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In vitro inhibitory activity of black seed oil and potassium chloride against the biomarkers of triple negative breast cancer

Tamaratina Jombai
Louisiana State University and Agricultural and Mechanical College, tjomba1@lsu.edu

IN VITRO INHIBITORY ACTIVITY OF BLACK SEED OIL AND POTASSIUM CHLORIDE AGAINST BIOMARKERS OF TRIPLE NEGATIVE BREAST CANCER

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DEDICATION

This thesis is dedicated to God for all He has been to me. I can never completely list the innumerable things He has done for me even during my time in graduate school but God has been my ever present help, my assurance and confidence through everything. I have been able to complete and achieve this and everything else I have achieved only by his unending grace and favor on my life which I cannot find the words to describe.

I am also dedicating this thesis to my family who are a great blessing to me. My parents, Sylvester and Elizabeth Jombai, have been faithful to God in their responsibilities as my parents and have done above and beyond, constantly making huge sacrifices all through my life to support and provide for me to have nothing but the best. Through their prayers, love, encouragement and support, I have come this far and I continue to run the course God has set before me to fulfill His purpose for my life. My siblings Betty, Godwin, Powela, Ben, Emi, and Ebike have been like best friends to me and have supported me in many different ways. The strength of their love breaks through the barriers of distance even though we are thousands of miles apart and they have always been there to provide a shoulder to lean on, ears to listen, encouragement or advice whenever I needed them.

My dearest Aunt, Chinyere Oparison was diagnosed with Cancer and passed away in August 2006. She was like a second mom to me and an example in different ways, I learned a lot from her. She made a strong and endless impact on my life. I appreciate her for all she was to me and I would like to also dedicate this thesis to her and her family.

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ABSTRACT

Breast cancer is the leading cause of death among women and is highly heterogeneous in nature. One very aggressive subtype of this disease is Triple Negative Breast Cancer. Triple Negative Breast Cancer (TNBC), is a very aggressive disease characterized by the lack of expression of estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor-2 (HER-2). TNBC patients account for approximately 15% of total breast cancer patients. This breast cancer subtype is characterized by high mortality rate, and is typically associated with poor prognosis. Current clinical methods have proven to have low success rates against TNBC, it is proposed that prevention by dietary approach with naturally occurring compounds in foods will be more effective. The objective of this research was to study the inhibitory activity of black seed oil and potassium chloride against the biomarkers of triple negative breast cancer. Hormone-independent MDA-MB-231 breast cancer cells were incubated with black seed oil, potassium chloride, or the combination of black seed oil with potassium chloride and incubated at 37 ºC and 5% CO_2 in a humidified incubator for 24, 48, or 72 h. Cell viability was measured by MTS assay. The effect of black seed oil on angiogenesis was determined using in vitro angiogenesis tube formation assay. Cancer cell cycle was analyzed by flow cytometry. The expression levels of biomarkers of TNBC cells were evaluated by Western blots. This study showed that black seed oil and potassium chloride proved efficient in down-regulating TNBC associated biomarkers including ALDH1, CD44, ESA, PI3K/Akt, Erk, COX-2, NFκB, Hsp90, p53, p21, Bax, and Bcl-2. The results indicate that black seed oil or KCl or their combination can attenuate the biomarkers of triple negative breast cancer MDA-MB-231, suggesting a potential preventive role against the disease.

CHAPTER 1:

INTRODUCTION

Breast cancer is the second most common cancer in the world and most common cancer among women (Key and others 2003). It is a leading cause of death in women and this disease is highly heterogeneous in nature. Triple Negative Breast Cancer (TNBC), which is a subtype of inflammatory breast cancer, is a very aggressive disease with poor prognosis. The malfunction in the DNA repair process leading to increased apoptosis and defective cell cycle, contributes to the poor prognosis of this disease.

The disease is characterized by a lack of targeted therapies, and is highly chemo-sensitive. There are current challenges faced by chemotherapy treatment for this disease, such as a lack of established targets, resulting in its low success rate. With the still unmet responses to the challenges of this disease, there arises strong need for more efficacious treatment options. The poor prognosis and low success rates of treatments associated with triple negative breast cancer implies that prevention by dietary approach would be better, thus the use of non-toxic naturally occurring bioactive compounds may be more effective and may be the best approach in targeting this disease.

Sustained angiogenesis is one of the known hallmarks of cancer. Angiogenesis is the formation of new blood vessels from pre-existing ones. It is a process that occurs normally in life but also plays a role in various disease conditions when there is an imbalance in the regular process of angiogenic stimulation and inhibition. The process of angiogenesis advances tumor growth and metastasis by the disruption of basement membrane of endothelial cells, cell proliferation, migration, and finally formation of new three dimensional tubular structures. Targeting angiogenic factors is one of the major approaches to cancer treatment and prevention.

It is well established that there are various mechanisms which may lead to an increased risk of breast cancer due to its inherent heterogeneity. One major risk factor is proved by the evidence that nutritional factors can affect breast cancer risk either positively or negatively through hormonal mechanisms; For example, saturated fat can pose negative risk (Key and others 2003). It is proposed that dietary bioactive compounds will carry out inhibitory activities against the growth and metastasis of cancerous cells without affecting normal breast cells. This will result in better patient treatment outcomes including the prevention of relapse, moreover improving prognosis, and ultimately increasing overall survival rates (Altenburg and others 2011; George and others 2011). Prevention has been recognized by world top oncologists as the best cure for cancer, food can prevent cancer from progressing and causing death.

Dietary bioactive compounds that promote cancer inhibition have been identified and studied. Some of which include curcumin, epigallocatechin, resveratrol, diosgenin, thymoquinone, etc. The focus for this study was black seed oil, extracted from black seed also known as black cumin seed, or *Nigella Sativa.* The main bioactive component in black seed oil is thymoquinone which has been found to possess inhibitory properties against cancer cell proliferation, and to induce apoptosis in various human cancers (Gali-Muhtasib and others 2006).

The main objective of this study was to investigate the interaction of black seed oil (BSO) or potassium chloride (KCl) or a combination of the oil with potassium chloride with the biomarkers of triple negative breast cancer. The steps taken to test this objective include:

1) Investigation of the cancer cell viability after treatments with BSO, KCl and the combination of both treatments.
2) Analyses on the effects of the treatments on cell cycle, angiogenesis, and signal transduction pathways associated with TNBC

CHAPTER 2:

LITERATURE REVIEW

2.1. Breast Cancer

Breast cancer is a complex disease which is heterogeneous in nature (Bosch and others 2010; Oakman and others 2010), with diverse outcomes and responses to treatment (Oakman and others 2010). It is the most common diagnosed malignancy in women (Giovannini and others 2010). It is the most common cancer in women and the leading cause of death (Irminger-Finger 2010; Irvin Jr and Carey 2008). The statistics in 2007 show that 1,300,000 women were diagnosed with breast cancer and 465,000 died from breast cancer worldwide. In the United States, 182,480 new diagnoses and 40,170 deaths were estimated in 2009 (Sun and others 2010). The American Cancer Society (ACS) estimated 207,090 new cases of invasive breast cancer diagnosis in women and 39,840 deaths for 2010. The trends show an increase in breast cancer diagnoses.

Various risk factors influence the development of breast cancer such as age, gender, race, dietary and life-style habits, physical activity, and breast cancer susceptibility gene mutation carriers (Tot 2011). Breast cancer cells have the ability to metastasize and spread to other sites in the body. One major way this occurs is through the lymphatic system. Most of the lymph vessels lead to axillary lymph nodes located under the arms (see Figure 2.1). The cells can enter the lymphatic vessels and begin to grow in lymph nodes and cause the lymph nodes to swell. There is higher likelihood that cancerous cells will be found in the blood stream and other parts of the body once the cancerous cells get into the axillary lymph nodes and if there are more lymph nodes with cancer cells (Cancer 2010). The many different types of breast cancer mainly

constitute of about 80% invasive ductal carcinomas, and 10-15% invasive lobular carcinomas while the rest (5-10%) are the additional rare types (Hwang-Verslues and others 2009).

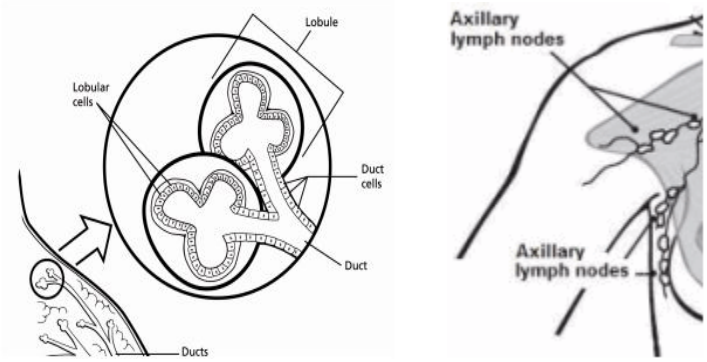


Figure 2.1: The Lymphatic system

Breast cancer has been defined and classified using a number of clinical and pathologic features (such as age, tumor size, hormonal receptor status, etc) for outcome predictions and response to treatment (Bosch and others 2010; Irvin Jr and Carey 2008). A validated classification based on gene-expression assays considered four breast cancer subtypes (Table 2.1.) The classes include: luminal, human epidermal growth factor receptor (HER2 or ERBB2) positive, normal breast, and basal like (Bosch and others 2010; Hwang-Verslues and others 2009).

Table 2.1: Breast cancer classification based on gene-expression

Breast Cancer Subtype	Characteristics
Luminal	High expression of many genes expressed by breast luminal cells cytokeratins (8/18)and estrogen receptors Does not overexpress ERBB2.
ERBB2 +	Overexpression of ERBB2 oncogene Low levels of estrogen receptor (ER) expressions and all genes associated with it.
Normal breast subtype	Expression of genes distinctive of basal epithelial and adipose cells Low expression of luminal cells gene clutter
Basal-like	High expression of cytokeratin 5/6 and 17, fatty acid binding protein7 Failure to express ER and most genes co-expressed with it

Breast cancer is also classified based on clinical manifestations such as inflammatory and non-inflammatory breast cancer. The highly aggressive form, inflammatory breast cancer, was the main focus for this study and will be discussed in more detail.

2.2. Inflammatory Breast Cancer

A discrete clinical and pathologic entity with rapid disease progression and poor prognosis describe a form of breast cancer among the most metastatic variants of breastcancer known as inflammatory breast cancer (IBC) (Cariati and others 2005; Lopez and Porter 1996). Though IBC represents less than 10% of all breast cancers, it is the most lethal form of the disease (Ben Hamida and others 2008). The lethality of IBC is ascribed to its high invasive nature (Hoffmeyer and others 2005).

The American Joint Committee on Cancer (AJCC) defines IBC as "a clinicopathologic entity characterized clinically by diffuse edema and erythema of the breast, over the majority of the breast, and often without an underlying mass. The clinical appearance is due to pathologic plugging of the dermal lymphatics of the breast, but pathologic involvement of the dermal lymphatics alone does not confirm the diagnostics" (Anderson and others 2005) .

The main features that distinguish inflammatory breast cancer from other forms of breast cancer are its distinct phenotype, which includes rapidly progressive breast inflammation, and a high tendency for metastasis to occur (Lerebours and others 2008; Zell and others 2009). This suggests a high propensity for tumor cells to migrate (Ben Hamida and others 2008). The survival rate is significantly different from the non-inflammatory forms of breast cancer: a 3-yr survival rate is about 40% compared to non-inflammatory breast cancers with 85% (Lerebours and others 2008).

Inflammatory-like symptoms such as redness, warmth, and edema prevent proper diagnosis of the disease. A change in breast skin texture with similarities to that of an orange peel is a characteristic of IBC (Hoffmeyer and others 2005). This does not occur as a result of true physiologic inflammatory response (Anderson and others 2005). It is due, rather to extensive invasion of the dermal lymphatics by IBC tumor cell emboli (Anderson and others 2005; Hoffmeyer and others 2005). Thus, by the time proper diagnosis is made, the cancer would have aggressively filtered through surrounding tissues and the lymphatic system, leading to poor prognosis (Hoffmeyer and others 2005).

The descriptions of several molecular alterations in IBC include frequent hormone receptor negativity, TP53 mutations and HER2 amplifications (Lerebours and others 2008). Most studies have focused on the role of estrogen and progesterone receptors for investigations on the molecular basis of IBC. The majority of IBCs are estrogen receptor negative (Cariati and others 2005).

2.3.Triple Negative Breast Cancer

A lack in the expression of estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor-2 (HER-2) results in an aggressive type of cancer referred to as Triple Negative Breast Cancer (TNBC) (Carotenuto and others 2010; Chen and Russo 2009; Liedtke and others 2008; Oakman and others 2010; Podo and others 2010). TNBC accounts for approximately 25% of the total breast cancer population, and mostly occurs in young women originating from African, African-American, and Latino ethnic groups (Carotenuto and others 2010; Chen and Russo 2009). This type of cancer is also dominant among obese women (Chen and Russo 2009). According to Hortobagyi (2008), forty percent of inflammatory breast cancers are likely to be triple negative. TNBC is typically associated with

poor prognosis due to aggressive tumor phenotype(s), only partial response to chemotherapy, and present lack of clinically established targeted therapies (Podo and others 2010). As seen in Table 2.2, TNBC subtype is highly heterogeneous in itself and is not one disease as it also has various subtypes (Adamo and Anders 2011; Oakman and others 2010).

Table 2.2: Various Subtypes of TNBC

Poor Prognosis
Invasive ductal carcinoma NOS
Invasive lobular carcinoma
Metaplastic carcinoma
Myoepithelial carcinoma
High grade neuroendocrine (oat-cell) carcinoma
Good Prognosis
Apocrine carcinoma
Medullary carcinoma
Secretory breast carcinoma
Adenoid cystic carcinoma
Metaplastic carcinoma (adenosquamous and fibromatosis-like)

The absence of expression of estrogen receptor, progesterone receptor, and HER2 (also referred to as ERBB2), and proving the worst outcomes with no therapeutic targets, makes the basal-like subtype of breast cancer one of relevance (Bosch and others 2010). Basal like breast cancer has characteristic features that include the triple negative phenotype however it has been stated that Triple Negative and Basal-like are not equivalent terms (Bosch and others 2010; Carotenuto and others 2010; Irvin Jr and Carey 2008; Oakman and others 2010). The term basal-like breast cancer was coined to describe the tumors that overexpressed genes characterizing breast basal epithelial cells while triple negative is based on clinical observations describing

breast cancers that do not stain for ER, PR, and HER2 in immunohistochemistry studies (Bosch and others 2010).

Research shows that patients with the basal-like subtype of breast cancer have poorer prognosis than patients with the Luminal subtype (Lorico and Rappa 2011). ER negative basal subtypes are associated shorter relapse and overall survival times with patients, than luminal ER positive subtypes (Rosen and others 2010). A study done with different breast cancer subtypes- luminal, ERBB2+, and basal-like showed shorter survival and overall disease-free survival with ERBB2+ and basal-like patients while it was longer for Luminal A subtype patients (Bosch and others 2010). The differences in survival could be explained by the overexpression of ERBB2 (an oncoprotein) and presence of TP53 mutations which are associated with poor survival, as TP53 mutation levels were higher in ERBB2+ and basal-like types but not in Luminal A subtype. This shows that TP53 mutation is related to the lack of expression of ER and poor prognosis as seen in TNBC (Bosch and others 2010).

TNBC patients are also associated with high or early relapse (Carotenuto and others 2010; Chen and Russo 2009). TN tumors have worse relapse-free and overall survival for patients (Bosch and others 2010; Stratford and others 2010). Women with TNBC are twice as likely as other women to develop distant metastases, causing a shorter survival in TNBC patients as this disease condition has high malignancy, poor differentiation and is very aggressive (Chen and Russo 2009). The lack of expression of ER, PR, and HER-2 makes treatment of TNBC difficult. Patients are mostly only treated with standard chemotherapy due to the lack of expression of ER, PR, and HER-2, preventing treatment with more specific targeted therapy (Chen and Russo 2009). ER and PR were the first clinically used predictive prognostic markers for breast cancer arising from the exposition of hormonal regulation while HER-2 gene

amplification and protein over-expression have been the first cytogenic predictor for breast cancer treatment (Podo and others 2010; Rosen and others 2010).

Estrogens are essential for normal growth and differentiation of human breast epithelial cells. However, exposure to excess estrogens for a prolonged time is an important etiological factor for the development of breast cancer. The presence of ER in breast tumors is used to predict response to endocrine therapies and disease prognosis. Progesterone and progesterone receptors are essential for estrogen promotion of breast cancer (Chen and Russo 2009).

Human epidermal growth factor receptor (HER) is essential for normal breast growth and development and plays a key role in tumor development (Rosen and others 2010). Furthermore, gene-amplification and/ or over expression of HER-2 are key hallmarks in ER positive breast cancer patients (Alvarez and Price 2010; Chen and Russo 2009). It has been shown that HER-2 over expression caused metastatic outgrowth of breast cancer cells in the brain (Chen and Russo 2009). The HER family mediates a wide range of signals controlling various cellular process such as cell proliferation, angiogenesis, and apoptosis; however, HER2 is the preferred signaling partner for other members of the HER family (Rosen and others 2010). Thus, it serves as a great treatment target. The evaluation of HER2 overexpression guides in treatment selection and efficiency of treatment (Alvarez and Price 2010; Rosen and others 2010). Therefore, the absence of these essential breast cancer markers makes this disease very difficult to treat.

Several features with breast cancer susceptibility gene (BRCA) outcome-associated breast cancer also occur with TNBC patients. For example, approximately 20% of breast cancer patients with mutations in BRCA-1 and BRCA-2 have deficiency in DNA repair because normal BRCA1 and BRCA2 have an important role in DNA repair (Bosch and others 2010; Chen and Russo 2009; Podo and others 2010). BRCA1 and BRCA2 gene mutations cause the disruption of

breast cell homeostasis which results in breast cancer. BRCA dysfunction can be an important mechanism in TNBC development (Bosch and others 2010). BRCA1 mutation is not only a prognostic marker, but also a predictive marker for chemotherapy response (Oakman and others 2010).

BRCA1 and BRCA2 play a role in cell-cycle control. The cell cycle is a series of coordinated events describing DNA replication and cell division (Collins and others 1997). The life cycle of the cells is divided in four phases based on DNA content and mitotic activity (Figure 2.2). The cells divide in the M phase (mitosis), proceed on to the G1 phase with constant DNA content, and then into the S phase where DNA is duplicated. They enter the G2 phase from the S phase remaining at constant DNA content before going back into the M phase, continuing the cycle (McGuire and Dressler 1985).

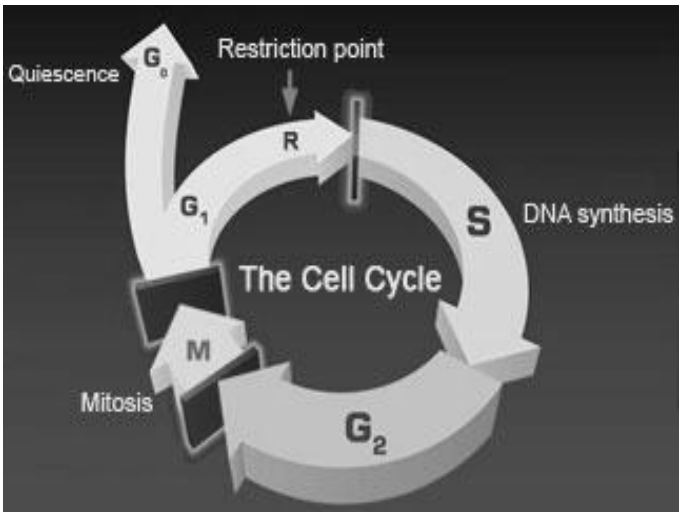


Figure 2.2: The cell cycle process

BRCA1 and BRCA2 regulate tumor suppressor protein p53 activity which functions in DNA repair. When DNA repair is not possible, cells go into apoptosis, which is the process of programmed cell death and it is regulated by genetically directed pathways. Apoptosis plays a

role in the formation of tumors and makes cells resistant to treatment. The mechanism of apoptosis is avoided by cancer cells in order for them to thrive (Podo and others 2010; Rosen and others 2010; Siddiqa and Marciniak 2008). In other words, TNBC patients with problems in DNA repair make the mutation carriers more sensitive to DNA- damaging agents, leading to shorter survival and altered expression of a number of proteins, oncogenes, and abnormal signaling pathways in TNBC patients (Chen and Russo 2009).

Though there is still a poorly understood association of TNBC with BRCA1 mutations (Podo and others 2010), there is definitely a significant overlap between TNBC, basal-like phenotype, and BRCA-1 mutated tumors (Oakman and others 2010). The heterogeneous characteristic of TNBC includes both basal and non-basal-like tumors, and triple negativity is sometimes used to identify basal-like tumors (Podo and others 2010). Only 71-91% of TNBC have basal-like phenotype, 77% of basal-like cancers are TNBC, while more than 80% of BRCA-1 mutations careers are triple negative (Chen and Russo 2009; Oakman and others 2010). In other words, not all BRCA-1 related breast cancers are TNBC or basal-like cancers (Bosch and others 2010).

The pathogenesis of TNBC is still not known to a high extent. This is a major challenge in developing therapies effective for treating this type of cancer (Chen and Russo 2009). The buildup of defects at the levels of gene amplification, methylation, mutations and other cellular regulation mechanisms is the underlying cause for disease heterogeneity and differential response to treatment. Understanding the relations between potential TNBC markers may help to realize the heterogeneity of these tumors and improve the response prediction to both single agents and combination treatments (Podo and others 2010). It may not be likely that a single biomarker will provide reliable prognostic information for a patient. Multiparameter

technologies such as proteomic or gene expression analyses give high expectations and will be more productive than analyzing a single gene or protein (Alvarez and Price 2010). The patient and tumor characteristics and treatment outcomes of TNBC are summarized in Table 2.3.

Table 2.3: Summary of clinical features of triple-negative tumors.

Patient characteristics	Younger age at diagnosis Predominantly African origin BRCA1 mutation carrier
Tumor characteristics	Ductal invasive carcinoma Rare histology (medullary, metaplastic) Negativity for ER, PR and HER-2 Elevated mitotic count Tumor necrosis Highly invasive Larger tumor size Axillary node involvement
Treatment/prognosis	Chemosensitive No clinically established specific targets Poorer prognosis Aggressive relapse (trend of relapse within first 3 years)

2.4. Angiogenesis and TNBC

Angiogenesis is the formation of blood vessels from a pre-existing one and is an important process of tumoregenesis. (Auerbach and others 2003; Chen and Russo 2009). This process is an integral part of both normal developmental processes and numerous pathologies including inflammation and tumor growth (Auerbach and others 2003). It is essential for organ

growth and repair; however an imbalance in the process results in numerous malignant, inflammatory, infectious and immune disorders (Carmeliet 2005).

The principal cells involved are endothelial cells which line all blood vessels and constitute virtually the entirety of capillaries. Figure 2.3 shows a summary of the angiogenesis process. For the formation of new blood vessels, endothelial cells must first break through the basement membrane, escaping from their stable location. Then the endothelial cells migrate toward an angiogenic stimulus (Auerbach and others 2003). Behind this migrating front, endothelial cells proliferate to form the necessary number of cells for making a new vessel, and then the new outgrowth of endothelial cells needs to reorganize into a patent three dimensional tube structure (Auerbach and others 2003).

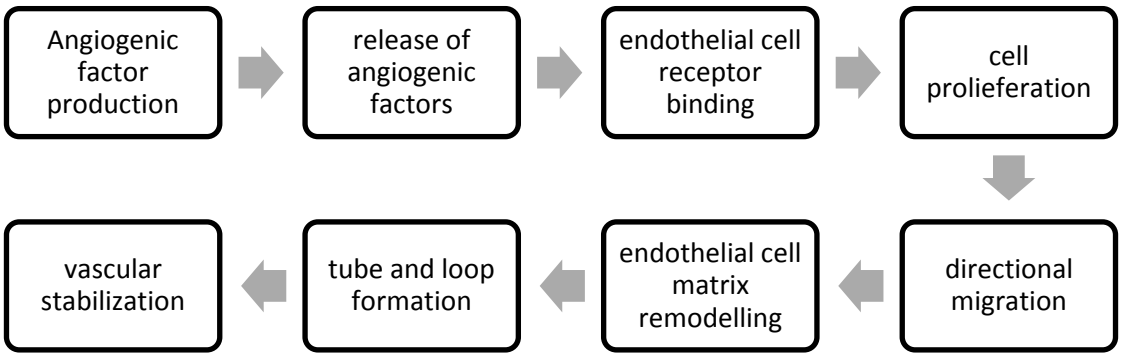


Figure 2.3: Summary of angiogenic process

Angiogenesis remains inactive during adulthood except in the cycling ovary and in the placenta during pregnancy. However, endothelial cells retain their ability to divide quickly in response to physicochemical stimulus such as hypoxia in blood vessels and in lymph vessels, and inflammation (Carmeliet 2005). In most disorders, this stimulus becomes excessive, resulting in an angiogenic switch due to an imbalance between stimulators and inhibitors. This is what occurs in most inflammatory disorders leading to additional processes responsible for diseases

such as obesity, asthma, diabetes, cirrhosis, multiple sclerosis, etc (Carmeliet 2005), whereas the stimulators are undersupplied in other situations causing the dysfunction of endothelial cells, vessel malformation or regression, or preventing healing and regeneration. Angiogenesis has been linked with over 70 disorders and the list is still growing (Carmeliet 2005).

Angiogenesis does not initiate malignancy, but promotes tumor progression and metastasis as the tumor progresses from micrometastases to lethal macrometastases which is what mostly leads to death in cancer patients (Carmeliet 2005; Rosen and others 2010; Siddiqa and Marciniak 2008). The angiogenesis process is a fundamental mechanism in tumor growth and development into detectable masses (Bosch and others 2010; Rosen and others 2010). Angiogenesis plays a role in the aggressive IBC, TNBC. High VEGF, which is the key factor in angiogenesis, is correlated with poor prognosis and response (which are the main characteristics of TNBC). VEGF levels are a prognostic factor for relapse-free and overall survival in patients; in addition, PDGFR-α has been connected with aggressiveness (Carvalho and others 2005; Relf and others 1997). Each step in neovascularization or angiogenesis, including the disruption of the basement membrane, cell proliferation, cell migration, and tube formation, can be a target for intervention (Auerbach and others 2003). Targeting just one step such as cell proliferation does not suffice in the formation of tumor mass (Rosen and others 2010).

2.5. TNBC Associated Biomarkers

Biomarkers are useful in the quantification of the course of malignancy and therapy response as well as in early identification of cancer patients and in therapy decision (Podo and others 2010). According to the U.S. Food and Drug Administration (FDA), a biomarker can be defined as “exploratory”, “probably valid” and “known valid”. A valid biomarker is “measured in an analytic test system with well-established performance characteristics, and for which there

is an established scientific framework or body of evidence that elucidates the physiologic, toxicological, pharmacological, or clinical significance of the test results" (Alvarez and Price 2010). The success of biomarkers depends on: (1) our ability to reveal critical cancer related molecular events and the mechanisms of action of targeted therapy, and (2) the efficiency of the relationship of specific biomarker to another and to a certain disease condition (Podo and others 2010).

A number of molecular changes in IBC/ TNBC which contribute to the inherently high metastasis of IBC have been described (Charafe-Jauffret and others 2010). Some markers which have been demonstrated to be useful in IBC/ TNBC are p53 and Hsp90. These biomarkers are useful for the prognostic evaluations, and for therapeutic response predictions which provide guidance for patient management (Podo and others 2010).

2.5.1. Breast Cancer Stem Cell Biomarkers

There have been no markers demonstrated to predict systemic metastasis development in IBC, however, there is increasing evidence that human breast cancers are driven by cancer stem cells (CSC) which initiate tumors with self renewal properties. CSCs have recently been validated by different studies to be a small cell population resistant to current anticancer therapies, antimitotic agents, and radiation. CSCs have the ability to sustain tumor formation and growth, giving rise to multi-lineage differentiated cells which form the tumor (Charafe-Jauffret and others 2010; Lorico and Rappa 2011). They are referred to as tumor-initiating cells which are resistant to therapy, and have been said to be probably responsible for the early recurrence in TNBC (Stratford and others 2010).

There are various breast cancer stem cell biomarkers. The different breast cancer cell lines and primary tumors show differences in expression of these CSC. The main cancer stem cells associated with TNBC include CD44, epithelial specific antigen (ESA), and aldehyde dehydrogenase (ALDH1) (Hwang-Verslues and others 2009).

2.5.2. TNBC Signal Transduction Biomarkers

Various processes occur either simultaneously or sequentially in tumor cells, producing signals. Signals from the environment can also lead to conflicting stimuli. Therefore organization of the processes requires firm coordination of the process events. For example, proteins like p53, control the expression of a large numbers of genes (Podo and others 2010). Table 2.4 shows some biomarkers associated with TNBC tumor and patient characteristics and their biological effects as related to the disease.

Table 2.4: TNBC associated biomarkers

Biomarker	Biological Effects
Akt	Cell proliferation Cell growth
ERK /MAPK	Cell proliferation Increased migration and invasion
Hsp90	Regulator of HER family Apoptosis
P53	Apoptosis/ Defective DNA repair Cell proliferation/ Cell cycle
Bax/ Bcl-2	Apoptosis
VEGF	Angiogenesis
NFκB	Inflammation

TNBC is known to have implications on cell signaling pathways, tumor metabolism, evasion of cell-cycle control, invasion, and metastasis. TNBC also affects a high number of genes associated with cell proliferation, frequent p53 mutations, reduced DNA repair capability, and increased angiogenesis (Oakman and others 2010).

2.6. Treatment Options and Responses

The main currently used treatment for breast cancer is chemotherapy. The cytotoxic effects of chemotherapeutic compounds occur by interference with DNA or RNA functions (Kitano 2004). It has been demonstrated that TNBC is a chemo-sensitive disease which escapes control by current standard treatments and with a lack of clinically established targets (Bosch and others 2010; Podo and others 2010). Some biological and chemotherapeutic agents are currently being tested either singly or in combination with other agents to increase treatment effectiveness. These agents directly target biomarkers associated with this highly metastatic breast cancer. Some treatment agents in trial are seen in Table 2.5. Though these therapies are being used, there are limitations and challenges being faced in using them.

Table 2.5: Possible Agents for TNBC Targeted Therapies

Biomarker/ Signal pathway	**Proposed therapeutic agents**
HER1 (EGFR)	*Cetuximab; Erlotinib; Gefitinib*
P53	Not available
VEGF/VEGFR	*Bevacizumab; Sunitinib; Sorafenib*
AKT	*Perifosine*
ERK/ MAPK	ERK kinase inhibitors
Hsp90	*Tanespimycin*

One major challenge in the development of treatment for this disease is the heterogeneity and complexity of this disease. Most cancer drugs aim at a limited number of targets, however there are numerous metastatic channels or signal pathways associated with the hallmarks of cancer which are evading metastasis, self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, tissue invasion and metastasis, and sustained angiogenesis. A lot of time, money, plus difficulty are involved in the search for targets that are valid and crucial to the disease (Siddiqa and Marciniak 2008; Tian and others 2010).

More challenges in TNBC treatment include drug resistance, low survival rate improvements, restrictions on therapies to a subset of patient populations (Rosen and others 2010) and low treatment success. The safety of anti-angiogenic treatment is a topic of emerging importance (Carmeliet 2005). It is a challenge to find the perfect targeted therapy that is specific for tumor cells without affecting the normal healthy tissues (Irminger-Finger 2010). One of the challenges is that chemotherapy or radiation can stimulate tumor initiating cells which could be a reason for early recurrence in TNBC. As a result of these challenges currently being faced with the treatment of this aggressive disease, new therapeutic agents are therefore being sought for TNBC (Stratford and others 2010).

2.7. Functional Foods and Breast Cancer

The present lack of efficacious clinically established targeted therapies demands a need for better prevention of IBC and TNBC. The currently used chemo-therapeutic agents have yielded low success rates with awful side-effects in breast cancer patients, leading to numerous studies on dietary components that will potentially be used in cancer prevention and treatment. Natural dietary compounds from foods will be less toxic to the human body than chemotherapy drugs (Lipski 2005). Lifelong dietary habits have a large influence on cancer development thus

dietary changes may prevent breast cancer development (Hanf and Gonder 2005; Tsubara et al. 2005; Kotsopoulos and Narod 2005).

Some of the various dietary components that have been and/ or are being studied for their anti-cancer activities are mentioned below. Resveratrol has been shown to have the qualities of natural chemo preventive agents including N-acetylcysteine, melatonin, reduced lipoic acid, and phytoestrogens (Chen and Russo 2009). Curcumin from turmeric has been shown to provide anti-inflammatory effects on biomarkers of IBC such as NF-κB and COX-2 (Jurenka 2009). Foods with polyphenolic compounds such as some mango varieties and cranberries have been studied for anti-carcinogenic effects on inflammatory breast cancer cells (Neto 2007; Noratto and others 2010). It has also been demonstrated that fenugreek has shown anti-cancer activities in the down-regulation of p53 (Shabbeer and others 2009). Oils from various food sources are also receiving focus in cancer prevention studies. For example, fish oil has been studied for its benefits in inhibiting tumor growth and progression (Mandal and others 2010).

2.8. Black Seed

One of the most expansively studied plants, as a result of the growing interest in naturally occurring compounds with anticancer potential, is *Nigella Sativa* known as Black Seed or Black Cumin Seed (Gali-Muhtasib and others 2006; Ramadan 2007). It is a dicotyledon grown in countries bordering the Mediterranean Sea, Pakistan, and India. The seeds have been used for thousands of years in a variety of foods such as bread, pastries, yogurt, pickles, soups, sauces, salad dressings, etc. It is also used externally on the skin or in cosmetics (Goreja 2003; Hajhashemi and others 2004).

It has been used for a very long time as a spice and food component, for its medicinal properties to promote health and prevent diseases, and to aid digestion (Ramadan 2007; Salem 2005; Tauseef Sultan and others 2009). Black seed provides a good source of essential nutrients with a diverse chemical composition comprising essential fatty acids, amino acids, carbohydrates, fiber, and minerals such as calcium, iron, etc (Gali-Muhtasib and others 2006; Tauseef Sultan and others 2009).

Figure 2.4: Black seed plant, black seed, black seed oil

Black seed oil is the spicy yellowish oil with an aromatic flavor and fresh flavored odor extracted from black cumin seed (Hajhashemi and others 2004; Ramadan 2007). Thymoquinone (TQ) is the major anticancer bioactive compound in the oil extracted from black seed (Figure 2.5) (Effenberger and others 2010; Gali-Muhtasib and others 2006; Hajhashemi and others 2004; Ramadan 2007; Velho-Pereira and others 2011).

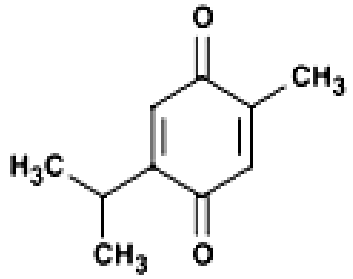


Figure 2.5: Chemical structure of Thymoquinone

It has been shown both in vivo and in vitro that thymoquinone inhibits the proliferation of various human cancers. Combining TQ with anti-cancer drugs has improved the results of chemotherapy and reduced their damaging effects to normal cells. Animal models have been used in numerous studies to demonstrate the promising effects of black seed oil anti-cancer activities (Gali-Muhtasib and others 2006). Though thymoquinone is the major bioactive compound of this oil, it has been shown that various compounds in the oil such as thymoquinone, terpineol, and carvacrol, work synergistically. Thus the importance of using the whole oil in studies is stressed. Studies have shown that the seed extract has low levels of toxicity (Ali and Blunden 2003; Effenberger and others 2010)

2.9. Potassium Chloride (KCl)

Western –style diets portray high salt and low potassium content, but the promotion of low sodium diets is on the rise. It has been reported that reducing salt intake reduces the risk of breast cancers (Strnad 2010). Potassium chloride (KCl) is the most widely used salt alternative among other alternatives to sodium chloride (NaCl) salts (Naidu 2000; Samapundo and others 2010). Potassium is an essential dietary component. It is also one of the essential minerals involved with cellular activities and heart stability in the body, at a normal range of 3.5-5.5 mmol/L (Zehender and others 1997). It is one of the three major electrolytes in the body that maintains blood pH. It is absorbed through the small intestine and excess potassium is excreted by the kidneys (Weatherby and Ferguson 2002).

One concern associated with potassium consumption is its toxicity level; however in normal conditions, the kidney functions in maintaining a normal potassium level in the blood. Thus, in a healthy population with normal kidney function, high potassium intake from foods poses no risk as the kidney readily excretes excess potassium. In contrast, overuse of

supplemental potassium can lead to toxicity in healthy individuals. The adequate intake of potassium has been set at 4.7g/day by an Institute of Medicine committee. Potassium intake of less than the adequate consumption amount is appropriate for individuals with impaired potassium excretion due to effects from hyperkalemia (Caballero 2009). Hyperkalemia occurs when elevated potassium levels surpass the amount the kidneys can excrete or when more than 18g of potassium is consumed orally at one time (Wilcox and Tisher 2009). An advantage of potassium consumption is seen in the inverse relationship between dietary potassium and blood pressure as an increase in daily consumption of potassium by 0.78g reduced blood pressure in hypertensive patients (D'Elia and others 2011).

Another concern with the consumption of potassium chloride is its flavor. It has a salty taste. However, when consumed in high amounts, KCl can impart bitterness and a metallic aftertaste. Since KCl provides sodium reduction advantages, it is blended with other ingredients to reduce its bitterness when used in formulations. Some KCl blends that have been used to reduce or eliminate the bitter taste from KCl include yeast; monopotassium glutamate, potassium citrate, potassium phosphate, L-glutamic acid, silicon dioxide, and KCl (Naidu 2000). Also, methyl and ethyl maltol which are mouth-feel enhancing ingredients mask the bitter taste of KCl in seasoned foods. Amino acids and organic acids are also said to reduce the bitterness of KCl when precipitated from aqueous solution (Roy 1997).

The anti-cancer activity of KCl is linked with the apoptotic activity of heat shock proteins (such as Hsp90) in the disease. Hsp90 is a chaperone protein which functions as a regulator of the HER family. Hsp90 is over-expressed in human tumor cells by up to ten fold due to ATPase activity. ATPase activity with Hsp90 has been proven to be reduced by KCl at 0.4% both *in vivo* and *in vitro* (Kamal and others 2003; Rosen and others 2010).

With the recent trend in using KCl as a substitute to NaCl in promoting low sodium diet, not many studies have been done showing the effects of KCl on cancer cells. To the best of our knowledge, no study has been done to see the effect of black seed oil combined with KCl on breast cancer cells. It is expected that the combination of KCl with black seed oil will enhance the inhibition activity of these dietary compounds against the highly aggressive TNBC.

CHAPTER 3:

MATERIALS AND METHODS

3.1. Cell Lines and Reagents

Breast cancer cell line MDA-MB-231 was obtained from American Type Culture Collection (ATCC, Manassas, VA). The cell line was propagated in Dulbecco's Modified Eagle's Medium (DMEM) high glucose containing sodium bicarbonate, 15 mM HEPES, fetal bovine serum to a final concentration of 10%, and nonessential amino acids. Cell propagation media were from Invitrogen (Carlsbad, CA). Protease inhibitor cocktail was purchased from Sigma Co (St. Louis, MO). Cold pressed extracted black seed oil was purchased from Botanic Oil Innovations (Spooner, WI). Electrophoresis gels and reagents, and PVDF membranes were from Invitrogen (Carlsbad, CA). Potassium chloride was purchased from EMD Chemical (Gibbstown, NJ). Primary antibodies (ALDH1, CD44, ESA, p-ERK, p53, NF-κB, Bax, and Bcl-2) and all secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibody (p21) was from Fisher Scientific (Fremont, CA). COX-2 was from Cayman Chemical Company (Ann Arbor, MI). Primary antibody p-Akt was from Cell Signaling Technology (Danvers, MA). Hsp90α was from Abcam (Cambridge, MA). All other reagents were of analytical grade.

3.2. Gas Chromatography Analysis on Black Seed Oil

The gas chromatography profile for black seed oil in this study was obtained by analysis on a Leco TOF GC/MS system equipped with an Agilent 6890 GC. A DB-5 (J &W Scientific, Albany, NY) 30 m by 0.25 mm I.D. with a 0.25 μm film thickness was used with a flow rate of 1.0 mL/min. One microliter injections were made in splitless mode at an injection temperature of 300 °C. The GC oven was initially held at 50 ºC for one minute then increased at 3 ºC/min to

80 °C and then at a rate of 10 °C/min to 340 °C. The mass spectrometer was operated in electron impact mode at 70 eV. Data was collected from m/z 35 to m/z 1000 and stored at 20 spectra/sec.

3.3. Cell Viability and Proliferation Assay

MDA-MB-231 cells (1 x 10^4 cells/well in 100 μL) were seeded in 96-well plates to adhere overnight. BSO, KCl, or BSO with KCl were prepared as stock solutions (BSO was prepared in DMSO and diluted in the cell culture media to achieve a final DMSO concentration of less than 0.1% in each well or plate used in this study). The BSO treatment ranged from 0-0.22% BSO per media solution. KCl treatments ranged from 0-0.2% KCl, the KCl was directly dissolved in cell culture media and pipetted on to cells in 96-well plates before incubation. The combination treatments were done with 0.22% BSO and concentrations of KCl from 0-0.2% KCl. The 96-well plates were incubated in the presence or absence of treatments followed by stimulation with 100 ng/mL of IGF-I and additional incubation for 24 h, 48 h or 72 h in serum-free medium at 37 °C, 5% CO_2 in a humidified incubator. After incubation, cell viability was determined by MTS assay. CellTiter 96 AQ_{ueous} One solution (Promega, Madison, WI) was used following the MTS Assay protocol from Promega. 20μl of the MTS solution was added to each well before incubation in a humidified 5% CO_2 incubator at 37^0C for a minimum of one hour. The absorbance was read at 490 nm using a Bio-Rad Model 680 micro plate reader (Hercules, CA). The percentage of surviving cells were determined by reduction of MTS [(3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-trazolium, innersalt)]. Cell viability was normalized to the levels in untreated control cells to determine the percentage of viable cells. Each experiment was performed in triplicate.

3.4. Cell Cycle Analysis by Flow Cytometry

The technique of clinical flow cytometric DNA was done using the florescence activated cell sorter (FACS), a flow cytometer that rapidly measures the fluorescence intensity based on DNA content of a cell population that has been stained with a fluorescent DNA dye (Morgan 2007). Cells were seeded at a density of 2.5 x 10^6 cells in T-25 flasks overnight, and treated with 0.22% BSO (using DMSO as a vehicle), or KCl at 0.2 % , or the combination of 0.22% BSO and 0.2% KCl for 72 h. To analyze the cell cycle after treatment period, cells were harvested by trypsinization, washed with PBS, and stained using the Beckman-Coulter DNA Prep Reagents Kit (cat # 6607055) following the manufacturers recommended staining procedure. Aliquot of cells were first incubated and vigorously vortexed for 30 s with reagent 1 (RNase and detergent) and stained for 1 h in the dark with reagent 2 (propidium iodide). The stained cells were read on a Beckman-Coulter Epics FC500 flow cytometer using CXP software for the initial acquisition. The data were then modeled using ModFit LT v3.2 software from Verity Software House (Topsham, ME).

3.5. *In vitro* Angiogenesis

The Angiogenesis Assay kit was obtained from Chemicon (Temecula, CA). Human umbilical vein endothelial cells (HUVEC) were used for this assay. The gel matrix was prepared following the manufacturer's instructions and then incubated at 37˚C to allow the matrix solution to solidify. HUVEC at 1 x 10^4 cells per well were seeded onto the surface of polymerized ECmatrix (Chemicon, Temecula, CA) alone, or treated with 0.22% BSO, or 0.2% KCl, or the combination treatment (0.22% BSO/0.2% KCl). The plate was then incubated at 37˚C for 6h. Tube formation was then inspected under an inverted light microscope (Olympus IX50) and photographed.

3.6. Total Cell, Cytoplasmic, and Nuclear Extracts Preparation and Protein Determination

Treated cells were grown as monolayer on T-75 flasks. Total cell extracts were prepared as follows. Briefly, cells (1×10^7 cells) were treated with DMSO as vehicle, BSO, or KCl, or the combination of BSO and KCl, washed with PBS, collected by scraping with RIPA buffer (50 mmol/L Tris-HCl, 150 mmol/L K NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, PMSF, orthovanadate, protease inhibitor mixture (added at a 1:100 dilution)) and incubated at 4 °C for 30 min. Lysates were centrifuged at 14,000 x *g* for 10 min and the supernatant recovered in other Eppendorf test tubes. Aliquots of supernatants were stored at -80 ºC for Western blot.

Cytoplasmic and nuclear extracts were prepared as follows. Briefly, cells were washed once with PBS and collected by scraping in 500 µL of hypotonic buffer (10 mmol/L HEPES pH 7.9, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.2 mmol/L PMSF, 0.5 mmol/L DTT, 5 mmol/L NaF, 1 mmol/L Na_3VO_4). Swollen cells were vortexed to shear cytoplasmic membranes. The mixture was centrifuged at 14,000 x *g* for 30 seconds and the supernatant was retained as cytoplasmic extracts. The nuclear pellet was resuspended in 50 µL of high-salt buffer (20 mmol/L HEPES pH 7.9, 25% glycerol, 1.5 mmol/L $MgCl_2$, 1.2 mol/L KCL, 0.1 mmol/L EDTA, 420 mmol/L NaCl, 0.5 mmol/L DTT, and 0.2 mmol/L PMSF) by pipetting up and down, vortexed 10 s at high setting, incubated for 30 min on ice, vortexed for 30 s at high setting, and centrifuged for 10 min at 14,000 x *g* in a micro-centrifuge pre-cooled at 4ºC. The supernatant (nuclear fraction) was transferred into other Eppendorf test tubes. Aliquots were kept at -80 ºC until use. Total protein in total cell extracts, cytoplasmic, or nuclear fractions was determined by the *DC* Protein assay (Bio-Rad, Hercules, CA).

3.7. Western Immunoblot

Equal amounts (50 µg) of cytoplasmic or nuclear fractions from treated cells were diluted with LDS sample buffer (Invitrogen, Carlsbad, CA), boiled, and loaded onto 4-12% Bis-Tris gels. Proteins were separated and transferred to PVDF membranes. Membranes were blocked with 5% BSA in TBS/Tween 20 (0.05%) following instructions from the antibody supplier, followed by an overnight incubation with a primary antibody in 5% BSA in TBS/Tween20 following the manufacturer's suggestion. Primary antibodies for stem cell surface markers including ALDH1, ESA, and CD44 were analyzed. Primary antibodies associated with cell proliferation including p-Akt, p-ERK, NFκB and COX-2 were also analyzed and furthermore, we analyzed Hsp90α and some of its co-chaperone proteins including p53, p21, Bax, and bcl-2. Visualization of the bound primary antibody was done by probing with alkaline phosphatase-conjugated secondary antibodies following the manufacturer's suggestion and exposure to chromogenic detection reagent BCIP.

3.8. Statistical Analysis

Statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS) software (version 15.0, 2006, SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) was conducted to examine the differences between treatments followed by *post-hoc* analysis (LSD). A P-value of < 0.05 was considered to be statistically significant.

CHAPTER 4:

RESULTS AND DISCUSSION

4.1. Gas Chromatography-Mass Spectroscopy (GC-MS) Profile of Black Seed Oil

To determine the chemical profile of BSO, gas chromatography-mass spectrometric analysis was performed on the oil. The results of GC-MS analysis of BSO indicated the presence of thymoquinone, p-cymene, carvacrol, terpinolene, longifolene, hexadecanoic (palmitic) acid, and linoleic (octadecanoic acid) as major compounds in the oil (Table 4.1). Black seed oil is a vegetable oil rich in bioactive compounds. The strong neutraceutical potential of black seed has been ascribed to its rich phytochemistry (Tauseef Sultan and others 2009).

Thymoquinone has been identified as the major bioactive component of black seed oil among other compounds that have been identified in the oil (Effenberger and others 2010; Gali-Muhtasib and others 2006; Velho-Pereira and others 2011). The concentration of TQ in the oil used for this study was 25.2 g/kg of oil. Thymoquinone has been demonstrated both *in vivo* and *in vitro* to possess anti-inflammatory and anti-oxidant characteristics, and to inhibit the proliferation of various human cancers (Gali-Muhtasib and others 2006; Salem 2005; Velho-Pereira and others 2011). For the study of the interactions of BSO and TNBC cells in vitro, 0.22% BSO containing 60 μM TQ was selected.

Thymoquinone works synergistically with other compounds in the oil such as carvacrol (which is a phenol), and terpenes (such as p-cymene, terpineol). Terpenes have been found to improve absorption and therefore aid absorption of black seed oil by the body (Harrewijn and others 2001). Carvacrol is a phenolic compound which serves as an excellent natural antioxidant in spices (Peter 2004). The bioactive compounds such as terpenes and phenols in black seed oil

are responsible for the pharmacological properties associated with the oil (Bhattacharjee and Sengupta 2009).

Table 4.1: GC-MS Profile of Black Seed Oil

Peak No.	RT	Area %	Library/ ID	Ref #	CAS#
1	6.47	3.87	5- Isopropyl-2-methyl-1,3-cyclohxadiene	32141	000099-83-2
2	9.38	16.60	p-cymene	31918	025155-15-1
3	11.98	2.14	Terpinolene	31925	000586-62-9
4	12.25	0.90	Decamethylcyclopentasiloxane	313067	000541-02-6
5	14.33	23.11	Thymoquinone	64415	000490-91-5
6	14.87	1.43	Morphinan-6-ol	290923	006703-27-1
7	14.97	0.73	Carvacrol	46681	000499-75-2
8	16.30	1.43	Longifolene	121403	000475-20-7
9	16.62	1.53	1-Methyl-3-(3,4-dimethoxyphenyl)-6,7-dimethoxyisochromene	291787	093772-22-6
10	18.02	1.11	3-(4-chlorophenyl)-4,6-dimethoxy-1-(prop-2'-enyl)indole-7-carbaldehyde	302379	000000-00-0
11	19.00	0.68	6-octenal, 3,7-dimethyl	52561	000106-23-0
12	19.18	0.63	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]	322218	003555-47-3
13	20.21	0.46	Octamethyl-cyclotetrasiloxane	244218	000556-67-2
14	20.59	0.64	n-Hexadecanoic acid	195432	000057-10-3
15	20.7	1.15	13-isopimaradiene	216672	001686-56-2
16	21.45	3.76	Endo-2,2,3-trimethylbicyclo[2.2.1]heptane	34362	020536-40-7
17	21.78	20.72	Linoleic acid	226097	000060-33-3
18	21.96	8.98	1H-Purin-6-amine[(2-flourophenyl)methyl]	177443	074421-44-6
19	24.82	10.11	2-chloro-4-(2'-furanyl)-6-(2'-thienyl)pyrimidine	202067	000000-00-0

The oil consists of several fatty acids of which over 80% are unsaturated fatty acids. The main fatty acids are linoleic acid, oleic acid, margaric acid, cis-11,14-eicosadeinoic acid, and stearic acid (Amin and others 2010; MM and others 2011). The saturated free fatty acids in black seed oil have been shown to induce apoptosis (Mu and others 2001). The absence of triglycerides as observed in the GC-MS profile of this oil (Table 4.1) is an essential aspect of the nutritional value of the oil. Breast cancer occurs less often in Asian countries such as India and China where black seed oil is included in diet that in western countries such as the United States where black seed oil is not popularly consumed (Lyn 2011).

4.2. Black Seed Oil or KCl or BSO with KCl Inhibits TNBC Cell Viability

To determine the efficacy of BSO or KCl or BSO with KCl against TNBC cells, MDA-MB-231 viability was screened in the presence of 0-0.22% of BSO or KCl at 0-0.2% or their combinations. MDA-MB-231 breast cancer cell line was used for this study. It was isolated from pleural effusion of a breast cancer patient. This cell line is well defined in its growth with invasive and metastatic characteristics. MDA-MB-231 cell line depicts triple negative characteristics of being ER, PR, and HER-2 negative (Bachmeier and others 2007). The cells have mesynchymal cell characteristics such as a strong ability to migrate (Yu and others 2009).

The measurement of cell viability is used to study integrated cellular changes. Cell viability is simply the determination of the total number of live cells in a sample. This assay is applied in the evaluation of treatment effectiveness against cancerous cells. Cell viability determines the change in the total number of cells before and after treatment (Wu 2010).

The MTS solution used for this analysis reacts with the cells in media solution producing a color change and the percentage of viable cells is relative to the color intensity of the cells in

media solution when read with a micro plate reader. BSO treated cells were reduced to approximately 20% at the highest concentration of treatment (0.22%) which contained 60µM TQ (Figure 4.1). Preliminary studies done in the lab showed that TQ at 60µM significantly reduced the percentage of viable cancer cells. In comparison to other concentrations of BSO used in this study (0.07% and 0.15%), the highest concentration of 0.22% was more effective. Also, the highest percentage reduction of viable MDA-MB-231 cells was observed after treatment for 72 h in comparison to 24 and 48 h of treatment. Therefore, we used 0.22% and 72 h treatment for all further analyses.

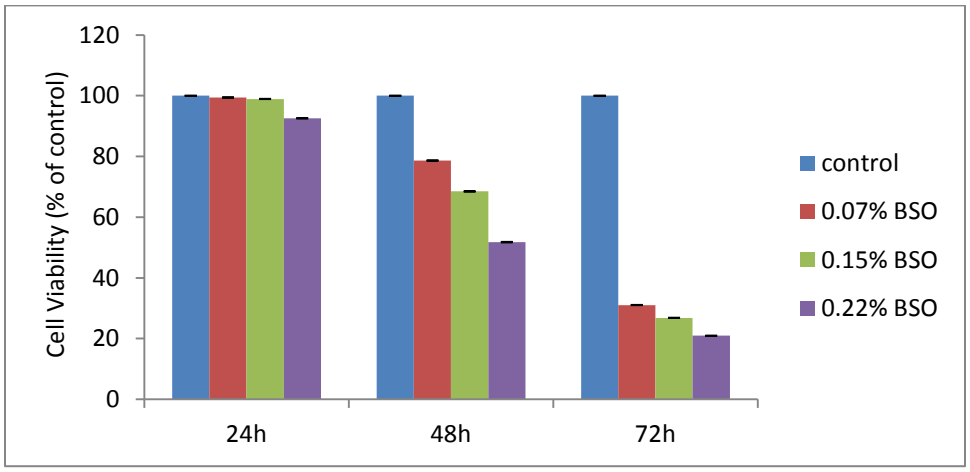


Figure 4.1: Viability of Control and BSO treated MDA-MB-231 cells

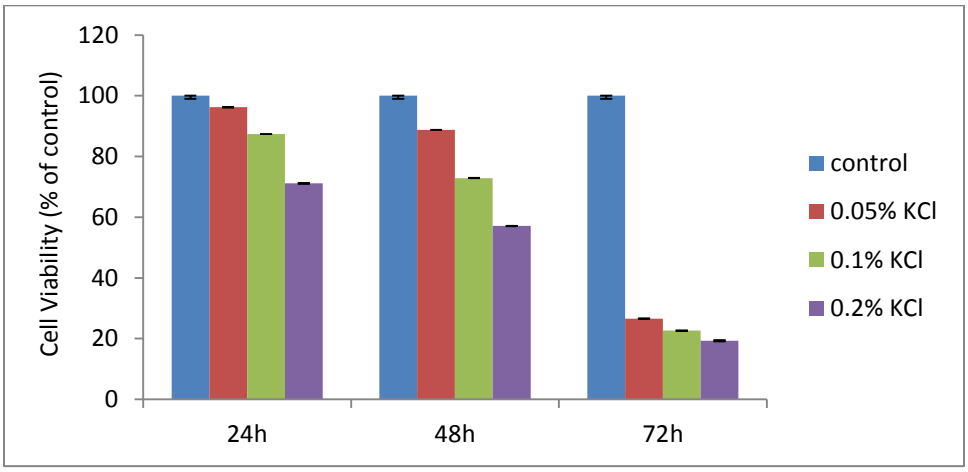


Figure 4.2: Viability of Control and KCl treated MDA-MB-231 cells

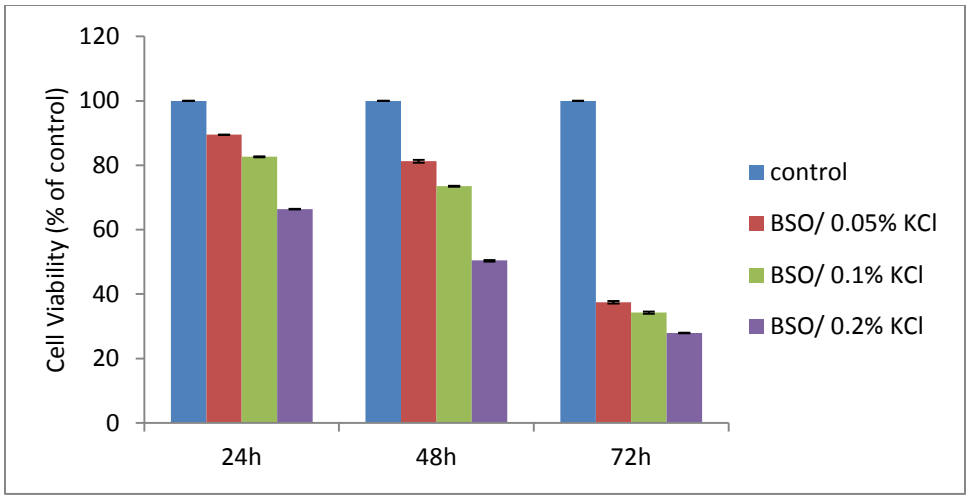


Figure 4.3: Viability of Control and BSO with KCl treated MDA-MB-231 cells

TNBC cells were also treated with 0.05, 0.1, and 0.2% KCl per media solution. The percentage of viable TNBC cells reduced with increased treatment time and concentration of KCl (Figure 4.2). After treatment with 0.2% KCl for 72 h, the percentage viable cells were reduced to 19%. There were only slight differences between the different concentrations of KCl in their percentage reduction of viable cells. The most effective treatment concentration and time, 0.2% KCl and 72 h, were selected for further analyses.

For the combination treatments 0.22% BSO per media solution was combined with three different concentrations of KCl (0.05, 0.1, and 0.2% KCl). A similar trend as with KCl treatments was observed as in increased reduction in percentage viable cells with increased treatment time and concentration of KCl (Figure 4.3).0.22% BSO combined with 0.2% KCl reduced percentage viable cells to approximately 27%, while treatments with 0.1 and 0.05% showed reduction to 34 and 37% viable cells respectively. BSO with 0.2% KCl which showed highest reduction in percentage viable cells was selected for further analyses.

4.3. Black Seed Oil or KCl or BSO with KCl Alters TNBC Cell Cycle Progression

To determine whether BSO or KCl or BSO with KCl induced reduction in TNBC cell viability was associated with cell cycle perturbation, cell cycle analysis by flow cytometry was performed and the distribution of cells in different phases of the cell cycle at 72 h of treatments was measured. Cell cycle analysis can be used for cell activation and proliferation measurement. Flow cytometry is used to determine cancer prognosis as abnormal DNA content would indicate high risk for tumor recurrence and worse prognosis (Hedley and others 1987).

The TNBC cells were fixed with methanol and stained with propidium iodide (PI). The PI intercalates into double stranded nucleic acids and can be excited by 488-nm lasers. The enzyme RNAse is used for degradation of double stranded RNA so that only double stranded DNA is left within the cell to bind to PI. When DNA binds to PI, florescence increases and PI peaks can be emitted. Flow cytometry analysis gives the percentage of cells in the phases of cell cycle (G0/G1, S, AND G2/M). Cell cycle analysis histograms report the G2 and M phases as combined in view of the fact that they cannot be distinguished on DNA content basis.

After analysis of control and BSO or KCl or BSO with KCl treated TNBC cells, it was observed that BSO treated MDA-MB-231 cells accumulated in the S-phase (from 6.24% in control cells to 27.28% in BSO-treated cells) and increased cells in G2/M phase while there was a decrease in G1 (Figure 4.5). The BSO with KCl combination treatment showed similar effect in cell population as the BSO- treated cells. The combination treatment showed an accumulation in the S phase with an increase from 6.24 in control to 17.4%, an increased population of cells in the G2/M phase and a decrease in the G1 phase (Figure 4.7). KCl treated cells also accumulated in the S phase with 21.58% and reduced in the G1 phase but showed a decrease in the G2/M phase (Figure 4.6).

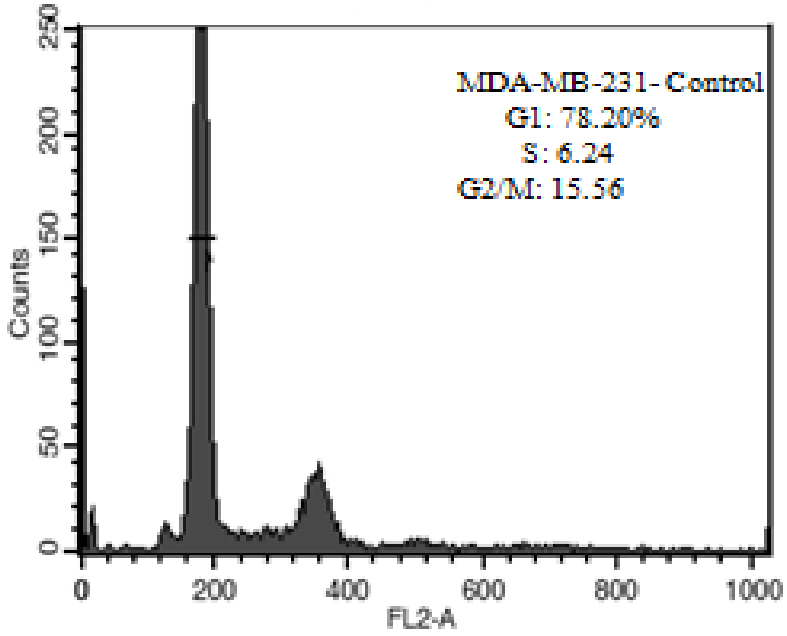


Figure 4.4: Flow cytometry histogram for MDA-MB-231

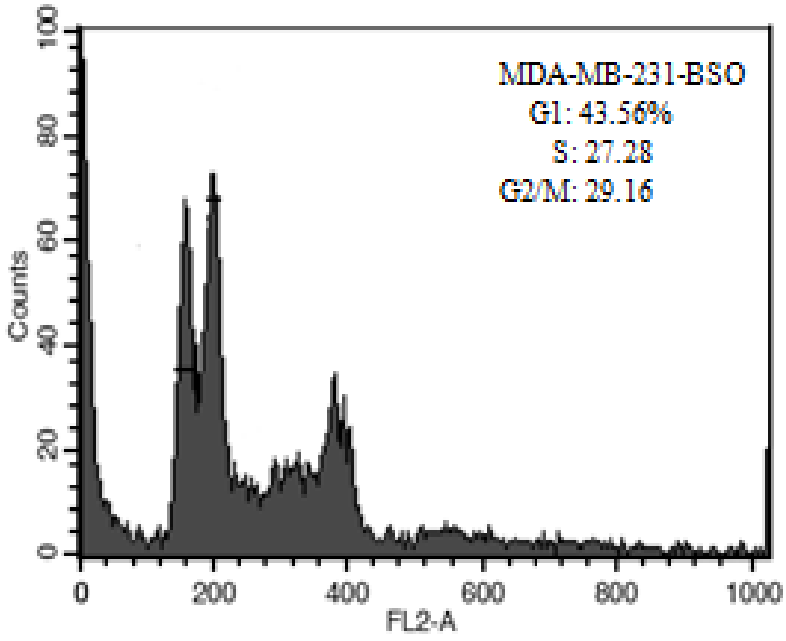


Figure 4.5: Flow cytometry histogram for BSO treated MDA-MB-231

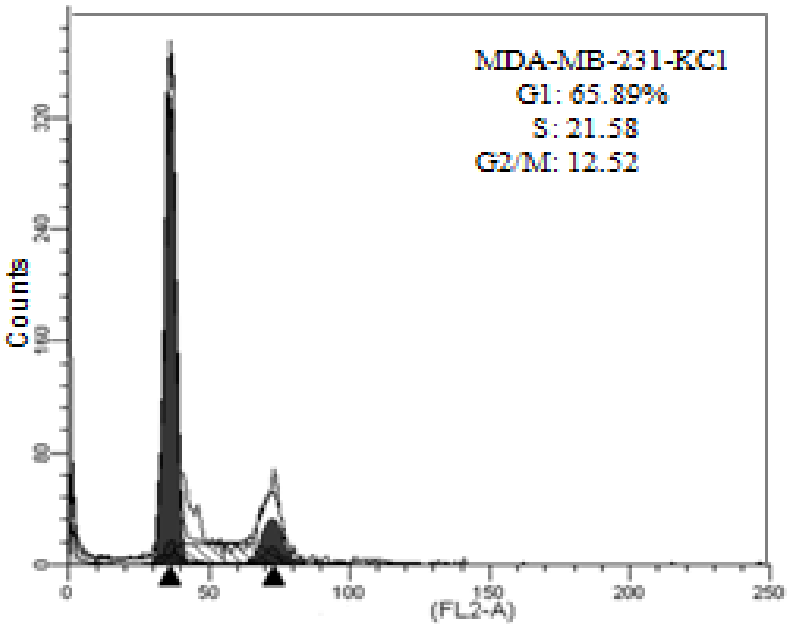


Figure 4.6: Flow cytometry histogram for KCl treated MDA-MB-231

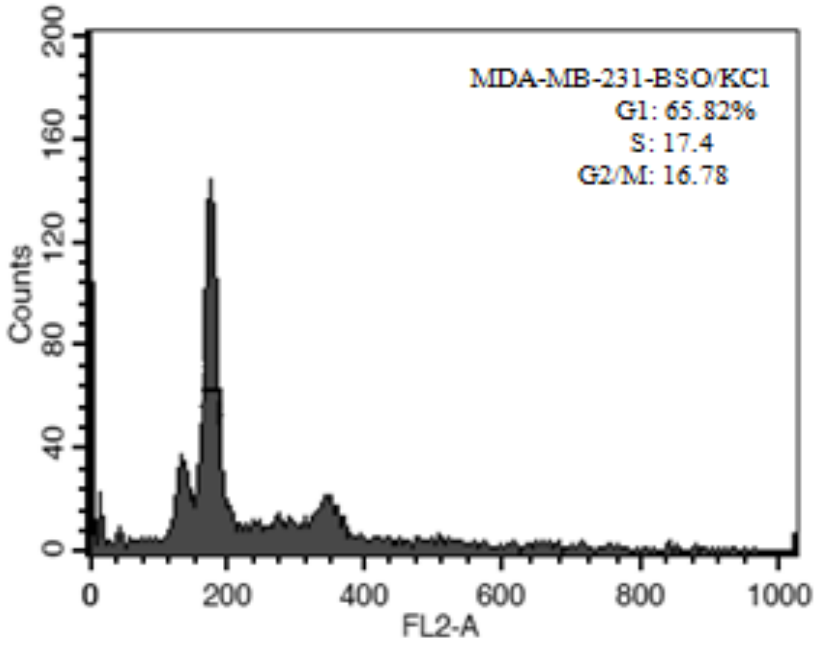


Figure 4.7: Flow cytometry histogram for BSO with KCl treated MDA-MB-231

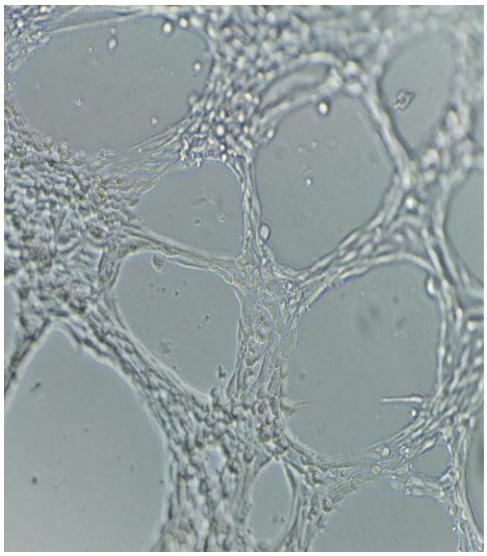
In conditions where there is adequate supply of nutrients, cell proliferation can occur. This usually takes place by default in the G0 phase for most non-tumorigenic cells and this is well controlled. However, genetic defects affect the cell cycle in cancer and lead to an uncontrolled cell division which is a hallmark of cancer. In most cancer cells, the control of progression in cell cycle is faulty, allowing cells with DNA errors (as with TNBC) to progress on to the next phase of the cycle. The cancer cells disregard cell cycle control and remain in the cycle causing more genetic diversity (Podo and others 2010). However based on the results obtained with treated cells, there was increased number of cells in the S phase in comparison to the control cells showing that BSO or KCl or BSO with KCl treatments interfere with the cell cycle progression machinery at the reputed S phase restriction point or checkpoint.

4.4. Black Seed Oil or KCl or BSO with KCl Inhibits Endothelial Cell Tube Formation

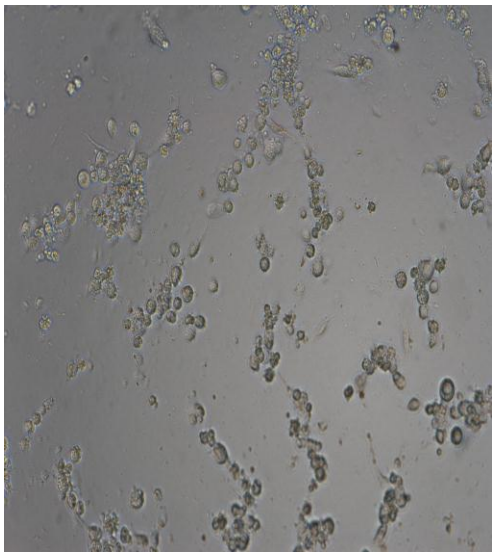
To determine the effect of BSO or KCl or BSO with KCl on endothelial cell tube formation, an *in vitro* angiogenesis tube formation assay was performed. Each of the steps in angiogenesis, including basement membrane disruption, cell migration, cell proliferation, and tube formation can be an intervention target and can be tested in vitro. Tube formation angiogenesis assay is a very specific test used for angiogenesis (Auerbach and others 2003). On a synthetic basement membrane matrix, cells are capable of morphological differentiation into an extensive network of capillary-like structures composed of highly organized three-dimensional cords. According to Bansode (2011), the alignment of endothelial cells in capillary-like structures is a functional trait that is crucial to angiogenesis.

In this assay, the ability of endothelial cells to form three-dimensional tubular structures was observed under a microscope. In the presence of 10ng/ml of VEGF, human umbilical vein

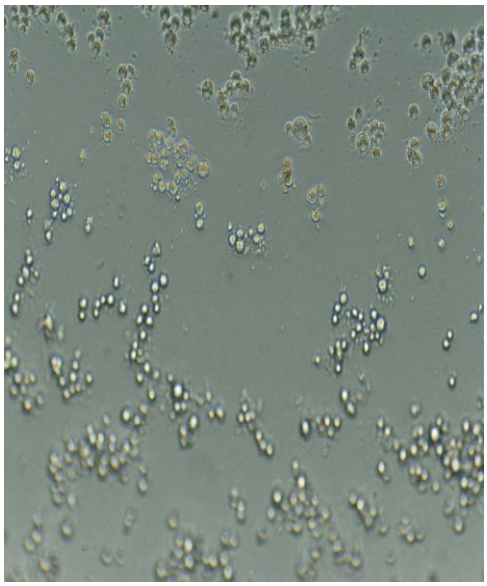
endothelial cells (HUVEC) plated on the EC matrix aligned and formed capillary-like three dimensional structures within 6h (Figure 4.8a). BSO or KCl or BSO with KCl inhibited tube formation within 6h. Treatment with BSO or KCl or BSO with KCl completely disrupted the capillary-like network of the ECmatrix (Figures 4.8b-d).



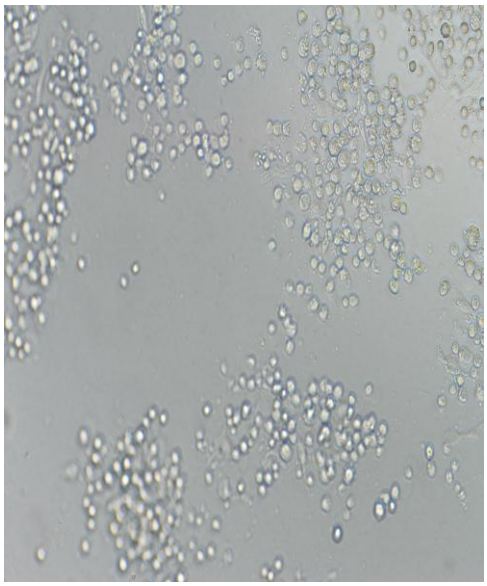
(a) Control HUVEC



(c) BSO treated cells



(b) KCl treated cells



(d) BSO with KCl treated cells

Figure 4.8: Control and BSO-, KCl-, and BSO with KCl- treated HUVEC

4.5. Black Seed Oil or KCl or BSO with KCl Down Regulates the Expression of Breast Cancer Stem Cell Markers

To further determine the molecular mechanism associated with BSO- or KCl- or BSO with KCl- mediated anti-proliferation activity on MDA-MB-231 cells, some relevant overexpressed or activated signaling proteins in the TNBC cells were investigated. Protein expression levels were determined by western blot analysis. Gene expression levels of breast cancer stem cell markers were analyzed.

There have been no markers demonstrated to predict systemic metastasis development in TNBC; however, there is increasing evidence that human breast cancers are driven by cancer stem cells (CSC), which initiate tumors with self renewal properties (Charafe-Jauffret and others 2010). CSCs have been validated by different studies to be a small cell population resistant to current anticancer therapies, antimitotic agents, and radiation. They are referred to as tumor-initiating cells which have been said to be probably responsible for the early recurrence in TNBC due to their resistance to therapy. The ability of CSCs to sustain tumor formation and growth gives rise to multi-lineage differentiated cells, which form the tumor (Hwang-Verslues and others 2009; Lorico and Rappa 2011).

There are various breast cancer stem cell biomarkers, different breast cancer cell lines and primary tumors show differences in expression of these CSC (Hwang-Verslues and others 2009). CD44, aldehyde dehydrogenase (ALDH1), and epithelial specific antigen (ESA) have been recognized as triple negative breast CSC. $CD44^{+}/CD24^{-}$ phenotype have shown high capacity for tumor initiation and for expression of ESA which is known to be a cell surface marker. ALDH is an enzyme involved in stem cell self-protection; tumors positive for ALDH expression have been associated with a lack of expression of ER and PR, and a poor clinical outcome (Croker and others 2009; Stratford and others 2010). BSO or KCl or BSO with KCl

down regulated the expression of TNBC cell surface markers including ALDH1, CD44, and ESA (Figure 4.9).

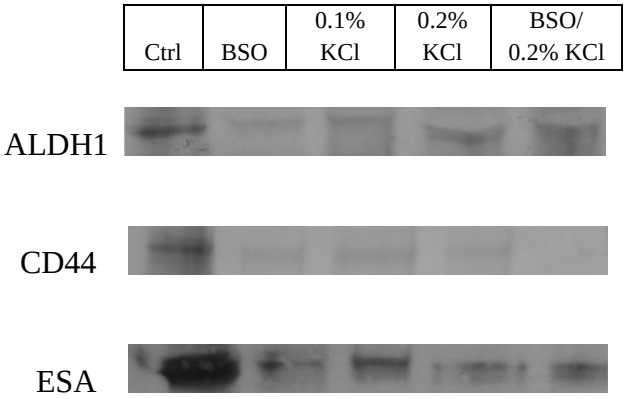


Figure 4.9: BSO or KCl or BSO with KCl treatments down regulate the expression of TNBC stem cell markers

4.6. Black Seed Oil or KCl or BSO with KCl Affects the Activation of Signaling Pathways Associated with Cell Proliferation and Metastasis

To determine the molecular mechanism associated with BSO or KCl or BSO with KCl mediated anti-proliferation activity on TNBC cells, some relevant overexpressed or activated apoptotic and cell survival signal transduction pathways associated with TNBC cells were evaluated. Cell proliferation in TNBC is stimulated by the phosphatidylinositol-3 kinase (PI3K)/Akt and/or the extracellular signal-regulated kinase (ERK) signaling pathways. BSO or KCl or BSO with KCl inhibited PI3K pathway and suppressed the phosphorylation of Akt, or ERK in MDA-MB-231 cancer cells (Figure 4.10). PI3K/Akt and NF-κB signaling pathways have been shown to be signaling pathways associated with the growth, migration, invasion, angiogenesis and metastasis in cancer progression (Rangaswami and others 2004; Wang and others 2009). The PI3K/Akt pathway promotes cell proliferation and survival by modulating several downstream signaling proteins including NF-κB. Results show that BSO or KCl or BSO with KCl down-regulated the levels of NF-κB in TNBC cells (Figure 4.10). Cyclooxygenase

(COX)-2 an inflammation marker associated with TNBC was also down-regulated by the treatments used in this study. Anti-inflammatory properties of black seed oil have been demonstrated in vivo and in vitro, and it was observed that the inflammation pathway cyclooxygenase (COX) was inhibited (Salem 2005). It is conceivable that COX-2 down-regulation was associated with the inhibition of NF-κB because the promoter sequence of COX-2 contains binding sites for NF-κB (Kaltschmidt 2002).

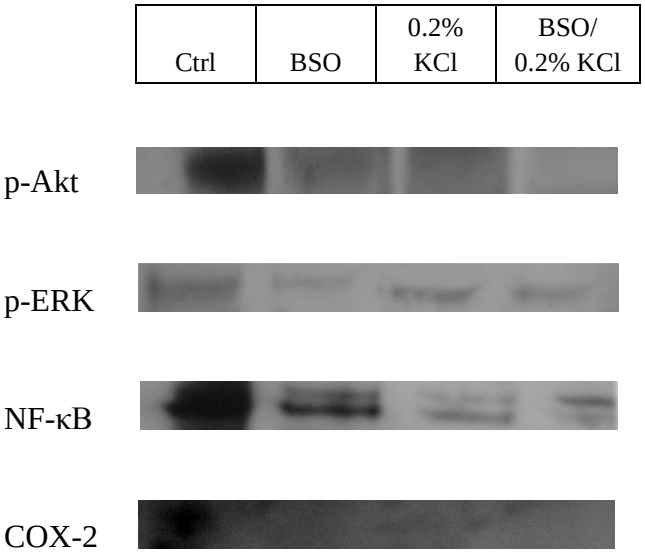


Figure 4.10: BSO or KCl or BSO with KCl treatments down regulate cell proliferation and progression markers

4.7. Black Seed Oil or KCl or BSO with KCl Affects the Activation of Signal Transduction by Hsp90 and Client Proteins

To determine whether BSO or KCl or BSO with KCl inhibits Hsp90 activity, Western blot analysis of Hsp90α was performed on MDA-MB-231 cells treated with BSO or KCl or BSO with KCl. Hsp90 has been recognized as a master regulator of signal transduction (Boulon and others 2008). Hsp90 is highly expressed in TNBC cells and assists in the conformational maturation and stability of a range of "client proteins". The overexpression of Hsp90 is mainly due to ATPase activity as the chaperone activity of Hsp90 is dependent on the activity of this

enzyme. Most Hsp90 client proteins are frequently mutated and include key mediator factors that regulate cell proliferation, inhibit apoptosis, regulate cell cycle, enhance inflammation, and sustain the survival of cancer cells. Inhibition or interruption of Hsp90 can destabilize these client proteins and lead to cancer cell apoptosis. The clinical significance of Hsp90 is that many of its client proteins are overexpressed in several types of cancer progression and proliferation including TNBC (Bagatell and Whitesell 2004; Kang and others 2010). Inhibiting Hsp90 disrupts Hsp90 interactions with co-chaperones and leads to the degradation of client proteins and weakening of processes fundamentally important to cancer cells including proliferation, cell cycle regulation, and avoidance of apoptosis (Powers and Workman 2006; Zhang and others 2008; Zuehlke and Johnson 2010).

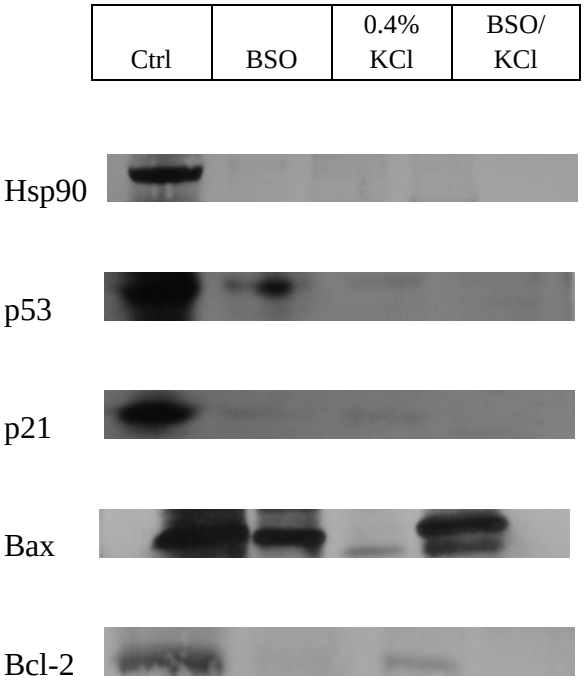


Figure 4.11: BSO or KCl or BSO with KCl treatments inhibit activity of Hsp90 and other apoptotic markers

Western blot analysis indicated that the expression of Hsp90α was down-regulated in all treated cells (Figure 4.11). The anti-cancer activity of KCl is linked with the apoptotic activity of heat shock proteins in the disease. ATPase activity with Hsp90 has been proven to be reduced by KCl at 0.2% both *in vivo* and *in vitro* (Kamal and others 2003).

The focus for this study was on Hsp90α, p53, NF-κB, bax, and *bcl*-2, because Hsp90 chaperones most of these proteins. Apoptosis induction is mediated by these proteins. Results in Figure 4.11 indicate that the levels of mutated p53 in BSO- or KCl- or BSO with KCl -treated TNBC cells were reduced compared to control cells. Thymoquinone from BSO is known to induce apoptosis by p53 pathways in cancer cell lines (Gali-Muhtasib and others 2006). The tumor suppressor protein p53 is a critical component of the apoptotic pathway as it controls the expression of large numbers of genes. P53 is mutated and non functional in most tumors and has been associated with poor prognosis of most cancers including TNBC. Due to a defective cell cycle, frequent p53 mutations have been reported in TNBC cells (Oakman and others 2010; Podo and others 2010). BSO or KCl or BSO with KCl decreased the levels of mutated p53 in MDA-MB-231 cells. However it was shown that KCl alone and the BSO/KCl combination treatments were more effective than BSO alone in down-regulating the expression of p53. A similar observation was made with p21, as it was also down-regulated in treated MDA-MB-231 cells.

The Bcl-2 family which includes both proapoptotic proteins such as Bax and antiapoptotic proteins such as Bcl-2 are associated with the regulation of cell growth and apoptosis. Overexpression of Bcl-2 enhances cell survival by apoptosis suppression on the other hand, Bax overexpression speeds up death (Wang and others 2009). Studies have shown that thymoquinone from black seed oil inhibits anti-apoptotic Bcl-2 (Ait Mbarek and others 2007). The levels of Bax proteins remained almost unchanged suggesting cell death by mixed apoptotic

and non-apoptotic ways. Down-regulation of Bcl-2 expression suggested that cell death occurred by apoptosis. The down-regulation of bcl-2 is likely linked to the ability of the treatments to induce apoptosis in TNBC cells. It was observed that bcl-2 levels were down-regulated by all treatments used (BSO or KCl or BSO/KCl). However unlike BSO and the BSO/KCl combination treatment, KCl treated cells did not show up-regulation of Bax as should be the case for effective induction of apoptosis. This shows an advantage of the combination treatment over the use of KCl alone.

CHAPTER 5:

SUMMARY AND CONCLUSION

The main objective of this study was to investigate the interaction of black seed oil (BSO) or potassium chloride (KCl) or a combination of the oil with potassium chloride with the biomarkers of triple negative breast cancer. To test this objective, the major bioactive compound in black seed oil, thymoquinone, was identified in the commercially available oil used for the study and cell viability, cell cycle, *in vitro* angiogenesis tube formation assays, as well as western immunoblots on TNBC associated biomarkers were performed to examine treatment effects on different aspects of TNBC. The expected results were that the treatments would inhibit the proliferation and anti-apoptotic activity of TNBC cells.

The results prove that black seed oil or KCl or BSO with KCl treatments inhibited the viability of MDA-MB-231 triple negative breast cancer cells after 72h exposure and led to down-regulation of several factors associated with cell proliferation and apoptotic signal transduction including Hsp90. Also the treatments disrupted tube formation in angiogenesis, and caused an interruption in TNBC cell cycle progression. This shows that the treatments were effective in targeting the hallmarks of breast cancer and characteristics of TNBC.

Overall, the analyses performed demonstrate that black seed oil and potassium chloride have a strong potential and several advantages as preventive or therapeutic agents for this aggressive deadly disease, triple negative breast cancer. However, further studies should be done to examine the interaction of these anticancer agents *in vivo*.

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VITA

Tamaratina Jombai was born on October 19, 1987, in the city of Warri in Delta State, Nigeria. Her parents are Elizabeth and Sylvester Jombai. She graduated from Presentation National High School in Benin City, Edo State, Nigeria, in July 2003 where she developed her interest in food science.

She came to Baton Rouge, Louisiana, U.S.A., in August 2004 to pursue a bachelor's degree in food science and Louisiana State University and graduated in May 2008. During her undergraduate study she gained internship experience at Kellogg's Eggo Company in Tennessee, U.S.A, and after graduation she worked in Quality Assurance for Community Coffee Company in Port Allen, Louisiana. She started the master's program in food science in August 2009 and is now a candidate for a Master of Science degree from the Department of Food Science in Louisiana State University and Agricultural and Mechanical College, which will be awarded in August 2011.

Tamaratina loves spending time with her family and loved ones. She has a lot of appreciation for the art of food as well as other forms of art such as music, painting, etc. She also loves to travel. Her greatest passion is worshipping Jesus and seeing the wonderful transformation that happens when people turn to Him and find their purpose for living.