EFFECTS OF *Kigelia africana*, *Ximenia caffra* AND *Mimusops zeyheri* SEED OILS ON HORMONE-INDEPENDENT (MDA-MB-231) AND HORMONE-DEPENDENT (MCF-7) BREAST CANCER CELL LINES

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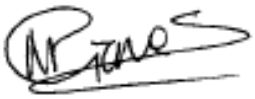
A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg in fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg, South Africa

10 May 2022

DECLARATION

I, Monica Nunes Gomes, declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



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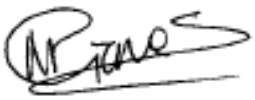
Monica N. Gomes

.....10th day of May 2022 in Parktown.....

I certify that all studies contained in this thesis have the approval of the Committee for Research on Humans of the University of the Witwatersrand (Clearance numbers: W-CJ-150422-1 and M160211).



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Monica N. Gomes

.....10th day of May 2022 in Parktown.....

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William Daniels (supervisor) Eliton Chivandi (supervisor)

..... day of May 2022 in Parktown.....

In loving memory of my mother

Maria da Conceição Nunes da Cruz Gomes

1959 - 2013

PRESENTATIONS (Poster)

In support of the present thesis, the work was presented at the following conference.

- Differential response of breast cancer cell lines to *Kigelia africana*, *Ximenia caffra* and *Mimusops zeyheri* seed oils. M. N. Gomes, T. N. Augustine, D. Moyo and E. Chivandi. University of the Witwatersrand 8th Cross Faculty Postgraduate Symposium, 25-26 October 2017, Parktown, Johannesburg, South Africa.

PUBLICATIONS

In support of the present thesis, the work included has resulted in the following publications.

- Gomes M, Augustine T, Moyo D., Chivandi E, 2019. Differential response of breast cancer cell lines to *Kigelia africana*, *Ximenia caffra* and *Mimusops zeyheri* seed oils. SAJB 121 (2019) 463–469. doi.org/10.1016/j.sajb.2018.12.017.
- Gomes MN, Fru P, Augustine T, Moyo D, Chivandi E, Daniels, WMU. Differential expression of platelet activation markers, CD62P and CD63, after exposure to breast cancer cells treated with *Kigelia africana*, *Ximenia caffra* and *Mimusops zeyheri* seed oils *in vitro.* Nutrition and Cancer ID: 2032215 DOI:10.1080/01635581.2022.2032215 (in press).

ABSTRACT

Breast cancer is the most prevalent type of cancer among women worldwide. The survival rate of breast cancer patients in developing countries is lower than that of their counterparts in first-world countries, since most do not have access to conventional medicine and treatment. Consequently, the health care of these women is heavily influenced by ethnomedicine.

In sub-Saharan Africa, traditional healers use parts of *Kigelia africana* (*K. africana*), *Ximenia caffra* (*X. caffra*) and *Mimusops zeyheri* (*M. zeyheri*) trees to treat various maladies. The seed oils of *K. africana*, *X. caffra* and *M. zeyheri* were shown to inhibit proliferation of human colon adenocarcinoma (Caco-2) and human embryonic kidney (HEK-293) cells *in vitro*. Consequently the effects of these seed oils on hormone-independent MDA-MB-231 and hormone-dependent MCF-7 breast cancer cells *in vitro* were investigated.

The seed oils' potential toxicities were tested using the lactate dehydrogenase cytotoxicity and neutral red cell viability assays. The possible mechanism(s) by which these seed oils induced cytotoxic/anti-proliferative effects on MDA-MB-231 and MCF-7 cells were subsequently investigated using flow cytometry and enzyme-linked immunoassays. The ability of the seed oils to reduce the thrombogenic ability of breast cancer cells was also studied using flow cytometry.

Analysis of the seed oils showed that *K. africana* seed oil had the highest concentration of α-linolenic acid (an essential omega-3 fatty acid) followed by *X. caffra* and lastly *M. zeyheri*. *M. zeyheri* had the highest concentration of linoleic acid (an essential omega-6 fatty acid) followed by *K. africana* and *X. caffra*. Our results point to the effects of *K. africana*, *X. caffra* and *M. zeyheri* seed oils being more dependent on the fatty acid composition, the duration of exposure, as well as phenotype of the breast tumors. *K. africana* and *X. caffra* seed oils induced the greatest cytotoxic effects on MDA-MB-231 cells, while *X. caffra* and *M. zeyheri* seed oils induced a growth inhibitory effect on MCF-7 cells. Analysis by flow cytometry suggested an apoptotic mediated cell death, however low levels of caspase-6 indicated the engagement of another pathway. *K. africana*-treated cells showed a significant reduction in the expression of LC3A in MDA-MB-231 cells suggesting that this seed oil may inhibit autophagic activity. *K. africana*, *X. caffra* and *M. zeyheri* seed oils decreased the thrombogenic ability of hormone-independent MDA-MB-231 breast cancer cells. These findings corroborate previous reports of plant seed oil's beneficial effects and supports the notion that plants can be considered for the

management of diseases such as breast cancer. However further investigations are required into the mechanisms by which these seed oils exert their effects.

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I would like to acknowledge several people, schools and institutes who contributed to this PhD thesis:

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- The National Research Foundation (NRF), the Faculty Research Council (FRC) and the University of the Witwatersrand for their financial support, which is greatly appreciated and acknowledged.
- Finally, I want to thank the Lord for his guidance and blessing me with the strength, both physical and mental, to see this to the end. I give You praise!

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LIST OF ABBREVIATIONS

12-HETE	12-hydroxyeicosatetraenoic acid
AA	Arachidonic acid
ADP	Adenosine diphosphate
AIF	Apoptosis-inducing factor
ANOVA	One-way analysis of variance
Apaf-1	Apoptotic protease activating factor 1
APC	Allophycocyanin
ARC	Agricultural Research Council
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X protein
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma-2
bFGF	Basic fibroblast growth factor
Bid	BH3-interacting domain death agonist
Bid	Bcl-2 homology 3 (BH3)-only proteins
Bik	Bcl-2 interacting killer
BRCA1	Breast cancer gene 1
BSA	Bovine serum albumin
CD-62	P-selectin
CMA	Chaperone-mediated autophagy
CML	Chronic myelogenous leukemia
COX 2	Cyclooxygenase 2
DAPk	Death-associated protein kinases
DC	Diluent control
DHA	Docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle's Medium

DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
ER-α	Oestrogen receptor-α
Fas	Fibroblast associated antigen
FBS	Foetal bovine serum
FCS	Flow Cytometry Standard
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
GF	Growth factor
HER2/ EGFR	Human epidermal growth factor receptor 2
Hsc-70	Heat shock cognate 70
HSD	Tukey Honestly Significantly different
IAP	Inhibitor of apoptosis protein
IL 1β	Interleukin 1β
IL-6	Interleukin-6
INT	Iodonitrotetrazolium
JNK1	cJun N-terminal kinase 1
***K. africana*/KA**	*Kigelia africana*
LAMP-2A	Lysosomal-associated membrane protein 2A
LDH	Lactate dehydrogenase
LIF	Leukaemia inhibitory factor

***M. zeyheri*/MZ**	*Mimusops zeyheri*
MC	Media control
mean±SD	Mean ± standard deviation
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n-3	Omega-3
n-6	Omega-6
NAD	Nicotinamide adenine dinucleotide
NFkB	Nuclear factor kappa light chain enhancer of activated B cells
NFTAGCB	New Fellowships to Tackle Africa's Growing Cancer Burden
NH_4Cl	Ammonium chloride
NR	Neutral red
NSCLC	Non-small cell lung cancer
PAF	Platelet-activating factor
PARP-1	Poly(ADP-ribose) polymerase-1
PCD	Programmed cell death
PE	Phosphatidylethanolamine
PE-Cy	Phycoerythrin cyanine
PerCP	Peridinin–chlorophyll–protein
PGs	Prostaglandins
PI	Propidium iodide
PUFAs	Polyunsaturated fatty acids
RIP1	Receptor-interacting protein 1
ROS	Reactive oxygen species
SMAC	Second mitochondrial activator of caspases
TF	Tissue factor
TMB	3,3',5,5"-tetramethylbenzidine
TNF-α	Tumour necrosis factor alpha
TRAF2	TNF-receptor-associated factor 2

TRAIL	TNF-related apoptosis inducing ligand
TxA_2	Thromboxane A2
VEGF	Vascular endothelial growth factor
VTE	Venous thromboembolism
WB	Whole blood
***X. caffra*/XC**	*Ximenia caffra*

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CHAPTER 1: LITERATURE REVIEW

1.0. INTRODUCTION

Cancer is a worldwide health public challenge (Nourazarian *et al*., 2014; Bray *et al*., 2018). It is estimated that out of 18.1 million people diagnosed with cancer in 2018, 9.6 million of these patients died (Bray *et al*., 2018). The survival rate of cancer patients varies according to the level of development of the country/region. The survival rate of cancer patients of developed countries is much higher than that of emerging economies (Kuete *et al*., 2013). Professor Nicholas Othieno-Abinya, an oncologist from the University of Nairobi Medical School, in a report on "New Fellowships to Tackle Africa's Growing Cancer Burden (NFTAGCB)" asserts that lack of medical resources and personnel with expertise in the diagnosis, treatment and management of cancer in Africa as major contributors to the abysmal cancer survival rate in Africa (NFTAGCB, 2016). South Africa, Morocco, Algeria, Tunisia and Egypt are the only African countries with functional programmes with regards to the training of oncologists (NFTAGCB, 2016).

Cancer is recognized as the accumulation of genetic changes in cells as a result of replication errors, environmental exposure and oncoviruses (Yokota, 2000; Fouad *et al*., 2017). Several factors are associated with the risk and/or increased risk for the development of tumours; among them internal and external factors (Anand *et al*., 2008). Regarding external factors, strong correlations between lifestyle and the environment have been implicated in increased worldwide tumour incidence (Anand *et al*., 2008). Internal factors, including inherited genetic mutations and immunodeficiency disorders, are associated with tumour formation (Anand *et al*., 2008). These genetic changes allow cells to develop heterogenetic morphology, increase proliferation, evade immune recognition and cell death, suppress immune reactivity, reprogram metabolism, invade tissue and metastasize (Hanahan and Weinberg, 2000; Lazebnik, 2010; Cavallo *et al*., 2011; Hanselmann and Welter, 2016; Fouad *et al*., 2017) as illustrated in Figure 1 below.

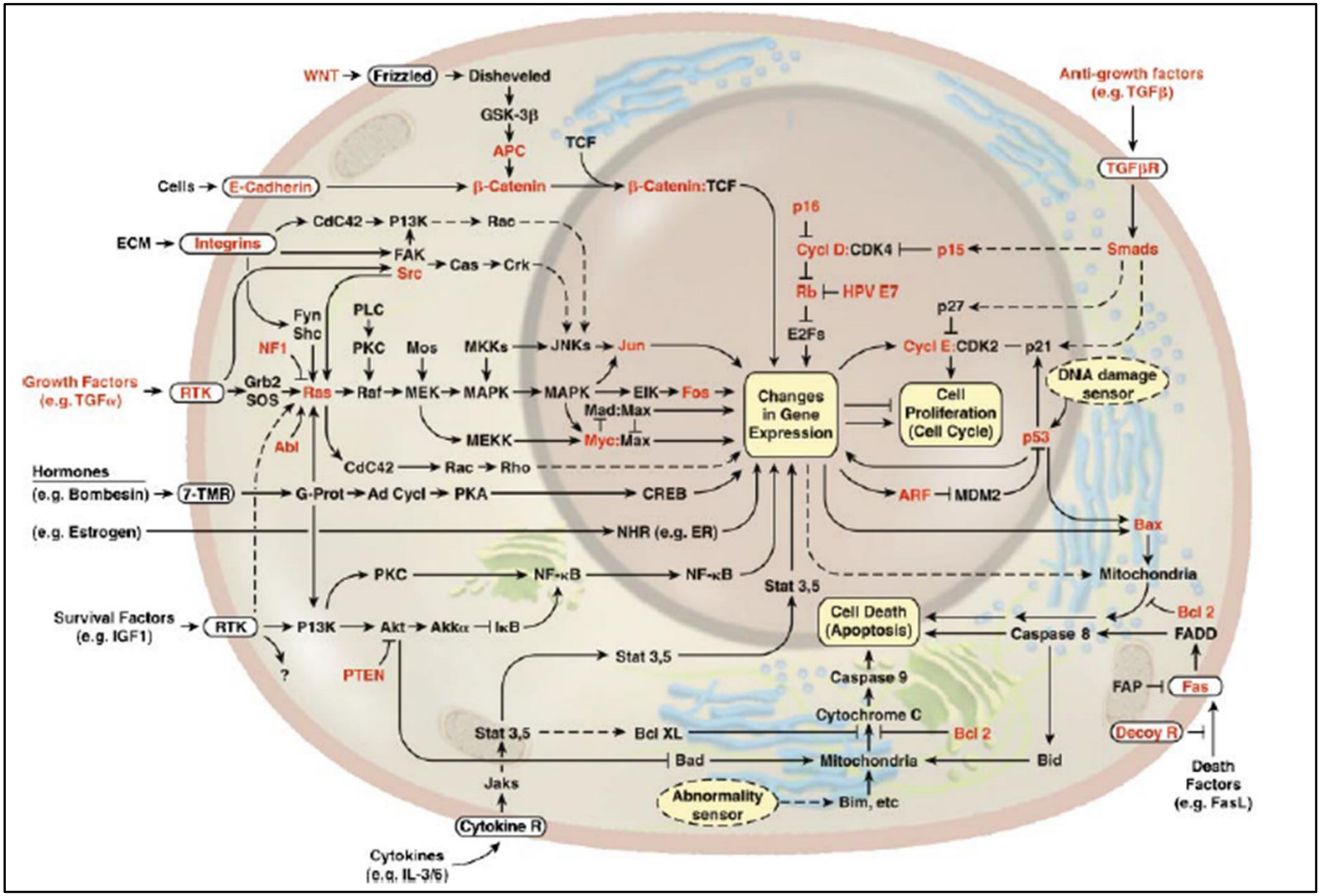

Figure 1.1: Signalling pathways within a cell and the genes functionally altered during cancer transformation (highlighted in red). Reprinted with permission from "The Hallmarks of Cancer", by D. Hanahan and R. A. Weinberg, 2000, Cell, 100, p59. Copyright 2000 by Elsevier.

Various types of cancer exist but globally, breast cancer is the most commonly diagnosed cancer and the foremost cause of cancer death amongst women (Monga *et al*., 2013; Bray *et al*., 2018).

1.1. Breast cancer

Although hereditary and genetic factors [mutations in breast cancer gene 1 (BRCA1), BRCA2 and other breast cancer susceptibility genes], account for 5-10% of breast cancer cases, differences observed in incidences between international and inter-ethnic migrants show that non-hereditary factors are the main cause (Bray *et al*., 2018). In the past few decades, the incidence of breast cancer has increased in most countries with some of the most rapid increases occurring in areas where incidences were previously relatively low (Bray *et al*., 2018). These trends are most likely the result of a combination of demographic factors related to social and economic development such as delayed childbearing, increased levels of obesity and physical inactivity as well as an increase in breast cancer screening and awareness (Bray *et al*., 2018). The survival rate for breast cancer in the US is almost 90% compared to 50% in Gambia, Uganda and Algeria (Kuete *et al*., 2013). The difference in the survival rate is ascribed to the lack of infrastructure, resources and specialized oncologists for routine screening mammography in emerging countries. This results in breast cancers being diagnosed at late stages of the disease. Due to the high cost and reduced availability of treatment options, women in such emerging economies receive inadequate treatment, pain relief or palliative care (Coughlin and Ekwueme, 2009).

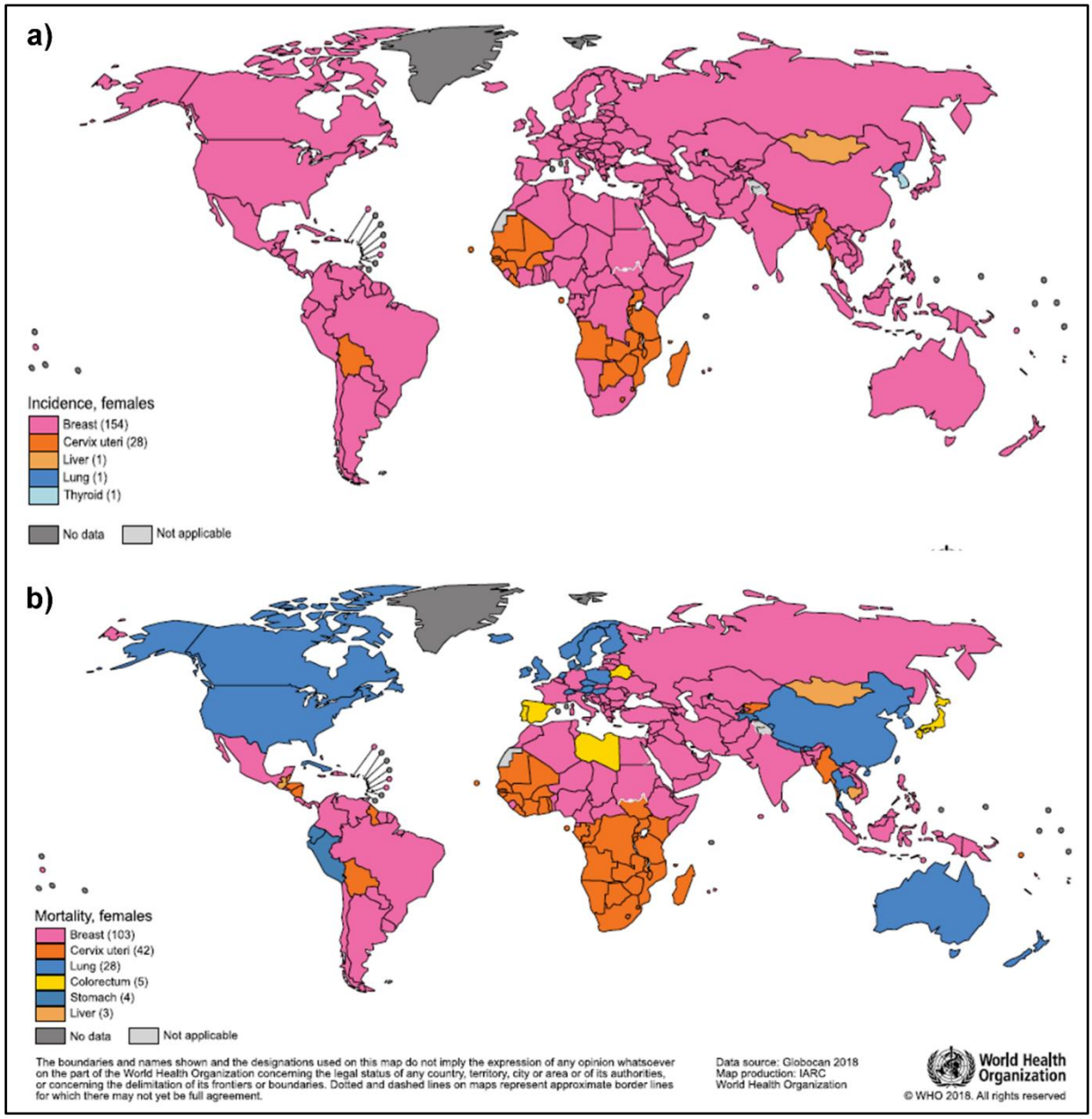

Figure 1.2: Global map presenting the most common types of cancer incidence (a) and mortality (b) among women in 2018. The number of countries represented in each ranking group are included in the legend. Reprinted with permission from "Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries", by F. Bray, J. Ferlay, Soerjomataram, R. L. Siegel, L. A. Torre and A. Jemal, 2018, CA: A Cancer Journal for Clinicians, 0, p8-9. Copyright 2018 by John Wiley & Sons, Inc.

1.1.1. Breast cancer subtypes

Breast cancer is a heterogeneous disease (Yersal and Barutca, 2014). The luminal phenotype, human epidermal growth factor receptor 2 (HER2 also referred to as EGFR) phenotype and basal-like phenotype are the 3 major subtypes of infiltrating ductal breast carcinoma (Polyak, 2011). Oestrogen and progesterone receptors are present in the luminal subtype whilst in the HER2 subtype, there is an increase in HER2 gene expression but oestrogen and progesterone

receptors are absent (Yersal and Barutca, 2014). The basal-like tumours have been found to contain neither hormone receptors nor HER2 and are referred to as triple-negative breast cancers (Polyak, 2011). Oestrogen receptor-α (ER-α) regulates cell growth and differentiation in the mammary gland and unfortunately promotes the survival of breast cancer cells in hormone-dependent breast cancer (Thomas and Gustafsson, 2015). In the HER2 subtype as well as the basal subtype, hormone receptors are absent and therefore oestrogen or progesterone are not the stimulators in these breast cancer phenotypes. Human EGFR is not only responsible for cell proliferation but cell motility, cell adhesion, invasion, cell survival and angiogenesis which is very important in tumour progression (Woodburn, 1999). Due to this heterogeneity, prognoses are unique and responsiveness to treatment differs (Barnard *et al.*, 2015).

1.1.2. Breast cancer treatment

Current treatment for breast cancer includes hormone therapy, chemotherapy (including neo adjuvant and adjuvant chemotherapy), radiotherapy and surgical removal of tumour tissue. Although chemotherapy in addition to surgery has proved useful in the treatment of breast cancer since it decreases the risk of recurrence and improves survival odds, it can cause severe side effects (Rampurwala *et al*., 2014). Chemotherapy, which induces apoptosis in highly proliferative cells (Payne and Miles, 2004), can also target healthy cells and has also been implicated in increasing the risk of thrombosis (Falanga *et al*., 2015). Tamoxifen, which was the first Food and Drug Administration (FDA) approved chemopreventive agent for reducing the risk of breast cancer, increases the risk of uterine cancer, ocular disturbances, hypocalcaemia, blood clots and strokes (Anand *et al*., 2008). Raloxifene, an osteoporosis drug, has fewer side effects and is as effective as tamoxifen in preventing oestrogen-receptor-positive breast cancer but, can still cause blood clots and strokes (Anand *et al*., 2008). A thrombotic risk exists for women on hormone replacement therapy (Sandset *et al*., 2009). This thrombotic risk is increased up to 7.1 fold when hormone therapy is used as adjuvant therapy in women with breast cancer following surgery (Haddad and Greeno, 2006).

Cancer treatment is exorbitantly expensive and drugs for the treatment thereof not always easily obtainable. Due to lack of infrastructure, medical resources and expertise, people in developing countries, especially in rural communities, still rely on traditional medicine (Ochwang'i *et al*., 2014).

1.2. PHYTOMEDICINE

Plants have been used for medicinal purposes by humans since long before recorded history (Park and Pezzuto, 2002). Herbal remedies offered by traditional healers are often prepared with two or three plants and/or different plant components (Sawadogo *et al*., 2012). Leaves, stem bark, root bark, fruit and combinations thereof are most commonly used and these are thought to have synergistic effects (Issa *et al*., 2006). Preparation of these herbal remedies include boiling and maceration or drying and grounding to powder (Ochwang'i *et al*., 2014). Because of the above, plants have played a major role in drug development (Voss *et al*., 2006). A number of compounds have been isolated and found to possess different anti-tumour effects employing various mechanisms of action (Payne and Miles, 2008; Page and Takimoto, 2010; Khazir *et al*., 2014). The "spindle poisons" also known as the vinca alkaloids and taxanes block the polymerisation of microtubules or promote the assembly and stabilization of the microtubules thereby inhibiting mitosis, respectively (Payne and Miles, 2008; Page and Takimoto, 2010; Khazir *et al*., 2014). The vinca alkaloids, Vincristine and Vinblastine, were isolated from the periwinkle plant, *Vinca rosea* and have been used in clinical oncology for almost 50 years (Payne and Miles, 2008; Page and Takimoto, 2010; Khazir *et al*., 2014). The taxane, Taxol (generic name Paclitaxel), was discovered from the bark of the Pacific Yew, *Taxus brevifolia* Nutt and is yet another example of how natural products can be a successful source of drugs (Payne and Miles, 2008; Page and Takimoto, 2010; Priyadarshini and Keerthi Aparajitha, 2012; Khazir *et al*., 2014). In addition to treating ovarian and breast cancers, Paclitaxel is used to treat non-small cell lung cancer (NSCLC) and other cancers like leukopenia and liver cancer (Priyadarshini and Keerthi Aparajitha, 2012; Khazir *et al*., 2014). The "topoisomerase inhibitors" consisting of two broad classes, prevent deoxyribonucleic acid (DNA) replication (Payne and Miles, 2008; Page and Takimoto, 2010; Khazir *et al*., 2014). By interrupting the elongation phase of DNA replication via inhibition of topoisomerase I, topoisomerase I inhibitors, such as Camptothecin which was isolated from the *Camptotheca acuminata* tree, prevent the elongation phase of DNA replication (Payne and Miles, 2008; Page and Takimoto, 2010; Khazir *et al*., 2014). Topoisomerase II inhibitors prevent the synthesis of DNA by stabilising the DNA-toposisomerase II complex (Payne and Miles, 2008; Page and Takimoto, 2010). Podophyllotoxin, obtained from *Podophyllum peltatum*, is an example of a topoisomerase II inhibitor (Payne and Miles, 2008; Page and Takimoto, 2010; Khazir *et al*., 2014). Other plant derived anticancer agents have been discovered, for example, Betulinic acid isolated from *Mauritiana rugosa* and *Mauritiana oenoplia* displays an assortment of biological activities while Silvestrol from *Aglaia foveolata*, appears to trigger the mitochondrial pathway

of apoptosis and Omacetaxine mepesuccinate, which was isolated from *Cephalotaxus harringtonia*, is being used for the treatment of chronic myelogenous leukemia (CML) (Khazir *et al*., 2014). It is unfortunate, however, that each of these natural products come with some degree of toxicity (Page and Takimoto, 2010; Khazir *et al*., 2014). The vinca alkaloids cause nausea, vomiting and bone marrow depression (Page and Takimoto, 2010). Paclitaxel causes peripheral neuropathy, mucositis and has been implicated in increasing the pro-thrombotic risk in cancer patients (Page and Takimoto, 2010; Zhang, *et al*, 2016; Kim *et al.*, 2018). Camptothecin's low aqueous solubility and severe toxicity prompted its discontinuation from clinical trials (Khazir *et al*., 2014). Fortunately, these "parent" compounds can be structurally manipulated chemically to develop new compounds, "semi synthetic analogues", that improve efficacy and solubility, reduce toxicity and/or lessen the severe side effects (Khazir *et al*., 2014). While plant-derived compounds may not always serve as drugs directly, they do provide clues for the production of potential anticancer drugs (Khazir *et al*., 2014).

Only a small portion of the diversity of plants on earth has been explored and therefore it is accepted that plants have the potential to provide potent bioactive compounds for the creation of new drugs against various diseases that are far more potent than conventional synthetic compounds (Khazir *et al*., 2014).

Although African traditional medicine is still poorly recorded, the rapid loss of many natural habitats due to human activity demands that the medicinal use of African plants be documented and scientifically researched (Sawadogo *et al*., 2012). *Kigelia africana (K. africana), Mimusops zeyheri (M. zeyheri)* and *Ximenia caffra (X. caffra)* are examples of such plants which have been used by traditional healers and because they are widespread in sub-Saharan Africa (Chivandi, 2012a), are of noteworthy interest to this study. Additionally, the fact that the seed oils from these plants have been shown to inhibit the proliferation of human colon adenocarcinoma (Caco-2) and human embryonic kidney (HEK-293) cells, further exploration of their potential medicinal (anti-cancer) benefits is essential (Chivandi *et al*., 2012b).

1.2.1. *Kigelia africana*

K. africana, also known as the Cucumber or Sausage tree because of its huge wooden berries (Figure 1.3a), is found largely in the wetter regions of sub-Saharan Africa specifically South, Central and West Africa (Owolabi and Omogbai, 2007; Saini *et al*., 2009; Chivandi *et al*., 2011b). The tree blossoms in spring (Saini *et al*., 2009). Its mature seeds are greyish-brown in

colour (Figure 1.3b) (Chivandi *et al*., 2011b) and flaky white, light yellow when deshelled (Figure 1.3c). Different parts of the *K. africana* have therapeutic properties such as being analgesic, anti-inflammatory (Owolabi and Omogbai, 2007) and anti-neoplastic (Naidoo *et al*., 2013). In Eastern Zimbabwe and Mozambique, a tea is brewed from the powdered stem bark or root bark of *K. africana* and used to treat stomach ailments (Owolabi and Omogbai, 2007). In West Africa, *K. africana* leaf decoctions are used for backache (Saini *et al*., 2009) while sexually-transmitted diseases and dysentery are treated using a macerate of stem bark (Houghton, 2002).

Figure 1.3: *K. africana* tree [Torrissen, BC (2012) A sausage tree inside the Serengeti National Park in Tanzania. [Online image] Available from https://commons.wikimedia.org/wiki/File:Kigelia-Africana-Serengeti.JPG Downloaded 22 September 2015], b) shelled and c) unshelled *K. africana* seeds.

1.2.2. *Ximenia caffra*

The large Sourplum (Figure 1.4a), *X. caffra*, is commonly found in dry wooded bushland although it is more abundant in coastal and lowland dry woodlands (Orwa *et al*., 2009). It is a relatively small tree reaching 6m high blooming creamy white flowers sometimes tinted pink or red in spring (Orwa *et al*., 2009). One large yellowish-brown oval-shaped seed (Figure 1.4b) can be found within each ripe fruit (Janick and Paull, 2008; Orwa *et al*., 2009). It is

approximately 2.5 cm long and 1 cm wide and once unshelled has a light yellow appearance (Figure 1.4c). The fruit pulp is rich in proteins and ascorbic acid (Roodt, 1998). The roots and stem bark of *X. caffra* are used to treat a variety of ailments including stomach aches, syphilis and hookworm (Orwa *et al*., 2009). The leaves of *X. caffra* are used to treat diarrhoea, dysentery, fever, cough and venereal diseases (Green *et al*., 2010). Zhen *et al*. (2015) found that extracts of *X. caffra* leaf demonstrate anti-proliferation and anti-inflammatory activities on murine RAW 264.7 macrophage cells, a model widely used for inflammation studies.

Figure 1.4: *X. caffra* tree [Dupont, B (2013) Sourplum. [Online image] Available from https://commons.wikimedia.org/wiki/File:Sourplum_(Ximenia_caffra)_(11905023854).jpg Downloaded 22 September 2015], b) shelled and c) unshelled *X. caffra* seeds.

1.2.3. *Mimusops zeyheri*

M. zeyheri (Figure 1.5a), family Sapotaceae, commonly known as Red Milkwood is widely distributed from Tanzania down to KwaZulu-Natal, South Africa and grows on rocky hillsides close to rivers (Department of Agriculture, Forestry and Fisheries, 2012). The tree is evergreen and grows up to 15m (Mashela *et al*., 2013). The edible fruit is usually a bright yellow fleshy berry containing a single seed with a shiny brown shell that is up to 2cm long with a distinct scar (Figure 1.5b) (TreeSA, 2017). Removal of the shell reveals a light yellow seed (Figure

1.5c). The fruit has a high ascorbic acid content (Janick and Paull, 2008). In Swaziland, candidiasis is treated using the roots which have been ground whilst the bark is used to treat ulcers and wounds (Amusan *et al*., 2002). *Mimusops elengi* Linn also from the family Sapotaceae has been shown to have anti-oxidant and anticancer effects (Baliga *et al*., 2001, Ganesh *et al*., 2013).

Figure 1.5: *M. zeyheri* tree [JMK (2013) Mimusops zeyheri. [Online image] Available from https://commons.wikimedia.org/wiki/File:Mimusops_zeyheri,_habitus,_Little_Eden,_b.jpg) Downloaded 22 September 2015], b) shelled and c) unshelled *M. zeyheri* seeds.

1.2.4. Seeds

Because most traditional medicines are based on leaf, root or stem bark extracts, research has mainly looked at their medicinal properties and pharmacological effects (Chivandi *et al*., 2012a). The seeds, however, contain many essential nutrients, including amino acids, fatty acids, minerals and vitamins (Chivandi *et al*., 2011a). *K. africana* seeds have been shown to contain minerals such as calcium, magnesium and phosphorus as well as vitamin E and essential amino acids such as arginine, histidine, isoleucine, leucine, lysine and valine (Chivandi *et al*., 2011b). The most abundant mineral and amino acid in the Sourplum (*X. caffra*) seed is phosphorus and glutamic acid, respectively and contains vitamin E (Chivandi *et al*., 2012c).

Glutamic acid was the most abundant amino acid while lysine and phenylalanine were also present in *M. zeyheri* seeds. Calcium was the most concentrated mineral found in *M. zeyheri* seeds (Chivandi *et al*., 2011a). The fatty acid profiles from each seed type differed (Chivandi *et al*., 2011a; Chivandi *et al*., 2011b; Chivandi *et al*., 2012c). Omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids (PUFAs) are contained in varying proportions in seed oils obtained from different plants (Huerta-Yépez *et al.*, 2016). In addition to their roles in cell membrane structure and function, omega-3 and omega-6 PUFAs are involved in cell signalling, gene expression regulation, and lipid mediator synthesis (Huerta-Yépez *et al.*, 2016). *De novo* synthesis of these PUFAs is impossible and must be obtained through one's diet (Berquin *et al.*, 2008).

Seed oils have been shown to possess anti-inflammatory, anti-oxidative, antimicrobial and anti-tumour properties. Constantini *et al*. (2014) found that breast cancer cells' viability and pro-inflammatory cytokines decreased substantially during treatment with pomegranate (*Punica granatum* L. which is native to Southeast Europe, Western Asia and the West Himalayas) seed oil while Hora *et al*. (2003) found pomegranate seed oil to be a chemopreventive drug for skin cancer that is safe and effective. Yamasaki *et al*. (2013) found that the oil extracted from *Jacaranda mimosifolia*, native to South America, caused leukaemia HL-60 cells to undergo oxidative stress *in vitro* which promoted cell death. *Portulaca oleracea* and *Pterodon pubescens* Benth. seed oil both showed anti-proliferative effects *in vitro* against human liver cancer (HepG2), human lung cancer (A-549) and human melanoma (SK MEL 37) cell lines respectively (Vieira *et al*., 2008; Al-Sheddi *et al*., 2015). *Moringa oleifera* (although native to India, Pakistan and Nepal, is found in South Africa) seed oil was shown to have anti-proliferative activity on a number of cell lines although some more than others (Elsayed *et al*., 2015). Similar findings were seen by Al-Oqail *et al*. (2013) when they tested the cytotoxicity of fenugreek seed oil against four cell lines - cell viability varied amongst the different cell lines. According to Al-Oqail *et al*. (2013) there was more blebbing and vacuolation in the treated cells, indicating that autophagy may be the cause of death. Unfortunately, most of the articles mentioned above have only looked at the cytotoxicity of the various oils and not the mechanisms by which these oils decrease cell viability or induce cell death. An important component of developing a strategy to treat human cancer is understanding the mechanisms involved in cell death induced by anti-proliferative drugs (La Porta, 2004).

1.3. MECHANISMS OF CELL DEATH

In addition to being vital to development and homeostasis, cell death also underlies several pathological conditions (Fiers *et al*., 1999). It is now clear that cell death may result from the activation of several signalling pathways and cellular mechanisms that may either be apoptotic or non-apoptotic (Fiers *et al*., 1999; de Bruin *et al*., 2008).

1.3.1. Apoptosis

Apoptosis, a form of programmed cell death (PCD), is regulated by a variety of molecular pathways (Edinger and Thompson, 2004; de Bruin and Medema, 2008). It occurs under different physiological and pathological circumstances that is distinctive in both its morphology and biochemistry (Kaufmann and Hengartner, 2001). During apoptosis, cells shrink, DNA is digested into 200 base pairs (bp) fragments, nuclear chromatin is condensed and apoptotic bodies are formed without breakdown of the plasma membrane (Hanahan and Weinberg, 2000; Saraste *et al*., 2000; Kaufmann and Hengartner, 2001; Edinger and Thompson, 2004; Ghavami *et al*., 2009; Loos and Engelbrecht, 2009). Phagocytic cells then detect and remove the apoptotic bodies (Hanahan and Weinberg, 2000; Saraste *et al*., 2000; Edinger and Thompson, 2004). This entire process occurs without signs of inflammation (Saraste *et al*., 2000; Edinger and Thompson, 2004; de Bruin and Medema, 2008).

Apoptosis is achieved following the activation of complex signalling pathways that are divided into an extrinsic, an intrinsic and a common executioner pathway (de Bruin *et al*., 2008; Ghavami *et al*., 2009). The extrinsic pathway, also known as the receptor triggered apoptotic pathway, was the first apoptotic pathway to be characterised (Ghavami *et al*., 2009). Extracellular ligands such as fibroblast associated antigen (Fas) ligand, TNF-α or TNF-related apoptosis inducing ligand (TRAIL) bind to their receptors located on the cell membrane and the intracellular death domains of these receptors are activated (Leist and Jäättelä, 2001; de Bruin and Medema, 2008). Adaptor proteins and initiator caspases-8 and -10 are recruited to initiate apoptosis directly by proteolytically activating downstream executioner caspases (caspase-3, -6 and -7) that break down cellular targets. Alternatively it can cleave the cytoplasmic protein BH3-interacting domain death agonist (Bid) activating the mitochondrial pathway (Green and Reed, 1998; Fiers *et al*., 1999; Kaufmann and Hengartner, 2001; de Bruin and Medema, 2008; Ghavami *et al*., 2009; McIlwain *et al*., 2013).

The intrinsic pathway, also known as the mitochondrial dependent pathway, is triggered through various cellular stresses (growth factor withdrawal, oxidative stress, DNA damage, or oncogene activation) resulting in mitochondrial membrane permeabilization (de Bruin and Medema, 2008; Ghavami *et al*., 2009; McIlwain *et al*., 2013). The intrinsic pathway, unlike the extrinsic pathway, is mediated by the insertion of Bcl-2-associated X protein (Bax)/ Bcl-2 homologous antagonist killer (Bak) into the mitochondrial membrane followed by the release of cytochrome c from its storage location into the cytosol (Kaufmann and Hengartner, 2001; Ghavami *et al*., 2009). The subsequent release of cytochrome c and other pro-apoptotic proteins [apoptosis-inducing factor (AIF), endonuclease G and second mitochondrial activator of caspases (SMAC)] disseminates the apoptotic signal leading to the formation of the apoptosome which is composed of cytochrome c, the apoptotic protease activating factor 1 (Apaf-1) and initiator caspase-9 (Green and Reed, 1998; Kaufmann and Hengartner, 2001; de Bruin and Medema, 2008). Activated caspase-9 triggers caspase-3 which results in a proteolytic cascade (Green and Reed, 1998; Kaufmann and Hengartner, 2001; de Bruin and Medema, 2008; Ghavami *et al*., 2009). This pathway is firmly controlled by proteins of the B-cell lymphoma-2 (Bcl-2) family which is comprised of (1) anti-apoptotic proteins (Bcl-2, Bcl-X_L), (2) pro-apoptotic pore-formers (Bax, Bak) and (3) pro-apoptotic Bcl-2 homology 3 (BH3)-only proteins [Bid, Bcl-2 interacting killer (Bik)] (Leist and Jäättelä, 2001; de Bruin and Medema, 2008).

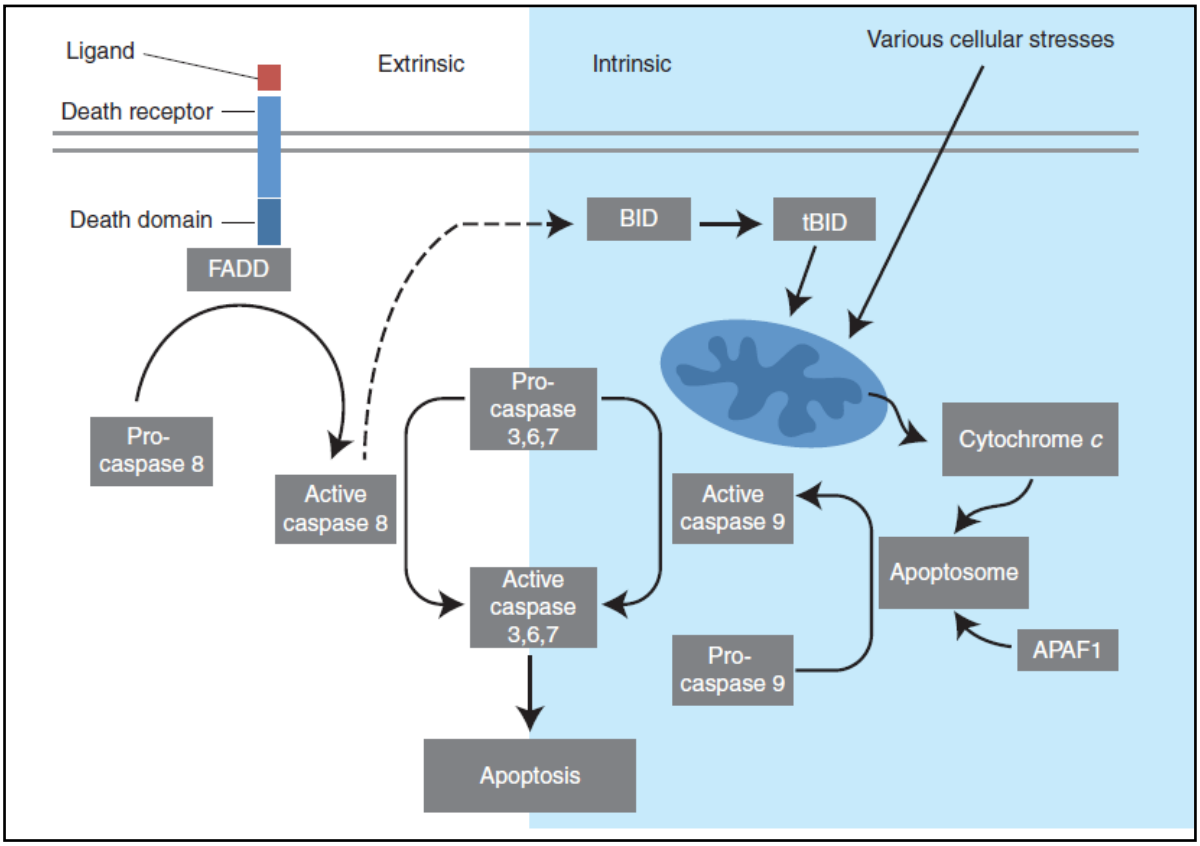

Figure 1.6: The extrinsic and intrinsic pathways of apoptosis. Reprinted with permission from "Caspase Functions in Cell Death and Disease", by D. R. McIlwain, T. Berger and T. W. Mak, 2013, Cold Spring Harb Perspect Biol, 5, p3. Copyright 2013 by Cold Spring Harbor Laboratory Press.

Another apoptotic pathway is the granzyme B pathway (Kaufmann and Hengartner, 2001; Ghavami *et al*., 2009). The granules of cytotoxic T cells contain granzyme B, an aspartate-directed protease that cleaves Bid, thereby activating the mitochondrial pathway (Kaufmann and Hengartner, 2001; Ghavami *et al*., 2009). Through these apoptotic pathways, caspase-3, -6 and -7 (members of the executioner pathway) lead to the demise of cells (de Bruin and Medema, 2008).

There are several mechanisms by which cancer cells become resistant to apoptosis (Hanahan and Weinberg, 2000). The most frequently mutated gene in human cancers is the p53 tumour suppressor gene which regulates protein expression and is involved in cell cycle arrest, DNA repair, apoptosis, and senescence (Hanahan and Weinberg, 2000; Gerl *et al*., 2005; de Bruin and Medema, 2008; Parrales and Iwakuma, 2016). Mutated p53 is unable to activate pro-apoptotic genes (Hanahan and Weinberg, 2000; Gerl *et al*., 2005). Cancer cells can also be found to exhibit other anti-apoptotic modifications, such as loss of functional pro-apoptotic Bax and Bak, or high expression levels of anti-apoptotic proteins (de Bruin and Medema, 2008).

Resistance to apoptosis may also be related to changes in the death receptor pathways and contribute to cancer therapy resistance (de Bruin and Medema, 2008; Ghavami *et al*., 2009).

Although cancer cells exhibit severe defects in classic apoptosis pathways as discussed above, they are still able to undergo cell death (Leist and Jäättelä, 2001). Following inhibition of caspase activity by a variety of factors [pharmacological inhibitors, energy depletion, mutations, oxidative stress, members of the inhibitor of apoptosis protein (IAP) family or a group of viral proteins that can suppress caspases], it was discovered that there are backup death pathways (Green and Reed, 1998; Leist and Jäättelä, 2001; Edinger and Thompson, 2004) that can be manipulated to achieve cell death.

1.3.2. Necrosis

Necrosis is a degenerative process that occurs as a result of physical and/or chemical stress (de Bruin and Medema, 2008; Loos and Engelbrecht, 2009). During necrosis, the cytoplasm vacuolates and the plasma membrane ruptures releasing intracellular contents and pro-inflammatory molecules into the surrounding milieu provoking an inflammatory response (Fiers *et al*., 1999; Edinger and Thompson, 2004; de Bruin and Medema, 2008; Ghavami *et al*., 2009; Loos and Engelbrecht, 2009). The protein kinase, receptor-interacting protein 1 (RIP1), is involved in death receptor-induced necrosis (Leist and Jäättelä, 2001; de Bruin and Medema, 2008). By targeting the mitochondria, RIP1 induces excessive production of reactive oxygen species (ROS), which are believed to be potent triggers of necrosis since ROS scavengers prevent necrosis induction by various treatments (Leist and Jäättelä, 2001; de Bruin and Medema, 2008).

Additionally, DNA damage can cause necrosis mediated by poly[adenosine diphosphate (ADP)-ribose] polymerase-1 (PARP-1) which depletes nuclear and cytoplasmic nicotinamide adenine dinucleotide (NAD) and inhibits glycolysis as a result (Edinger and Thompson, 2004; de Bruin and Medema, 2008). Consequently, glycolysis-dependent cells rapidly run out of adenosine triphosphate (ATP) and die by necrosis (Edinger and Thompson, 2004). Rapidly proliferating cancer cells require glucose metabolism to produce ATP because they use amino acids and lipids to synthesize proteins and membranes, respectively. In contrast ATP levels of resting non-cancerous cells can also be sustained by protein and lipid catabolism (Edinger and Thompson, 2004). Activation of PARP in resting non-cancerous cells is able to repair DNA damage without causing lethal ATP depletion (Edinger and Thompson, 2004). RIP1 and the

molecules TNF-receptor-associated factor 2 (TRAF2) and cJun N-terminal kinase 1 (JNK1) are required for PARP-1-mediated necrosis (de Bruin and Medema, 2008).

1.3.3. Autophagy

Autophagy has traditionally been considered a distinct type of non-apoptotic death different from necrosis (Edinger and Thompson, 2004). Autophagy can either serve as a degradative system eliminating damaged organelles and long-lived proteins or as an intracellular catabolic system in which proteins and organelles are degraded to generate energy during starvation and the resultant amino acids used to build new proteins (Edinger and Thompson, 2004; Mizushima, 2007; de Bruin and Medema, 2008; Loos and Engelbrecht, 2009). The recycling of carbohydrates and lipids by or during autophagy is not known (Mizushima, 2007). Autophagy can be induced by several stresses including depletion of essential amino acids, endoplasmic reticulum (ER) stress and hypoxia (Gozuacik and Kimchi, 2004; Mizushima, 2007).

Chaperone-mediated autophagy (CMA) involves selective proteins which are transported across the lysosomal membrane with the assistance of chaperone proteins such as heat shock cognate 70 (Hsc-70) (Mizushima, 2007; Loos and Engelbrecht, 2009; Glick *et al*., 2010). These proteins are subsequently identified by the lysosomal-associated membrane protein 2A (LAMP-2A) receptor where they are then unfolded and degraded (Glick *et al*., 2010). Micro-autophagy entails the invagination of the lysosomal membrane to capture and engulf cytosolic components directly by the lysosome (Loos and Engelbrecht, 2009; Glick *et al*., 2010). Macro-autophagy provides the mechanism for delivery of cytoplasm to lysosomes, and is generally referred to as autophagy (Mizushima, 2007; Loos and Engelbrecht, 2009). During autophagy, double membrane vesicles which contain cytosolic organelles (autophagosomes) are formed and then these autophagosomes merge with lysosomes (Gozuacik and Kimchi, 2004; Edinger and Thompson, 2004; Mizushima, 2007; de Bruin and Medema, 2008; Glick *et al*., 2010). The contents are subsequently degraded by lysosomal acid proteases and recycled for building macromolecules and for metabolism (Edinger and Thompson, 2004; Mizushima, 2007; Glick *et al*., 2010).

Modification of the mammalian target of rapamycin (mTOR) signalling pathway, caused by depletion of essential nutrients, triggers autophagy (de Bruin and Medema, 2008). Under growth factor (GF) conditions, autophagy is repressed and as such autophagy has often been thought of as an adaptive survival mechanism. However it has also been established that

autophagy can lead to cell death once it passes a certain threshold (Gozuacik and Kimchi, 2004; de Bruin and Medema, 2008; Loos and Engelbrecht, 2009). A group of proteins related to autophagy has been identified in humans (Atg) and contribute to the formation of autophagosomes through two conjugation systems (de Bruin and Medema, 2008; Glick *et al*., 2010). Beclin 1 is involved in the formation of autophagic vesicles during the early stages of autophagy (Edinger and Thompson, 2004). Atg12 is attached to the membrane while Atg8/LC3 is linked to phosphatidylethanolamine (PE) which is incorporated in the membrane (Mizushima, 2007; de Bruin and Medema, 2008; Glick *et al*., 2010). Proteins such as these have become hallmarks of autophagy.

The autophagy process is so important for the removal of cytoplasmic contents that defects can cause a variety of cellular malfunctions - neurodegeneration and tumorigenesis (Mizushima, 2007). In human neurodegenerative disorders such as Alzheimer's and Parkinson's disease, the accumulation of autophagic vacuoles has been observed. Findings such as these have led to the opinion that autophagy may not only be an adaptive response to nutrient deficient conditions, but may also be involved in the degeneration of cells resulting in pathological conditions (Edinger and Thompson, 2004; Mizushima, 2007).

In the early stages of tumorigenesis, autophagy may play a tumour suppressor role by inducing cell cycle arrest and preventing genome instability, but it may also protect tumour cells against damage caused by stressful conditions during the later stages of tumour growth (de Bruin and Medema, 2008; Glick *et al*., 2010). As tumour cells grow beyond their blood supply, they are exposed to nutrient-limiting or ischaemic conditions which enables them to activate autophagy which allows them to survive until angiogenesis is established (Edinger and Thompson, 2004; Mizushima, 2007; de Bruin and Medema, 2008). Normally such metabolic stress would induce apoptosis but apoptosis-defective cells are able to withstand such conditions (Mizushima, 2007). Ultimately, autophagy may play a role in cancer depending on the type of cell and/or stage of the disease (Glick *et al*., 2010). Regardless of some evidence between autophagy and tumorigenesis, the lack of markers that can detect autophagy *in vivo* limits research of human tumour tissue (de Bruin and Medema, 2008).

Numerous studies have indicated that, in some settings, apoptosis and autophagy may be interconnected and even activated simultaneously by the same trigger, resulting in different outcomes (Gozuacik and Kimchi, 2004). The interaction between apoptosis and autophagy shows itself in different ways or may also manifest in a mutually exclusive manner. As a

consequence of the context and stimulus within which autophagy occurs, autophagy can facilitate apoptosis by initiating and maintaining it (Gozuacik and Kimchi, 2004). In some situations, autophagy delays apoptosis by eliminating damaged mitochondria and in others, autophagy and apoptosis may act as backup mechanisms supporting each other to accomplish irreversible cell death (Gozuacik and Kimchi, 2004). Inhibition of autophagy in cells may switch death signals from autophagic cell death to apoptotic cell death. Death-associated protein kinases (DAPk) are able to trigger both apoptosis and autophagy depending on cellular circumstances suggesting that they may function as molecular switches or integrators of both types of cell death (Gozuacik and Kimchi, 2004). Mitochondria play a key role in the integration of apoptosis and autophagy (Gozuacik and Kimchi, 2004). Interestingly, cells whose apoptosis and autophagy pathways are inhibited, have a reduced chance of surviving (Mizushima, 2007).

Some pathways are specific to certain cell-types, but both dormant/inactive and activated pathways can be present in the same cell but be activated at different times in response to different stimuli (Fiers *et al*., 1999). Cell death is a much more complex phenomenon having a greater dynamic and molecular overlap than previously believed and that by combining stimuli that activate different death-inducing proteases *in vitro* increases tumour cell death, suggesting that therapies based on multiple programmed cell death pathways simultaneously, could also be effective in the clinic (Leist and Jäättelä, 2001; Loos and Engelbrecht, 2009).

Previously in our laboratory, the seed oils of *K. africana*, *X. caffra* and *M. zeyheri* demonstrated anti-proliferative effects on human colon adenocarcinoma (Caco-2) and human embryonic kidney (HEK-293) cells *in vitro*. Consequently, we decided to determine whether the effects of these seed oils occur on hormone-independent (MDA-MB-231) and hormone-dependent (MCF-7) breast cancer cells. If affirmative, we then wished to establish the mechanisms by which these seed oils suppress cell proliferation. Since cancer patients are at a higher risk for thrombosis when receiving treatment, a preliminary investigation was also conducted to determine if *K. africana*, *X. caffra* and *M. zeyheri* seed oils are able to reduce the thrombogenic ability of breast cancer cells.

1.4. AIM OF STUDY

Thus the aim of this study was to determine if *K. africana*, *M. zeyheri* and *X. caffra* seed oils have anti-tumour effects on hormone-independent (MDA-MB-231) and hormone-dependent (MCF-7) breast cancer cells.

The specific objectives of this study were to:

1) extract and characterise the oils from the seeds of *K. africana, X. caffra* and *M. zeyheri*;
2) assess cell viability and cytotoxicity using different concentrations and incubation times of *K. africana*, *X. caffra* and *M. zeyheri* seed oils on breast cancer cells (MDA-MB-231 and MCF-7);
3) determine the mechanisms through which *K. africana*, *X. caffra* and *M. zeyheri* seed oils bring about cell death in breast cancer cells by examining 1) apoptosis and 2) autophagy;
4) to investigate the effects of *K. africana, X. caffra* and *M. zeyheri* seed oils on platelet activation following the co-incubation of breast cancer cells, seed oils and human whole blood and analyzing the expression of the platelet activation markers, CD62P and CD63.

The null hypothesis of this study was therefore that *K. africana, M. zeyheri* and *X. caffra* seed oils do not have anti-tumour or anti-thrombogenic effects on hormone-independent (MDA-MB-231) and hormone-dependent (MCF-7) breast cancer cells.

CHAPTER 2

Differential response of breast cancer cell lines to *Kigelia Africana, Ximenia caffra* and *Mimusops zeyheri* seed oils

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Differential response of breast cancer cell lines to *Kigelia Africana, Ximenia caffra* and *Mimusops zeyheri* seed oils

2.0. ABSTRACT

Breast cancer is the most common cancer among women worldwide. The survival rate of breast cancer is higher in first world countries than in developing countries, which still rely on ethnomedicine. The seed oils of *Kigelia africana*, *Mimusops zeyheri* and *Ximenia caffra* previously demonstrated anti-proliferative effects on Caco-2 and HEK-293 cells *in vitro*. We determined the effects of these seed oils on hormone-dependent (MCF-7) and hormone-independent (MDA-MB-231) breast cancer cells Different seed oil concentrations and different incubation times were evaluated by making use of the neutral red cell viability assay as well as the lactate dehydrogenase cytotoxicity assay. Analysis of the seed oils showed that *Kigelia africana* seed oil had the highest concentration of α-linolenic acid (an essential omega-3 fatty acid). *Mimusops zeyheri* had the highest concentration of linoleic acid (an essential omega-6 fatty acid) followed by *Kigelia africana* and *Ximenia caffra*. While the seed oils concentrations had no significant cytotoxicity or effect on MDA-MB-231 and MCF-7 viability; oil type and incubation time showed significant effects. *K. africana* and *X. caffra* seed oils induced the greatest cytotoxic effects on hormone-independent MDA-MB-231 breast cancer cells, while *X. caffra* and *M. zeyheri* seed oils induced a growth inhibitory effect on hormone-dependent MCF-7 breast cancer cells.

Key words: breast cancer, ethnomedicine, *Kigelia africana*, *Mimusops zeyheri*, *Ximenia caffra*, seed oils

2.1. INTRODUCTION

Breast cancer is the most common cancer amongst women globally (Monga *et al*., 2013). Of the 1 million women diagnosed with breast cancer per year worldwide, 140 000 die (Coughlin and Ekwueme, 2009; Youlden *et al*., 2012). Breast cancer is a heterogeneous disease (Gazinska *et al*., 2013) and consequently different treatment strategies are required (Yersal and Barutca, 2014). The survival rate for breast cancer is almost 90% in the US compared to 50% in developing African countries, e.g. Gambia, Uganda and Algeria (Kuete *et al*., 2013). The comparatively lower survival rate in the developing African countries is ascribed to the lack of essential infrastructure and resources to facilitate routine screening mammography. This lack of infrastructure results in breast cancers being diagnosed at late stages of the disease. Due to the heterogeneity of breast cancer, high cost and reduced availability of treatment options, women in such emerging countries receive inadequate treatment, pain relief or palliative care (Coughlin and Ekwueme, 2009). They, therefore, to a large extent, depend on ethnomedicine to treat and/or manage breast cancer (Ochwang'i *et al*., 2014). For many centuries, ethnomedicine has been used for prophylaxis and in the treatment of various ailments including cancer and as a result all phytochemical extracts should be scientifically corroborated for their alleged usefulness, safety and toxicity (Ochwang'i *et al*., 2014). The vinca alkaloids, Vinblastine and Vincristine, isolated from the Madagascar periwinkle plants (*Vinca rosea*) (Moudi *et al*., 2013), are used largely to treat Hodgkin's lymphoma and leukaemia (Khazir *et al*., 2014) by blocking the polymerization of tubulin molecules resulting in metaphase arrest and apoptosis (Voss *et al*., 2006). Paclitaxel, a standard chemotherapy agent impeding mitotic progression of ovarian and breast cancer (Priyadarshini and Keerthi-Aparajitha, 2012; Khazir *et al*., 2014), was initially extracted from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. Camptothecin, from the Chinese tree *Camptotheca acuminata* Decne, is used to treat stomach, rectal, colon and liver cancer (Lee, 2004).

Kigelia africana (K. africana), *Mimusops zeyheri (M. zeyheri)* and *Ximenia caffra (X. caffra)*, which are widely distributed in the sub-Saharan Africa region, are used by traditional healers for the treatment of a number of ailments (Chivandi *et al*., 2012a). The fruit and stem bark extracts of *K. africana* (also known as the Cucumber or Sausage tree because of its huge wooden berries) are used for treatment of melanoma and other skin neoplasms (Houghton, 2002; Saini *et al*., 2009; Bello *et al*, 2016) and in the management of endomentrial cancer (Ashidi *et al*., 2010). Although *M. zeyheri* (commonly known as Red Milkwood; family Sapotaceae), is used in Swaziland to treat candidiasis, ulcers and wounds (Amusan *et al*., 2002), a dearth of

information exists regarding its possible anticancer properties. The leaves of *X. caffra* (also known as the large Sourplum) are used to treat diarrhoea, dysentery, fever, cough and venereal diseases (Green *et al*., 2010). Extracts of the *X. caffra* leaf were also shown to have anti-proliferative and anti-inflammatory activities on murine RAW 264.7 macrophage cells (Zhen *et al*., 2015), a model widely used for inflammation studies.

Besides the beneficial properties of leaf extracts and/or macerations, seeds from indigenous fruit-bearing trees contain important amino acids, fatty acids and vitamins (Chivandi *et al*., 2012a) and are implicated in anti-inflammatory (Constantini *et al*., 2014) and anti-proliferative activities (Vieira *et al*., 2008; Seal *et al*., 2012; Al-Sheddi *et al*., 2015; Guo *et al*., 2016) that may influence tumour progression. In *vitro* investigations of a number of seed oils have highlighted their ability to affect pro-tumorigenic processes. Pomegranate (*Punica granatum* L.) seed oil reduces the production of the pro-angiogenic compound vascular endothelial growth factor (VEGF), and pro-inflammatory cytokines (IL-2, IL-6 and TNF-α) by breast cancer cells (Constantini *et al*., 2014). *Pterodon pubescens* Benth. seed oil inhibits melanoma cell growth (Vieira *et al*., 2008), while *Litsea cubeba* and *Portulaca oleracea* seed oils are able to inhibit tumour cell progression through the cell cycle (Seal *et al*., 2012; Al-Sheddi *et al*., 2015; Guo *et al*., 2016).

Recent research has shown that *K. africana*, *M. zeyheri* and *X. caffra* seed oils suppress the proliferation of human colon adenocarcinoma (Caco-2) and human embryonic kidney (HEK-293) cells (Chivandi *et al*., 2012b); however the possible anticancer activity of these seed oils on human breast cancer cells have not been explored. We thus investigated the effects of these three seed oils on cell viability and the induction of cytotoxicity at various concentrations and incubation times on hormone-dependent (MCF-7) and hormone-independent (MDA-MB-231) breast cancer cells.

2.2. MATERIALS AND METHODS

2.2.1. Seed source

K. africana seeds were obtained from the ripe fruits from trees in Gutu District, Chitsa Communal area, Zimbabwe which is located at latitude and longitude of 19º41′S and 31º09′E respectively. It is characterized by granitic soils and has annual rainfall and temperature ranges of 650-800 mm and 20.5-30.0ºC, respectively (Chivandi, 2012a). *M. zeyheri* seeds were

harvested from fruits off trees found in the Gokwe District, in North West Zimbabwe, in the Machakata Community Forest area of the Mapfungautsi Plateau which is located at latitude and longitude of 20º25′S and 28º29′E respectively. It is characterized by shallow sandy, lithosol soil and has an annual rainfall of +/- 609 mm and temperature of +/- 25.9ºC (Chivandi, 2012a). *X. caffra* seeds were collected from ripe fruit of the Zhombe District, Zimbabwe which is located at latitude and longitude of 14º45′S and 26º50′E respectively. The area has annual rainfall of 550 mm and a mean annual temperature of 26ºC (Chivandi, 2012a). The seeds from which the seed oils were extracted, were imported into the Republic of South Africa under the permit number P0039683 following confirmation of the trees' identities by the National Botanical Gardens of Zimbabwe.

2.2.2. Seed oil extraction and storage

Seeds were dehulled and then the hulled hemp seeds were crushed in a blender (Waring; Lasec Pty Ltd, Johannesburg, South Africa). Hexane was used to extract the oil from each of the seed meals as described by Yamasaki *et al*. (2013). Briefly, 800 mL of hexane was added to 160 g of each seed meal and then mixed overnight using a magnetic stirrer (VELP Scientifica, Lombardy – Italy). The mixture was then filtered through filter paper (Whatman No.1) and using a rotary vacuum, the hexane was evaporated at 65ºC and the extracted oils were kept in brown sample bottles at -20ºC before analysis (Yamasaki *et al*., 2013).

2.2.3. Fatty acid profile determination

Fatty acid profiling of the extracted seed oils was conducted at the Agricultural Research Council's (ARC) Irene Institute of Animal Production Analytical Services Laboratories, using gas chromatography. Briefly, a methanol-sodium hydroxide solution was used to trans-methylate the seed oils. The resultant methyl esters were extracted using heptane, filtered and dried under nitrogen as described by Christopherson and Glass (Christopherson and Glass, 1969). The fatty acids were then separated using a gas chromatograph (HP680GC - Hewlett Packard, Bristol, UK).

2.2.4. Cell culture

Ethical clearance was obtained from the Human Research Ethics Committee Clearance Certificate number M160211, University of the Witwatersrand, Johannesburg. The two human breast cancer cell lines (MCF-7 and MDA-MB-231) used in the experiments were donated by

Dr. R Duarte (School of Clinical Medicine, University of the Witwatersrand) from American Type Culture Collection (ATCC) (Virginia, USA). MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 5% foetal bovine serum (FBS) and 100 µg/mL streptomycin, 100 units/mL penicillin. MDA-MB-231 cells were cultured in DMEM/Ham's F-12 nutrient mixture with 10% FBS and 100 µg/mL streptomycin, 100 units/mL penicillin. Both cell lines were incubated at 37^{o}C in a humidified atmosphere of 5% CO_2.

2.2.5. Seed oil concentrations and treatment of cells

The seed oils of *K. africana*, *X. caffra* and *M. zeyheri* were not completely soluble in aqueous medium solution, therefore stock solutions (10 mg/mL) of all the oils were prepared in 100% absolute alcohol and diluted in culture medium to reach the desired concentrations (20 µg/mL, 40 µg/mL, 80 µg/mL, 100 µg/mL and 120 µg/mL). The concentration of absolute alcohol in culture medium was not more than 5%. A diluent control consisting of 100% absolute alcohol diluted in culture medium was included.

Confluent cells were subcultured by 0.25% trypsin/edta. MCF-7 cells (P35) and MDA-MB-231 cells (P23) were plated in 24 well plates at 6.3 x 10^4 cells/well and 3.1 x 10^4 cells/well respectively in their respective media. Both cell lines were incubated at 37^{o}C in a humidified atmosphere of 5% CO_2 for 24 h. The cell culture media from each well was then removed and cells were rinsed with Dulbecco's phosphate-buffered saline (DPBS). Cells were treated using 200 µL of the various seed oil concentrations (20 µg/mL, 40 µg/mL, 80 µg/mL, 100 µg/mL and 120 µg/mL) in matched culture media and incubated at 37^{o}C in a humidified atmosphere of 5% CO_2 for 24, 48 and 72 h. In all experiments untreated and diluent treated cells were included as controls.

2.2.6. Lactate dehydrogenase assay

Cytotoxicity induced by the various seed oil concentrations was measured using the lactate dehydrogenase (LDH) cytotoxicity assay kit [ScienCell Research Laboratories, Carlsbad – California catalogue number 8078)]. After each time point the plates were centrifuged for 5 min at 400 g and 150 µL of supernatant from each well of the 24-well plate transferred to the corresponding well of a 96-well test plate. LDH assay reagent (60 µL) was added to each well and the test plate was incubated for 20 min at room temperature in darkness. The reaction was terminated using 20 µL sodium oxymate and absorbance read at 492 nm using a microtiter plate

reader (Labsystems Multiscan *Ascent* plate reader). A negative and positive control were included. The negative control consisted of cells and cell culture media. The positive control consisted of cells and 1 % triton X-100. Cytotoxicity was then calculated as follows:

$$\%\ cytotoxicity = \frac{[OD(test\ sample) - OD(negative\ control)]}{[OD(positive\ control) - OD(negative\ control)]} X\ 100$$

2.2.7. Neutral red assay

Cell viability induced by the various seed oil concentrations was determined using the neutral red (3-amino-*m*-dimethylamino-2-methyl-phenazine hydrochloride) (NR) dye. The method of Borenfreund and Puerner (1984) was used with modifications. After each time point, the media was removed and 150 µL of neutral red dye (100 µg/mL) dissolved in serum free medium (DMEM or DMEM/Ham's F-12 for MCF-7 and MDA-MB-231 cells, respectively) was added to each well. The cells were incubated for a further 3 h at 37°C in a humidified atmosphere of 5% CO_2. Cells were then washed twice with DPBS and 150 µL elution media (EtOH/AcCOOH, 50%/1%) was added followed by gentle shaking for 60 min so that complete dissolution could be achieved. The resulting solutions (150 µL) were then transferred to 96-well plates and the absorbance at 540 nm was read using a microtiter plate reader (Labsystems Multiscan *Ascent* plate reader). Blank and control samples were included. The blank sample contained cell culture media only while the control sample contained cells and cell culture media. Cell viability was then calculated as follows:

$$\%\ cell\ viability = \frac{[OD(test\ sample) - OD(blank)]}{[OD(control) - OD(blank)]} X100$$

2.2.8. Data analysis

Data are presented as mean ± standard deviation (mean±SD). The Shapiro-Wilk test was used to determine if the raw data was normally distributed. For the lactate dehydrogenase assay results, statistical analyses were carried out by one-way analysis of variance (ANOVA). For the neutral red assay results, the raw data were log transformed to a symmetric distribution and this was followed by a one-way ANOVA. The Bonferroni's post hoc test was then used to express any difference among the groups. STATA 13.1 (Statacorp, Texas – USA) statistical package was used to analyse data. Significance was set at 95%.

2.3. RESULTS

2.3.1. Lipid yield and fatty acid profiles of the seed oils

Table 2.1 highlights the lipid yield and fatty acid profile from each of the seed meals. *X. caffra* seed meal yielded the highest amount of oil (39.16%) followed by *K. africana* (34.30%) and lastly *M. zeyheri* seed meal (14.04%). *K. africana* seed oil had the highest concentration of α-linolenic acid (59.03%) followed by *X. caffra* (1.27%) and lastly *M. zeyheri* (0.10%). *M. zeyheri* had the highest concentration of linoleic acid (18.87%) followed by *K. africana* (0.14%) and *X. caffra* (0.10%).

Table 2.1: Lipid yield (%) and fatty acid composition (%) of the tree seed oils.

Parameter	***K. africana***	***X. caffra***	***M. zeyheri***
Lipid yield	34.30	39.16	14.04
Fatty acid profile			
Saturated			
C12:0 (lauric acid)	0.015	0.028	Nd
C14:0 (myristic acid)	0.025	0.029	0.063
C15:0 (pentadecanoic acid)	0.007	0.000	0.037
C16:0 (palmitic acid)	9.712	1.006	12.717
C17:0 (margaric acid)	Nd	Nd	0.143
C18:0 (stearic acid)	5.995	1.044	9.278
C20:0 (arachidic acid)	0.952	0.494	0.847
C21:0 (heneicosanoic acid)	0.031	0.059	Nd
C22:0 (behenic acid)	0.366	2.028	0.592
C23:0 (tricosanoic acid)	0.047	0.219	Nd
C24:0 (lignoceric acid)	0.000	3.665	0.225
TSFA	**17.150**	**8.572**	**23.902**
Monounsaturated			
C14:1n7 (myristoleic acid)	0.000	0.079	Nd
C16:1n7 (palmitoleic acid)	0.080	0.081	0.137
C17:1n8 (8-heptadecenoic acid)	0.033	0.030	0.020
C18:1n9 (oleic acid)	21.798	59.388	55.732
C20:1 (11-eicosenoic acid)	0.337	1.080	0.559
C22:1n9 (erucic acid)	0.013	1.556	0.032
C24:1n9 (nervonic acid)	0.000	4.926	0.087
TMUFA	**22.261**	**67.140**	**56.567**
Polyunsaturated			
C18:2n6 (linoleic acid)	0.137	0.104	18.870
C18:3n3 (α-linolenic acid)	59.033	1.275	0.100
C18:3n6 (γ-linolenic acid)	0.339	<0.000	0.009
C20:2n6 (11,14-eicosadienoic acid)	0.273	0.000	Nd
C20:3n3 (11,14,17-eicosatrienoic acid)	0.079	22.331	0.085
TPUFA	**59.861**	**23.710**	**19.064**
Cis fatty acids	21.935	59.492	74.602
Omega-3	59.112	23.606	0.185
Omega-6	0.505	0.519	19.324
Omega-9	21.838	61.021	55.764
TPUFA:TSFA	3.490:1	2.766:1	0.798:1
n3PUFA:n6PUFA	78.921:1	226.981:1	0.0098:1

TSFA: total saturated fatty acids; TMUFA: total monounsaturated fatty acids; TPUFA: total polyunsaturated fatty acids; n3PUFA: omega-3 polyunsaturated fatty acids; n6PUFA: omega-6 polyunsaturated fatty acids. nd: not detected.

2.3.2. Cytotoxicity

The lactate dehydrogenase cytotoxicity assay measures the cytosolic enzyme lactate dehydrogenase (LDH) released in the media by dying cells possessing compromised cell membranes. The released LDH oxidizes lactate to pyruvate that converts iodonitrotetrazolium (INT) present in the assay reagent to formazan which can be colorimetrically quantified at 490 nm (Weyermann *et al*., 2005).

No significant cytotoxic effect was induced by differing seed oil concentrations with each cell line (Figure 2.1). No significant cytotoxic effect was induced by the diluent control which comprised of 100% absolute alcohol diluted in culture medium (Figure 2.1).

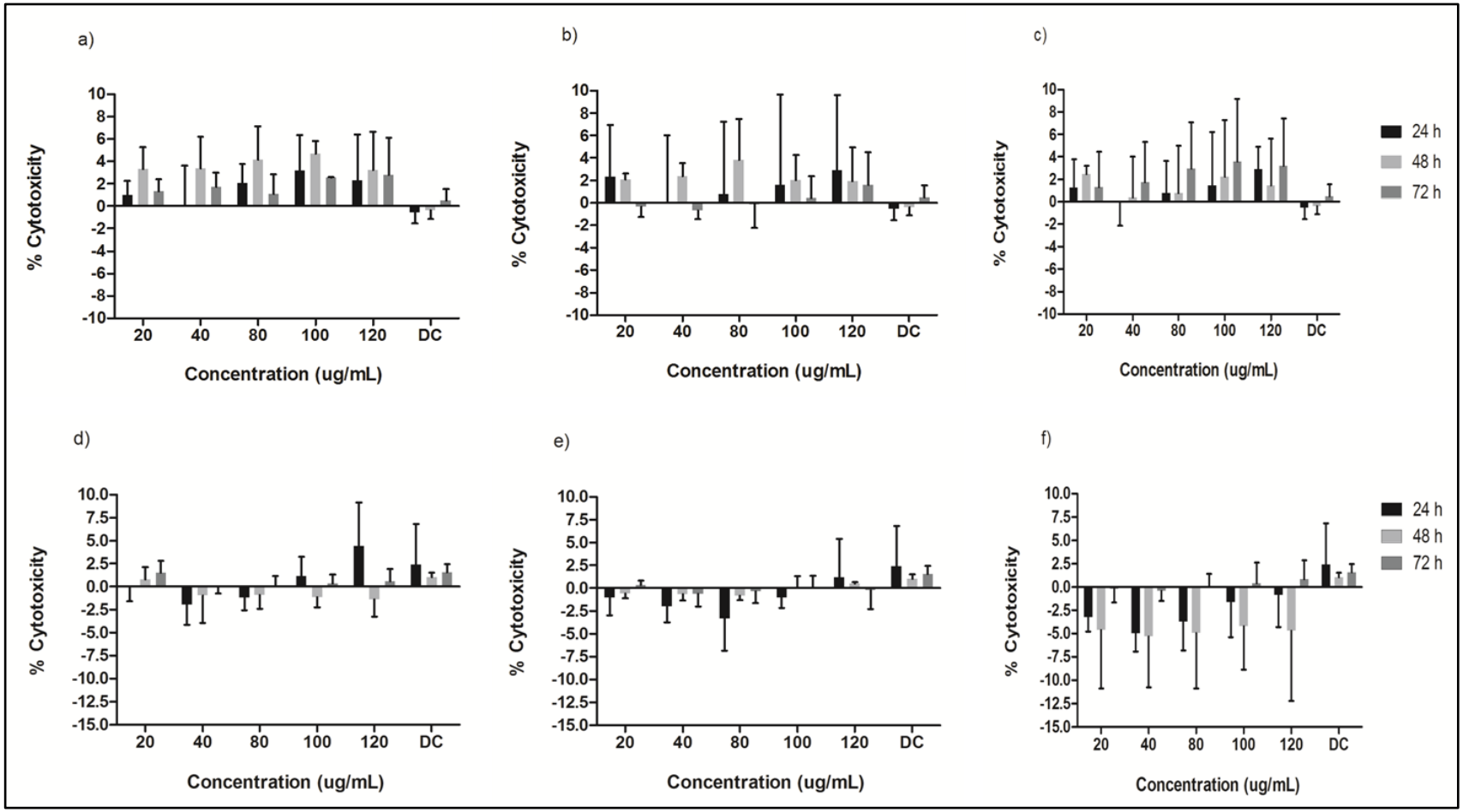

Figure 2.1: The cytotoxic effects of differing concentrations of *K. africana* (a, d), *X. caffra* (b, e) and *M. zeyheri* (c, f) seed oils and diluent control (DC) on MDA-MB-231 (a, b, c) and MCF-7 (d, e, f) breast cancer cells at 24, 48 and 72 h incubation.

However, further analysis of the results for the seed oils identified significant cytotoxic effects based on the type of seed oil and incubation time employed with each cell line (Figure 2.2).

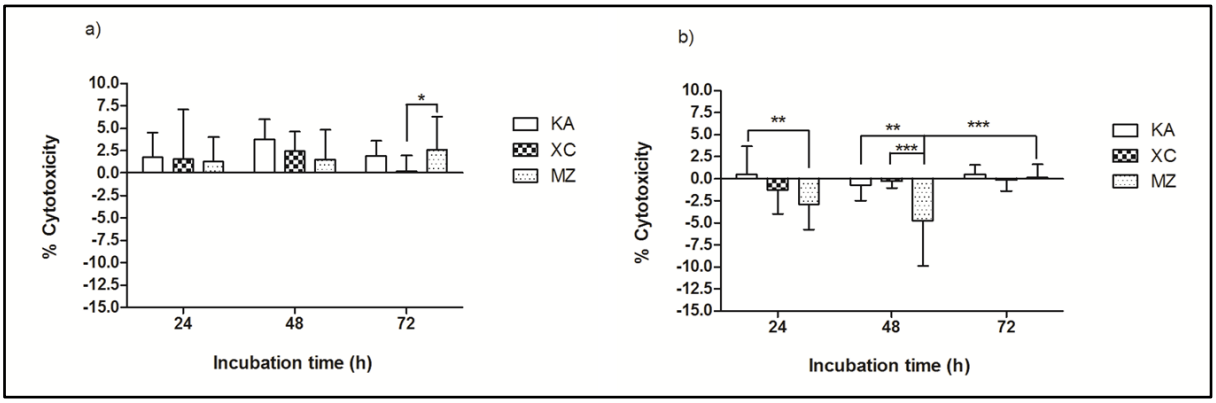

Figure 2.2: Comparison of the cytotoxicity effects of the different seed oils (KA = *K. africana*; XC = *X. caffra* and MZ = *M. zeyheri*) on (a) MDA-MB-231 and (b) MCF-7 breast cancer cells after 24, 48 and 72 h incubation. The overall cytotoxicity effect of *K. africana*, *X. caffra* and *M. zeyheri* seed oils on MDA-MB-231 breast cancer cells (a) was less than 5% whereas with MCF-7 breast cancer cells was less than 1%. The three seed oils induced a cytotoxic effect on MDA-MB-231 breast cancer cells but a negative cytotoxic effect on the MCF-7 breast cancer cells indicating cell growth inhibition. The bars represent standard deviations where * = $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.001$.

The cytotoxic effect of *K. africana*, *X. caffra* and *M. zeyheri* seed oils on MDA-MB-231 breast cancer cells (Figure 2.2a) was less than 5% with *K. africana* seed oil having the greater cytotoxic effect at 48 h incubation. A significant ($p < 0.05$) statistical difference was observed between *X. caffra* and *M. zeyheri* seed oils at 72 h incubation, where *M. zeyheri* induced a greater cytotoxic effect than *X. caffra* seed oil on MDA-MB-231 breast cancer cells (Figure 2.2a; $p \leq 0.05$). The three seed oils elicited a differential response from the MCF-7 breast cancer cells (Figure 2.2b). There was a slight cytotoxic effect (less than 1%) when MCF-7 breast cancer cells were treated with *K. africana* seed oil at 24 h and 72 h incubation. *X. caffra* and *M. zeyheri* seed oils produced negative cytotoxic effects at 24 h and 48 h incubation and no cytotoxic effects (< 0.5%) at 72 h. Significant ($p < 0.05$) statistical differences were found between *K. africana* and *M. zeyheri* seed oils at 24 h and at 48 h (Figure 2.2b, $p \leq 0.01$). *M. zeyheri* seed oil produced a greater negative cytotoxic effect than *K. africana* seed oil. There was a significant ($p < 0.05$) statistical difference between *X. caffra* and *M. zeyheri* oil at 48 h incubation on MCF-7 breast cancer cells (Figure 2.2b; $p \leq 0.001$). *M. zeyheri* seed oil produced a larger negative cytotoxic effect than *X. caffra* seed oil (-4.76% vs. -0.27%). Additionally, *M. zeyheri* seed oil effects showed a time-dependent effect (Figure 2.2b, $p \leq 0.001$), eliciting a negative cytotoxic effect at 48 h, followed by recovery of the MCF-7 breast cancer cells at 72 h (-4.76% vs. 0.13%).

Comparison between each cell line showed differential responses to the seed oils, potentially related to tumour cell phenotype (Figure 2.3).

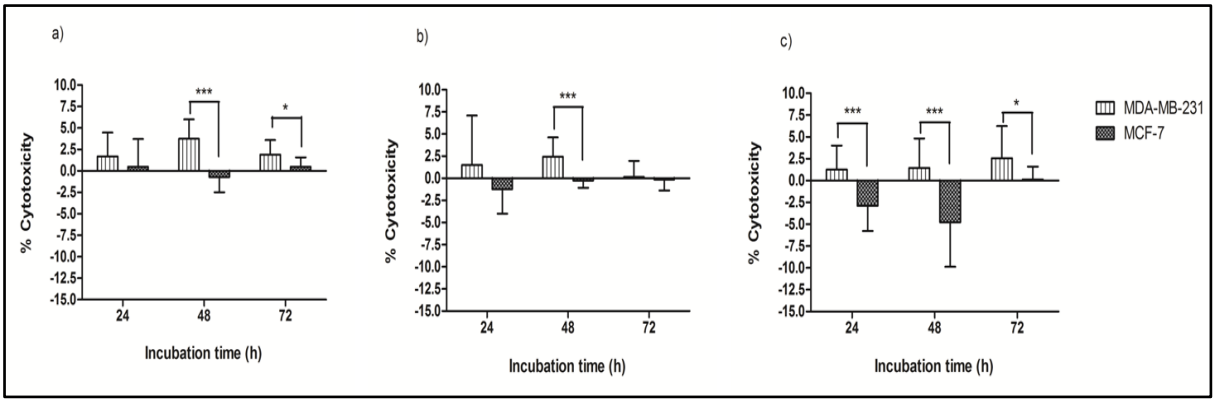

Figure 2.3: Comparison of the cytotoxicity effects between MDA-MB-231 and MCF-7 breast cancer cells after 24, 48 and 72 h incubation with (a) *K. africana*; (b) *X. caffra* and (c) *M. zeyheri* seed oils. A cytotoxic effect was observed on MDA-MB-231. A negative cytotoxic effect was observed for MCF-7 breast cancer cells at 48 h. The bars represent standard deviations where * = $p \leq 0.05$ and *** = $p \leq 0.001$.

Significant ($p < 0.05$) differences were found between the two cell lines when incubated with *K. africana* seed oil at 48 h and 72 h (Figure 2.3a; $p \leq 0.001$ and $p \leq 0.05$ respectively). *K. africana* seed oil induced a cytotoxic effect on MDA-MB-231 breast cancer cells but a negative cytotoxic result was found for the MCF-7 breast cancer cells. At 48 h, *X. caffra* seed oil induced a higher cytotoxic effect on MDA-MB-231 breast cancer cells than MCF-7 breast cancer cells (2.46% vs. -0.41%) (Figure 2.3b; $p \leq 0.001$). When the two cell lines were incubated with *M. zeyheri* seed oil, a significant ($p < 0.05$) statistical difference was found between the cell lines at 24-, 48- and 72- h incubation (Figure 2.3c; $p \leq 0.001$, $p \leq 0.001$ and $p \leq 0.05$ respectively). *M. zeyheri* seed oil induced a cytotoxic effect on MDA-MB-231 breast cancer cells at 24-, 48- and 72- h incubation but a negative cytotoxic result was found for the MCF-7 breast cancer cells at 24 h and 48 h incubation (1.28% vs. -2.89% and 1.47% vs. -4.76% respectively).

2.3.3. Cell Viability

In this colorimetric cytotoxicity assay, viable cells take up and store the neutral red dye in lysosomes, which is released in the presence of solubilisation buffer (Weyermann *et al.*, 2005). Healthy cells with intact lysosomes store more neutral red dye than dead cells or cells undergoing cell death.

Similar to the results obtained with the cytotoxicity assay (Figure 2.1) no significant effect on cell viability was induced by changes in seed oil concentrations or the diluent control, regardless of cell type (data not shown). However, further analysis identified that MCF-7 cells and MDA-MB-231 cells responded differently to a collective effect of type of seed oil and incubation time employed (Figure 2.4).

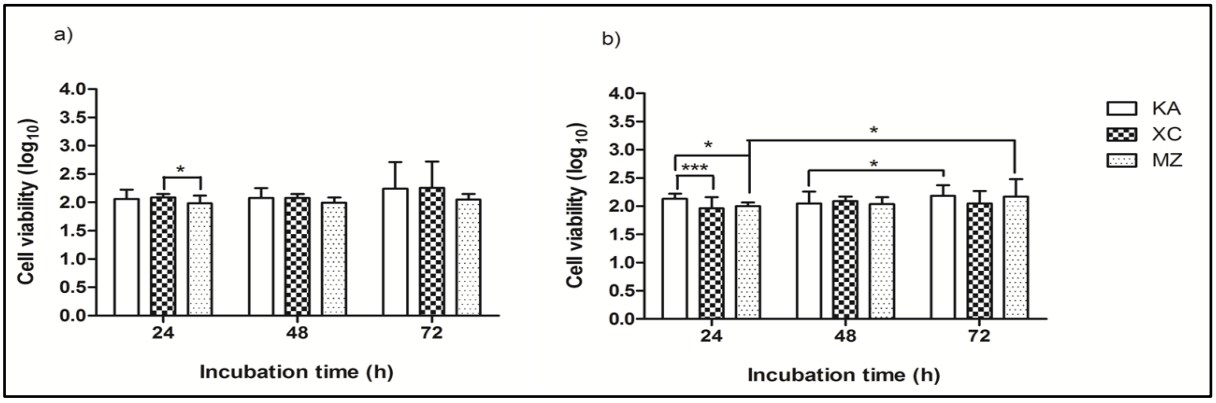

Figure 2.4: Comparison of the different seed oils (KA = *K. africana*; XC = *X. caffra* and MZ = *M. zeyheri*) on cell viability of (a) MDA-MB-231 and (b) MCF-7 breast cancer cells after 24, 48 and 72 h incubation. *M. zeyheri* seed oil induced a decrease in MDA-MB-231 cell viability (a) compared to *K. africana* and *X. caffra* seed oil at 24, 48, 72 h. Incubation times had an effect on cell viability of MCF-7 cells (b). The bars represent standard deviations where * = $p \leq 0.05$ and *** = $p \leq 0.001$.

M. zeyheri seed oil induced a decrease in cell viability at all incubation times compared to other seed oils; however, only significantly ($p \leq 0.05$) compared to *X. caffra* at 24 h incubation (Figure 2.4a). The hormone-dependent MCF-7 cell line showed a markedly different response to the seed oils and incubation periods (Figure 2.4b). At 24 h incubation, in comparison to the effect of *K. Africana* seed oil, *X. caffra* seed oil induced the most significant decrease in MCF-7 cell viability ($p \leq 0.001$), followed by *M. zeyheri* seed oil (Figure 2.4b). Additionally, the response of MCF-7 cells to the seed oils were shown to be dependent on exposure time. *M. zeyheri* seed oil treatment induced a significant ($p \leq 0.05$) increase in cell viability by 72 h. MCF-7 cell viability under *K. Africana* treatment reduced at 48 h, followed by recovery at 72 h.

No statistically significant difference was observed between the two cell lines when treated with *K africana* or *M. zeyheri* seed oil. Only *X. caffra* seed oil showed an effect, with a significantly higher level of cell viability for MDA-MB-231 than MCF-7 cells at 24 h incubation (Figure 2.5b; $p \leq 0.05$).

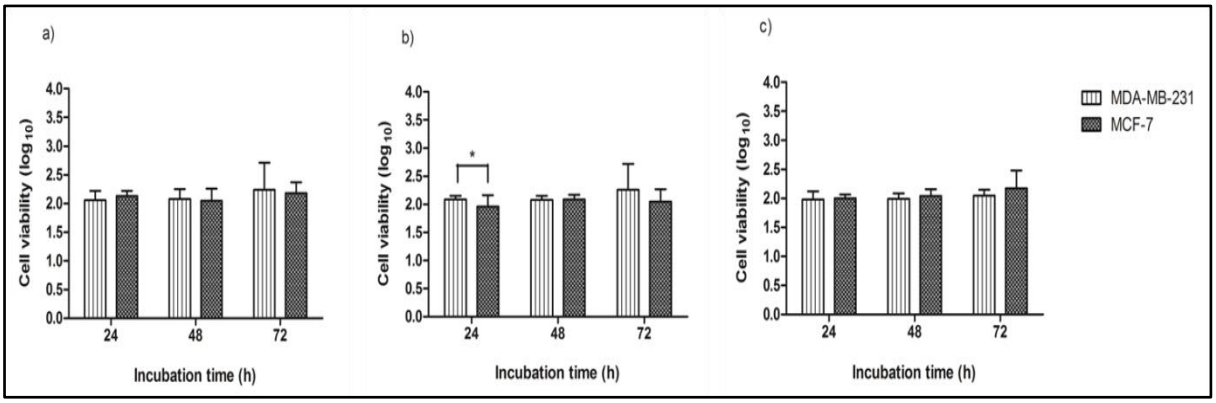

Figure 2.5: Comparison between MDA-MB-231 and MCF-7 breast cancer cells' viability after 24, 48 and 72 h incubation with (a) *K. africana*; (b) *X. caffra* and (c) *M. zeyheri* seed oils. No significant statistical difference was observed between the two cell lines when treated with *K. africana* or *M. zeyheri* seed oil. Only *X. caffra* showed an effect. The bars represent standard deviations where * = $p \leq 0.05$.

K. africana seed oil had the highest concentration of α-linolenic acid (59.03%) while *M. zeyheri* had the highest concentration of linoleic acid (18.87%). *K. africana*, *X. caffra* and *M. zeyheri* seed oils had a cytotoxic effect on hormone-independent MDA-MB-231 breast cancer cells but a growth inhibitory effect on hormone-dependent MCF-7 breast cancer cells.

2.4. DISCUSSION

With the incidence of breast cancer in developing countries on the increase, coupled with the use of ethnomedicines, determining if commonly used seed oils have any effect on breast cancer is essential. *K. africana* seed oil has previously been shown to elicit a growth inhibitory effect on Caco-2 and HEK-293 cells at a range of concentrations, whereas *M. zeyheri* and *X. caffra* seed oils exerted a similar effect only at 80 µg/mL (Chivandi *et al*., 2012b). Our results, however, indicate that matched concentrations of these seed oils have no significant impact on breast cancer cell growth inhibition, despite the highest concentration being 6 times as large as that of the lowest concentration. This is an interesting phenomenon, given that multiple studies have previously identified dose-dependent effects in cell lines (Al-Sheddi *et al*., 2015; Guo *et al*., 2016), which may indicate that the range of concentrations employed in our study were too low to elicit an effect in, specifically, breast cancer cells. This result highlights the possible induction of differential effects by seed oils in different tumour types, a postulation supported by findings that pomegranate seed oil decreases breast cancer cell viability, with no effect on colon (HT29 and HCT116), hepatic (HepG2 and Huh7) or prostate (DU145) cancer cell lines (Constantini *et al*., 2014). Our results point to the effects of *K. africana*, *M. zeyheri* and *X.*

caffra seed oils being more dependent on fatty acid composition, the duration of exposure, as well as phenotype of the breast tumours.

Hormone-dependent MCF-7 cells are a weakly metastatic breast cancer cell line (Pogash *et al*., 2015; Flodrova *et al*., 2016) that is phenotypically and behaviourally different to the more aggressive, hormone-independent triple-negative MDA-MB-231 cell line (Pogash *et al*., 2015; Flodrova *et al*., 2016). This was reflected by MDA-MB-231 cell viability being least affected and MCF-7 cells being more responsive to the seed oils. MCF-7 cells displayed a reduction in viability associated with exposure time to *M. zeyheri* and *K. africana*; however, cell viability peaked at 72 h.

Cytotoxicity tests are used to screen the cytotoxic potential of compounds present in extracts that affect cellular functions (Ganesh *et al*., 2013). Criteria used to establish cell damage depend on the techniques used and can be divided into changes in cell morphology, cell function impairment and increased permeability of the plasma membrane (Danpure, 1984). The assessment of cell viability was based on use of the neutral red assay, which is dependent on this supravital dye diffusing through the plasma membrane of viable, metabolising cells and concentrating in lysosomes (Borenfreund and Puerner, 1984; Weyermann *et al*., 2005). The amount of dye extracted from the cells using solubilisation buffer is directly proportional to the number of viable cells (Weyermann *et al*., 2005). Since studies have shown variability between different assays, which may be based on different modes of cell death (Weyermann *et al*., 2005), we also employed the LDH assay to determine the permeability of the plasma membrane which is an indication of necrotic cells and which can be quantified by measuring the release of the LDH enzyme (Chan *et al*., 2013). This dual approach provides more information regarding seed oil effects than the trypan blue exclusion method based on manual haemocytometer cell counting employed by Chivandi *et al*. (2012b). A method which is time consuming, has user-to-user variation and does not differentiate between apoptotic and necrotic cells (Altman *et al*., 1993).

By monitoring the integrity of the plasma membrane, we found that MDA-MB-231 cells were more susceptible to a cytotoxic effect than MCF-7 cells. Specifically, *K. africana* and *X. caffra* seed oils elicited the greatest cytotoxic effects on MDA-MB-231 cells at 48 h incubation; however, cells showed recovery capacity at 72 h. While this may be related to the doubling time (41.9 h) of these hormone-independent cells (Dolfi *et al*., 2013; Kuksin and Chan, 2013), this phenomenon might also be related to these compounds requiring additional time to induce cell

death (Smith *et al*., 2011), or recovery of cell membrane integrity (Chivandi *et al*., 2012b). Moreover, while *K. africana* seed oil produced a positive, albeit low, cytotoxic effect on MCF-7 cells, *X. caffra* and *M. zeyheri* seed oils elicited a negative cytotoxic effect. This finding is essentially linked to the basis of defining levels of cytotoxicity using the LDH assay. It is proposed that the cells within the control group continue to divide and multiply whereas exposure to seed oils halted cell cycle resulting in no division and multiplication of cells, the result of which being quantitatively expressed as a "negative" cytotoxic effect (Smith *et al*., 2011). As such the negative cytotoxic effect observed in our results is indicative of MCF-7 cell growth inhibition, as opposed to induction of cell death alone.

While an association between the effects of seed oils and seed oil composition were identified, in comparison with profiles reported by Chivandi (2012a), our study found a lower lipid yield. These differences might be attributable to different extraction methods or conditions, different cultivars and climatic conditions (Sorice *et al*., 2016; Bala *et al*., 2018).

Variance in the fatty acid profile of each seed oil may explain the differences in induced effects on each cell line. *K. africana*, which yielded particularly high levels of α-linolenic acid, had a greater cytotoxic effect on MDA-MDA-231 cells than MCF-7 cells. Flaxseed oil, which contains approximately 57% α-linolenic acid, an omega-3 polyunsaturated fatty acid, successfully reduced breast cancer cell growth (Wiggins *et al*., 2015). Direct α-linolenic acid treatment has been shown to reduce prostaglandin-E2 in MDA-MB-231 by lowering the level of cyclooxygenase (COX) 2 expression (Horia and Watkins, 2005). COX2, which is required for the conversion of arachidonic acid to prostaglandins (Smith *et al*., 2000), is highly expressed in MDA-MB-231 cells compared to MCF-7 cells (Horia and Watkins, 2005) and is associated with tumour initiation and progression (Smith *et al*., 2000). Additionally, linolenic acid-derived components eicosapentaenoic acid (EPA) and docosahenxaenoic acid (DHA) are associated with growth-inhibitory effects on MDA-MB-231 and MCF-7 breast cancer cells (Chamras *et al*., 2002; Schley *et al*., 2005). Growth inhibition was identified in our study only in the MCF-7 line, specifically for *M. zeyheri* seed oil and to a lesser extent, *X. caffra*, both of which conversely presented with low levels of α-linolenic acid. *M. zeyheri* seed oil presented a comparatively higher level of linoleic acid, as well as total omega-6 and omega-9 fatty acids than *K. africana* seed oil.

In conclusion, we observed that the seed oils had a cytotoxic effect on MDA-MB-231 breast cancer cells but a growth inhibitory effect on MCF-7 breast cancer cells. This was only detected by the LDH assay, with the results from the neutral red assay being inconclusive due to the mechanisms by which these seed oils exert their effects and which will require further investigation. While inferences can be drawn regarding the composition of these seed oils in relation to their effects, more work remains to be done to clarify these inferences.

CHAPTER 3

Kigelia africana, Ximenia caffra and *Mimusops zeyheri* seed oils induce apoptosis and/or autophagy in MDA-MB-231 and MCF-7 breast cancer cell lines

The data in this chapter will soon be published in a suitable journal.

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***Kigelia africana, Ximenia caffra* and *Mimusops zeyheri* seed oils may induce apoptosis and/or autophagy in hormone-independent and hormone dependent breast cancer cells**

3.0. ABSTRACT

Parts of the *Kigelia africana*, *Ximenia caffra* and *Mimusops zeyheri* trees are used by sub-Saharan Africa traditional healers to treat a variety of ailments. Seed oils derived from these trees have been shown to inhibit the proliferation of hormone-independent MDA-MB-231 and hormone dependent MCF-7 breast cancer cell lines. However, the possible mechanism of action by which these seed oils exert their effects have not been elucidated. In this study, we investigated the possible mechanism(s) by which the seed oils inhibit the growth of hormone-independent MDA-MB-231 and hormone dependent MCF-7 breast cancer cell lines. *Kigelia africana* seed oil, characterised by higher levels of α-linolenic acid compared to *Ximenia caffra* and *Mimusops zeyheri*, induced apoptosis in hormone-independent MDA-MB-231 cells and to a lesser degree, in hormone-dependent MCF-7 breast cancer cells. The *Kigelia africana* seed oil induced effects were not associated with significant increases in the levels of caspase 6 expression suggesting that caspase 6 does not play a key role in *Kigelia africana*-induced MDA-MB-231 cell death. Treatment of cells with *Kigelia africana* seed oil caused a significant reduction ($p \leq 0.001$) in the expression of LC3A, a marker of autophagic activity in MDA-MB-231 breast cancer cells. However, overall MCF-7 cells had lower LC3A expression levels compared to their MDA-MB-231 counterparts. With the lowered LC3A concentration, *K. africana* seed oil is possibly blocking the formation of autophagosomes, thereby preventing autophagy to occur in these cells.

Key words: breast cancer, ethnomedicine, indigenous plant-derived seed oils, apoptosis, caspase 6, autophagy, LC3A

3.1. INTRODUCTION

Apoptosis or programmed cell death (PCD) is a physiological mechanism that plays an important role in the maintenance and order of normal cells and organs (Kroemer, 2003; Azadmehr *et al*., 2015). Biochemical indicators of apoptosis include activation of specific enzymes such as caspases, chromatin condensation and exposure of phosphatidylserine on the cell membrane (Leist and Jäättelä, 2001; Gerl and Vaux, 2005). Dysregulation of apoptosis has been associated with a variety of pathological conditions for example neurodegenerative diseases (Stefanis *et al*., 1997), autoimmune diseases (Eguchi, 2001), heart diseases (Lee and Gustafson, 2009) as well as cancer (Gerl and Vaux, 2005; Azadmehr *et al*., 2015).

Autophagy is another physiological process that either entails the breakdown of intracellular proteins into amino acids which are then used to synthesise new proteins, or the degradation of damaged proteins and organelles for elimination (Maycotte and Thorburn, 2014; Ueno *et al*., 2016). Autophagy, similar to apoptosis, has been involved in a number of diseases including liver disease, diabetes, kidney disease, heart diseases, inflammatory diseases, neurodegenerative disease and cancer (Maycotte and Thorburn, 2014; Ueno *et al*., 2016; Ichimiya *et al.*, 2020). It is therefore interesting to note that both apoptosis and autophagy may be active in cancer.

The most common cancer in women worldwide is breast cancer and this has been shown to be the primary cause of cancer-related deaths among women (Shahali *et al*., 2018). In 2018, 2.09 million women were diagnosed with breast cancer and 627 000 deaths were reported (Bray *et al*., 2018). The survival rate for breast cancer in the US is much higher than in African countries (Kuete *et al*. 2013). In countries like Uganda, Ghana and Algeria where resources are scarce and treatment options limited, women receive inadequate treatment, pain relief or palliative care. In these cases patients become dependent on ethnomedicine to treat and/or manage breast cancer (Coughlin and Ekwueme, 2009; Ochwang'i *et al*., 2014).

The use of phytoceutics for a variety of ailments, including cancer, has been the practise for many centuries (Park and Pezzuto, 2002; Khazir *et al*., 2014). For example Hodgkin's lymphoma and leukaemia are treated using vinblastine and vincristine, compounds isolated from the Madagascar periwinkle plants (*Vinca rosea*) (Moudi *et al*., 2013, Khazir *et al*., 2014). Initially extracted from the bark of American Yew, Taxus brevifolia Nutt, paclitaxel is a standard chemotherapy agent impeding mitotic progression of ovarian and breast cancer

(Priyadarshini and Keerthi-Aparajitha, 2012; Khazir *et al*., 2014). In the sub-Saharan Africa region, traditional healers are using parts of the *Kigelia africana* (*K. africana*), *Ximenia caffra* (*X. caffra*) and *Mimusops zeyheri* (*M. zeyheri*) plants to treat fungal infections, psoriasis, dysentery, candidiasis, fever and malaria (Amusan *et al*., 2002; Bello *et al*., 2016; Maroyi, 2016). Historically, a tea made from the powdered bark of *K. africana* has been shown to have both analgesic and anti-inflammatory (Owolabi and Omogbai, 2007) and anticancer properties (Naidoo *et al*., 2013). Leaf extracts of *X. caffra* have been shown to inhibit proliferation and inflammation in murine RAW 264.7 macrophages (Zhen *et al*. 2015), demonstrating that this plant extract may have anti-cancer benefits. Despite Swaziland's traditional use of *M. zeyheri*'s roots to treat candidiasis and bark for treating ulcers and wounds (Amusan *et al*., 2002), little is known about its possible cancer-fighting properties.

Although leaf extracts and/or macerations of indigenous fruit-bearing trees contain beneficial compounds, seeds from these trees also contain amino acids, fatty acids, and vitamins (Chivandi *et al*., 2011a). It has been shown that seeds may have anti-inflammatory (Constantini *et al*., 2014) and anti-proliferative activities (Vieira *et al*., 2008; Seal *et al*., 2012; Al-Sheddi *et al*., 2015; Guo *et al*., 2016) that may influence tumour development and growth. Chivandi *et al*. (2012b) showed that *K. africana, X. caffra* and *M. zeyheri* seed oils suppress the proliferation of human colon adenocarcinoma (Caco-2) and human embryonic kidney (HEK-293) cells. In a previous study, we showed that these seed oils are also able to suppress the proliferation of hormone-independent (MDA-MB-231) and hormone dependent (MCF-7) breast cancer cells (Gomes *et al*., 2019). The mechanism by which these seed oils exert their effects have not been elucidated so in the present study we investigated the possible mechanism(s) by which the seed oils of *K. africana, X. caffra* and *M. zeyheri* may inhibit the growth of hormone-independent (MDA-MB-231) and hormone dependent (MCF-7) breast cancer cells.

3.2. MATERIALS AND METHODS

3.2.1. Seed source

K. africana seeds were obtained from the ripe fruits from trees in Gutu District, Chitsa Communal area, Zimbabwe which is located at latitude and longitude of 19º41′S and 31º09′E respectively. It is characterized by granitic soils and has annual rainfall and temperature ranges of 650-800 mm and 20.5-30.0ºC, respectively (Chivandi, 2012a). *M. zeyheri* seeds were harvested from fruits off trees found in the Gokwe District, in North West Zimbabwe, in the Machakata Community Forest area of the Mapfungautsi Plateau which is located at latitude and

longitude of 20º25′S and 28º29′E respectively. It is characterized by shallow sandy, lithosol soil and has an annual rainfall of +/- 609 mm and temperature of +/- 25.9ºC (Chivandi, 2012a). *X. caffra* seeds were collected from ripe fruit of the Zhombe District, Zimbabwe which is located at latitude and longitude of 14º45′S and 26º50′E respectively. The area has annual rainfall of 550 mm and a mean annual temperature of 26ºC (Chivandi, 2012a). The seeds from which the seed oils were extracted, were imported into the Republic of South Africa under the permit number P0039683 following confirmation of the trees' identities by the National Botanical Gardens of Zimbabwe.

3.2.2. Seed oil extraction and storage

Seeds were dehulled and subsequently crushed in a blender (Waring; Lasec Pty Ltd, Johannesburg, South Africa) to form a seed meal. Hexane was used to extract the oil from each of the seed meals as described by Yamasaki *et al*. (2013). Briefly, 800 mL of hexane was added to 160 g of each seed meal and then mixed overnight using a magnetic stirrer (VELP Scientifica, Lombardy, Italy). The mixture was then filtered through filter paper (Whatman No.1) and the hexane was evaporated using a rotary vacuum set at 65ºC. The extracted oils were kept in brown sample bottles at -20ºC before use (Yamasaki *et al*., 2013).

3.2.3. Cell culture

A Human Ethics Waiver Certificate (W-CJ-150422-1) was obtained from the Human Research Ethics Committee, University of the Witwatersrand, Johannesburg (South Africa). The two human breast cancer cell lines (MCF-7 and MDA-MB-231) used in the experiments were obtained from ATCC (Virginia, USA) and donated by Dr R Duarte (School of Clinical Medicine, University of the Witwatersrand). The reason for choosing these two cells lines is that it will allow us to differentiate the effects of the seed oils on hormone-dependent (MCF-7) and hormone-independent (MDA-MB-231) breast cancer cells. MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 5% foetal bovine serum (FBS) and 100 µg/mL streptomycin, 100 units/mL penicillin. MDA-MB-231 cells were cultured in DMEM/Ham's F-12 nutrient mixture with 10% FBS and 100 µg/mL streptomycin, 100 units/mL penicillin. Both cell lines were incubated at 37ºC in a humidified atmosphere of 5% CO_2.

3.2.4. Seed oil concentrations and treatment of cells

Stock solutions (10 mg/mL) of the seed oils were prepared in 100% absolute alcohol and diluted in culture medium to reach the desired concentration (120 µg/mL). The concentration of absolute alcohol in culture medium was not more than 5%. A diluent control consisting of absolute alcohol diluted in culture medium was included.

Confluent cells were subcultured by 0.25% trypsin/EDTA. MCF-7 cells (P35) and MDA-MB-231 cells (P23) were plated in 24 well plates at 6.3 x 10^4 cells/well and 3.1 x 10^4 cells/well respectively in their respective media. Both cell lines were incubated at 37ºC in a humidified atmosphere of 5% CO_2 for 24 h. The cell culture media from each well was then removed and cells were rinsed with Dulbecco's phosphate-buffered saline (DPBS). Cells were treated using 200 µL of the seed oil concentration (120 µg/mL) in matched culture media and incubated at 37ºC in a humidified atmosphere of 5% CO_2 for 48 h. In all experiments untreated and diluent treated cells were included as controls.

3.2.5. Apoptosis

3.2.5.1. FITC Annexin V Apoptosis Detection Assay

The fluorescein isothiocyanate (FITC) Annexin V apoptosis detection kit (556547, BD Pharmingen^{TM} California – USA) allows for the identification of early apoptotic, advanced apoptotic, necrotic and viable cells. FITC Annexin V staining occurs before the cell loses its membrane integrity while the inclusion of the vital dye, propidium iodide (PI, as the kit component), which enters cells with compromised membranes indicates advanced apoptosis/necrosis.

After 48hrs, seed oil treatments were removed and cells detached by 0.25% trypsin/EDTA. Cells were monitored for detachment every 5-10minutes. As soon as the cells were detached, an equal volume of complete media was added to the trypsin/EDTA in order to neutralise the action of the trypsin/EDTA. The cell suspension was then collected into a flow tube and centrifuged for 5 minutes at 400 x g. The supernatant was discarded and the cells were re-suspended in 500 µL complete media and incubated for 30 minutes to allow cells to recover from trypsinization. After the incubation, the cell suspension was centrifuged for 5 minutes at 400 x g and the supernatant discarded. Cells were then rinsed in 1 mL DPBS supplemented with 10% FBS, centrifuged and supernatant was discarded. Cells were subsequently re-suspended in 100 µL 1x binding buffer supplemented with 10% FBS. FITC Annexin V (2 µL)

and PI (2 µL) were then added to the respective tubes. Tubes were vortexed, incubated for 15 minutes at room temperature in the dark and 200 µL 1x binding buffer supplemented with 2% FBS and 1mM EDTA was then added. Samples were placed on ice and analysed using the LSR Fortessa Analyser (San Diego, CA, USA) within 1 hour.

3.2.5.1.1. Flow cytometry

A total of 30,000 events were acquired per sample on the flow cytometer using the BD FACSDiva software after instrument quality control and as previously described (Fru *et al*., 2021). The events were recorded at a forward scatter voltage of 50V and a side scatter voltage of 246V for cell size and granularity respectively. The BD FACSDiva software was used to detect FITC positive signals for apoptotic cells (Annexin V positive) on a 530/30 filter and peridinin–chlorophyll–protein (PerCP) positive signals for necrotic cells (PI positive) on a 610/20 band pass filter.Compensation controls included unlabelled cell samples and single antibody staining of samples to determine levels of autofluorescence and fluorescence overlap for gating purposes. Exported Flow Cytometry Standard (FCS) files were analysed using FlowJo V10 (FlowJo, LLC, Ashland, OR 97520, USA).

3.2.5.2. Caspase 6 Assay

For the preparation of samples for the caspase 6 assay, the seed oil treatments were removed after 48 hrs and the cells detached using 0.25% trypsin/EDTA. An equal volume of complete media was added to the trypsin/EDTA in order to neutralise the action of the trypsin/EDTA as soon as the cells were detached. The cell suspension was then collected and centrifuged for 5 minutes at 400 x g. The supernatant was subsequently discarded and DPBS (1 mL) added. The number of cells was then determined using an automated cell counter (BioRad TC20, California - USA). The tube was re-centrifuged for 5 minutes at 400 x g, the supernatant discarded and cells washed twice with pre-cooled DPBS (500 µL). After the final wash, pre-cooled DPBS (300 µL) was added to keep the cells suspended. The cell suspension was then transferred into an Eppendorf tube and frozen at -80^{o}C for 10 minutes. The cell suspension was then thawed and mixed. This freeze/thaw cycle was repeated five times. After the final thaw, the cell suspension was centrifuged for 10 minutes at 1500 x g at 4^{o}C. The supernatant was collected and stored at -80^{o}C for subsequent caspase 6 analysis.

Samples were thawed and their total protein concentration was determined using the Bradford method. In brief, a 1 mg/mL bovine serum albumin (BSA) protein standard stock solution was

made and used in the preparation of a protein dilution series. Protein standards and samples (5 µL) were pipetted into allocated wells of a 96-well plate. Bradford reagent (250 µL) was added to each well. The plate was then mixed and incubated for 10 minutes at room temperature. The optical density was measured spectrophotometrically at a wavelength of 595nm.

Caspase 6 concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) kit bought from Elabscience, USA. Standards and samples (100 µL) were pipetted into allocated wells of a coated 96-well ELISA plate. The plate was then incubated for 90 minutes at 37^{o}C. Standards and samples were removed from wells and biotinylated antibody specific for Human Caspase 6 (100 µL) was added in all the wells. The plate was incubated for 60 minutes at 37^{o}C and then washed thrice. Avidin-horseradish peroxidase conjugate (100 µL) was subsequently added to the wells and the plate was incubated for 30 minutes at 37^{o}C. Plate was washed again (5 times) and substrate reagent (90 µL) was added to each well. The plate was incubated for 15 minutes at 37^{o}C. Stop solution (50 µL) was then added to the wells and the optical density was measured spectrophotometrically at a wavelength of 450nm. Caspase 6 concentrations were determined using the standard curve generated and percentage caspase 6 from total protein concentration is shown.

3.2.5.3. LC3A Autophagy Assay

After 48hrs, treatments were removed and cells detached by 0.25% trypsin/EDTA. An equal volume of complete media was added to neutralise the action of the trypsin/EDTA as soon as the cells were detached. The cell suspension was collected into a centrifuge tube and centrifuged for 5 minutes at 400 x g. The supernatant was discarded and DPBS (1 mL) added. The number of cells was then determined using an automated cell counter (BioRad TC20, California - USA). The tube was re-centrifuged for 5 minutes at 400 x g, the supernatant discarded and the cells washed twice with pre-cooled DPBS (500 µL). After the final wash, 1x cell extraction buffer PTR (300 µL) was added to solubilise the cell pellet (1 x 10^{6}cells). The cell suspension was then transferred into an Eppendorf tube and incubated on ice for 20 minutes. After the incubation, the cell suspension was centrifuged for 20 minutes at 16 000 x g at 4^{o}C. The supernatant was transferred into clean tubes and stored at -80^{o}C.

Total protein concentration was determined using the bicinchoninic acid (BCA) protein method. In brief, a 1 mg/mL BSA protein standard stock solution was made and used in the preparation of a protein dilution series. Protein standards and samples (25 µL) were pipetted

into allocated wells of a 96-well plate. BCA (200 μL) was added to each well. The plate was then mixed and incubated for 30 minutes at 37^{o}C. The optical density was then measured spectrophotometrically at 540nm.

LC3A concentrations were determined using an ELISA kit bought from Abcam, UK. Standards and samples (50 μL) were pipetted into allocated wells of a coated 96-well ELISA plate. An antibody cocktail (50 μL) was then added to all wells. The plate was incubated for 60 minutes at room temperature on a plate shaker set to 400rpm and subsequently washed thrice. 3,3',5,5”-tetramethylbenzidine (TMB) development solution (100 μL) was added to all wells and the plate was incubated for 10 minutes in the dark on a plate shaker set to 400rpm. Stop solution (100 μL) was added to all the wells and the plate mixed for 1 minute on a plate shaker set to 400rpm. The optical density was measured spectrophotometrically at a wavelength of 450nm. LC3A concentrations were determined using the standard curve generated and percentage LC3A from total protein concentration is shown.

3.2.6. Data analysis

Data are presented as mean±standard deviation of four independent experiments. All analyses were done using Python 3.9.5 software (Python Software Foundation - OR, USA). The Shapiro-Wilk test was used to test for normality. Non-parametric one-way analysis of variance (ANOVA) followed by Tukey Honestly Significantly different (HSD) test was used to analyse the data. A *P* value of <0.05 was considered statistically significant.

3.3. RESULTS

3.3.1. Quantitative analysis of apoptotic cells by Annexin V/propidium iodide staining

To determine apoptosis of breast cancer cell lines, FITC-conjugated Annexin V and PI double staining (detected by flow cytometry) were used. Representative data, scatter plots showing viable MDA-MB-231 and MCF-7 breast cancer cells, cells in early apoptosis, cells in advanced apoptosis and necrotic (dead) cells are shown in Figure 3.1 and 3.2 respectively.

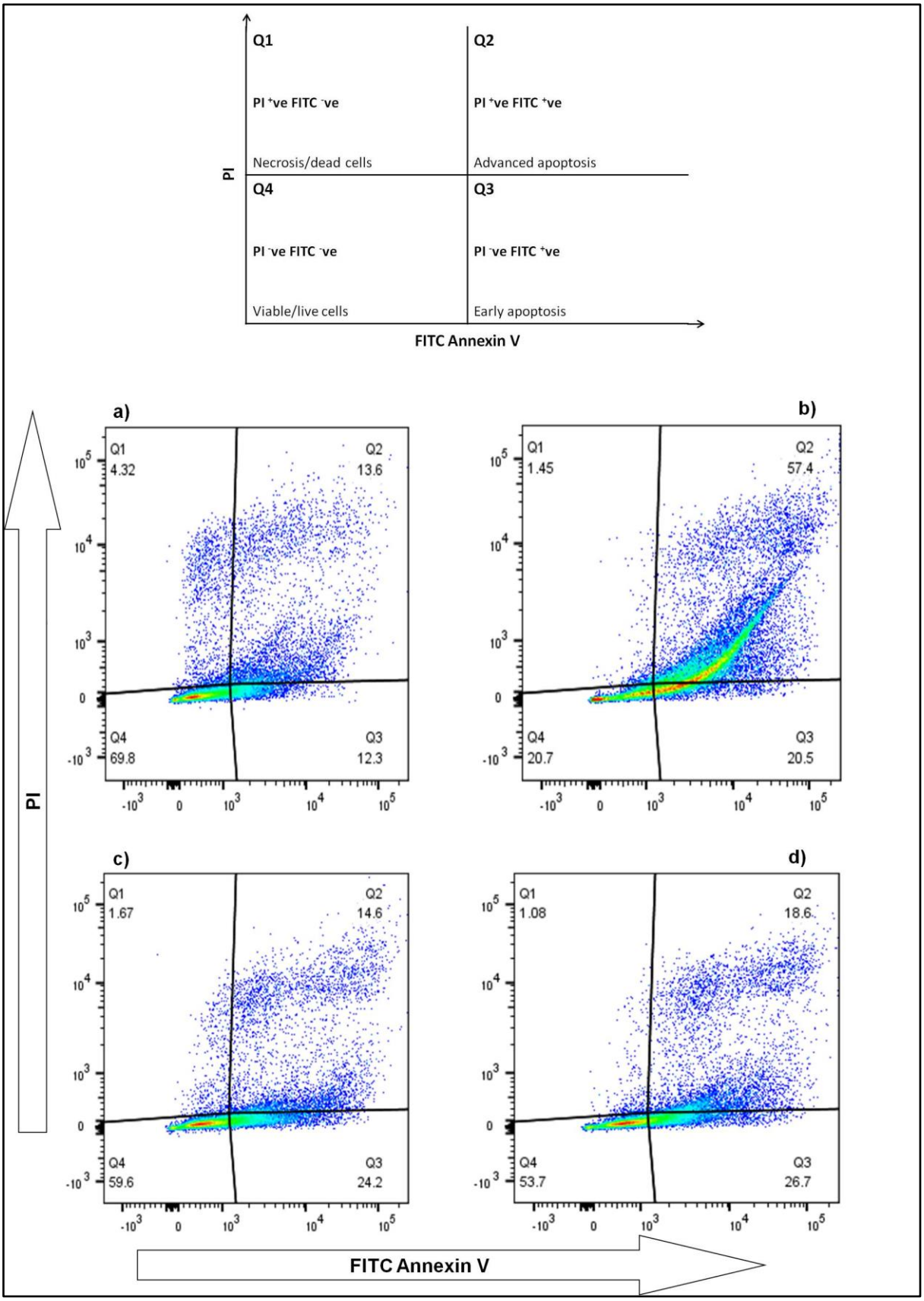

Figure 3.1: Scatter plots of viable MDA-MB-231 breast cancer cells, cells in early apoptosis, cells in advanced apoptosis and dead cells: (a) untreated breast cancer cells, (b) breast cancer cells treated with KA seed oil, (c) breast cancer cells treated with XC seed oil and (d) breast cancer cells treated with MZ seed oil.

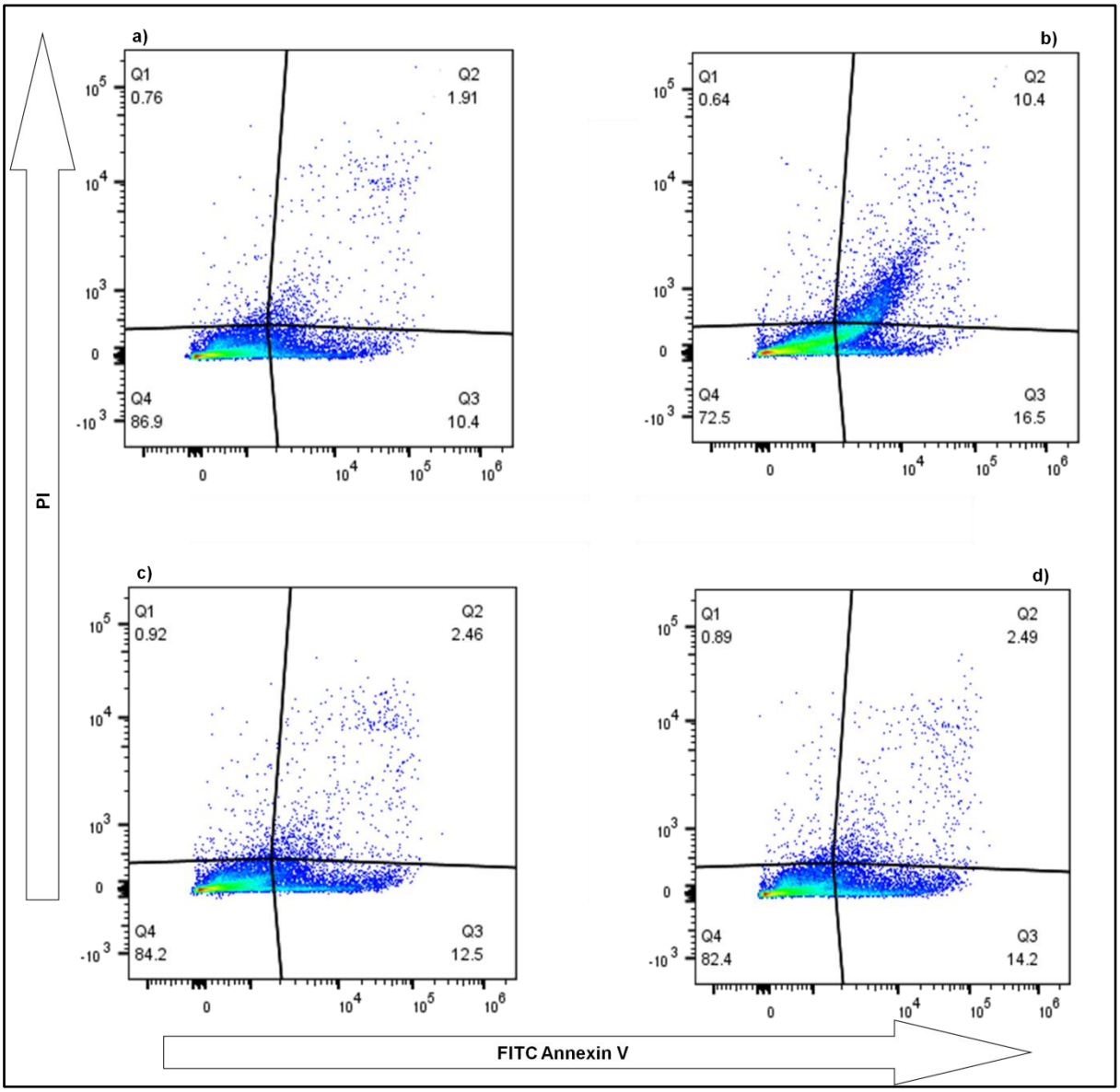

Figure 3.2: Scatter plots of viable MCF-7 breast cancer cells, cells in early apoptosis, cells in advanced apoptosis and dead cells: (a) untreated breast cancer cells, (b) breast cancer cells treated with KA seed oil, (c) breast cancer cells treated with XC seed oil and (d) breast cancer cells treated with MZ seed oil.

K. africana seed oil was most potent in stimulating apoptosis of MDA-MB-231 breast cancer cells when compared to *X. caffra* and *M. zeyheri* seed oils (Figure 3.3) resulting in a higher number of MDA-MB-231 breast cancer cells being in the advanced apoptosis stage (Figure 3.3). A significant statistical difference was observed between *K. africana* and the other seed oils, including the media control (Figure 3.3).

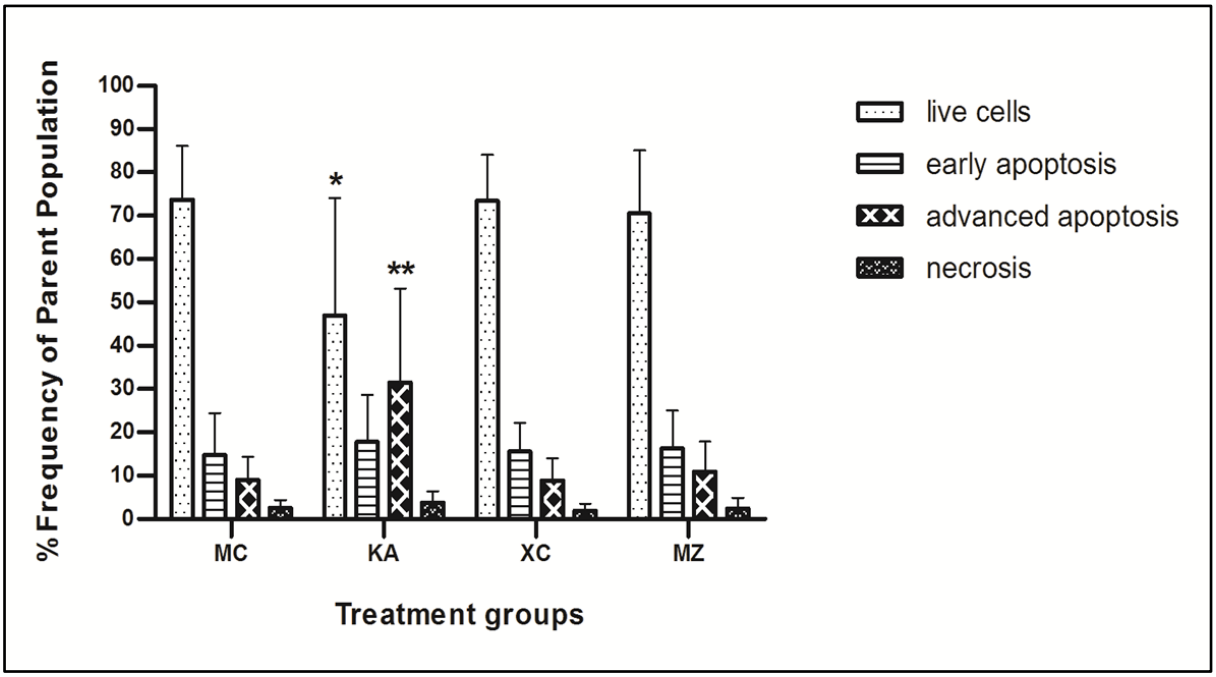

Figure 3.3: The apoptotic stages induced by the different treatment groups on MDA-MB-231 breast cancer cells. MC = cells treated with cell culture media only, KA = cells treated with *K. africana* seed oil, XC = cells treated with *X. caffra* seed oil and MZ = cells treated with *M. zeyheri* seed oil. The bars represent means ± standard deviations where * = $p \leq 0.05$ (significantly different from MC, XC and MZ) and ** = $p \leq 0.01$ (significantly different from MC, XC and MZ).

Significant statistical differences were observed when MCF-7 breast cancer cells were exposed to the various treatments (Figure 3.4). Similar to MDA-MB-231 cells, *K. africana* seed oil again showed greatest activation of apoptosis in MCF-7 breast cancer cells in comparison to *X. caffra* and *M. zeyheri* seed oils (Figure 3.4). However in contrast to MDA-MB-231 cells, there were more MCF-7 breast cancer cells in the early apoptosis stage than in the advanced apoptosis stage when treated with *K. africana* seed oil (Figure 3.4).

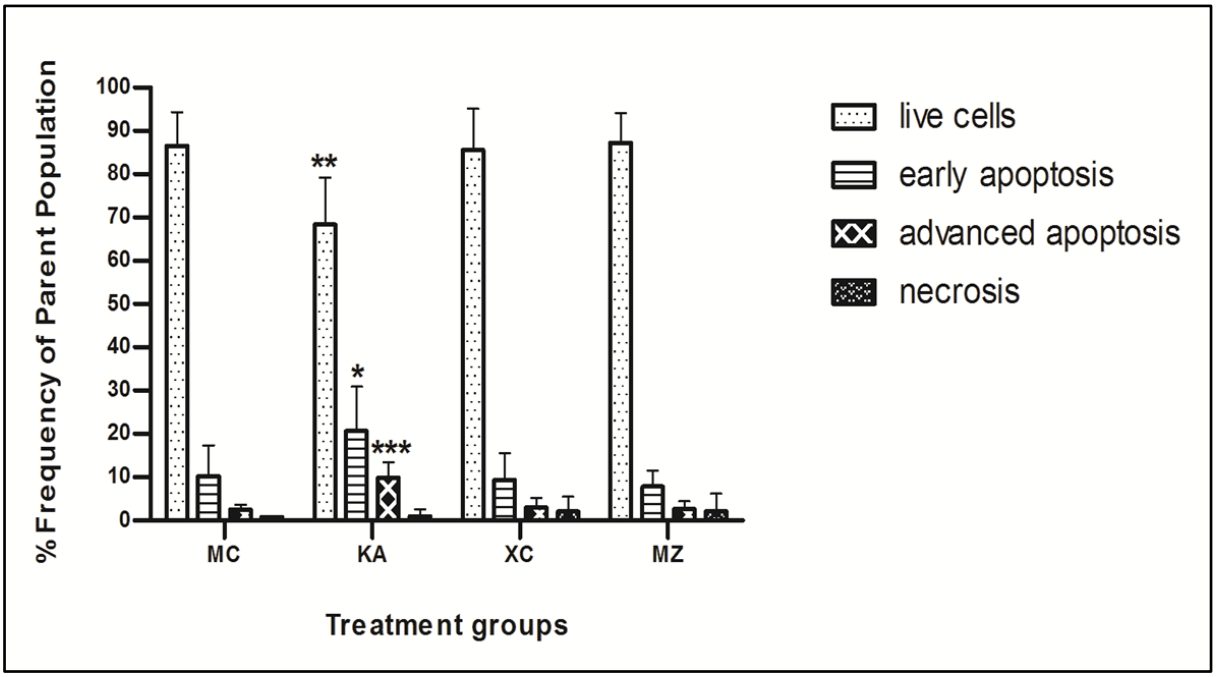

Figure 3.4: The apoptotic stages induced by the different treatment groups on MCF-7 breast cancer cells. MC = cells treated with cell culture media only, KA = cells treated with *K. africana* seed oil, XC = cells treated with *X. caffra* seed oil and MZ = cells treated with *M. zeyheri* seed oil. The bars represent means ± standard deviations where * = $p \leq 0.05$ (significantly different from XC and MZ), ** = $p \leq 0.01$ (significantly different from MC, XC and MZ) and *** = $p \leq 0.001$ (significantly different from MC, XC and MZ).

Other differences in how the two cancer cell lines responded to the various treatments, were also observed (Figures 3.3 and 3.4). *K. africana* seed oil induced significantly more cell death of MDA-MB-231 than MCF-7 breast cancer cells (Figures 3.3 and 3.4). The number of necrotic cells was higher in all the treated groups of MDA-MB-231 breast cancer cells (with *K. africana*-treated being the highest - 4.18 % MDA-MB-231 vs. 1.00 % MCF-7) compared to all the treated groups of MCF-7 breast cancer cells.

3.3.2. Involvement of caspase 6 in apoptosis induced by *K. africana, X. caffra* and *M. zeyheri* seed oils

To test whether caspase 6 is involved in the induction of apoptosis by *K. africana*, *X. caffra* and *M. zeyheri* seed oils on MDA-MB-231 and MCF-7 breast cancer cells, the expression levels of caspase 6 were analysed. Caspase 6 was specifically chosen as this enzyme is expressed in both cell lines and is part of the common executioner pathway of apoptosis.

Treatment of MDA-MB-231 breast cancer cells with all 3 seed oils resulted in elevated levels of caspase 6. However this increase was only significant in the groups that were treated with *X.*

caffra and *M. zeyheri* seed oils when compared to untreated MDA-MB-231 cells (Figure 3.5a). There was no significant difference in caspase 6 levels between the treated groups of MCF-7 breast cancer cells when compared to untreated MCF-7 cells (Figure 3.5b).

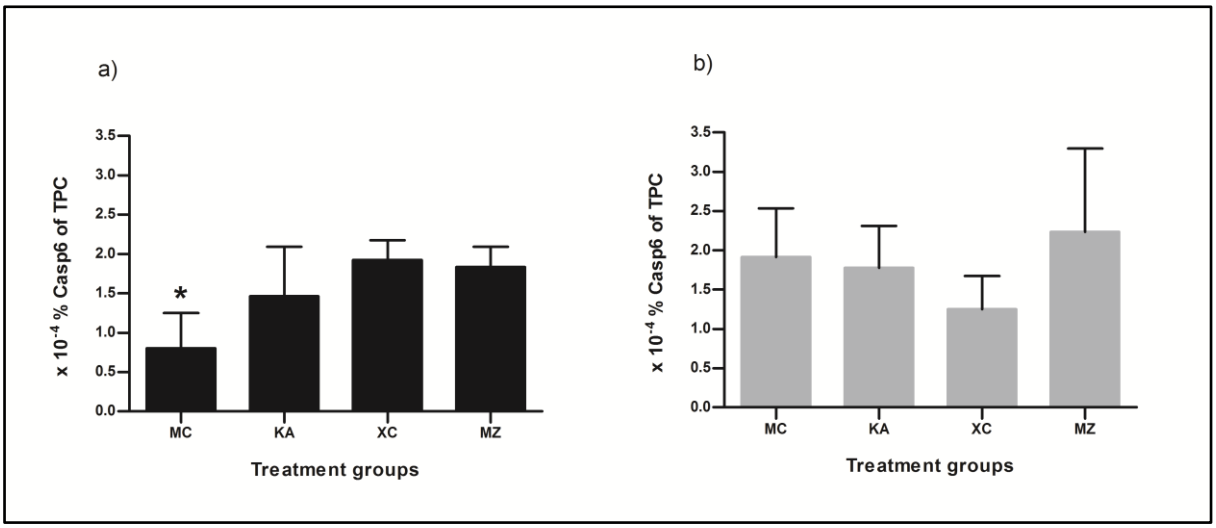

Figure 3.5: Percentage caspase 6 expression, induced by the different treatment groups, in comparison to total protein concentration in a) MDA-MB-231 and b) MCF-7 breast cancer cells. MC = cells treated with cell culture media only, KA = cells treated with *K. africana* seed oil, XC = cells treated with *X. caffra* seed oil and MZ = cells treated with *M. zeyheri* seed oil. The bars represent means ± standard deviations where * = $p \leq 0.05$ (significantly different from XC and MZ).

3.3.3. Involvement of LC3A in autophagy induced by *K. africana*, *X. caffra* and *M. zeyheri* seed oils

K. africana treated MCF-7 breast cancer cells induced a significant decrease in LC3A expression when compared to untreated (MC), *X. caffra*- and *M. zeyheri*- treated MDA-MB-231 breast cancer cells (Figure 3.6a). No significant statistical differences were found among the different treatment groups and untreated MCF-7 breast cancer cells (Figure 3.6b). Significant statistical differences were found between the two different cell lines (Figure 3.6c). MDA-MB-231 breast cancer cells showed significantly higher LC3A expression levels in MC, *X. caffra*- and *M. zeyheri*- treated cells when compared to MCF-7 cells. The opposite was observed in *K. africana*-treated cells where the LC3A expression levels in MDA-MB-231 cells were significantly less than in MCF-7 cells.

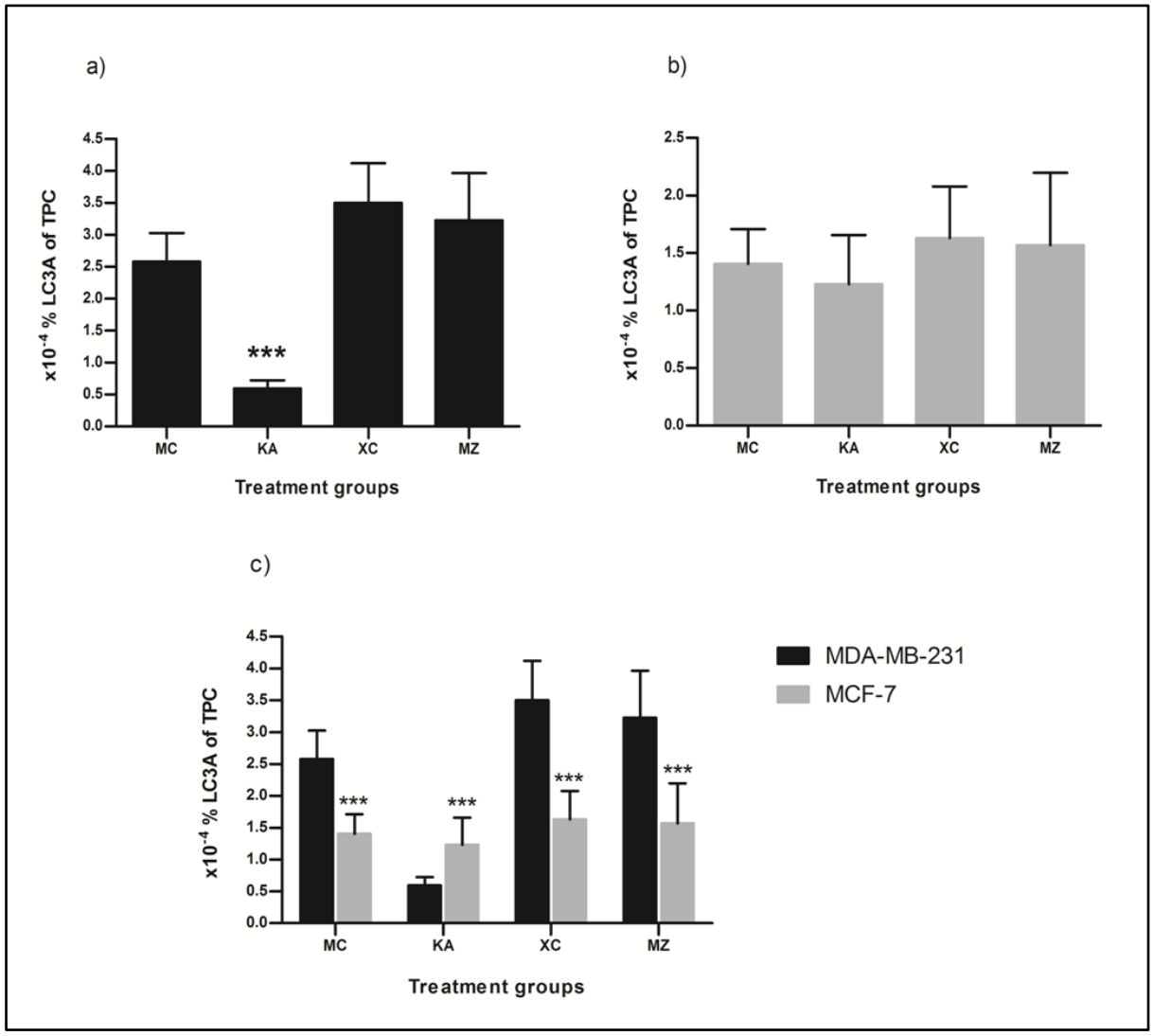

Figure 3.6: Percentage LC3A expression, induced by the different treatment groups, in comparison to total protein concentration in a) MDA-MB-231 and b) MCF-7 breast cancer cells. The bars represent means ± standard deviations where *** = $p \leq 0.001$ (significantly different from MC, XC and MZ). Figure 3.6c shows the LC3A expression levels of the two breast cancer cell lines, induced by the different treatment groups in comparison to total protein concentration. The bars represent means ± standard deviations where *** = $p \leq 0.001$ (significantly different from MDA-MB-231 cells). MC = cells treated with cell culture media only, KA = cells treated with *K. africana* seed oil, XC = cells treated with *X. caffra* seed oil and MZ = cells treated with *M. zeyheri* seed oil.

3.4. DISCUSSION

There are numerous examples for looking to nature for drug discovery, as naturally occurring compounds, particularly those found in plants, have demonstrated potential value in cancer treatment for many years (Cheah *et al*., 2008). According to the US Food and Drug Administration, more than half of all anticancer drugs approved since 1960 have been derived from plants (Mann, 2002; Treasure, 2005). Previously, we showed that *K. africana*, *X. caffra* and *M. zeyheri* seed oils are able to suppress the proliferation of hormone-independent MDA-

MB-231 and hormone dependent MCF-7 breast cancer cells to varying degrees (Gomes *et al*., 2019). The present study was designed to examine the possible mechanism of action by which these seed oils exert their effects on hormone-independent MDA-MB-231 and hormone dependent MCF-7 breast cancer cells.

In summary our main findings were that (i) *K. africana* was able to significantly reduce the number of live MDA-MB-231 cells and increase the number of these cells in the advanced apoptotic stage; (ii) these *K. africana* effects were greater in MDA-MB-231 cells than in MCF-7 cells; (iii) these *K. africana* effects were not associated with significant increases in caspase 6 expression levels; (iii) treating with *X. caffra* and *M. zeyheri* seed oils did not lead to significant cell death even though the expression of caspase 6 levels in these cells were significantly higher than controls; (iv) *K. africana* treatment led to significantly low expression levels of LC3A, and (v) MCF-7 cells in general (except *K. africana*-treated MDA-MB-231 cells) had lower LC3A expression levels than MDA-MB-231 breast cancer cells.

K. africana seed oil treatment, causing a significant decrease in live MDA-MB-231 and MCF-7 breast cancer cells, demonstrates this seed oil's ability to induce cell death in these cells. The fact that many cells were found to be in the advanced apoptosis stage suggested *K. africana* seed oil to have potent apoptotic inducing ability in hormone-independent MDA-MB-231 and hormone-dependent MCF-7 breast cancer cells, and that apoptosis may be a mode of cell death employed by this seed oil. This suggestion is in line with previous findings that also show an increase in apoptosis in MDA-MB-231 and MCF-7 breast cancer cells after treatment with flaxseed oil (Sorice *et al*., 2016). Interestingly the effect of *K. africana* seed oil was greater in MDA-MB-231 cells than in MCF-7 cells. Similar findings were reported by Yang *et al*. (2007) where growth reduction was greater in MDA-MB-231 than in MCF-7 cells when these cells were exposed to genistein, a major soy isoflavone. It is suspected that the absence of caspase 3 in MCF-7 cells (Yang *et al*., 2007; Shahali *et al*., 2018) is partially protecting against the toxic effects of *K. africana* seed oil, thereby minimizing the impact of the seed oil on breast cancer cell wellbeing.

In the present study the expression levels of the apoptosis associated protein, caspase 6, were significantly increased in MDA-MB-231 cells when exposed to *X. caffra* and *M. zeyheri* seed oils but not to *K. africana* seed oil. These observations suggest that caspase 6 does not play a key role in *K. africana*-induced MDA-MB-231 cell death. It is therefore likely that *K. africana* seed oil modulates MDA-MB-231 cell apoptosis via an alternative mechanism. Interestingly

the observed high levels of caspase 6 expression in *X. caffra* and *M. zeyheri* treated MDA-MB-231 cells did not lead to advance apoptosis (Figure 3.3). In these cases, it may be possible that the seed oils were able to activate caspase 6 but that this activation did not necessarily lead to apoptosis. These results point to the importance of having multiple indicators of apoptosis in order to unequivocally state that this mode of cell death is involved in the observed seed oil-induced reduction in cell number. In line with this reasoning caspase 6 also did not seem to play a key role in MCF-7 cell death either. Caspase 6 was chosen as it is expressed in both cell lines whilst caspase 3 is only expressed in MDA-MB-231 breast cancer cells (Yang *et al*., 2007; Cheah *et al*., 2008) and not in MCF-7 breast cancer cells due to a 47-base-pair deletion (Yang *et al*., 2007; Shahali *et al*., 2018). Because caspase 3 is an important mediator in apoptosis cell death, the absence of caspase 3 in MCF-7 cells could be a further explanation for the decrease in cytotoxic response to *K. africana* seed oil. The presence of oestrogen receptors as well as the status of the tumour suppressor gene, p53, are different between the two cell lines - MDA-MB-231 cells are oestrogen receptor negative and express mutant p53, while MCF-7 cells are oestrogen receptor positive and express wild type p53 (Yang *et al*., 2007; Singh *et al*., 2011). Breast tumours that are oestrogen receptor negative and p53 mutant are more aggressive and have fewer treatment alternatives (Singh *et al*., 2011). The selective toxic effects of *K. africana* seed oil on MDA-MB-231 cells are therefore noteworthy.

Autophagy is the process by which intracellular proteins and organelles are degraded within lysosomes (Tanida *et al*., 2004; Mizushima, 2007). During autophagy, parts of the cytoplasm as well as intracellular organelles are packaged into double-membrane-bound structures known as autophagosomes (Tanida *et al*., 2004; Kondo *et al*., 2005). These autophagosomes then fuse with lysosomes and the contents are subsequently degraded by lysosomal hydrolases (Tanida *et al*., 2004; Kondo *et al*., 2005; Ito *et al*., 2005). LC3A is involved in the formation of the autophagosome and in the elongation of the phagophore membrane (Tanida *et al*., 2004; Ito, *et al*., 2005; Koukourakis *et al*., 2015). The expression level of LC3A is therefore a useful indicator of autophagic activity. We measured the levels of LC3A in untreated and treated MDA-MB-231 and MCF-7 breast cancer cells to determine whether the process of autophagy contributes to the observed plant seed oil toxic effects. Overall MCF-7 cells had lower LC3A expression levels than MDA-MB-231 cells. Further *K. africana* seed oil was the only treatment that caused a significant reduction in LC3A expression and this effect was only present in MDA-MB-231 cells. In contrast higher expression levels of LC3A were obtained when *X. caffra* and *M. zeyheri* seed oils were used to treat the cells, with a greater response seen in MDA-MB-231 than in MCF-7 breast cancer cells. With the lowered LC3A concentration, *K.*

africana seed oil is probably blocking the formation of autophagosomes, thereby preventing autophagy to occur in these cells. Although the mechanism by which *K. africana* seed oil could act is not clear, factors that affect membrane fluidity and signal transduction have been implicated (Wiggins *et al.*, 2015). Nevertheless, autophagy has also been described as a mechanism employed during cellular rescue (Gozuacik and Kimchi, 2004; Mizushima, 2007). Reducing autophagy while stimulating apoptosis, is therefore a plausible explanation for the increase in cell death seen in *K. africana* seed oil -treated cells.

K. africana seed oil had a different effect on the two breast cancer cells than *X. caffra* and *M. zeyheri* seed oils. Variations in the fatty acid composition of each seed oil may explain the different effects observed. *K. africana* seed oil has higher levels of α-linolenic acid (omega-3 polyunsaturated fatty acid) than *X. caffra* and *M. zeyheri* seed oils (Gomes *et al*., 2019). Alpha (α)-linolenic acid has been shown to reduce breast cancer cell growth (Schley *et al*., 2005; Wiggins *et al*., 2015). *M. zeyheri* has higher levels of linoleic acid (omega-6 polyunsaturated fatty acid) (Gomes *et al.*, 2019). Omega-3 and omega-6 polyunsaturated fatty acid (PUFAs) are essential fatty acids necessary for human health and are involved in many aspects of cellular physiology (Berquin *et al*., 2008; Huerta-Yépez *et al*., 2016). Schley *et al*. (2005) found that a combination of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both omega-3 polyunsaturated fatty acids, induced apoptosis of MDA-MB-231 breast cancer cells through increased activity of caspase proteins (although they don't specify which caspases) and loss of mitochondrial membrane potential.

Many hypotheses have been proposed by which PUFAs exert their effects - from membrane fluidity to increased lipid peroxidation and reactive oxygen species (Wiggins *et al*., 2015). Schley *et al*. (2005) hypothesized that omega-3 PUFAs decrease proliferation and increase apoptosis of human breast cancer cells through the inhibition of the Akt/NFκB cell survival signalling pathway. Our data suggest that other pathways may also be involved in seed oil-induced cell death. It is therefore clear that further investigation is required into the molecular mechanisms by which seed oils exert their effects. Furthermore, it is evident that treatment of different cancers demand diverse approaches and as such the usefulness other plants and/or seeds in the management of tumours should continue to be evaluated.

CHAPTER 4

Differential expression of platelet activation markers, CD62P and CD63, after exposure to breast cancer cell lines treated with *Kigelia africana*, *Ximenia caffra* and *Mimusops zeyheri* seed oils *in vitro*

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Differential expression of platelet activation markers, CD62P and CD63, after exposure to breast cancer cells treated with *Kigelia africana*, *Ximenia caffra* and *Mimusops zeyheri* seed oils *in vitro*

4.0. ABSTRACT

Cancer patients, including breast cancer patients, live in a hypercoagulable state. Chemo- and hormone- therapy used in the treatment of breast cancer increases the risk of thrombosis. Due to differences in health care services between developed and developing countries, the survival rate of women with breast cancer in developing countries is low. Consequently, ethnomedicines are used and their efficacy as potential alternatives are being scientifically explored. The seed oils of *Kigelia africana*, *Ximenia caffra* and *Mimusops zeyheri* have anti-proliferative effects on hormone-dependent (MCF-7) and cytotoxic effects on hormone-independent (MDA-MB-231) breast cancer cells. In this study, we determined if these seed oils reduce the thrombogenic ability of breast cancer cells by measuring the platelet surface expression of the activation-specific antigens CD62P and CD63. MDA-MB-231 and MCF-7 cells were pre-treated with the seed oils before being exposed to whole blood of human female volunteers. An increase in CD62P and CD63 expression following whole blood exposure to untreated breast cancer cells was observed. Treated MDA-MB-231 cells reduced CD62P and CD63 expression while treated MCF-7 cells increased CD62P and decreased CD63 expression. *Kigelia africana, Ximenia caffra* and *Mimusops zeyheri* seed oils are able to reduce the thrombogenic ability of MDA-MB-231 breast cancer cells.

Key words: breast cancer; thrombosis; seed oils; ethnomedicine

4.1. INTRODUCTION

The relationship between cancer and thrombosis was established in the 1900s by Armand Trousseau when he observed malignancy in some patients after they had suffered thrombotic episodes (Varki, 2007; Karimi and Cohan, 2010). Venous thromboembolism (VTE) is a common complication in cancer patients (Karimi and Cohan, 2010; Cooke *et al.*, 2013; Riedl *et al.*, 2014), with the likelihood of VTE occurrence being four- to seven- times higher in cancer patients than in non-cancer patients (Elyamany *et al.*, 2014). This high prevalence of thrombosis in cancer patients has been attributed to the activation of the patient's haemostatic system. Tumours can activate the host's haemostatic system through multiple mechanisms (Elyamany *et al.*, 2014). One such mechanism entails the production of tissue factor that has been shown to play an important role in the initiation of the coagulation process (Grignani and Maiolo, 2000). Tissue factor (TF) is a transmembrane glycoprotein that binds to factor VII/VIIa resulting in the activation of the extrinsic pathway of the clotting cascade to generate thrombin and eventually fibrin (Grignani and Maiolo, 2000; Kasthuri *et al*., 2009). Reports have shown that cancer cells have the ability to release vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Kasthuri *et al*., 2009; Riedl *et al*., 2014). These pro-angiogenic cytokines not only upregulate tissue factor production but also enhance the expression of endothelial cell adhesion molecules that activate localised clotting factors, thereby promoting thrombosis formation (Karimi and Cohan, 2010).

Platelets mainly function to regulate haemostasis (Plantureux *et al.*, 2018) and hence they are central to the formation of thrombi (Breet *et al.*, 2011; Yun *et al.*, 2016). These anucleate blood cell fragments (2-4 μm) generated from megakaryocytes in the bone marrow, contain a variety of granules and proteins that equip them for their function (Harrison and Cramer, 1993; Riedl *et al.*, 2014; Yun *et al.*, 2016; Plantureux *et al.*, 2018). For instance upon stimulation by TF, platelets release glycoproteins and coagulation factors from alpha granules as well as calcium ions, serotonin and ATP from dense granules to facilitate thrombus formation (Nash *et al*., 2002). Interestingly tissue factor-activated platelets also express P-selectin (CD-62P) and β-thromboglobulin, proteins shown to be important for tumour growth and metastasis (Goubran *et al.*, 2013, Lal *et al.*, 2013). CD62P (α-granule membrane protein) together with CD63, a lysosomal integral membrane protein, are often used for the detection of platelet activation in investigations using flow cytometry (Taylor *et al.*, 1995; Murakami *et al.*, 1996; Marquardt *et al.*, 2002).

The most common cancer in women worldwide is breast cancer (~ 25%) with 2.09 million cases and 627 000 deaths reported in 2018 (Bray *et al.*, 2018). Current treatment for breast cancer includes hormone therapy, chemotherapy (including neo adjuvant and adjuvant chemotherapy), radiotherapy and surgical removal of tumour tissue (Miller *et al.*, 2014; Rampurwala *et al.*, 2014; Elomrani *et al.*, 2015). Although chemotherapy in addition to surgery has proved useful in the treatment of breast cancer since it decreases the risk of recurrence and improves survival odds, it can cause severe side effects (Ochwang'i *et al.*, 2014). Chemotherapy, which induces apoptosis in highly proliferative cells (Payne and Miles, 2004), also targets healthy cells and has subsequently been implicated in increasing the risk of thrombosis (Haddad and Greeno, 2006; Falanga *et al.*, 2015). A thrombotic risk also exists for women on hormone replacement therapy (Sandset *et al.*, 2009). This risk is increased 1.5 to 7.1 fold when hormone therapy is used as adjuvant therapy in women with breast cancer following surgery (Deitcher and Gomes, 2004; Haddad and Greeno, 2006). It is therefore clear that current therapeutic strategies are not ideal and hence the search for better management approaches has to continue.

Besides present day cancer treatment being inadequate, it is also inaccessible due to unaffordable costs to many cancer patients. In developing countries, approximately 75% of women diagnosed with breast cancer are in clinical stages III and IV, whereas in North America 70% of newly diagnosed women with breast cancer are in clinical stages 0 and 1 (Coughlin and Ekwueme, 2009). Five-year survival comparative rates estimate 12% from different parts of Africa to almost 90% in North America (Youlden *et al*., 2012). Reasons for these huge discrepancies include cultural barriers, and the lack of infrastructure and resources for early detection, proper diagnosis and treatment (Coughlin and Ekwueme, 2009; Youlden *et al*., 2012; Bray *et al.*, 2018). As a result women with breast cancer in low- and middle-income countries are frequently misdiagnosed and/or receive insufficient treatment (Coughlin and Ekwueme, 2009). In these communities ethnomedicine has been explored as a potential alternative to help women with breast cancer (Ochwang'i *et al.*, 2014).

Ethnomedicine has historically been used for the prevention and treatment of various diseases (Park and Pezzuto, 2002). For instance Hodgkin's lymphoma and leukaemia are treated with compounds isolated from the Madagascar periwinkle plants (*Vinca rosea*) – Vinblastine and Vincristine (Moudi *et al.*, 2013; Khazir *et al.*, 2014), while Paclitaxel, originally extracted from the bark of the Pacific Yew, *Taxus brevifolia* Nutt, is frequently used in the treatment of ovarian and breast cancer (Priyadarshini and Keerthi-Aparajitha, 2012; Khazir *et al.*, 2014). However the use of some of these herbiceuticals has been controversial. A concern has been raised over

a possible Paclitaxel-induced pro-thrombotic risk as a number of reports of thrombotic episodes in cancer patients being treated with this chemotherapy plant alkaloid have been reported (Zhang, *et al*, 2016; Kim *et al.*, 2018).

Kigelia africana (K. africana), Mimusops zeyheri (M. zeyheri) and *Ximenia caffra (X. caffra)* are commonly found in southern Africa (Chivandi *et al.*, 2012a). Traditional healers use various parts of these trees for the treatment of a number of diseases (Chivandi *et al.*, 2012a). For example, a tea brewed from the powdered bark of *K. africana* has been shown to have analgesic and anti-inflammatory (Owolabi and Omogbai, 2007) and, more recently, anti-neoplastic (Naidoo *et al.*, 2013, Bello *et al.*, 2016) effects. Similarly the bark of *M. zeyheri* have been used to treat ulcers and wounds, while its roots have been used to treat candidiasis (Amusan *et al.*, 2002). Proliferation of murine RAW 264.7 macrophage cells are suppressed by extracts of the *X. caffra* leaf suggesting anti-inflammatory properties of this plant species (Zhen *et al.* 2015).

The health benefits of plants are not limited to extracts of leaf or bark material, but also includes that of seeds (Chivandi *et al.*, 2011a). Seeds of plants such as *Glycine max* (soyabean), *Gossipium hirstum* (cotton seed), *Helianthus annuus* (sunflower) and *Sesamun indicum* (sesame seed) contain essential amino acids, fatty acids and vitamins (Chivandi *et al.*, 2011a) that may afford some of the medicinal properties of seeds. Subsequently oils extracted from seeds have been shown to possess anti-inflammatory (Constantini *et al.*, 2014**)** and anti-proliferative (Vieira *et al.*, 2008; Seal *et al.*, 2012; Al-Sheddi *et al.*, 2015; Guo *et al.*, 2016) properties. Anti-cancer effects of some seed oils have been indicated by (i) a decrease in the production of VEGF by breast cancer cells treated with Pomegranate (*Punica granatum* L.) seed oil (Constantini *et al.*, 2014), (ii) inhibition of melanoma cell growth by *Pterodon pubescens* Benth. seed oil (Vieira *et al.*, 2008), (iii) the prevention of tumour cell progression through the cell cycle by *Litsea cubeba* and *Portulaca oleracea* seed oils (Seal *et al.*, 2012; Al-Sheddi *et al.*, 2015; Guo *et al.*, 2016), and (iv) the decrease of proliferation of human colon adenocarcinoma (Caco-2) cells by *K. africana, X. caffra* and *M. zeyheri* seed oils (Chivandi *et al.*, 2012b).

While the literature suggests seed oils of some plants to have prominent anti-tumour potential, data from our laboratory indicated that these results need to be interpreted with caution. We recently showed that *K. africana, M. zeyheri* and *X. caffra* seed oils, while having anti-proliferative effects on hormone-dependent breast cancer cells (MCF-7), exert cytotoxic effects on hormone-independent breast cancer cells (MDA-MB-231) (Gomes *et al.*, 2019). Given the

high dependency on ethnomedicines to manage diseases in low- to middle-income countries, it is evident that more research is required to affirm the medicinal benefit of seed oils. In the present study an investigation was conducted to determine if *K. africana*, *X. caffra* and *M. zeyheri* seed oils could reduce the thrombogenic ability of breast cancer cells by measuring the ability of these seed oils to inhibit breast cancer cell-induced activation of platelets. Two human breast cancer cell lines (MDA-MB-231 and MCF-7) were pre-treated with the respective seed oils prior to being exposed to whole blood of healthy human female volunteers. The anti-thrombogenic capacity of the seed oils were determined by measuring the platelet surface expression of the activation-specific antigens CD62P and CD63.

4.2. MATERIALS AND METHODS

4.2.1. Seed source

K. africana seeds were obtained from the ripe fruits from trees in Gutu District, Chitsa Communal area, Zimbabwe which is located at latitude and longitude of 19º41′S and 31º09′E respectively. It is characterized by granitic soils and has annual rainfall and temperature ranges of 650-800 mm and 20.5-30.0ºC, respectively (Chivandi, 2012a). *M. zeyheri* seeds were harvested from fruits off trees found in the Gokwe District, in North West Zimbabwe, in the Machakata Community Forest area of the Mapfungautsi Plateau which is located at latitude and longitude of 20º25′S and 28º29′E respectively. It is characterized by shallow sandy, lithosol soil and has an annual rainfall of +/- 609 mm and temperature of +/- 25.9ºC (Chivandi, 2012a). *X. caffra* seeds were collected from ripe fruit of the Zhombe District, Zimbabwe which is located at latitude and longitude of 14º45′S and 26º50′E respectively. The area has annual rainfall of 550 mm and a mean annual temperature of 26ºC (Chivandi, 2012a). The seeds from which the seed oils were extracted, were imported into the Republic of South Africa under the permit number P0039683 following confirmation of the trees' identities by the National Botanical Gardens of Zimbabwe.

4.2.2. Seed oil extraction and storage

Seeds were dehulled and crushed in a blender (Waring; Lasec Pty Ltd, Johannesburg, South Africa). Hexane was used to extract the oil from each of the seed meals as described by Yamasaki *et al.* (2013). In short, 800 mL of hexane was added to 160 g of each seed meal and then mixed overnight using a magnetic stirrer (VELP Scientifica, Lombardy – Italy) at room temperature. The mixture was then passed through filter paper (Whatman No.1) and the hexane

evaporated off using a rotary vacuum at 65^{o}C. The extracted oils were kept in brown sample bottles at -20 ^{o}C before use (Yamasaki *et al.*, 2013).

4.2.3. Cell culture

A Human Ethics Waiver Certificate (W-CJ-150422-1) was obtained from the Human Research Ethics Committee, University of the Witwatersrand, Johannesburg. The two human breast cancer cell lines (MDA-MB-231 and MCF-7) used in the experiments were sourced from ATCC (Virginia, USA) and donated by Dr. R Duarte (School of Clinical Medicine, University of the Witwatersrand). MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 5% foetal bovine serum (FBS) and 100 µg/mL streptomycin, 100 units/mL penicillin. MDA-MB-231 cells were cultured in DMEM/Ham's F-12 nutrient mixture with 10% FBS and 100 µg/mL streptomycin, 100 units/mL penicillin. Both cell lines were incubated at 37^{o}C in a humidified atmosphere of 5% CO_2.

4.2.4. Seed oil concentrations and treatment of cells

Stock solutions (10 mg/mL) of the seed oils were prepared in 100% absolute alcohol and diluted in culture medium to reach the desired concentration (120 µg/mL). The concentration of absolute alcohol in the culture medium was not more than 5%. The control consisted of 100% absolute alcohol diluted in culture medium to obtain a 5% final solution.

Confluent cells were harvested in 0.25% trypsin/EDTA. MCF-7 cells (P44) and MDA-MB-231 cells (P19) were plated in 24 well plates at 6.3 x 10^4 cells/well and 3.1 x 10^4 cells/well, respectively in their specific media. Both cell lines were incubated at 37^{o}C in a humidified atmosphere of 5% CO_2 for 24 h. The cell culture media from each well was then removed and cells rinsed with Dulbecco's phosphate-buffered saline (DPBS). Cells were treated with 200 µL of the seed oil at a concentration of 120 µg/mL in matched culture media and incubated at 37^{o}C in a humidified atmosphere of 5% CO_2 for 48 h. In all experiments untreated and diluent treated cells were included as controls.

4.2.5. Blood collection

Ethical clearance was obtained from the University of the Witwatersrand's Human Ethics Committee (Clearance Certificate Number: M160211) for the collection of blood from healthy female participants between the ages of 19-30 years (n = 8) (23.6±2.8 years, mean age ± SD).

Exclusion criteria for participants included pregnancy, contraceptive use, smoking, previous history of cancer, presence of autoimmune diseases, immunodeficiency and the use of anti-platelet/anti-coagulation medication. After giving informed consent, blood was collected from participants on days 5.4±2.7 (mean days ± SD) of their menstrual cycle. Circulating oestrogen and progesterone levels are low between days 1 and 10 of the menstrual cycle, consequently the potential effects of these hormones on breast cancer cells are limited (Pather *et al*., 2019).

Peripheral blood was obtained by venipuncture into 0.109M (3.2%) sodium citrate vacutainers (Lasec, South Africa). The first 2 mL of blood drawn was discarded to minimise the inclusion of mechanically activated platelets in the study (Leytin *et al.*, 2000). Blood samples were processed within 1 h of collection.

4.2.6. Blood co-culture and platelet labelling

After the 48 h incubation period, media were removed from the wells. The remaining cells were then incubated with 200 µL whole blood for 2.5 min. For analysis of platelets, 100 µL whole blood was removed from the wells and incubated in 1 mL cold ammonium chloride (NH_4Cl) erythrocyte lysis buffer for 10 min at room temperature. Samples were subsequently centrifuged at 200 × g for 5 min and the supernatant discarded. The pellet was gently resuspended in 150 µL cold Tyrode's buffer and incubated with their respective pre-titrated antibodies for 15 min: 10 µL allophycocyanin (APC)-conjugated mouse anti-human CD41a (559777; BD Pharmingen) was used as a tagging antibody for platelets whilst 10 µL fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD62P (555523; BD Pharmingen) and 2.5 µL phycoerythrin cyanine (PE-Cy) mouse anti-human CD63 (561982; BD Pharmingen) were used to detect platelet activation. The samples were then fixed with 1% paraformaldehyde (PFA) for 10 min and subsequently centrifuged at 200 × g for 5 min and the supernatant discarded. The pellet was again gently resuspended in 500 µL cold Tyrode's buffer and transferred into tubes appropriate for use in flow cytometry. Platelets activated by 0.1U thrombin were used as a positive control (Augustine *et al.*, 2016; Pather *et al.*, 2019).

4.2.7. Flow cytometry

Samples were incubated on ice before data acquisition on an LSRFortessa (BD Biosciences) equipped with four lasers (Blue, Red, Violet and UV). Events (100 000 events per sample) were acquired and pre-analysed using the BD Biosciences FACSDiva software. The events were recorded at a forward scatter voltage of 240V and a side scatter voltage of 210V. Compensation

controls included unlabelled whole blood samples and pre-titrated single antibody staining with CD41a-APC, CD62P-FITC and CD63 PE-Cy to determine the levels of autofluorescence and fluorescence overlap. All samples were analysed in duplicate. Exported Flow cytometry Standard (FCS) files were analysed using FlowJo V10 (FlowJo, LLC, Ashland, OR 97520, USA).

Results are expressed as percentage change in CD62 and CD63 expression after exposure to non-treated/treated breast cancer cells relative to whole blood alone and was calculated as follows:

$$\%\ change = \frac{Frequency\ of\ CD62P\ or\ CD63\ positive\ platelets\ from\ WB\ or\ WB\ exposed\ to\ untreated\ or\ treated\ breast\ cancer\ cells}{Frequency\ of\ CD62P\ or\ CD63\ positive\ platelets\ from\ whole\ blood\ (WB)} X\ 100$$

4.2.8. Statistical data analysis

The individual data of each participant is presented together with the average for the respective groups which is depicted as mean ± SD. Data were analysed using SPSS (IBM SPSS Statistics for Windows, Version 25.0, Chicago, USA). All the numerical values were tested for normality using the Shapiro-Wilk Test. Differences between groups were evaluated using the two-way analysis of variance (ANOVA). A p value of <0.05 was considered significant.

4.3. RESULTS

4.3.1. Flow Cytometry

The gating strategy to detect platelet activation using representative data is shown in Figure 4.1.

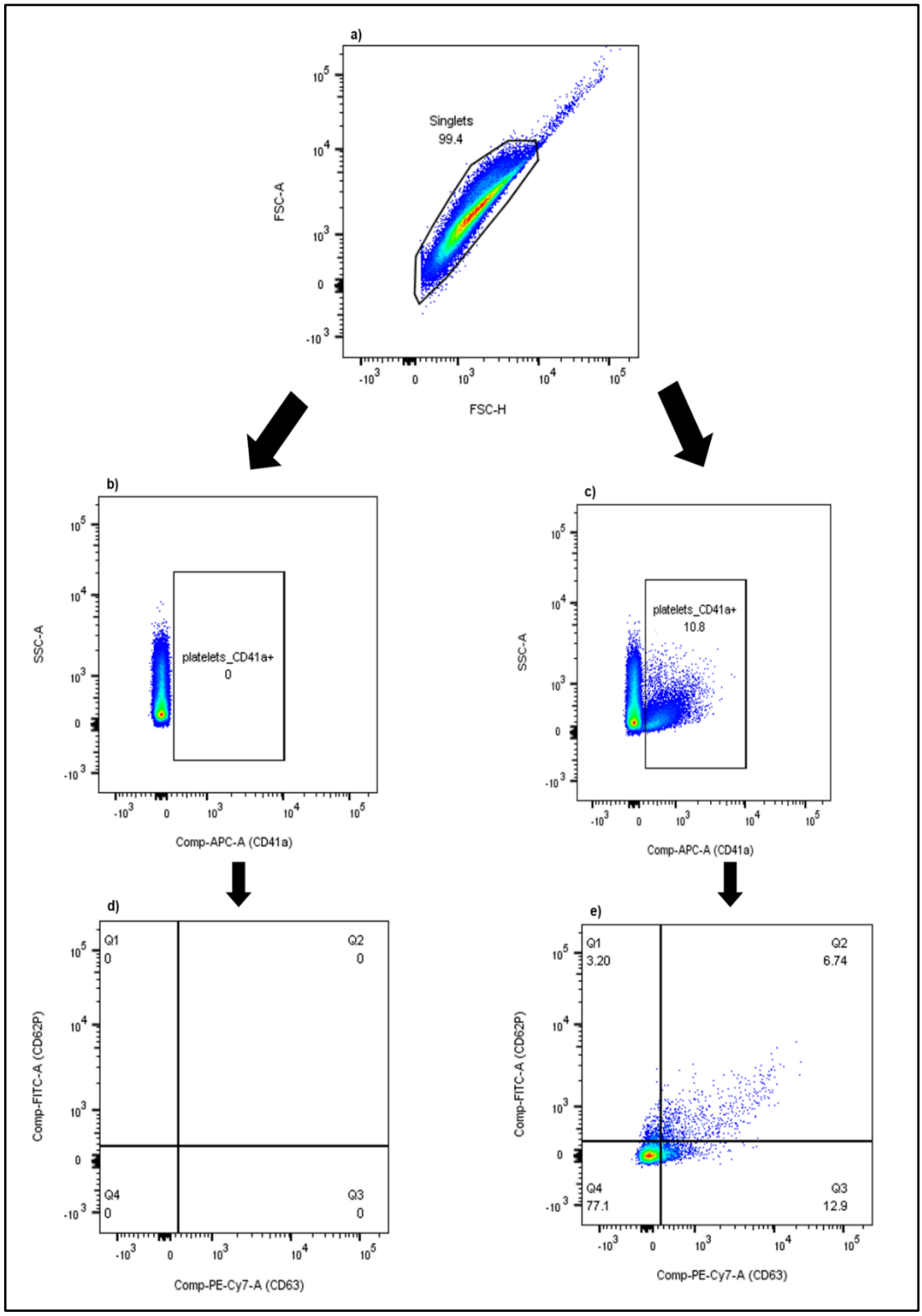

Figure 4.1: Gating strategy used to detect platelet activation using unstained (a, b, d) and stained (c, e) whole blood. (a) A singlet population of whole blood was selected using forward scatter height versus forward scatter area density plot. To discriminate platelets from other cells and debris, a single platelet gate was positioned on the basis of side scatter and platelet marker (APC-conjugated mouse anti-human CD41a) (b & c). Platelet activation was then determined using FITC-conjugated mouse anti-human CD62P and phycoerythrin cyanine (PE-Cy) mouse anti-human CD63 antibodies as shown in the quadrant plot in (d) and (e).

Using representative data, scatter plots showing the expression of platelet activation markers after whole blood was exposed to untreated and treated breast cancer cells (MDA-MB-231), are shown in Figure 4.2.

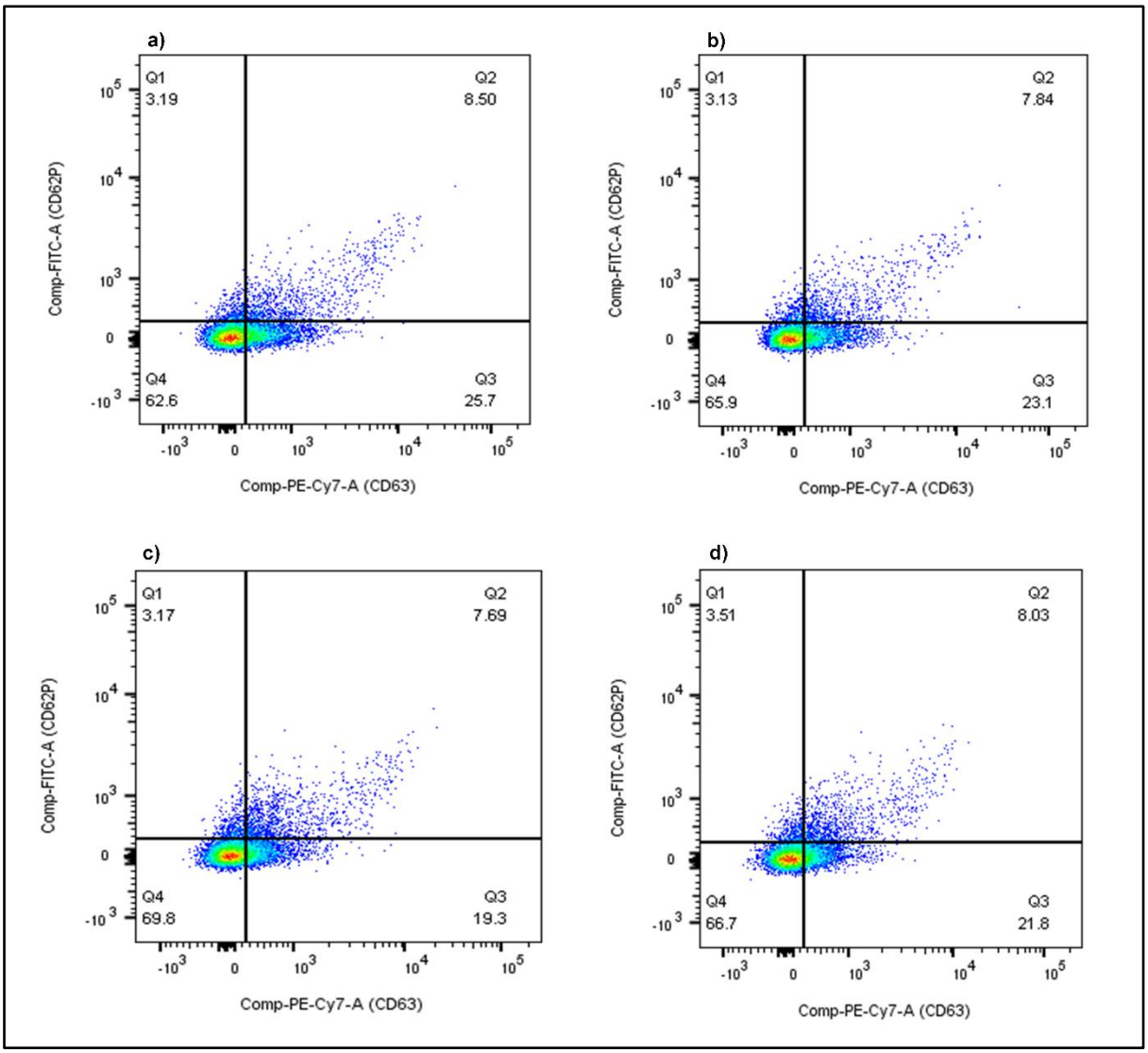

Figure 4.2: Scatter plots of the expression of platelet activation markers after exposure to untreated and treated MDA-MB-231 breast cancer cells. (a) Whole blood exposed to untreated cells, (b) whole blood exposed to cells treated with KA seed oil, (c) whole blood exposed to cells treated with XC seed oil and (d) whole blood exposed to cells treated with MZ seed oil.

4.3.2. Percentage change of CD62P expression after whole blood was exposed to untreated and treated MDA-MB-231 breast cancer cells

Out of eight participants, platelets of five participants (P1, P3, P4, P6 and P8) showed an increase in the percentage change of CD62P expression after their whole blood was exposed to untreated MDA-MB-231 breast cancer cells (Figure 4.3a), while that of one participant (P5)

was reduced and two others (P2 and P7) basically remained unchanged (Figure 4.3a). The increase in the percentage change of CD62P expression in the five participants was not observed after their whole blood was exposed to KA-treated MDA-MB-231 breast cancer cells (Figure 4.3b), while the percentage change of CD62P expression was increased in P5 and continued to be unchanged in P2 and P7. As a group, the average percentage change of CD62P expression increased by 24.45% when the whole blood was exposed to untreated MDA-MB-231 breast cancer cells, but was reduced when whole blood was exposed to KA-treated MDA-MB-231 breast cancer cells. However these changes did not reach statistical significance.

Similarly, the percentage change of CD62P expression levels that were previously high in the five participants following exposure of their whole blood to untreated MDA-MB-231 breast cancer cells (Figure 4.3a), were decreased when their whole blood was exposed to XC-treated MDA-MB-231 breast cancer cells (Figure 4.3c). Interestingly P5 that previously showed a reduced percentage change of CD62P expression when exposed to untreated MDA-MB-231 breast cancer cells, displayed a further reduction when exposed to XC-treated MDA-MB-231 breast cancer cells (Figure 4.3c), while P2 and P7 that exhibited an unchanged percentage change of CD62P expression when exposed to untreated MDA-MB-231 breast cancer cells, showed a differential response to XC-treated MDA-MB-231 breast cancer cells with P2 showing an increase and P7 a decrease in the percentage change CD62P expression (Figure 4.3c). On average whole blood exposure to untreated MDA-MB-231 breast cancer cells led to an increase in the percentage change of CD62P expression levels that were not seen when the whole blood was exposed to XC-treated MDA-MB-231 breast cancer cells (Figure 4.3c).

Exposure of the whole blood of the participants to MZ-treated MDA-MB-231 breast cancer cells, yielded comparable results with P1, P3, P4, P6 and P8 showing reduced, P2 unchanged, and P5 and P7 a further reduction in the percentage change of CD62P expression (Figure 4.3d). On average, the percentage change of CD62P expression of the group decreased from 124.45% (the percentage change of CD62P expression from whole blood exposed to untreated MDA-MB-231 breast cancer cells) to 79.47% after whole blood exposure to MZ-treated MDA-MB-231 breast cancer cells (Figure 4.3d).

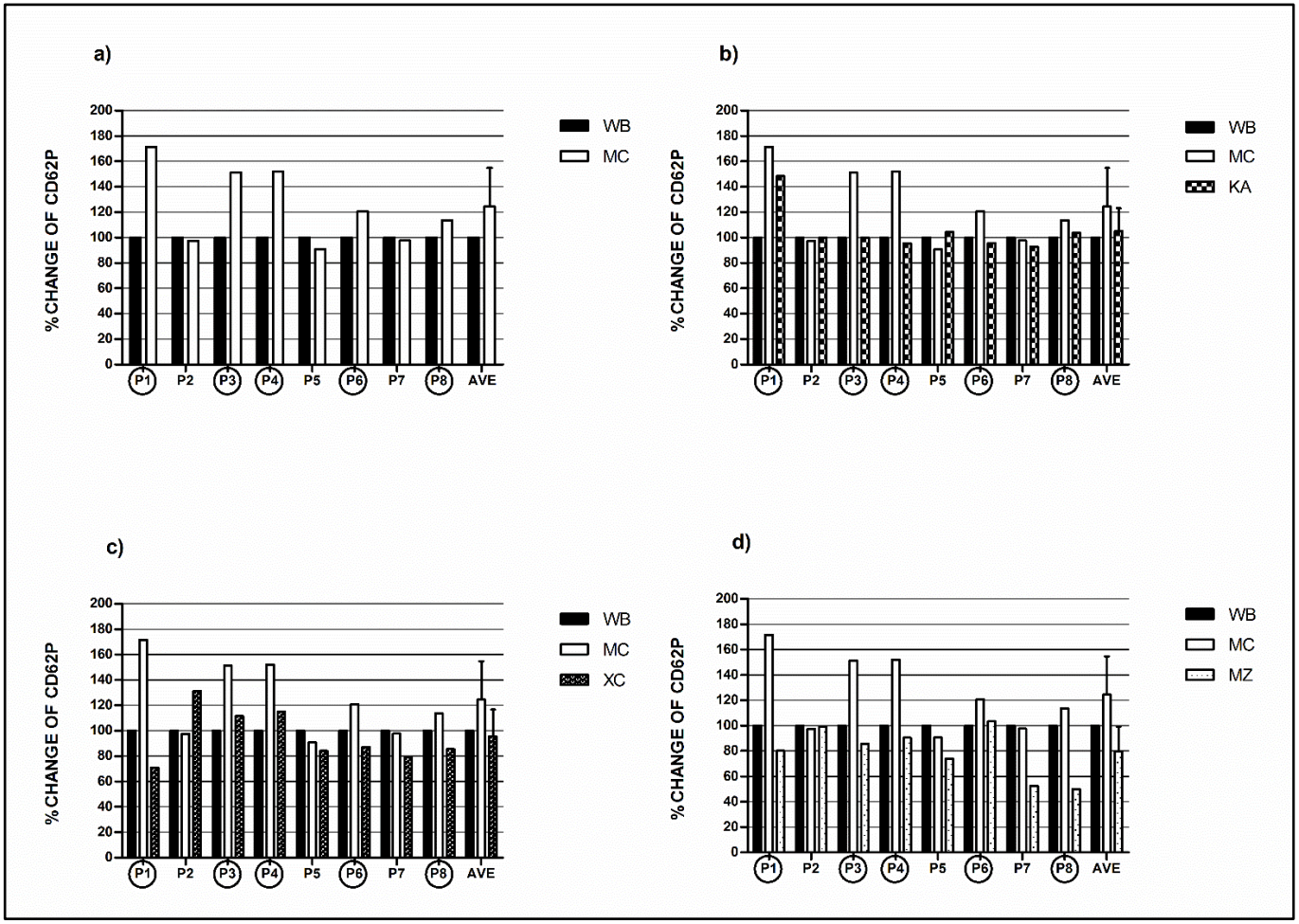

Figure 4.3: Percentage change of the platelet activation marker, CD62P, of each participant's whole blood sample after exposure to untreated (a) and treated MDA-MB-231 breast cancer cells (b, c, d). WB = whole blood only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil. P1, P2, P3 ... = Participant 1, Participant 2, Participant 3, etc. Encircled participants indicate an increase in CD62P expression after their WB was exposed to untreated MDA-MB-231 breast cancer cells.

The box plot shown in Figure 4 shows the pooled data of all the participants' response to the platelet activation marker CD62P to untreated and treated MDA-MB-231 breast cancer cells. Overall, MZ-treated MDA-MB-231 breast cancer cells had a decrease in percentage change of CD62P expression.

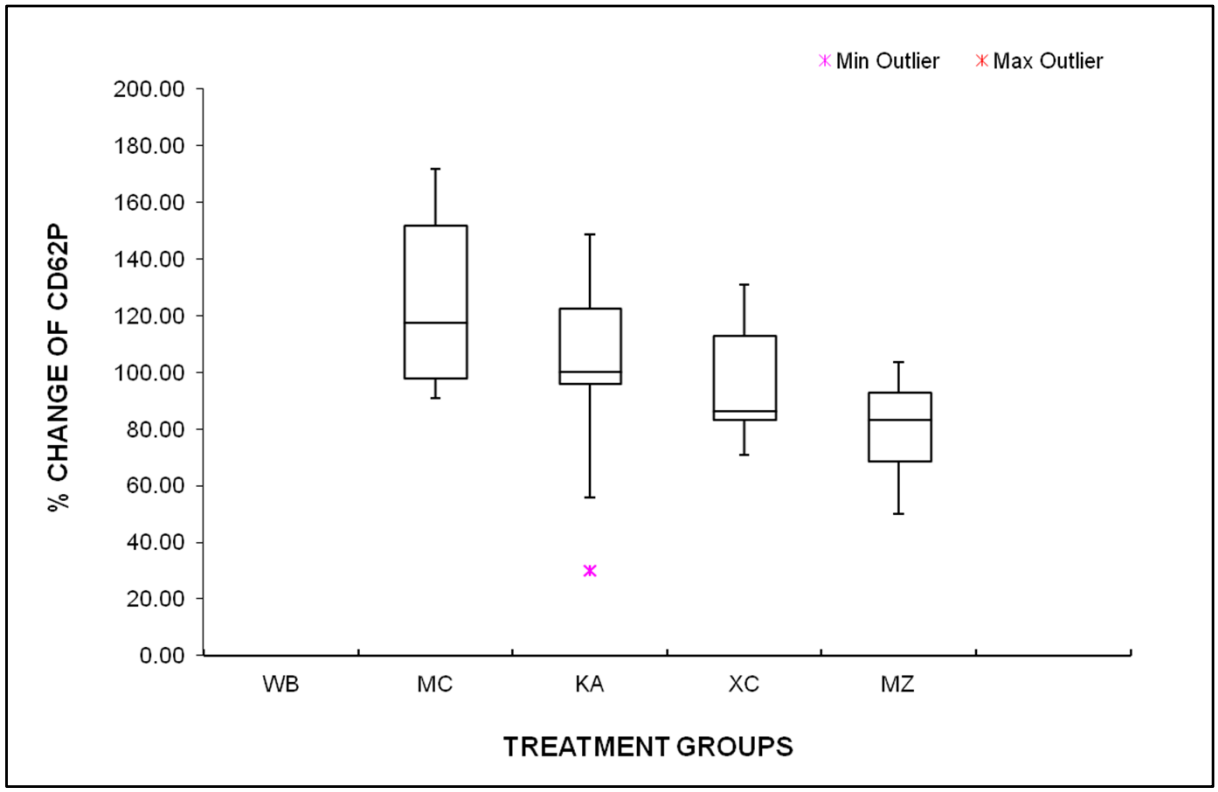

Figure 4.4: Box plot of pooled data of platelet activation marker CD62P data after exposure of whole blood samples to untreated and treated MDA-MB-231 breast cancer cells. WB = whole blood only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil.

4.3.3. Percentage change of CD62P expression after whole blood was exposed to untreated and treated MCF-7 breast cancer cells

Out of the same eight participants, the percentage change of CD62P expression of four participants increased after whole blood exposure to untreated MCF-7 breast cancer cells relative to whole blood alone (P3, P4, P5 and P6), while that of two participants (P1 and P2) were unchanged and another two (P7 and P8) were decreased (Figure 4.5a). The increase in the percentage change of CD62P expression observed in participants P3, P4, P5 and P6, was not evident after whole blood was exposed to KA-treated MCF-7 breast cancer cells (Figure 4.5b). The percentage change of CD62P expression was unchanged in P1 and decreased in P2 when

their whole blood was exposed to KA-treated MCF-7 breast cancer cells. In contrast, the percentage change of CD62P expression in P7 and P8 was dramatically stimulated when their whole blood was exposed to KA-treated MCF-7 breast cancer cells. As a group, the average percentage change of CD62P expression was increased when the whole blood was exposed to untreated and KA-treated MCF-7 breast cancer cells. This increase was found not to be statistically significant.

The percentage change of CD62P expression levels that were previously high in the four participants following exposure of their whole blood to untreated MCF-7 breast cancer cells (Figure 4.5a), were decreased (with the exception of P6) when their whole blood was exposed to XC-treated MCF-7 breast cancer cells (Figure 4.5c). There was an increase in the percentage change of CD62P expression for P1, P6, P7 and P8 while the percentage change of CD62P expression remained unchanged for P2 when their whole blood was exposed to XC-treated MCF-7 breast cancer cells. As a group, the average percentage change of CD62P expression increased by 9.35% after whole blood exposure to XC-treated MCF-7 breast cancer cells. This increase was found not to be statistically significant.

The percentage change of CD62P expression levels that were previously high in the four participants following exposure of their whole blood to untreated MCF-7 breast cancer cells (Figure 4.5a), were decreased (with the exception of P6) when their whole blood was exposed to MZ-treated MCF-7 breast cancer cells (Figure 4.5d). There was an increase in the percentage change of CD62P expression for P1, P6, P7 and P8 while the percentage change of CD62P expression remained unchanged for P2 when their whole blood was exposed to MZ-treated MCF-7 breast cancer cells. As a group, the average percentage change of CD62P expression increased by 22.45% after whole blood exposure to MZ-treated MCF-7 breast cancer cells.

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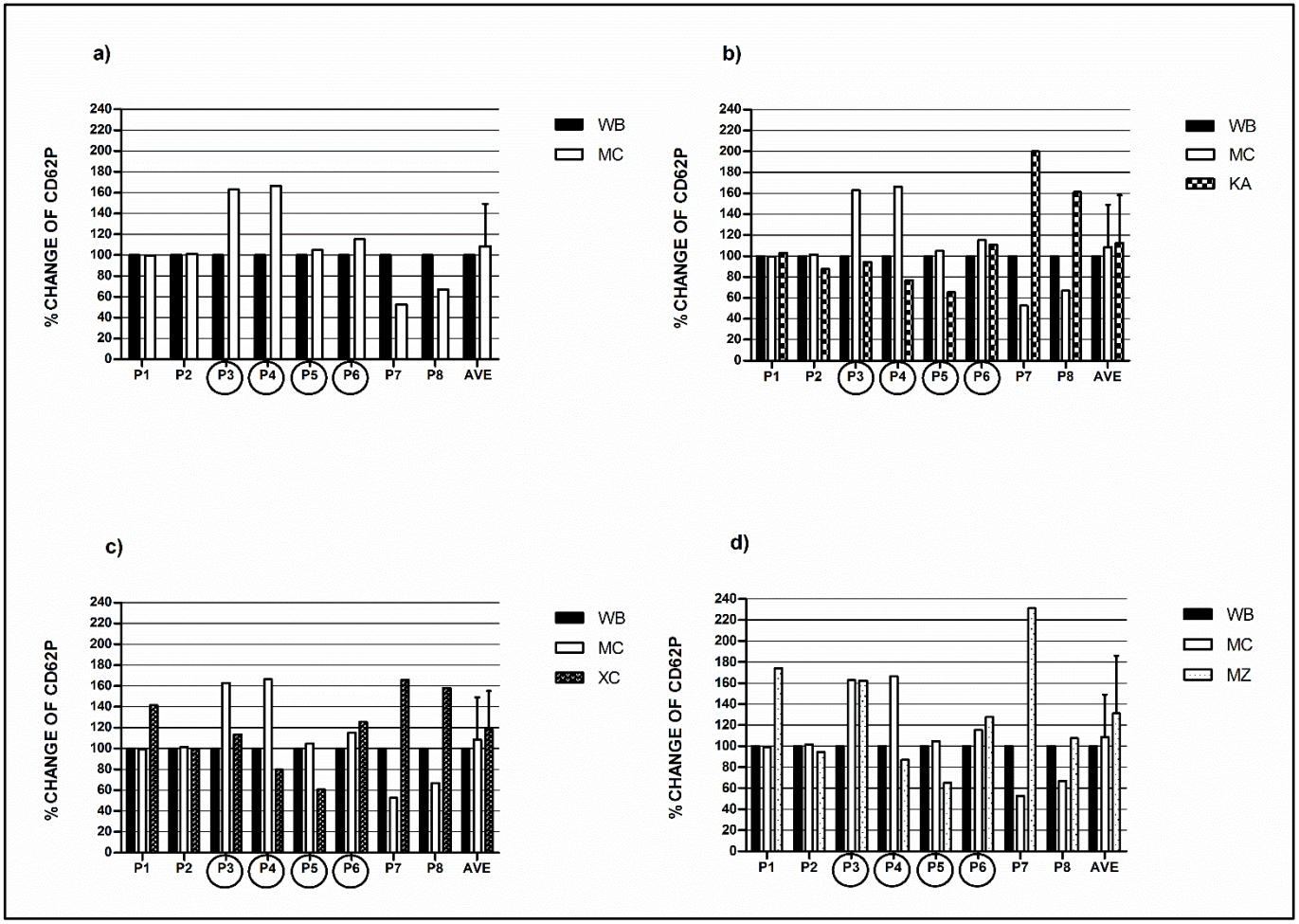

Figure 4.5: Percentage change of the platelet activation marker, CD62P, of each participant, as well as the group average, after their whole blood sample was exposed to untreated (a) and treated MCF-7 breast cancer cells (b, c, d). WB = whole blood only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil. P1, P2, P3 ... = Participant 1, Participant 2, Participant 3, etc. Encircled participants indicate an increase in CD62P expression after their WB was exposed to untreated MCF-7 breast cancer cells.

The box plot shown in Figure 6 shows the pooled data of all the participants' response to the platelet activation marker CD62P to untreated and treated MCF-7 breast cancer cells. Overall, MZ-treated MCF-7 breast cancer cells had an increase in percentage change of CD62P expression.

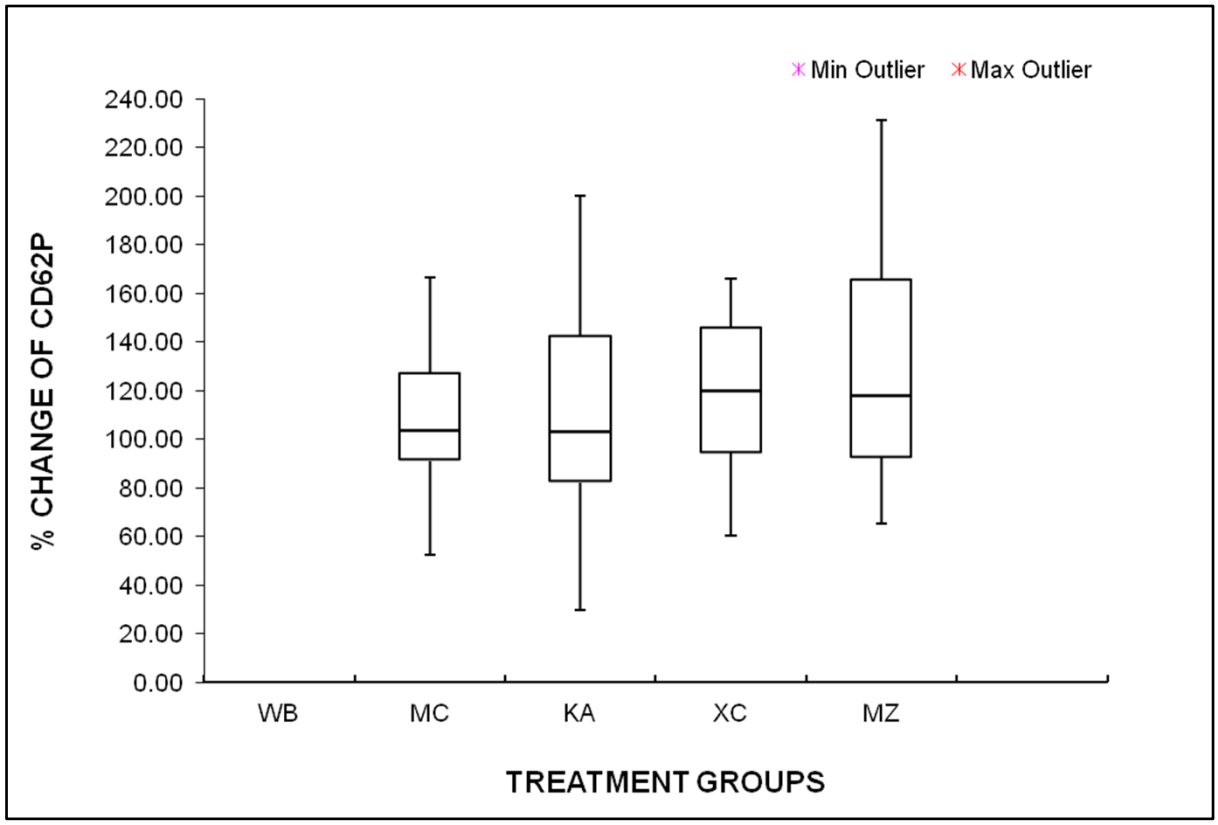

Figure 4.6: Box plot of pooled platelet activation marker CD62P after exposure of whole blood samples to untreated and treated MCF-7 breast cancer cells. WB = whole blood only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil.

4.3.4. Percentage change of CD63 expression after whole blood was exposed to untreated and treated MDA-MB-231 breast cancer cells

Investigating the percentage change of CD63 expression in the same eight participants showed that four participants had an increase in the percentage change of CD63 expression levels after whole blood exposure to untreated MDA-MB-231 breast cancer cells relative to whole blood alone (P5, P6, P7 and P8), while that of one participant (P2) was unchanged and another three (P1, P3 and P4) were decreased (Figure 4.7a). The increase in the percentage change of CD63 expression in the four participants was not observed after their whole blood was exposed to KA-treated MDA-MB-231 breast cancer cells (Figure 4.7b) while the percentage change of CD63 expression was increased in P2 and P3 but decreased in P1 and P4. As a group, the

average percentage change of CD63 expression increased when the whole blood was exposed to untreated MDA-MB-231 breast cancer cells but was reduced when the whole blood was exposed to KA-treated MDA-MB-231 breast cancer cells.

Similarly, the percentage change of CD63 expression levels that were previously high in the four participants following exposure of their whole blood to untreated MDA-MB-231 breast cancer cells (Figure 4.7a), were decreased when their whole blood was exposed to XC-treated MDA-MB-231 breast cancer cells, while the percentage change of CD63 expression was increased for P1, P2 and P3 but decreased for P4 (Figure 4.7c). On the average, whole blood exposure to untreated MDA-MB-231 breast cancer cells led to an increase in the percentage change of CD63 expression levels that were not seen when the whole blood was exposed to XC-treated MDA-MB-231 breast cancer cells (Figure 4.7c).

The percentage change of CD63 expression levels that were previously high in the four participants following exposure of their whole blood to untreated MDA-MB-231 breast cancer cells (Figure 4.7a), were decreased (with the exception of P7) when their whole blood was exposed to MZ-treated MDA-MB-231 breast cancer cells (Figure 4.7d). There was an increase in the percentage change of CD63 expression for P2, P3 and P7, while the percentage change of CD63 expression was decreased for P1 when their whole blood was exposed to MZ-treated MDA-MB-231 breast cancer cells. As a group, the average percentage change of CD63 expression decreased after whole blood exposure to MZ-treated MDA-MB-231 breast cancer cells.

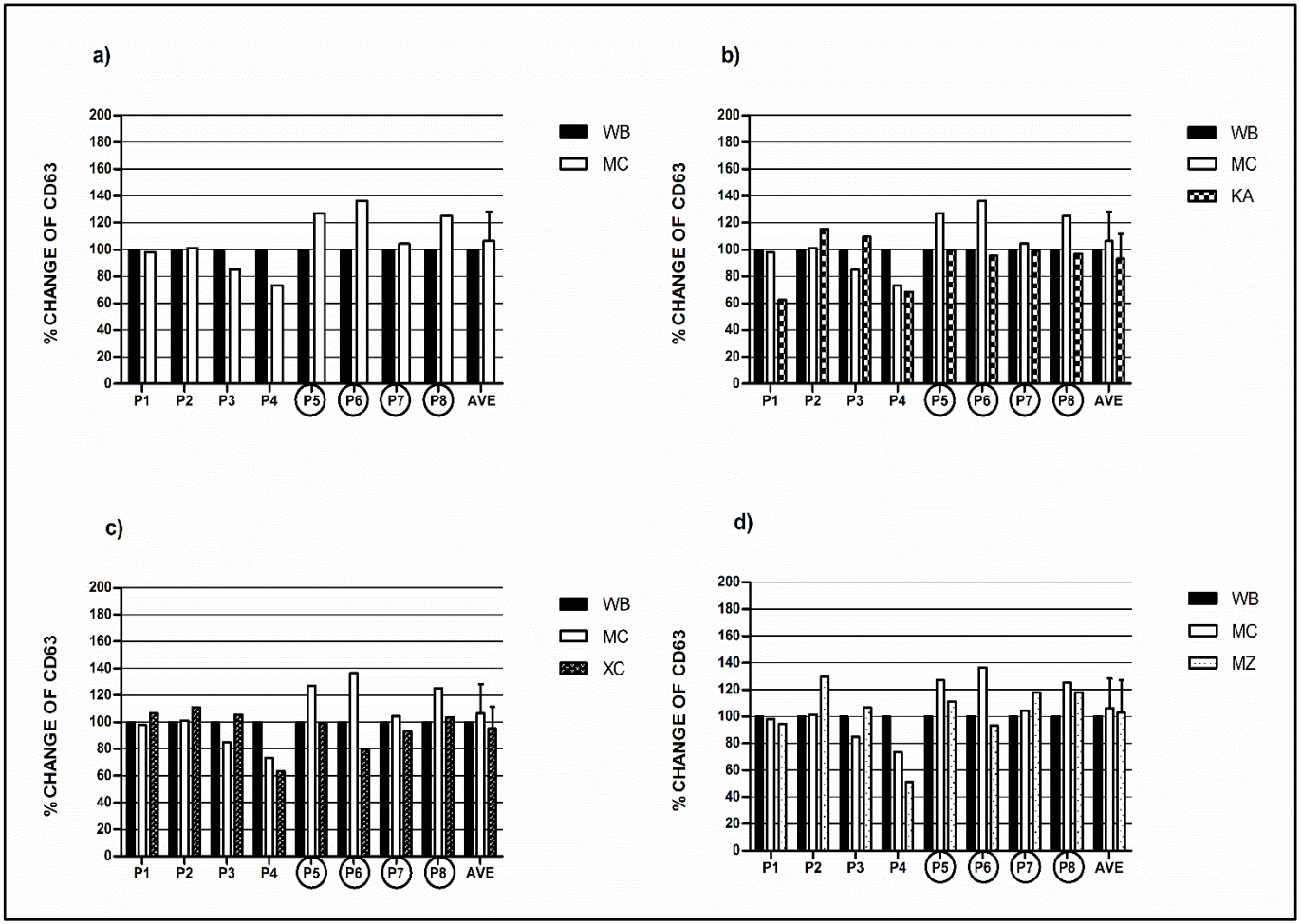

Figure 4.7: Percentage change of the platelet activation marker, CD63, of each participant, as well as the group average, after their whole blood sample was exposed to untreated (a) and treated MDA-MB-231 breast cancer cells (b, c, d). WB = whole blood only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil. P1, P2, P3 ... = Participant 1, Participant 2, Participant 3, etc. Encircled participants indicate an increase in CD63 expression after their WB was exposed to untreated MDA-MB-231 breast cancer cells.

The box plot shown in Figure 8 shows the pooled data of all the participants' response to the platelet activation marker CD63 to untreated and treated MDA-MB-231 breast cancer cells. Overall, KA-treated MDA-MB-231 breast cancer cells had a decrease in percentage change of CD63 expression.

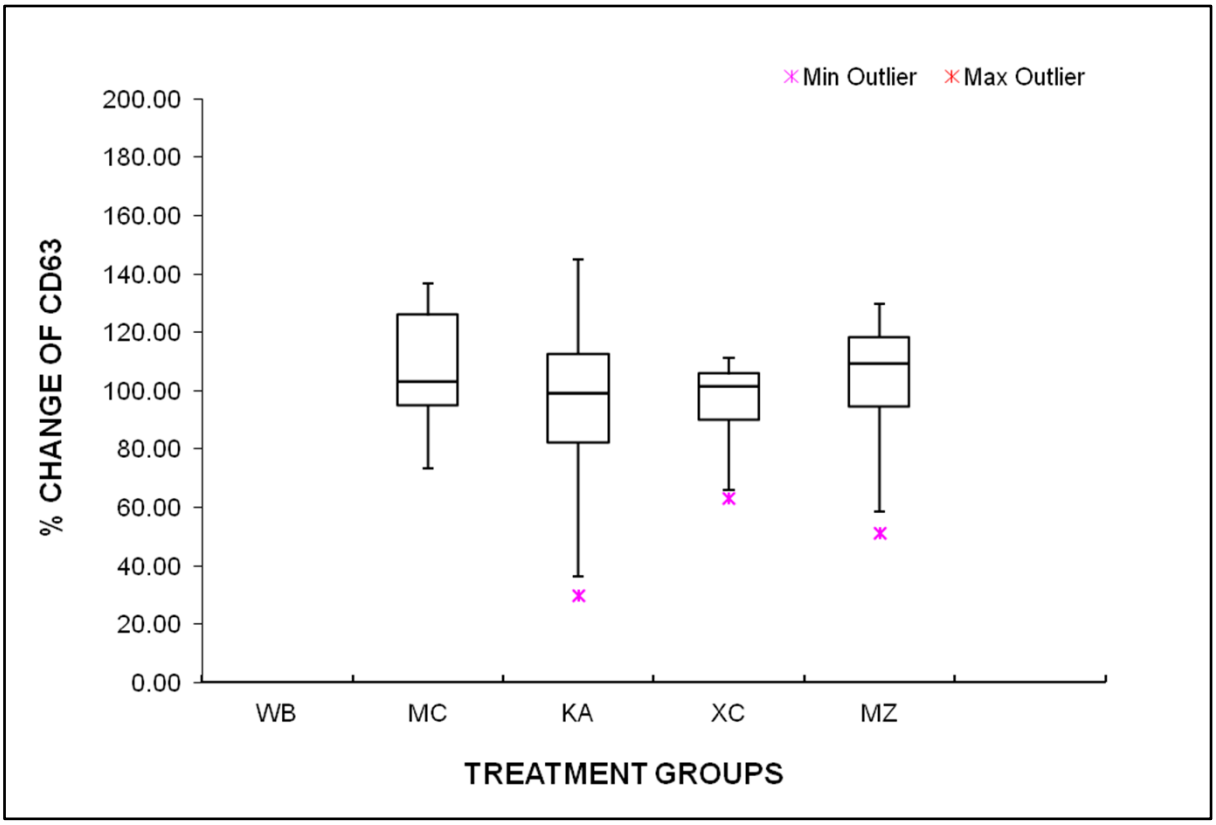

Figure 4.8: Box plot of pooled platelet activation marker CD63 data after exposure of whole blood samples to untreated and treated MDA-MB-231 breast cancer cells. WB = whole blood only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil.

4.3.5. Percentage change of CD63 expression after whole blood was exposed to untreated and treated MCF-7 breast cancer cells

Out of the same eight participants, platelets of five participants (P1, P2, P6, P7 and P8) showed an increase in the percentage change of CD63 expression after their whole blood was exposed to untreated MCF-7 breast cancer cells (Figure 4.9a) while the percentage change of CD63 expression remained unchanged in one participant (P3) and decreased in two other participants (P4 and P5). The increase in the percentage change of CD63 expression in the five participants was not observed after their whole blood was exposed to KA-treated MCF-7 breast cancer cells (Figure 4.9b) while the percentage change of CD63 expression was increased in P4, the percentage change of CD63 expression remained unchanged for P5 and decreased for P3. As a

group, the average percentage change of CD63 expression increased when whole blood was exposed to untreated MCF-7 breast cancer cells but was reduced when whole blood was exposed KA-treated MCF-7 breast cancer cells.

Similarly, a decrease in the percentage change of CD63 expression was observed for these participants (P1, P2, P6, P7 and P8) including P3, while the percentage change of CD63 expression was increased in P4 and P5 after their whole blood was exposed to XC-treated MCF-7 breast cancer cells (Figure 4.9c). On average, the percentage change of CD63 expression decreased after whole blood exposure to XC-treated MCF-7 breast cancer cells (Figure 4.9c).

Exposure of the whole blood of the participants to MZ-treated MCF-7 breast cancer cells, yielded comparable results with P1, P2, P6, P7 and P8 showing a reduced percentage change of CD63 expression including P3, while P4 and P5 showed an increase in the percentage change of CD63 expression (Figure 4.9d). On average, the percentage change of CD63 expression of the group decreased after whole blood exposure to MZ-treated MCF-7 breast cancer cells (Figure 4.9d).

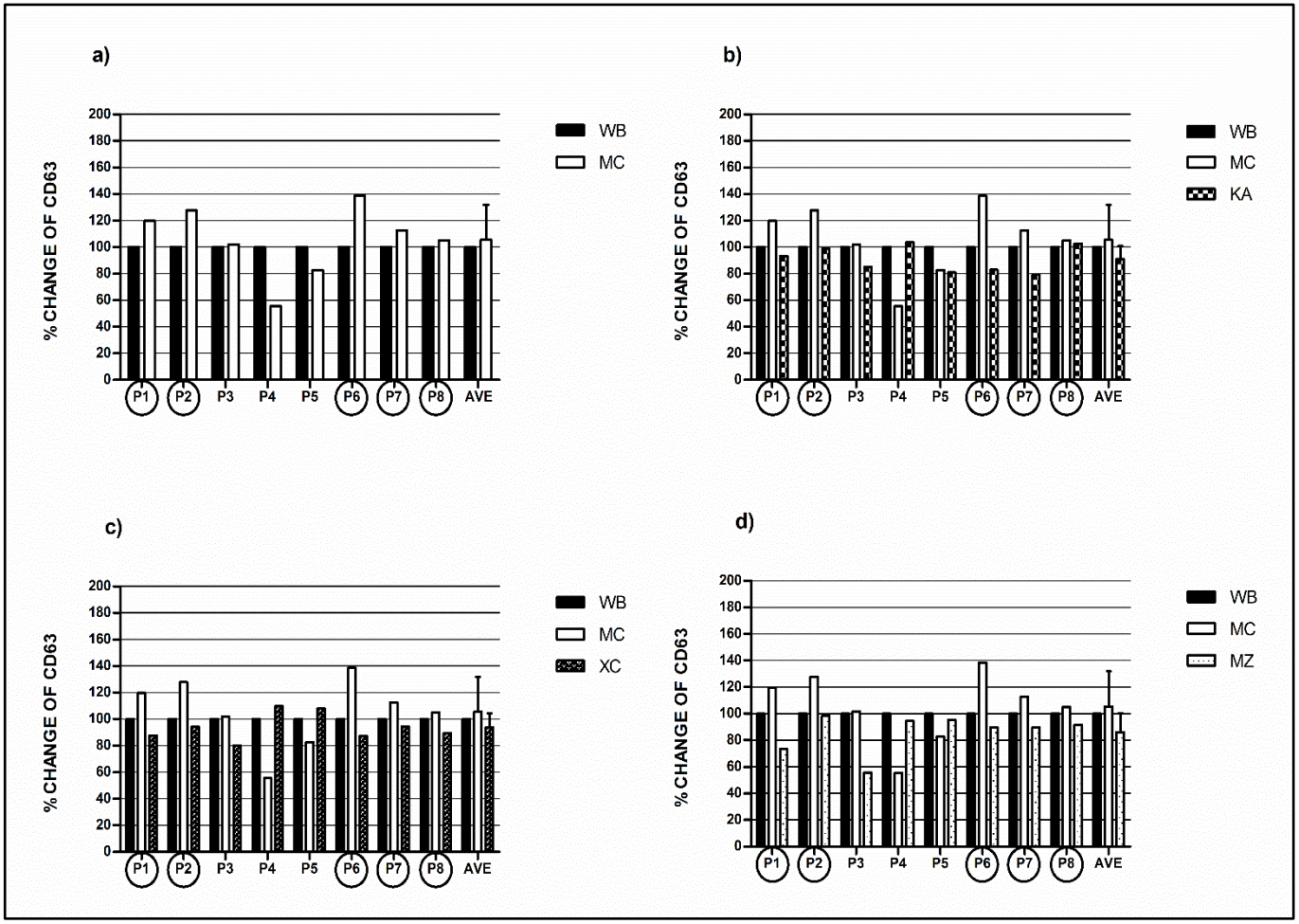

Figure 4.9: Percentage change of the platelet activation marker, CD63, of each participant, as well as the group average, after their whole blood sample was exposed to untreated (a) and treated MCF-7 breast cancer cells (b, c, d). WB = whole blood only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil. P1, P2, P3 ... = Participant 1, Participant 2, Participant 3, etc. Encircled participants indicate an increase in CD63 expression after their WB was exposed to untreated MCF-7 breast cancer cells.

The box plot shown in Figure 10 shows the pooled data of all the participants' response to the platelet activation marker CD63 to untreated and treated MCF-7 breast cancer cells. Overall, MZ-treated MCF-7 breast cancer cells had a decrease in percentage change of CD63 expression.

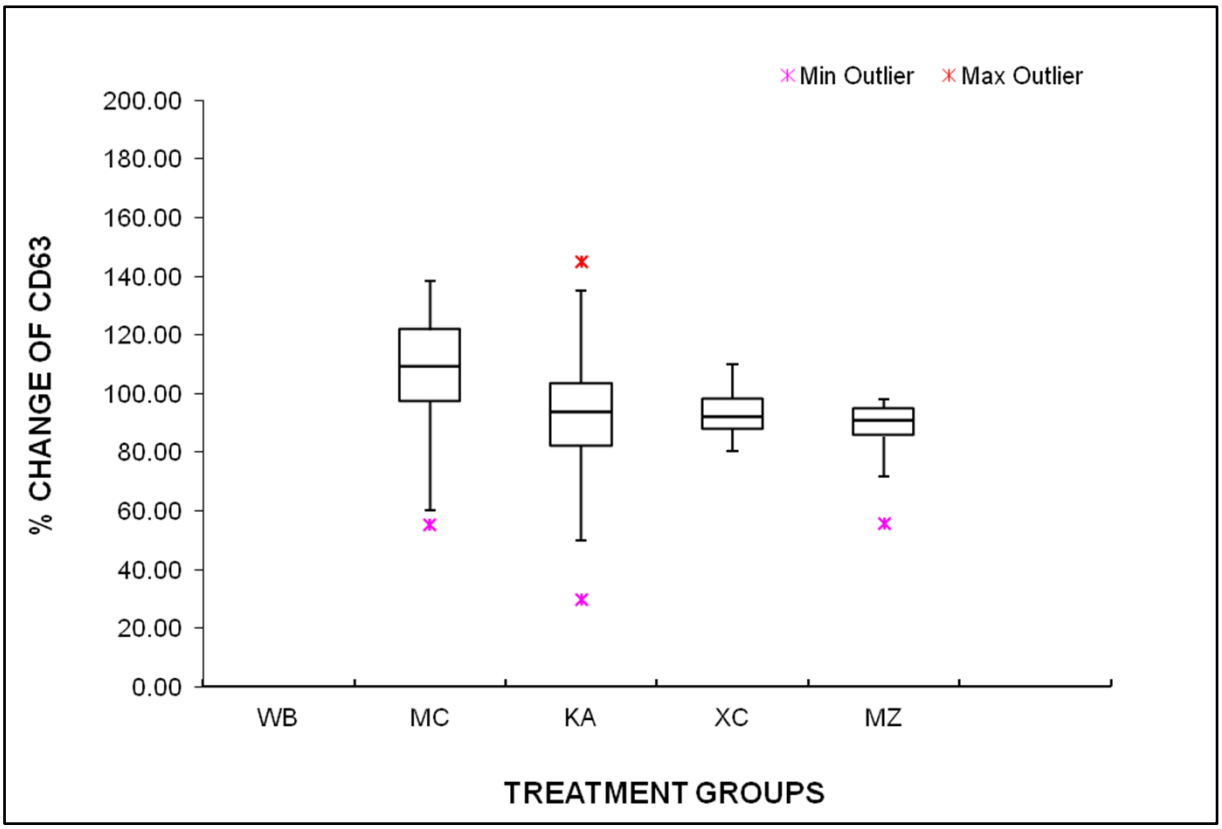

Figure 4.10: Box plot of pooled platelet activation marker CD63 data after exposure of whole blood samples to untreated and treated MCF-7 breast cancer cells. WB = whole blood only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil.

4.4. DISCUSSION

Cancer patients, including breast cancer patients, live in a hypercoagulable state, either because cancer cells produce and secrete TF, cancer procoagulant and fibrinolytic proteins (Lee, 2002; Karimi and Cohen, 2010; Ruf, 2012; Falanga *et al.*, 2015) or due to the treatments cancer patients receive (Deitcher and Gomes, 2004; Haddad and Greeno, 2006; Oppelt *et al.*, 2015). Platelets are activated by cancer cells either by the above-mentioned secreted factors or by the adhesion of the cancer cells to circulating platelets (Bambace and Holmes, 2011; Riedl *et al.*, 2014; Plantureux *et al.*, 2018). Once activated, important granule membrane molecules, such as P-selectin (CD62P) and CD63 become evident on the platelet cell surface which subsequently aid tumour growth and metastasis (Sedlmayr *et al.*, 1996; Goubran *et al.*, 2013,

Lal *et al.*, 2013). Consequently, platelets play an important role in cancer associated thrombosis and cancer spread (Goubran *et al.*, 2013; Zarà *et al.* 2018).

In this preliminary investigation, we found differential expression of the platelet activation markers, CD62P and CD63, when whole blood of healthy females was exposed to untreated or treated breast cancer cells. While the majority of our participants' blood showed an overall increase in platelet activation when their blood was exposed to untreated breast cancer cells, some participants showed no effect and others showed a decrease in the percentage change of CD62P and CD63 expression (Figures 4.3, 4.5, 4.7 and 4.9). The response of platelets between participants (and/or after exposure of their whole blood to untreated breast cancer cells) has not been reported before although Mirlashari *et al*. (1996) mentions, but doesn't show, that "no differences could be demonstrated with platelets from some "donors" but whilst in other "donors" striking differences in the kinetics of release of the two granule types were observed" when they were comparing secretion of platelet granule constituents with surface expression of the granule membrane markers CD62P and CD63.

As stated earlier, overall, the platelets of the whole blood samples displayed an increase in the percentage change of CD62P and CD63 expression following exposure to untreated breast cancer cells, i.e. both cells lines (MDA-MB-231: hormone-independent and MCF-7: hormone-dependent cells) were able to promote platelet activity as exhibited by the increase in the percentage change of CD62P and CD63 expression levels. These findings are in line with that reported by others. Zarà *et al*. (2018) found that MDA-MB-231 and MCF-7 breast cancer cells are able to stimulate platelets to release microparticles and growth factors (VEGF, transforming growth factor, platelet derived growth factor) (Plantureux *et al*., 2018) in order to initiate migration and invasion. Unfortunately the expression of platelet activation markers was not investigated by Zarà *et al*. (2018) and, as a result, making a comparison is difficult. Zarà *et al*. (2018) also found that there is more TF in MDA-MB-231 than in MCF-7 breast cancer cells. More recently, Pather (2019; 2020) found that untreated MCF-7 breast cancer cells induce a higher CD62P expression while untreated T47D breast cancer cells induce a higher CD63 expression. A further increase in CD62P and CD63 expression was found after whole blood exposure to Tamoxifen-treated MCF-7 cells while a significant increase in CD62P was observed after whole blood exposure to Tamoxifen-treated T47D cells.

Exposing whole blood to MDA-MB-231 and MCF-7 breast cancer cells treated with the different seed oils induced varying levels of CD62P and CD63 expression. Overall, exposing

whole blood to KA-, XC- and MZ- treated MDA-MB-231 breast cancer cells reduced the percentage change of CD62P expression but treating MCF-7 breast cancer cells with these oils had the opposite effect - an increase in the percentage change of CD62P expression was observed. On the whole, there was a decrease in the percentage change of CD63 expression following exposure of whole blood to KA-, XC- and MZ- treated MDA-MB-231 and MCF-7 breast cancer cells.

The different fatty acid profile of each seed oil could explain the differences observed (Gomes *et al.*, 2019). *K. africana* seed oil has a higher concentration of α-linolenic acid (omega-3 polyunsaturated fatty acid) while *M. zeyheri* has a higher level of linoleic acid (omega-6 polyunsaturated fatty acid) (Gomes *et al.*, 2019). In line with some of our findings, Bazán-Salinas *et al.* (2015) found a decrease in platelet aggregation when subjects consumed *Vitis vinifera* (grape) or *Arachis hypogaea* (peanut) oils which contained high levels of linoleic acid and oleic acid respectively. Once again, the expression of platelet activation markers (CD62P, CD63) was not investigated by Bazán-Salinas *et al.* (2015) since they used the Born turbidimetric method to determine platelet aggregation. Consequently, comparing results is difficult. Seed oils extracted from different plants contain varying concentrations of omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids (PUFAs). Omega-3 and omega-6 PUFAs are involved in cell membrane structure and function, cell signalling, gene expression regulation and are substrates for the synthesis of lipid mediators (Huerta-Yépez *et al.*, 2016). These PUFAs cannot be synthesized *de novo* and must be obtained from the diet (Berquin *et al.*, 2008). Omega-3 PUFAs include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) isolated from marine sources and α-linolenic acid from leafy vegetables, nuts, and oils (Anderson and Ma, 2009; Huerta-Yépez *et al.*, 2016). EPA and DHA can be synthesized by the conversion of α-linolenic acid *in vivo* (Huerta-Yépez *et al.*, 2016). Omega-6 PUFAs include linoleic acid present in grains, meats and plant seeds (Covington, 2004). The exact mechanism by which these omega-3 PUFAs inhibit platelet activation has not been entirely elucidated but factors that affect membrane fluidity (Akiba, *et al.*, 2000), signal transduction and thromboxane metabolism have been implicated (Cohen *et al.*, 2011). Arachidonic acid (AA), an omega-6 fatty acid, is metabolised to prostaglandin thromboxane A2 (TxA_2), a potent platelet activator and vasoconstrictor (Cohen *et al.*, 2011; DeFilippis *et al.*, 2014). Omega-3 PUFAs suppress the conversion of AA to TxA_2 and act as competitors for the TxA_2 specific receptor (Akiba, *et al.*, 2000; Cohen *et al.*, 2011; DeFilippis *et al.*, 2014) preventing platelet activation and subsequent platelet aggregation.

Different intracellular pathways are triggered when platelets are activated by different agonists, as was observed by Taylor *et al.* (1995) and Murakami *et al.* (1996). When platelets were activated using platelet-activating factor (PAF), AA or collagen, CD63 expression was greater than CD62P expression (Taylor *et al.*, 1995) and that under these conditions, there may be a preferential secretion of lysosomal content compared to α-granules. The above observations demonstrate that the activation markers expressed on the platelet surface varies with different tumour phenotypes (Holmes *et al*., 2016; Pather *et al*., 2019; Pather *et al*., 2020) and/or agonists resulting in different expression of platelet activation markers.

In the present study, platelets were neither washed nor treated directly with the different seed oils; rather breast cancer cells were pre-treated with the different seed oils prior to incubation with whole blood to emulate *in vivo* platelet-tumour cell interactions and probable breast tissue changes due to consumption of dietary seed oils. The effects of *in vitro* seed oil pre-treatments on breast cancer cells and the impact thereof on platelet activation have not been previously investigated in this manner.

While this preliminary study yielded promising results, a few limitations also need to be recognised. Despite substantial effect sizes in some instances, the relatively small samples sizes could have caused changes to be not statistically significant. Secondly, circulating human platelets show differences with respect to size and age (Mangalpally *et al*., 2010). Platelets are extremely sensitive and if samples are not taken carefully or processed quickly, *in vitro* activation of platelets may occur resulting in erroneous conclusions (Murakami *et al.*, 1996; Marquandt *et al.*, 2002; Mangalpally *et al*., 2010; Picker, 2011; Augustine *et al.*, 2016). Future experiments should therefore consider using scanning electron microscopy to assess platelet morphology (Augustine *et al.*, 2016; Pather *et al.*, 2019) and/or determine platelet factor 4 and β-thromboglobulin levels (Murakami *et al.*, 1996) which are released from platelets during activation to compliment results obtained by flow cytometry.

Further experiments are also required to determine the factors which are responsible for the differential expression of the platelet activation markers, CD62P and CD63 specifically targeting pathways relating to cancer and platelet lipid metabolism.

CHAPTER 5: CONCLUDING DISCUSSION

Cancer remains one of the major health challenges facing the world. Approximately 25% of cancer cases among women is breast cancer, making it the most prevalent type of cancer among women worldwide (Bray *et al*., 2018). Women living in developing countries who suffer from cancer do not often have access to specialised oncologists, conventional medicine and treatment. Consequently, their survival rate is lower than that of their counterparts in first world countries. As a result, these women's health care, especially in rural areas, depends largely on ethnomedicine (Kuete *et al*., 2013).

The use of plants for medicinal purposes has been around for centuries and as a result, plants have played a significant role in the development of drugs. In spite of the rich diversity of plants only a fraction has been explored for their medicinal benefits (Khazir *et al*., 2014). The potential of plants to be alternative sources of therapeutics is further threatened by increasing human activity which is destroying plants' natural habitats. These two factors call for the urgent need of scientific documentation and research on the medicinal uses of plants, not only to address the paucity in current literature but also to ensure continued preservation of this valuable resource (Sawadogo *et al*., 2012). The present study therefore is part of a broad endeavour to highlight the usefulness of African flora in combating pathologies focusing on cancer.

Different parts of the *Kigelia africana* (*K. africana*), *Ximenia caffra* (*X. caffra*) and *Mimusops zeyheri* (*M. zeyheri*) trees are often used by traditional healers in sub-Saharan Africa to treat a variety of ailments. In a previous study, the seed oils of *K. africana*, *X. caffra* and *M. zeyheri* were shown to inhibit the proliferation of human colon adenocarcinoma (Caco-2) and human embryonic kidney (HEK-293) cells *in vitro* (Chivandi *et al*., 2012b). Consequently we decided to determine the effects of these seed oils on hormone-independent (MDA-MB-231) and hormone-dependent (MCF-7) breast cancer cells *in vitro*. Firstly, we wanted to establish if the seed oils were cytotoxic by assessing their effects on the cell viability on these two breast cancer cell lines. Accordingly, different seed oil concentrations and different incubation times were evaluated using the LDH cytotoxicity and NR cell viability assays. We found that oil type and incubation time showed significant effects. *K. africana* and *X. caffra* seed oils induced the greatest cytotoxic effects on MDA-MB-231 cells, while *X. caffra* and *M. zeyheri* seed oils induced a growth inhibitory effect on MCF-7 cells.

As a result of these findings, we subsequently investigated the possible mechanism(s) by which these seed oils induced cytotoxic/anti-proliferative effects on MDA-MB-231 and MCF-7 cells by looking at apoptosis using the FITC Annexin V apoptosis detection kit as well as

determining the expression level of caspase 6. In addition, the expression levels of LC3A, an indicator of autophagic activity, were also determined. K. *africana* was more potent in reducing the number of live MDA-MB-231 cells compared to its effect on MCF-7 cells. These *K. africana* effects were not associated with significant increases in caspase 6 expression levels for both cell lines. *X. caffra* and *M. zeyheri* seed oils treatment failed to cause significant cell death despite significantly higher caspase 6 levels than in control cells. These findings suggest that caspase 6 does not play a key role in *K. africana*-induced MDA-MB-231 cell death and that an alternative cell death mechanism may have been stimulated. MCF-7 cells are unable to express caspase 3 (Yang *et al*., 2007; Shahali *et al*., 2018) and are therefore partially protected from the lethal effects of *K. africana* seed oil. Also, the presence of oestrogen receptors as well as the status of the tumour suppressor gene, p53, are different between the two cell lines - MDA-MB-231 cells are oestrogen receptor negative and express mutant p53, while MCF-7 cells are oestrogen receptor positive and express wild type p53 (Yang *et al*., 2007; Singh *et al*., 2011). It is known that breast tumours that are oestrogen receptor negative and p53 mutant, are more aggressive and have fewer treatment alternatives (Singh *et al*., 2011). The selective toxic effects of *K. africana* seed oil on MDA-MB-231 cells are therefore noteworthy. Overall MCF-7 cells had lower LC3A expression levels than MDA-MB-231 cells. Further *K. africana* seed oil was the only treatment that caused a significant reduction in LC3A expression and this effect was only present in MDA-MB-231 cells. With the lowered LC3A concentration, *K. africana* seed oil is probably blocking the formation of autophagosomes, thereby preventing autophagy to occur in these cells. The mechanism(s) by which *K. africana* seed oil is possibly blocking the formation of autophagosomes is currently unknown but the seed oil has high levels of α-linolenic acid which is an omega-3 PUFA. It has been demonstrated that incorporating omega-3 PUFAs into cell phospholipids alters membrane structure and permeability (Chajès *et al*., 1995; Berquin *et al*., 2008; Bougnoux *et al*., 2010). Omega-3 PUFAs have anti-inflammatory and anti-oxidative properties which could reduce LC3A expression via the decrease of inflammatory markers such as IL-1, TNF-α, IL-6, IFN-γ, IL-2 and TGF-β (Larsson *et al*., 2004; Anderson and Ma, 2009; Lorin *et al*., 2013). Also, Singh *et al.* (2009) found that when intracellular lipid levels rise abnormally, autophagic clearance is reduced, which impairs cellular survival and this was indicated by the decreased lipid droplet/LAMP 1 co-localization and the absence of autophagic activation in hepatocytes cultured with lipids.

Aside from causing severe side effects, cancer patients are at risk for thrombosis when receiving chemotherapy. Furthermore, tumours are also capable of activating the host's haemostatic system thereby increasing the risk of thrombosis (Elyamany *et al.*, 2014). A preliminary

investigation was therefore conducted to determine if *K. africana*, *X. caffra* and *M. zeyheri* seed oils could reduce the thrombogenic ability of breast cancer cells by measuring the ability of these seed oils to inhibit breast cancer cell-induced platelet activation. The anti-thrombogenic capacity of the seed oils were determined by measuring the platelet surface expression of the activation-specific antigens CD62P and CD63. We found differential expression of the platelet activation markers, CD62P and CD63, when whole blood was exposed to untreated or treated MDA-MB-231 and MCF-7 cells. An increase in CD62P and CD63 expression following whole blood exposure to untreated breast cancer cells was observed while *K. africana*-, *X. caffra*- and *M. zeyheri*- treated MDA-MB-231 breast cancer cells reduced CD62P expression but treated MCF-7 breast cancer cells increased CD62P expression. CD63 expression decreased following exposure of whole blood to *K. africana*-, *X. caffra*- and *M. zeyheri*- treated MDA-MB-231 and MCF-7 cells. These findings indicate that *K. africana*, *X. caffra* and *M. zeyheri* seed oils are able to reduce the thrombogenic ability of hormone-independent MDA-MB-231 breast cancer cells and could be considered as a supplement to conventional breast cancer treatment.

Limitations and future experiments

While the current study addressed our initial objectives adequately, a number of limitations were also identified, some of which could direct future experiments. For instance cytotoxicity assays depend on various cellular mechanisms causing cell death (Weyermann *et al*., 2005; Fotakis and Timbrell, 2006). In the present study the integrity of the cell membrane, one of the standard parameters of cell death, was measured by LDH release. Previous data showed that MDA-MB-231 cells were more susceptible to a cytotoxic effect than MCF-7 cells (Weyermann *et al*., 2005). However LDH is only released into the media when the cells are at a late enough stage of demise. As such measuring LDH is considered a useful method for detecting necrosis (Smith *et al*., 2011; Chan *et al*., 2013). Another parameter for cell death is to determine the metabolic activity of viable cells which can be ascertained by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), NR and ATP assays. In our study we ran the NR assay as it was convenient and readily available. The obtained data were inconclusive probably due to the mechanisms by which the seed oils exert their effects. Knowing that *K. africana* seed oil modulates MDA-MB-231 cell apoptosis via an alternative mechanism such as the mitochondrial pathway, perhaps the MTT assay would have yielded clearer results. The MTT assay is based on the ability of metabolically active cells to transform a water-soluble dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] which is metabolized by mitochondrial succinic dehydrogenase into an insoluble purple formazan product by the

mitochondria of viable cells. The MTT assay therefore provides a direct read-out of the function of mitochondria and hence greater insight into the metabolic activity of cells.

Another shortcoming in my study was that I only investigated the expression levels of caspase 6 as an indicator of apoptotic activity in seed oil-treated cells. By measuring more enzyme markers of apoptotic pathways would have given me a more definitive explanation for the toxic effects observed. Such additional information would also have strengthened my hypothesis that *K. africana* seed oil modulated MDA-MB-231 cell apoptosis via an alternative mechanism. For example knowing the levels of caspase 3 levels would have been useful since Ruemmele *et al.* (2003) attributed butyrate-induced apoptosis in Caco-2 cells to be mediated via the mitochondrial pathway as evidenced by Bak upregulation, translocation of cytochrome c, and the eventual activation of caspase 3.

Previous work by Chivandi *et al*. (2012b) and others (Liu and Bodmer, 2006; Yang *et al*., 2007; Singh *et al*., 2011) demonstrated that both human colon adenocarcinoma (Caco-2) cells and MDA-MB-231 breast cancer cells express mutant p53. It may be possible that mutations in p53 render cancer cell lines susceptible to *K. africana* seed oil toxicity. It would therefore be interesting to see whether *K. africana* seed oil have similar effects on other cell lines with p53 mutations. Determining whether the mutation is at the same location on the p53 gene and establishing the gene sequencing of these p53 mutations would contribute significantly to my current understanding of *K. africana* seed oil-induced cell death.

Autophagy functions as an intracellular degradative system that removes impaired organelles and old proteins from cells. It also serves as a catabolic mechanism that generates energy during periods of cellular starvation as well as promote the synthesis of new proteins from “old” amino acids (Edinger and Thompson, 2004; Mizushima, 2007; de Bruin and Medema, 2008; Loos and Engelbrecht, 2009). In the context of cancer, autophagy has been suggested to play an integral role in tumour growth because of its ability to provide nutrients to cells. In 2007, Mizushima stated that "the recycling of carbohydrates and lipids by or during autophagy is not known" but it has been found, however, that cellular lipids stored in lipid droplets are hydrolysed to fatty acids under starvation conditions for energy and this process is known as lipophagy - selective degradation of lipid droplets (Ichimiya *et al*., 2020). Evidently different mechanisms are being employed depending on the type of cell and/or stage of tumour growth (de Bruin and Medema, 2008; Glick *et al*., 2010; Ichimiya *et al*., 2020). We measured the levels of LC3A in untreated and treated MDA-MB-231 and MCF-7 breast cancer cells to determine whether the process of

autophagy contributes to the observed plant seed oil toxic effects. Overall MCF-7 cells had lower LC3A expression levels than MDA-MB-231 cells. *K. africana*-treated MDA-MB-231 breast cancer cells were the only cells that had a significant reduction in LC3A expression. LC3A plays a role in autophagosome formation and phagophore membrane elongation (Tanida *et al*., 2004; Ito, *et al*., 2005; Koukourakis *et al*., 2015). It is suspected that although MCF-7 cells had lower LC3A expression levels, MCF-7 cells might have had higher levels of Beclin 1. Breast carcinoma cells frequently express low levels of endogenous Beclin 1 protein unlike normal breast tissue which exhibits high levels of endogenous Beclin 1 protein expression (Liang *et al*., 1999). Overexpression of Beclin 1 inhibits proliferation and tumour formation in MCF-7 breast cancer cells (Liang *et al*., 1999; Maycotte and Thorburn, 2014). This may account for our observations of growth inhibition on MCF-7 cells with the cytotoxicity assays. Measuring Beclin 1 levels would have offered an alternative explanation for the decrease proliferation of MCF-7 cells.

Other parameters that could also be considered to confirm an autophagic mode of cell death include cathepsin proteases B and D which are needed for the maturation of the autolysosome and LAMP 1 and 2 which are essential for functional autophagy (Glick *et al*., 2010).

A more recent approach in assessing tumor autophagy dynamics has been the use of quantitative proteomics to detect selected factors in plasma secreted by cancer cells with high autophagy levels and compared this to cancer cells with low autophagy levels (Kraya *et al*., 2015). Unique factors such as leukaemia inhibitory factor (LIF) and interleukin 1β (IL 1β), which are involved in immunity and inflammation (Kraya *et al*., 2015), may serve as prognostic biomarkers and should be considered in future studies to assist in patients' selection in need of autophagy inhibition (Bortnik and Gorski, 2017).

A study was conducted to determine if *K. africana*, *X. caffra* and *M. zeyheri* seed oils could inhibit breast cancer cell-induced platelet activation. Although promising results were obtained - *K. africana*, *X. caffra* and *M. zeyheri* seed oils reduced the thrombogenic ability of hormone-independent MDA-MB-231 breast cancer cells - it was not without challenges. Even though the effect sizes were substantial in some cases, the relatively small sample size may have compromised the detection of statistically significant differences. The small sample size was the result of applying strict exclusion criteria that included pregnancy, contraceptive use, smoking, previous history of cancer, presence of autoimmune diseases, immunodeficiency and the use of anti-platelet/anti-coagulation medication. These exclusion criteria made recruiting of

female participants difficult. Blood could also only be collected when the participant was on her menstrual cycle. Human platelets are extremely sensitive and vary in size and age in circulation (Mangalpally *et al*., 2010). False positive results can occur when samples are not taken carefully or processed immediately (Murakami *et al.*, 1996; Marquandt *et al.*, 2002; Mangalpally *et al*., 2010; Picker, 2011; Augustine *et al.*, 2016). Augustine *et al*. (2016) and Pather *et al*. (2019) used scanning electron microscopy to assess platelet morphology during the different stages of platelet activation. Future studies should consider implementing scanning electron microscopy to confirm biochemical findings. Expanding the repertoire of parameters such as platelet factor 4 and β-thromboglobulin that are released during platelet activation (Murakami *et al*., 1996) could be determined to supplement the results obtained by flow cytometry. Additional experiments that focus on the factors responsible for the differential expression of the platelet activation markers, CD62P and CD63, would elaborate on the association between the metabolism of lipids in platelets and cancer. For instance the release of arachidonic acid (AA) from membrane phospholipids by phospholipase A2 is an important step in the thromboxane (Tx) A2 formation pathway (Akiba *et al*., 2000; DeFilippis *et al*., 2014). Cyclooxygenase (COX), also called prostaglandin endoperoxide synthase, is the enzyme responsible for the production of prostaglandins (PGs) and related eicosanoids from AA (Liu and Rose, 1996). Inhibition of COX by omega-3 fatty acids inhibits the formation of TxA2 from AA and obstructs the binding of TxA2 to its receptor (Akiba *et al*., 2000). As a result of lipoxygenase, omega-3 fatty acids get metabolised into 11- and 14-hydroxyl isomers which have been shown to inhibit TxA2-dependent platelet aggregation (Akiba *et al*., 2000). Long chain omega-3 PUFA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are linolenic acid-derived components, inhibit TxA2 formation by competing with AA for COX and by directly inhibiting COX activity, respectively (Capone *et al*., 1997; Akiba *et al*., 2000; DeFilippis *et al*., 2014). Platelets with high concentrations of EPA and DHA have decreased abilities to bind fibrinogen, release their granules and produce thrombin on their surfaces (Larson *et al*., 2011). An important metabolite produced by lipoxygenase in platelets is 12-hydroxyeicosatetraenoic acid (12-HETE). Measuring 12-HETE from platelets can therefore serve as a reliable read-out of the effects of omega-3 fatty acids on the lipoxygenase pathway. It would be extremely useful if the effects of *K. africana*, *X. caffra* and *M. zeyheri* seed oils on the lipoxygenase pathway are compared to those of irreversible cyclooxygenase inhibitors and ADP receptor inhibitors (Akiba *et al*., 2000; Bazán-Salinas *et al*., 2015).

In addition to being essential fatty acids necessary for human health, omega-3 and omega-6 PUFAs play a vital role in cellular physiology (Berquin *et al*., 2008; Huerta-Yépez *et al*., 2016).

Membrane structure and permeability can be changed by including PUFAs into cell phospholipids which subsequently increases protein kinase C activity (Chajès *et al*., 1995; Berquin *et al*., 2008; Bougnoux *et al*., 2010). A number of signalling pathways are distinctively influenced by omega-3 and omega-6 PUFAs which are important to tumour progression and carcinogenesis (Berquin *et al*., 2008). *K. africana* seed oil has higher levels of α-linolenic acid than *X. caffra* and *M. zeyheri* seed oils whilst *M. zeyheri* has higher levels of linoleic acid (omega-6 PUFA) (Gomes *et al*., 2019). Hormone-independent MDA-MB-231 breast cancer cells display low expression levels of COX-1 but high expression levels of COX-2 while hormone-dependent MCF-7 breast cancer cells display high expression levels of COX-1 (Liu and Rose, 1996). MDA-MB-231 cells demonstrate higher expression levels of COX-2 than MCF-7 cells which is needed for the conversion of AA to PGs, a process that promotes tumour growth (Smith *et al*., 2000; Horia and Watkins, 2005). Hormone-independent tumours with high metastatic potential normally possess high levels of PGE_2 (Liu and Rose, 1996). MDA-MB-231 cells treated with α-linolenic acid showed decreased levels of PGE_2 as a result of lowered COX-2 expression (Horia and Watkins, 2005). EPA and DHA also exhibit growth inhibition in breast cancer cells MDA-MB-231 and MCF-7, and are known to enhance cytotoxic effects of numerous anti-cancer drugs (Chamras *et al*., 2002; Schley *et al*., 2005; Bougnoux *et al*., 2010). Breast cancer cells are believed to be susceptible to apoptosis induced by omega-3 PUFAs via the inhibition of the Akt/NFκB signalling pathways (Schley *et al*., 2005). The ability of long-chain omega-3 PUFAs to inhibit growth and/or induce apoptosis in tumour cells has been explained by the increased vulnerability of these cells to lipid peroxidation (Chamras *et al*., 2002; Berquin *et al*., 2008; Bougnoux *et al*., 2010). Subsequent experiments should include the potential mechanisms of action of these seed oils on lipid peroxidation, determining expression levels of COX-1 and COX-2 and monitoring of prostaglandin production.

A further advancement of the study would be to replicate our findings of the seed oils in an *in vivo* model. Mammary tumours could be induced in rodents after which the animals could be subjected to treatment with the various seed oils. The impact of the seed oils on tumour growth or shrinkage could then be evaluated. Alternatively the rodents could first be exposed to the seed oils followed by the induction of mammary tumours. Such an approach would be attractive as a strategy to prevent the development of the tumour.

Lastly, it would be interesting to conduct an epidemiological study examining cancer distribution patterns among populations living near these trees and/or those who seek treatment from traditional healers who use these plants.

Cancer, especially breast cancer with all of its different subtypes, is a giant puzzle with all these pieces (as indicated above) that need to be put together. It's definitely not an one-person investigation but a collaborative effort that eventually needs to come together because there is a common denominator - lipids and their metabolism. Lipids are involved in all aspects of cellular physiology and pathologies. Regardless of genomic alterations associated with breast cancer, lifestyle and nutritional factors influence survival rates.

In conclusion, *K. africana* seed oil has potent apoptotic inducing ability in hormone-independent MDA-MB-231 and to a lesser degree in hormone-dependent MCF-7 breast cancer cells. Patients with breast tumours that have a p53 mutation and lack oestrogen receptors have less treatment options. These cancers tend to be more aggressive as well (Singh *et al*., 2011). The discriminatory toxic effects of *K. africana* seed oil on MDA-MB-231 cells are therefore significant. *K. africana*, *X. caffra* and *M. zeyheri* seed oils are able to reduce the thrombogenic ability of hormone-independent MDA-MB-231 breast cancer cells suggesting that these oils are possibly able to decrease the risk of thrombosis too. It is clear that further investigations are required into mechanisms by which these seed oils exert their effects as mentioned above and that different types of cancer require different approaches in treatment therapies and as such, other plants and/or seeds should be continually evaluated for their potential use in tumour management.

APPENDICES

Appendix A

Ethical Clearance Certificates

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: Tobias Health Sciences Building, 2nd floor Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

Ref: W-CJ-150422-1 22/04/2015

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Monica Gomes (student no A0011669)**

Project title: ***Kigelia africana*, *Mimusops zeheri* and *Ximenia caffra* seed oils: effects on normal mammary cells (HMEC) and on MCF-7 and MDA-MBV-231 breast cancer cells.**

Reason: This laboratory study will use established cell lines listed in the project title. There are no human participants

UNIVERSITY OF THE WITWATERSRAND
2015 -04- 22
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu, Langutani Masingi.

R14/49 Ms Monica Gomes

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M160211

NAME: (Principal Investigator)	Ms Monica Gomes
DEPARTMENT:	School of Physiology School of Anatomical Sciences School of Clinical Medicine
PROJECT TITLE:	Effects of Kigelia Africana (K.africana), Mimusops Zeyheri (M. zeyheri) and Ximenia caffra (X. caffra) Seeds and Oils on Hormone-Dependant (MCF-7) and Hormone-Independent (MDA-MB-231) Breast Cancer Cells
DATE CONSIDERED:	26/02/2016
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	De Elton Chivandi and Dr Tanya Augustine
APPROVED BY:	Professor P. Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	24/06/2016

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004,10th floor, Senate House/2nd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the he conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated,from the research protocol as approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. in this case, the study was initially review in February and will therefore be due in the month of February each year.

Principal Investigator Signature

Date 28/6/2016

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B

"Turn-It-In" Plagiarism Report

The similarity index is 47% but 23% is from our publication (please see turn-it-in report below).

ORIGINALITY REPORT

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1	M.N. Gomes, T.N. Augustine, D. Moyo, E. Chivandi. "Differential response of breast cancer cell lines to Kigelia africana, Ximenia caffra and Mimusops zeyheri seed oils", South African Journal of Botany, 2019 Publication	23%
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Appendix C

Publication

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Differential response of breast cancer cell lines to *Kigelia africana*, *Ximenia caffra* and *Mimusops zeyheri* seed oils

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ABSTRACT

Breast cancer is the most common cancer among women worldwide. The survival rate of breast cancer is higher in first-world countries than in developing countries, which still rely on ethnomedicine. The seed oils of *Kigelia africana*, *Mimusops zeyheri* and *Ximenia caffra* previously demonstrated anti-proliferative effects on Caco-2 and HEK-293 cells in vitro. We determined the effects of these seed oils on hormone-dependent (MCF-7) and hormone-independent (MDA-MB-231) breast cancer cells. Different seed oil concentrations and different incubation times were evaluated. While the seed oils concentrations had no significant cytotoxicity or effect on MDA-MB-231 and MCF-7 viability; oil type and incubation time showed significant effects. *Kigelia africana* and *X. caffra* seed oils induced the greatest cytotoxic effects on hormone-independent MDA-MB-231 breast cancer cells, while *X. caffra* and *M. zeyheri* seed oils induced a growth inhibitory effect on hormone-dependent MCF-7 breast cancer cells. These effects are dependent on oil type/composition, duration of exposure and cell phenotype.

1. Introduction

Breast cancer is the most common cancer among women globally (Monga et al., 2013). Of the 1 million women diagnosed with breast cancer per year worldwide, 140,000 die (Coughlin and Ekwueme, 2009; Youlden et al., 2012). Breast cancer is a heterogeneous disease (Gazinska et al., 2013) and consequently different treatment strategies are required (Yersal and Barutca, 2014). The survival rate for breast cancer is almost 90% in the US compared to 50% in developing African countries, e.g., Gambia, Uganda and Algeria (Kuete et al., 2013). The comparatively lower survival rate in the developing African countries is ascribed to the lack of essential infrastructure and resources to facilitate routine screening mammography. This lack of infrastructure results in breast cancer being diagnosed at late stages of the disease. Due to the heterogeneity of breast cancer, high cost and reduced availability of treatment options, women in such emerging countries receive inadequate treatment, pain relief or palliative care (Coughlin and Ekwueme, 2009). They, therefore, to a large extent, depend on ethnomedicine to treat and/or manage breast cancer (Ochwang'i et al., 2014). For many centuries, ethnomedicine has been used for prophylaxis and in the treatment of various ailments including cancer and as a result all phytochemical extracts should be scientifically corroborated for their alleged usefulness, safety and toxicity (Ochwang'i et al., 2014). The vinca alkaloids, Vinblastine and Vincristine, isolated from the Madagascar periwinkle plants (*Vinca rosea*) (Moudi et al., 2013), are used largely to treat Hodgkin's lymphoma and leukaemia (Khazir et al., 2014) by blocking the polymerization of tubulin molecules resulting in metaphase arrest and apoptosis (Vass et al., 2006). Paclitaxel, a standard chemotherapy agent impeding mitotic progression of ovarian and breast cancer (Khazir et al., 2014; Priyadarshini and Ilavenil-Aparajitha, 2012), was initially extracted from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. Camptothecin, from the Chinese tree *Camptotheca acuminata* Decne, is used to treat stomach, rectal, colon and liver cancer (Lee, 2004).

Kigelia africana (*K. africana*), *Mimusops zeyheri* (*M. zeyheri*) and *Ximenia caffra* (*X. caffra*), which are widely distributed in the sub-Saharan Africa region, are used by traditional healers for the treatment of a number of ailments (Chivandi et al., 2012). The fruit and stem bark extracts of *K. africana* (also known as the Cucumber or Sausage tree because of its huge wooden berries) are used for treatment of melanoma and other skin neoplasms (Bello et al., 2016; Houghton, 2002; Saini et al., 2009) and in the management of endometrial cancer (Ashidi et al., 2010). Although *M. zeyheri* (commonly known as Red Milkwood; family Sapotaceae), is used in Swaziland to treat candidiasis, ulcers and wounds (Amusan et al., 2002), a dearth of information exists regarding its possible anticancer properties. The leaves of *X. caffra* (also known as the large Sourplum) are used to treat diarrhoea, dysentery,

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fever, cough and venereal diseases (Green et al., 2010). Extracts of the *X. caffra* leaf were also shown to have anti-proliferative and anti-inflammatory activities on murine RAW 264.7 macrophage cells (Zhen et al., 2015), a model widely used for inflammation studies.

Besides the beneficial properties of leaf extracts and/or macerations, seeds from indigenous fruit-bearing trees contain important amino acids, fatty acids and vitamins (Chivandi et al., 2011) and are implicated in anti-inflammatory (Constantini et al., 2014) and anti-proliferative activities (Al-Sheddi et al., 2015; Gao et al., 2016; Seal et al., 2012; Vieira et al., 2008) that may influence tumor progression. In vitro investigations of a number of seed oils have highlighted their ability to affect pro-tumorigenic processes. Pomegranate (*Punica granatum* L.) seed oil reduces the production of the pro-angiogenic compound vascular endothelial growth factor (VEGF), and pro-inflammatory cytokines (IL-2, IL-6 and TNF-α) by breast cancer cells (Constantini et al., 2014). *Pterodon pubescens* Benth. seed oil inhibits melanoma cell growth (Vieira et al., 2008), while *Litsea cubeba* and *Persimmon oleiferum* seed oils are able to inhibit tumor cell progression through the cell cycle (Al-Sheddi et al., 2015; Gao et al., 2016; Seal et al., 2012).

Recent research has shown that *K. africana*, *M. zeyheri* and *X. caffra* seed oils suppress the proliferation of human colon adenocarcinoma (Caco-2) and human embryonic kidney (HEK-293) cells (Chivandi et al., 2012); however the possible anticancer activity of these seed oils on human breast cancer cells have not been explored. We thus investigated the effects of these three seed oils on cell viability and the induction of cytotoxicity at various concentrations and incubation times on hormone-dependent (MCF-7) and hormone-independent (MDA-MB-231) breast cancer cells.

2. Materials and methods

2.1. Seed source

Kigelia africana seeds were obtained from the ripe fruits from trees in Guta District, Chitsa Communal area, Zimbabwe. *Mimusops zeyheri* seeds were harvested from fruits off trees found in the Golowe District, in North West Zimbabwe, in the Machakata Community Forest area of the Mapfungautsi Plateau, while *X. caffra* seeds were collected from ripe fruit of the Zhombe District, Zimbabwe.

2.2. Seed oil extraction and storage

Seeds were dehulled and then the hulled hemp seeds were crushed in a blender (Waring; Lasec Pty Ltd., Johannesburg, South Africa). Hexane was used to extract the oil from each of the seed meals as described by Yamasaki et al. (2013). Briefly, 800 mL of hexane was added to 160 g of each seed meal and then mixed overnight using a magnetic stirrer (VELP Scientifica, Lombardy – Italy). The mixture was then filtered through filter paper (Whatman No. 1) and using a rotary vacuum, the hexane was evaporated at 65 °C and the extracted oils were kept in brown sample bottles at −20 °C before analysis (Yamasaki et al., 2013).

2.3. Fatty acid profile determination

Fatty acid profiling of the extracted seed oils was conducted at the Agricultural Research Council's (ARC) Irene Institute of Animal Production Analytical Services Laboratories, using gas chromatography. Briefly, a methanol–sodium hydroxide solution was used to trans-methylate the seed oils. The resultant methyl esters were extracted using heptane, filtered and dried under nitrogen as described by Christopherson and Glass (1969). The fatty acids were then separated using a gas chromatograph (HP6890GC – Hewlett Packard, Bristol, UK).

2.4. Cell culture

Ethical clearance was obtained from the Human Research Ethics Committee Clearance Certificate number M160211, University of the Witwatersrand, Johannesburg. The two human breast cancer cell lines (MCF-7 and MDA-MB-231) used in the experiments were donated by Dr. R Duarte (School of Clinical Medicine, University of the Witwatersrand) from ATCC (Virginia, USA). MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 5% fetal bovine serum (FBS) and 100 μg/mL streptomycin, 100 units/mL penicillin. MDA-MB-231 cells were cultured in DMEM/Ham's F-12 nutrient mixture with 10% FBS and 100 μg/mL streptomycin, 100 units/mL penicillin. Both cell lines were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

2.5. Seed oil concentrations and treatment of cells

The seed oils of *K. africana*, *X. caffra* and *M. zeyheri* were not completely soluble in aqueous medium solution, therefore stock solutions (10 mg/mL) of all the oils were prepared in 100% absolute alcohol and diluted in culture medium to reach the desired concentrations (20, 40, 80, 100 and 120 μg/mL). The concentration of absolute alcohol in culture medium was not more than 5%. A diluent control consisting of 100% absolute alcohol diluted in culture medium was included.

Confluent cells were subcultured by 0.25% trypsin/edta. MCF-7 cells (P35) and MDA-MB-231 cells (P23) were plated in 24-well plates at 6.3×10^4 and 3.1×10^4 cells/well, respectively, in their respective media. Both cell lines were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h. The cell culture media from each well was then removed and cells were rinsed with Dulbecco's phosphate-buffered saline (DPBS). Cells were treated using 200 μL of the various seed oil concentrations (20, 40, 80, 100 and 120 μg/mL) in matched culture media and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 24, 48 and 72 h. In all experiments untreated and diluent treated cells were included as controls.

2.6. Lactate dehydrogenase assay

Cytotoxicity induced by the various seed oil concentrations was measured using the lactate dehydrogenase (LDH) cytotoxicity assay kit (ScienCell Research Laboratories, Carlsbad – California catalogue number 8078). After each time point the plates were centrifuged for 5 min at 400 g and 150 μL of supernatant from each well of the 24-well plate transferred to the corresponding well of a 96-well test plate. LDH assay reagent (80 μL) was added to each well and the test plate was incubated for 20 min at room temperature in darkness. The reaction was terminated using 20 μL sodium oxyrnate and absorbance read at 492 nm using a microtiter plate reader (Labsystems Multiscan Ascent plate reader). Cytotoxicity was then calculated as follows:

$$\% \text{ cytotoxicity} = \frac{[OD(\text{test sample}) - OD(\text{negative control})]}{[OD(\text{positive control}) - OD(\text{negative control})]} X\ 100$$

2.7. Neutral red assay

Cell viability was determined using the neutral red (3-amino-n-dimethylamino-2-methyl-phenazine hydrochloride) (NR) dye. The method of Borenfreund and Puerner (1984) was used with modifications. After each time point, the media was removed and 150 μL of neutral red dye (100 μg/mL) dissolved in serum free medium (DMEM or DMEM/Ham's F-12 for MCF-7 and MDA-MB-231 cells, respectively) was added to each well. The cells were incubated for a further 3 h at 37 °C in a humidified atmosphere of 5% CO_2. Cells were then washed twice with DPBS and 150 μL elution media (EtOH/AcOOH, 50%/1%) was added followed by gentle shaking for 60 min so that complete

dissolution could be achieved. The resulting solutions (150 μL) were then transferred to 96-well plates and the absorbance at 540 nm was read using a microtiter plate reader (Labsystems Multiscan Ascent plate reader). Cell viability was then calculated as follows:

$$\%\ \text{cell viability} = \frac{[OD(\text{test sample}) - OD(\text{blank})]}{[OD(\text{control}) - OD(\text{blank})]} \times 100$$

2.8. Data analysis

Data are presented as mean ± standard deviation (mean ± SD). For the lactate dehydrogenase assay results, statistical analyses were carried out by one-way analysis of variance (ANOVA). For the neutral red assay results, the Shapiro–Wilk test was used to determine if the raw data were normally distributed. The raw data were subsequently log transformed to a symmetric distribution and this was followed by a one-way ANOVA. The Bonferroni's post hoc test was then used to express any difference among the groups. STATA 13.1 statistical package was used to analyze data. Significance was set at 95%.

3. Results

3.1. Lipid yield and fatty acid profiles of the seed oils

Table 1 highlights the lipid yield and fatty acid profile from each of the seed meals. *Ximenia caffra* seed meal yielded the highest amount of oil (39.16%) followed by *K. africana* (34.30%) and lastly *M. zeyheri* seed meal (14.04%). *Kigelia africana* seed oil had the highest concentration of α-linolenic acid (50.03%) followed by *X. caffra* (1.27%) and lastly *M. zeyheri* (0.10%). *Mimusops zeyheri* had the highest concentration of linoleic acid (18.87%) followed by *K. africana* (0.14%) and *X. caffra* (0.10%).

Table 1
Lipid yield (%) and fatty acid composition (%) of the tree seed oils.

Parameter	*K. africana*	*X. caffra*	*M. zeyheri*
Lipid yield	34.30	39.16	14.04
Fatty acid profile			
Saturated			
C12:0 (lauric acid)	0.015	0.028	Nd
C14:0 (myristic acid)	0.025	0.029	0.053
C15:0 (pentadecanoic acid)	0.007	0.000	0.017
C16:0 (palmitic acid)	9.712	1.006	12.717
C17:0 (margaric acid)	Nd	Nd	0.143
C18:0 (stearic acid)	5.995	1.064	9.278
C20:0 (arachidic acid)	0.872	0.494	0.847
C21:0 (heneicosanoic acid)	0.021	0.059	Nd
C22:0 (behenic acid)	0.348	2.028	0.592
C23:0 (tricosanoic acid)	0.047	0.219	Nd
C24:0 (lignoceric acid)	0.000	3.855	0.225
TSFA	17.150	8.572	23.902
Monounsaturated			
C14:1n7 (myristoleic acid)	0.000	0.079	Nd
C16:1n7 (palmitoleic acid)	0.080	0.081	0.127
C17:1n8 (9-heptadecenoic acid)	0.021	0.020	0.020
C18:1n9 (oleic acid)	21.788	58.288	52.732
C20:1 (11-eicosenoic acid)	0.337	1.080	0.559
C22:1n9 (erucic acid)	0.013	1.556	0.082
C24:1n9 (nervonic acid)	0.000	4.826	0.087
TMUFA	22.261	67.140	56.567
Polyunsaturated			
C18:2n6 (linoleic acid)	0.137	0.104	18.870
C18:2n3 (α-linolenic acid)	50.033	1.275	0.100
C18:3n6 (γ-linolenic acid)	0.339	<0.000	0.009
C20:2n6 (11,14-eicosadienoic acid)	0.270	0.000	Nd
C20:3n3 (11,14,17-eicosatrienoic acid)	0.078	22.331	0.085
TPUFA	50.861	23.710	19.064
Cis fatty acids	21.925	59.492	74.602
Omega-3	50.112	23.606	0.185
Omega-6	0.505	0.519	19.324
Omega-9	21.788	61.021	53.764
TMUFA:TSFA	1.490:1	2.366:1	0.768:1
nPUFA:nmPUFA	78.921:1	226.981:1	0.009:1

TSFA: total saturated fatty acids; TMUFA: total monounsaturated fatty acids; TPUFA: total polyunsaturated fatty acids; nPUFA: omega-3 polyunsaturated fatty acids; nmPUFA: omega-6 polyunsaturated fatty acids; nd: not detected.

3.2. Cytotoxicity

The lactate dehydrogenase cytotoxicity assay measures the cytosolic enzyme lactate dehydrogenase (LDH) released in the media by dying cells possessing compromised cell membranes. The released LDH oxidizes lactate to pyruvate that converts iodonitrotetrazolium (INT) present in the assay reagent to formazan which can be colorimetrically quantified at 490 nm (Weyermann et al., 2005).

No significant cytotoxic effect was induced by differing seed oil concentrations with each cell line (Fig. 1). No significant cytotoxic effect was induced by the diluent control which comprised of 100% absolute alcohol diluted in culture medium (Fig. 1).

However, further analysis of the results for the seed oils identified significant cytotoxic effects based on the type of seed oil and incubation time employed with each cell line (Fig. 2).

The cytotoxic effect of *K. africana*, *X. caffra* and *M. zeyheri* seed oils on MDA-MB-231 breast cancer cells (Fig. 2a) was less than 5% with *K. africana* seed oil having the greater cytotoxic effect at 48 h incubation. A significant statistical difference was observed between *X. caffra* and *M. zeyheri* seed oils at 72 h incubation, where *M. zeyheri* induced a greater cytotoxic effect than *X. caffra* seed oil on MDA-MB-231 breast cancer cells (Fig. 2a; $p \leq 0.05$). The three seed oils elicited a differential response from the MCF-7 breast cancer cells (Fig. 2b). There was a slight cytotoxic effect (less than 1%) when MCF-7 breast cancer cells were treated with *K. africana* seed oil at 24 and 72 h incubation. *Ximenia caffra* and *M. zeyheri* seed oils produced negative cytotoxic effects at 24 and 48 h incubation and no cytotoxic effects (<0.5%) at 72 h. Significant statistical differences were found between *K. africana* and *M. zeyheri* seed oils at 24 h and at 48 h (Fig. 2b, $p \leq 0.01$). *Mimusops zeyheri* seed oil produced a greater negative cytotoxic effect than *K. africana* seed oil. There was a significant statistical difference between *X. caffra* and *M. zeyheri* oil at 48 h incubation on MCF-7 breast cancer cells (Fig. 2b; $p \leq 0.001$). *Mimusops zeyheri* seed oil produced a larger negative cytotoxic effect than *X. caffra* seed oil (−4.76% vs. −0.27%). Additionally, *M. zeyheri* seed oil effects showed a time-dependent effect (Fig. 2b, $p \leq 0.001$), eliciting a negative cytotoxic effect at 48 h, followed by recovery of the MCF-7 breast cancer cells at 72 h (−4.76% vs 0.13%).

Comparison between each cell line showed differential responses to the seed oils, potentially related to tumor cell phenotype (Fig. 3).

Significant differences were found between the two cell lines when incubated with *K. africana* seed oil at 48 and 72 h (Fig. 3a; $p \leq 0.001$ and $p \leq 0.05$ respectively). *Kigelia africana* seed oil induced a cytotoxic effect on MDA-MB-231 breast cancer cells but a negative cytotoxic result was found for the MCF-7 breast cancer cells. At 48 h, *X. caffra* seed oil induced a higher cytotoxic effect on MDA-MB-231 breast cancer cells than MCF-7 breast cancer cells (2.46% vs. −0.41%) (Fig. 3b; $p \leq 0.001$). When the two cell lines were incubated with *M. zeyheri* seed oil, a significant statistical difference was found between the cell lines at 24-, 48- and 72-h incubation (Fig. 3c; $p \leq 0.001$, $p \leq 0.001$ and $p \leq 0.05$ respectively). *Mimusops zeyheri* seed oil induced a cytotoxic effect on MDA-MB-231 breast cancer cells at 24-, 48- and 72-h incubation but a negative cytotoxic result was found for the MCF-7 breast cancer cells at 24 and 48 h incubation (1.28% vs. −2.89% and 1.47% vs. −4.76%, respectively).

3.3. Cell viability

In this colorimetric cytotoxicity assay, viable cells take up and store the neutral red dye in lysosomes, which is released in the presence of

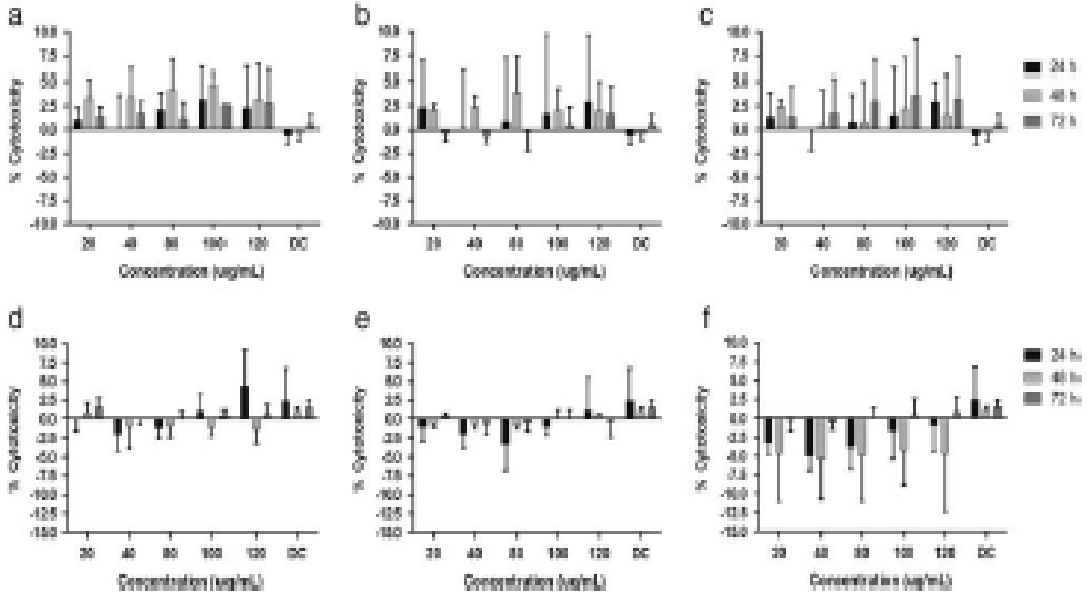

Fig. 1. The cytotoxic effects of differing concentrations of *K. africana* (a, d), *X. caffra* (b, e) and *M. zeyheri* (c, f) seed oils and diluent control (DC) on MDA-MB-231 (a, b, c) and MCF-7 (d, e, f) breast cancer cells at 24, 48 and 72 h incubation.

solubilization buffer (Weyermann et al., 2005). Healthy cells with intact lysosomes store more neutral red dye than dead cells or cells undergoing cell death.

Similar to the results obtained with the cytotoxicity assay (Fig. 1) no significant effect on cell viability was induced by changes in seed oil concentrations or the diluent control, regardless of cell type (data not shown). However, further analysis identified that MCF-7 cells and MDA-MB-231 cells responded differently to a collective effect of type of seed oil and incubation time employed (Fig. 4).

Mimusops zeyheri seed oil induced a decrease in cell viability at all incubation times compared to other seed oils; however, only significantly compared to *X. caffra* at 24 h incubation ($p \leq 0.05$) (Fig. 4a). The hormone-dependent MCF-7 cell line showed a markedly different response to the seed oils and incubation periods (Fig. 4b). At 24 h incubation, in comparison to the effect of *K. Africana* seed oil, *X. caffra* seed oil induced the most significant decrease in MCF-7 cell viability ($p \leq 0.001$), followed by *M. zeyheri* seed oil (Fig. 4b). Additionally, the response of MCF-7 cells to the seed oils were shown to be dependent on exposure time. *Mimusops zeyheri* seed oil treatment induced a significant increase ($p \leq 0.05$) in cell viability by 72 h. MCF-7 cell viability under *K. Africana* treatment reduced at 48 h, followed by recovery at 72 h.

No statistically significant difference was observed between the two cell lines when treated with *K. africana* or *M. zeyheri* seed oil. Only *X. caffra* seed oil showed an effect, with a significantly higher level of cell viability for MDA-MB-231 than MCF-7 cells at 24 h incubation (Fig. 5b; $p \leq 0.05$).

Kigelia africana seed oil had the highest concentration of α-linolenic acid (50.03%) while *M. zeyheri* had the highest concentration of linoleic acid (18.87%). *Kigelia africana*, *X. caffra* and *M. zeyheri* seed oils had acytotoxic effect on hormone-independent MDA-MB-231 breast cancer cells but a growth inhibitory effect on hormone-dependent MCF-7 breast cancer cells.

4. Discussion

With the incidence of breast cancer in developing countries on the increase, coupled with the use of ethnomedicines, determining if commonly used seed oils have any effect on breast cancer is essential. *Kigelia africana* seed oil has previously been shown to elicit a growth inhibitory effect on Caco-2 and HEK-293 cells at a range of concentrations, whereas *M. zeyheri* and *X. caffra* seed oils exerted a similar effect only at 80 μg/mL (Chivandi et al., 2012). Our results, however, indicate that matched concentrations of these seed oils have no significant impact on breast cancer cell growth inhibition, despite the highest concentration being 6 times as large as that of the lowest concentration. This is an interesting phenomenon, given that multiple studies have

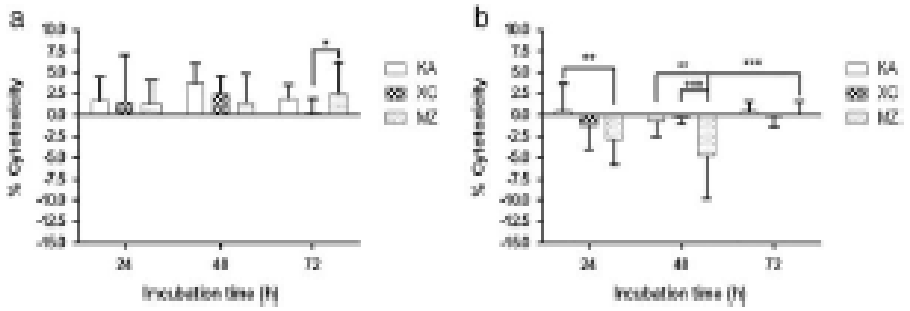

Fig. 2. Comparison of the cytotoxicity effects of the oil from seed oils (KA = *K. africana*; XC = *X. caffra* and MZ = *M. zeyheri*) on (a) MDA-MB-231 and (b) MCF-7 breast cancer cells after 24, 48 and 72 h incubation. The overall cytotoxicity effect of *K. africana*, *X. caffra* and *M. zeyheri* seed oils on MDA-MB-231 breast cancer cells (a) was less than 5% whereas with MCF-7 breast cancer cells was less than 15. The three seed oils induced acytotoxic effect on MDA-MB-231 breast cancer cells but a negative cytotoxic effect on the MCF-7 breast cancer cells indicating cell growth inhibition. The bars represent standard deviations where * = $p \leq 0.05$; ** = $p \leq 0.01$ and *** = $p \leq 0.001$.

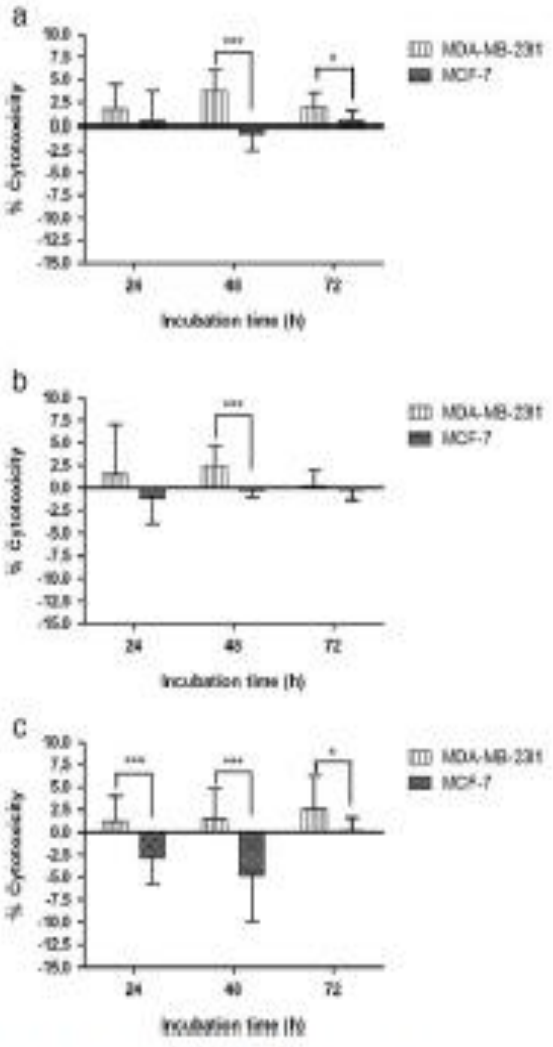

Fig. 3. Comparison of the cytotoxicity effects between MDA-MB-231 and MCF-7 breast cancer cells after 24, 48 and 72 h incubation with a) *X. africana*; b) *X. caffra* and c) *M. zeyheri* seed oils. A cytotoxic effect was observed on MDA-MB-231. A negative cytotoxic effect was observed for MCF-7 breast cancer cells at 48 h. The bars represent standard deviations where * = $p \leq 0.05$ and ** = $p \leq 0.001$.

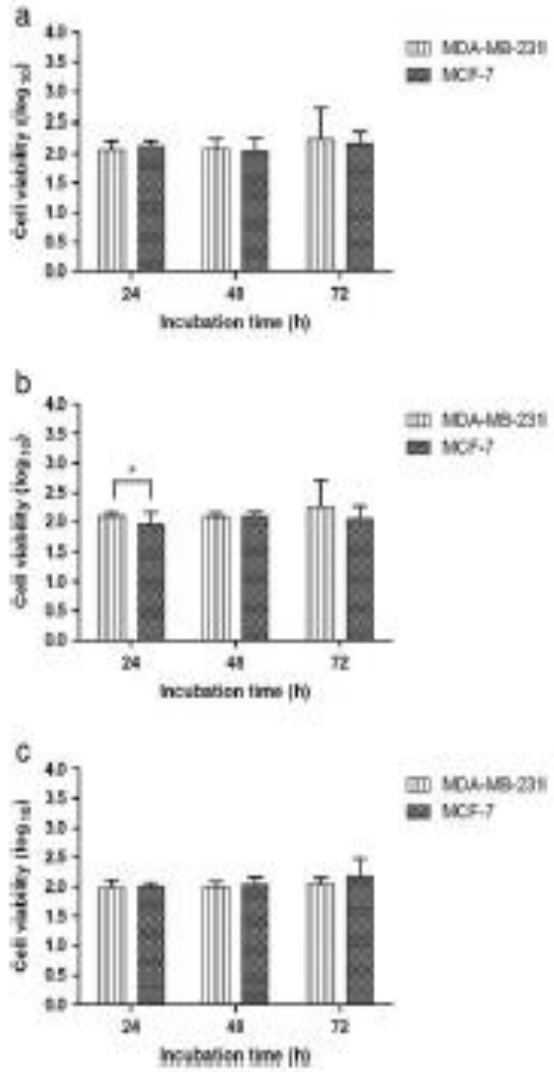

Fig 5. Comparison between MDA-MB-231 and MCF-7 breast cancer cell viability after 24, 48 and 72 h incubation with a) *X. africana*; b) *X. caffra* and c) *M. zeyheri* seed oils. No significant statistical differences were observed between the two cell lines when treated with *X. africana* or *M. zeyheri* seed oil. Only *X. caffra* showed an effect. The bars represent standard deviations where * = $p \leq 0.05$.

previously identified dose-dependent effects in cell lines (Al-Sheddi et al., 2015; Guo et al., 2016), which may indicate that the range of concentrations employed in our study were too low to elicit an effect in, specifically, breast cancer cells. This result highlights the possible induction of differential effects by seed oils in different tumor types, a postulation supported by findings that pomegranate seed oil decreases breast cancer cell viability, with no effect on colon (HT29

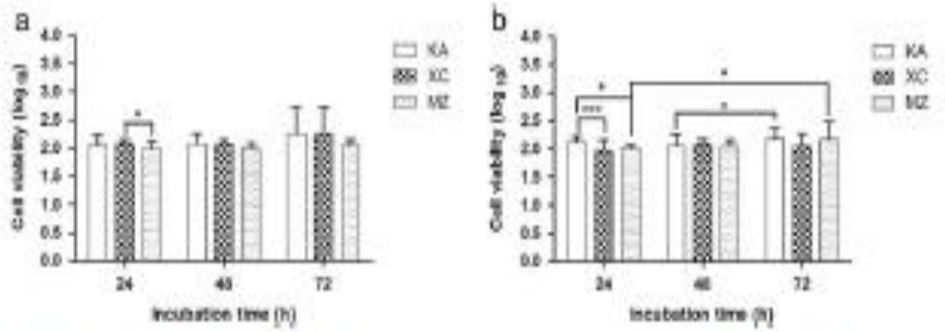

Fig. 4. Comparison of the different seed oils (KA = *X. africana*; KC = *X. caffra* and MZ = *M. zeyheri*) on cell viability of (a) MDA-MB-231 and (b) MCF-7 breast cancer cells after 24, 48 and 72 h incubation. *M. zeyheri* seed oil induced a decrease in MDA-MB-231 cell viability (a) compared to *X. africana* and *X. caffra* seed oil at 24, 48, 72 h. Incubation time had an effect on cell viability of MCF-7 cells (b). The bars represent standard deviations where * = $p \leq 0.05$ and ** = $p \leq 0.001$.

and HCT116), hepatic (HepG2 and Huh7) or prostate (DU145) cancer cell lines (Constantini et al., 2014). Our results point to the effects of *K. africana*, *M. zeyheri* and *X. caffra* seed oils being more dependent on fatty acid composition, the duration of exposure, as well as phenotype of the breast tumors.

Hormone-dependent MCF-7 cells are a weakly metastatic breast cancer cell line (Flodrova et al., 2016; Pogash et al., 2015) that is phenotypically and behaviourally different to the more aggressive, hormone-independent triple-negative MDA-MB-231 cell line (Flodrova et al., 2016; Pogash et al., 2015). This was reflected by MDA-MB-231 cell viability being least affected and MCF-7 cells being more responsive to the seed oils. MCF-7 cells displayed a reduction in viability associated with exposure time to *M. zeyheri* and *K. africana*; however, cell viability peaked at 72 h.

Cytotoxicity tests are used to screen the cytotoxic potential of compounds present in extracts that affect cellular functions (Ganesh et al., 2013). Criteria used to establish cell damage depend on the techniques used and can be divided into changes in cell morphology, cell function impairment and increased permeability of the plasma membrane (Danpure, 1984). The assessment of cell viability was based on use of the neutral red assay, which is dependent on this supravital dye diffusing through the plasma membrane of viable, metabolising cells and concentrating in lysosomes (Borenfreund and Puerner, 1984; Weyermann et al., 2005). The amount of dye extracted from the cells using solubilization buffer is directly proportional to the number of viable cells (Weyermann et al., 2005). Since studies have shown variability between different assays, which may be based on different modes of cell death (Weyermann et al., 2005), we also employed the LDH assay to determine the permeability of the plasma membrane which is an indication of necrotic cells and which can be quantified by measuring the release of the LDH enzyme (Chan et al., 2013). This dual approach provides more information regarding seed oil effects than the trypan blue exclusion method based on manual hemocytometer cell counting employed by Chivandi et al. (2012). A method which is time-consuming, has user-to-user variation and does not differentiate between apoptotic and necrotic cells (Altman et al., 1993).

By monitoring the integrity of the plasma membrane, we found that MDA-MB-231 cells were more susceptible to a cytotoxic effect than MCF-7 cells. Specifically, *K. africana* and *X. caffra* seed oils elicited the greatest cytotoxic effects on MDA-MB-231 cells at 48 h incubation; however, cells showed recovery capacity at 72 h. While this may be related to the doubling time (41.9 h) of these hormone-independent cells (Dolfi et al., 2013; Kulsin and Chan, 2013), this phenomenon might also be related to these compounds requiring additional time to induce cell death (Smith et al., 2011), or recovery of cell membrane integrity (Chivandi et al., 2012). Moreover, while *K. africana* seed oil produced a positive, albeit low, cytotoxic effect on MCF-7 cells, *X. caffra* and *M. zeyheri* seed oils elicited a negative cytotoxic effect. This finding is essentially linked to the basis of defining levels of cytotoxicity using the LDH assay. It is proposed that the cells within the control group continue to divide and multiply whereas exposure to seed oils halted cell cycle resulting in no division and multiplication of cells, the result of which being quantitatively expressed as a "negative" cytotoxic effect (Smith et al., 2011). As such the negative cytotoxic effect observed in our results is indicative of MCF-7 cell growth inhibition, as opposed to induction of cell death alone.

While an association between the effects of seed oils and seed oil composition were identified, in comparison with profiles reported by Chivandi et al. (2012), our study found a lower lipid yield. These differences might be attributable to different extraction methods or conditions, different cultivars and climatic conditions (Bala et al., 2018; Sorice et al., 2016).

Variance in the fatty acid profile of each seed oil may explain the differences in induced effects on each cell line. *Kigelia africana*, which yielded particularly high levels of α-linolenic acid, had a greater cytotoxic effect on MDA-MDA-231 cells than MCF-7 cells. Flaxseed oil, which contains approximately 57% α-linolenic acid, an omega-3 polyunsaturated fatty acid, successfully reduced breast cancer cell growth (Wiggins et al., 2015). Direct α-linolenic acid treatment has been shown to reduce prostaglandin-E2 in MDA-MB-231 by lowering the level of cyclooxygenase (COX) 2 expression (Horia and Watkins, 2005). COX2, which is required for the conversion of arachidonic acid to prostaglandins (Smith et al., 2000), is highly expressed in MDA-MB-231 cells compared to MCF-7 cells (Horia and Watkins, 2005) and is associated with tumor initiation and progression (Smith et al., 2000). Additionally, linolenic acid-derived components eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are associated with growth-inhibitory effects on MDA-MB-231 and MCF-7 breast cancer cells (Chamras et al., 2002; Schley et al., 2005). Growth inhibition was identified in our study only in the MCF-7 line, specifically for *M. zeyheri* seed oil and to a lesser extent, *X. caffra*, both of which conversely presented with low levels of α-linolenic acid. *Mimusops zeyheri* seed oil presented a comparatively higher level of linoleic acid, as well as total omega-6 and omega-9 fatty acids than *K. africana* seed oil.

The mechanism of apoptosis caused by Bcl2, Caspase 3 and Bax, is currently being investigated by flow cytometry and real time PCR. Subsequent experiments will include the potential mechanisms of action of these seed oils on lipid peroxidation, altering cell membrane receptor (EGFR, ERα and HER2) expression and activation.

In conclusion, we observed that the seed oils had a cytotoxic effect on MDA-MB-231 breast cancer cells but a growth inhibitory effect on MCF-7 breast cancer cells. This was only detected by the LDH assay, with the results from the neutral red assay being inconclusive due to the mechanisms by which these seed oils exert their effects and which will require further investigation. While inferences can be drawn regarding the composition of these seed oils in relation to their effects, more work remains to be done to clarify these inferences.

Declarations of interest

None.

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