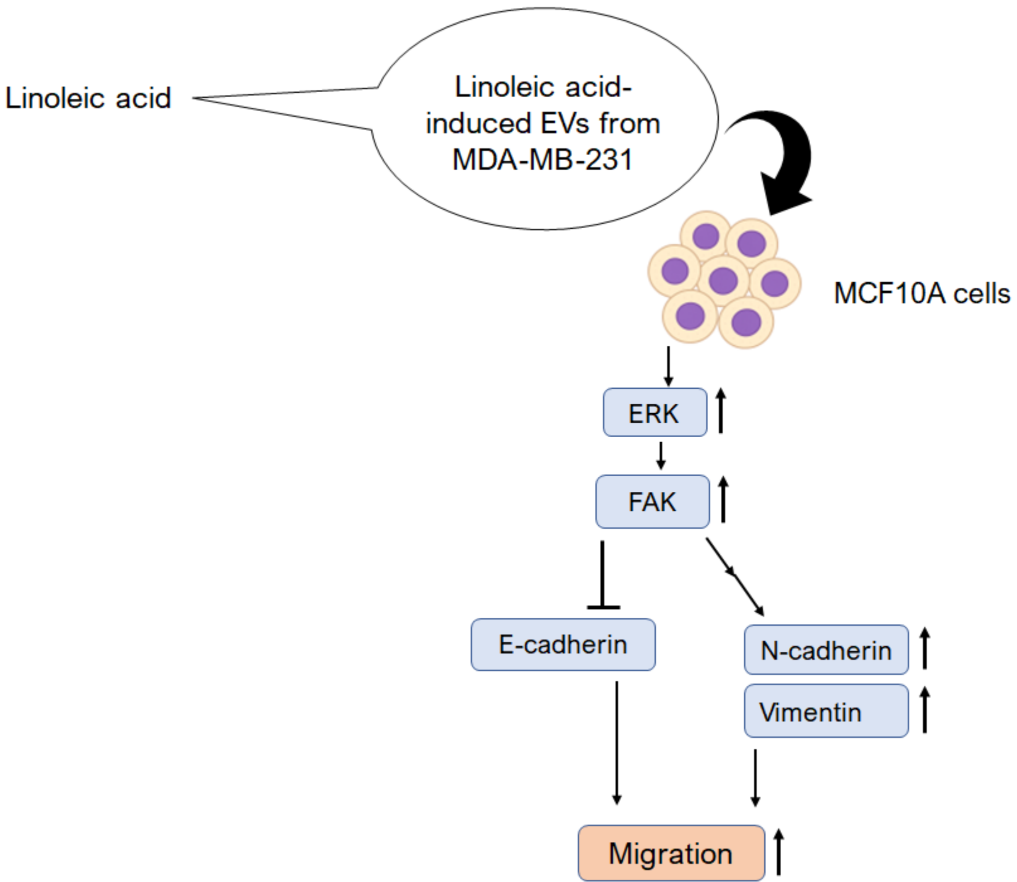


Figure 6.3.1: Overview of Proposed Mechanisms for Enhanced Migration in MDA-MB-231 cells and MCF10A cells induced by Extracellular Vesicles of MDA-MB-231 cells

Based on evidence from the literature, migration in MDA-MB-231 cells is thought to be increased by one of the major lipophilic components of the hexane extract, linoleic acid, through several mechanisms. Top image represents how linoleic acid, and its metabolite arachidonic acid, might have been involved in increased migration in MDA-MB-231 cells. The significant increase in MMP9 expression without a significant difference in invasion in this cell line could be due to TIMP1 blocking its activity (represented by question marks), which needs further validation. Bottom image demonstrates the tumourigenic, pro-migratory effect of EVs collected from linoleic acid-induced MDA-MB-231 cells on non-tumourigenic MCF10A cells. Both diagrams were constructed and modified from 05200 N-Pathways in Cancer map from KEGG based on literature findings. Images downloaded from www.biorender.com.

6.3.2 Potential Mechanisms of Action in MDA-MB-468 Cells

Availability of data in the literature on the effects of fatty acids on MDA-MB-468 cell migration is very limited. However, it is thought that cell migration was reduced as a result of modifications induced by α-linolenic acid, the major component of the hexane and methanol extracts identified in the present study. As discussed in Chapter 4, the competitive incorporation of

α-linolenic acid into the plasma membrane could disrupt the bilayer, forcing signalling receptors, including overexpressed EGFR, to move to non-lipid rafts and become non-functional. This would subsequently inactivate the downstream signalling pathways, including PI3K/Akt and Rho-Rac family which also modulate migration (Deng et al., 2022).

The role of EGFR in regulating migration was also previously reported. Knockout of the tripartite motif 66 (TRIM66), whose altered expression is associated with increased malignancy of cancers, resulted in a significant reduction in migration in MDA-MB-468 cells through downregulation of EGFR-dependent pathway (H. Zhang, Zheng, & Zhang, 2021).

It is possible that amplification of EGFR, which is an oncogene, predisposes MDA-MB-468 cells to be over-reliant on EGFR-driven signalling pathways, a phenomenon known as 'oncogene addiction'. According to this concept, in cancer cells, a given oncogene may play a more critical role in a given pathway compared with its role in normal, non-malignant cells (Weinstein & Joe, 2008). Therefore, EGFR could have behaved like a hyper-sensitive signalling receptor in MDA-MB-468 cells.

Nevertheless, behaviour of MDA-MB-468 cells in the presence of various plant extracts or individual bioactive compounds, including fatty acids, should be studied further. This would help provide more comparative insight between a Caucasian origin cell line, such as MDA-MB-231, and a black origin cell line, such as MDA-MB-468.

As described in Chapter 1, although not identified in the GC-MS analysis in the present study, quercetin and chlorogenic acid were previously found to be present in the alcoholic extracts of *C. olitorius* leaves and therefore, may also account for the observed effects in TNBC cell lines used in this study.

A study which investigated the anti-migratory effects of 10 and 50 µM quercetin on MDA-MB-231 and MDA-MB-468 cells reported reduced migration in both cell lines through Akt-dependent signalling pathway.

Quercetin was found to downregulate Akt and its downstream effector, β-catenin. β-catenin is a structural protein which binds E-cadherin to maintain cell-to-cell adhesion. High levels of β-catenin have been associated with invasive types of breast cancer (Srinivasan et al., 2016).

β-catenin is thought to be inhibited by quercetin through another axis which involves Hu-antigen R protein (HuR). HuR is a post-transcriptional regulator of gene expression whose increased cytoplasmic levels are positively associated with disease deterioration in various cancers (Umar et al., 2022; Wu & Xu, 2022). It was proposed that HuR regulates the expression of β-catenin in MDA-MB-231 cells and that quercetin treatment impairs cell migration in this cell line via downregulating HuR-dependent expression of β-catenin (Umar et al., 2022).

Other studies investigated the anti-migratory effects of chlorogenic acid and its isomer, isochlorogenic acid C, on MDA-MB-231 cells. In two studies in which wound healing assay was used, both chlorogenic acid and isochlorogenic acid C inhibited migration of MDA-MB-231 cells significantly compared to the control in a dose-dependent manner over 48 hours (J. K. Yu et al., 2018; Zeng et al., 2021). In the presence of isochlorogenic acid C, the inhibition was reported to be around 70% (Yu et al., 2018).

These results contrast with the findings of the present study in MDA-MB-231 cells, suggesting that other bioactive compounds in the methanol extract, particularly linoleic acid, may have contributed to an overall pro-migratory effect. They also highlight the distinction between the effects of individual compounds and combined effects observed in plant extracts.

Future research could be aimed to quantifying protein content and assessing the expression of PI3K, Akt and E-cadherin using Western blotting in both cell lines after treatment with the hexane and methanol extracts. This would be useful to confirm the role of PI3K/Akt signalling pathway in cell migration.

Figure 6.3.2 summarises the potential mechanisms and signalling pathways involved in suppressing migration in MDA-MB-468 cells using evidence from the literature, as discussed above. The diagram was constructed using KEGG 05200 N-Pathways in Cancer map as a reference and it was modified according to literature findings relevant to the present study.

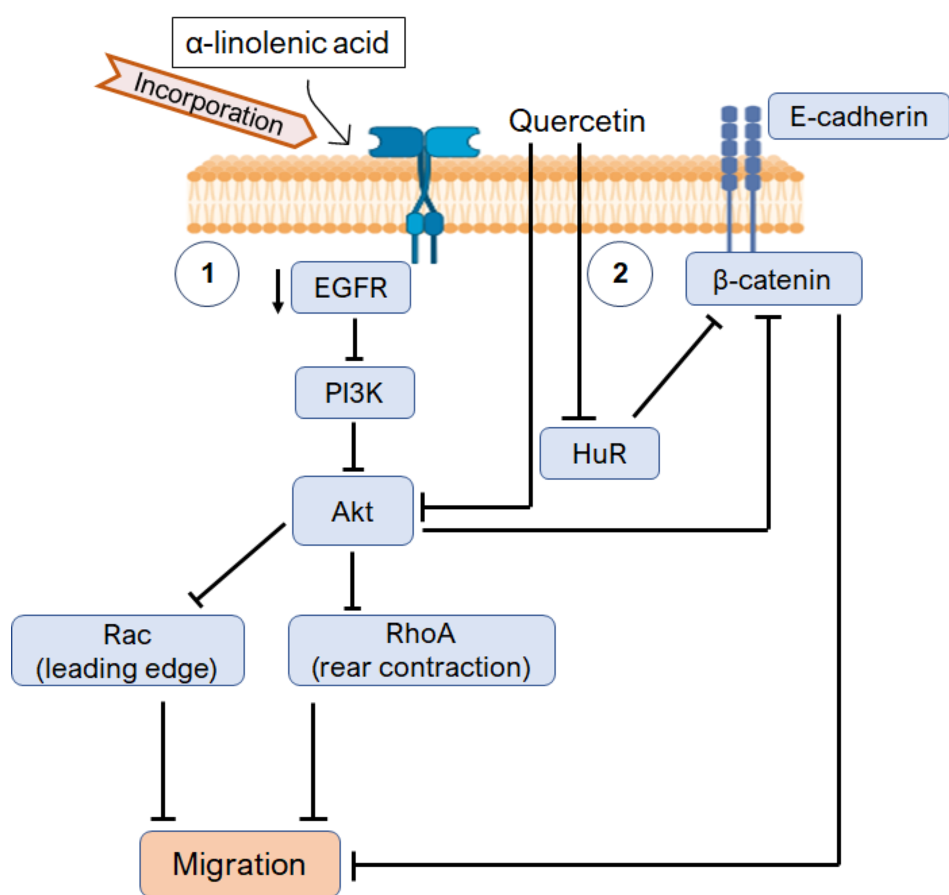


Figure 6.3.2: Overview of Proposed Mechanisms for Reduced Migration in MDA-MB-468 cells

Based on limited evidence from the literature, migration is thought to be reduced through several mechanisms in MDA-MB-468 cells by α-linolenic acid, the major lipophilic component identified in the hexane and methanol extracts, and quercetin as another major compound of *C. olitorius* leaves identified in previous studies. Mechanism 1 represents the potential incorporation of α-linolenic acid into the lipid bilayer and its downstream effects. Mechanism 2 represents the likely involvement of HuR protein after quercetin treatment in suppressing β-catenin activity, anchoring it to E-cadherin, resulting in reduced migration. The diagram was constructed and modified from KEGG, 05200 N-Pathways in Cancer map based on literature findings. Image downloaded from www.biorender.com.

6.4 Conclusion

Investigation of migration as a characteristic of metastatic cancer cells may reveal important information about how it can be prevented. In this assay, behaviour of two metastatic cell lines, MDA-MB-231 and MDA-MB-468, was evaluated using leaf extracts of *C. olitorius*.

In summary, findings of this study suggest that a moderate concentration of the hexane extract significantly increased migration in MDA-MB-231 cells. However, in MDA-MB-468 cells, the strongest concentrations of the hexane and methanol extracts reduced migration.

The behavioural differences observed in MDA-MB-231 and MDA-MB-468 cells in the present study indicate that, even though both are TNBC cell lines, plant extracts affect different signalling pathways and result in an overall differential response due to their molecular differences. This was also shown in their MMP9 expression, as MDA-MB-231 cells had a significantly higher expression, whereas MDA-MB-468 cells did not show any statistical difference from the control cells after a 24-hr incubation with the hexane and methanol extracts.

These findings also indicate the potential synergistic effect of highly polar and non-polar bioactive compounds in *C.olitorius* leaves on suppressing migration in MDA-MB-468 cells, which needs further investigation.

It was previously suggested that in addition to cell-dependent factors, dynamic tumour microenvironment may also be responsible for regulating the migration mode in cancer cells (Friedl & Alexander, 2011). It should be noted that other bioactive compounds in the hexane and methanol extracts, which could not be identified through GC-MS, could potentially exert their effects promoting pro-migratory or anti-migratory pathways in MDA-MB-231 and MDA-MB-468 cells, respectively. Therefore, other factors should be considered while interpreting the observations of this assay.

Chapter 7 - Effects of *C. olitorius* Extracts on Invasion of Breast Cancer Cells and CD24 Expression

7.1 Introduction

Invasion is a complex process in which tumour cells penetrate into normal tissue. Gaining the ability to spread to adjacent or distant tissues is one of the hallmarks of cancer (Friedl et al., 2012; Hanahan & Weinberg, 2011).

Although the first proposed model of spread was via migration of monoclonal (single type) tumour cells, there is substantial clinical evidence that tumour cells can also invade collectively as a polyclonal cluster of genetically different cells. Invasion of the surrounding stroma as cohesive clusters is referred to as 'collective invasion'. Collective invasion is seen in certain types of cancers including breast cancer. It is thought to be an efficient characteristic of tumour cells in terms of metastasising and colonising distant organs (Cheung et al., 2016; Majidpoor & Mortezaee, 2021).

Recently, CD24 protein expression has been investigated for its potential role in the metastasis of most cancers. Previously, it has been identified as a ligand for an adhesion receptor on endothelial cells (H. J. Kim et al., 2011). Evidence from both in vitro and clinical studies suggest that its expression in breast cancer cells might enhance the metastatic potential of tumour cells due to its role in adhesion and invasion and eventually result in poor prognosis in breast cancer (Jang, Kang, Jang, Paik, & Kim, 2016; H. J. Kim et al., 2011)

Since invasion occurs in some types of breast cancer, invasive abilities of the metastatic cell lines, MDA-MB-231 and MDA-MB-468, were investigated in this study. Wound healing method was used in this assay where the well plates were coated with collagen to provide the cells with a surface to adhere on. Another layer of collagen gel was loaded on top of

the cells on the day of the scratch to evaluate whether they would invade the gel. In association with the invasiveness of these cell lines, their expression of CD24 was also evaluated.

7.2 Results

Wound-healing assay was carried out to investigate the effects of the extracts on the invasive abilities of the metastatic cell lines, MDA-MB-231 and MDA-MB-468. Cells were grown in collagen-coated wells. An additional top layer of collagen was added to examine whether they would invade in the presence of different leaf extracts in acetone, hexane and methanol compared to the control. A negative control was used which consisted of cells in 0.1% DMSO/media containing 1% serum but no extract. Photographs of the control cells and treated cells were taken at t=0 hr and t=48 hr to observe the invasive ability of the cells.

The assay was repeated three times per extract and photos were taken for evaluation. The change in wound width was measured using ImageJ. Percent change in wound width between t=0 hr and t=48 hr in the extract-treated cells were calculated using Microsoft Excel compared to the control mean with ± S.D. These data were statistically analysed on GraphPad Prism 10 using unpaired t-tests to compare the means of the control and the tested concentration of the extracts. Tested concentrations were 1.50 mg/mL, 0.40 mg/mL and 0.72 mg/mL for the acetone, hexane and methanol extract, respectively.

Figure 7.2a and Figure 7.2b show the photos of MDA-MB-231 and MDA-MB-468 controls at t=0 hr and t=48 hr, respectively.

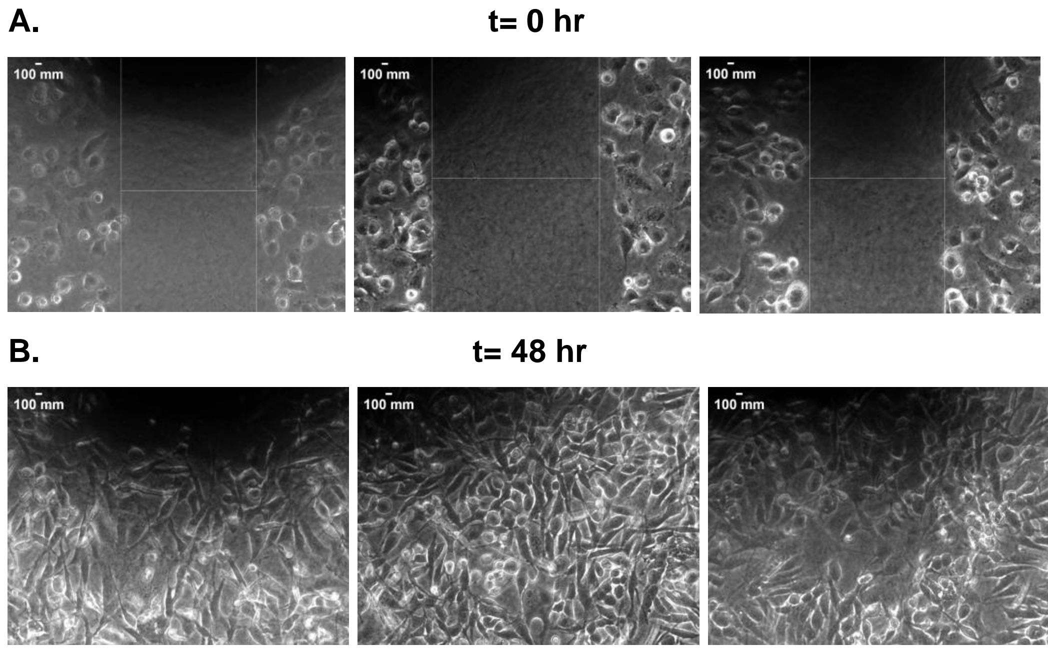


Figure 7.2a: MDA-MB-231 control cells at 0 hr and 48 hr after wound scratch

A. represents control cells at t=0 hr of the wound with the length of the wound width shown. Each experiment was repeated three times. Gap width was measured as the distance from one end of the wound to the other using NIH Image J2 software. **B.** shows the closure of the wound by the control cells at t=48 hr.

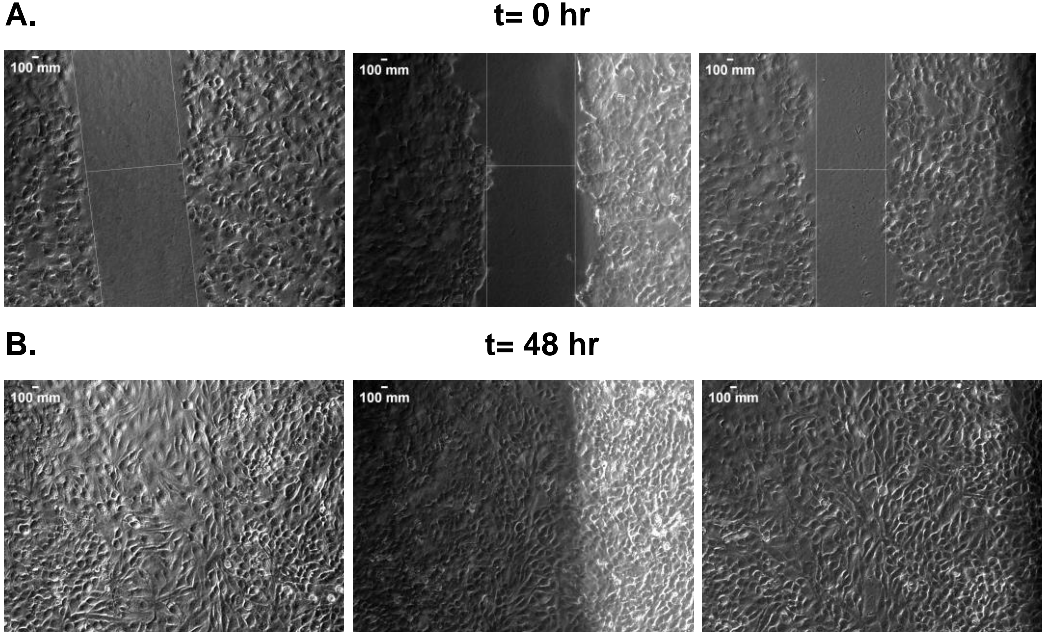


Figure 7.2b: MDA-MB-468 control cells at 0 hr and 48 hr after wound scratch

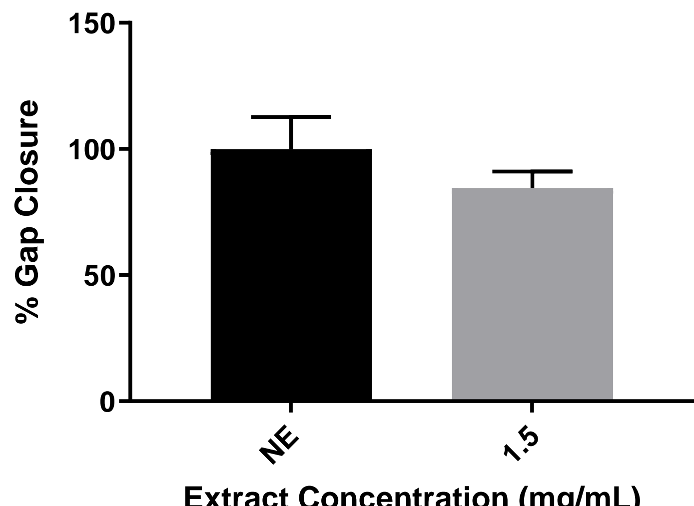
A. represents control cells at t=0 hr of the wound with the length of the wound width shown. Each experiment was repeated three times. Gap width was measured as the distance from one end of the wound to the other using NIH Image J2 software. **B.** shows the closure of the wound by the control cells at t=48 hr.

7.2.1 Effects of the Acetone Extract on Breast Cancer Cell Lines

Acetone extract was loaded on top of the collagen layer to examine whether it would stop MDA-MB-231 and MDA-MB-468 cells from invading the layer and closing the wound.

In MDA-MB-231 cells, 48 hours after the scratch, wound closure was observed with 1.5 mg/mL of the acetone extract (Figure 7.2.1.1). Percent change in wound width compared to the control mean was 84.59% which was not statistically significant from the control. In other words, acetone extract did not inhibit invasion of MDA-MB-231 cells (Graph 7.2.1.1).

In MDA-MB-468 cells a similar pattern was observed and at t=48 hr the gap between the cells has been completely closed (Figure 7.2.1.2). The percent mean change in wound width in the extract-treated cells was 116.89%. However, this was not significantly different from the control. Therefore, the acetone extract did not significantly prevent invasion of MDA-MB-468 cells (Graph 7.2.1.2).



Graph 7.2.1.1: Anti-invasive Effect of the Acetone Extract on MDA-MB-231 cells

Effects of the acetone extract (1.5 mg/mL) were evaluated on the invasion of MDA-MB-231 cells. The negative control used, represented as NE (no extract), consisted of cells in media but no extract. Data are presented as a percent of control mean ± S.D of three replicates indicating the closure of the wound width at t=48 hr. No statistically significant difference was observed between the control and the extract-loaded cells.

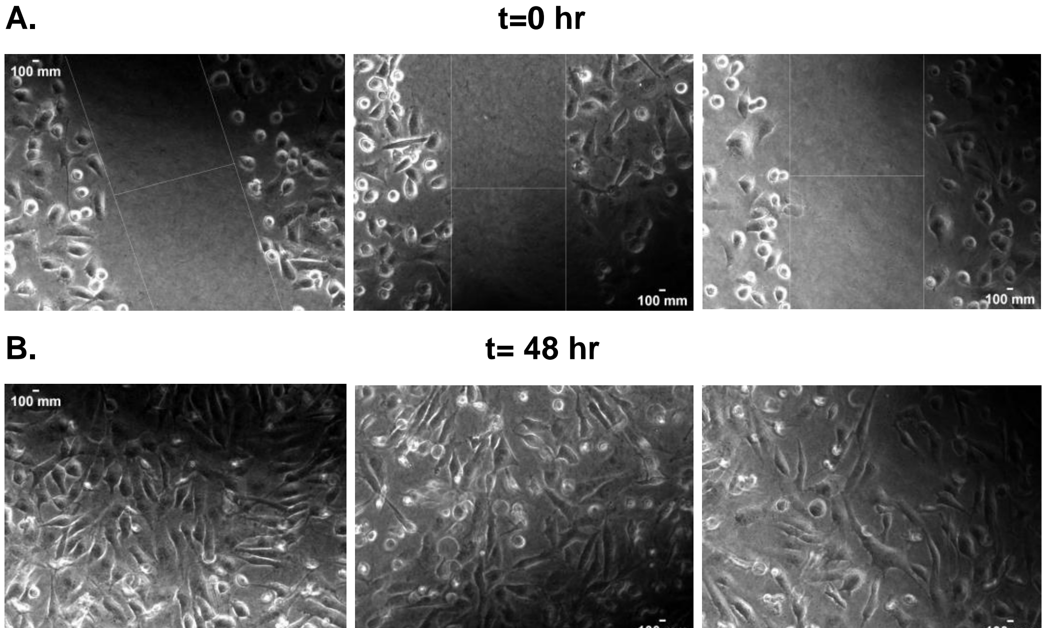
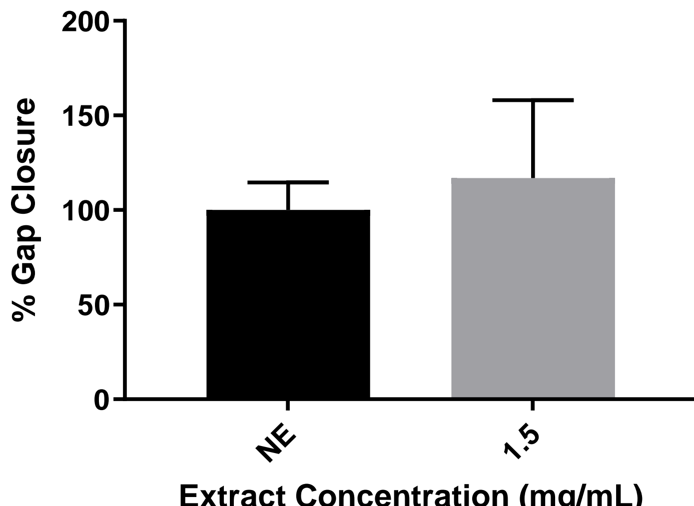


Figure 7.2.1.1: Effects of 1.5 mg/mL Acetone Extract on MDA-MB-231 Cells

A. represents the cells treated with the acetone extract at 1.50 mg/mL, t=0 hr. The length of the wound width was measured as the distance from both ends of the wound using Image J2. **B.** shows the distance travelled by the cells and the closure of the wound 48 hours after the scratch and extract loading. Each experiment was repeated three times.



Graph 7.2.1.2: Anti-invasive Effect of the Acetone Extract on MDA-MB-468 cells

Effects of the acetone extract (1.5 mg/mL) were evaluated on the invasion of MDA-MB-468 cells. A negative control, represented as NE (no extract), was used which consisted of cells in media but no extract. Data are presented as a percent of control mean ± S.D of three repeats showing the closure of the wound width at t=48 hr. No statistically significant difference was observed between the control and the extract-treated cells and the wound has completely closed in both.

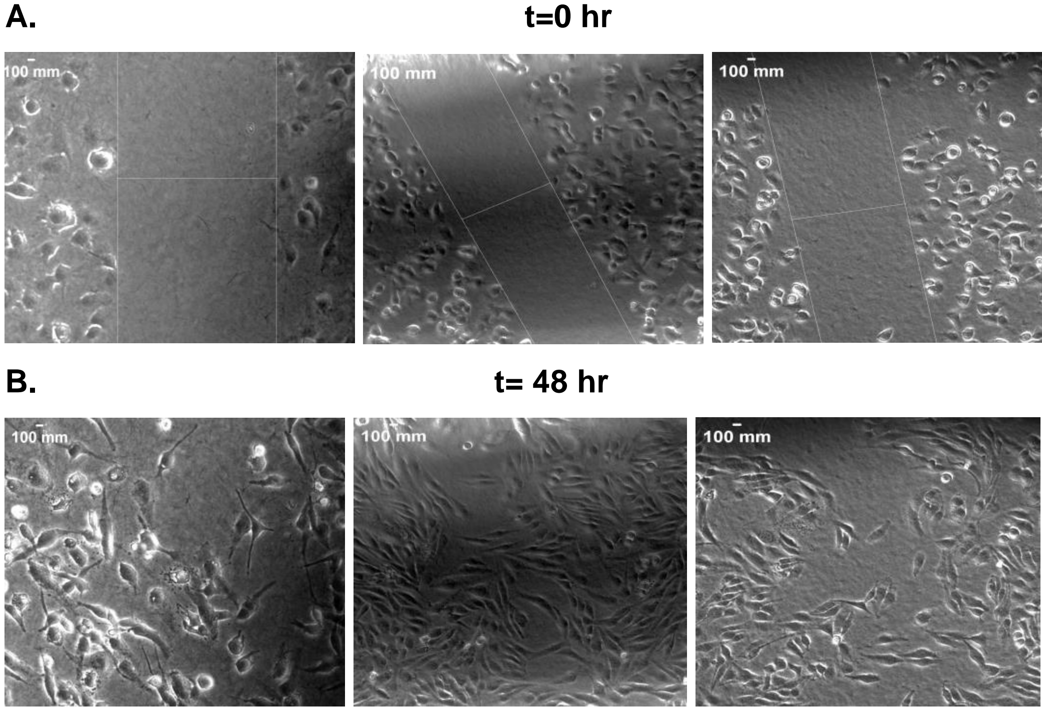


Figure 7.2.1.2: Effects of 1.5 mg/mL Acetone Extract on MDA-MB-468 Cells

A. represents the cells treated with 1.50 mg/mL of the acetone extract at t=0 hr. The length of the wound width was measured as the distance from both ends of the wound using Image J2. **B.** shows the distance travelled by the cells and the closure of the wound 48 hours after the scratch and extract loading. Each experiment was repeated three times.

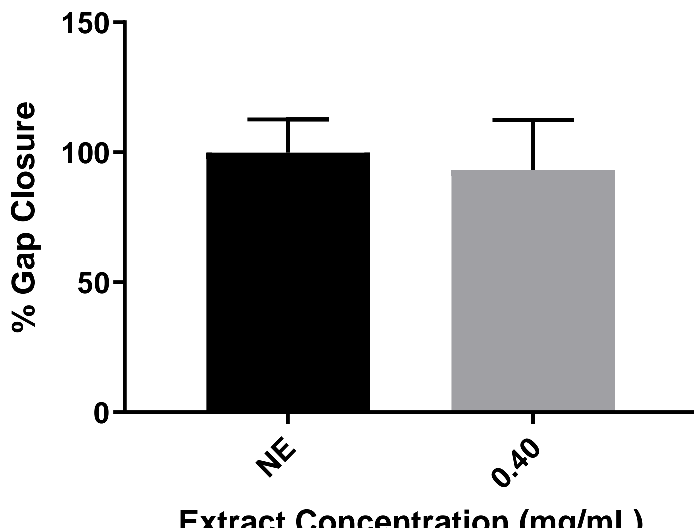
7.2.2 Effects of the Hexane Extract on Breast Cancer Cell Lines

Hexane extract of *C. olitorius* was also used to evaluate anti-invasive behaviour of MDA-MB-231 and MDA-MB-468 cells. In both cell lines, there was no significant difference between the control cells and the experimental cells.

In MDA-MB-231 cells, wound closure was observed at t=48 hr (Figure 7.2.2.1) and percent change in wound width compared to the control mean was 93.21% (Graph 7.2.2.1). Thus, the hexane extract did not have any inhibitory effect on the invasiveness of this cell line.

In MDA-MB-468 cells, although 48 hours after the scratch the wound has not closed completely, there was no significant difference between the extract-treated cells and the control cells (Figure 7.2.2.2). Percent change in wound width in the cells containing the extract was 79.41% (Graph 7.2.2.2).

In short, addition of the hexane extract did not show any significant inhibitory effect on the invasion of the metastatic cell lines investigated.



Graph 7.2.2.1: Anti-invasive Effect of the Hexane Extract on MDA-MB-231 cells

Effects of the hexane extract were evaluated on the invasion of MDA-MB-231 cells. A negative control, represented as NE (no extract), was used which consisted of cells in media without extract. Data are presented as a percent of control mean ± S.D of three replicates showing the closure of the wound width at t=48 hr. No statistically significant difference was observed between the control and the extract-treated cells.

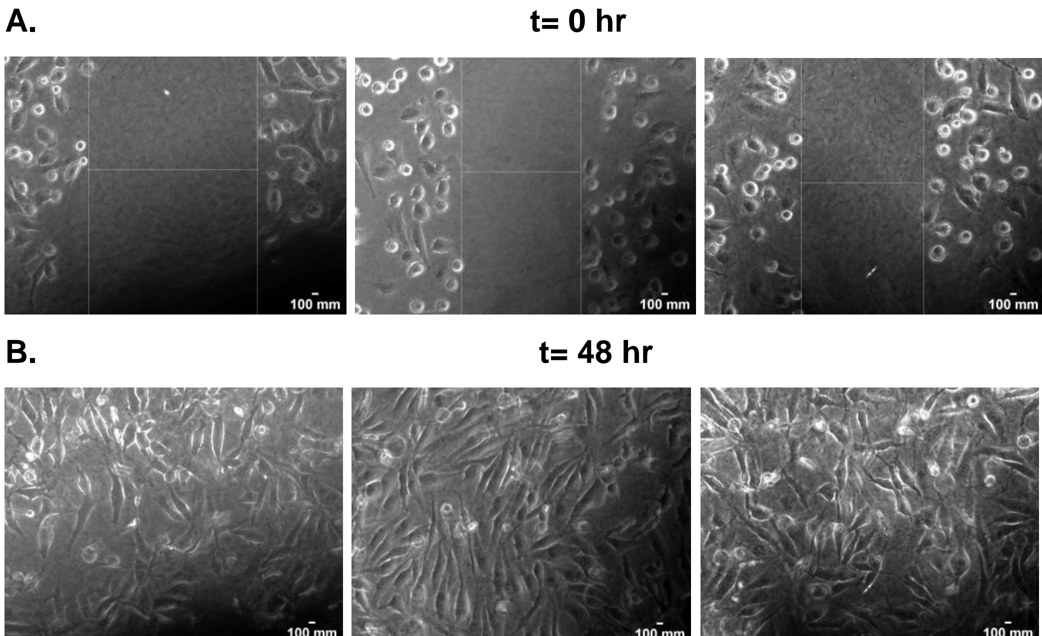
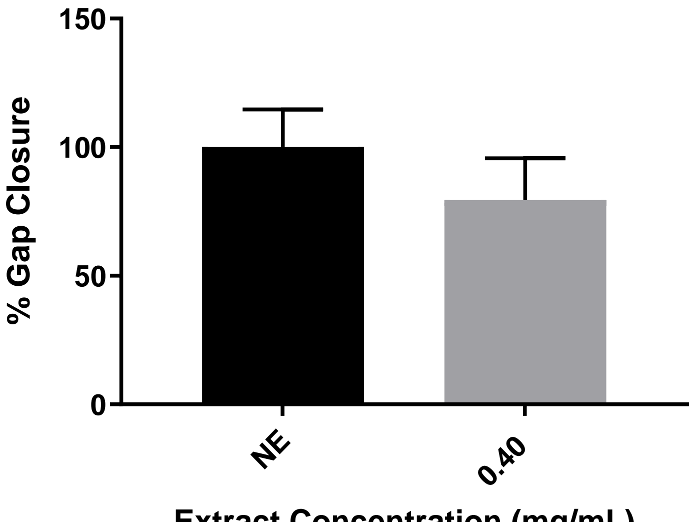


Figure 7.2.2.1: Effects of the Hexane Extract on MDA-MB-231 Cells

A. represents the cells treated with the hexane extract at 0.40 mg/mL, t=0 hr. The length of the wound width was measured as the distance from both ends of the wound using Image J2. **B.** shows the distance travelled by the cells and the closure of the wound 48 hours after the scratch and extract loading. Each experiment was repeated three times.



Graph 7.2.2.2: Anti-invasive Effect of the Hexane Extract on MDA-MB-468 cells

Effects of the hexane extract were evaluated on the invasion of MDA-MB-468 cells. The negative control used, represented as NE (no extract), consisted of cells in media but no extract. Data are presented as a percent of control mean ± S.D of three repeats at t=48 hr. No statistically significant difference was observed between the control and the treated cells.

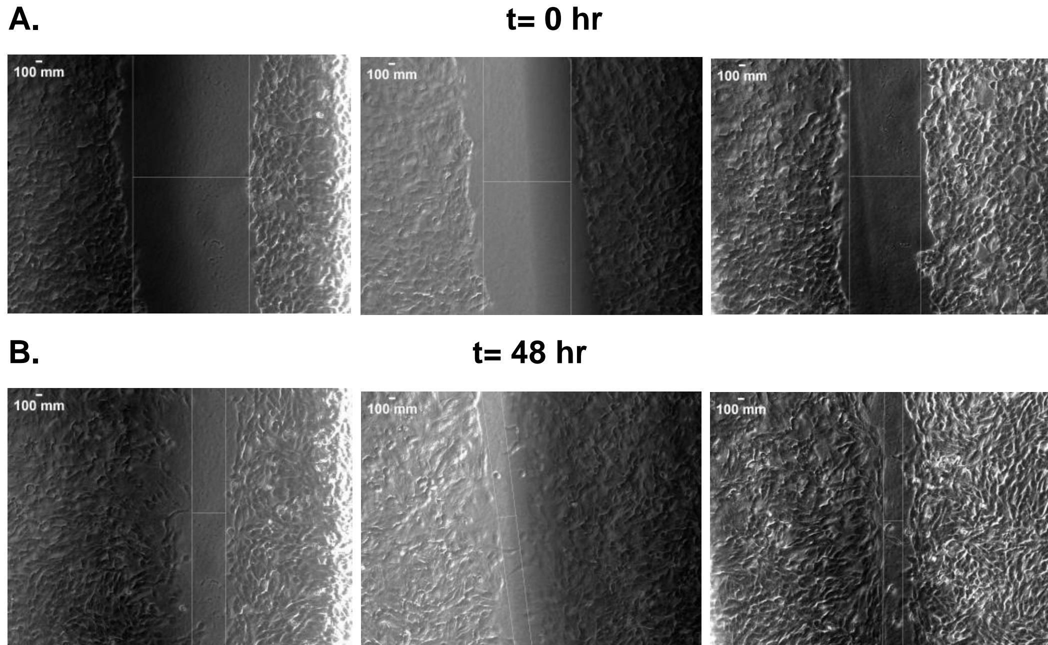


Figure 7.2.2.2: Effects of the Hexane Extract on MDA-MB-468 Cells

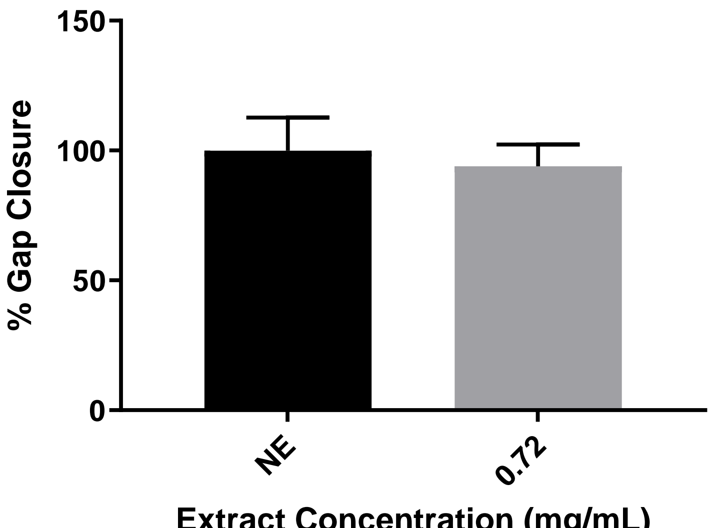
A. represents MDA-MB-468 cells treated with the hexane extract at 0.40 mg/mL, t=0 hr. The length of the wound width was measured as the distance from both ends of the wound using Image J2. **B.** shows the distance travelled by the cells and the closure of the wound 48 hours after the scratch and extract loading. The change in wound width was not significant compared to the control. Each experiment was repeated three times.

7.2.3 Effects of the Methanol Extract on Breast Cancer Cell Lines

Effects of the methanol extract on the invasion of MDA-MB-231 and MDA-MB-468 cells were evaluated and similar to the acetone and hexane extracts, no significant difference was observed between the control cells and the extract-treated cells.

In MDA-MB-231 cells, a total wound closure was observed 48 hours after the scratch (Figure 7.2.3.1). Percent change in wound width was 93.88% and this was not statistically different from the control mean (Graph 7.2.3.1). Therefore, addition of the methanol extract did not have any significant effect on the invasive ability of this cell line.

In MDA-MB-468 cells, wound closure was partial 48 hours after the scratch (Figure 7.2.3.2:). Percent change in wound width in the extract-treated cells was 85.96% compared to the control mean, however, this did not result in a significant difference between the control mean and the experimental cells (Graph 7.2.3.2).



Graph 7.2.3.1: Anti-invasive Effect of the Methanol Extract on MDA-MB-231 cells

Effects of the methanol extract were evaluated on the invasion of MDA-MB-231 cells. A negative control, represented as NE (no extract), was used which consisted of cells in media without extract. Data are presented as a percent of control mean ± S.D of three replicates showing the closure of the wound width at t=48 hr. No statistically significant difference was observed between the control and the treated cells.

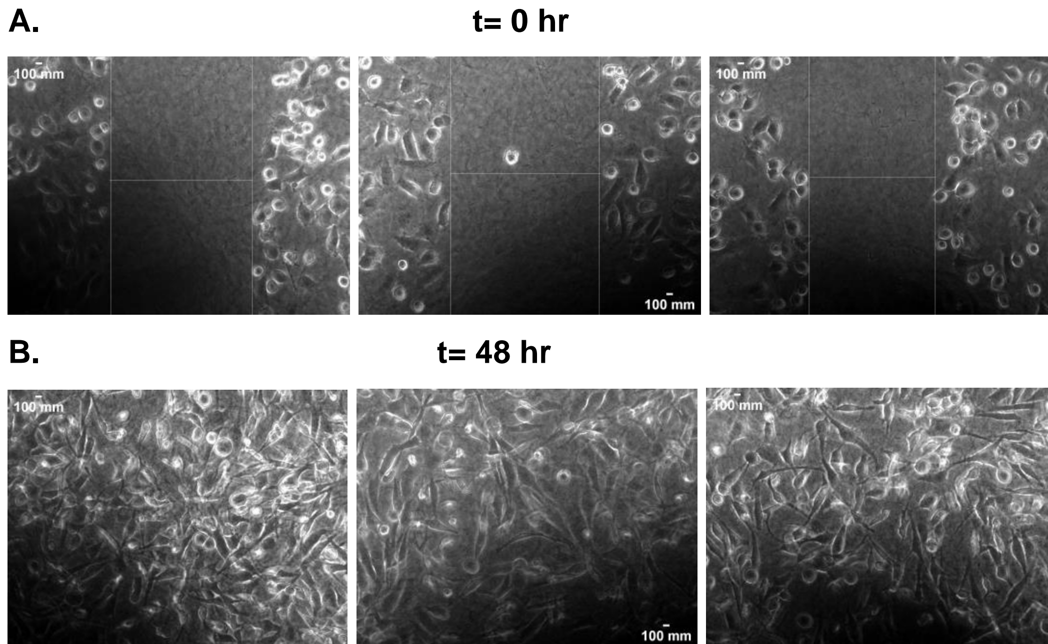
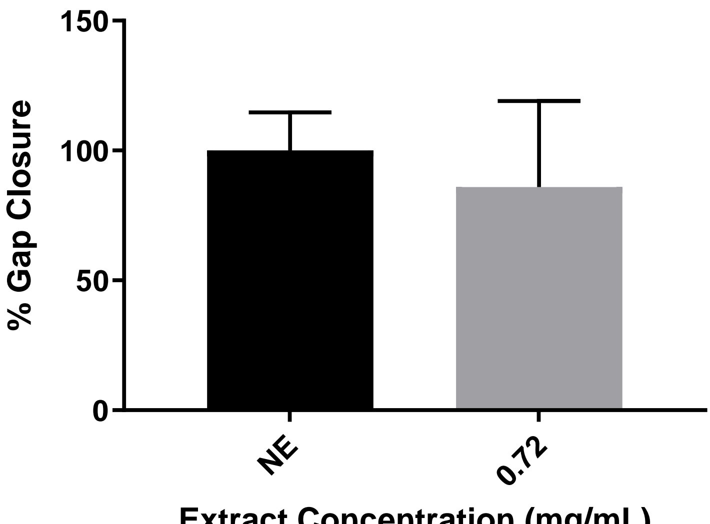


Figure 7.2.3.1: Effects of the Methanol Extract on MDA-MB-231 Cells

A. represents the cells treated with the methanol extract at 0.72 mg/mL, t=0 hr. The length of the wound width was measured as the distance from both ends of the wound using Image J2. **B.** shows the distance travelled by the cells and the closure of the wound 48 hours after the scratch and extract loading. Each experiment was repeated three times.



Graph 7.2.3.2: Anti-invasive Effect of the Methanol Extract on MDA-MB-468 cells

Effects of the methanol extract were evaluated on the invasion of MDA-MB-468 cells. The negative control used, represented as NE (no extract), consisted of cells in media but no extract. Data are presented as a percent of control mean ± S.D of three repeats. No statistically significant difference was observed between the control and the extract-loaded cells.

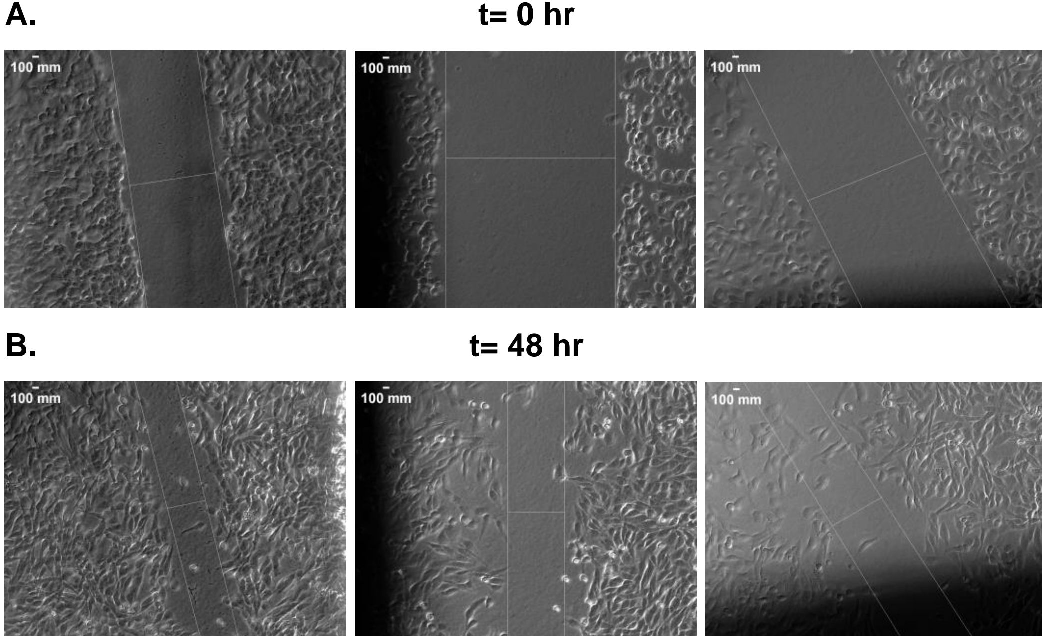


Figure 7.2.3.2: Effects of the Methanol Extract on MDA-MB-468 Cells

A. represents the cells treated with the methanol extract at 0.72 mg/mL, t=0 hr. The length of the wound width was measured as the distance from both ends of the wound using Image J2. **B.** shows the distance travelled by the cells and the closure of the wound 48 hours after the scratch and extract loading. Total wound closure did not occur, however, the distance travelled by the cells was not statistically significant from the control. Each experiment was repeated three times.

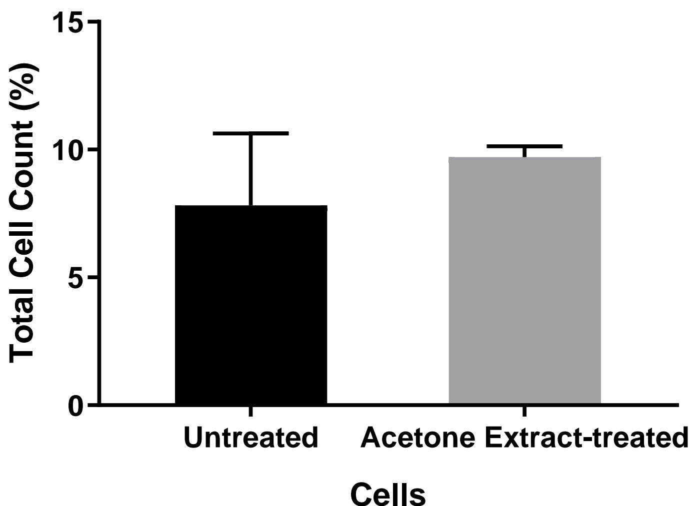
7.2.4 Expression of CD24

Anti-CD24 is an antibody labelled with the fluorochrome, PE, which emits yellow-orange fluorescence at 575 nm (Abcam, 2022). Flow cytometry was used to measure the fluorescence emission of the antibody. For each cell line, a negative control and experimental cells were prepared. The negative control consisted of antibody-labelled cells in PBS whereas experimental cells were labelled with antibody and treated with the extracts. Cell concentration (cells/mL) and percent of total cell count were recorded.

In MDA-MB-231 cells, no significant difference was observed in the percentage of total labelled cells between the experimental and control cells ($p>0.05$). The mean percentage of control cells labelled with the antibody was 7.81%, whereas for the experimental cells, it was 9.70%, 9.04% and 8.94% with acetone, hexane and methanol extracts, respectively (Graph 7.2.4.1, Graph 7.2.4.2, Graph 7.2.4.3).

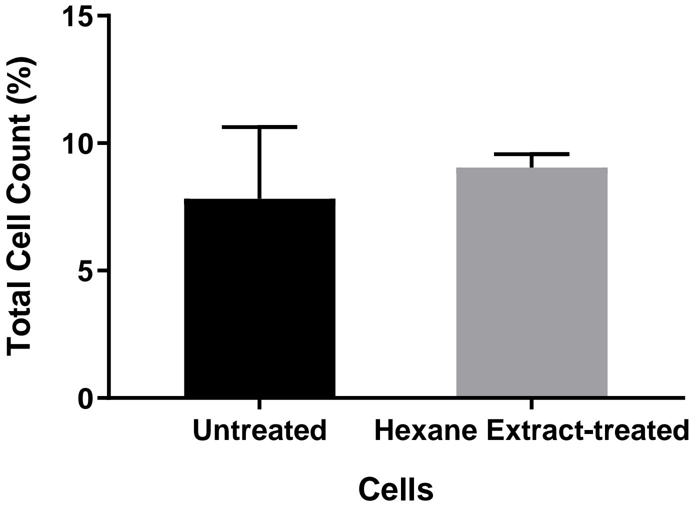
In MDA-MB-468 cells, even though the percent cell count was slightly higher in the treated cells than in the control cells, the difference was not statistically significant ($p>0.05$). The mean percentage of control cells labelled with the antibody was 65.42%. This was followed by the methanol extract, 67.55%, hexane extract, 67.92% and finally by the acetone extract, 69.16% (Graph 7.2.4.4, Graph 7.2.4.5, Graph 7.2.4.6).

Cell concentrations per cell line are tabulated in Appendix 2.3.8. Plots showing yellow fluorescence emission of PE are given below.



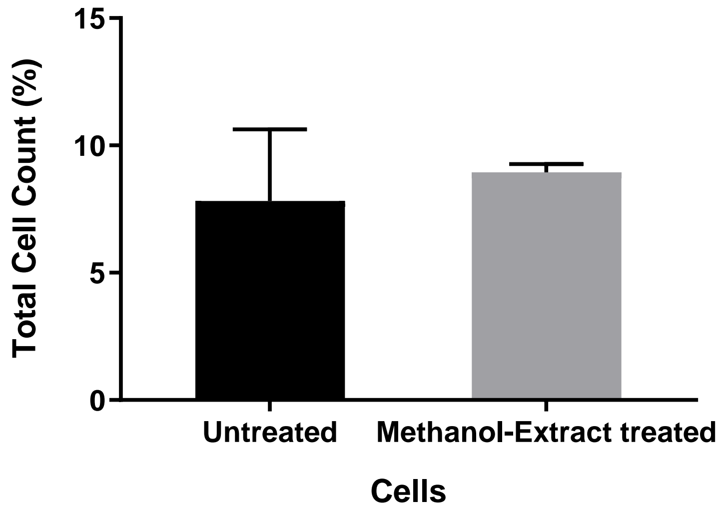
Graph 7.2.4.1: Antibody-labelled MDA-MB-231 cells in Acetone Extract vs Control

Effects of the acetone extract on the count of labelled cells were evaluated in MDA-MB-231 cells. A negative control of cells in PBS labelled with antibody was used without extract. Data are presented as a percent of control mean ± S.D of three repeats. No statistically significant difference was observed between the control and the experimental cells.



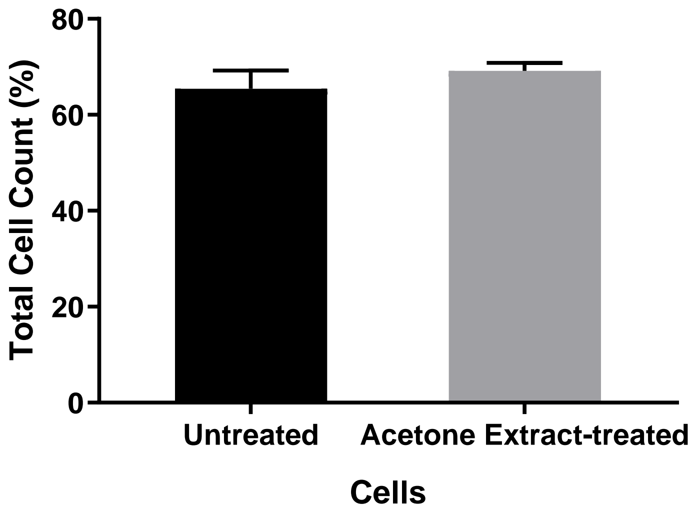
Graph 7.2.4.2: Antibody-labelled MDA-MB-231 cells in Hexane Extract vs Control

Effects of the hexane extract on the count of labelled cells were evaluated in MDA-MB-231 cells. A negative control of cells in PBS labelled with antibody was used without extract. Data are presented as a percent of control mean ± S.D of three repeats. No statistically significant difference was observed between the control and the extract-treated cells.



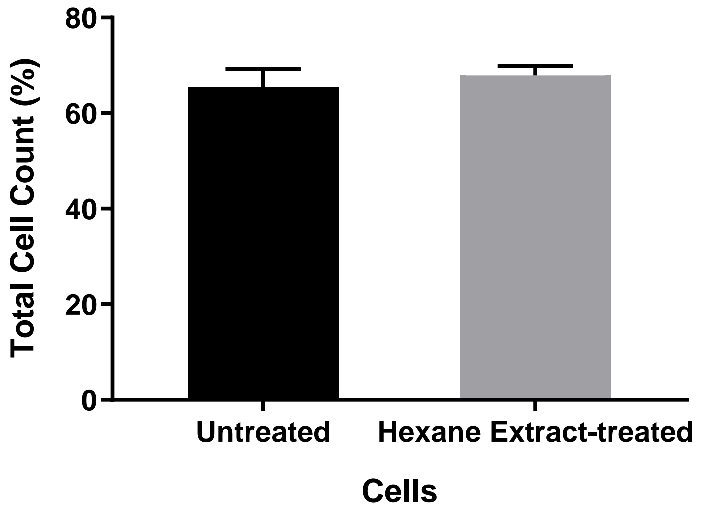
Graph 7.2.4.3: Antibody-labelled MDA-MB-231 cells in Methanol Extract vs Control

Effects of the methanol extract on the count of labelled cells were evaluated in MDA-MB-231 cells. A negative control of cells in PBS labelled with antibody was used without extract. Data are presented as a percent of control mean ± S.D of three repeats. No statistically significant difference was observed between the control and the treated cells.



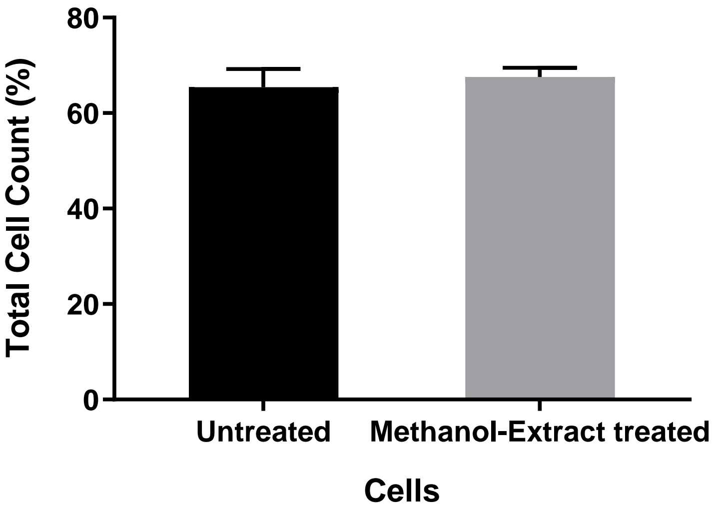
Graph 7.2.4.4: Antibody-labelled MDA-MB-468 cells in Acetone Extract vs Control

Effects of the acetone extract on the count of labelled cells were investigated in MDA-MB-468 cells. A negative control of cells in PBS labelled with antibody was used without extract. Data are presented as a percent of control mean ± S.D of three repeats. No statistically significant difference was observed between the control and the treated cells.



Graph 7.2.4.5: Antibody-labelled MDA-MB-468 cells in Hexane Extract vs Control

Effects of the hexane extract on the count of labelled cells were investigated in MDA-MB-468 cells. A negative control of cells in PBS labelled with antibody was used without extract. Data are presented as a percent of control mean ± S.D of three repeats. No statistically significant difference was observed between the control and the extract-treated cells.



Graph 7.2.4.6: Antibody-labelled MDA-MB-468 cells in Methanol Extract vs Control

Effects of the methanol extract on the total count of labelled cells were investigated in MDA-MB-468 cells. A negative control of cells in PBS labelled with antibody was used without extract. Data are presented as a percent of control mean ± S.D of three repeats. No statistically significant difference was observed between the control and extract-treated cells.

7.3 Discussion

Invasion is one of the most critical steps in metastasis and two metastatic breast cancer cell lines, MDA-MB-231 and MDA-MB-468, were investigated for their invasive potential when treated with the acetone, hexane and methanol extracts of *C. olitorius* leaves in this study. In order to study the effects of the extracts with a more focused perspective, only one concentration per extract has been chosen. For the acetone and hexane extracts, a mid-range concentration with 1 in 20 dilution was chosen. Therefore, 1.5 mg/mL and 0.40 mg/mL were the extract concentrations used, respectively. For the methanol extract, previous experiments using a concentration of 1:20 dilution were found to be toxic to cells, causing changes in cell morphology and cell death which could have confounding effects on cell behaviour and invasiveness. Thus, a more diluted, i.e., 1:100, concentration of the methanol extract was used, which was 0.72 mg/mL.

Findings of the invasion assay indicate that none of the extracts had any statistically significant anti-invasive effect on MDA-MB-231 (Graph 7.2.1.1, Graph 7.2.2.1, Graph 7.2.3.1) or MDA-MB-468 cells (Graph 7.2.1.2, Graph 7.2.2.2, Graph 7.2.3.2).

In MDA-MB-231 cells, with all three extracts examined, full wound closure was observed 48 hours after the scratch had been created. In MDA-MB-468 cells, those treated with the acetone extract also closed the wound at t=48 hr. In cells treated with the hexane and methanol extracts, full wound closure was not observed. However, the percent change in wound width was not significantly different from that in the control cells.

In the acetone extract, there might not be a synergistic anti-invasive effect of the plant compounds. In the hexane and methanol extracts, the bioactive compounds might have exerted subtle anti-invasive effects on MDA-MB-468 cells which did not translate to a significant reduction in invasion. Alternatively, the bioactive compounds in these extracts may not work synergistically. In other words, even if there are phytochemicals that

possess anti-invasive properties in the extracts, their effect may be cancelled out by other compounds which stimulate invasion.

Furthermore, the anti-invasive phytochemicals may not possess a very strong effect within a mixture of plant compounds but if they are isolated, concentrated and tested on breast cancer cells, they may show their inhibitory effects. Therefore, isolation of the major components identified and evaluation of their effects either alone or in various combinations on TNBC cell lines could also be assessed alongside to gain a deeper understanding of the plausible mechanisms involved.

In a previous study in which A-375 (human melanoma), AGS (gastric cancer) and SUIT-2 (pancreatic cancer) cells were investigated, cells were treated with an aqueous extract of *C. olitorius* and its isolated components, chlorogenic acid and isoquercetin, for 48 hours to evaluate their effects on angiogenesis. Significant inhibition of angiogenesis was observed with all of the extracts and isolates in the study. Even though cell invasion was not specifically investigated in this study, angiogenesis is one of the prior steps of invasion and therefore, its inhibition is critical in the development and spread of cancer (Tosoc et al., 2021).

The anti-invasive effects of quercetin and lutein were shown on TNBC cells, MDA-MB-231 and MDA-MB-157, at varying concentrations. In the same studies it was suggested that impaired EMT and suppression of cell motility markers, including MMP2 and MMP9, and vascular endothelial growth factor was responsible for cell invasion inhibition in these cells upon phytochemical treatment (Jia et al., 2018; Y. Li et al., 2018).

Based on the findings of previous studies, the major phytochemicals in the *C. olitorius* leaf extracts may suppress similar signalling pathways in cell migration and invasion. However, in the present study this was not among the observations. The differences in observation could be due to the fact that in most studies concentrated isolates of individual bioactive compounds were used, whereas in this study, a mixture of different

phytochemicals was present in the extracts which might have had antagonistic effects.

As discussed in Chapter 6, although MDA-MB-231 cells had a significantly higher expression of MMP9 after treatment, it seems that the higher expression did not translate to a higher enzymatic and ECM-degrading proteolytic activity, which resulted in the absence of a significant change in the invasion assay. As suggested earlier, TIMP1 could be responsible for blocking MMP9 activity, which requires further verification.

The non-significant MMP9 expression in MDA-MB-468 cells could be due to an MMP9-independent mechanism involved in a non-significant reduction in invasion. Additionally, the non-significance could be linked to the extract concentrations used in this study. Therefore, the effects could be examined using alternative extract concentrations.

Studying CD24 expression in tumour cells has gained a lot of interest in the past few decades since it is thought to be a marker of cancer stem cells (Kraus et al., 2015). CD24 is normally expressed in haematopoietic cells, such as B-cell precursors and neutrophils, neuronal tissue and in certain types of epithelial cells (Altevogt, Sammar, Hüser, & Kristiansen, 2021; Baumann et al., 2005). It is involved in cell to cell interaction through binding to its only ligand, P-selectin (Kristiansen, Sammar, & Altevogt, 2004). CD24 stimulates the adhesion of monocytes or neutrophils to activated endothelial cells and platelets as they both express P-selectin on their surface. This suggests a possible mechanism in which CD24 could mediate the invasion of the vasculature and adherence to endothelial cells in a distant site leading to metastasis. It is these findings that make it an ideal candidate as a prognostic marker in breast cancer (Ju et al., 2011; Kraus et al., 2015; Kristiansen et al., 2004).

It was previously suggested that ER-positive MCF-7 cells express CD24 as a cell surface marker and that this expression could correlate with ER-positivity in primary breast cancers (Yang, Ross, Kuang, Brown, & Weigel, 1999). This was also supported in another study demonstrating that a

subtype MCF-7 can exhibit CD24 expression (H. J. Kim et al., 2011). However, in another study it was stated that, although statistically non-significant (p=0.21), MCF-7 cells had lower CD24 expression and this could be due to oestrogen potentially repressing CD24 expression in this cell line (Sorbello et al., 2003). Even though there is conflicting evidence, MCF-7 is characterised by oestrogen and/or progesterone dependence, showing high proliferative activity but lower metastatic potential (Chekhun et al., 2013).

Additionally, in another study, it was shown that high expression of CD24 as a cell marker was correlated with high levels of lymph node metastasis suggesting that CD24 is associated with more aggressive and metastatic types of breast cancer (Kwon et al., 2015). Therefore, in this study, expression of CD24 was investigated as part of the more invasive and metastatic cancer cells, MDA-MB-231 and MDA-MB-468, in immunofluorescent analysis.

CD24 immunofluorescence assay was performed using flow cytometry. The initial adjustment of the measurements in flow cytometry was performed using unlabelled cells in PBS for each cell line. Yellow fluorescence plots show clusters of cells as red squares (data not shown). In these plots, cells are normally expected to be more evenly spread out rather than clustered very tightly together. This type of clustering of cells could be due to the initial voltage settings in the adjustment stage of the analysis. Even though the adjustments were initiated with a lower voltage setting gradually going up as described in the manuals, the optimal adjustment requires a lot of hands-on experience and a good knowledge of the use of the flow cytometer. Therefore, the clustered cells in the plots could be a result of limited practical experience with the device.

Another expected observation in these plots is the similar shape and sizes of the squares. This gives information about how well the experiment was set up methodologically and whether or not the washing step was successfully performed to remove dead cells and debris from the cell suspension. Smaller and/or narrower squares could indicate presence of

debris or non-specific binding of the antibody to dead cells. Despite the clustered cells in both MDA-MB-231 and MDA-MB-468 cells, the dispersed cells generally appear to be similar in shape and size with only some having different sizes.

Unpaired t-test revealed no significant difference between the control cells and the extract-treated cells in CD24 analysis, indicating that the fluorescent antibody was bound to almost the same percentage of cells in both cell lines. Even though in both MDA-MB-231 and MDA-MB-468, the mean percentages of cells were higher than their controls, the differences were not significant. This finding could suggest that the extracts did not reduce the number of cells expressing CD24. This could be due to the concentration of extracts used in this analysis. A number of different extract concentrations could be tested in the immunofluorescence assay to see if the extract concentration is one of the reasons for the non-significance.

Although cells were mostly clustered together in yellow fluorescence plots, the flow cytometric analysis of CD24 may support the non-significant differences seen in the invasion assay between the controls and the experimental cells in both of the metastatic cell lines. This, too, could point out that trying different concentrations of extracts and also working out different cell concentrations might provide more information about the optimal concentration in each case. It could also mean that the signalling pathways involved in invasion were not affected by these extracts in MDA-MB-231 and MDA-MB-468 cells.

The immunofluorescent analysis of CD24 in MDA-MB-231 and MDA-MB-468 cells also demonstrated that CD24 expression is low in MDA-MB-231 and high in MDA-MB-468. The mean percentage of total cell count of the control cells was 7.81% in MDA-MB-231 cells whereas this was 65.42% in MDA-MB-468 cells. These findings are consistent with previous findings (Calaf et al., 2018; Chekhun et al., 2013; Ju et al., 2011) and may suggest that MDA-MB-468 cells express more CD24 on their surface.

7.4 Conclusion

Invasion assay using the wound scratch method was carried out using metastatic cell lines, MDA-MB-231 and MDA-MB-468. The same cell lines were also investigated for their CD24 cell surface marker expression. No significant difference was observed in wound closure after 48 hours of incubation with the extracts in these cell lines when compared to the controls. Similarly, no difference was observed in CD24 expression in the untreated control cells and extract-treated cells.

There could be a number of reasons for the above observations. Although all experiments were repeated several times using the same extract concentrations to confirm the findings, a varied working concentration range for each extract could show a potentially significant change in the behaviour of the cell lines tested. In other words, perhaps the extract concentrations used in this study were below the threshold of causing a statistically significant difference between the control cells and the experimental breast cancer cell lines. This may explain the non-significant difference between the control cells and the treated cells in the hexane and methanol extracts with MDA-MB-468 cell line.

Additionally, the use of a flow cytometer requires a deeper knowledge of the device controls. Therefore, gaining more practical experience and developing better knowledge of the initial adjustments of the flow cytometer would largely improve the methodology and consequently, results.

Chapter 8 - Investigation of the Relationship between Breast Cancer and Eating Habits of Women in Northern Cyprus

8.1 Introduction

Non-communicable diseases, including breast cancer, are multifactorial however, nutrition is considered to be one of the significant modifiable causes (Buja, Pierbon, Lago, Grotto, & Baldo, 2020; De Cicco et al., 2019). Whereas some nutritional factors may increase the risk of breast cancer, such as a high-fat dietary pattern, other factors, such as higher consumption of fruits and vegetables, can reduce the risk (Chlebowski et al., 2020).

In the European Prospective Investigation into Cancer and Nutrition (EPIC) study, the largest cancer investigation cohort in Europe, evidence suggested a negative association between adherence to a Mediterranean diet and breast cancer risk. Therefore, a diet rich in plant-based foods with high fibre and a rich antioxidant content, such as β-carotene and Vitamin C, was found to be protective against breast cancer in general (Ubago-Guisado et al., 2021). In an earlier study carried out in Southern Cyprus, a protective effect of a dietary pattern rich in vegetables, legumes, fish and olive oil against breast cancer was reported. The authors also highlighted the potential combined effects of various foods in a diet that confer protection against breast cancer (Demetriou et al., 2012). The beneficial effects of olive oil have been attributed to its oleic acid and antioxidant polyphenolic content which protect against DNA damage and repress HER2 activity thereby reducing breast cancer risk and its severity (Alegre, Knowles, Robison, & O'Neill, 2013).

Based on the updated evidence from the EPIC study, a clear, strong and dose-dependent positive association was found between the consumption of alcohol and breast cancer risk in both pre- and post-menopausal women

(Ubago-Guisado et al., 2021). Furthermore, in a recent cohort study, a strong association between alcohol intake and ER-positive breast cancers was reported. In the same study, it was stated that although the mechanism in this relationship was not fully clear, an interplay of hormones, rather than the action of a single sex steroid hormone, was likely to be responsible for the association (Assi et al., 2020).

These findings were also previously reported by the World Cancer Research Fund and American Institute for Cancer Research. In the same joint report, body fat and weight gain in adulthood were found to be associated with a higher risk of breast cancer in post-menopausal women (World Cancer Research Fund/American Institute for Cancer Research, 2018).

Since Cyprus is an island in the Mediterranean, eating habits of the smaller and less-known Turkish Cypriot community were investigated as part of this research. Overall dietary behaviours of women living in Northern Cyprus were examined in a pilot study. In particular, their consumption of the Mediterranean diet components - such as olive oil, fibre-rich vegetables and grains and low saturated fat (Trichopoulou et al., 2014) was assessed. The differences in the eating habits of breast cancer patients and the control group were also evaluated.

8.2 Results

A total of 67 women joined the survey with 12 (17.9%) having a previous breast cancer history and forming the case group, and 55 (82.1%) making up the control group without a history of breast cancer. Forty-one participants were recruited from the coastal cities of Famagusta and Kyrenia and twenty-six from the capital city, Nicosia. Participants were recruited from medical clinics, hospitals and via social media. Summary of demographic information and characteristics of study participants are given in Table 8.2.1.

Anthropometric measurements and relevant BIA readings are tabulated in Table 8.2.2. Mann-Whitney U test was used to compare the differences between controls and cases at p<0.05. According to the findings, cases were significantly different from the controls in weight (p=0.001), BMI (p=0.001), waist circumference (<p=0.001), body fat (p=0.003) and body water (p=0.004). In other words, patients had a significantly higher weight, BMI, waist circumference and body fat percentage than controls. Proportionally, body water percentage was statistically higher in the control group. Patients and controls did not have a statistically significant difference in height (p>0.05).

In Table 8.2.3, general characteristics, anthropometry and body composition details of both groups were statistically analysed at p<0.05. As seen in the table, a significantly higher percentage of controls were in the 18-44 age category (p<0.001) than cases. A higher percentage of controls had a postgraduate degree (p=0.001), had alcohol regularly (p=0.001) and had a healthy BMI (p=0.002) than cases, which is under 25 kg/m^2. On the other hand, a significantly higher proportion of cases had other health problems (p=0.022) and a waist circumference above 80cm (p=0.002).

In Table 8.2.4, reproductive health of cases and controls was evaluated using different parameters. According to the findings, only the menstruation and pregnancy status were significantly different between groups with p<0.001 and p=0.020, respectively. Whereas 83.6% of the controls still had menstrual period ongoing at the time of the survey, this was the case for only 8.3% of the patients. On the other hand, only 38.2% of the controls had a previous pregnancy while this was true for 75% of the cases. The rest of the parameters did not show any significant difference.

Overall breast health of both groups was examined and results are given in Table 8.2.5. Among different parameters evaluated, annual breast check-up was found to be significantly different between the cases and the control group (p<0.001). A majority of women in the control group, 72.7%, said that they had never had an annual breast check-up or would start in

the later years while most of the cases, 91.7%, were doing their check-ups annually.

Results of the dietary habits of cases and controls are given in Table 8.2.6. Based on the findings, main meal and snack consumption were not significantly different between groups. However, 81.8% of cases had a major change in their diet after their diagnosis, such as reducing energy intake and cutting down on desserts and sugar-rich foods, compared to no change in the control group ($p<0.001$). Likewise, 58.3% of them followed a specific diet, which was a weight loss diet (data not shown), compared to only 9.3% of those in the control group ($p=0.001$).

Frequency of use of different cooking methods is given in Table 8.2.7. Boiling and grilling were the two most diversely used cooking methods. The least preferred cooking techniques were, respectively, steaming, roasting and deep frying. Among the three methods, 69.7% of participants said they never used steaming as a way of cooking or used it less than once a month. This number was 56.1% for roasting and 43.9% for deep frying. Further details about cooking method frequencies are given in the same table.

Evaluation of energy, macronutrient and fibre intake of both groups is tabulated in Table 8.2.8. These findings suggest that energy intake and the amount of protein, fat, carbohydrate (CHO) and fibre consumed by the cases were significantly higher than the controls ($p<0.05$). However, percent intake of protein and fat was not found to be statistically significant.

More detailed analysis of fatty acid and cholesterol intakes was also carried out. According to the findings shown in Table 8.2.9, intakes of saturated fatty acids (SFA), polyunsaturated fatty acids (PUFA) and Omega 6 fatty acids (FA) were statistically higher in the case group than the control group ($p<0.01$). Cholesterol intake was not statistically different.

Mineral intake of both groups was compared to the Recommended Dietary Allowances (RDA) using one sample t-test, as seen in Table 8.2.10. Sodium intake was not significantly different in either cases or controls.

However, intakes of potassium, phosphorus and zinc were significantly higher than the RDAs whereas zinc intake was significantly lower ($p<0.05$). In the case of calcium and iron, the RDAs change for women over 50 years of age and for magnesium, the cut off age is 30. Since there were women over 30 and 50 in both groups, both RDA values were used to investigate intakes for each mineral. For those aged under 50, calcium intake was significantly higher than the RDA in the control group ($p<0.003$) only. However, for those over 50 years of age, where the RDA increases to 1200 mg of calcium a day, there was no statistically significant difference in the intakes in either group (data not shown in the table). For magnesium, the RDA increases from 310 mg/day to 320 mg/day after the age of 30, however, this increment did not change the significantly higher intakes of magnesium in women older than 30. The RDA for iron drops from 18 mg/day to 8 mg/day after 50 and in both age groups, controls had significantly different intakes than recommended ($p<0.001$). In the case group, the decrease in the recommended amount resulted in a significantly higher intake of iron ($p=0.001$).

Vitamin intake was also assessed relative to the RDAs and results are given in Table 8.2.11. Intakes of vitamin A, B12, C, E and riboflavin were all found to be significantly higher than the recommended allowances ($p<0.05$). For carotene and retinol, which are plant- and animal-food precursors of vitamin A, no RDA was determined. Therefore, they were not compared. Thiamine, niacin and folate intakes were significantly higher in the case group ($p<0.05$), but not in the control group. The RDA for pyridoxine increases to 1.5 mg/day after the age of 50, however, both cases and controls still consumed significantly higher amounts than recommended whether they were under or over 50 ($p<0.05$). Intake of Vitamin D through diet was statistically lower than the RDA.

Table 8.2.12 summarises the treatments received by the patients of the study. All patients had an operation and received some sort of treatment, radiotherapy being the main one (91.7%). At the time of the survey, 75% of the patients were still receiving ongoing treatment. Patients who

participated in the survey were at different stages of breast cancer such that 54.5% of them were in the earlier stages (Stage 1 and 2).

Table 8.2.1: Demographics of Study Participants

	Health Condition		**Age Group**		**Highest Qualification**		**Monthly Income**	
	Control	**Case**	**18-44**	**45+**	**University Degree or below**	**PG Degree**	**<5.000 TL**	**>5.000 TL**
N	55	12	44	23	28	39	34	28

N: number of participants, PG: Postgraduate

Table 8.2.2: Anthropometry and Body Composition of Participants

	Controls (n=54)+		**Cases** (n=12)		
	Mean ± SD	**Median (Min-Max)**	**Mean ± SD**	**Median (Min-Max)**	**p-value**
Height (m)	1.6 ± 0.2	1.6 (1.5 - 1.7)	1.6 ± 0.1	1.6 (1.5 - 1.7)	0.907
Weight (kg)	59.5 ± 11.3	57.4 (35.7 - 90.5)	72.5 ± 12.7	72.6 (56.9-95.7)	0.001*
BMI (kg/m²)	22.8 ± 4.3	22.2 (15.3 - 34.1)	27.8 ± 4.4	28.4 (20.1 - 34.7)	0.001*
Waist circum. (cm) +	72.3 ± 10.4	70.0 (55.0 - 96.0)	88.9 ± 11.2	89.0 (70.0 - 105.0)	<0.001*
Body Fat (%) +	27.7 ± 9.0	27.1 (2.2 - 44.4)	36.6 ± 6.3	35.3 (26.2 - 45.9)	0.003*
Body Water (%)+	51.9 ± 6.8	53.2 (23.6 - 61.6)	46.4 ± 4.5	47.3 (39.1 - 54.0)	0.004*

Waist Circum.: Waist Circumference

*+Number of participants in the control group whose waist circumference and body fat and body water measurements were taken was 51 and 52, respectively. Those parameters with a significant difference at p<0.05 are represented with an *.*

Table 8.2.3: Characteristics of Participants

		Controls (n)	Controls	Cases (n)	Cases	p-value
Age	18-44	43	78.2%	1	8.3%	<0.001*
	45+	12	21.8%	11	91.7%	
Highest Qualification	University Degree or below	18	32.7%	10	83.3%	0.001*
	PG Degree	37	67.3%	2	16.7%	
Income	<5.000 TL	28	56.0%	6	50.0%	0.708
	>5.000 TL	22	44.0%	6	50.0%	
Smoking Status	Never Smoked	46	83.6%	10	83.3%	1.000
	Smoked	9	16.4%	2	16.7%	
Exercise Habits	Regular Exercise	24	45.3%	7	58.3%	0.414
	No Exercise	29	54.7%	5	41.7%	
Alcohol Intake	Regular Drinker	29	54.7%	0	0.0%	0.001*
	No Alcohol Intake	24	45.30%	12	100%	
Other Health Problems	Exist	20	38.5%	9	75.0%	0.022*
	No Other Health Problems	32	61.5%	3	25.0%	
BMI	Healthy BMI (<25 kg/m^2)	39	75.0%	3	25.0%	0.002*
	Overweight/Obese (≥25 kg/m2)	13	25.0%	9	75.0%	
Waist Circumference	<80 cm	38	74.5%	3	25.0%	0.002*
	≥80 cm	13	25.5%	9	75.0%	
Body Fat	<35%	40	76.9%	6	50.0%	0.080
	≥ 35%	12	23.1%	6	50.0%	

*General characteristics and body composition of cases and controls were statistically analysed at p<0.05 level. Those with significance are represented with an *.*

Table 8.2.4: Reproductive Health of Participants

		Controls (n)	Controls	Cases (n)	Cases	p-value
Age of Onset of Menstruation	<13	19	38.0%	6	50.0%	0.521
	≥13	31	62.0%	6	50.0%	
Menstruation Status	Ongoing	46	83.6%	1	8.3%	<0.001*
	Stopped	9	16.4%	11	91.7%	
End of Menstruation Reason	Naturally Stopped	8	88.9%	5	45.5%	0.070
	Other Reason	1	11.1%	6	54.5%	
Pregnancy	Previous Pregnancy	21	38.2%	9	75.0%	0.020*
	No Previous Pregnancy	34	61.8%	3	25.0%	
Age of First Pregnancy	<30	13	72.2%	6	60.0%	0.677
	≥30	5	27.8%	4	40.0%	
Number of Miscarriages	One	5	83.3%	2	50.0%	0.500
	Two or more	1	16.70%	2	50.0%	
Breastfeeding History	Breastfed	14	77.8%	8	88.9%	0.636
	No breastfeeding	4	22.2%	1	11.1%	
Fertility Medication	Used	3	6.3%	2	22.2%	0.173
	Not used	45	93.8%	7	77.8%	
OC Use	Used	12	22.6%	3	27.3%	0.710
	Not used	41	77.4%	8	72.7%	
HRT Use	Used	3	5.7%	0	0.0%	1.000
	Not used	50	94.3%	11	100.0%	

*OC: Oral Contraceptive, HRT: Hormone Replacement Therapy. Reproductive health of cases and controls were compared and statistically analysed at p<0.05 level. Those with significance are represented with an *.*

Table 8.2.5: Breast Health of Participants

		Controls (n)	Controls	Cases (n)	Cases	p-value
Deodorant Use	Used	53	96.4%	10	83.3%	0.144
	Never Used	2	3.6%	2	16.7%	
Bra Cup Size	A-B	36	65.5%	4	33.3%	0.054
	C or above	19	34.5%	8	66.7%	
Annual Breast Check-up	Yes	15	27.3%	11	91.7%	<0.001*
	No/Not yet	40	72.7%	1	8.3%	
Breast Density	Dense Breast	7	13.5%	3	25.0%	0.381
	No Dense Breast	45	86.5%	9	75.0%	
Other Breast Condition	Yes	6	12.5%	1	8.3%	1.000
	No	42	87.5%	11	91.7%	
Family History of BC before Age 50	Yes	11	20.0%	4	33.3%	0.444
	No	44	80.0%	8	66.7%	
Relationship	Close Family	4	36.4%	3	75.0%	0.282
	Distant Family	7	63.6%	1	25.0%	

*BC: Breast Cancer. Breast health of cases and controls were statistically analysed at p<0.05 level. Those with significance are represented with an *.*

Table 8.2.6: Eating Habits of Participants

		Controls (n)	Controls	Cases (n)	Cases	p-value
Daily Main Meal Frequency	Three times	45	81.8%	8	66.7%	0.257
	Other	10	18.2%	4	33.3%	
Skipped Meals	Breakfast	22	62.9%	2	50.0%	0.631
	Other Main Meals	13	37.1%	2	50.0%	
Daily Snack Frequency	1-2 times	34	63.0%	7	70.0%	1.000
	>2 times	20	37.0%	3	30.0%	
Diets Followed	Specific Diet followed	5	9.3%	7	58.3%	0.001*
	No Specific Diet	49	90.7%	5	41.7%	
Major Change in Eating Habits	Change in Habits	0	0.0%	9	81.8%	<0.001*
	No Change	30	100.0%	2	18.2%	

*Significant differences in general eating habits of cases and controls at p<0.05 are represented with an *.*

Table 8.2.7: Frequency of Cooking Methods used by Participants

	Boiling		Steaming		Deep Frying		Sautéing		Grilling		Roasting		Barbecue	
	N	**Percent (%)**	**N**	**Percent (%)**	**N**	**Percent (%)**	**N**	**Percent (%)**	**N**	**Percent (%)**	**N**	**Percent (%)**	**N**	**Percent (%)**
Never or less than once a month	3	4.5	46	69.7	29	43.9	6	9.1	3	4.5	37	56.1	7	10.6
1-3 times a month	12	18.2	9	13.6	21	31.8	10	15.2	6	9.1	15	22.7	38	57.6
1-2 times a week	20	30.3	7	10.6	9	13.6	27	40.9	26	39.4	6	9.1	20	30.3
3-4 times a week	16	24.2	3	4.5	6	9.1	18	27.3	21	31.8	7	10.6	1	1.5
5-6 times a week	5	7.6					5	7.6	6	9.1	1	1.5		
Once a day	2	3.0			1	1.5			3	4.5				
2-3 times a day	6	9.1	1	1.5					1	1.5				
4-5 times a day	2	3.0												

Table 8.2.8: Daily Energy, Macronutrient and Fibre Intake of Participants

		Energy (kcal)	Protein (g)	Protein (%)	Fat (g)	Fat (%)	CHO (g)	CHO (%)	Fibre (g)
Controls (n=55)	**Mean ± SD**	1805.4 ± 615.5	84.7 ± 26.6	19.8 ± 3.2	85.3 ± 33.9	42.2 ± 7.8	167.5 ± 68.7	38.1 ± 7.7	31.6 ± 14.0
	Min-Max	857.4 - 3403.5	41.6 - 160.8	11.0 - 27.0	27.8 – 179.4	26.0 - 59.0	66.0 - 359.5	20.0 - 55.0	6.4 - 68.3
Cases (n=12)	**Mean ± SD**	2882.7 ± 950.4	129.2 ± 50.5	18.8 ± 3.9	119.6 ± 46.9	37.3 ± 6.8	307.4 ± 106.9	44.3 ± 7.5	51.7 ± 20.7
	Min-Max	1504.8 - 4879.3	68.7 - 239.2	12.0 - 27.0	53.8 - 223.0	26.0 - 49.0	129.8 - 463.3	34.0 - 58.0	24.9 - 97.2
	p-value	<0.001*	0.001*	0.357	0.013*	0.048	<0.001*	0.021*	0.001*

*CHO: Carbohydrate. Significant differences between cases and controls at p<0.05 are represented with an *.*

Table 8.2.9: Daily Fatty Acid and Cholesterol Intake of Participants

		SFA (g)	MUFA (g)	PUFA (g)	Oleic Acid (g)	Omega 3 FA (g)	Omega 6 FA (g)	Cholesterol (mg)
Controls (n=55)	**Mean ± SD**	28.3 ± 12.4	33.43± 14.6	15.3 ± 8.2	31.6 ± 13.8	1.8 ± 1.0	13.4 ± 7.6	298.4 ± 146.8
	Min-Max	9.7 - 76.2	10.5 - 69.8	4.3 - 45.8	9.7 - 65.3	0.6 - 5.2	3.5 - 43.1	79.1 - 798.6
Cases (n=12)	**Mean ± SD**	45.2 ± 23.2	41.8 ± 16.5	21.1 ± 5.4	39.3 ± 16.0	2.0 ± 0.7	18.9 ± 5.0	439.7 ± 315.7
	Min-Max	11.5 - 95.4	20.7 - 81.3	14.1 - 29.1	19.5 - 78.1	1.1 - 3.6	11.8 - 26.0	142.3 - 1083.6
	p-value	0.009*	0.100	0.004*	0.120	0.125	0.003*	0.295

*SFA: Saturated Fatty Acid, MUFA: Mono-unsaturated Fatty Acid, PUFA: Polyunsaturated Fatty Acid, FA: Fatty Acid. Significant differences between cases and controls at p<0.05 are represented with an *.*

Table 8.2.10: Daily Mineral Intake of Participants

		Sodium (mg)	Potassium (mg)	Calcium (mg)	Magnesium (mg)	Phosphorus (mg)	Iron (mg)	Zinc (mg)	Selenium (µg)
Controls (n=55)	**Mean ± SD**	1337.4 ± 701.1	3626.4 ± 1333.7	1191.5 ± 455.4	374.8 ±143.0	1477.5 ± 454.3	12.0 ± 4.8	11.3 ± 3.6	12.2 ± 9.8
	Min-Max	520.2 – 4071.0	1549.6 - 6722.2	495.9 - 2612.9	156.7 - 812.0	697.3 - 2544.8	4.1 - 27.0	5.3 - 18.5	0.2 - 47.4
	p-value	0.091	<0.001*	0.003*	0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Cases (n=12)	**Mean ± SD**	2998.3 ± 1920.7	5785.61± 2309.0	1983.6 ± 1380.8	578.5 ± 257.7	2261.9 ± 1056.4	20.1 ± 9.1	17.9 ± 7.3	14.1 ± 14.3
	Min-Max	966.2 - 7862.8	3227.3 - 11435.4	614.5 - 5715.1	332.5 - 282.1	1088.6 – 5076.0	10.5 - 42.2	11.0 - 34.8	0 - 41.1
	p-value	0.021	0.001*	0.031	0.004*	<0.001*	0.446	0.001*	<0.001*
	RDA	**1500 mg/d**	**2600 mg/d**	**1000 mg/d** $^{+}$	**310 mg/d** $^{++}$	**700 mg/d**	**18 mg/d** $^{+}$	**8 mg/d**	**55 µg/d**

RDA: Recommended Dietary Allowance

$^{+}$ *For women over 50, the RDA for calcium is 1200 mg/d and iron 8mg/d*

$^{++}$ *For women over 30 years of age, the RDA for magnesium is 320 mg/d.*

** represents significant differences at p<0.05.*

Table 8.2.11: Daily Vitamin Intake of Participants

	Controls (n=55)			**Cases** (n=12)			
	Mean + SD	**Min-Max**	**p-value**	**Mean + SD**	**Min-Max**	**p-value**	**RDA**
Vitamin A (µg)	1843.0 + 986.3	305.9 - 3921.0	<0.001*	3901.9 + 3033.8	803.0 - 9728.8	0.004*	**700 µg/d+**
Carotene (mg)	8.5 + 4.7	0.6 - 21.9	-	17.4 + 15.5	3.5 - 56.6	-	**ND++**
Retinol (µg)	607.5 + 608.0	118.5 - 2941.2	-	1327.2 +1724.9	85.5 - 5003.0	-	**ND++**
Thiamine (mg)	1.1 + 0.4	0.5 - 2.7	0.537	1.8 + 0.6	1.10 - 3.10	0.003*	**1.1 mg/d**
Riboflavin (mg)	1.7 + 0.6	0.7 - 3.4	<0.001*	2.6 + 1.2	1.30 - 4.70	0.001*	**1.1 mg/d**
Niacin (mg)	13.5 + 5.3	4.5 - 28.7	0.509	21.0 + 7.9	10.7 - 34.4	0.011*	**14 mg/d**
Pyridoxine (mg)	1.8 + 0.7	0.6 - 3.6	<0.001*	2.6 + 0.9	1.3 - 4.5	0.001*	**1.3 mg/d+++**
Folate (µg)	375.1 + 159.0	117.8 - 775.8	0.251	673.0 + 304.4	380.9 - 1483.8	0.010*	**400 µg/d**
Vitamin B12 (µg)	5.3 + 2.5	1.30 - 14.0	<0.001*	8.8 + 6.5	2.8 - 22.7	0.006*	**2.4 µg/d**
Vitamin C (mg)	236.1 + 130.8	35.2 - 588.1	<0.001*	369.2 + 176.8	153.9 - 775.9	<0.001*	**75 mg/d**
Vitamin D (µg)	4.1 + 2.8	1.0 - 13.9	<0.001*	4.2 + 3.2	0.2 - 9.2	<0.001*	**15 µg/d**
Vitamin E (mg)	20.7 + 11.0	5.2 - 60.2	<0.001*	28.3 +8.7	12.3 - 41.0	<0.001*	**15 mg/d**

RDA: Recommended Dietary Allowance,

+*As retinol activity equivalents (RAE) to account for different absorption rates of retinols and carotenoids*
(School of Public Health Harvard University, 2023)

++ *ND: Not determined, RDA was not determined separately for carotenoids and retinol*

+++ *For women over 50, the RDA for pyridoxine is 1.5 mg/d*

* *represents significant differences at p<0.05.*

Table 8.2.12: Details of Patients

Treatment Received	Number of Cases (n)	Percent of Cases (%)
Operation	12	100.0
Radiotherapy	11	91.7
Chemotherapy	10	83.3
Hormone Therapy	10	83.3
Other Therapy	3	25.0
Ongoing Treatment	9	75.0
Stage of BC		
Stage 1 or 2	6	54.5
Stage 3 or 4	5	45.5
Age of First BC Diagnosis		
< 50	5	50.0
$\geq$ 50	5	50.0

BC: Breast Cancer

8.3 Discussion

It has long been known that diet is one of the factors which has a significant impact on overall health. One of the objectives of this pilot study was to collect preliminary data about the relationship between dietary habits of women and risk of breast cancer and to further improve the study design. Therefore, the number of participants recruited was 67, 12 of whom were women with a history of breast cancer.

According to the results, cases weighed significantly higher than the controls, with a higher body fat percentage, waist circumference and BMI. Furthermore, they were mostly in the 45+ years of age group. As individuals age, body fat content increases inevitably and the distribution of fat tends to be around the abdominal organs (St-Onge & Gallagher, 2010). Body fat content is also inversely proportional to the body water content which explains the lower body water of the cases. Although the number of cases was low in the study, these findings suggest that the older age of the cases could be related to the higher body fat around the abdominal area, hence the higher waist circumference, resulting in a higher weight and BMI. Higher BMI and waist circumference indicate that patients were either overweight or obese. It could also be due to post-op medication used by the patients causing higher body fat and weight. It has long been known that overweight and obesity are a risk factor for breast cancer in postmenopausal women (Amadou, Hainaut, & Romieu, 2013; Krebs et al., 2006). There are several mechanisms suggested for the association between adiposity and risk of postmenopausal breast cancer.

One of the pathways involved is thought to be the endogenous oestrogen synthesis by the adipose tissue, including breast preadipocytes which are precursors of fat cells (Amadou et al., 2013; W. Guo, Key, & Reeves, 2018; Krebs et al., 2006; H. Zhao et al., 2018). Before menopause, oestrogen is synthesised by the aromatase enzyme primarily in the ovaries. However, aromatase activity ceases after menopause and the expression of aromatase in breast preadipocytes becomes the major source of oestrogen.

The over-expressed oestrogen in women with more adipose tissue is thought to drive ERα-positive breast cancers (H. Zhao et al., 2018).

Even though specific receptor positivity was not questioned in the present survey to identify whether they had an ERα-positive breast cancer, the cases had a higher waist circumference and BMI showing abdominal adiposity. This may be implicated with their diagnosis.

Having a waist circumference above 80 cm is also associated with an increased risk of chronic diseases including cardiovascular diseases and all-cause death (Darsini, Hamidah, Notobroto, & Cahyono, 2020; R. Ross et al., 2020). A significantly higher number of cases with a waist circumference >80 cm could also explain the other health conditions they had and the weight loss diet they followed at the time (Table 8.2.6).

Abdominal adiposity also stimulates chronic low-grade inflammation. The release of inflammatory cytokines, such as Tumour Necrosis Factor-α (TNF-α), interleukin-6 (IL-6), C-reactive protein (CRP), result in oxidative stress which may cause carcinogenesis (Amadou et al., 2013; W. Guo et al., 2018). Additionally, this inflammation may stimulate cancer progression through cell proliferation, apoptosis, angiogenesis and migration. It may even contribute to enhanced aromatase expression in adipocytes adding further to the endogenous synthesis of oestrogen (Amadou et al., 2013; Dashti et al., 2022).

Individuals with abdominal adiposity tend to have decreased insulin sensitivity and high fasting insulin. Insulin may increase cancer promotion by enhancing the bioavailability of IGF- 1. High plasma levels of IGF-1 may trigger carcinogenesis in several pathways including stimulation of cell proliferation and increased aromatase expression (Amadou et al., 2013; Dashti et al., 2022).

Leptin is a protein secreted by adipocytes which normally controls food intake. However, in obese individuals, it acts as a pro-inflammatory cytokine stimulating chronic inflammation (Ouchi, Parker, Lugus, & Walsh,

2011). Decreased leptin sensitivity in chronic inflammation is also thought to contribute to breast cancer development (Dashti et al., 2022; Shapira, 2017).

Another finding of the study was the significant non-use of alcohol in patients before the diagnosis. As stated earlier, alcohol is among the well-established risk factors associated with breast cancer and results of this study correlates with previous studies showing the avoidance of alcohol use by cancer patients (Hagen, Aas, Kvaløy, Søiland, & Lind, 2018; Vance, Campbell, Mccargar, Mourtzakis, & Hanning, 2014).

Smoking and low physical activity, which are also risk factors particularly for post-menopausal breast cancer cases (Hanaoka, Yamamoto, Sobue, Sasaki, & Tsugane, 2005; Sanderson et al., 2015), were not found to be statistically significant between the groups. This could be due to the limited number of participants, which could be focused more on a larger scale epidemiological study.

Table 8.2.4 summarises the reproductive factors associated with breast cancer questioned in this survey. Early onset of menarche (before the age of thirteen), late pregnancies (after 30 years of age), late menopause, breastfeeding history, use of hormonal medication are among the reproductive risk factors (Sanderson et al., 2015; Sieri et al., 2014). In this study, a significant difference was observed in menstrual status and pregnancy history of the groups. More specifically, there were more participants in the control group with ongoing menstruation than those in the case group. Additionally, more than half of the control group had no previous pregnancy whereas this was the opposite in the case group. This could be due to a larger number of participants with a younger age in the control group. When approached, patients of younger age were unwilling to talk about their illness and participate in the survey. Therefore, patients in their 30s or younger could not be recruited to the study.

Certain conditions related to breast health were also assessed in the study, as given in Table 8.2.5. In some sources, there is a debate about the potential anti-perspirant/deodorant use and higher risk of breast cancer due to the presence of different chemicals applied underarm (Darbre, 2005, 2009). However, in this study no significant difference was observed between the cases and controls. Other breast-related concerns that cause predisposition to cancer, such as high breast density, large breasts, other breast conditions or even close family history of breast cancer before the age of 50, were not significantly different between the groups. A noticeable increase was observed in the number of patients attending their annual breast check-ups, with more patients adhering to a regular schedule. This was anticipated, as periodic breast examinations are crucial for monitoring disease progression.

Overall comparison of meal consumption of cases and controls suggests that both groups mostly consume three main meals with one or two snacks daily. The main differences observed between the cases and controls were having a major change in their diet and the type of specific diet they followed, which was a weight loss diet. A weight reduction diet could be recommended by their physicians. Use of medication after surgery, stress and anxiety caused by the disease, limited physical activity due to fatigue or pain or prolonged side effects of therapy could be some of the factors leading to overweight and obesity in patients. It was also suggested that excess body weight could exacerbate these undesirable outcomes in a vicious cycle (Reeves, Terranova, Eakin, & Demark-Wahnefried, 2014). There is evidence from meta-analyses that excess body weight in patients is associated with an increased risk of second primary cancers of breast, endometrial and colorectal and mortality (Druesne-Pecollo et al., 2012). In another study, it was found that following a weight loss diet could be achieved and maintained well among patients and could improve the negative outcomes of the disease (Reeves et al., 2014). It was also suggested that combining exercise with a well-balanced diet would improve

overall health in both obese and non-obese cancer survivors (Druesne-Pecollo et al., 2012).

Use of different cooking methods by participants was also investigated in this study Table 8.2.7. Based on the findings, boiling was the most diversely used cooking technique followed by grilling. Among other methods, steaming and roasting were the least popular with more participants using them never or less than once a month. Different cooking methods have different effects on foods and consequently on health. It was reported that some cooking techniques, such as boiling and even steaming, may cause extensive loss of the heat labile vitamin, vitamin C with some plant foods. Vitamin C possesses antioxidant activity, which is known to be protective against oxidative reactions in the body that may result in cancer. On the other hand, in the same study it was found that steaming eased and increased the release of other, secondary antioxidant plant chemicals (Kinyi, Tirwomwe, Ninsiima, & Miruka, 2022).

This was also supported in another study which found that boiling, and especially steaming, increased extraction of carotenoids, another group of antioxidant plant constituents, from various vegetables enhancing their bioavailability after consumption (Castro et al., 2021). Therefore, it can be suggested that methods like boiling and steaming are generally safe cooking techniques which may enhance the release of various antioxidants from plants leading to positive health outcomes. Therefore, although boiling was diversely used by the participants in this study, steaming may also be recommended as a healthy cooking technique.

On the contrary, there are other cooking methods which do not involve water while cooking and may potentially result in burning of foods, particularly meat products. Such methods include grilling, smoking, barbecuing, and deep frying where repeatedly heated vegetable oils are used. Some studies suggest that the use of these methods cause formation of carcinogenic chemical compounds on the surface of muscle meats, such as polycyclic aromatic hydrocarbons and heterocyclic amines, which may

cause breast cancer (Ganesan & Xu, 2020; White et al., 2016). It was also previously shown that genetic polymorphisms in enzymes that detoxify these carcinogens may results in an elevated risk of breast cancer (Zheng et al., 2002). Although the frequency of use of these methods and the amount of exposure to these chemicals by the participants is not clear in the present study, it is recommended to use these cooking techniques less frequently and prefer healthier methods as mentioned above.

Comparison of energy, macronutrient and fibre intake of both groups has shown that all statistically significant parameters were higher in the case group (Table 8.2.8). Total amounts of protein, fat and carbohydrate (CHO) intake of the cases were significantly higher than the control group resulting in a significantly higher energy intake. Even though the amount of fat and CHO consumed by the case group were higher, the percent proportions were opposite resulting in the control group consuming a higher CHO as a percentage than fat. Another significant difference was observed in fibre consumption. The case group consumed almost 1.5 times more fibre than the control group. This could be due to a higher consumption of CHO in this group. They may have consumed more fruits and whole grain products, such as whole wheat bread, pasta or cracked wheat, in their diet at the time. Alternatively, their diet may have included more vegetables with a high fibre content.

Macronutrient and fibre intake of participants were also compared against the RDAs. According to these guidelines, 45-65% of daily energy intake must come from CHO for adults. Similarly, the recommended daily intakes for protein and fat are 10-35% and 20-35%, respectively (National Academies of Sciences, Engineering, 2019). In this study, protein intake of both groups was within the recommended range, however, fat intake was higher and CHO intake was lower than recommended. It must also be emphasised that, it is not only the amount of fat that has an impact on health, but also the type consumed.

A diet high in saturated fat and omega-6 polyunsaturated fatty acids (PUFA) increases the risk of developing an ER/PR-positive breast cancer (Kiyabu et al., 2015; Sieri et al., 2014). It was suggested that arachidonic acid, which is a major component of animal fat, and lipids derived from its altered metabolism, mainly eicosanoids, play a key role in inflammation and breast cancer progression via upregulation of aromatase activity and increased oestrogen synthesis (Dieli-Conwright, Lee, & Kiwata, 2016; Wang & Dubois, 2010).

On the other hand, intake of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are the omega-3 fatty acids found in fish, is found to be inversely associated with breast cancer (Hagen et al., 2018; Kiyabu et al., 2015). In the present study, the case group had a significantly higher intake of SFA, PUFA and omega-6 PUFA (Table 8.2.9). However, all PUFA and omega-6 PUFA consumption of patients still fall within the recommended range. Even though there is not a specific recommendation for SFA intake, in both international and regional guidelines it is stated to be 'consumed as little as possible' (National Academies of Sciences, Engineering, 2019; TC Sağlık Bakanlığı, 2022). Oleic acid, the main monounsaturated fatty acid (MUFA) in olive oil, was also investigated in the study. No difference in consumption was observed between the groups. However, this was not a very surprising finding. Olive oil is used extensively in Cyprus for cooking and in salads. Therefore, this was an expected finding of the study.

Comparison of mineral and vitamin intakes of cases and controls with both internationally accepted RDAs and regional recommendations demonstrated that in overall, both groups exceeded the recommendations except for Vitamin D and selenium (National Academies of Sciences, Engineering, 2019; TC Sağlık Bakanlığı, 2022). Whereas the widely accepted daily recommendation for vitamin D is 15 µg, both groups consumed around 4.1 µg per day, which may be considered fairly low. However, it must be noted that the main source of vitamin D is sunlight that

initiates the activation of the vitamin's pre-form in the skin. Therefore, dietary sources of vitamin D generally act as secondary sources. Even so, it is not uncommon to have vitamin D deficiencies worldwide. There is also growing evidence showing that low plasma levels of vitamin D are associated with various cancers, including breast cancer. This is because vitamin D has receptors in most tissues and cells and acts as a transcription factor modulating gene expression (Atoum & Alzoughool, 2017; Holick et al., 2011). Even though the association of breast cancer with the intake of vitamin D was not evaluated in this study, regular and physician-controlled vitamin D supplementation after testing may need to be considered as an important measurement to lower deficiencies.

Women in both groups having higher intakes of minerals and vitamins than the recommendations could be attributed to a diversified diet composed of the elements of the Mediterranean Diet. These may include food groups such as grains, fruits and vegetables and animal products. All these groups are rich in minerals and vitamins. A high intake of vitamin B12 in both groups indicates that participants were consuming adequate amounts of animal products at the time of the survey since this vitamin is only found in animal foods. This finding is also supported by the adequate or high consumption of protein and fats as mentioned above.

On the other hand, even though selenium is normally rich in meat, fish, seafoods and some plant foods (Navarro-Alarcon & Cabrera-Vique, 2008), its intake was significantly lower than the recommendations in this study. Selenium is attached to the protein fraction in foods, thus foods high in protein are also good sources of selenium. However, several factors determine the amount of selenium accumulated in foods. Geographical region, seasonal changes, selenium concentration of the soil where plants grow and type of soil are among these factors (Kieliszek, 2019). Food preparation and processing, method of cooking used may also cause losses in selenium content of foods (Navarro-Alarcon & Cabrera-Vique, 2008).

Another finding of this study was the difference observed in iron intake. The RDA for iron is 18 mg/day for women aged under 50, whereas this drops to 8 mg/day around the time of menopause and after the age of 50 (National Academies of Sciences, Engineering, 2019). Since the majority of women were younger in the control group than in the case group, their significantly lower dietary intake of iron could result in iron deficiency anaemia (IDA). IDA has been recognised as the most prevalent nutritional deficiency by the WHO, most commonly seen in menstruating women (A. Kumar, Sharma, Marley, Samaan, & Brookes, 2022). Pregnant women are also susceptible to low iron intake. IDA affects foetal development and is implicated in maternal consequences throughout and after labour (Skolmowska, Głąbska, Kołota, & Guzek, 2022). In this study, it has been demonstrated that women of childbearing age in the control group, had a higher risk of developing IDA due to their significantly lower dietary iron intake. This could also affect their pregnancy outcomes if they were planning pregnancy at the time of the survey. However, it must be emphasised that dietary supplements were not included in nutrient analysis calculations. These observations are based merely on the intakes through food.

Lastly, Table 8.2.12 summarises details about patients. It can be seen that all patients had a surgery of some sort with majority having had hormone-dependent breast cancer. Half of the patients had their first diagnosis before the age of 50 and almost half of the patients had an early-stage cancer. However, one patient did not know the stage of her disease (data not given). During the interviews, it was also observed that patients could not tell most of the details of their condition, such as the stage or associated risks, without their medical documents. There may be a number of reasons for this. One of them could be physicians not explaining the disease and its prognosis to their patients clearly. Even though cancer is a disease that may sound complex and frightening, clear communication between the physician and the patient helps the patient understand their condition better and deal with the emotional or psychological aspects better.

8.4 Conclusion

One of the limitations of the study was the lower number of younger cases recruited in the study. The nutrient analysis software also had some limitations. Although some locally used recipes were added to the software programme after thorough research and consideration, most of the default recipes were developed using generalised recipes. Since these are fixed recipes and cannot be changed, they may not fully represent the true consumption by the participants. Furthermore, regional differences in recipes may slightly affect the final calculated intakes.

Additionally, dietary nutrient intakes were based on an FFQ in this study. FFQs may potentially be biased since they are reported subjectively by the participants. Also, food supplements could not be recorded in the software programme, so the results of this study may only reflect a portion of the participants' total nutrient intake.

However, despite the limitations encountered throughout the study, there were some important conclusions. One of them was the use of boiling as the most frequently used cooking method by the participants. Boiling is one of the recommended cooking techniques over frying and other dry heat methods that do not use water and increase the chance of burning food. However, if the boiling time is prolonged, it can cause loss of vitamins, particularly in vegetables.

It was also observed that steaming was not very frequently used, even though it is one of the safest and nutrient-reserving methods. As part of public health nutrition, use of steaming, especially for vegetables, could be encouraged.

Lastly, the percent amount of CHO consumed by both groups was found to be low. Increasing the consumption of CHO sources, ideally in the form of whole grains and legumes, would reduce the intake of fat-rich foods and balance the proportion of these macronutrients.

Chapter 9 – General Discussions

9.1 Justification of Methods

One of the aims of the present study was to investigate the anti-cancer effects of *C. olitorius* leaves extracted with acetone, hexane and methanol. These solvents were chosen based on their ability to extract plant compounds of different polarities. Whereas methanol was used to extract highly polar phytochemicals, hexane was used for non-polar and acetone was used for compounds of mid-range polarity.

Extraction of bioactive compounds from plants is important in research as it provides valuable insight about their structure and functions in biological systems as well as potential health benefits and uses in therapy. These bioactive substances might offer an alternative solution to chemotherapy with minimal side effects.

As mentioned in Chapter 1, *C. olitorius* leaves have been shown to contain polyphenolic compounds including flavonoids in previous studies. In this study, total phenol and flavonoid content of the extracts was measured to compare the results with literature findings. TPC and TFC assays are inexpensive, convenient and reproducible assays that provide information about the polyphenolic content of the leaf extracts. Free radical scavenging activity of the extracts was also determined in the present study using the DPPH assay, which reflects the antioxidant potential of the extracts. The results were found to be correlated with the polyphenol content of the extracts. In this study, the methanol extract was shown to have high TPC and TFC values while having the lowest IC_{50} value in the DPPH assay (Table 3.2.2c, Table 3.2.3c, Table 3.2.4), consistent with evidence reported in the literature. Although the DPPH assay measures only one aspect of antioxidant activity, which is free radical scavenging, as a widely used cost-effective, rapid and relatively sensitive technique, it still provides insights about the presence of various bioactive compounds that possess antioxidative activity.

In order to confirm the compound content of each extract and further assess the antioxidant compounds in the extracts, GC-MS was performed. As stated in Chapter 3, GC-MS is a highly sensitive and specific analytical method used to identify compounds that can be volatised in plant extracts. With an access to standardised online compound databases, such as NIST and Wiley as used in this study, it allows for the identification of a vast array of unknown compounds in plants. In the present study, the GC-MS analysis has identified 86-91% of the moderately polar acetone and non-polar hexane extracts with $\geq$80% library matching quality, while this was around 19% for the methanol extract. However, more polyphenolic compounds are thought to be present in the methanol extract, despite their lower matching quality, as explained earlier.

The GC-MS analysis has shown that the major compounds identified across the extracts are fatty acids, particularly α-linolenic, linoleic and palmitic acid, phytol, α-tocopherol and several smaller phenolic compounds. As discussed in Chapter 3, these results are consistent with previous findings reported (Azuma et al., 1999; Hasan & Kadhim, 2018; T. Nguyen et al., 2016; Oboh et al., 2009). It is also worth mentioning that the content of the bioactive compounds in plant leaves may differ depending on several factors. Geographical location of the plant species, soil type and content, genetics of the plant itself and other environmental factors such as sun exposure, water availability etc., may all affect the content and availability of phytochemicals in a plant. Additionally, the binding of phytochemicals to other plant components in a food matrix and their interactions may also have a role in the bioavailability of phytochemicals (Aguilera, 2019; Arfaoui, 2021).

In contrast to most studies in which individual isolates of phytochemicals were used, in the present study leaf extracts were preferred. The main reason was to observe the potential synergistic or antagonistic interactions of the bioactive compounds in a plant extract and how they would affect various cellular pathways as a whole. Since there are not any studies which investigated the anti-cancer effects of *C. olitorius* leaf extracts on breast

cancer cells *in vitro*, this study provides important preliminary information about the plant's possible anti-proliferative, anti-adhesive and anti-migratory effects on breast cancer cell lines, particularly MDA-MB-468 cells.

For instance, in a recent study, it was reported that a seed extract of *Bixa orellana L.* has shown greater anti-proliferative activity on MCF-7 cells than isolated bixin, which is a major carotenoid in the plant (Kusmita, Franyoto, Mutmainah, Puspitaningrum, & Nurcahyanti, 2022). This suggests that it is equally important to evaluate the overall effects of plant extracts as a mixture of various phytochemicals, not just the concentrated isolates of individual compounds.

Furthermore, examining the combined effects of whole extracts, rather than isolated compounds, provides a more nutritionally relevant perspective. Plant-based foods are consumed as complex mixtures of phytochemicals that interact synergistically or antagonistically within the body. Therefore, studying extracts as a whole better reflects dietary intake and the true biological impact of plant foods than studies limited to individual compounds, offering real-life insights.

The anti-cancer effects of the extracts were assessed through proliferation, adhesion, migration and invasion assays on hormone receptor-positive and triple negative breast cancer cell lines to gain a deeper understanding of the potential behavioural mechanisms via widely studied assays in cancer research.

For example, as described in Chapter 4, CV staining assay is a simple, low-cost, widely used technique which uses a DNA-binding dye to estimate the number of proliferating cells in a population. It is an ideal method for comparing the anti-proliferative effects across various extracts on viable cells. Similarly, its use in the adhesion assay allows quantification of adherent cells on to a collagen-rich surface which mimics the ECM environment, providing deeper insights into the metastatic potential of the cell lines investigated. Since various cell concentrations were initially tested to determine the ideal working cell concentration with multiple repeats in

absorbance measurements, the CV assay provides a reliable estimation for proliferating and adherent cancer cells. Furthermore, the results of the adhesion assay in MDA-MB-231 and MDA-MB-468 cells appear to be related to those of the migration assay, suggesting the importance of conducting these assays in parallel. This provides complementary insights into the metastatic behaviour of these cell lines and a comprehensive evaluation of the anti-cancer effect of extracts.

The wound healing assay is one of the simplest and inexpensive methods to study cell behaviour *in vitro* as it can mimic, to some extent, migration along a 2D surface and invasion and intrusion into a 3D ECM network by cancer cells (Rodriguez, Wu, & Guan, 2005). Therefore, in the migration assay, cells were left to collectively migrate on a collagen-coated two-dimensional surface for 24 hrs but in the invasion assay, another layer of collagen was added on top of cells to examine their ability to penetrate through this layer over 48 hrs.

This assay was chosen since it is a widely accepted assay in the literature, particularly relevant in this study for comparing MDA-MB-231, an aggressively invasive cell line which is moderately responsive to chemotherapy and MDA-MB-468, a moderately aggressive TNBC cell line which is more responsive to therapeutic drugs. Observations of their migratory and invasive behaviour after treatment in the wound healing assay offers an early phase yet valuable insight into the potential therapeutic effects of the extracts, while also highlighting parallels with their response to chemotherapy.

As discussed in Chapter 4 and 6, a sandwich ELISA assay was used to measure the expression of caspase 3 and 7 and validate whether reduced cell proliferation following extract treatment in MDA-MB-468 cells could be attributed to increased apoptosis, since these are the executioner caspases in programmed cell death. Similarly, MMP9 expression was measured to strengthen the interpretation of the findings from the migration and invasion assays. Overall, both caspase and metalloprotease quantification assays were used to serve as complementary approaches

to the proliferation, migration and invasion assays and gain further insights into the possible cellular responses.

This type of ELISA assay is a highly sensitive and quantitative method, widely used in measuring protein expressions in biomedical research (Alhajj, Zubair, & Farhana, 2023).

9.2 Integration of Findings

The results of this study suggest that the hexane and methanol extracts of the jute plant contain bioactive compounds that exhibit stronger effects than those in the acetone extract.

Table 9.2.1 and Table 9.2.2 summarise the results from the cell culture and ELISA assays in the present study. Colour coding in Table 9.2.1 was used to highlight the differences in observed effects, where green coding indicates potential anti-cancer effects and red coding represents cancer-stimulating effects of the extracts on specific cell lines. As stated earlier, MCF-7 cells were not used in the migration and invasion assays due to their non-metastatic profile, shown by grey shading.

Table 9.2.1: Summary of In vitro Findings

		Observed Effects by Assay Type			
Cell Line	**Extract**	**Proliferation**	**Adhesion**	**Migration**	**Invasion**
MCF-7	Acetone	↔	↑	-	-
	Hexane	↔	↑	-	-
	Methanol	↔	↓	-	-
MDA-MB-231	Acetone	↔	↑	↔	↔
	Hexane	↔	↑	↑	↔
	Methanol	↔	↑	↔	↔
MDA-MB-468	Acetone	↔	↓	↔	↔
	Hexane	↓	↓	↓	↔
	Methanol	↓	↓	↓	↔

Arrows represent the effect observed by the extract and assay type; ↑ - increase, ↓ - decrease, ↔ - no significant change and – not applicable. Green shading indicates anti-cancer effects while red shading indicates pro-cancer effects.

Table 9.2.2: Summary of ELISA Assay Findings

	Caspase 3		**Caspase 7**		**MMP9**	
Cell Line	**Hexane**	**Methanol**	**Hexane**	**Methanol**	**Hexane**	**Methanol**
MDA-MB-231	↑	↑	↑	↑	↑	↑
MDA-MB-468	↑	↑	↑	↔	↔	↔

Arrows represent the effect observed on protein expression by the hexane or methanol extracts; ↑ - increase, ↓ - decrease, ↔ - no significant change.

In this study, metastatic MDA-MB-468 was the cell line most impacted by the extracts in all assays. Particularly the hexane and methanol extracts significantly reduced cell proliferation, adhesion and migration in this cell line. Even in the invasion assay, although non-significant, hexane and methanol extracts suppressed cell invasion to a limited degree. MDA-MB-231 cells were observed to have moderately increased adhesive and migratory behaviour, especially when treated with the hexane extract. In both assays, MDA-MB-231 cells appear to have a higher response with modest concentrations in the hexane and methanol extracts. MCF-7 cells were the least affected by the extracts, showing enhanced adhesion in the acetone and hexane extracts but reduced adhesion in the methanol extract, with no significant effect in proliferation.

These results also highlight the complexity of investigating the effects of different types of plant extracts.

The molecular profile of MCF-7 may account for the observed effects. MCF-7 cells have mutations in *CDKN2A* and *PIK3CA* genes, which encode for proteins regulating cell cycle progression and cell proliferation (Hollestelle et al., 2010; Kenny et al., 2007; Kresty et al., 2002). Mutations in these proteins stimulate cell cycle progression and continued proliferation in this cell line. Therefore, even if the extracts had exerted any anti-proliferative effect, these mutations could have minimised their anti-proliferative signalling, resulting in an overall non-significant difference in the proliferation assay. Another possible explanation could be - as discussed earlier in Chapter 4 - that the growth and survival of MCF-7 cells

was unaffected by the major component identified in all three extracts, which is α-linolenic acid.

The lack of a particular trend across the extracts in the adhesion assay could be attributed to the phenotypic heterogeneity in gene and hormone receptor expression and signalling pathways affected in a subgroup of MCF-7 cells (Comşa et al., 2015). Additionally, the complex interplay between the fatty acids and polar polyphenols in the methanol extract, which might not have been present in the other extracts, could account for reduced adhesion whereas adhesion was enhanced in the acetone and hexane extracts.

Even though MDA-MB-468 and MDA-MB-231 cells are both metastatic TNBC cell lines, this study demonstrates differences in their behaviour due to the variations in their molecular profile. Expression of certain signalling proteins and receptors may account for the differences between these two metastatic cell lines.

For instance, as stated in Chapter 5, E-cadherin and claudin protein expression is higher in MDA-MB-468 cells than in MDA-MB-231 cells while MDA-MB-231 has a higher β1 integrin expression than MDA-MB-468. Another example is the expression of EGFR. MDA-MB-468 cells are known to exhibit amplification of EGFR in contrast to MDA-MB-231 cells (Chavez et al., 2010; Holliday & Speirs, 2011). Lastly, differences in Ki67 expression could be one of the reasons explaining the differences observed with the TNBC cells. MDA-MB-468 cells have a higher expression of Ki67, highlighting their more proliferative nature than MDA-MB-231 cells (Holliday & Speirs, 2011; Uxa et al., 2021).

Table 9.2.3 outlines the molecular and behavioural differences between MDA-MB-231 and MDA-MB-468 cells based on reported evidence and this study further examined how these two TNBC cell lines differing in phenotype responded to the extracts. Including TNBC cell lines of different phenotypes enables a more comprehensive comparison of their molecular

and behavioural characteristics, providing deeper insight into the variability of responses observed with these cell lines.

Table 9.2.3: Molecular and Behavioural Comparison of the MDA-MB-231 and MDA-MB-468 Cell Lines

Molecular Profile	Roles and/or Pathways Involved	MDA-MB-231	MDA-MB-468	References
*B-Raf**	Involved in cell survival and metastasis Regulator of MAPK/ERK signalling pathway	Mutated	No change	*(Y. Guo et al., 2020; Hollestelle et al., 2010; Kenny et al., 2007; O. Li et al., 2023)*
*CDKN2A**	Encodes the proteins p16INK4a & p14ARF Cell cycle regulation Cell cycle arrest through p53 stabilisation (p14ARF)	Deleted	No change	*(Hollestelle et al., 2010; Kenny et al., 2007; Kresty et al., 2002)*
*K-Ras**	Involved in cell survival and metastasis Regulator of MAPK/ERK signalling pathway	Mutated	No change	*(Chavez et al., 2010; Y. Guo et al., 2020; Kenny et al., 2007; O. Li et al., 2023)*
*TP53**	Encodes a tumour suppressor Regulator of cellular stress response, cell cycle, DNA repair, apoptosis, angiogenesis	Mutated	Mutated	*(Chavez et al., 2010; Flöter et al., 2017; Hollestelle et al., 2010; Kenny et al., 2007; Sana & Malik, 2015)*
*PTEN**	Encodes a tumour suppressor Inhibitor of cell growth and cell cycle progression Regulator of PI3K/Akt pathway	No change	Deleted	*(Chavez et al., 2010; Glaviano et al., 2023; Minami, Nakanishi, Ogura, Kitagishi, & Matsuda, 2014)*
Claudin	Tight junction protein Cell-to-cell adhesion	Lower expression	Higher expression	*(Holliday & Speirs, 2011; Murakami-Nishimagi et al., 2023)*
E-cadherin	Adherens junction protein	Lower expression	Higher expression	

	Cell-to-cell adhesion			*(Chekhun et al., 2013; Holliday & Speirs, 2011)*
EGFR	Regulation of cell growth Activator of PI3K/Akt pathway	No change	Higher expression	*(Chavez et al., 2010; Holliday & Speirs, 2011; Kenny et al., 2007; Orofiamma et al., 2022)*
β1 integrin	Cell-to-ECM adhesion Regulator of cell adhesion and migration	Higher expression	Lower expression	*(Baltes et al., 2020; Ziperstein et al., 2015)*
Ki67	Proliferation marker	Lower expression	Higher expression	*(Beresford et al., 2006; Holliday & Speirs, 2011)*
Other Characteristics				
Epithelial/ Mesenchymal Mode	-	Mesenchymal	Epithelial	*(Chekhun et al., 2013; Dongre & Weinberg, 2019)*
Migration Speed	-	Fast	Fast	*(Ziperstein et al., 2015)*
Invasiveness	-	Aggressively invasive	Moderately invasive	*(Ziperstein et al., 2015)*

** represents the genes affected in TNBC cell lines used in this study.*

Evidence from the literature examining the effects of *C. olitorius* leaf extracts is very limited as earlier studies largely focused on isolated individual plant compounds. However, in the present study, GC-MS analysis identified major compounds in the leaf extracts, providing insight about the potential effects and interactions of these compounds.

As detailed in Table 9.2.3, increased adhesion and migration observed in MDA-MB-231 cells highlight their mesenchymal and more invasive profile with a higher expression of β1 integrin than MDA-MB-468 and the findings of the present study may suggest that the extracts, especially the hexane extract, could have enhanced the signalling along the β1-integrin-FAK axis. It may be possible that linoleic acid, as one of the major compounds identified in the hexane extract, is responsible for the effects observed in MDA-MB-231 cells. As discussed in Chapter 5 and 6, there is consistent evidence from the literature proposing that linoleic acid activates FAK-Src dependent pathways leading to stronger adhesion, migration and invasion in MDA-MB-231 cells. Nevertheless, the interactions of different bioactive compounds in plant extracts and their combined effect, whether synergistic, antagonistic or additive, requires further investigation and validation.

As the literature suggests, MDA-MB-468 cells have a more proliferative, epithelial and less invasive phenotype than MDA-MB-231 cells (Table 9.2.3) and seem to rely more heavily on EGFR-PI3K/Akt pathway. It may be possible that the extracts, the hexane and methanol extracts in particular, disrupted this overexpressed and over-sensitive pathway, subsequently resulting in reduced proliferation, adhesion and migration. The reported effects of the major compounds identified in these extracts along with the polyphenolic content of the methanol extract, shown by a high TPC value and high DPPH radical scavenging activity, could suggest a beneficial effect on MDA-MB-481 cells.

As discussed in Chapter 4, the observed growth inhibition in MDA-MB-468 cells could be attributed to the insertion of α-linolenic acid, present in the extracts, into the lipid bilayer of cell membranes. Earlier studies show that omega-3 fatty acids have the ability to insert into the plasma membrane

which ultimately cause alterations in membrane lipid composition, redistribution of receptor proteins including EGFR to non-lipid rafts and modulation of downstream intracellular signalling which result in growth inhibition and cell death (Corsetto et al., 2012; Germain et al., 1998; Virk et al., 2024). Additionally, lipid peroxidation and ferroptosis could be proposed as other mechanisms causing cell death in this cell line (Tan et al., 2025). It could be possible that the incorporation of α-linolenic acid into the membrane, together with increased levels of intracellular iron typically found in TNBC cells, enhanced lipid peroxidation through ROS generation which overwhelmed glutathione peroxidases and resulted in cell death.

To further clarify the observed effects and investigate potential mechanisms in future work, expression and activity of key proteins, such as E-cadherin, FAK and Akt, could be measured in both cell lines. These data would provide stronger evidence linking the hexane- and methanol-treated cells to specific molecular pathways. Lipid peroxidation could also be measured using the thiobarbituric acid reactive substances (TBARS) assay following extract treatment in MDA-MB-468 cells. This widely accepted colorimetric assay reacts with lipid peroxidation end products, such as malondialdehyde, which can be measured and quantified spectrophotometrically (Deshpande et al., 2013). Furthermore, in MDA-MB-468 cells, the expression of ACSL4, glutathione peroxide and the levels of iron could also be measured to assess the potential role of ferroptosis in reducing proliferation.

One of the important findings of the study was the difference in the observations of cell migration and invasion assays. Even though there was a significant difference between the experimental and control cells in both TNBC cell lines in the migration assay, no significance was observed in invasion. This could be explained by the differences in these processes.

Migration assays measure cell motility on a two-dimensional flat geometry without any obstructive ECM network. Therefore, cell migration is largely a barrier-free movement of cells. In some cases, cells can rapidly change their shape through cytoskeletal remodelling and squeeze through narrow

gaps of the ECM in the surrounding environment to change their position (Jia shun Wu et al., 2021).

Migrating cells primarily display directional cell polarity with a leading edge at the front supported by a rear end contraction as a consequence of the rearrangement of cytoskeletal components, including actin, microtubules and intermediate filaments. As stated in Chapter 6, changes in the cytoskeleton during cell movement are regulated by several proteins. Among these are Rac and RhoA, where Rac enhances actin activity at the leading edge forming lamellipodia and growing filopodia, while RhoA stimulates rear retraction. Migration also involves integrin-mediated focal adhesion turnover in which new transient adhesion occurs at the leading edge while the rear end detaches from the surface (Jia shun Wu et al., 2021; X. Zhao & Guan, 2011).

Since ECM degradation is not necessarily a requirement for migration, it is not an MMP-dependent process and it may occur without leading to invasion (Kramer et al., 2013; Jia shun Wu et al., 2021).

Invasion, however, is a more complex process as it involves penetrating through the three-dimensional basal membrane and a dense network of connective tissue in the ECM. The fibre-dense 3D matrix imposes space limitations against the moving cell. This causes the cells to undergo modifications in their morphology and strong interactions and remodelling of the ECM, eventually leading to its proteolytic degradation. Unlike migration, invasion strongly relies on degradation of the ECM (Friedl & Wolf, 2010; Kramer et al., 2013). Whereas migrating cells grow lamellipodia and filopodia at the leading edge to help the cell move, invading cells grow actin-rich protrusions called invadopodia underneath the cell body which pass through the ECM by secreting proteases, such as MMPs (Bourgot et al., 2020; Friedl & Wolf, 2010; Majidpoor & Mortezaee, 2021). For invasion to take place, adhesion, migration and proteolytic degradation of the surrounding ECM are prerequisites. Unlike migration, invasion is an MMP-dependent process (Kramer et al., 2013).

As discussed in Chapter 6, a significantly high MMP9 expression in the ELISA assay seen in the MDA-MB-231 cell line might not have converted to an active enzyme but could rather reflect a higher measure of the inactive form, pro-MMP9. This could explain the non-significant effects observed in invasion, as active MMP9 is required for degradation of the ECM in invading cells. In future work, invasion assay could be coupled with MMP9 and TIMP1 activity assays to gain a deeper understanding of possible mechanisms involved.

It was previously proposed that the level of adhesion on a surface impacts the rate of migration and that highest migration is achieved with a biphasic adhesion strength in cells. This means that if adhesion is low, cells cannot acquire sufficient traction to move in the direction of the leading edge and if it is too strong, it prevents detachment of the rear end. However, intermediate adhesion enables the traction at the front to detach the rear end and promote cell movement (Gupton & Waterman-Storer, 2006). This could explain the parallel observations of adhesion and migration assays in both cell lines.

In MDA-MB-231 cells, adhesion strength via integrin-ECM interactions might have been optimal to facilitate cell motility at the leading edge but too strong for cells to detach the rear end and remodel the ECM for invasion to take place. In other words, the extracts might have pushed the cells to a range of adhesion strength which favoured 2-D migration but suppressed invasion, resulting in an overall non-significant change in the invasion assay. Therefore, it can be suggested that the extracts impacted adhesion dynamics, enhanced the activity of integrin-FAK signalling and cytoskeletal remodelling in migration, however, proteolytic ECM degradation was not activated to enable significant invasion in this cell line.

MDA-MB-468 cells have a lower baseline level of MMP9 and are only moderately invasive compared with MDA-MB-231, as stated in Chapter 6. Owing to their more epithelial type, characterised by stronger cell–cell interactions relative to cell–ECM and a possible over-reliance on EGFR-dependent signalling, the extracts might have reduced adhesion and

migration without exerting a significant effect on ECM degradation, invadopodia formation and consequently invasion. The slight but non-significant reduction in invasion observed in MDA-MB-468 cells could suggest the involvement of an MMP-independent mechanism or limitations related to the concentration range of the extracts used in the present study.

In both cell lines, these observations suggest that the extracts appear to have impacted adhesion dynamics and migratory remodelling to achieve 2-D migration without having a strong effect on protease- and invadopodia-dependent mechanisms required for 3-D invasion.

In order to further elucidate the mechanisms involved in the observed effects, time-lapse images of live cells could be recorded throughout the migration and invasion assays. These images would provide insights about the morphological changes cell may undergo, including the formation of lamellipodia and/or invadopodia, and cell directionality (L. Y. Yu, Lin, Hsu, Kao, & Tsai, 2023).

Figure 9.2.1 summarises the potential anti-proliferative, anti-adhesive and anti-migratory mechanisms observed with the MDA-MB-468 cell line in the hexane and methanol extracts as discussed in detail in Chapter 4, 5 and 6. Proposed mechanisms are based on reported evidence from the literature.

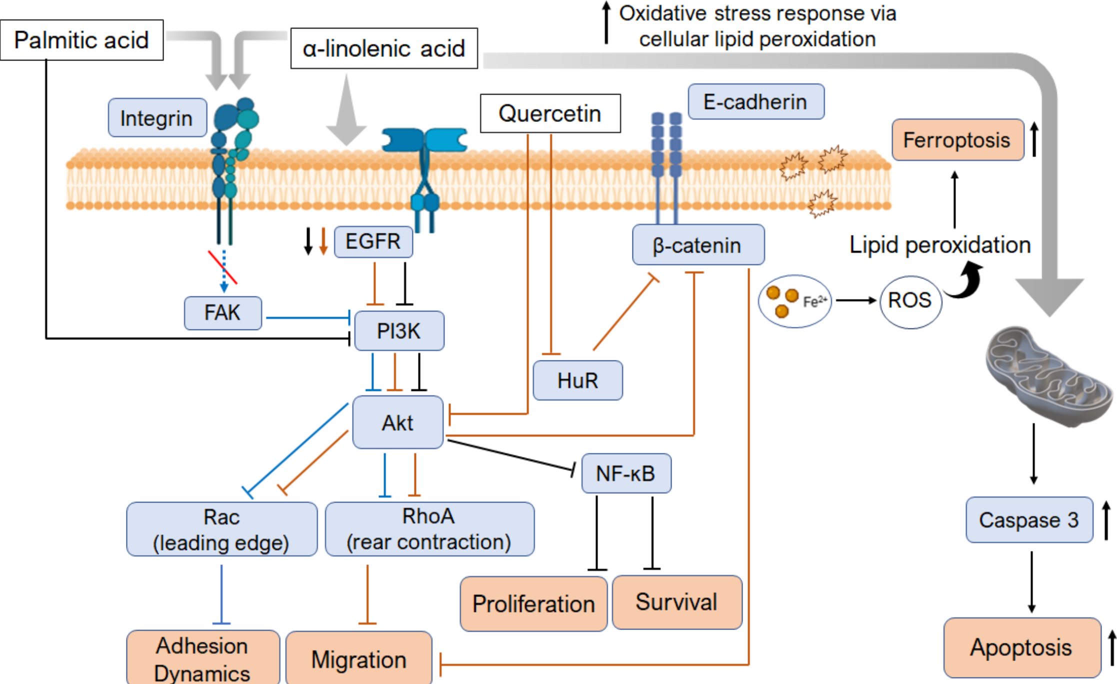


Figure 9.2.1: Summary of Proposed Anti-cancer Mechanisms in MDA-MB-468 cells

Based on evidence from the literature, potential anti-cancer effects of α-linolenic acid, palmitic acid and quercetin, as major compounds in *C. olitorius* extracts, on MDA-MB-468 cells are collectively summarised. Black lines suggest anti-proliferative effects on signalling pathways whereas blue and orange lines propose anti-adhesive and anti-migratory mechanisms, respectively. The red line crossing the dashed arrow represents the plausible inhibition of the already weak integrin-FAK signalling in this cell line. Images downloaded from www.biorender.com.

Another aim of the present study was to investigate the overall dietary habits of adult women with and without breast cancer history in Northern Cyprus. A detailed survey, including an FFQ and questions about meal consumption with cooking techniques, was designed to collect comparative data on women's eating habits in both groups. Other sections of the survey included questions about breast and reproductive health and women's history of or predisposition to breast cancer. These questions were designed to collect and evaluate data from a public health perspective and also to determine if there could be a parallel correspondence between the survey findings and the *in vitro* experiments in the study.

For instance, in the survey, intakes of omega-3 and omega-6 fatty acids were assessed (Table 8.2.9). In Western diets, both α-linolenic acid and linoleic acid are the most abundant PUFAs (Fabian, Kimler, & Hursting,

2015). These fatty acids were also among the major components of the extracts identified by GC-MS and literature review was primarily focused on the potential anti-cancer effects of these fatty acids to interpret the *in vitro* findings in the present study.

The ratio of dietary omega-6 to omega-3 fatty acids has been linked to several diseases including cardiovascular diseases, inflammation and cancer. Although the recommended omega-6:omega-3 ratio associated with lower incidences of diseases is 1:1 or 2:1, due to the increased use of plant oils and ultra-processed foods in most diets, this ratio has risen up to around 15:1 (Fabian et al., 2015; Simopoulos, 2002).

In the present study, even though omega-3 intake included EPA and DHA as marine sources in addition to α-linolenic acid, the ratio of omega-6 to omega-3 was nearly 7.5:1 in the control group whereas this was 9.5:1 in the case group. This reflects a dietary pattern characterised by omega-6 dominance in both groups, particularly in the case group.

Previously, fatty acid composition in breast adipose tissue, which was used a marker of past dietary intake of fatty acid, was examined in a case-control study. Fatty acids were measured as a percentage of total fatty acid content in biopsy tissues and an inverse relationship between omega-3 intake and risk of breast cancer was reported, suggesting a protective effect of higher dietary intake of omega-3 fatty acids (Maillard et al., 2002).

In the present study, the survey data may provide support for the *in vitro* findings. As discussed in Chapter 5 and 6, linoleic acid is hypothesised to be the major compound increasing adhesion and migration in MDA-MB-231 cells, consistent with studies suggesting increased dietary intakes of omega-6 could stimulate cancer progression. On the other hand, in MDA-MB-468 cells, α-linolenic acid is thought to be primarily responsible for reducing proliferation, adhesion and migration. Together, these findings suggest a link between dietary patterns and disease mechanism, proposing that dietary intakes of bioactive food compounds and cellular responses are not independent but interconnected.

Future epidemiological work could be designed to assess how reduced dietary omega-6 to omega-3 ratio of the female population in Northern Cyprus would affect their disease risk and whether it would be an effective strategy in the prevention of breast cancer.

Another important observation of the survey was the less frequent use of healthier cooking techniques. For example, steaming was found to be a less preferred method of cooking among women. However, cooking vegetables with moist heat methods, such as steaming and simmering, at lower temperatures preserves the maximum amount of nutrients. To enhance the bioavailability of fat-soluble vitamins and phytochemicals, such as carotenoids, olive oil could be added. These methods are also known to be the least cancer-associated cooking methods as they contain water and do not burn foods.

It is known that bioavailability and absorption of nutrients and phytochemicals show variability among different plant foods and it is difficult to estimate how much can be taken up and used by the body. Therefore, general Healthy Eating Guidelines recommend eating a variety of vegetables to achieve maximum health benefits and reduce the risk of chronic diseases.

The methodological design used in the present study is graphically summarised in Figure 9.2.2. The study framework is composed of experimental and epidemiological components. The experimental procedures briefly consisted of extract preparation from *C. olitorius* leaves, polyphenol and flavonoid content analyses, assessment of antioxidant properties, compound identification and cell culture assays. The epidemiological component of the study comprised a detailed case-control survey in which eating habits of women in Northern Cyprus were assessed to find potential association between dietary intakes of bioactive compounds and their link to cellular processes, integrating combined findings.

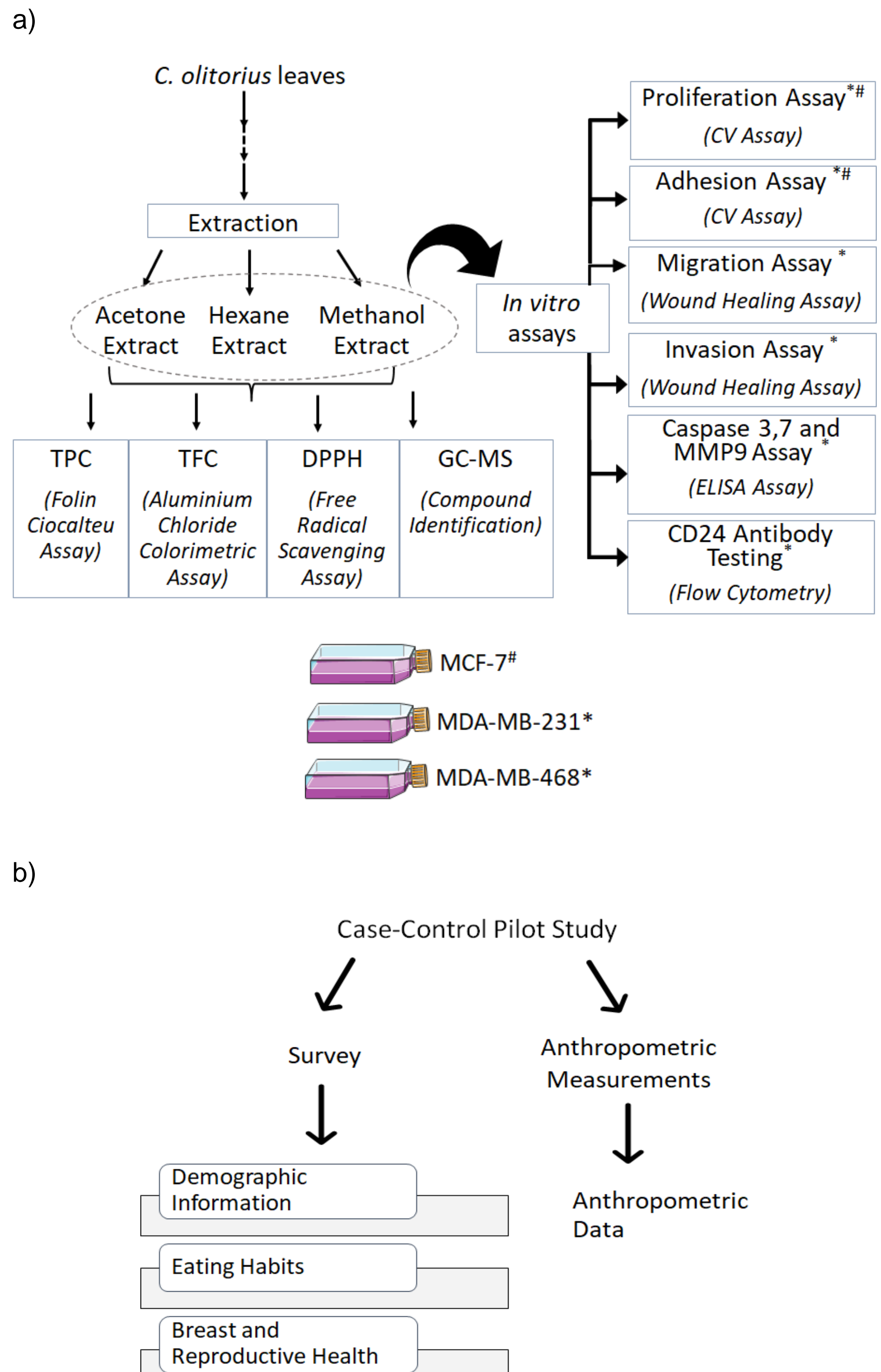


Figure 9.2.2: Outline of the Study Design

a) *C. olitorius* leaves were dried, ground and solvent extracted using acetone, hexane and methanol, which were then evaporated to obtain crude extracts (For simplicity, a dashed arrow was used to represent the multi-step process). TPC, TFC and DPPH free

radical scavenging activity of the extracts were measured and compound identification was performed via GC-MS. The extracts were examined for potential anti-cancer effects on MCF-7, MDA-MB-231 and MDA-MB-468 cells through proliferation and adhesion assays. MDA-MB-231 and MDA-MB-468 cell lines were also assessed in migration and invasion assays, along with the expression of CD24 and caspase 3, 7 and MMP9 quantification assays. [Image downloaded from www.smart.servier.com] b) The epidemiological study involved a case-control survey in which eating habits, breast and reproductive health, demographic and anthropometric information were collected and analysed.

9.3 Limitations of the Study

It should be noted that cell culture studies come with limitations. *In vitro* conditions are strictly controlled, artificial environments in which a single cell line is grown. In such environments, cells can mimic *in vivo* conditions only to a certain extent as they lack the actual complexity of *in vivo* conditions. Additionally, interactions of a particular cell line with non-malignant cells in a tumour environment may not be replicated in a desirable way in cell culture studies.

Another potential limitation could be introduced by the passage number of the cells being used. Even though each cell line used in the present study had a low initial passage number, replication and growth in a culture environment over time could result in genetic variations which may not fully represent the characteristics and behaviour of the original cell line.

Nevertheless, cell culture studies provide an opportunity to understand the primary interactions in cellular pathways which may be investigated further in higher models. Cell lines are also easier than animal models to work with in ethical terms.

Lack of a positive control was another limitation of the study. Even though a positive control was included in the TPC, TFC, DPPH and ELISA assays, one of the limitations of this study was the lack of use of a positive control in the cell culture experiments.

A positive control in an assay determines whether an assay procedure is performing as expected, strengthening the reliability and reproducibility of test results (Petersen et al., 2021). Several compounds, as anti-cancer drugs, were used as a positive control in previous studies investigating the effects of plant extracts or individual phytochemicals on MCF-7, MDA-MB-231 and other cancer cell lines for anti-proliferative effects. Studies that specifically used previously established MMP inhibitors, such as marimastat or ilomastat (Augoff, Hryniewicz-Jankowska, Tabola, & Stach, 2022), as positive controls in migration assays were not encountered in the literature.

One of those studies used doxorubicin (0.5 µM), a well-known drug for proliferation inhibition, on MCF-7 and MDA-MB-231 cells (Venkataswamy, Karunakaran, Islam, & Meriga, 2023) as a positive control. Another study carried out with sarcoma 180 and human leukaemia (HL-60) cell lines also used doxorubicin but at a higher concentration (6 µM) (Alencar et al., 2023). Staurosporine was another anti-proliferative drug which was used as a positive control (10 µM) on MDA-MB-231 cells for caspase activation (Naso et al., 2014). The other anti-proliferative drug used as a positive control was cisplatin (7.5 µg/mL) in a study which assessed the effects of the ethanol extract of C. olitorius leaves on HepG2 cells (C. Li et al., 2012).

It is worth mentioning that these studies did not include all three cell lines used in the current study. Additionally, concentrations of the anti-proliferative drugs showed variations on different cell lines. While a positive control would have provided additional validation of the findings, it was not included in this study due to several practical limitations and considerations.

Although many different compounds may inhibit cancer-related behaviours, such as migration and proliferation, each compound must first be tested to determine its optimal working concentration on specific cell lines before it can be validated as a positive control for comparison with a test compound or plant extract.

Besides, a single positive control compound may have differential effects on the cell lines used in a study, making interpretation of the results more complex. In this study, the use of a positive control was limited by practical constraints including the high cost of drug compounds, the restricted experimental timeline and limited availability of suitable compounds within the limited timeframe.

Furthermore, the primary aim of this study was to investigate the early-phase, preliminary effects of different extracts of C. olitorius leaves on certain cancer behaviours of breast cancer cell lines. Given the exploratory nature of the study, the synergistic biological activity of phytochemicals in the plant extracts was evaluated against cells treated with DMSO only as controls, rather than against a known positive control drug. In addition, no studies were found in the literature which assessed the anti-cancer effects of C. olitorius leaves on the breast cancer cell lines used in this study, making it difficult to compare study designs.

Another consideration was that the negative control cells behaved as expected in the assays. For instance, in the wound healing assay, the incubation period after the scratch is 24-48 hrs in the standard protocol (Fraser et al., 2003; Liang, Park, & Guan, 2007). In this study, untreated control cells closed the gap within 24 hrs. For the proliferation assay, a prior test was performed to determine an appropriate cell concentration in DMSO which would not lead to excessive apoptosis. In addition, the findings of the assays were aligned across independent experiments, indicating that the assay procedure was functioning as expected.

In the literature, there are a number of studies which investigated plant extracts or individual plant compounds focusing on their biological effects on cancer cells which used cells treated with DMSO only as controls.

In a study, in which MDA-MB-231 and MDA-MB-468 cells were investigated for cell proliferation and migration, cells treated with DMSO only were used to compare the effects of treatment with curcumin or quercetin (Kundur et al., 2018). In another study, which evaluated the

effects of a polyphenol mixture composed of curcumin, resveratrol, epigallocatechin gallate and quercetin on the proliferation of MDA-MB-231 and MCF-7 cells, cells treated with DMSO only were used as controls in the assay (Alqarni et al., 2022).

In several other studies investigating the anti-cancer effects of lycopene and β-carotene (Gloria et al., 2014); quercetin (Ranganathan, Halagowder, & Sivasithambaram, 2015); resveratrol, quercetin and catechin as a mixture or quercetin alone (Rivera Rivera, Castillo-Pichardo, Gerena, & Dharmawardhane, 2016) and methanolic extracts from *Juniperus sabina* and *Ferula communis* leaves (Kavaz & Faraj, 2023) on MDA-MB-231 and MCF-7 cells, cells treated with DMSO only were used as controls.

Building on the early-phase findings of this study, future in vitro research evaluating the effects of C. olitorius leaf extracts should incorporate a positive control to validate the results and strengthen the study design.

Another limitation encountered in the study was the lack of access to an LC-MS instrument with an accessible online library for unknown compound identification. Even though a major part of the acetone (~86%) and hexane extracts (~91%) were more readily identified with GC-MS analysis, only 19% of the methanol extract could be identified with $\geq$80% library matching quality via this method due to its polar nature not being largely suitable for GC-MS analysis. However, despite having a low match score in GC-MS analysis, the methanol extract likely contained more phenolic compounds than those identified, which potentially accounted for the higher IC_{50} value in the DPPH assay and a higher TPC value than the other extracts.

In the survey study, patients of younger age (35 or below) could not be recruited to the study as they were not open to discussing their condition, which resulted in an age difference between the case group and the control. However, the survey was already planned as a pilot study with a small sample size to collect preliminary data about the relationship between dietary habits and risk of breast cancer. In the future, an improved pilot study design could be planned with controls and patients adjusted for age,

body weight, height and menopausal status along with more detailed data collected about the diagnosis of patients. This would enable a more specific comparison between the two groups.

9.4 Conclusions and Future Research

The findings of *in vitro* assays suggest that the hexane and methanol extracts of *C. olitorius* leaves exert moderate anti-proliferative, anti-adhesive and anti-migratory effects on MDA-MB-468 cells and that the bioactive compounds in the hexane extract appear to stimulate adhesion and migration in MDA-MB-231 cells. In addition, the expression of caspase 3, as the main apoptotic executioner, was significantly higher in both TNBC cell lines whereas MMP9 was only significantly higher in MDA-MB-231 cells.

In order to gain a deeper understanding of the molecular pathways and mechanisms involved in the observed effects, activity of several proteins, along with their expression, could be examined in future work after hexane- and methanol-extract treatment in the proliferation, adhesion and migration assays. Among these are caspase 3 and its substrate PARP-1, integrin, FAK, PI3K, Akt and E-cadherin. Furthermore, MMP9 activity, in combination with TIMP1 quantification, could be further investigated in the MDA-MB-231 cell line which may provide deeper insights about the increased MMP9 expression that did not translate to a significant change in invasion. In MDA-MB-468 cells, ferroptosis-mediated cell death could also be assessed further. If possible, functional proteomics techniques, such as LC-MS/MS combined with affinity purification approaches, could be performed to identify unknown proteins and their interactions with other protein complexes. This approach increases the accuracy of the analysis and covers a broader spectrum of proteins involved in signalling pathways at the molecular level (Cui, Cheng, & Zhang, 2022; Monti, Orrù, Pagnozzi, & Pucci, 2005).

Although the initial cell culture assays were planned with only a negative control, future experiments should be designed using a suitable, affordable

and validated positive control. This would strengthen the reliability of further findings.

The DPPH assay has shown that the methanol extract possesses free radical scavenging ability with an IC_{50} value of 51.70 µg/mL (Table 3.2.4). The antioxidant potential of the extracts could be further investigated through complementary antioxidant assays, such as nitric oxide radical scavenging or oxygen radical absorbance capacity assays, to substantiate the current findings. Furthermore, the effects of the extracts, especially the methanol extract, could be measured on the expression and/or activity of glutathione peroxidase in MDA-MB-468 cells to complement the DPPH assay findings.

In the present study, due to a lack of access to an LC-MS equipment and online library of compounds, the phytochemical constituents of the methanol extract were identified via GC-MS. Even though more lipophilic leaf components were readily identified through this method in the methanol extract, an LC-MS or LC-MS/MS analysis of the methanol extract will help identify the more polar compounds including various polyphenols.

Future research may also involve assessing the possible synergistic effects of the plant extracts with an existing therapeutic drug targeting MDA-MB-468 cells. This would provide information about the possible additive effects of a dualistic therapy. For instance, the anti-proliferative effects of doxorubicin with the hexane and methanol extracts and the role of lipid peroxides in the observed effects on MDA-MB-468 cells could be assessed.

Using animal models, *in vivo* assessment of extract efficacy could be carried out on breast cancer progression markers, such as tumour size and growth rate, expression of cancer-specific proteins, and metastasis of tumours. These studies would help provide valuable information about the effective extract concentrations with anti-cancer effects that are non-cytotoxic to normal cells.

Lastly, since epidemiological data is largely missing in Northern Cyprus, a prospective cohort study could be designed to assess the association between jute consumption and breast cancer risk in the female population. Alternatively, an intervention study with reduced dietary omega-6:omega-3 ratio could be conducted to investigate its impact on prevention of breast cancer.

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Appendix 2.3.3.1.

Calculated cell concentrations and absorbance measurements are given in the tables below for three of the cell lines.

Table 2.3.3.1a: Cell concentrations and absorbance readings for MCF-7 Cells

Cell Concentration	1st Dilution (1 x 10^5 cells/mL)	2nd Dilution (0.5 x 10^5 cells/mL)	3rd Dilution (0.25 x 10^5 cells/mL)	4th Dilution (0.125 x 10^5 cells/mL)
Equivalent volumes	0.806 mL cells + 4.194 mL media	0.403 mL cells + 4.597 mL media	0.200 mL cells + 4.800 mL media	0.100 mL cells + 4.900 mL media
Absorbance at 570 nm	3,310	3,129	2,991	1,005
	3,375	3,375	3,170	1,339
	3,375	3,375	2,593	1,342
	3,372	3,375	2,686	1,212

The number of MCF-7 cells obtained initially to determine the working cell concentration was 6.2 x 10^5 cells/mL. Equivalent volumes that were calculated and absorbance readings are given in the table for each dilution tested.

Table 2.3.3.1b: Cell concentrations and absorbance readings for MDA-MB-231

Cell Concentration	1st Dilution (1 x 10^5 cells/mL)	2nd Dilution (0.5 x 10^5 cells/mL)	3rd Dilution (0.25 x 10^5 cells/mL)	4th Dilution (0.125 x 10^5 cells/mL)
Equivalent volumes	0.500 mL cells + 4.500 mL media	0.250 mL cells + 4.750 mL media	0.125 mL cells + 4.875 mL media	0.062 mL cells + 4.938 mL media
Absorbance at 570 nm	3,348	2,200	1,611	0,521
	3,348	1,993	1,562	0,799
	3,348	1,884	1,547	0,698
	3,238	1,860	1,274	0,495

The number of MDA-MB-231 cells obtained initially to determine the working cell concentration was 10 x 10^5 cells/mL. Equivalent volumes that were calculated and absorbance readings are given above for each dilution tested.

Table 2.3.3.1c: Cell concentrations and absorbance readings for MDA-MB-468

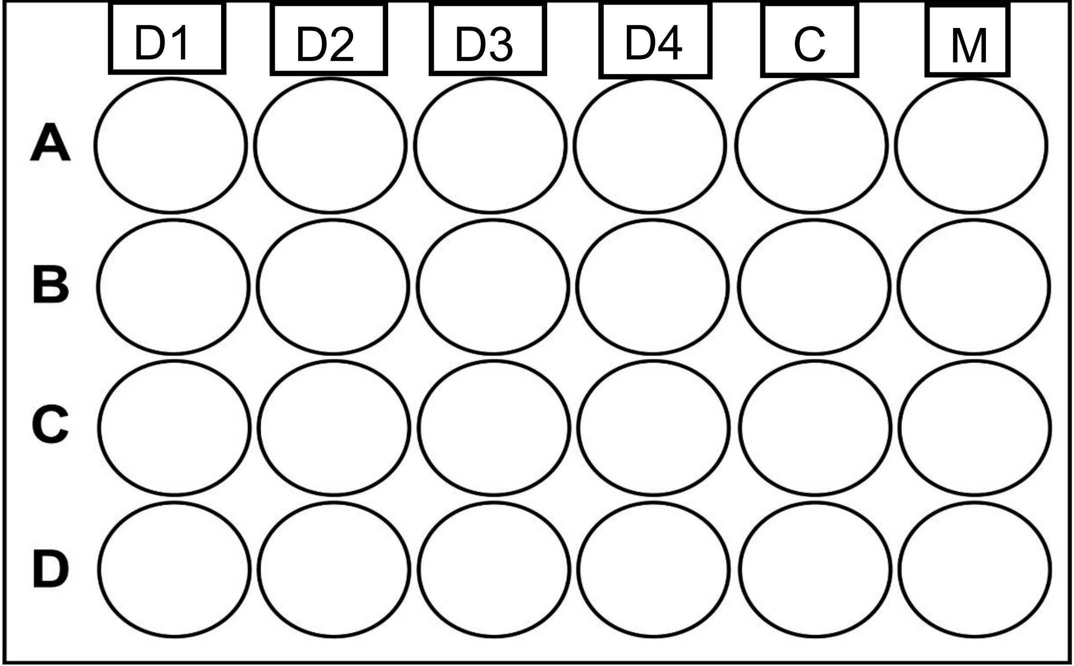
Cell Concentration	1st Dilution (1 x 10^5 cells/mL)	2nd Dilution (0.5 x 10^5 cells/mL)	3rd Dilution (0.25 x 10^5 cells/mL)	4th Dilution (0.125 x 10^5 cells/mL)
Equivalent volumes	0.781 mL cells + 4.219 mL media	0.391 mL cells + 4.609 mL media	0.195 mL cells + 4.805 mL media	0.098 mL cells + 4.902 mL media
Absorbance at 570 nm	2,971	2,151	0,917	0,535
	3,313	2,529	1,041	0,564
	2,876	2,117	1,033	0,608
	3,313	1,638	1,142	0,449

The number of MDA-MB-468 cells obtained initially to determine the working cell concentration was 6.4 x 10^5 cells/mL. Equivalent volumes that were calculated and absorbance readings are given above for each dilution tested.

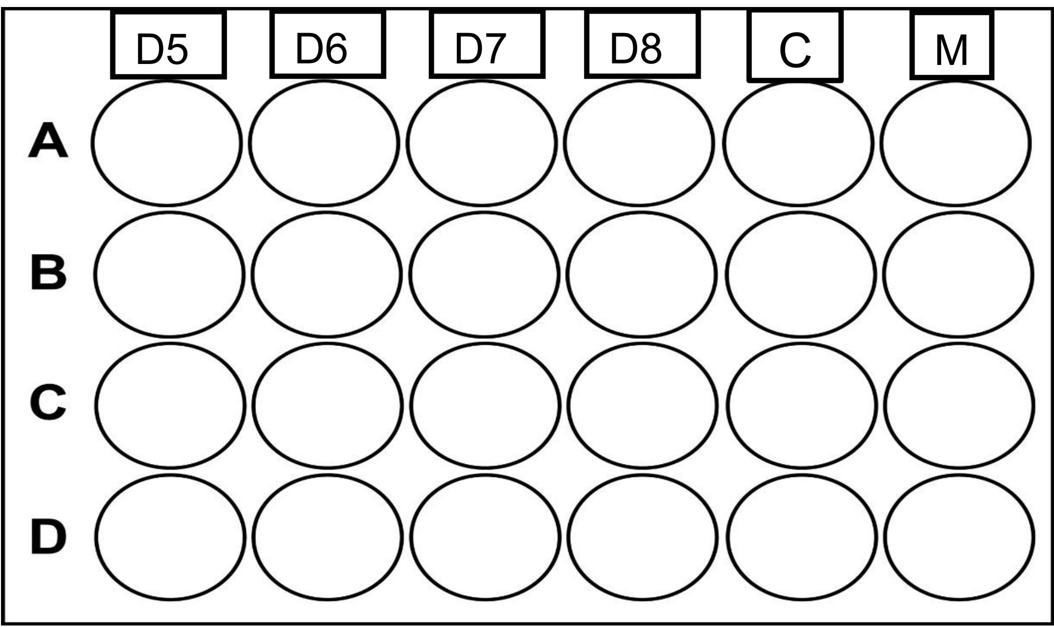
For MCF-7 cells, 3rd dilution (0.25 x 10^5 cells/mL) and for MDA-MB-231 and MDA-MB-468 cells, 2nd dilution (0.5 x 10^5cells/mL) were chosen for further experiments since in the previous dilutions maximum growth of cells was reached.

Appendix 2.3.3.3.

Two sets of D1-D4 and D5-D8 plates were prepared for each cell line with each extract.



D1: Dilution 1, D2: Dilution 2, D3: Dilution 3, D4: Dilution 4, C: Cells only, as negative control, M: Media only, as a blank [template downloaded and modified from www.sigmaaldrich.com, 25.07.18]



D5: Dilution 5, D6: Dilution 6, D7: Dilution 7, D8: Dilution 8, C: Cells only, as negative control, M: Media only, as a blank [template downloaded and modified from www.sigmaaldrich.com, 25.07.18]

Table 2.3.3.3: Concentrations of extracts used in the proliferation assay

ACETONE		HEXANE		METHANOL	
Name on Plate	**Concentration of Extract (mg/mL)**	**Name on Plate**	**Concentration of Extract (mg/mL)**	**Name on Plate**	**Concentration of Extract (mg/mL)**
D1	0.3000	**D1**	0.1600	**D1**	1.4400
D2	0.1000	**D2**	0.0533	**D2**	0.4800
D3	0.0333	**D3**	0.0178	**D3**	0.1600
D4	0.0111	**D4**	0.0059	**D4**	0.0533
D5	0.0037	**D5**	0.0020	**D5**	0.0178
D6	0.0012	**D6**	0.0007	**D6**	0.0059
D7	0.0004	**D7**	0.0002	**D7**	0.0020
D8	0.0001	**D8**	0.0001	**D8**	0.0007

Appendix 2.3.4.

Table 2.3.4a: Concentrations of the extracts used in adhesion assay

ACETONE		HEXANE		METHANOL	
Name of Extract	**Concentration of Extract (mg/mL)**	**Name of Extract**	**Concentration of Extract (mg/mL)**	**Name of Extract**	**Concentration of Extract (mg/mL)**
D1	0.3000	**D1**	0.1600	**D1**	1.4400
D2	0.1000	**D2**	0.0533	**D2**	0.4800
D3	0.0333	**D3**	0.0178	**D3**	0.1600
D4	0.0111	**D4**	0.0059	**D4**	0.0533

Table 2.3.4b: Equivalent volume of MCF-7 cells used in adhesion assay

Name of Extract	Concentration of Extract (mg/mL)	Equivalent volume of cells in 8 mL (µL)	Volume of media (µL)
ACETONE (D1-D4)	0.3000	3333	4667
	0.1000	1739	6261
	0.0333	1778	6222
	0.0111	2857	5143
HEXANE (D1-D4)	0.1600	1481	6519
	0.0533	1212	6788
	0.0178	1356	6644
	0.0059	1333	6667
METHANOL (D1-D4)	1.4400	860	7140
	0.4800	1667	6333
	0.1600	2162	5838
	0.0533	825	7175
Control	-	3478	4522

Table 2.3.4c: Equivalent volume of MDA-MB-231 cells used in adhesion assay

Name of Extract	Concentration of Extract (mg/mL)	Equivalent volume of cells in 8 mL (µL)	Volume of media (µL)
ACETONE (D1-D4)	0.3000	2105	5895
	0.1000	1667	6333

	0.0333	1379	6621
	0.0111	1905	6095
HEXANE (D1-D4)	0.1600	1860	6140
	0.0533	1860	6140
	0.0178	1818	6182
	0.0059	1905	6095
METHANOL (D1-D4)	1.4400	2000	6000
	0.4800	1905	6095
	0.1600	1860	6140
	0.0533	1600	6400
Control	-	2000	6000

Table 2.3.4d: Equivalent volume of MDA-MB-468 cells used in adhesion assay

Name of Extract	**Concentration of Extract (mg/mL)**	**Equivalent volume of cells in 8 mL (µL)**	**Volume of media (µL)**
ACETONE (D1-D4)	0.3000	909	7091
	0.1000	1000	7000
	0.0333	6154	1846
	0.0111	1000	7000
HEXANE (D1-D4)	0.1600	1250	6750
	0.0533	964	7036
	0.0178	1067	6933
	0.0059	1212	6788
METHANOL (D1-D4)	1.4400	6154	1846
	0.4800	3200	4800
	0.1600	5714	2286
	0.0533	2963	5037
Control	-	1111	6889

Appendix 2.3.6.

Table 2.3.6: Concentrations of extracts used in the migration assay

ACETONE		HEXANE		METHANOL	
Name of Extract	**Concentration of Extract (mg/mL)**	**Name of Extract**	**Concentration of Extract (mg/mL)**	**Name of Extract**	**Concentration of Extract (mg/mL)**
D1	3.00	**D1**	0.80	**D1**	7.20
D2	1.50	**D2**	0.40	**D2**	3.60
D3	0.30	**D3**	0.08	**D3**	0.72

Appendix 2.3.8.

Table 2.3.8a: Cell concentrations of MDA-MB-231 in CD24 staining assay

	Percent of Total Cell Count (%)			
MDA-MB-231	**Control**	**Acetone**	**Hexane**	**Methanol**
	4.56	9.78	9.16	9.30
	9.52	9.24	9.50	8.86
	9.36	10.08	8.46	8.66
	Cell Concentration (Cells/mL)			
	Control	**Acetone**	**Hexane**	**Methanol**
	1.04E+03	1.19E+04	1.12E+04	1.28E+04
	1.49E+04	1.24E+04	1.16E+04	1.26E+04
	1.44E+04	1.37E+04	1.11E+04	1.31E+04

Table 2.3.8b: Cell concentrations of MDA-MB-468 in CD24 staining assay

	Percent of Total Cell Count (%)			
MDA-MB-468	**Control**	**Acetone**	**Hexane**	**Methanol**
	61.03	68.44	70.20	65.96
	67.52	67.94	66.42	69.74
	67.72	71.10	67.14	66.96
	Cell Concentration (Cells/mL)			
	Control	**Acetone**	**Hexane**	**Methanol**
	1.16E+04	1.03E+05	9.51E+04	8.84E+04
	9.88E+04	1.03E+05	9.59E+04	9.78E+04
	9.75E+03	1.09E+05	9.33E+04	9.22E+04

Appendix 2.3.9.

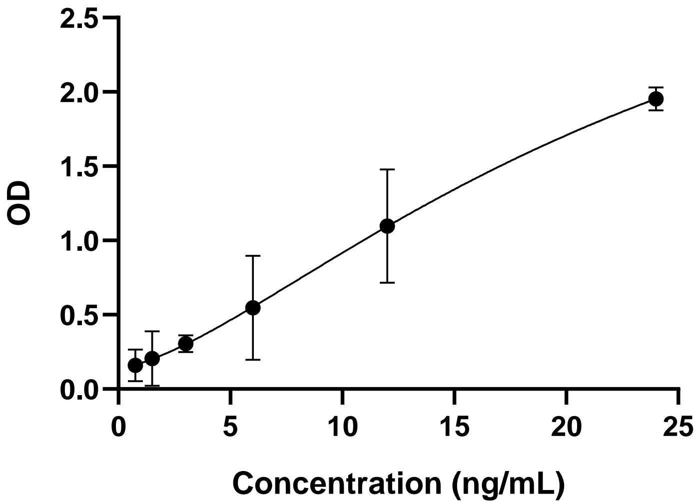


Figure 2.3.9a: Standard Curve for Caspase 3 in MDA-MB-231 cells

Six standard solutions were prepared to generate a standard curve from which the concentration of caspase 3 was calculated using 4PL sigmoidal non-linear regression model on GraphPad Prism. O.D (Optical Density) represents absorbance by the standards at 450 nm.

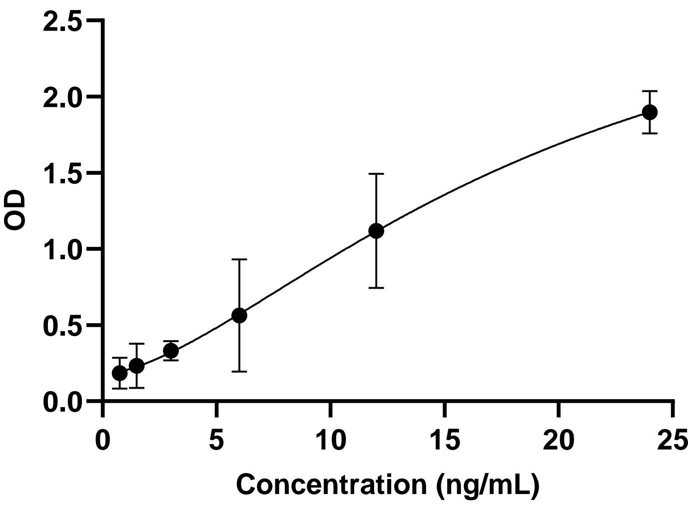


Figure 2.3.9b: Standard Curve for Caspase 3 in MDA-MB-468 cells

Six standard solutions were prepared to generate a standard curve from which the concentration of caspase 3 was calculated using 4PL sigmoidal non-linear regression model on GraphPad Prism. O.D (Optical Density) represents absorbance by the standards at 450 nm.

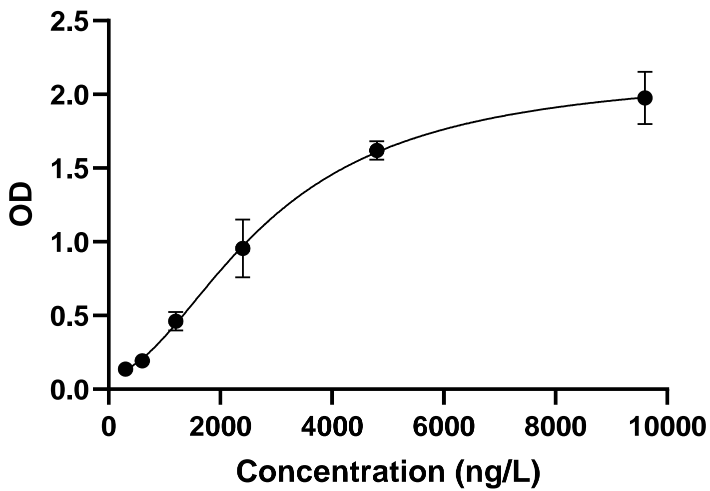


Figure 2.3.9c: Standard Curve for Caspase 7 in MDA-MB-231 cells

Six standard solutions were prepared to generate a standard curve from which the concentration of caspase 7 was calculated using 4PL sigmoidal non-linear regression model on GraphPad Prism. O.D (Optical Density) represents absorbance by the standards at 450 nm. Some error bars that are too small and appear hidden by the data points cannot be shown.

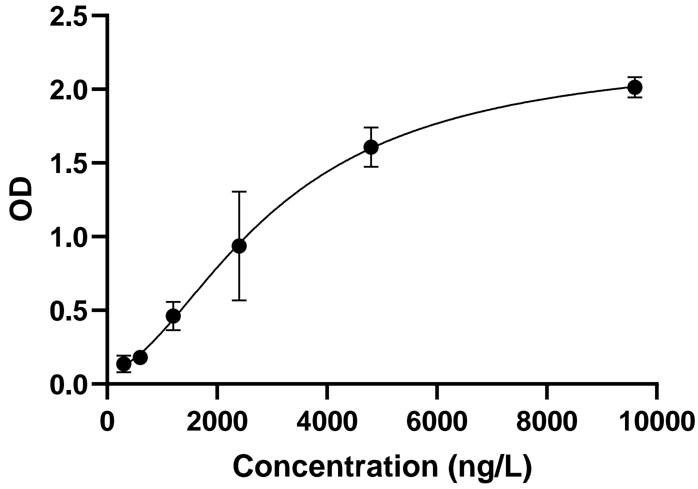


Figure 2.3.9d: Standard Curve for Caspase 7 in MDA-MB-468 cells

Six standard solutions were prepared to generate a standard curve from which the concentration of caspase 7 was calculated using 4PL sigmoidal non-linear regression model on GraphPad Prism. O.D (Optical Density) represents absorbance by the standards at 450 nm. Some error bars that are too small and appear hidden by the data points cannot be shown.

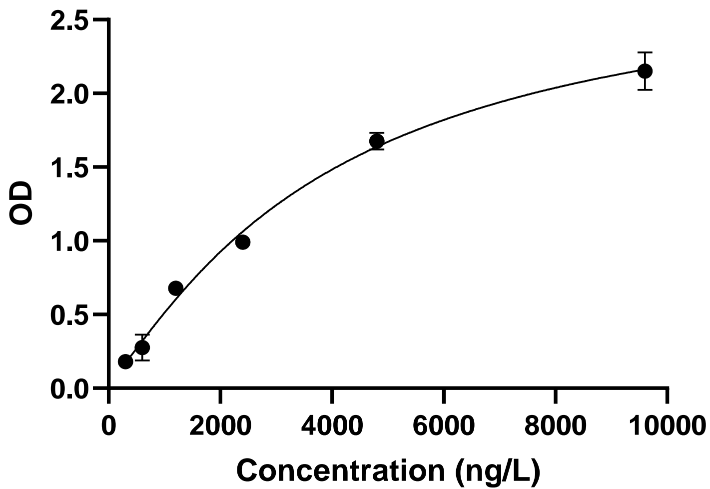


Figure 2.3.9e: Standard Curve for MMP9 in MDA-MB-231 cells

Six standard solutions were prepared to generate a standard curve from which the concentration of matrix metalloprotease 9 (MMP9) was calculated using 4PL sigmoidal non-linear regression model on GraphPad Prism. O.D (Optical Density) represents absorbance by the standards at 450 nm. Some error bars that are too small and appear hidden by the data points cannot be shown.

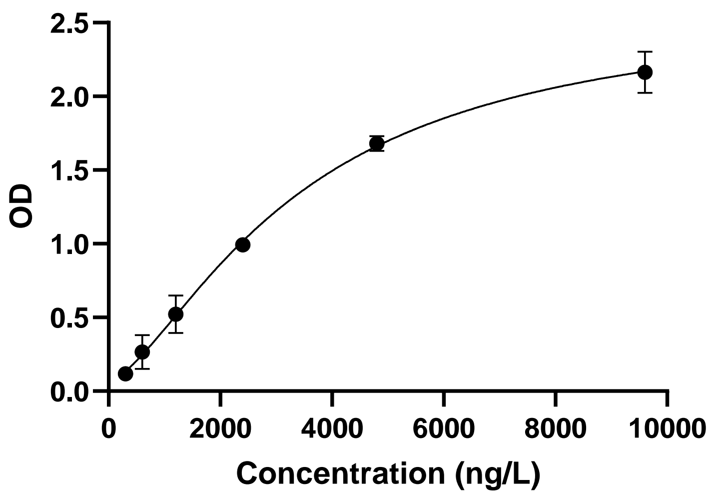


Figure 2.3.9f: Standard Curve for MMP9 in MDA-MB-468 cells

Six standard solutions were prepared to generate a standard curve from which the concentration of matrix metalloprotease 9 (MMP9) was calculated using 4PL sigmoidal non-linear regression model on GraphPad Prism. O.D (Optical Density) represents absorbance by the standards at 450 nm. Some error bars that are too small and appear hidden by the data points cannot be shown.

Appendix 2.4.

INVESTIGATION OF DIETARY HABITS OF WOMEN IN NORTHERN CYPRUS

Questionnaire No:

PART I

SECTION 1: General Information

Please read each question carefully and indicate your answer.

1. Which of the following is your age group?

1. ☐ 18-24 2. ☐ 25-34 3. ☐ 35- 44

4. ☐ 45-54 5. ☐ 55-64

2. What is your nationality?

1. ☐ Turkish Cypriot 2. ☐ Turkish 3.☐ Other (please specify) ________

3. What is your highest level of education?

1. ☐ Secondary School Diploma 2. ☐ High School Diploma

3. ☐ Bachelor's Degree 4. ☐ Master's Degree

5. ☐ Doctoral/Higher Degree

4. What is your occupation?

1. ☐ Student 2. ☐ Self-employed 3. ☐ Public sector 4. ☐ Private sector 5. ☐ Retired 6. ☐ Other (please specify:_____)

5. What is your monthly income?

1. ☐ 2000 – 2500 TL 2. ☐ 2500 - 3500 TL 3. ☐ 3500 - 5000 TL

4. ☐ Over 5000 TL

6. What is your marital status?

1. ☐ Single 2. ☐ Married 3. ☐ Other (Please specify:_______)

7. In which town do you live?

1. ☐ Nicosia 2. ☐ Kyrenia 3. ☐ Famagusta

8. Are you a smoker?

1. ☐ Yes 2. ☐ No 3. ☐ Stopped smoking

If you stopped smoking, please state the duration and number of cigarettes smoked daily:

Duration: ______ months or years

Number of cigarettes: _______ per day

9. Do you exercise regularly?

1.☐ Yes 2. ☐ No

If yes, please state:

Type of exercise:________

Duration of exercise: _______ hours per day

Frequency: _______ times per week

10. Do you drink alcohol?

1. ☐ Yes 2. ☐ No

If yes, please state:

Type of alcohol:_________

Frequency: ______ times per day or_______ times per week

Amount: ______ glasses per day or _______ glasses per week

11. Do you have any medical condition?

1. ☐ Yes (please specify the condition: ___________) 2. ☐ No

3. ☐ I don't know

<u>SECTION 2: Anthropometric Measurements</u>

FOR INVESTIGATOR ONLY

Measurements taken	Reading (cm)
Height (m)	
Weight (kg)	
Body Mass Index (BMI) (kg/m^2)	
Waist Circumference (cm)	

BIA Readings

Measurements taken	Reading
Body Fat %	
Body Water %	
Muscle Mass (kg)	

Visceral Fat Rating	

SECTION 3: Reproductive Health

12. Do you remember your age when your period began?

1. ☐ Yes 2. ☐ No 3. ☐ I don't know

If yes, please state your age: __________

13. Do you still have your period?

1. ☐ Yes (regular) 2. ☐ Yes (irregular) 3. ☐ No

If 'No', please state your age your periods stopped completely: ______

14. If 'No', did your period stop naturally?

1. ☐ Yes 2. ☐ No 3. ☐ I don't know

15. Have you ever received treatment because of problems with your period?

1. ☐Yes 3. ☐ No

If you answered 'Yes', please specify:

The problem: ____________

The duration of treatment: ___________ days/months/years

Type of treatment received: __________

16. Have you tried to be pregnant?

1. ☐ Yes 2. ☐ No (*Go to* ***Q19***)

How old were you when you got pregnant? _________

17. How many pregnancies have you had?

1. ☐ 1 2. ☐ 2 3. ☐ 3 4. ☐ More than 3

5. ☐ I had an abortion (Number of abortions: ________)

18. Did you breast feed?

1. ☐ Yes 2. ☐ No 3. ☐ Breast fed and bottle fed together

Duration of breast feeding: _________ days/months/years

19. If you have not ever been pregnant, was it because:

1. ☐ Never tried 2. ☐ You had problems

3. ☐ Partner had problems 4. ☐ Other reasons

20. Were you ever given fertility drugs to help you get pregnant?

1. ☐ Yes 2. ☐ No 3. ☐ I don't know

If 'Yes', please state:

Age when you started: ________

Duration of use: ______ days/months/years

21. Have you taken oral contraceptives (birth control pills)?

1. ☐ Yes 2. ☐ No (*Go to **Q23***)

22. At what age did you first start using oral contraceptives?

1. ☐ Age:________ 2. ☐ I don't remember

For how long have you taken them? __________ days/months/years

23. Have you received hormone replacement therapy (HRT)?

1. ☐ Yes 2. ☐ No (*Go to **Q26***) 3. ☐ I don't know

24. Do you know the type of HRT you have received?

1. ☐ Yes – Oestrogen only
2. ☐ Yes – Oestrogen and progestin combined (continuous)
3. ☐ Yes – Oestrogen and progestin combined (cyclical)
4. ☐ No

25. For how long have you received HRT? ________ months/years

26. Have you had a health condition with your ovaries and/or womb?

1. ☐ Yes – one ovary only 2. ☐ Yes – both ovaries
3. ☐ Yes – Womb only 4. ☐ Yes – Ovaries and womb
5. ☐ No

If 'Yes', what was the condition? _________

SECTION 4: Breast Health

27. Do you use deodorants regularly?

1. ☐ Yes 2. ☐ No (*Go to **Q31***) 3. ☐ I stopped

If you stopped, please state:

Date you stopped:______

Duration you used:_____ years

28. Which type of deodorants do you use?

1. ☐ Antiperspirants 2. ☐ Other deodorants 3. ☐ Combination of both
4. ☐ Other underarm cosmetics (please specify: _______)
5. ☐ I don't know

29. How long have you been using them?

1. ☐ 5-10 years 2. ☐ 10-15 years 3. ☐ More than 20 years

30. How often do you use these products?

1. ☐ Once a day 2. ☐ More than once a day 3. ☐ 3-4 days a week

31. How many hours in a day do you wear a bra?

1. ☐ < 8 hours 2. ☐ 8-12 hours 3. ☐ 12-16 hours 4. ☐ 24 hours 5. ☐ I don't wear a bra *(Go to **Q34**)*

32. How many days in a week do you wear a bra?

1. ☐ 1-3 2. ☐ 3-5 3. ☐ 5-7

33. What size of bra do you wear?

1. ☐ A 2. ☐ B 3. ☐ C 4. ☐ D or larger

At what age did you begin wearing a bra? _________

34. Do you have breast check-ups annually?

1. ☐ Yes 2. ☐ No 3. ☐ Will do at a later age

35. Has your doctor told you that you have 'dense breasts'?

1. ☐ Yes 2. ☐ No 3. ☐ I don't know

36. Have you had any breast disease other than cancer before?

1. ☐ Yes 2. ☐ No

If yes, please specify: ___________

Your age when you had the diagnosis: _________

37. Do you have a family history of breast cancer before the age of 50?

1. ☐ Yes 2. ☐ No 3. ☐ I don't know

38. If 'Yes', what was your relation to the person with breast cancer in the family?

1. ☐ Mother 2. ☐ Daughter 3. ☐ Sister
4. ☐ First-degree cousin 5. ☐ Second-degree cousin
6. ☐ Other (please specify)______

39. Have you ever been tested for *BRCA1/ BRCA 2* mutations?

1. ☐ Yes 2. ☐ No 3. ☐ I don't know

40. Have you been diagnosed with breast cancer?

1. ☐ Yes 2. ☐ No (Go to ***Section 6***)

If 'Yes', please indicate:

Your age at the time of first diagnosis: ___________

41. What was the stage of your diagnosis?

1. ☐ Stage 1 2. ☐ Stage 2 3. ☐ Stage 3

4. ☐ Stage 4
5. ☐ I don't know

42. What treatment did you receive? Please tick all that apply.

1. ☐ Didn't receive any treatment 2. ☐ Surgery 3. ☐ Radiotherapy 4. ☐ Chemotherapy 5. ☐ Hormone Therapy 6. ☐ Other therapy (please specify: ________________)

43. Are you on any active treatment at the moment?

1. ☐ Yes (Please specify the type of treatment: ___________)
2. ☐ No

PART II

SECTION 6: Eating Habits

In the questions below, please try to choose the best answer considering your average eating habits ***BEFORE YOUR DIAGNOSIS.***

44. How many main meals did you have in a day?

1. ☐ 1 2. ☐ 2 3. ☐ 3 4. ☐ 4
5. ☐ 5 6. ☐ More than 5

45. If you skipped any meals, which one was the most frequently skipped meal?

1. ☐ Breakfast 2. ☐ Lunch 3. ☐ Dinner

46. How many times did you eat snacks in a day?

1. ☐ 1 2. ☐ 2 3. ☐ 3 4. ☐ 4 5. ☐ 5 6. ☐ More than 5

47. How many glasses of water did you drink daily?

1. ☐ Less than 5 2. ☐ 5-8 3. ☐More than 10

48. Were you living:

1. ☐ with family 2. ☐ alone

49. Did you cook for yourself?

1. ☐ Yes 2. ☐ Yes – I also cooked for family
3. ☐ No - Someone else cooked for me

50. Did you follow a special diet?

1. ☐ Yes (please specify: ______________)
2. ☐ No

51. For this question please try to estimate how often you used a particular cooking method by putting a tick (√) for the best option.

	Frequency of Using the Cooking Method								
Cooking Method:	**1**.Never or less than once a month	**2**. 1-3 times a month	**3**. 1-2 times a week	**4**. 3-4 times a week	**5**. 5-6 times a week	**6.** Once a day	**7**. 2-3 times a day	**8**. 4-5 times a day	**9**. 6 or more times a day
Boiling									
Steaming									
Deep-Frying									
Sautéing									
Grilling									
Roasting									
Barbecue									

52. Have you had a major change in your daily eating habits since your diagnosis?

1. ☐ Yes 2. ☐ No

SECTION 7: Food Frequency Questionnaire

For this section, please try to estimate how often and how much you consumed a particular food ***BEFORE YOUR DIAGNOSIS.***

Food Item	**1.**Never or less than once a month	**2.**Once a month	**3.** Twice a month	**4.** 1-2 times a week	**5.** 3-4 times a week	**6.**5-6 times a week	**7.** Every day	**8.** Every meal	**Serving Size**
GRAINS									
Bread – White									
Bread – Brown									
Rice									
Pasta/Noodles – White									
Bulgur									
Potatoes – boiled									
Potatoes – fried									
Cereals – Muesli, oats etc (……..)									

Wheat flour – White/Brown (pitta bread etc)									
DAIRY FOOD									
Milk – Whole									
Milk – Semi skimmed									
Fatty Cheese									
Low-fat cheese									
Processed cheese									
Halloumi									
Kefir									
Ayran									
Yogurt – Full fat/low fat									
Cream									
Butter									
Margarine									

MEAT,FISH, EGGS & PULSES									
Red Meat – Full fat									
Red Meat – Low fat									
Poultry/Turkey – Full fat									
Poultry/Turkey – Low fat									
Fish (Name:…………)									
Processed meat products (salami, sausage etc)									
Organ meats (liver etc)									
Egg									
Pulses (black eyed beans, chickpeas, lentils, beans etc)									
OILS & FATS									
Olive oil									
Other vegetable oils (Name:…………..)									

Animal fat									
Olives – Green/Black									
Nuts (The most consumed: …………..)									
FRUITS & VEGETABLES									
Blue/Purple									
Grapes									
Plum									
Red onion									
Red cabbage									
Beet									
Aubergine									
Red									
Cherry									
Pomegranate									
Watermelon									

Tomato									
Red pepper									
Radish									
Yellow/ Orange									
Orange/ Tangerine									
Apricot									
Peach/ Nectarine									
Carrot									
Lemon									
Melon									
White									
Cauliflower									
Garlic									
Onion/leek									
Banana									

Green									
Kiwi									
Avocado									
Green grapes									
Apple/ pear									
Lettuce									
Cucumber									
Zucchini									
Green beans									
Broccoli									
Okra									
Green pepper									
Asparagus									
Peas									
Spinach									

Celery									
Artichoke									
Vine leaf									
Swiss chard									
Jute									
SWEETS & FAST FOOD									
Dried fruits (Name:…………..)									
Table sugar (Coffee, tea etc.)									
Coke/Sweetened Beverages									
Jams									
Molasses									
Cakes/ Biscuits/ Crackers/ Wafers									
Pastry (puff pastry etc.) (…………)									
Chocolate									

Desserts with milk (pudding, custard etc.)									
Syrup desserts (baklava etc.)									
Fast food (hamburger, pizza, fried chicken, doner kebab etc.)									

Appendix 2.5.

PARTICIPANT INFORMATION SHEET

Full title of Project: Investigation of Dietary Habits of Turkish Cypriot Women

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully.

What is the purpose of the study?

This study will look at the eating habits of women in Northern Cyprus and the association of dietary behaviour with the risk of breast cancer. We will do this by asking you a list of questions about your age, education, general health condition including your menstrual and breast health, the analysis of fat and muscle content of your body, your daily eating habits and how often you consume certain foods. With the analysis of the information collected, the link between dietary habits and breast cancer development will be evaluated.

Why have I been invited to participate?

You have been invited to take part because you meet the study requirements. Only women living in Northern Cyprus will be included in the study. You also meet the age range.

Do I have to take part?

No, you do not have to take part in the study. It is up to you to decide whether to take part. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason.

What will happen to me if I take part?

If you wish to take part, we will arrange a convenient time and you will be asked some questions about general information about you such as your age, where you live, educational background etc, your health including your menstrual and breast health and your routine eating habits which should take no longer than 45 minutes. We will also carry out an analysis of your body to determine the fat, muscle and water content. We will measure your height and waist with a tape measure. Your weight, body water, muscle and body fat will also be measured by stepping on a measuring device which will only take a few minutes. You will not feel anything when the device is on and measuring. Measurements will be taken three hours after rising or eating without any prior exercise or caffeine, nicotine, alcohol and any diuretic medication intake. Since the device gives a weak current, if you have a pacemaker or an implant, we will only measure your weight, height and waist. At the end of the survey, you will be given a copy of your measurements for your own records.

What are the possible disadvantages and risks of taking part?

There should be no major risks or disadvantages involved in this study as it will involve answering questions about health and diet and no testing of new medication. Also analysis of body fat, muscle and water content is expected to cause minimal burden, if any.

What are the possible benefits of taking part?

Joining the study would offer an opportunity for obtaining answers to questions relating to your condition and getting immediate feedback. The benefits also include the educational opportunities in healthy eating. These opportunities may encourage you to understand the importance of healthy eating habits in obtaining overall health. You also will receive your own record of your body composition measurements.

In addition, your participation will help us understand the link between dietary habits and the development of breast cancer and try to prevent it in the future, particularly in women living in Northern Cyprus.

Will my data be kept confidential?

We shall require your consent to access your medical records, but this will be kept to a minimum and on a strictly 'need-to-know' basis. All information collected about you will be kept strictly confidential. Access to the data will only be by researchers working on this study. Computer files will be password protected and all data, codes and identifying information will be kept in locked filing cabinets. The findings generated during the research will be kept securely for a period of ten years after the completion of this project.

What should I do if I want to take part?

If you would like to take part in this study, you can do so by contacting the researchers at the address, phone number or email address given at the end of this information sheet. It is also important to confirm that you have read and understood this project and that you agree to participate and sign it below to give us your consent before joining.

What will happen to the results of the research study?

The results of this research will be published in a scientific journal. Your identity will not be recognisable from this. If you would like a copy of the published research you can contact the researchers at the address, phone number or email address given below following completion of the study.

Who is organising and funding the research?

The study is self-funded by the researcher and supervised by staff researchers at London Metropolitan University.

Who has reviewed the study?

This research has been approved by London Metropolitan University Research Ethics Committee.

Contact for Further Information:

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N7 8DB

Eliz Arter
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Email: dietitianeliz@gmail.com
Eastern Mediterranean University
Famagusta

I am interested in this research and I give my consent to participate.

Patient Name- Signature:

Date: