Immunomodulatory and anticancer potential of a traditional medicine product from a traditional healer against MCF-7 breast cancer cells.

By

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List of abbreviations

ATM	African Traditional Medicine
BCS	Breast conserving surgery
BCSC	Breast cancer stem cells
CANSA	Cancer Association of South Africa
CPT	Campthothecin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ELISA	Enzyme Linked Immunosorbent Assay
ER	Estrogen Receptor
FCS	Heated-inactivated fetal calf serum
FDA	Food and Drugs Administration
GM-CSF	Granulocytes macrophage colony-stimulating factor
GSH	Glutathione
HER2	Human epidermal growth factor receptor
ICPI	Immune Checkpoint Inhibitor
IL	Interleukin
IP	Intellectual Property
NCI	National Cancer Institute
OBSC	Oncoplastic breast conserving surgery
PBMCs	Human peripheral blood mononuclear cells
PBS	Phosphate Buffer Saline

PHA	Phytohaemagglutinin
PR	Progesterone receptor
PSF	Penstreptomycin
RLU	Reflective Light Units
RPMI-1640	Roswell Park Memorial Institute
TNBC	Triple negative breast carcinoma
WHO	World Health Organization

Abstract

Introduction:

The incidence of breast cancer in South Africa is expected to rise dramatically in the forthcoming decade. In 2018, approximately 2 088 849 new cases and 626 679 cases of deaths from breast cancer alone were reported worldwide. Among African countries, the incidence of cancer is higher in southern Africa with an estimate standard rate of 235.9 per 1000, 0000 people. Currently breast cancer is treated with either chemotherapy, surgery, radiotherapy or immunotherapy. All this conventional treatment options available for breast cancer have some limitations and drawbacks. Therefore, there is an urgent need for the development of novel breast cancer therapy that will potential kill tumour cells more effective and selective. The use of traditional medicine as an alternative source of healthcare is gaining an increasing interest in many countries including South Africa. This is due to their accessibly and perceived effectiveness. However, the majority of these traditional medicines have not been scientifically validated.

Aim:

The purpose of this study was to determine immunomodulatory and anti-cancer activity of an African traditional medicine product from a traditional healer (Product Nkadinde, a traditional medicine composed of four medicinal plants) on MCF-7 breast tumour cells.

Methods:

Normal peripheral blood mononuclear cells (PBMCs) were used initially to establish the IC_{50} dose of Product Nkabinde using the Celltiter-Glo™ ATP assay. Conditioned media from PBMCs was then prepared by treating a separate set of normal PBMCs using the IC_{50} dose and the positive control, camptothecin (1μM). For the anticancer assays, the Celltiter-Glo™ ATP assay was used to determine the cytotoxicity effects of conditioned media from PBMCs with Product Nkabinde against MCF-7 breast cancer cells. Antioxidant levels (changes in intracellular glutathione (GSH) levels) were measured using GSH-Glo™ Glutathione assay kit. The mechanism of cell death was determined using the Caspase 3/7 and DNA fragmentation assays. Immunomodulation studies was carried out using a multi analyte cytokine ELISA assay kit.

Results:

The results obtained from this study revealed that Product Nkabinde was cytotoxic to MCF-7 and an IC_{50} dose was established at 344 µg/ml in treatment PBMCs. Product Nkabinde was able to induce caspase 3/7 activity, decrease intracellular GSH levels and induce DNA fragmentation comparative to the positive control, thus showed potential to induce apoptosis in breast cancer cells. Inflammatory cytokines secretion studies indicated that Product Nkabinde did not significantly change the secretion of all 12 inflammatory cytokines analyzed in treated PBMCs. However, Product Nkabinde was shown to increase the secretion of IL 1β, IL 8 and GM-CSF in treated MCF-7 cells, although this was not significant.

Conclusion:

The results of this study showed that Product Nkabinde induced immunomodulatory effects through changes in cytokines secretion and direct anticancer effects through activation of caspase 3/7 enzymes, decreasing GSH levels and induction of DNA fragmentation in treated MCF-7 cells. This traditional medicine has the potential to be developed and optimized as an alternative anticancer medicine.

Chapter 1

Introduction

1.1 Background

Cancer is one of the non-communicable diseases that has a high mortality rate worldwide (Alshammari, 2019; Sun et al., 2018). Cancer has been regarded as the second leading cause of death globally in the 21st century (Bray et al., 2018; Pawar et al., 2019). This is a disease that is characterized by uncontrollable growth of abnormal cells due to genetic impairment (Ashiq, 2018). Breast cancer is one of the most common form of cancer mostly diagnosed among woman worldwide with 2.4 million cases (Alshammari, 2019). The World Health Organization (WHO) has reported that approximately 627 000 women died from breast cancer in 2018 (WHO, 2018). It has been reported that the incident rate of breast cancer is low in Africa (Espina et al., 2017; Jedy-Agba et al., 2016). However, survival rate from breast cancer is poorer in less developed countries than developed countries (Espina et al., 2017). The incidence of breast cancer in less developed countries is expected to rise dramatically in the forthcoming decade (Bray et al., 2012; Jedy-Agba et al., 2016). This is largely due to population growth as well as lifestyle changes (Pervaiz and Faisal, 2017). Among African countries, the incidence of cancer is higher in southern Africa with an estimate standard rate of 235.9 per 100 000 people (Pervaiz and Faisal, 2017). The rate of cancer in South African continues to rise every year (Manu and Kutten, 2008; Sylla et al., 2012). In 2014, approximately 38 000 people died from cancer in South Africa (Made et al., 2017). It is also estimated that about 115 000 people are diagnosed with cancer each year in South Africa (CANSA, 2018).

Currently cancer is treated with either radiotherapy, chemotherapy, surgery, biological therapy and/or immunotherapy (Ashiq, 2018). The most common and effective method for treatment of cancer are surgery, radiotherapy as well as chemotherapy (Nagle et al., 2018). Specifically for breast cancer, the most common method of treatment is surgery which involves mastectomy and lumpectomy (Elkayal et al., 2015). Despite these significant improvements in cancer research and treatments in the past decade, there remain a need for development of new cancer therapies (Ju et al., 2018). This is largely due to fact that most of the current treatment methods have some limitations and drawbacks to a certain extent (Safarzadeh et al., 2014; Qi et al., 2015). For example,

it is well documented that the radiotherapy and chemotherapy are associated with various severe side effects and poor prognosis (Elkayal et al., 2015; Kim et al., 2019; Li et al., 2012, Li et al., 2016; Ochwangi et al., 2014). These treatment methods fail to remove all or only tumorous cells inside the patient but also result in cellular damage of healthy cells (Ashiq, 2018).

In the past 20 years immunotherapy was not recommended for cancer treatment because breast cancer was not regarded as immunogenic (Disis and Standton, 2018). The use of immunotherapy to treat tumours has however, become part of the new innovation in the sphere of oncology (Hu et al., 2019). There are several immunotherapies that have been developed including cytokines, T-cell therapy and cancer vaccines (Qi et al., 2015). These therapies can modulate immune cells to improve their immune activity (Zavala and Kalergis, 2015). It is important to note that prices of immune therapies is very high, and most people cannot afford it (Qi et al., 2015). Immune Checkpoint Inhibitor (ICPI) is one of the promising approaches that has the potential to treat tumours with less side effect (Hu et al., 2019). However, these immunotherapies are still at trial stages and some of them have shown severe side effects similar to those exhibited by currently prescribed treatments.

Such problems have stimulated researchers around the world to strive for the development of cancer treatment from natural products (Kim et al., 2017; Sakthive et al., 2012; Sulistyani, 2017). There is an increasing scientific and commercial interest in researching natural products as sources of new anti-cancer agents (Fouche et al., 2008; Li et al., 2016). Complementary medicines such as traditional medicines are increasingly being used for treatment of cancer worldwide (Ochwangi et al., 2014). However, herbal therapies have not been extensively studied or tested (Ochwangi et al., 2014).

The modification of the immune response by tumour cells results in rapid progression of cancer (Razali et al., 2016). This problem can be mitigated by modulating the immune response within the host, this will subsequently trigger the host's own immune system which will lead to eradication of tumor cells (Razali et al., 2016). A substance that modifies the activity of the immune system is called an immunomodulator (Manu and Kuttan, 2008). This substance can act as either an immunosuppressant or immunostimulator (Nagarathna et al., 2013). This study focused on a plant-based African traditional medicine product with immunomodulatory properties as a potential immunomodulator against breast cancer *in vitro*.

The WHO reported that approximately eighty percent (80%) of the emerging world's population utilized traditional herbal medicines as their primary source of healthcare (WHO, 2013). This is largely due to the common belief that herbal medicine possesses less to no side effects (Rashid et al., 2018). Utilization of herbal medicines in Africa has been largely due to inadequate access to western form remedies and most people cannot afford to access Western treatments (Mahomoodally, 2013). In South Africa, traditional medicine has been used by traditional healers to treat cancer and this knowledge system has pass from generation to generation (Koduru et al., 2007; Mahomoodally, 2013). However, the knowledge and experience of these herbalists has not been scientifically validated (Koduru et al., 2007; Ochwangi et al., 2014). Therefore, this study aimed to evaluate the immunomodulatory potential of African traditional medicine against breast cancer cells.

1.2 Rationale, Aim, Objectives and Hypothesis

1.2.1 Rationale of the study

Breast cancer is a malignant disease originating from epithelial tissue of mammary breasts (Feng et al., 2018). Current estimates showed that 2 088 849 new cases of breast cancer case were reported worldwide in 2018 (Song et al., 2020). South Africa has the highest cancer incidence rate amongst African countries with an estimate of 35.0 breast cancer incidence rate per 100,000 population (Pervaiz and Faisal, 2017). The incidence rate of breast cancer in South Africa is expected to rise in the forthcoming decade (Joffe et al., 2018). This is largely due to the expected population growth as well as lifestyle changes. Currently breast cancer is treated with surgery, chemotherapy, radiotherapy and/or immunotherapy (Yu et al., 2017). All these types of treatments are expensive, have some limitations and drawbacks (Safarzadeh et al., 2014). These treatments also fail to eradicate breast cancer completely especially when cancer is at stage 4 and are associated with various side effects (Chang et al., 2019). Therefore, there is an urgent need for the development of novel breast cancer therapies that will potentially kill tumour cells more effectively and be more selective in their actions (Gai et al., 2018).

Recent studies indicate that traditional medicines possess anticancer activity (Hashim et al., 2014; Rungruangmaitree and Jiraungkoorskul. 2017). These traditional medicines are also thought to modulate numerous components of the immune system to fight against cancer cells. *In vitro* study have demonstrated that *Artemisia annua* extract inhibit cancer growth in a dose and time dependent manner (Mashati et al., 2019). *Trametes versicolor* extract was found to inhibit invasion and migration of cancer cells (Roca-Lema et al., 2019). *Ganoderma lucidum* extract possessed anti-tumour and immunomodulatory activities (Lin and Zhang, 2004). Liu et al. (2020) has demonstrated the immunomodulation effects of traditional Chinese medicine against cancer. However, the effectiveness of African traditional medicine (ATM) against breast cancer has not been extensively studied or tested in *in vitro* or clinical models. Therefore, the present study aimed to evaluate the immunomodulatory potential of African traditional medicine against breast cancer cells.

1.2.2 Aim of the study:

The aim of this study was to determine immunomodulatory and anti-cancer activity of an African traditional medicine from a traditional healer, Product Nkabinde, on MCF-7 breast tumour cells.

1.2.3 Specific objectives:

- To determine cytotoxicity effect of traditional medicine on normal PBMCs and MCF-7 breast cancer cells;
- To evaluate the apoptosis inducing potential of this traditional medicine on breast cancer cells;
- To determine the immunomodulatory effects of the traditional medicine on breast cancer cells.

1.2.4 Hypothesis:

The study hypothesized that the traditional medicine from the traditional healer has immunomodulatory and anticancer activities on breast cancer cells.

Chapter 2

Literature review

2.1 Breast cancer

Breast cancer is defined as a heterogeneous group of neoplasm tissue which arise or develop from epithelial cells covering the milk duct (Polyak et al., 2011). Changes in genetic and epigenetic genes that typically control the functioning of epithelial cells covering milk duct result in tumor initiation (Jiang and Shapiro, 2014). However, the main molecular mechanism of breast cancer carcinogenesis and progression is still not fully elucidated (Guo et al., 2019). Breast cancer possesses stem cells that share similar properties present in normal stem cells such as differentiation as well as self-renewal (Ayob and Ramasamy, 2018). Breast cancer stem cells (BCSC) are minute populations of tumour cells that possess the following characteristics: differentiation, self-renewal, specific surface marker and tumorogenicity (Ghasemi et al., 2019). These characteristics make BCSC responsible for tumour initiation, metastasis, progression, local recurrence and relapse of current therapy (Orlova et al., 2018). Differentiation of BCSC results in the formation each types of breast cancer (Orlova et al., 2018). BCSC have the ability to migrate via blood circulation and metastasize at distant sites to form secondary tumours (Al-hajj et al., 2003; Butti et al., 2019). They are many factors that may cause breast cancer and these include internal factors and external factors. The internal factors are the immune system, genetic mutation, and metabolic system mutation and external factors may include radiation and chemicals (Hermawan and Putri, 2018). Family history and woman age have also been described as risk factors for breast tumors (Hartmann et al., 2005; Secretariat, 2010).

2.2 Types of Breast cancer

Breast cancer is not a single disease but a heterogeneous disease that originates from the cells of the breast (Said et al., 2018; Weigelt et al., 2010). It can affect both women and men, and it is most frequent among women (Said et al., 2018). According to classification, breast cancer can either be invasive or non-invasive. Invasive breast cancer means that the tumor has metastasized to the surrounding part or tissue of the breast, while non-invasive or ductal carcinoma in *situ* means that breast tumour stay within mammary duct. It has been reported that metastasis is one of the

major causes of cancer related death (Liang et al., 2019). Invasive breast carcinomas have been classified into five groups which are Luminal A, Luminal B, basal-like, normal breast-like and human epidermal growth factor receptor (HER2) (Livasy et al., 2006). This classification was based on a microarray technique which contradicts histological classification (Anders and Carey 2009). Livasy et al. (2007) reported that little is known concerning the development and treatment of the basal-like subtype breast cancer. Tumour cells are classified as basal-like because of the similarity in gene expression found in normal basal breast cells (Lehmann and Pietenpol, 2014). Basal-like tumour are biologically and clinically aggressive (Anders and Carey, 2009; Lehmann and Pietenpol, 2014) and represent 10%-25% of all carcinoma (Perou, 2011). Some studies suggest that basal-like breast carcinoma is triple negative due to lack of progesterone receptor (PR) expression (Tran and Bedard et al., 2011). Triple negative breast carcinoma (TNBC) typically results from mutation in the p53 gene. The p53 gene is recognized by its role which involves regulating the cell cycle (Bae et al., 2018). It has been reported that patients with TNBC are at high risk of developing visceral or brain metastasis (Castrellon et al., 2018). Basal-like and HER2 subtypes are estrogen receptor (ER) negative and these two subtypes have poor prognosis (Cancello et al., 2012) and high proliferation rate (Livasy et al., 2006). Liminal A and Luminal B breast tumours are ER positive (Tran and Bedard et al., 2011). Luminal A breast carcinoma has a good prognosis and studies have shown that this subtype can be treated by hormone therapy (Cancello et al., 2012). Unlike Luminal A, Luminal B tumours have a poor prognosis and there are no specific therapies recommended or available for this subtype (Cancello et al., 2012). MCF-7 cells are classified as Luminal breast cancer cells (Pogash et al., 2015), because this cell line is ER positive (Prat et al., 2013).

2.3 Epidemiology of breast cancer

The Cancer Association of South Africa (CANSA) has reported that cancer has social and economic impact, with around US$1.6 trillion spent on prevention and control of this disease annually (CANSA, 2016; WHO, 2018). Previous studies documented that cancer constitute approximately 2-3 % of annual deaths globally (Shareef et al., 2016). It has been reported that cancer incidence rate as well as mortality rate are growing globally (Bray et al., 2018). In 2018, an estimate of 2 088 849 new cases and 626 679 number of deaths from breast cancer alone were

reported worldwide (Bray et al., 2018). While in 2012, about 1 671 149 new cases of breast carcinoma and 527 907 cases of death occurred worldwide (Ghoncheh et al., 2016). These statistics indicate the need for development of cancer treatments that will be accessible to everyone. In 2008, 68,000 women were diagnosed with breast cancer and 37,000 deaths were recorded in Africa (Abdulrahman et al., 2012). Breast cancer also occurs in males and this accounts for less than 1% of all breast tumour cases (Giunta et al., 2017). Breast cancer is more prevalent in older women, 45% of all breast cancer cases occur in women over the age of 65-year-old and 33% arise occur in women over the age 70 years old (Esposito et al., 2015; Reginelli et al., 2014). It has been reported that amongst the population in the Sub-Saharan African region, breast cancer incidence rates are higher in Southern African women (Jemal et al., 2012). South Africa was reported to have a high cancer incidence rate amongst the middle to high income population (35.0 breast cancer incidence rate per 100,000 population) in July 2009 (Wadler et al., 2011).

2.4 Currently available treatment for Breast cancer

2.4.1. Surgery

There are two types of surgery treatments currently available for patients with early breast cancer, namely breast conserving surgery (BCS) or oncoplastic breast conserving surgery (OBCS) and mastectomy (Abdelshafy et al., 2018). During breast conserving surgical operation, local anesthesia with intravenous sedation is typically employed for definitive wide local excision (Apantaku, 2002). The excision is normally made directly over the tumour, only malignant carcinoma or affected tissues are removed during operation (Apantaku, 2002). The main goal of BCS is to excise breast cancer cells with clear margins yet providing a good cosmetic outcome at the same time (Clough et al., 2015; Khan et al., 2017). The cosmetic result is typically influenced by the size of the tumour to be excised (Clough et al., 2015). Patients with small cancers have good cosmetic outcomes than those with larger cancers (Khan et al., 2017). BCS has been regarded as a gold standard (Khafagy et al., 2012; Zhang et al., 2018), and the most effective method for treatment of patients with early breast cancer (Knuttel et al., 2017; Pajak et al., 2018). Most patients prefer BSC because it is more effective in controlling cancer with cosmetic results that are preferable by patients (Ogawa, 2014). The surgery for BSC has been described as “preference sensitive care’’ (Gu et al., 2018). However, patients who have undergone BCS carry high risk of local recurrence of breast tumour for life (Fajdic et al., 2013).

Mastectomy has been regarded as standard surgical approach for treatment of in-breast local recurrence of carcinoma (Kuerer et al., 2004). Mastectomy procedure involves removal of the entire (one or both) breast that have tumours (Menon and Mohony, 2019). Surgeons typically uses standard electrocautery-based dissection kit to suture skin and subcutaneous tissue (Mazouni et al., 2015). The skin incision is subsequently stitched in a double layer manner after complete removal of the breast. Mastectomy is the most invasive surgical procedure for treating breast carcinoma (Kauer-Dorner et al., 2012; Menon and Mahony, 2019). It has been indicated that mastectomy is associated with various side effects and these may include changes in body image as well as high surgical site complication rates (Crown et al., 2019). Patients who undergo mastectomy with reconstruction have the worse body image than patients who have received BSC (Collins et al., 2011). Mastectomy has the negative effect on quality of life as most patients after treatment suffer from emotional and physical distress (Kauer-Dorner et al., 2012). Hamelinck et al. (2018) has reported that the risk of undergoing second surgery are slimmer in mastectomy.

2.4.2. Radiotherapy

Radiotherapy involves the use of X-rays to deliver high dose of radiation to control or destroy the growth of malignant tissues (Ronckers et al., 2004). There are two ways that has been proposed to deliver radiation to the location of tumours. It can either be via external beam radiation or internal brachytherapy (Baskar et al., 2012). Radiotherapy has the potential to reduce the risk of local recurrence in the affected breast (He et al., 2018; Reginelli et al., 2014). Radiation therapy is recommended for treatment of breast cancer after the patient has undergone mastectomy or BSC (Flatley and Dodwell, 2016; Kundrat et al., 2019; Rather et al., 2014). It has been previously reported that radiotherapy is a cost-effective treatment for breast cancer (Baskar et al., 2012). Women who have been treated with radiotherapy are at high risk of developing new primary cancer because radiotherapy is also carcinogenic (Rather et al., 2014) and has toxic effects on normal tissues (Ebeid et al., 2016). Radiation does not only destroy abnormal cell but also destroy even normal cells. Johnson et al. (2019) suggested that toxicity associated with breast tumour radiotherapy can be reduced by identification of genes that affect “circadian rhythm”.

2.4.3. Chemotherapy

Chemotherapy is also a treatment option available for patients with breast cancer. Chemotherapy typically uses antineoplastic agents to destroy cancer cells by interfering with cellular functioning (Singh et al., 2018). Majority of chemotherapy drugs normally target rapidly dividing cancerous cells (Ramakrishnan and Gabrilovich, 2011). These drugs have the ability to cause immunologic active cancer cell death and has immunosuppressive effect on the immune system (Mattarollo et al., 2011; Ramakrishnan and Gabrilovich, 2011). There are several side effects that are associated with chemotherapy (Soliman and Osman, 2018; Zu et al., 2018). Side effects may include alopecia, myelosuppression, emesis and nausea, (Partridge et al., 2001). All these side effects affect the ability of patients to complete or adhere to chemotherapy (Marx et al., 2017; Windebank and Grislod, 2008). Steenkamp and Gouws (2006) indicated that the majority of chemotherapy drugs have negative effects on normal cells. The toxic effects of chemotherapy on normal cells limits its effectiveness on breast cancer treatment (Ochwangi et al., 2014). Some studies suggest that a combination of natural based products with chemotherapy may overcome cytotoxicity effects associated with chemotherapy on normal cells (Zu et al., 2018).

2.4.4. Immunotherapy

Immunotherapy has been defined as a new approach to treat breast cancer either by augmenting or generating an immune response against it (Khalil et al., 2016; Qi et al., 2015). Immunotherapies influence various features of the immune systems such as boosting tumour antigen presentation as well enhancing T cell activation (Koury et al., 2018). There are few types of T-cells that are capable of destroying tumours, namely Type 1 phenotype and CD4 T-cells (Disis and Stanton, 2018). In the tumour microenvironment CD4 T-cells normally secret type 1 cytokines such as tumour necrosis factor-alpha and interferon-gamma (Disis and Stanton, 2018). Interleukin-6 (IL-6) is another inflammatory cytokine that is secreted or released by several cells as well as cancerous cells in tumour microenvironment (Masjedi et al., 2018). These cytokines play an essential role in the induction of cell-mediated immune response that plays a role in the development of cancer. An accurate dosage of cytokines in cancer treatment remains challenging (Patwardhan and Gautam, 2005). Immunotherapy has been shown to be effective in a minority of patients (Huber et al., 2017).

They are two strategies that have been developed to harness or strengthen the immune system to treat or eradicate breast carcinoma. These include vaccines and drugs that usually modulate T-cell inhibitory checkpoints (Soliman and Osman, 2018; Sanchez et al., 2016). Immune checkpoint inhibitor agents have the ability to allow tumour educated T-cells to identify cancer and inhibit tumour growth (Ramsay et al., 2013). There are several immunotherapies that have been approved by the Food and Drugs Administration (FDA) and other agencies (Nathan and Scmid, 2018). The FDA has approved the use of Tecentriq and Abraxane for patients with advanced TNBC. According to the National Cancer Institute (NCI), immunotherapy is associated with several side effects the commonest ones being pain, swelling, rash and soreness (NCI, 2018). The shortfall with immunotherapy is that they are costly and most cancer patients cannot access them (Qi et al., 2015).

2.5 Traditional medicines as alternatives against breast cancer

Medicinal plants possess secondary metabolites that are active ingredients for folk medicine (Sawadogo et al., 2012). Approximately 70% of anticancer compounds are derived or isolated from medicinal plants (Ahmad et al., 2017; Singh et al., 2004). These compounds are used as active ingredients for formulation of cancer drugs, and many lead to development of novel anticancer drugs. Recent studies indicate that most of the patients diagnosed with cancer use traditional medicine even during chemotherapy or in combination with other therapies (Ahmad et al., 2017; Damery et al., 2011). It has been recently reported that approximately 27 million people in South Africa still rely on traditional medicine as their primary source of health care (Twilley et al., 2020). In South Africa, there are few reports or studies on medicinal plant/s or traditional medicines that are typically used in cancer treatment (Steenkamp and Gouws, 2006).

Many women in South Africa seek treatment for various diseases from traditional healers (Steenkamp, 2003). The use of traditional medicine as an alternative source of healthcare is gaining an increasing interest in many countries including South Africa due to their accessibly (Mahwasane and Boaduo, 2013). However, the safety and effectiveness of African traditional medicines against many ailments has not been rigorously tested (Ochwangi et al., 2014). It has been reported that *Equisetum hyemale* extract inhibits breast cancer cell proliferation. The inhibitory activity of this plant extract on breast cancer cell line was shown to be dose dependent

(Li et al., 2012). Similar results were also demonstrated on the MCF-7 cell line where certain dosages of *Wrightia tomentosa* extract inhibited proliferation of this breast cancer cell line (Chakravarti et al., 2012). *In vitro* studies have shown that traditional medicine treats carcinomas by inhibiting tumour cell growth (Hu et al., 2018; Tripathy and Pradhan, 2013; Qin et al., 2019). The inhibition of breast cancer stem cells proliferation by traditional medicine (Curcumin extract) suppresses metastasis and subsequently limits tumour formation (Liu et al., 2018). Other studies have shown that at low dosages traditional Chinese medicine induces apoptosis in breast cancer cells (Zu et al., 2018). Recent study indicate that the Flaxseed *(Linum usitatissimum)* extract induces apoptosis in dose and time dependent manner (Hu et al., 2018).A recent study indicated that the majority of South African medicinal plants have anti-tumour activities (Fouche, 2008). Other findings have shown that medicinal plants used in the formulation of traditional medicine exhibit high toxicity activity against breast cancer cell lines (Itharat et al., 2004). While Singh et al. (2019) reported that the *Amoora rohituka* extract has anticancer activity with less toxicity effect in normal cells. A recent study from Chinese traditional medicine has demonstrated that Chinese herbal extract Qinghoufu can prevent migration as well as proliferation of the MCF-7 cell line (Hu et al., 2018).

2.6 Traditional medicine as immunomodulators against cancer

Recent studies have demonstrated that traditional medicines can modify the host immune response and subsequently make the immune system more capable in eradicating tumours (Otsuki et al, 2010; Razali et al., 2016). Traditional medicine modulates numerous components of the immune system, this includes NK cells, antigen presenting cells, T and B lymphocytes (Lin and Zhang, 2004). In addition, some traditional medicines modulate the expression of various cytokines (Spelman et al., 2006). The study by Ngcobo and Gqaleni (2016) demonstrated that African traditional medicine can stimulate the secretion of pro-inflammatory cytokines. TNF cytokines are the main immumoregulatory molecules with immunomodulatory and antitumor properties (Imir et al., 2016). Reports from previous studies indicate that the anti-tumour effects of traditional medicines might be implemented by boosting the immune system (Imir et al., 2016). Traditional Chinese Medicine is thought to have the ability to enhance anticancer immunity by synchronizing the balance between anti- and pro-inflammatory cytokines (Ma et al., 2012). Some medicinal

plants are also known to possess both immunosuppressive and immunostimulatory properties (Upadhayay et al., 2012).

2.7 Conclusion

The incidence of breast cancer in South Africa is expected to rise in the forthcoming decades. Current cancer treatments are not effective in eradicating breast cancer cells in cancer patients. Most South Africans rely on traditional medicine as their primary source of healthcare including for cancer treatment. Most of these traditional medicines have not been scientifically validated for their effectiveness against breast cancer and there is a need for them to be extensively studied or tested. This study therefore aimed to evaluate the immunomodulatory effects of an African traditional medicine product prescribed by a traditional healer on MCF-7 breast cancer cells.

Chapter 3

Materials and Methods

3.1 Ethical clearance

The present study received ethical clearance from Biomedical Research Ethic Committee of University of KwaZulu-Natal (BREC reference number: BE452/19). A copy of current BREC approval certificate in Appendix A.

3.2 Study area

The evaluation of the immunomodulatory and anticancer effects of an African traditional medicine product prescribed by a traditional healer on MCF-7 breast cancer cells were conducted in the Traditional Medicine Laboratory, School of Nursing and Public Health, University of KwaZulu-Natal.

3.3 Botanical identification

This project was funded by the Department of Science and Innovation and was done in collaboration with the Durban University of Technology and the University of Pretoria. The traditional medicine was supplied by Mr. Siphathimandla Nkabinde, a traditional healer from Dundee in KwaZulu Natal. He prepared the ATM by drying, grinding, extracts from 4 individual plants (see Table 1) and combined of all four medicinal plants at a specific ratio. After cooling, the solid material was filtered and the decoction (water mixture) was used. Plants samples as used by Mr. Nkabinde were delivered to the Department of Chemistry, University of Pretoria and botanically identified by the curator of the University Herbarium and coded (see Table 1 for the codes). The identities of the 4 medicinal plants will be withheld until the intellectual property rights of the traditional healer are secured by the Department of Science and Innovation. For our experiments, the filter-sterilized crude extract was freeze dried to powder as described below in order to prepare standardized concentrations for laboratory experiments.

Table 1: Medicinal plants used by the herbalist to make the traditional medicine.

Plant names	Plant part	Nkabinde Code
	Stem bark	MGN
	Roots	SPN
	Roots	SDK
	Roots	CML
Mixture as used by Mr Nkabinde	Mixture (roots and stem bark)	As prescribed to his patients

3.4 Preparation of the traditional medicine

The ATM was prepared for laboratory experiments as follows: the ATM extract as prepared by Mr. Nkabinde was first filtered by high speed centrifugation in 50 ml tubes for a period of 10 minutes. This was followed by filtration using a 0.45 µm Corning filtration system. The supernatant was subsequently poured into Scholtz freeze drying bottles and temporally stored in a -20 ºC refrigerator for 24 hours, and then transferred further to -80 ºC freezer for another 24 hours for proper freezing. The ATM was then freeze dried using a VirTis SP Scientific freeze dryer. The powdered extract was stored at -20 ºC until used.

To prepare the stock solution for tissue culture experiments, 10 ml of phosphate buffered saline (PBS) was used to dissolve 100 mg of the freeze-dried ATM to make a stock solution of 10 mg/ml, which was then mixed by vortexing. This stock solution was subsequently filter-sterilized using a 0.22 µm Corning filtration system. For experiments, the ATM stock solution was diluted in complete culture media to make working concentrations ranging from 100 to 2000 µg/ml.

3.5 Cell culture

Human peripheral blood mononuclear cells (PBMCs) (ATCC, United State) were cultured at 1 X 10^6 cells/ml in Roswell Park Memorial0 Institute (RPMI)-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1 % penstreptomycin (PSF) in humidified incubator at 37^{o}C and 5% CO_2.

The human breast cancer cell line, MCF-7, was obtained from the Sigma Aldrich (South Africa). The MCF-7 breast cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with 10% FCS and 1% PSF in a humidified incubator at 37^{o}C and 5% CO_2. Cell were sub cultured twice a week and growth medium were also changed twice a week.

3.6 Treatment of PBMCs

Confluent PBMCs (1 X 10^6) were aliquoted into 24 well plates and were treated with different doses of Product Nkabinde (100 to 2000 µg/ml). RPMI medium was used as a negative control. Plates were subsequently incubated for 24 hours at 37^{o}C and 5% CO_2. Cell viability was then quantified using the CellTiter-Glo luminescent cell viability assay as described below. This was done to establish the IC_{50} dose of the ATM on treated PBMCs.

3.7 Preparation of conditioned media from PBMCs

Exponential growing cultured PBMCs (1.0 x 10^6 cells/ml) were treated with the IC_{50} dose of the ATM as calculated. Phytohemagglutinin (PHA) at a dose of 5 µg/ml was used as the positive control for cytokines secretion. Campthothecin (CPT) at a dose of 1 µM was used as positive control for anticancer assays. Untreated PBMCs in complete culture media were used as a negative control and DMSO (1%) was used as a negative control for the preparation of CPT. These sets of treated PBMCs were incubated for 24 hours at 37 ^{o}C and 5% CO_2. At the end of incubation period, the treated cells were centrifuged to pellet the cells. The supernatants were then collected in separate cryovials and stored at -20 ^{o}C until further use for anticancer assays and cytokines secretion assays. Pelleted cells were re-suspended in 400 µl RPMI, this was followed by cell viability assay using a CellTiter-Glo luminescent cell viability assay as described below.

3.8 Treatment MCF-7 breast cancer cells

Confluent wells of MCF-7 cells (1.0 X 10^6 cells/well) were treated with conditioned media collected from treated PBMCs as described above. The different sets of treated MCF-7 cell wells included wells treated with conditioned media from IC_{50} dose of the ATM, PHA (5 µg/ml) and CPT (1 µM). Conditioned medium from untreated PBMCs was used as negative control. This was followed by incubation for 24 hours at 37 ^oC and 5% CO_2. At the end of incubation period, MCF-7 cells were detached from wells using a working solution of trypsin and cell viability was evaluated using CellTiter-Glo luminescent cell viability as described below.

3.9 Cell viability (ATP) assay

The CellTiter-Glo® Luminescent cell viability assay is a homogeneous and luminescence-based method designed to measure cell viability in cultured cells, based on quantitation of the ATP present. The ATP that is present in a culture signal the presence of metabolic active cells. The assay uses Cell-Titre Glo from Promega, which results in a luminescence signal, produced by the luciferase enzyme reaction:

$$ATP + d\text{-}Luciferan + O_2 \rightarrow Oxyluciferan + AMP + PPi + CO_2 + Light.$$

The addition of the ATP (CellTitre-Glo) reagent to cells, results in cell lysis. This generates luminescence, which is proportional to the ATP reaction in cells, and ultimately indicates the number of viable cells (Promega, 2015).

Briefly for this study, 100 µl of treated/control PBMCs/MCF-7 cells were pipetted into three different wells of a white opaque 96-well plate. The working CellTiter-Glo reagent was prepared immediately before use and was added to the wells at the final volume of 100 µl per well. The plate was then shaken on a plate shaker for 2 minutes at 150 *g*. The plate was then incubated in darkness for 10 minutes at room temperature. After incubation, the plate was placed on luminometer for reading. From the result obtained, background signals from the negative control (culture media plus traditional medicine) was subtracted from each average reading. A dose response curve was generated for the ATP levels using reflective light units (RLU) versus different

concentrations of samples/control. The cell viability assay was repeated twice before the follow up assays were undertaken.

3.10 Antioxidant activity as measured by changes in glutathione (GSH) levels

The glutathione (GSH) assay kit was utilised to measure the changes in GSH levels in all samples. The GSH assay is a luminescence-based assay designed to measure and quantify levels glutathione (GSH) in cultured cells. GSH is a major intra-cellular antioxidant that is found in eukaryotic cells, made up of three-amino-acid peptide (Terpstra et al., 2003). A change in GSH levels is important in the assessment of toxicological responses and is an indicator of oxidative stress, potentially leading to apoptosis or cell death. The assay is based on the conversion of a luciferin derivative into luciferin in the presence of GSH. The reaction is catalyzed by a glutathione S-transferase (GST) enzyme supplied in the kit. The luciferin formed is detected in a coupled reaction using Ultra-Glo™ Recombinant Luciferase that generates a glow type luminescence that is proportional to the amount of glutathione present in cells (Promega, 2015).

MCF-7 cells were plated and incubated overnight to allow them to attach on 24 well-plate. The following day, confluent cells (1 X 10^6 cells/well) were treated with traditional medicine in conditioned media and CPT was used as positive control. Medium only from untreated PBMCs was used as a negative control. The cells were then incubated for 24 hours at 37°C and 5% CO_2. After incubation period, antioxidant levels (changes in intracellular GSH levels) were measured using GSH-Glo glutathione assay kit.

To perform the assay in this study, samples (100 µl) of resuspended MCF-7 cells was transferred into labelled eppendorf tubes and centrifuged to pellet the cells. After the removal of the media, the cells were re-suspended in PBS and pipetted into triplicate wells of white opaque 96-well plate. This was followed by the addition of the GSH-Glo™ reagent in all wells with cell samples and the plate was incubated at room temperature for 30 minutes. At the end of this incubation, a 100 µl of reconstituted Luciferin Detection Reagent was added into each well and incubated at room temperature for 15 minutes. Luminescence was then read using a GLO-Max luminescent reader. Background signals from the negative control (PBS) was subtracted from each average reading. A dose response curve was be generated using a GSH standard curve in order to calculate GSH levels in treated/control samples using reflective light units (RLU).

3.11 Caspase 3/7 assay

The Caspase-Glo® 3/7 assay kit is a homogenous, luminescent based assay designed to quantity caspase-3/7 activity in cultured cells (Promega, 2015). Caspase-3/7 are members of cysteine aspartic acid-specific proteases (caspase) family, playing a key effector role in apoptosis in mammalian cells (Walsh et al., 2008). Adding the single Caspase-Glo® 3/7 reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate. This liberates free aminoluciferin, which is consumed by the luciferase, generating a "glow-type" luminescent signal that is proportional to caspase-3/7 activity. The luminescence that is generated by the addition of a single Caspase-Glo® 3/7 reagent in an add-mixture format is proportional to the amount of caspase activity present (Promega, 2015).

For this study, MCF-7 cells were plated at the density of 1 X 10^6 cells/well in each well and incubated for 24 hours at 37ºC and 5%CO_2. After 24 hours, cells were treated with conditioned media (ATM or CPT) and further incubated for 24 hours at 37ºC and 5%CO_2. After incubation, cells were harvested via trypsinization and re-suspended in DMEM. Then Caspase-Glo 3/7 assay kit was used to measure caspase activity in treated and untreated cells according to manufacture instructions. To perform the assay, 100 µl of treated and control cell suspensions were pipetted into 96-well plate in triplicates. The working Caspase-Glo 3/7 assay reagent was immediately added to the wells at 100 µl per well. The plate was shaken on a plate shaker for 2 minutes at 150 *g* and then incubated in the dark for 30 minutes at room temperature. After incubation period, caspase activity was measured using plate reader luminometer. The triplicate luminescence readings were averaged and the background signals of cell culture media was subtracted from each average reading. The results were expressed as the means ± standard deviation of the 3 readings for each treatment.

3.12 DNA fragmentation

DNA fragmentation was detected using Hoechst 33342 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate) solution. Hoechst 33342 is a cell-permeable DNA stain that is excited by ultraviolet light and emits blue fluorescence at 460 to 490 nm. This assay is employed for distinguishing apoptotic cells from healthy cells. Cell death that results from the induction of apoptosis typically displays fragmented nuclei (Crowley et al., 2018).

For this study, MCF-7 cells were seeded into 24 well plates at the density of 1 X 10^6 cells/well and incubated for 24 hours. After 24 hours, the culture medium was removed and replaced with fresh medium. Cells were subsequently treated with conditioned media (ATM or CPT) and incubated for 24 hours at 37ºC and 5%CO_2. After incubation, the conditioned media that was used to treat the cells was removed and the cells were washed with PBS. The washed cells and controls were then suspended in Hoechst 33342 solution initially diluted with PBS (containing 2% FCS) at a final concentration of 15 µg/ml. The plate was then incubated at room temperature for 15 minutes. After incubation, the Hoechst stain was removed and the cells were washed once with PBS and re-suspended in 100 µl of PBS and examined under a fluorescent cell imager microscope (ZOE™ Cell Imager BIO-RAD).

3.13 Inflammatory cytokines secretion

Analyses of inflammatory cytokines secretion from treated PBMCs and MCF-7 cells was carried out using the Multi-Analyte Profiler ELISArray assay kit (Qaigen, USA) and was performed according to the provided protocol. The Multi-Analyte ELISArray assay kit is a cost-effective method designed to detect the levels of multiple cytokines and/or chemokines in a 96-well ELISA microplate, using a simple sandwich-based enzyme-linked immunosorbant assay. Each Multi-Analyte ELISA microplate is coated with twelve target-specific capture antibodies for the same cytokine or chemokines (QIAGEN, 2011).

To perform this assay, 50 µl of supernatants from PBMCs and MCF-7 treated with conditioned media and control samples were separately added to each appropriate 96 well plate of the ELISA plate followed by incubation for 2 hours. After washing away unbound protein with wash buffer, biotinylated detection antibodies (50 µl) were added to the wells to also bind the captured analyte and incubated for another hour. Following another wash, an avidin-horseradish peroxidase conjugate (100 µl) was added and incubated for another 30 minutes. The wells were again washed and the colorimetric substrate solution was added, developing to a blue colour in direct proportion to the amount of protein analyte present in the initial sample. The colour development was stopped by adding the stop solution, and the absorbance was read at 450 nm with reference at 570 nm in a microplate reader as per manufacturer's instructions. Secretion of cytokines was measured in duplicates and two independent experiments were done.

3.14 Statistical analyses

Initial data analysis was done on Microsoft Excel and all data was expressed as means ± standard error of means (SEM). Further statistical analysis was performed using GraphPad Prism Instat Software (version 5.00, GraphPad Software, San Diego, California, USA). One-way analysis of variance (ANOVA) followed by Tukey-Kramer was used for analysis of differences between control and experimental groups.

Chapter 4

Results

4.1 Cell viability assays on PBMCs

PBMCs were treated with various doses of Product Nkabinde and cell viability was evaluated after 24 hours of incubation. This ATM was shown inhibit PBMCs growth in dose dependent manner, with cytotoxicity increasing with an increase in treatment doses (Fig. 1). Compared to untreated, the observed effect of traditional medicine on PBMCs cells proliferation was significant ($p<0.0001$, Fig. 1). The calculated IC_{50} value established be 344 µg/ml and this concentration was used for further experiments.

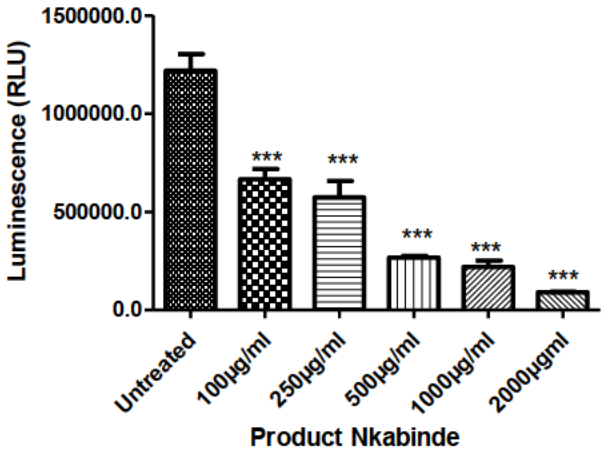

Figure 1: Inhibitory effect of Product Nkabinde (ATM) on the growth of PBMCs cells over 24 hours. The results are expressed as mean ± SEM (n=3). The calculated IC_{50} was 344 µg/ml (***$p< 0.0001$).

4.2 Preparation of conditioned media from PBMCs

Conditioned media from all these samples was separated from these samples by centrifugation and stored in marked cryovials at -20 degrees until use. The PBMCs from the different treatments were used for the cell viability assay. There was a statistically significant difference in the cell viability of PBMCs treated with the IC_{50} concentration of ATM and campthothecin (CPT) versus the

untreated PBMCs as expected ($p < 0.05$, Fig. 2). Phytohemagglutinin (PHA) had no cytotoxicity effect on the viability of PBMCs compare with untreated PBMCs cells ($p > 0.05$). There was no significant difference in cell growth between PBMCs treated with Product Nkabinde and PBMCs treated with CPT ($p > 0.05$). Since CPT was dissolved in DMSO, the cytotoxicity effect of DMSO on cell growth was also investigated. It was found that DMSO had no cytotoxicity on PBMCs proliferation ($p > 0.05$).

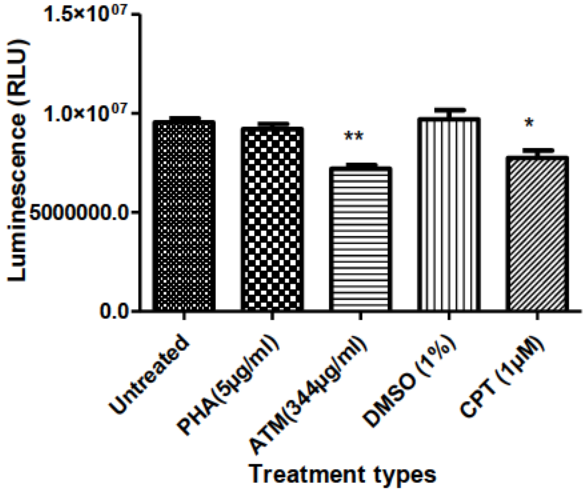

Figure 2: Cytotoxicity effect of Product Nkabinde (ATM), PHA, CPT and 1% DMSO on PBMCs over 24 hours. The results are represented as mean ± SEM (n= 3). (*$p<0.05$, **$p < 0.001$).

4.3 Cytotoxicity of conditioned media on MCF-7 cells

The result showed that conditioned media from the IC_{50} concentration of Product Nkabinde and that from CPT significantly inhibited the growth of MCF-7 cells ($p = 0.0001$, Fig. 2). There was no statistically difference between cytotoxicity effect of Product Nkabinde and CPT on MCF-7 growth ($p > 0.05$). It was found that PHA stimulated the growth of MCF-7, however, there was no statistically different between the growth of MCF-7 treated with PHA and those that were untreated.

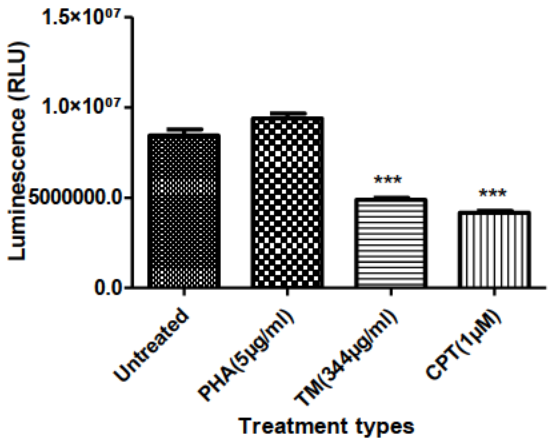

Figure 3: Effects of conditioned media from Product Nkabinde (ATM), PHA, and CPT on MCF-7 cells growth over 24 hours. Results are expressed as mean ± SEM (n=3) (***$p < 0.0001$).

4.4 Caspase3/7 activity

To determine whether Product Nkabinde induced apoptosis in MCF-7 cells, caspase 3/7 enzyme was evaluated. Conditioned media with Product Nkabinde was shown to significantly induced caspase 3/7 enzyme activity after 24 hours when compared to conditioned media from untreated and CPT cells ($p< 0.0001$, Fig.3). PHA was found to have no significant effect on caspase 3/7 enzyme activation ($p> 0.05$, Fig. 3).

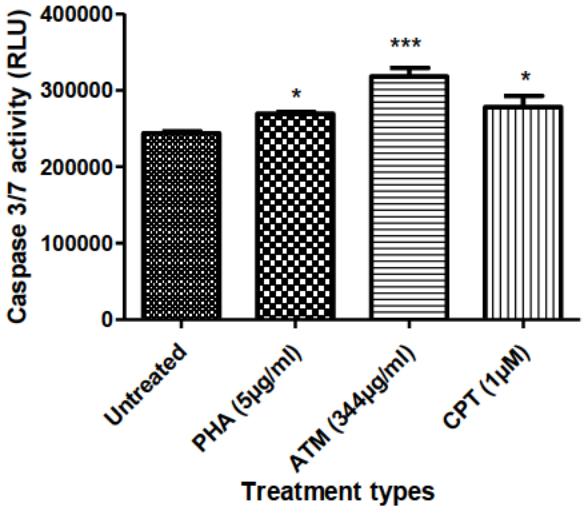

Figure 4: The effects of conditioned media from Product Nkabinde (ATM), PHA and CPT on caspase 3/7 enzyme activity in MCF-7 cells. . The results are expressed as mean ± SEM (n=3) (*$p < 0.05$, **$p < 0.001$ and ***$p < 0.0001$).

4.5 Antioxidant activity

The effect of Product Nkabinde on GSH levels in MCF-7 breast cancer cells was evaluated using Glutathione-Glo™ assay kit. The result obtained showed that Product Nkabinde significantly decreased GSH levels when compared to untreated and CPT treated MCF-7 cells ($p<0.001$, Fig. 5).

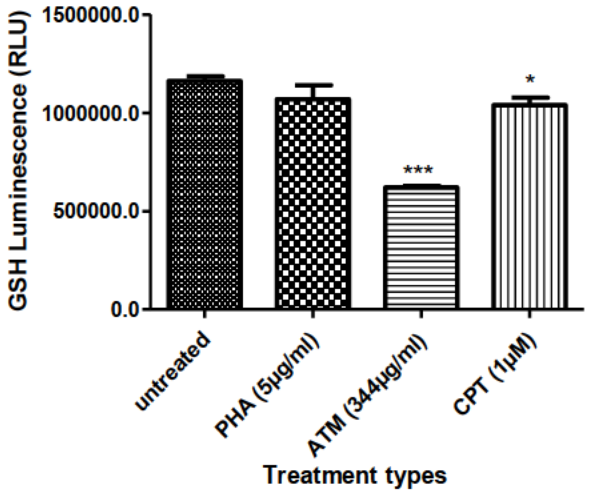

Figure 5: The effect of conditioned media with Product Nkabinde (ATM), PHA and CPT on GSH levels in MCF-7 cells. The results are expressed as mean ± SEM (n=3). (*$p < 0.05$ and ***$p < 0.0001$).

4.6 Hoechst staining

After 24 hours, the cells were stained with Hoechst 33342 stain solution and imaged under a fluorescent microscope. Conditioned media with Product Nkabinde induced DNA fragmentation as indicated by the intense brightness of blue colour of stained cells under the microscope (micrograph B, Fig. 6). However, CPT showed a higher number of intensely bright cells indicating DNA fragmentation than cells treated with Product Nkabinde or untreated cells (micrograph C, Fig. 6). DNA fragmentation was also observed in untreated cells, which indicates a form of cell death occurring in cultured cells.

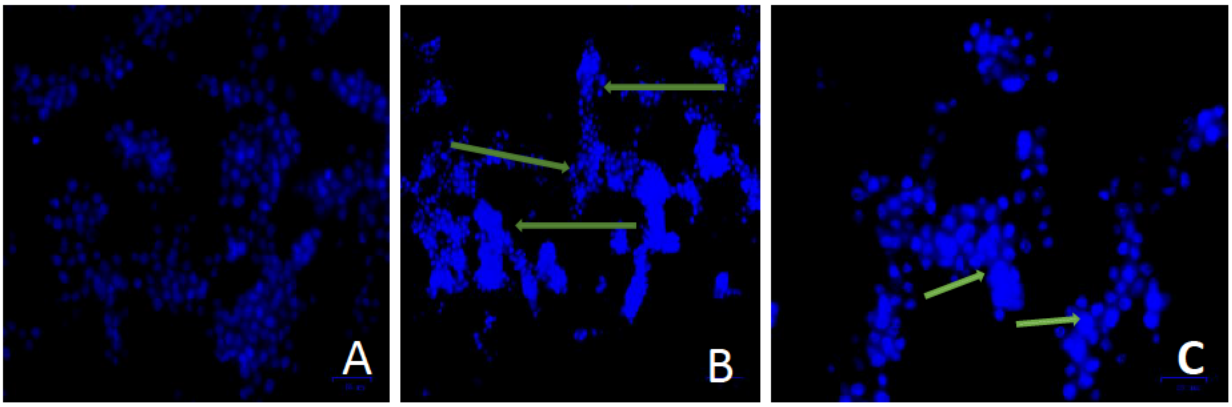

Figure 6: The effects of Product Nkabinde and CPT on DNA integrity in treated MCF-7 cells over 24 hours. The intense bright blue colour represents the intercalation of Hoechst stain in fragmenting DNA strands. Micrograph (A) represents untreated MCF-7 cells, (B) are MCF-7 cells treated with Product Nkabinde and (C) are MCF-7 cells treated with CPT.

4.7 Immunomodulatory effects on PBMCs and MCF-7 cells

The result obtained from PBMCs treated with the IC_{50} dose of Product Nkabinde demonstrated that traditional medicine did not have a statistically significant effect on the secretion of any of the inflammatory cytokines analyzed ($p>0.05$, Fig. 7). However, there were significant differences in the secretion of IL-12 between CPT versus the untreated control, PHA and Product Nkabinde ($p<0.05$, Fig. 7).

The harvested conditioned media from treated PBMCs was then used to treat MCF-7 cells and changes in the secretion of inflammatory cytokines was evaluated. Results showed that conditioned media with Product Nkabinde increased the secretion of IL 1β, IL 8 and GM-CSF in treated MCF-7 cells when compared to the untreated control, PHA and CPT. However, the results were not statistically significant ($p> 0.05$, Fig. 8). PHA and CPT significantly decreased the secretion of IL 1α and IL 4 when compared to the untreated control ($p< 0.05$). Similarly, Product Nkabinde significantly decreased the secretion of IL 4 when compared to the untreated control ($p< 0.05$). Product Nkabinde significantly increased the secretion of IL 1β when compared to CPT ($p< 0.05$). Product Nkabinde significantly increased the secretion of GM-CSF when compared to all other treatments but this was shown to be not significant ($p> 0.05$).

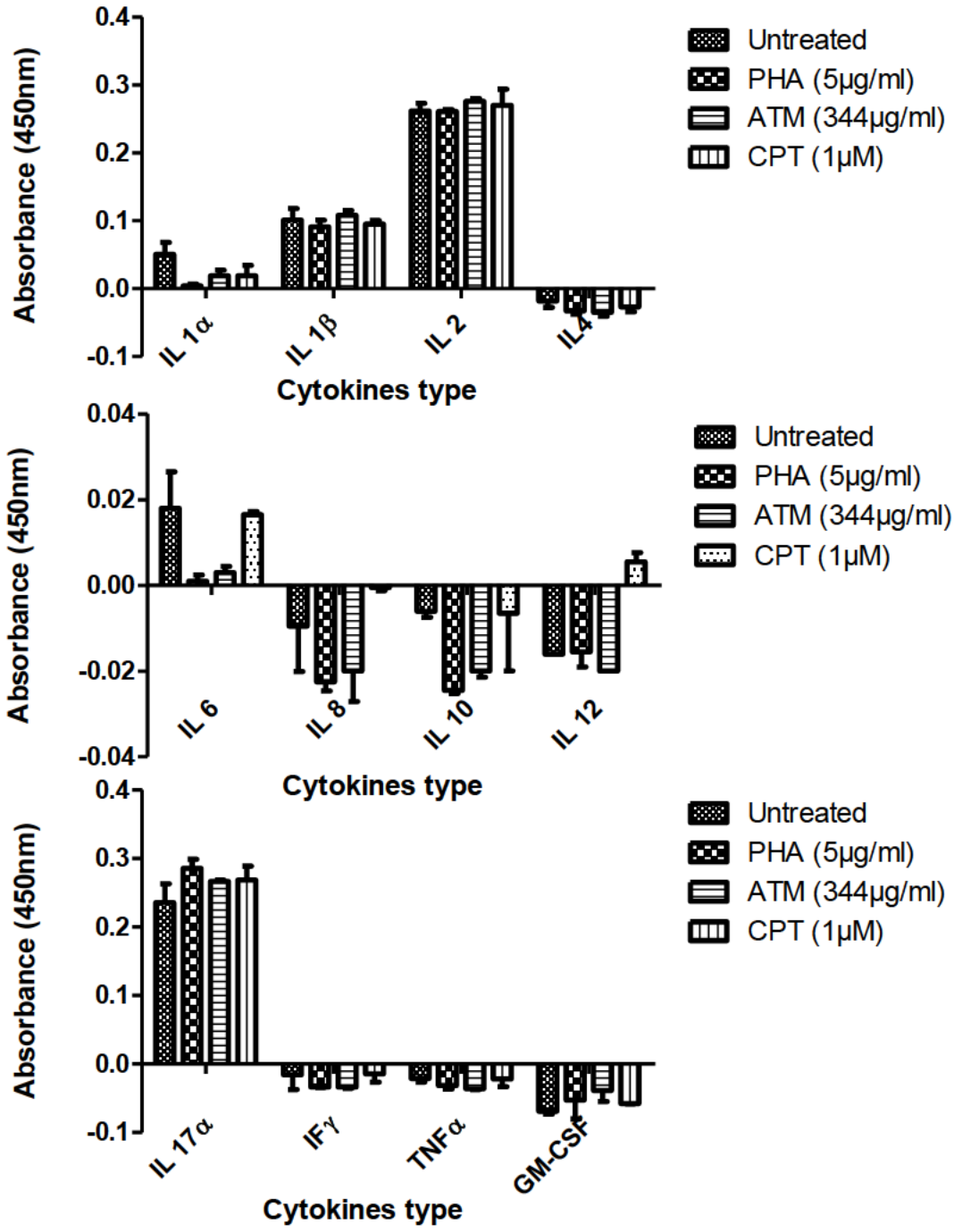

Figure 7: Immunomodulatory effects of Product Nkabinde, PHA and CPT on secretion of inflammatory cytokines on treated PBMCs over 24 hours. Results are expressed as mean ± SEM (n=2).

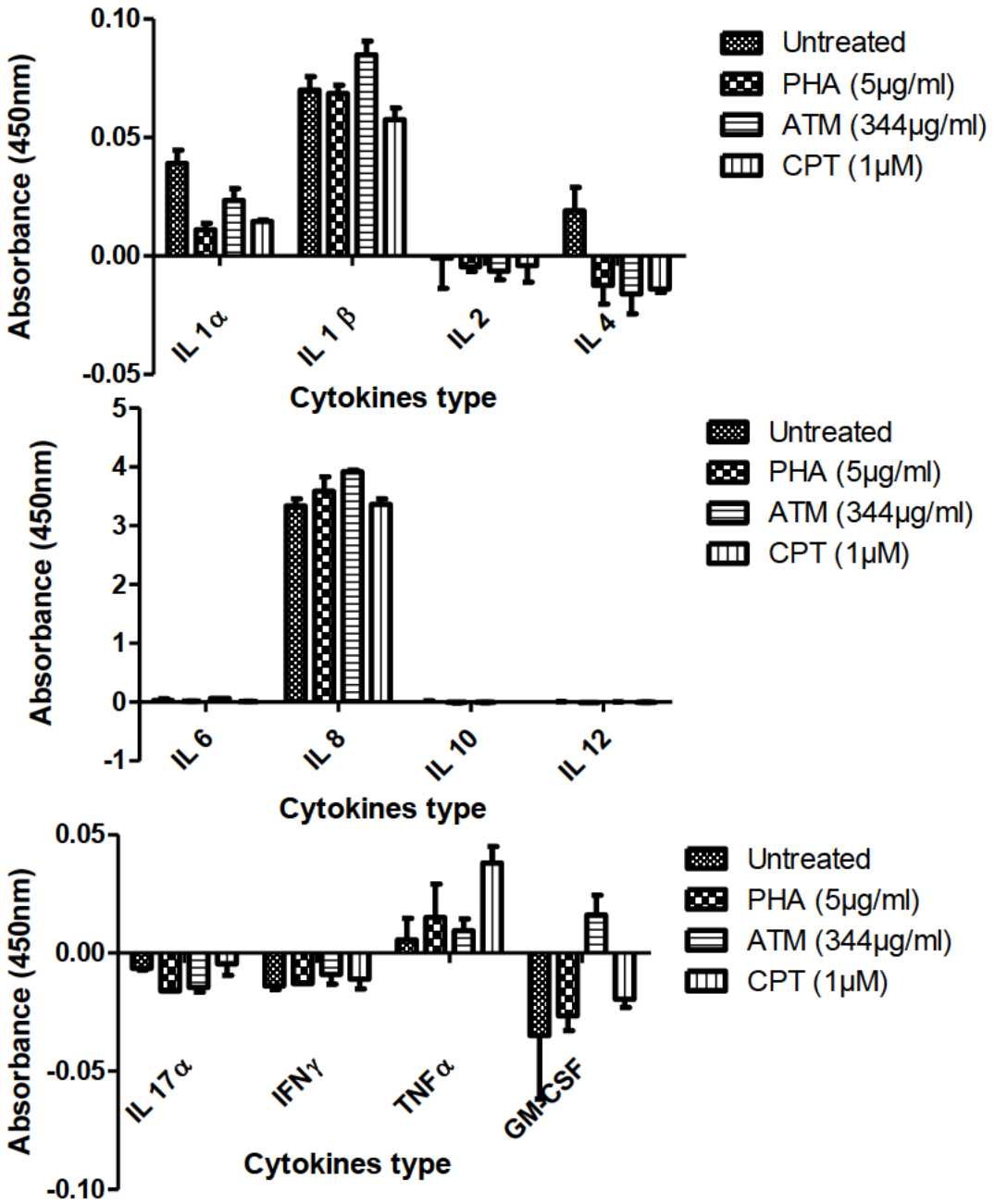

Figure 8: The effects of conditioned media from PBMCs treated with Product Nkabinde, PHA and CPT on inflammatory cytokines secretion in treated MCF-7 cells. Results are expressed as mean ± SEM (n=2).

Chapter 5

Discussion

In order to circumvent the side effects associated with conventional anti-cancer treatments such as chemotherapy and radiotherapy, it is crucial to identify new sources of natural remedies with fewer side effects and high specificity (Zarei and Yaghoobi, 2019). Traditional medicine has the potential to destroy tumour cells less intensively and more naturally, which then reduced the toxic side effects associating with current treatment (Li et al., 2016). In this study, we investigated the immunomodulatory and anti-cancer properties of Product Nkabinde, a traditional medicine that composed of four medicinal plants, on PBMCs and MCF-7 cells. The first step looked at the cytotoxicity of this traditional medicine on normal PBMCs. Product Nkabinde was shown to have a dose dependent cytotoxic effect on treated PBMCs with an IC_{50} value of PBMCs was established at 344 µg/ml. The relatively high IC_{50} of this traditional medicine is consistent with extracts prepared in the traditional method of boiling medical plants in water. Most traditional medicines, including ATM, are prepared using boiling water to extract herbal materials for medicinal prescriptions (Huie, 2002).

According to the classification of *in vitro* cytotoxicity of compounds by the World Health Organization (WHO), compounds with IC_{50} concentrations above 90 µg/ml are not considered cytotoxic (https://www.who.int/tdr/grants/workplans/en/cytotoxicity_invitro.pdf). Herbal medicines have been shown to exhibit the highest cytotoxicity effects against breast cancer cells (Alsaraf et al., 2019). In this study, the anti-proliferation effect of Product Nkabinde against breast cancer cells was compared with a positive control, CPT, a known inhibitor of breast cancer cells growth *in vitro*. The conditioned media prepared from normal PBMCs using the IC_{50} (344 µg/ml) of Product Nkabinde exhibited the highest cytotoxicity effect towards MCF-7 breast cancer cells (Fig. 2). Other studies have demonstrated that some traditional medicines used to treat cancer do not have growth inhibitory effects on normal cell but have been shown to inhibit breast cancer cell proliferation in a dose and time dependent manner (Bosio et al., 2015; Zhang et al., 2018). This was also observed in the current study where Product Nkabinde was less cytotoxic to normal PBMCs while in MCF-7 cells the cytotoxicity was more pronounced. This is what is expected

from an effective cancer drug, a drug must display less toxicity to normal cells and show the highest cytotoxicity to cancer cells (Azadmehr el al., 2015).

Cancer cells do not die due to their resistance to apoptosis, a process that is essential for mortality and removal of abnormal cells during human development (Mahassni and Al-Reemi, 2013). When normal cells are damage or converted into cancer cells, apoptosis plays a major role in eliminating these cancer cells. The main function of apoptosis is to maintain tissue homeostasis. Resistance of breast cancer cells to apoptosis allows them to proliferate (Dou et al., 2019). Apoptosis is usually characterized by condensation and fragmentation of DNA, blebbing of plasma membranes, cell rounding and cell shrinkage (Shoja et al., 2015). One of the objectives of this study was to evaluate the direct apoptosis inducing potential of conditioned media with Product Nkabinde against MCF-7 cells. To the best of our knowledge, this is the first study to demonstrate the anti-cancer effects of Product Nkabinde against MCF-7 cells through induction of apoptosis. Apoptosis may proceed either by intrinsic (mitochondrial) or extrinsic (death receptor) pathways (Thi-kim Nguyen et al., 2019). Both intrinsic and extrinsic pathways lead to caspases activation (Hamsa and Kuttan, 2011). Previous studies have demonstrated that traditional medicines have the ability to activate caspase 3/7 in MCF-7 cells, thus inducing apoptosis (Shoja et al., 2015). Caspase 3/7 are members of cysteine proteases which are responsible for execution of apoptosis and their activation has been regarded as hallmarks of apoptosis (Samarghandian et al., 2019). This study has demonstrated that Product Nkabinde significantly (p <0.05) induced activation of caspase 3/7 enzymes when compared to untreated and CPT treated cells. These results suggest that the decrease in cell viability observed in MCF-7 after treatment with Product Nkabinde can be attributed to apoptosis. Our findings are also consistent with previous report which demonstrated that traditional medicine can promote apoptosis through activation of caspase 3/7 enzymes (Shoja et al., 2015). A similar study using traditional Chinese medicine found that induction of caspase 3/ 9 activity in a dose dependent manner in MCF-7 cells was part of the mechanisms of actions (Lan et al., 2019). Caspase 3 has been regarded as one of the biomarkers in breast cancer treatment and prevention, since deficiency and downregulation of caspase 3 is associated with breast carcinogenesis (Elumalai et al., 2012).

Glutathione (GSH) is a non-enzymatic antioxidant synthesized by cells and plays an important role in protecting cells from oxidative stress (Forman et al., 2009). Antioxidants have the ability to

scavenge free radicals which are harmful molecules produced as metabolic by-products by natural cells, hence inducing cellular protection as well reducing the risk of cancer (Dhanasekaran, 2020). Cancer cells have high levels of GSH content, which indicates its importance in their continued survival (Moghtaderi et al., 2018). It has been previously reported that African traditional medicine has an effect on GSH levels in cancer cells (Lawal et al., 2017; Gulumian et al., 2018). Wageesha et al. (2017) suggested that an increase in GSH levels as well activity of its related enzymes contribute to resistance of tumour cells to either radiotherapy or chemotherapy. The effect of Product Nkabinde on GSH levels in MCF-7 cells was evaluated in the present study. The results obtained from the current study demonstrate that Product Nkabinde depletes GSH levels in breast cancer cells after 24 hours of treatment. Similar effects were reported by You and Park (2010), who demonstrated that gallic acid from medicinal plants has the ability to decrease GSH levels in lung cancer cells. Wageesha et al. (2017) also reported similar effects of a poly herbal traditional medicine on GSH levels on HepG2 and HeLa cells. The IC_{50} concentration of Product Nkabinde showed a significant decrease in GSH than the positive control, CPT. These results suggest that one of the mechanisms Product Nkabinde inhibits breast cancer cells growth is by reducing levels of GSH on MCF-7 cells and thereby allowing the process of cell death through apoptosis to take place.

Morphological analysis of MCF-7 cells treated with Product Nkabinde through staining with the Hoechst stain confirmed that this ATM product promotes DNA fragmentation. The findings from this study is in line with other studies which have previously reported that traditional medicine increases DNA fragmentation in breast cancer cells (Gomez et al., 2016). Tanih and Ndip (2013) also reported that traditional medicine induced apoptosis which results in morphological changes in MCF-7 cells. This evidence of DNA fragmentation induced by Product Nkabinde in this study, along with the demonstrated ability to increase the activity of caspase 3/7 enzymes and the observed decrease in GSH levels, supports the conclusion that Produce Nkabinde induces apoptosis in the treated breast cancer cells. The findings in this study suggest that Product Nkabinde is a very effective inducer of apoptosis in MCF-7 breast cancer cells. Induction of apoptosis in MCF-7 cells has been regarded as the most effective strategy to treat breast cancer (Haque et al., 2019). The IC_{50} concentration of Product Nkabinde has demonstrated strongest direct anti-cancer activity against breast MCF-7 cells.

Plant extracts have been used for centuries to modulate the immune system to treat diseases. The immune system plays a critical role in treatment of cancer as it can either promote or inhibit the growth of cancer cells (Jamali et al., 2020; Jiang, 2014). Recent studies have suggested that the immune system promotes breast cancer progression through secretion of chemokines and inflammatory cytokines (Boyle and Kochetkova, 2014). In the present study, the aim was to determine the immunomodulatory effects of Product Nkabinde against both PBMCs and MCF-7 breast cancer cells. Cytokines are small soluble proteins that regulate immunity; when unregulated they contribute to tumour pathogenesis (Johdi et al., 2017). This study has showed that Product Nkabinde did not significantly stimulate the secretion of inflammatory cytokines in treated PBMCs. The conditioned media generated from the treated PBMCs was used to treat MCF-7 cells over a 24 hours period and modulation of inflammatory cytokines was then evaluated. It has been reported that cannabidiol derived from *Cannabis sativa* modulates the production of pro-inflammatory cytokines in breast cancer cells (Elbaz et al., 2015). The result from present study has also indicated that Product Nkabinde modulates the secretion of proinflammatory cytokines in MCF-7 cells. The extract was able to stimulate the secretion of IL 1β, IL 8 and GM-CSF. IL-8 is a proinflammatory chemokine that is overexpressed in many types of cancer. The function of this chemokine correlated to tumour resistant therapy (Tan et al., 2018). In contrast to the present study, Esquivel-Garcia et al. (2020) demonstrated that pyrolytic oils from *Amphipterygium adstringens* inhibit production of IL 8 and IL 17. Another study found that some plant extracts used against hematological tumors in traditional medicine of Jordan can inhibit secretion of proinflammatory cytokine IL 8, while at the same time inducing secretion of IL 1β cytokines in cancer cells (Assaf et al., 2013). It has been reported that high levels of IL 1β contribute to breast cancer cell proliferation. This suggests that in an ideal situation, expression and secretion of IL 1β in MCF-7 cells should be inhibited (Chukiatsiri et al., 2020). Ye et al. (2019) showed that traditional Chinese medicine formula, RP, has the potential to reduce the expression of IL 1β in breast cancer metastasis. Over-expression of GM-CSF in cancer cells promotes tumour proliferation and growth (Thomas et al., 2019). Product Nkabinde stimulated secretion of all the above proinflammatory cytokines in MCF-7 cells and therefore can be assumed to assist cancer cells in evading the immune response. Further, Product Nkabinde was shown to significantly inhibit the secretion of IL 4 in treated MCF-7 cells. IL 4 is a known anti-inflammatory cytokine and its inhibition confirms the pro-inflammatory effects of this traditional medicine in treated MCF-7 cells. Based on these

results in this study, Product Nkabinde was shown to have pro-inflammatory immunomodulatory effects on treated MCF-7 cells *in vitro* and these effects can have negative effects on treatment of cancer as they facilitate immune evasion.

Chapter 6

Conclusion and recommendations

The results of this study showed that Product Nkabinde has direct anticancer effects through direct cytotoxicity, activation of caspase 3/7 enzymes, decreasing GSH levels and induction of DNA fragmentation in treated MCF-7 cells. Product Nkabinde exhibited the highest cytotoxic activity in MCF-7 cells compared to untreated cells and was less cytotoxic to normal PBMCs. Based on the observed activation of caspase enzymes, decrease in GSH levels and DNA fragmentation using a Hoechst fluorescent dye, it is safe to conclude that Product Nkabinde induces cell death in treated MCF-7 cells through apoptosis. However, further mechanism of action studies using flow cytometry to confirm the occurrence of apoptosis in treated breast cancer cells is needed. In the immunomodulation assays, Product Nkabinde was shown to increase the secretion of pro-inflammatory cytokines and decrease the secretion of anti-inflammatory cytokines in MCF-7 cells. Such effects are generally undesirable in cancer therapy as they may facilitate an inflammatory environment, which is conducive for the growth of cancer cells. Further studies looking at intracellular expression of these inflammatory cytokines are needed to confirm the observed pro-inflammatory properties of Product Nkabinde.

Based on the above findings, it is recommended that further studies on the anticancer and immunomodulatory effects of Product Nkabinde should be conducted using other *in vitro* and *in vivo* models. These studies should focus on improving the formulation of this traditional medicine product, improving the extraction method used to prepare the traditional medicine, studies on individual plants out of the four medicinal plants used by the traditional healer, determining the chemical profile of the original product as prepared the traditional healer and possible focus on isolating the anticancer active compounds from these medicinal plants. Other studies may focus on the anti-cancer molecular mechanisms of Product Nkabinde. In general, Product Nkabinde has promising anti-cancer effects and warrants the need for further studies to develop this traditional medicine as a possible alternative treatment against breast cancer.

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Appendix A

Ethics approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

25 June 2019

Mr S Sithole (211513812)
School of Nursing and Public Health
College of Health Sciences
211513812@stu.ukzn.ac.za

Dear Mr Sithole

Protocol: Immunomodulatory potential of traditional medicine against breast tumour cells
Degree: MMedSc
BREC Ref No: BE452/19

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received 18 June 2019.

The conditions have been met and the study is given **full ethics approval** and may begin as from 25 June 2019. **Please ensure that site permissions are obtained and forwarded to BREC for approval before commencing research at a site.**

This approval is valid for one year from **25 June 2019.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **noted** by a full Committee at its next meeting taking place on **09 July 2019.**

Yours sincerely

[redacted]

Prof D Wassenaar
Acting Chair: Biomedical Research Ethics Committee

cc: Postgrad Officer: ramlalm@ukzn.ac.za Supervisor: ngcobom3@ukzn.ac.za

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Appendix B

Turnitin report

Immunomodulatory and anticancer potential of a traditional medicine product from a traditional healer against MCF-7 breast cancer cells.

ORIGINALITY REPORT

9%	6%	5%	2%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Submitted to University of KwaZulu-Natal Student Paper	1%
2	academic.oup.com Internet Source	1%
3	bmccomplementmedtherapies.biomedcentral.com Internet Source	<1%
4	worldwidescience.org Internet Source	<1%
5	synapse.koreamed.org Internet Source	<1%
6	Sneha Sheokand, Umashanker Navik, Arvind K. Bansal. "Nanocrystalline solid dispersions (NSD) of hesperetin (HRN) for prevention of 7, 12-dimethylbenz[a]anthracene (DMBA)-induced breast cancer in Sprague-Dawley (SD) rats", European Journal of Pharmaceutical Sciences, 2019 Publication	<1%