2013

Efficacy of tamoxifen and thymoquinone on tumor development, lipid peroxidation, and total antioxidant capacity in DMBA induced mammary carcinoma in female Sprague-Dawley rats

Nadiah S. Alotaibi
Eastern Washington University

EFFICACY OF TAMOXIFEN AND THYMOQUINONE ON TUMOR DEVELOPMENT, LIPID PEROXIDATION, AND TOTAL ANTIOXIDANT CAPACITY IN DMBA INDUCED MAMMARY CARCINOMA IN FEMALE SPRAGUE- DAWLEY RATS

A Thesis

Presented To

Eastern Washington University

Cheney, Washington

In Partial Fulfillment of the Requirements

For the Degree

Master of Science in Biology

By

Nadiah S. Alotaibi

Fall 2013

THESIS OF NADIAH S. ALOTAIBI APPROVED BY

_____ DATE _____

KAREN CARLBERG, PH.D., GRADUATE STUDY COMMITTEE CHAIR

_____ DATE _____

JOANNA JOYNER-MATOS, PH.D., GRADUATE STUDY COMMITTEE

_____ DATE _____

YVES NIEVERGELT, PH.D., GRADUATE STUDY COMMITTEE

THESIS FOR MASTER OF SCIENCE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Eastern Washington University, I agree that JFK Library shall make copies freely available for inspection. I further agree that copying of this project in whole or in part is allowable only for scholarly purposes. It is understood, however, that any copying or publication of this thesis for commercial purposes, or for financial gain, shall not be allowed without my written permission.

Signature_____

Date_____

ABSTRACT

Tamoxifen (TAM) is a drug used for treatment and prevention of breast cancer. However, TAM has been reported to induce liver tumors in several animal models due to overproduction of free radicals and lipid peroxidation. Thymoquinone (TQ), from *Nigella sativa* seeds, is a Middle East natural medicine and it has been used to treat a number of diseases, including cancer. The aim of my study was to investigate the effect of TQ and TAM on tumor development, lipid peroxidation level, and total antioxidant capacity in DMBA treated female Sprague-Dawley rats. Five groups of 10 rats (control, DMBA, TAM, TQ, and TAM+TQ) were used. Mammary tumors were induced by DMBA which was given by oral gavage to four groups: DMBA, DMBA+ TAM, DMBA+ TQ, and DMBA+ TAM & TQ. The control group received tap water only. Starting three weeks later, TAM and/or TQ were administered daily for 10 weeks. Mammary tumors were found in two DMBA treated animals. There was coloration in the mammary glands in four other DMBA treated animals. No mammary tumors or mammary gland coloration was observed in other groups. Rats treated with TAM showed a significantly higher level of lipid peroxidation when compared to control ($P<0.05$). However, the administration of TQ together with TAM showed significant decrease in lipid peroxidation level compared to treatment with TAM alone ($P<0.05$). Total antioxidant capacity did not differ among the groups ($P=0.791$). Average body weight gain during 12 weeks differed significantly among the groups ($P<0.001$). TAM treated rats had a lower body weight gain when compared with other animals and this was not ameliorated by TQ. In conclusion, both TAM and TQ appeared to prevent tumor induction by DMBA.TAM had two adverse effects on the rats: increasing in lipid peroxidation levels and reduction in body weight gain. TQ ameliorated the adverse effect of TAM on lipid peroxidation but not body

weight gain. My study is the first to show the anticancer/antitoxic effects of TQ and TAM together on mammary tumor and lipid peroxidation level. Data from this study suggested that the combination therapy of TQ and TAM could be a safer anticancer drug than TAM alone.

ACKNOWLEDGMENTS

I would like to thank my Graduate Advisor Dr. Karen Carlberg for her support and guidance through my project, as well as my other committee members, Dr. Joanna Joyner-Matos and Dr. Yves Nievergelt.

Thank you to Dr. Mike Satterwhite for his guidance in care and housing of my project animals.

Thank you to David French for his assistance with project supplies.

Thank you to Dr. Tom Hancock for the use of his spectrophotometer.

Thank you to my partner Abrar Sindi for her assistance with animal care and assay kits.

Thank you to my family for their support and encouragement to complete my education.

Table of Contents

Tables

Figures

Introduction

Breast Cancer

Breast cancer is the most common cancer among women, with more than 210,000 cases diagnosed in the United States alone every year (Siziopikou, 2013). Breast carcinoma is a disease in which abnormal cells in the mammary glands divide and multiply in an uncontrolled way. This disease is divided into two major types:

Non-invasive breast cancer (*in situ*): is an abnormal growth of the cells still within the area in which it started. It is classified as ductal carcinoma *in situ* or lobular carcinoma *in situ*.

- Ductal carcinoma *in situ*: is the most common type of non-invasive that presents in the duct and does not go through the duct walls into the surrounding tissues.
- Lobular carcinoma *in situ*: is a very early form of breast cancer that develops within the lobules of the breast (Richie & Swanson, 2003).

A. Invasive breast cancer: is a type of breast cancer that starts in the ducts or lobules and spread to nearby or distant tissue of the body. Paget's disease is a rare form of invasive breast cancer and it begins in the ducts and spreads to the skin of the nipple and areola (Muttarak et al., 2011).

Reactive Oxygen Species and Oxidative Stress

Reactive oxygen species (ROS) have been implicated in the development of breast cancer. ROS are products of normal cellular metabolism (Karihtala & Soini, 2007). ROS are divided into free radicals and non-radical ROS. Free radicals, which contain one or more unpaired electrons, include superoxide anion radical ($O\cdot\square_2$), the highly reactive

hydroxyl radical ($^{\cdot}OH$), nitric oxide ($^{\cdot}NO$), and alkoxyl- ($RO^{\cdot}$) or peroxyl-($ROO^{\cdot}$) radicals. Non-radical ROS include hydrogen peroxide (H_2O_2), hypochloride (HOCI), and organic hydroperoxide (ROOH) (Storz, 2005).

Reactive oxygen species are generated in our body by various endogenous factors such as many enzymatic activities like proteins within the plasma membrane, lipid metabolism within the peroxisomes, as well as the activity of various cytosolic enzymes such as cyclooxygenases (Droge, 2002; Klaunig et al., 2010; Shinde et al., 2012). Although all these endogenous sources contribute to the ROS production, the major source of ROS is the mitochondria.

Most the energy that our cells need to live depends on a mitochondrial process that requires oxygen (O_2). In this process, which is called oxidative phosphorylation, most of O_2 (>95%) is used by the mitochondria to produce ATP. ATP generation is coupled with a reaction in which O_2 is reduced to H_2O. Under certain conditions, some of oxygen (<0.1% to 5%) is not completely oxidized to water but rather to the production of reactive oxygen species, primarily the superoxide anion (Clark, 2002).

On the other hand, exogenous factor may also contribute to the production of ROS. For example, ionizing radiation causes the formation of water radiolysis products that contain some reactive oxygen species (Yamamori et al., 2012).

Reactive oxygen species normally exist in living cells in balance with biochemical antioxidants. This balance is essential for the health and survival of organisms. Oxidative stress occurs when this critical balance is disrupted because of excess ROS and/or depletion of antioxidant or repair enzymes (Halliwell & Gutteridge, 2007). The results of oxidative stress may include increased cell proliferation, cell injury,

senescence, and cell death (Halliwell & Gutteridge, 2007). In addition, oxidative stress plays an important role in the development of chronic diseases such as cancer, arthritis, autoimmune disorders, cardiovascular and neurodegenerative diseases (Shinde et al., 2012).

Oxidative Stress and Cancer

Reactive oxygen species play a major role in tumor promotion by direct chemical reaction or alteration of cellular processes (Vina et al., 2006). Cancer is a multistage process defined by at least three stages: initiation, promotion, and progression (Figure 1).

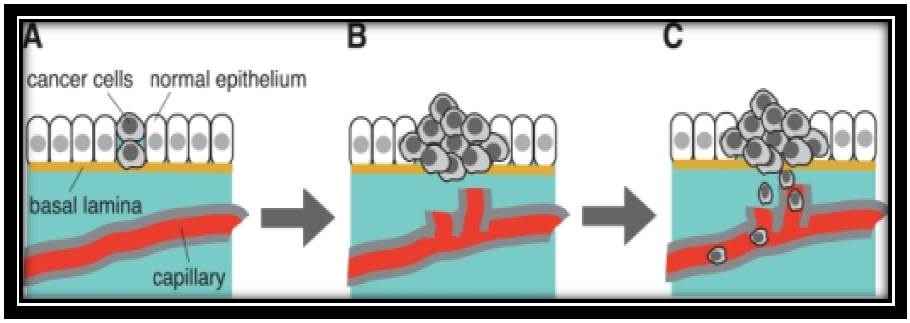

Figure 1. Multistage process of cancer. A. initiation, B. promotion, and C. progression (Sato, 2008).

Oxidative stress interacts with all three stages of this process. In the initiation stage, which is caused by DNA alteration, ROS may produce DNA damage by introducing gene mutations and structural alterations of the DNA. During the promotion stage, which is caused by the expression of a latent phenotype of initiated cells, ROS can contribute to abnormal gene expression, blockage of cell to cell communication, and modification of second messenger systems. This results in an increase of cell proliferation or a decrease in apoptosis of the initiated cell population. In the progression stage, defined as the development of a premalignant lesion into a malignant one,

oxidative stress may participate by adding more DNA alterations to the initiated cell population (Halliwell & Gutteridge, 2007; Klauniget et al., 1998).

Furthermore, reactive oxygen species can activate a variety of transcription factors like nuclear factor kappa B (NF-κB), activating protein-1 (AP-1), and β-catenin. The activation of these transcription factors contributes to tumor development by the regulation of cellular proliferation, angiogenesis, and metastasis. For example, ROS have been shown to have an impact on NF-κB regulation. Expression of NF-κB has been shown to promote cell proliferation, whereas inhibition of NF-κB activation blocks cell proliferation (Reuter et al., 2010). Additionally, cell lines from different cancers, including colon, breast, pancreas, and squamous cell carcinoma, have all been reported to express activated NF-κB (Bours et al., 1994).

Oxidative Stress and Lipid Peroxidation

Lipid peroxidation is defined as the oxidative damage of polyunsaturated lipids in cell membranes, which contain two or more carbon-carbon double bonds (Evans & Burdon, 1993). Many toxic products result from the formation of lipid peroxidation and these have an impact at a site away from the area of their generation. Membrane lipids such as mitochondrial membrane lipids and plasma membranes are more susceptible to lipid peroxidation than other lipids. Therefore, the damage caused by lipid peroxidation affects the function of the cells and their survival (Devasagayam et al., 2003).

Reactive oxygen species can change the membrane lipids, which contain a high concentration of polyunsaturated fatty acid, and cause the lipid peroxidation (Barrera, 2012). The formation of lipid peroxidation results in reactive aldehydes, including malondialdehde (MDA) and 4-hydroxy-2-noneal (4-HNE), which demonstrate high

reactivity with protein and DNA (Klaunig et al., 2010). Malondialdehdes also react with DNA bases and can introduce mutagenic lesions (Halliwell & Gutteridge, 2007).

Lipid peroxidation plays a major role in the pathogenesis of several diseases, including atherogenesis, ischemia, and cancer (Halliwell & Gutteridge, 2007). Karihtala et al. (2011) reported that the level of 4-hydroxy-2-nonenal, which is considered as a marker of lipid peroxidation, increased during breast carcinogenesis. Several studies suggested that lipid peroxidation products act as bioactive molecules in physiological and pathological conditions. These compounds may affect and modulate several cell functions including signal transduction, gene expression, cell proliferation, and the response of the target cell (Parola et al., 1999).

Antioxidant Defense

An antioxidant is defined as a substance that when present at low concentrations compared with those of an oxidizable substrate, such as fats, proteins, carbohydrates or DNA, significantly delays or prevents oxidation of that substrate (Halliwell, 1990). Some antioxidants, such as glutathione, and uric acid, are produced endogenusly. Other antioxidants, like vitamins A, C and E, beta carotene, and lycopene, are acquired in food (Lobo et al., 2010).

Antioxidants are divided into two groups based on mechanism of action: (1) chain-breaking antioxidants, such as vitamin E and beta-carotene, break the chain of free radical formation by donating an electron to stabilize existing free radical; and (2) preventive antioxidants are enzymes that scavenge initiating radicals before they start an oxidation chain (Scheibmeir et al., 2005).

Antioxidant enzymes include superoxide dismutase, glutathione reductase, glutathione-S-transferase, and glutathione peroxidase. Nonenzymatic antioxidants include ascorbic acid, glutathione, melatonin, vitamin E, and uric acid (Halliwell & Gutteridge, 2007). The main role of superoxide dismutase is to catalyze the dismutation of superoxide to hydrogen peroxide, which may prevent further generation of free radicals. Glutathione interacts with free radicals by donating a hydrogen atom. This reaction provides protection by neutralizing reactive hydroxyl radicals that are thought to be a major source of free radical pathology, including cancer (Clark, 2002).

Antioxidants are consumed rapidly during the scavenging of ROS, so they need to be regenerated or replaced by new endogenous formation or dietary - derived compounds (Ghiselli et al., 2000). Antioxidants are provided by a healthy diet that includes variety of fruits, vegetables, and herbs.

Nigella sativa and Thymoquinone

Nigella sativa, commonly known as black seed, is a Middle Eastern natural medicine. In Islam, it is regarded as one of the greatest forms of healing medicine available. The Prophet Muhammad, peace be upon him, once stated that the black seed can heal every disease except death. *Nigella sativa* is an annual herbaceous plant belonging to dicotyledon of the Ranunculaceae family. It grows in the Middle East, Western Asia, and Europe. It has been employed for many years as a spice and food preservative (Salama, 2010). The black seeds have been used in herbal medicine by various cultures and civilizations to treat a number of diseases and as a supplement to maintain good health (Butt & Sultan, 2010). *Nigella sativa* seed is used in folk medicine as an antispasmodic, antihelminthic, antiseptic, antiarthritic, nerve tonic, appetizer,

emmenagogue, and for the treatment of ascites, asthma and pustular dermatitis (Kanter et al., 2003).

Nigella sativa contains both fixed and essential oils, proteins, alkaloids and saponin. The major component of the essential oil is thymoquinone (TQ). Many studies have indicated that TQ has antioxidant effects and has been shown to protect against heart, liver and kidney damage in animal studies (Ali & Blunden, 2003).

Antioxidant /Anticancer Activities of *Nigella sativa* and Thymoquinone

The anticancer effects of *Nigella sativa* seed and its extracts have been investigated by a large number of researchers using both *in vivo* animal models and *in vitro* cancer cell lines. Bourgou et al. (2010) investigated the antioxidant and anticancer activities of the essential oil from Tunisian *Nigella sativa* seed and its main components. The results of this study indicated that the essential oil significantly inhibited ROS production and thus exhibited the ability to protect cells from oxidative stress. In addition, the anticancer activity of *Nigella sativa* was evaluated against human lung carcinoma A-549 and colon adenocarcinoma DLD-1 cell lines. The oil was found to significantly inhibit the growth of these cancer cell lines.

Topical application of *Nigella sativa* seed extract inhibited dimethylbenz [a] anthracene/croton oil induced skin carcinogenesis in mice, delayed the onset of papilloma formation and reduced the mean number of papillomas per mouse (Salomi et al., 1991). In addition, the oral administration of *Nigella sativa* seeds gave protection against methylnitrosourea induced oxidative stress and carcinogenesis in 80% of rats and *Nigella sativa* seeds with honey together in 100% of Sprague-Dawley rats (Mabrouk et al., 2002). In another study, the oral administration of *Nigella sativa* was shown to inhibit the

induction and development of 1, 2- dimethylhydrazine (DMH) -induced aberrant crypt foci, putative preneoplastic lesions for colon cancer, without any pathological changes in the liver, kidneys, and spleen (Salim & Fukushima, 2003).

TQ has been reported to possess an anti-cancer effect against a large number of cancer cell lines as well as in animal models. Li et al. (2010) evaluated the anticancer effects of TQ through inhibition of the STAT3 signaling pathway in multiple myeloma (MM) cells. Excess STAT3 protein may contribute to the growth of cancers by allowing abnormal cells to grow and divide uncontrollably. The results of this study showed that TQ could suppress both constitutive and inducible STAT3 activation in MM cells. Also, they found that TQ inhibited the proliferation and accumulation of cells in sub-G1 phase and apoptosis.

TQ was also reported to possess antitumor effect on MCF-7, MDA-MB-231 and BT-474 breast cancer cell lines. TQ was able to reduce the migration and invasion of these cells (Woo et al., 2011). Moreover, TQ showed promising anti-cancer activity against hepatocellular carcinoma by the inhibition of HepG2 cells in a dose-dependent manner (Ahmed et al., 2008).

Additionally, TQ was shown to inhibit the development of 1, 2-dimethyl-hydrazine (DMH)-induced oxidative stress during initiation and promotion of colon cancer in Wistar rats (Harzallah et al., 2012). TQ also was found to be effective in protecting mice against imidacloprid (IMI)-induced oxidative stress by enhancing antioxidant defense mechanisms (Ince et al., 2013). In another study, TQ supplementation prevented the development of diethylnitrosamine (DENA)-induced initiation of liver cancer by decreasing oxidative stress (Ahmed et al., 2010).

When TQ is combined with other anticancer drugs, it may enhance the performance of these drugs. For example, Jafri et al. (2010) evaluated the antineoplastic effect of TQ in combination with cisplatin, which is the most active chemotherapeutic agent for the treatment of non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) using *in vitro* and *in vivo* techniques. The results showed that the administration of TQ and cisplatin together inhibited proliferation of NSCLC and SCLC cell lines. Additionally, the combination of TQ and cisplatin significantly reduced tumor volume and tumor weight in a mouse model.

Another study by Arafa et al. (2011) examined the anti-proliferative effects of TQ in doxorubicin-resistant human breast cancer cells. Doxorubicin is one of the clinically most important anti-neoplastic agents and possesses a wide spectrum of anti-cancer activity against various solid tumors, including breast cancer. However, the development of doxorubicin resistance limits its use in treating breast cancer patients. Drug resistance is associated with activation of signaling pathways such as the phosphatidylinositol-3 kinase (PI3K)/Akt or suppression of tumor suppressor genes, including PTEN. The results of this study showed that TQ induces apoptosis in doxorubicin-resistant breast cancer cells through up-regulation of PTEN at transcription level. The up-regulated PTEN, in turn, inhibited the PI3K/Akt pathway and induced p53 and p21 protein expression, thereby inducing G2/M cell cycle arrest and apoptosis.

Thymoquinone and Lipid Peroxidation

Lipid peroxidation is an important step in the pathogenesis of several disease states. It is generated in small amounts by the effect of reactive oxygen species (Mylonas & Kouretas, 1999). Antioxidants may delay the start or slow the rate of the lipid

peroxidation reaction by inhibiting the formation of free radicals (Dauqan et al., 2011). TQ has been reported to inhibit lipid peroxidation in several studies. Mansour et al. (2002) reported that the oral administration of TQ reduced the hepatic and cardiac lipid peroxidation in Swiss albino mice. In another study, the oral intake of *Nigella sativa* oil for four weeks prior to induction of hepatotoxicity by D-galactosamine or carbon tetrachloride gave complete protection against D-galactosamine (El-Dakhakhny et al., 2000). In the same study, *Nigella sativa* oil showed a favorable effect on the serum lipid pattern by decreasing serum total cholesterol, low density lipoprotein, and triglycerides, and increasing high density lipoprotein level.

Tamoxifen

Tamoxifen (TAM) is an antiestrogen agent first synthesized in 1966 by Harper and Walple at the Imperial Chemical Industries in Great Britain. The compound was developed first as an antifertility drug but its antiestrogenic properties led to its evaluation in the treatment of breast cancer in 1970 (Legha, 1988). In 1977, TAM was approved for the prevention and treatment of breast cancer by the United States Food and Drug Administration (Haskell, 2003).

TAM is used to treat women whose breast cancers express estrogen receptors (ER) (White, 1999). Estrogens are steroid compounds that are important to develop and maintain female reproductive organs. The primary source of estrogens is ovaries. The granulosa cells, which surround an ovum within an ovarian follicle, secrete large amount of estrogens during the days before ovulation, when the ovum and follicle are maturing (Carlberg, 2011).

The targets of estrogens include breast, all the other reproductive organs, brain, bone, and liver. The tissues affected by estrogen molecules contain estrogen receptors, which are protein molecules. Therefore, when estrogens circulate through the bloodstream, they exert effects only on cells that contain estrogen receptors. There are three known types of estrogen receptors: estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), and GPER1 (Carlberg, 2011).

Estrogen receptors normally reside in the cell's nucleus, along with DNA molecules. Therefore, when an estrogen molecule enters a cell and passes into the nucleus, the estrogen binds to its receptor, activating it by altering its shape slightly. This estrogen-receptor complex then binds to specific DNA sites, called estrogen response elements. After it has attached to estrogen response elements in DNA, this estrogen-receptor complex binds to coactivator proteins and nearby genes become active. The active genes produce molecules of messenger RNA, which guide the synthesis of specific proteins. These proteins can then influence cell behavior in different ways, depending on the cell type involved. In breast tissue, for example, estrogen triggers the proliferation of cells lining the milk glands in the breast, thereby preparing the breast to produce milk if the woman should become pregnant (Carlberg, 2011; National Cancer Institute).

Unfortunately, estrogen in some cases may contribute to the growth of breast cancer by stimulating breast (cancer and normal) cell proliferation. That occurs when the breast cells already possess DNA mutations that increase the risk of developing cancer. Approximately 75% of all breast cancers are ER- positive, the type of breast cancer that sensitive to estrogen (National Cancer Institute).

A class of drug called selective estrogen receptor modulators (SERMs) can be used to prevent or treat diseases of estrogen-responsive tissues (Carlberg, 2011). TAM is the oldest and most prescribed SERM. TAM is chemically similar to estrogen, and binds in the same site on the estrogen receptor as the natural hormone. Therefore, TAM exerts its action by fitting into the estrogen receptors and blocking estrogen from acting on the cells (Gadducci et al., 2005). This action is responsible for the pharmacological effects of TAM.

Side Effects of Tamoxifen

TAM has been used in the therapy of advanced breast cancer since 1970. Prevention trails have been initiated in which TAM is given to healthy women at high risk of developing breast cancer. However, several studies indicated that the long-term exposure to TAM may be associated with some side effects. TAM has been shown to increase the incidence of liver tumors in several strains of rat. In humans, long-term treatment of TAM is associated with an increased risk of endometrial cancer (Karki et al., 2000).

TAM is metabolized by the human cytochrome P450s 3A4, 2C9, 2B6, 2C8, 2C19, 2D6, 1A1, 1A2, and 2A6 and is also an effective inducer of P450s 2B2, 2B1, and 3A in rat liver at doses comparable with the therapeutic doses used in humans (Dehal & Kupfer, 1997). Cytochromes P450 are hemoproteins that catalyze the metabolism of a diverse group of xenobiotics (drugs, environmental pollutants, pesticides, and herbicides) and several biologically active endogenous compounds (steroids, fatty acids) (Sridar et al., 2002). The major products of the metabolism of TAM are tamoxifen-N-oxide, formed

by flavin containing monooxygenase, and N-desmethyl and 4-hydroxytamoxifen, formed by cytochrome P450.

TAM has been shown to be a hepatocarcinogen in rats due to overproduction of free radicals and lipid peroxidation during TAM metabolism (Ahotupa et al., 1994; Nazarewicz et al., 2007; Tabassum et al., 2006). The hepatotoxicity-inducing mechanism of TAM appears to involve mitochondrial injury, which causes steatosis because of impaired beta-oxidation of fatty acids, and leads to generation of ROS and ATP depletion (Farrel, 2002). Tabassum et al. (2006) reported that TAM induced lipid peroxidation and inhibited enzymes of the antioxidant defense system in Swiss albino mice. Caballero et al. (2001) reported that long- term treatment with TAM enhanced hepatocarcinogenesis induced by p- dimethylaminoazobenzene in mice. In another study, the strong hepatocarcinogenic effect of TAM was observed in the high-dose female rats (Williams et al., 1993).

Administration of an antioxidant, such catechin, has been effective in preventing the toxicity of TAM (Tabassum et al., 2007). El-Beshbishy (2005) reported that the oral administration of dimethyl dimethoxy biphenyl dicarboxylate, which is a traditional Chinese medicine, together with TAM resulted in increasing the antioxidant enzyme activities and decreasing lipid peroxidation in female albino rats. Parvez et al. (2008) investigated the effect of pre-treatment with taurine, a natural amino acid known to possess antioxidant activity, on the toxicity of TAM in mouse liver mitochondria. The results showed the pre-treatment of Swiss albino mice with taurine markedly lowered mitochondrial lipid peroxidation, and restored enzymatic and non-enzymatic antioxidants of mitochondria.

Summary

In summary, breast cancer is the most common cancer in women worldwide. TAM is a drug used for treatment and prevention of breast cancer. However, TAM has been reported to induce liver tumors in several animal models due to overproduction of free radicals and lipid peroxidation. On the other hand, TQ, from *Nigella sativa* oil, shows promising effects against neoplastic activity in various tumor cell lines *in vitro* and *in vivo*. This activity may be attributed to its inhibitory effects on cancer cell growth and its capability for inducing apoptosis. In addition, TQ been reported to have antioxidant effects and has been shown to protect against liver damage in several animal studies. TQ also has been reported to enhance the performance of other anticancer drugs.

In my study, the combination of TAM and TQ may improve the performance of TAM and expand the use of TAM in breast cancer treatment by overcoming the side effects of TAM. Mammary tumors were induced in my study by 7, 12-dimethylbenz (a)anthracene (DMBA). Additionally, rats' livers were removed and weighed to investigate the side effects of TAM.

Induction of Mammary Tumors in Rats

Mammary glands of several rat strains, mainly Sprague-Dawley and Wistar-Furth, are susceptible to transformation induced by chemical carcinogens. The most widely used active chemical inducer of mammary carcinogenesis is 7, 12-dimethylbenz (a)anthracene (DMBA) (Barros et al., 2004). DMBA is metabolized by P450 enzymes in the rat liver. The oxidation of DMBA leads to the formation of reactive metabolites that bind to DNA causing mutations and cancer initiation (Soujanya et al., 2011).

Hypotheses

The purpose of my study was to investigate the efficacy of a combination of thymoquinone and tamoxifen against 7, 12- dimethylbenz(a) anthracene (DMBA) induced mammary carcinogenesis in female Sprague- Dawley rats. By measuring the lipid peroxidation and total antioxidant capacity, as well as evaluating the number and the sizes of tumors, I evaluated the effect of these drugs in preventing mammary cancer.

1. My first hypothesis was that the combination of TAM and TQ would decrease the lipid peroxidation level in experimental groups more than TAM alone.
2. My second hypothesis was that the combination of TAM and TQ would increase the total antioxidant capacity in TAM+ TQ group more than TAM alone.
3. Finally, I hypothesized that the combination of TQ and TAM would lead to a decrease in the number or the size of tumors in experimental groups.

Materials and Methods

Animals

Fifty female Sprague-Dawley rats were used in this study. All the animals came from the Eastern Washington University Animal Facility. At the beginning of this study the animals were 12 weeks old. IACUC approval was granted through Eastern Washington University.

The rats were randomly assigned to five groups of 10 rats each. Mishra et al. (2011) used six rats for their TAM group. The power of their experiment was 0.05 which is so low due to the sample size. On the other hand, Singh et al. (2011) used 18 rats for their TAM group. The power of their experiment was 1. I concluded that 10 rats for each group would be sufficient for determining the effect of the drugs on the rats.

The 50 animals used in this study were randomly placed into five groups of 10.The groups were as follows:

TAM group: received a single dose of DMBA, and after three weeks they received TAM daily for 10 weeks.

TQ group: received a single dose of DMBA, and after three weeks they received TQ daily for 10 weeks.

TAM+ TQ group: received a single dose of DMBA, and after three weeks they received TAM + TQ daily for 10 weeks.

DMBA group: received a single dose of DMBA, and tap water daily for 10 weeks

Control group: received tap water only, daily for 10 weeks.

All animals were housed in the Eastern Washington University Animal Facility. The animals were placed on 12 hours light/dark cycle. The room temperature was

26±1ºC, and humidity was 25-30%. The animals were housed in pairs in standard cages and allowed free access to water and Teklad 8640 Standard Rodent Diet. The cages were cleaned every seven days and changed every four weeks. Body weight was measured on all animals every seven days to calculate the volume of drug doses.

Drugs

DMBA administration. Mammary tumors were induced by 7, 12-dimethylbenz(a) anthracene (DMBA) (Barros et al., 2004). A single dose of DMBA dissolved in corn oil was given by oral gavage to four groups: DMBA, TAM, TQ, TAM+TQ. Starting three weeks later, TAM and/or TQ were administered daily for 10 weeks by oral gavage using the syringe and needles in Dr. Carlberg's lab. In the fourth week, the same dose of DMBA was given again to the same groups of animals due to an error in needle length measurement.

DMBA was purchased from Sigma Aldrich and dissolved in corn oil. The concentration of the solution was 20 mg DMBA per 1 ml corn oil (Barros et al., 2004). The volume given to each rat was equal to its weight in kg times 1 ml. For example, if the rat weight was 250 g the volume was 0.25 ml of the solution.

For oral gavage, I extended the rat's head back using an index finger on top of the head to raise the head so the esophagus was in a straight line. Next, I inserted the needle into the rat's mouth. Once the gavage needle was properly placed, the rounded end of the needle was in the rat's stomach, I slowly administered the dose. After administration, I removed the needle from esophagus (University of Delaware, 2010).

DMBA is a carcinogenic agent, so some protection steps were performed before, during, and after the DMBA administration. Before the administration, I covered all the

surfaces with protective paper, and I wore a disposable lab coat with face shield mask. Also, I placed absorbent sheets in the animal cages so I could tell if they vomited. Moreover, DMBA administration was given under the hood that was also was covered by protective paper. After the administration, I removed all the protective paper from the surfaces, and the absorbent sheets from the cages. I put the disposal lab coat, face shield mask, protective paper, and absorbent sheets in the hazard bags. Also, I cleaned all the surfaces with soap and alcohol. Two weeks later, all the cages were washed twice in the cage washer to ensure a clean environment for the animals. Furthermore, all the rats were washed in warm water and placed under a heat lamp to make sure they dried completely.

Tamoxifen and thymoquinone administration. TAM was purchased from Cayman Chemical. The drug arrived in a powder form, and I kept it at -20°C during the experiment. The dose of TAM was 10 mg/kg /day (Soujanya et al., 2011). The total volume of TAM needed for all of the 20 rats was determined. For an average animal weight of 250 g the total volume of TAM was 50 mg. Therefore, I prepared a solution containing 50 mg of TAM dissolved in 5 ml water daily. The dose for each rat was calculated as follows: the rat weight in mg/1000.

TQ was purchased from Sigma Aldrich. The drug arrived in a crystal form, and I kept it at room temperature in the lab drawers. The dose of TQ was 20 mg/kg/day (Jafri et al., 2010). The total volume of TQ needed for all of the 20 rats was determined. For an average animal weight of 250 g the total volume of TQ was 100 mg. I prepared a solution containing 100 mg of TQ mixed with 10 ml of corn oil daily. The dose for each rat was calculated as follows: the rat weight in mg/1000.

The TAM + TQ group received the same volume of TAM and TQ in the same oral gavage at the same time. The volume for 250 g rat was 0.25 ml of TAM solution and 0.25 of TQ solution.

The gavage needles were flushed with alcohol between animals. At the end of each day, rats were observed for one hour after drug administration in case they vomited. Surfaces, drugs containers, and needles were washed with soap and alcohol.

Evaluation of Mammary Tumors

I inspected all rats for mammary tumors at 3 weeks after administering DMBA. The second evaluation was after five weeks of treatment and the last one was after 10 weeks of treatment. To begin this procedure, I held the rat by grasping the whole body with my palm over the rat's back, with forefinger behind the head and the thumb and second finger under the opposite axilla. I turned the rat and palpated to detect any mammary tumors (Assis et al., 2010).

Blood Collection

After 10 weeks I collected blood samples (up to 3 ml) from each rat by cardiac puncture (Beeton et al., 2007). Before this process some preparation steps were performed. I added 5 ml of distilled water to a heparin bottle. Needles 21G X 1.5 inch with 3cc syringes were coated with heparin to prevent blood clot formation. I put paper towels in the two chambers that I used for anesthesia and euthanasia. I added to the anesthesia chamber 6 ml of isoflurane, and 15 ml of isoflurane to the euthanasia chamber. Centrifuge tubes were labeled with rat identification numbers.

Each rat was anesthetized with isoflurane in the first chamber. When the rat was unconscious, I removed it from the chamber and placed it on its back, with its nose in a

nose cone filled with isoflurane-soaked cotton. Then I inserted a 21G X 1.5 inch heparin-coated needle connected to a 3 cc syringe below and slightly to the left of the xiphoid cartilage at the base of the sternum, at a 20° to 30° angle. I advanced the needle slowly, applying very slight negative pressure on the barrel of the syringe. Blood flowed into the needle when the tip entered a chamber of the heart. When collection was completed, the rat was euthanized with a higher dose of isoflurane in the second chamber.

Blood samples were centrifuged at 600 ´ *g*, at 4°C for 10 minutes. I removed the plasma and aliquoted it into three microtubes per rat, one for the lipid peroxidation assay, one for the total antioxidant capacity assay, and a third one for Abrar Sindi's study. For the lipid peroxidation assay, I added 5 µL of antioxidant Butylated hydroxytoluene (BHT) to each plasma sample to prevent further oxidation of lipid. Samples were labeled and stored at -80°C until analysis.

Tumor Evaluation

After euthanasia rats were dissected and evaluated for tumors. Mammary tumors were removed using a dissection tray, a scalpel, and a scissors. Then, I used a caliper to measure the width, length, and height of the tumor. Tumor volume was calculated using the formula: Volume =1/6πabc, where 'a'= width, 'b' = length and 'c' = height (Assis et al., 2010). Also, photos were taken of the mammary tumors, as well as the coloration in mammary glands. Moreover, whole livers from rats were removed and weighted.

Lipid Peroxidation Assay

Evidence of lipid peroxidation in plasma was measured by the OxiSelect TBARS assay kit (Cell Biolabs, Inc., San Diego, California). According to the product manual the thiobarbituric acid reactive substances (TBARS) assay kit is a tool for the direct

quantitative measurement of malondialdehyde (MDA) in biological samples. MDA is a secondary product of lipid peroxidation that is widely used as an indicator of lipid peroxidation. The TBARS assay measured MDA in plasma samples using thiobarbituric acid (TBA), which forms an adduct with MDA when incubated at 95 °C. The MDA-TBA complex forms a pink color that can be read spectrophotometrically. The MDA level in plasma samples was quantified by comparison to an MDA standard curve.

Preparation of standard curve. I prepared a dilution series of MDA standards in the concentration range of 125 µM – 0 µM by diluting the MDA Standard in deionized water according to Table 1 below.

Standard tubes	MDA Standard (µL)	Water (µL)	MDA Standard(µM)
1	125 µL	875 µL	125
2	250 µL of tube # 1	250 µL	62.5
3	250 µL of tube # 2	250 µL	31.25
4	250 µL of tube # 3	250 µL	15.63
5	250 µL of tube # 4	250 µL	7.81
6	250 µL of tube # 5	250 µL	3.91
7	250 µL of tube # 6	250 µL	1.95
8	250 µL of tube # 7	250 µL	0.98
9	0 µL	250 µL	0.0

Table 1: Preparation of MDN standards.

Preparation of reagents.

TBA acid diluent: 2X TBA diluent was diluted (1:2) with deionized water.

TBA regent: I prepared a 5.2 mg/mL solution of TBA regent by weighing out 260 mg of thiobarbituric acid (TBA). 2X TBA diluent was added and mixed vigorously until the powder was dissolved. I adjusted the pH of the solution to pH 3.5 with sodium hydroxide solution.

Assay protocol. Each MDA-containing sample and standard was assayed in duplicate.

1- 100 µL of MDA standard and plasma was added to separate centrifuge tubes.
2- 100 µL of the SDS lysis solution was added to each tube and incubated for 5 minutes at room temperature.
3- 250 µL of TBA regent was added to each plasma and MDA standard.
4- Each tube was closed and incubated at 95°C in a water bath for 60 minutes.
5- All the tubes were placed in an ice bath for 5 minutes, and then centrifuged at 3000 rpm for 15 minutes.
6- The supernatant was removed from the plasma and MDA standard.
7- 300 µL of the supernatant was added to the new tube.
8- 300 µL of n-Butnol was added to each plasma and standard to prevent the interference of hemoglobin and its derivatives.
9- All sample tubes were placed in a vortex mixer for 2 minutes.

10- After that the tubes were centrifuged at 10,000´*g* in the cold room for 5 minutes.

11- 200 µL each plasma and MDA standard was transferred to 96-well flat-bottomed microplate.

12- The absorbance was read at 532 nm using the spectrophotometer. The reaction between MDA and thiobarbituric acid (TBA) yielded a pink color, which peaks at 532 nm.

Total Antioxidant Capacity Assay

The total antioxidant capacity (TAC) of plasma was measured by the OxiSelect Total Antioxidant Capacity (TAC) assay kit (Cell Biolabs, Inc., San Diego, California). According to the product manual the TAC assay is based on measurement of the reduction of copper (II) to copper (I) by viable antioxidants in a sample within a 96-well microtiter plate. Upon reduction, the copper (I) ion will react with a coupling chromogenic reagent that produces a color with a maximum absorbance at 490 and is read in a spectrophotometer. The absorbance values are then compared to a standard curve of uric acid antioxidant activity.

Preparation of uric acid standard curve. A uric acid standard stock solution was prepared by weighing out uric acid powder for a 10 mg/mL or 60 mM solution in 1N NaOH. The stock solution was used to prepare serial dilutions of uric acid in deionized water, with the highest concentration at 1 mM diluted to 9 different concentrations with 0.0039 mM as the lowest, and the 10th sample as a blank sample containing only water.

Tubes	2 mM Uric Acid Antioxidant Standard (µL)	Deionized water (µL)	Resulting Uric Acid Concentration (mM)
1	500	500	1
2	500 of tube # 1	500	0.5
3	500 of tube # 2	500	0.25
4	500 of tube # 3	500	0.125
5	500 of tube # 4	500	0.0625
6	500 of tube # 5	500	0.03125
7	500 of tube # 6	500	0.0156
8	500 of tube # 7	500	0.0078
9	500 of tube # 8	500	0.0039
10	0	500	0.0

Table 2: Preparation of uric acid standards.

Preparation of reagents.

1X reaction buffer: reaction buffer was diluted (1:100) with phosphate buffered saline.

1X copper ion regent: copper ion reagent was diluted (1:100) with deionized water.

1X stop solution: stop solution was diluted (1:10) with deionized water.

Assay protocol. Each uric acid or plasma sample was tested in triplicate.

1- 20 µL of each uric acid standard or plasma was added to the 96-well plate.

2- 180 µL of the reaction buffer was added to each sample and standard and mixed with a vortexer.

3- An initial, baseline absorbance was read at 490 nm wavelength.

4- The reaction started when 50 µL of the copper ion reagent was added into each well, and then incubated for 5 minutes on an orbital shaker.
5- The reaction was terminated with the addition of 50 µL of the stop solution.
6- The plate was read at 490 nm wavelength.
7- The baseline readings were subtracted from these values to produce the net absorbance values.
8- Antioxidant capacities of the samples were calculated first into "mM uric acid equivalents" by comparison against the uric acid standard concentration curve.
9- This value was then be used to obtain the "copper reducing equivalents" by multiplying the uric acid equivalent concentrations by 2189 (µM Cu2+/mM uric acid).
10- This final value was proportional to the sample's total antioxidant capacity and was used for data analysis.

Statistical Analysis

The number of rats with tumors and the number of rats with mammary gland coloration were counted. No statistical analysis was performed.

Data for body weight gain between weeks one and 13, and liver weight were determined to be normally distributed by using Shapiro-Wilk test. Therefore, the one-way ANOVA test was used to analyze body weight gain and liver weight data.

For total antioxidant capacity, a mean of triplicate measurements was calculated for each animal. Data for total antioxidant capacity were not normally distributed. Therefore, the Kruskal-Wallis test was used to analyze total antioxidant capacity data.

For lipid peroxidation levels, a mean of duplicate measurements was calculated for each rat. Data for lipid peroxidation were not normally distributed. Therefore, the Kruskal-Wallis test was used.

Lipid peroxidation, total antioxidant capacity, body weight gain, and liver weight data were analyzed using Sigma Plot software. Values of $P<0.05$ were considered significant.

Results

Three rats from TQ group died, probably due to an error in TQ administration. TQ was mixed with corn oil and that caused the needle to become slippery. The gavage needle may have been inserted by mistake into the trachea instead of the esophagus. All three rats had difficulty breathing after TQ administration and died the next day.

During the three times of mammary tumor inspection, I was unable to feel any lumps that felt like tumors. After the dissection, there were mammary tumors in two DMBA treated rats. No tumors were found in the other groups (Figure 2, Table 3). The tumor volumes were 299.5 mm^3, 351.86 mm^3 . Additionally, there was coloration in mammary glands in four other DMBA treated animals and no coloration was observed in the other groups. Red to dark green color was noticed in mammary glands of four DMBA treated animals (Figure 3).

For lipid peroxidation, the Kruskal-Wallis test showed that lipid peroxidation levels differed significantly among groups ($P=0.017$, Figure 4). Dunn's Multiple Comparison test showed that the lipid peroxidation level was significantly higher in the TAM group when compared with control group ($P<0.05$). Animals receiving TQ + TAM treatment showed significantly lower lipid peroxidation levels than the TAM-treated group ($P<0.05$).

For total antioxidant capacity, the Kruskal-Wallis test showed there was no significant difference among the groups ($P=0.791$, Figure 5)

For liver weight, the one way ANOVA showed there was no significant difference among the groups ($P=0.163$, Figure 6). In addition, liver looked normal and there was no visible sign of liver tumor.

Average body weight gain differed significantly among the groups ($P<0.001$, Figure 7). Pairwise Multiple Comparison procedures showed that treatment of animals with TAM resulted in a significantly lower body weight gain when compared with control ($P<0.001$), DMBA ($P<0.001$) and TQ ($P=0.012$) groups. Animals receiving TQ +TAM treatment showed a significantly lower body weight gain when compared with control ($P<0.001$), DMBA ($P<0.001$) and TQ ($P=0.002$) groups.

	Control	DMBA	TAM	TQ	TAM+TQ
Tumor	0	2	0	0	0
Coloration	0	4	0	0	0
Total	0/10	6/10	0/10	0/7	0/10

Table3. Table shows the number of rats with mammary tumors or mammary gland coloration in control, DMBA, TAM, TQ, TAM+TQ groups.

Figure 2. Mammary tumor in one of DMBA treated rats.

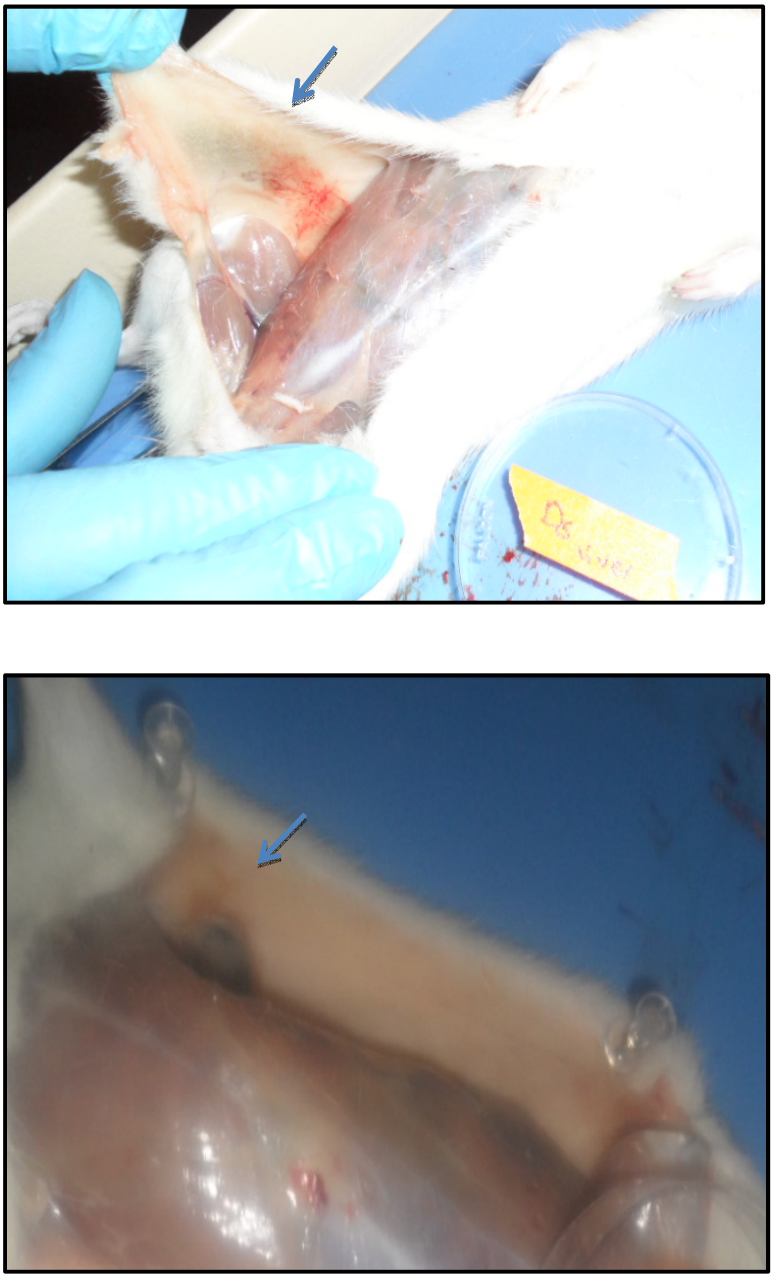

Figure 3. Mammary gland coloration in two of the DMBA trated rats.

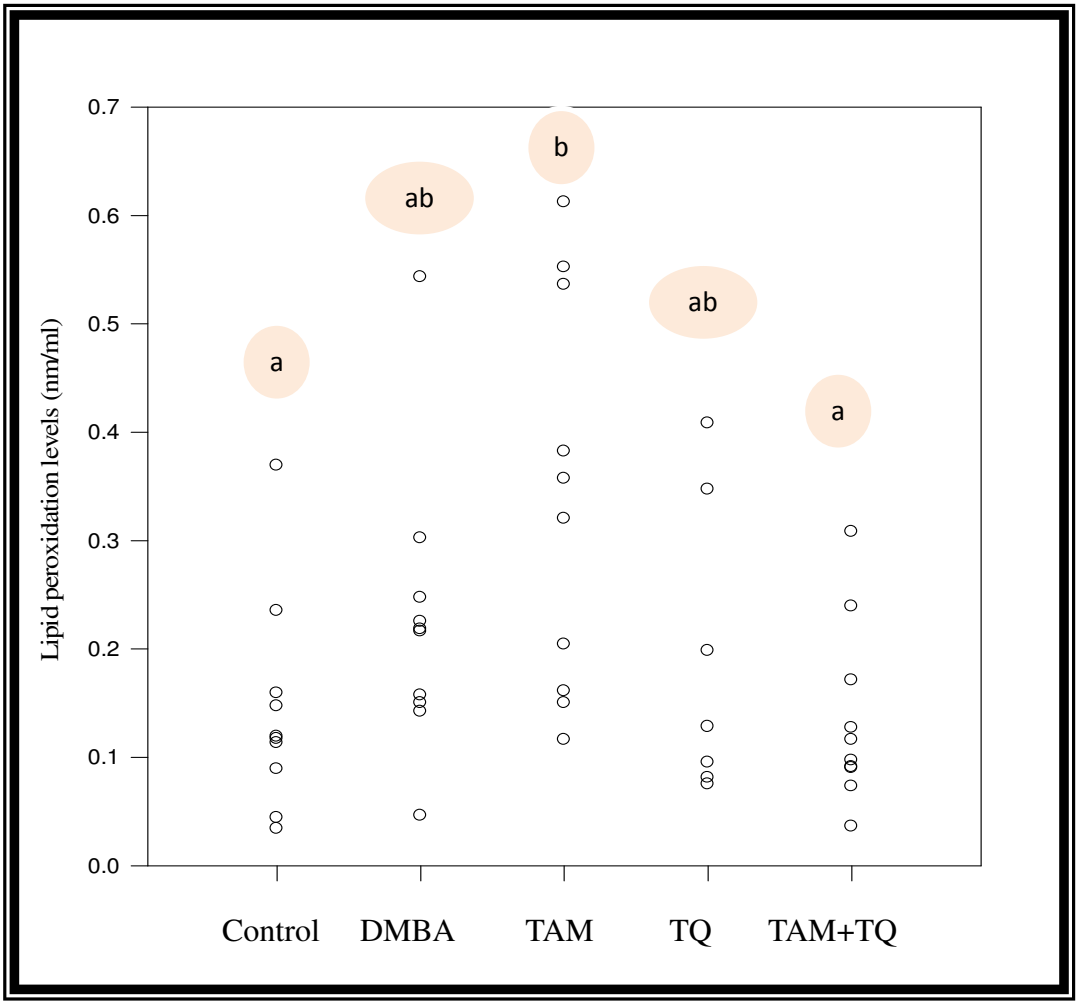

Figure 4. Lipid peroxidation levels in control, DMBA, TAM, TQ, TAM+TQ groups. The Kruskal-Wallis test showed that there was a significant difference among the groups (P=0.017). Lipid peroxidation level was significantly higher in the TAM group when compared to control and TAM +TQ groups (P<0.05). Treatment groups that do not share letters (a or b) are significantly different.

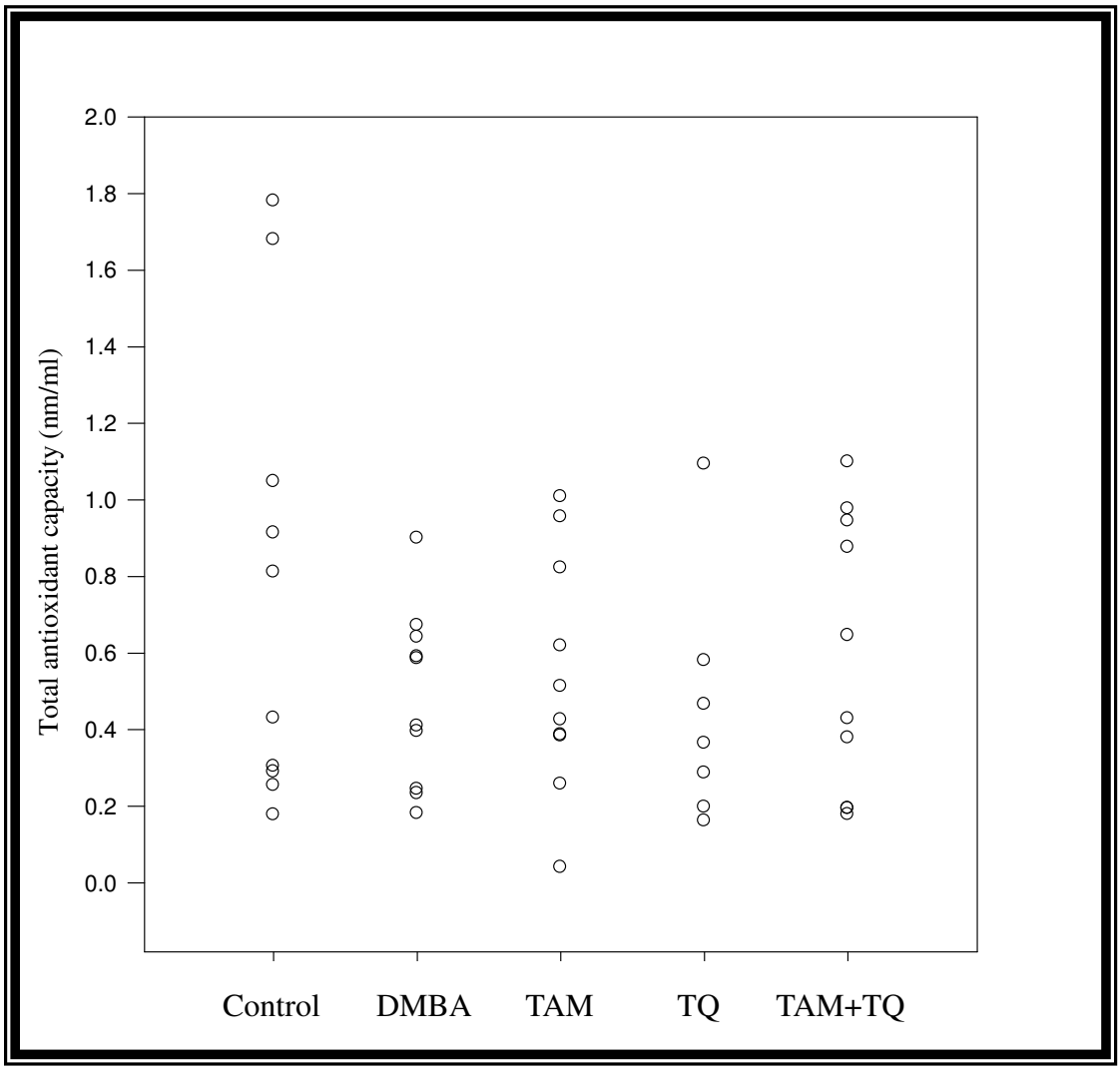

Figure 5. Total antioxidant capacity in control, DMBA, TAM, TQ, TQ+TAM groups. The Kruskal-Wallis test showed there was no significant difference among the groups (P=0.791)

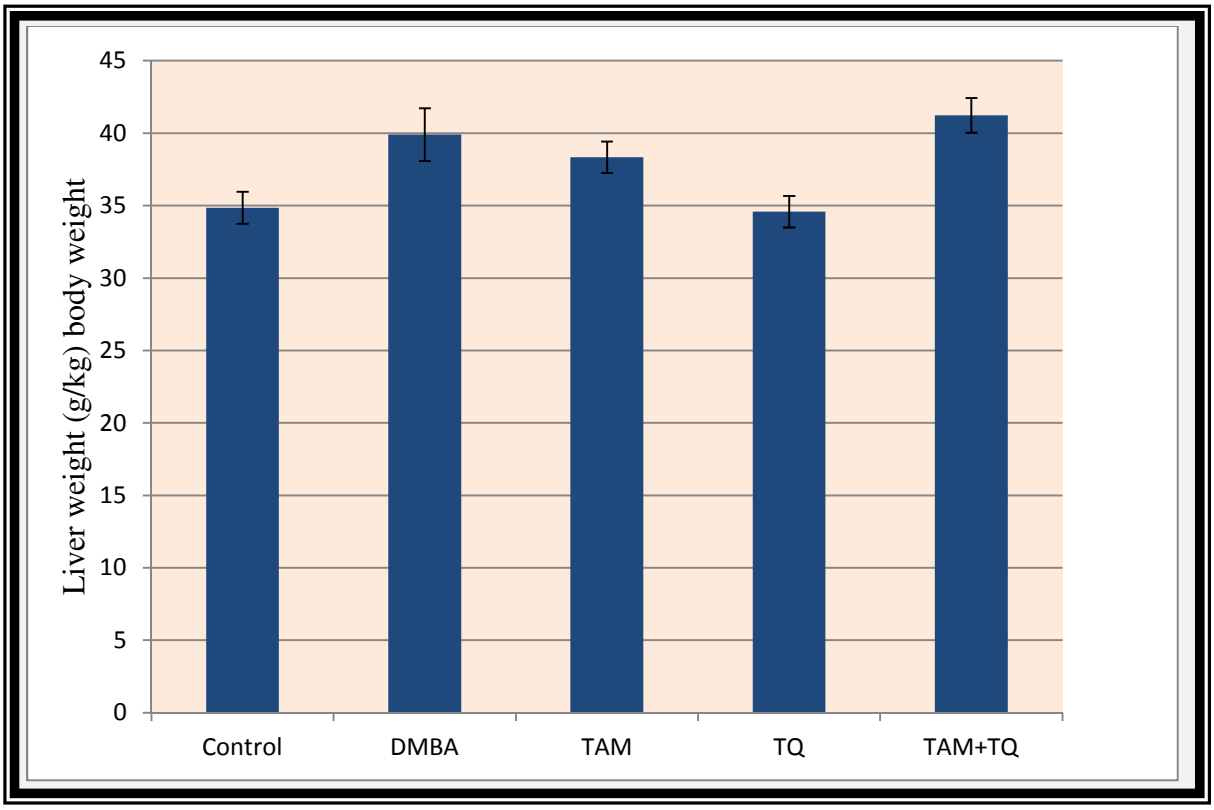

Figure 6. Values are mean and ± S.D. for liver weight in scaled to body weight in control, DMBA, TAM, TQ, TQ+TAM groups. One-way ANOVA showed there was no significant difference among the groups (P=0.163).

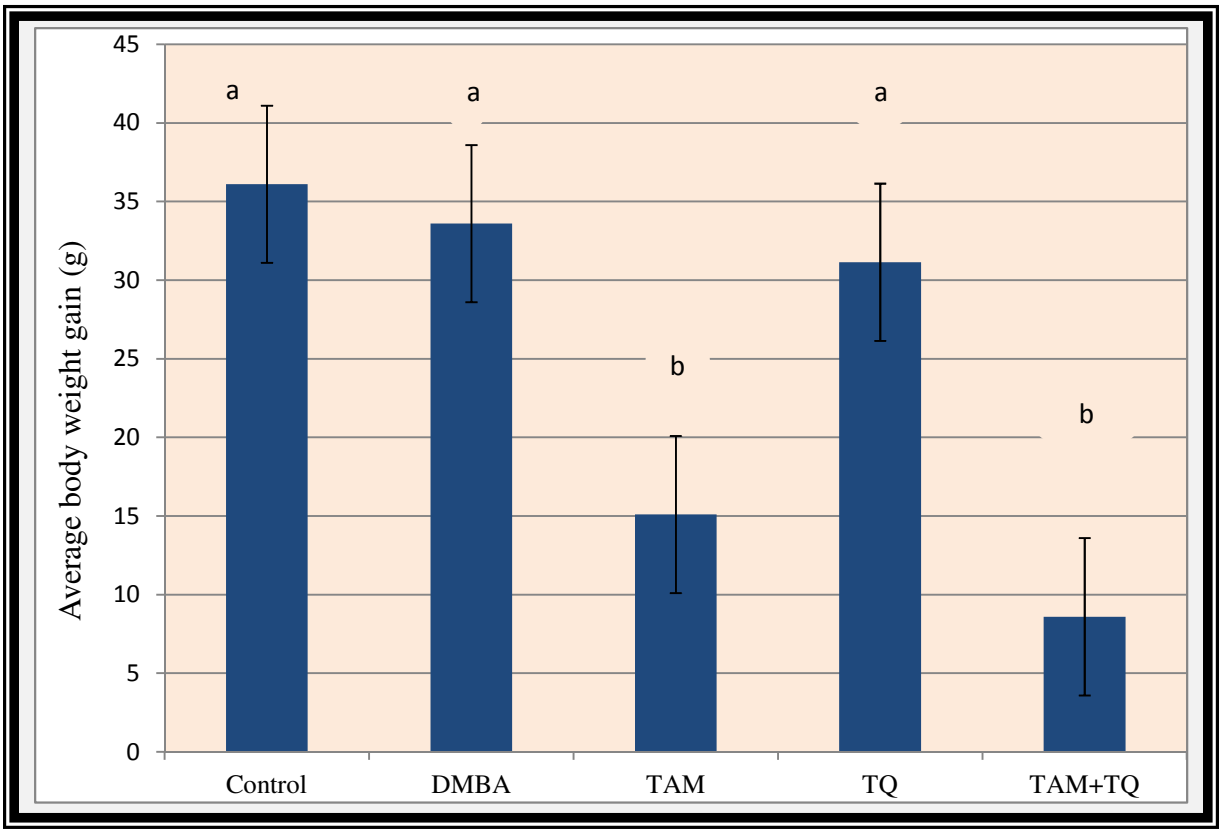

Figure 7. Values are mean and ± S.D. for body weight gain during 12 weeks. One-way ANOVA showed a significant difference among the groups (P<0.001). Treatment of animals with TAM resulted in a significantly lower body weight gain when compared with control (P<0.001), DMBA (P<0.001) and TQ (P=0.012) groups. Animals receiving TQ +TAM treatment showed a significantly lower body weight gain when compared with control (P<0.001), DMBA (P<0.001) and TQ (P=0.002) groups. Treatment groups that share letters (a or b) are similar to one another.

Discussion

Mammary Tumors

In my study, I used DMBA as a carcinogen to induce mammary tumors in female Sprague-Dawley rats. The mechanism by which DMBA causes cancer is based on the metabolism of DMBA. Soujanya et al. (2011) reported that DMBA is metabolized in the microsomes of both liver and mammary gland. The toxic manifestation of DMBA is associated with its oxidative metabolism leading to the formation of reactive metabolites capable of generating free radicals, which play a major role in the etiology of cancer.

The results of my study showed that there were mammary tumors in two DMBA treated animals. Additionally, there were four other DMBA treated rats with mammary gland coloration. Perumal et al. (2005) identified tumor cells in their study by the appearance of a red mass under the epithelial layer of mammary pad. Therefore, 60% of my DMBA treated rats developed mammary cancer. However, there were no mammary tumors or mammary gland coloration in control, TAM, TQ, TAM+TQ groups, potentially due to the protective role of both TAM and TQ.

TAM is widely used in the prevention and treatment of breast cancer. TAM works against breast cancer by interfering with the activity of estrogen. Estrogen stimulates the growth of breast cancer cells. TAM binds to estrogen receptors and blocks estrogen from acting on the cells. In animal models, TAM was shown to be effective in preventing the occurrence of mammary tumors (Soujanya et al., 2011). Perumal et al. (2005) reported that the administration of TAM with riboflavin, niacin and coenzyme Q_{10}, which are known to have antioxidant activity, resulted in decreasing mammary tumors in female Sprague-Dawley rats.

On the other hand, many studies have indicated that TQ has an anti-cancer effect against different carcinogenic agents or cancer cell lines. Woo et al. (2011) reported that TQ possesses an anti-tumor effect on MCF-7, MDA-MB-231 and BT-474 breast cancer cell lines. TQ was able to reduce the migration and invasion of these cells. Ahmed et al. (2010) reported that TQ supplementation prevented the development of diethylnitrosamine (DENA)-induced initiation of liver cancer by decreasing oxidative stress.

Additionally, the combination of TQ with other anticancer drugs was shown to improve the performance of these drugs. Jafri et al. (2010) evaluated the antineoplastic effect of TQ in combination with cisplatin, which is the most active chemotherapeutic agent for the treatment of non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) using *in vitro* and *in vivo* techniques. The results showed that the administration of TQ and cisplatin together inhibited proliferation of NSCLC and SCLC cell lines. Also, the combination of TQ and cisplatin significantly reduced tumor volume and tumor weight in a mouse model.

The results of my study indicated that both TQ and TAM alone and together have a protective role against DMBA-induced mammary tumors in female Sprague-Dawley rats. Additionally, the results of this study show for the first time that the combination therapy of TQ and TAM prevented the development of mammary cancer. Therefore, my data provide evidence to implicate TQ alone or in combination with TAM in breast cancer prevention and treatment.

Lipid Peroxidation Levels

Lipid peroxidation is generated by the effect of ROS and it is a common feature of several diseases, including cancer. Lipid peroxidation levels were assessed using TBARS assay kit. This assay depends on the measurement of MDA in plasma samples. MDA is a result of lipid peroxidation. It has been reported that MDA may cause DNA damage (Halliwell & Gutteridge, 2007).

The results of my study showed that the lipid peroxidation level was significantly higher in the TAM group compared with the control group. In addition, animals receiving TQ + TAM treatment showed significantly lower lipid peroxidation levels than the TAM-treated group.

Many studies have shown that TAM could be a hepatocarcinogen due to overproduction of free radicals and lipid peroxidation during hepatic TAM metabolism. Tabassum et al. (2006) reported that TAM induced lipid peroxidation and inhibited enzymes of the antioxidant defense system in Swiss albino mice. Caballero et al. (2001) reported that long- term treatment with TAM enhanced hepatocarcinogenesis induced by p- dimethylaminoazobenzene in mice. However, administration of an antioxidant, such catechin, has been effective in preventing the toxicity of TAM (Tabassum et al., 2007). El-Beshbishy (2005) reported that the oral administration of dimethyl dimethoxy biphenyl dicarboxylate, which is a traditional Chinese medicine, together with TAM resulted in lower lipid peroxidation in female rats.

In contrast, TQ has been reported to decrease lipid peroxidation levels in several studies. TQ and other antioxidants delay the start of the lipid peroxidation reaction by inhibiting the formation of ROS. Mansour et al. (2002) reported that the oral

administration of TQ reduced hepatic and cardiac lipid peroxidation in Swiss albino mice. In another study, the oral intake of *Nigella sativa* oil for four weeks prior to induction of hepatotoxicity by D-galactosamine gave complete protection against D-galactosamine (El-Dakhakhny et al., 2000). In the same study, *Nigella sativa* oil showed a favorable effect on the serum lipid pattern by decreasing serum total cholesterol, low density lipoprotein, and triglycerides, and increasing high density lipoprotein level.

In my study, TAM did not affect liver weight which suggested that there was no liver tumor. However, TAM treated animals showed a significantly higher level of lipid peroxidation when compared to control. The administration of TQ together with TAM showed significant decrease in lipid peroxidation level compared to treatment with TAM alone. Data from this study show for the first time that the combination therapy of TQ and TAM may help to overcome this side effect of TAM. TQ is a natural medicine and many studies have indicated that there were no side effects observed with TQ administration. Therefore, the combination therapy could be a powerful and safe drug in prevention and treatment of breast cancer.

Total Antioxidant Capacity

Total antioxidant capacity was assessed using the OxiSelect Total Antioxidant Capacity assay kit. Antioxidant defense helps to protect the body from free radicals and their effects. The result of my study showed that total antioxidant capacity did not differ among the groups. There could be two reasons that total antioxidant capacity did not differ among groups. The dosage of TQ used, 20 mg/kg, may not have been sufficient to increase antioxidant level. Alternatively, the assay kit may not have measured the total antioxidant capacity accurately in plasma samples.

Several studies have indicated that TQ possesses antioxidant activity and that plays a major role in inhibiting the development of tumors. Harzallah et al. (2012) reported that TQ was shown to inhibit the development of 1, 2-dimethyl-hydrazine (DMH)-induced oxidative stress during initiation and promotion of colon cancer in Wistar rats. In the same study, TQ treated rats showed a significantly higher level of the antioxidant enzymes catalase, superoxide dismutase, and glutathione peroxidase levels when compared to control rats. Ince et al. (2013) reported that TQ was effective in protecting mice against imidacloprid (IMI)-induced oxidative stress by enhancing superoxide dismutase, catalase, and glutathione peroxidase levels.

In contrast, long-term administration of TAM is accompanied by toxicity and poor antioxidant defenses. However, El-Beshbishy (2005) reported that the toxicity of TAM was abolished when combined with dimethyl dimethoxy biphenyl dicarboxylate, which is a traditional Chinese medicine. The oral administration of combination therapy resulted in increasing SOD, CAT and GPX levels in female rats.

In my study, TQ did not improve the antioxidant activity of TAM+TQ group. In future studies an alteration in TQ doses will be useful to determine the effect of TQ in improving the antioxidant activities. Choosing a different assay kit might lead to different results.

Body Weight Gain

Total body weight gain over 12 weeks differed significantly among groups of animals. Treatment of animals with TAM in both TAM and TAM+TQ groups resulted in a significantly lower body weight gain when compared with other groups. TQ by itself

did not reduce body weight gain, but also did not affect TAM-induced reduction in weight gain.

Wade & Heller (1993) reported that the daily administration of TAM prevented body weight gain in rats and this was associated with a significant reduction in daily food intake. In addition, the reduction of body weight gain may be related to other effects of TAM on body composition, adipose tissue, and energy expenditure reported in rats. However, the effect of TAM on body weight in women is controversial. Data from two clinical trials indicated that at least obese women treated with TAM had lower body mass index than those not receiving the drug (Manni, et al., 2010). The results of my study showed that TAM reduced weight gain and this effect was not ameliorated by TQ.

Conclusion

The purpose of my study was to investigate the effects of TQ and TAM on tumor development, lipid peroxidation level, and total antioxidant capacity in DMBA treated female Sprague-Dawley rats. My study showed that both TQ and TAM successfully prevented the development of mammary tumors. Moreover, the oral administration of TAM for 10 weeks increased the level of lipid peroxidation. Administration of TQ along with TAM ameliorated this effect of TAM. Therefore, the combination therapy of TQ and TAM could be a safer anti-cancer drug than TAM alone. In addition, the daily administration of TAM resulted in reduction in weight gain. TQ, however, did not affect body weight gain by itself, and did not prevent the reduction in weight gain caused by TAM. Total antioxidant capacity did not differ among the groups.

My study shows for the first time that the combination of TQ and TAM successfully prevents mammary tumor development and reduces lipid peroxidation

induced by TAM. My data suggest that TQ alone or in combination with TAM have potential utility in breast cancer prevention and treatment. Clinical studies are required to evaluate the efficacy of adding TQ to TAM for treatment of breast cancer in women.

References

Ahmed, W.A., Hassan, S. A., Galeb, F.M., El-Taweel, M. A., & Abu-Bedair, F. A. (2008). The *in vitro* promising therapeutic activity of thymoquinone on hepatocellular carcinoma (HepG2) cell line. *Global Veterinaria 2*(5), 233–241.

Ahotupa, M., Hirsimaki, P., Pairssinen, R., & Mantyla, E. (1994). Alterations of drug metabolizing and antioxidant enzyme activities during tamoxifen-induced hepatocarcinogenesis in the rat. *Carcinogenesis, 15*(5), 863-868.

Ali, B. H., & Blunden, G. (2003). Pharmacological and toxicological properties of nigella sativa. *Phytotherapy Research, 17*(4), 299-305.

Arafa, E. A., Zhu, Q., Shah, Z. I., Wani, G., Barakat, B. M., Racoma, I., & Wani, A. A. (2011). Thymoquinone up-regulates PTEN expression and induces apoptosis in doxorubicin-resistant human breast cancer cells. *Mutation Research: Fundamental & Molecular Mechanisms of Mutagenesis, 706*(1), 28-35.

Barrera, G. (2012). Oxidative stress and lipid peroxidation products in cancer progression and therapy. *ISRN Oncology,*1-21. doi:10.5402/2012/137289.

Barros, A. D., Muranaka, E.K., Mori, L. J., Pelizon, C.T., Iriya, K., Giocondo, G. & Pinotti, J. A. (2004). Induction of experimental mammary carcinogenesis in rats with 7, 12-dimethylbenz(a)anthracene.*Revista Do Hospital Das Clínicas,* 59(5), 257-261.

Beeton, C., Garcia, A., & Chandy, K.G. (2007). Drawing blood from rats through the saphenous vein and by cardiac puncture. *Journal of Visualized Experiments,* 7, 266.

Betteridge, D. J. (2000). What is oxidative stress?. *Metabolism: Clinical and Experimental, 49*(2), 3-8.

Bourgou, S., Pichette, A., Marzouk, B., & Legault, J. (2010). Bioactivities of black cumin essential oil and its main terpenes from Tunisia. *South African Journal of Botany,76*(2), 210-216.

Bours, V., Dejardin, E., Goujon-Letawe, F., Merville, M. P., & Castronovo, V. (1994). The NF-kappa B transcription factor and cancer: high expression of NF-kappa B- and I kappa B-related proteins in tumor cell lines. *Biochemical Pharmacology, 47*(1), 145-9.

Butt, M. S., & Sultan, M. T. (2010). Nigella sativa: Reduces the risk of various maladies. *Critical Reviews in Food Science & Nutrition, 50*(7), 654-665.

Caballero, F., Gerez, E., Oliveri, L., Falcoff, N., Batlle, A., & Vazquez, E. (2001). On the promoting action of tamoxifen in a model of hepatocarcinogenesis induced by p-dimethylaminoazobenzene in CF1 mice. *The International Journal of Biochemistry & Cell Biology, 33*(7), 681-90.

Carlberg , K (2011). *Biology of women.* Cheney: Eastern Washington University.

Clarke, R., Liu, M. C., Bouker, K. B., Gu, Z., Lee, R. Y., Zhu, Y., Wang, Y. (2003). Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling. *Oncogene, 22*(47), 7316-7339.

Clark, S. F. (2002). The biochemistry of antioxidants revisited. *Nutrition in Clinical Practice,17* (1), 5-17.

Custodio, J., Dinis, T., Almeida, L., & Madeira, V. (1994). Tamoxifen and hydroxytamoxifen as intramembraneous inhibitors of lipid peroxidation. Evidence

for peroxyl radical scavenging activity. *Biochemical Pharmacology, 47*(11), 1989-1998.

Dauqa, M.A., Sani, H. A., & Abdullah, A. (2011). Natural antioxidants, lipid profile, lipid peroxidation, antioxidant enzymes of different vegetable oils. *Advance Journal of Food Science and Technology, 3*(4), 308-316.

De, A. S., Warri, A., Idalia, C. M., & Hilakivi-Clarke, L. (2010). Changes in mammary gland morphology and breast cancer risk in rats. *Journal of Visualized Experiments,* 44.

Dehal, S. S., & Kupfer, D. (1997). CYP2D6 catalyzes tamoxifen 4-hydroxylation in human liver. *Cancer Research, 57*(16), 3402-6.

Devasagayam, T. P. A., Boloor, K. K., & Ramasarma, T. (2003). Methods for estimating lipid peroxidation: An analysis of merits and demerits. *Indian Journal of Biochemistry and Biophysics, 40,* 300-308.

Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiological Reviews, 82*(1), 47-95.

El-Beshbishy, H. A. (2005). The effect of dimethyl dimethoxy biphenyl dicarboxylate (DDB) against tamoxifen-induced liver injury in rats: DDB use is curative or protective. *Journal of Biochemistry and Molecular Biology, 38*(3), 300-6.

El-Dakhakhny, M., Mady, N. I., & Halim, M. A. (2000). Nigella sativa L. oil protects against induced hepatotoxicity and improves serum lipid profile in rats. *Arzneimittel-forschung, 50*(9), 832-6.

Farrel G. (2002). Drugs and steatohepatitis. *Seminars in Liver Disease, 22,* 85–194.

Gadducci, A., Biglia, N., Sismondi, P., & Genazzani, A. R. (2005). Breast cancer and sex steroids: Critical review of epidemiological, experimental and clinical investigations on etiopathogenesis, chemoprevention and endocrine treatment of breast cancer. *Gynecological Endocrinology, 20*(6), 343-360.

Ghiselli, A., Serafini, M., Natella, F., & Scaccini, C. (2000). Total antioxidant capacity as a tool to assess redox status: Critical view and experimental data. *Free Radical Biology & Medicine, 29*(11), 1106-1114.

Halliwell, B. (1990). How to characterize a biological antioxidant. *Free Radical Research, 9*(1), 1-32.

Halliwell, B., & Gutteridge, J. M. C. (2007). *Free radicals in biology and medicine*. Oxford: Oxford University Press.

Haskell, S. G. (2003). Selective estrogen receptor modulators. *Southern Medical Journal, 96*(5), 469-76.

Hosseinzadeh, H., Parvardeh, S., Asl, M. N., Sadeghnia, H. R., & Ziaee, T. (2007). Effect of thymoquinone and nigella sativa seeds oil on lipid peroxidation level during global cerebral ischemia-reperfusion injury in rat hippocampus *Phytomedicine, 14*(9), 621-627.

Ince, S., Turkmen, R., Kucukkurt, I., Demirel, H. H., Zemheri, F., & Akbel, E. (2013). The role of thymoquinone as antioxidant protection on oxidative stress induced by imidacloprid in male and female Swiss albino mice. *Toxicological and Environmental Chemistry, 95*(2), 318-329.

Jafri, S. H., Glass, J., Shi, R., Zhang, S., Prince, M., & Kleiner-Hancock, H. (2010). Thymoquinone and cisplatin as a therapeutic combination in lung cancer: *In vitro* and *in vivo*. *Journal of Experimental & Clinical Cancer Research, 29*, 87-97.

Jrah-Harzallah, H., Ben-Hadj-Khalifa, S., Almawi, W. Y., Maaloul, A., Houas, Z., & Mahjoub, T. (2012). Effect of thymoquinone on 1, 2-dimethyl-hydrazine-induced oxidative stress during initiation and promotion of colon carcinogenesis. *European Journal of Cancer, 49*(5), 1127-35.

Kanter, M., Meral, I., Dede, S., Cemek, M., Ozbek, H., Uygan, I., & Gunduz, H. (2003). Effects of nigella sativa L. and urtica dioica L. on lipid peroxidation, antioxidant enzyme systems and some liver enzymes in CCl_4-treated rats. *Journal of Veterinary Medicine A, 50*(5), 264-268.

Karki, A., Mantyla, E., Hirsimaki, Y., Karlsson, S., Toikkanen, S., & Hirsimaki, P. (2000). Comparison of the effects of tamoxifen and toremifene on rat hepatocarcinogenesis. *Archives of Toxicology*, *74*(4), 249.

Klaunig, J. E., Kamendulis, L. M., & Hocevar, B. A. (2010). Oxidative stress and oxidative damage in carcinogenesis. *Toxicologic Pathology, 38*(1), 96-109.

Klaunig, J. E., Xu, Y., Isenberg, J. S., Bachowski, S., Kolaja, K. L., Jiang, J., Stevenson, D. E., Walborg, E. F. J. (1998). The role of oxidative stress in chemical carcinogenesis. *Environmental Health Perspectives, 106,* 289-95.

Legha, S. S. (1988). Tamoxifen in the treatment of breast cancer. *Annals of Internal Medicine, 109*(3), 219-28.

Li, F., Rajendran, P., & Sethi, G. (2010). Thymoquinone inhibits proliferation, induces apoptosis and chemosensitizes human multiple myeloma cells through

suppression of signal transducer and activator of transcription 3 activation pathway. *British Journal of Pharmacology, 161*(3), 541-554.

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews, 4*(8), 118-26.

Mabrouk, G. M., Moselhy, S. S., Zohny, S. F., Ali, E. M., Helal, T. E., Amin, A. A., & Khalifa, A. A. (2002). Inhibition of methylnitrosourea (MNU) induced oxidative stress and carcinogenesis by orally administered bee honey and nigella grains in Sprague Dawley rats. *Journal of Experimental & Clinical Cancer Research, 21*(3), 341-6.

Manni, A., Xu, H., Washington, S., Aliaga, C., Cooper, T., Richard, J.P., Bruggeman, R., Prokopczyk, B., Calcagnotto, A., Trushin, N., Mauger, D., Verderame, M.F., & El-Bayouny, K. (2010). The impact of fish oil on the chemopreventive efficacy of tamoxifen against development of N-methyl-N-nitrosourea-induced mammary carcinogenesis. *Cancer Prevention Research,3*, 322.

Mansour, M. A., Nagi, M. N., El-Khatib, A. S., & Al-Bekairi, A. M. (2002). Effects of thymoquinone on antioxidant enzyme activities, lipid peroxidation and DT-diaphorase in different tissues of mice: a possible mechanism of action. *Cell Biochemistry and Function, 20*(2), 143-51.

Mishra, R., Bhadauria, S., Murthy, P. K., Murthy, P.S.R. (2011) Glycine soya diet synergistically enhances the suppressive effect of tamoxifen and inhibits tamoxifen-promoted hepatocarcinogenesis in 7,12-dimethylbenz[a]anthracene induced rat mammary tumor model. *Food and Chemical Toxicology,* 49:434–440.

Muttarak, M., Siriya, B., Kongmebhol, P., Chaiwun, B., & Sukhamwang, N. (2011). Paget's disease of the breast: Clinical, imaging and pathologic findings: A review of 16 patients. *Biomedical Imaging & Intervention Journal, 7*(2), 1-7.

Mylonas, C., & Kouretas, D. (1999). Lipid peroxidation and tissue damage. *In Vivo,13*,3.

Nazarewicz, R. R., Zenebe, W. J., Parihar, A., Larson, S. K., Alidema, E., Choi, J., & Ghafourifar, P. (2007). Tamoxifen induces oxidative stress and mitochondrial apoptosis via stimulating mitochondrial nitric oxide synthase. *Cancer Research, 67*(3), 1282-90.

Office of Laboratory Animal Medicine. (2010). Oral gavage – mouse & rat. *University of Delaware.* Retrieved from http://www.udel.edu/research/pdf/Oral-Gavage-Mouse-and-Rat.pdf

Parola, M., Bellomo, G., Robino, G., Barrera, G., & Dianzani, M. U. (1999). 4-Hydroxynonenal as a biological signal: Molecular basis and pathophysiological implications. *Antioxidants & Redox Signaling, 1*(3), 255-84.

Parvez, S., Tabassum, H., Banerjee, B. D., & Raisuddin, S. (2008). Taurine prevents tamoxifen- induced mitochondrial oxidative damage in mice. *Basic & Clinical Pharmacology & Toxicology, 102*(4), 382-7.

Perumal, S. S., Shanthi, P., & Sachdanandam, P. (2005). Augmented efficacy of tamoxifen in rat breast tumorigenesis when gavaged along with riboflavin, niacin, and CoQ10: effects on lipid peroxidation and antioxidants in mitochondria. *Chemico-Biological Interactions, 152*(1), 49-58.

Quinn, G.P., & Keough, M.J.(2002). *Experimental design and data analysis for biologists*. New York, NY: Cambridge University Press.

Reuter, S., Gupta, S. C., Chaturvedi, M. M., & Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: How are they linked? *Free Radical Biology and Medicine, 49*(11), 1603-1616.

Rice-Evans, C., & Burdon, R. (1993). Free radical-lipid interactions and their pathological consequences. *Progress in Lipid Research, 32*(1), 71-110.

Richie, R. C., & Swanson, J. O. (2003). Breast cancer: a review of the literature. *Insurance Medicine, 35*(2), 85-101.

Salama, R. H. M. (2010). Clinical and therapeutic trials of nigella sativa. *TAF Preventive Medicine Bulletin, 9*(5), 513-522.

Salim, E. I., & Fukushima, S. (2003). Chemopreventive potential of volatile oil from black cumin (nigella sativa L.) seeds against rat colon carcinogenesis. *Nutrition & Cancer, 45*(2), 195-202.

Salomi, M. J., Nair, S. C., & Panikkar, K. R. (1991). Inhibitory effects of nigella sativa and saffron (Crocus sativus) on chemical carcinogenesis in mice. *Nutrition and Cancer, 16*(1), 67-72.

Sato, K. (2008). Signal transduction of fertilization in frog eggs and anti-apoptotic mechanism in human cancer cells: Common and specific functions of membrane microdomains. *The Open Biochemistry Journal, 2*, 49-59.

Sayed-Ahmed, M. M., Aleisa, A. M., Al-Rejaie, S. S., Al-Yahya, A. A., Al-Shabanah, O. A., Hafez, M. M., & Nagi, M. N. (2010). Thymoquinone attenuates diethylnitrosamine induction of hepatic carcinogenesis through antioxidant signaling. *Oxidative Medicine and Cellular Longevity, 3*(4), 254-261.

Scheibmeir, H., Christensen, K., Whitaker, S., Jegaethesan, J., Clancy, R., & Pierce, J. (2005). A review of free radicals and antioxidants for critical care nurses. *Intensive and Critical Care Nursing, 21*(1), 24-28.

Shinde, A., Ganu, J., & Naik, P. (2012). Effect of free radicals & antioxidants on oxidative stress. *Journal of Dental & Allied Sciences 1*(2), 63-66.

Singh, B., Bhat, N. K., & Bhat, H. K. (2011). Partial inhibition of estrogen-induced mammary carcinogenesis in rats by tamoxifen: Balance between oxidant stress and estrogen responsiveness. *Plos One, 6*(9), 1-12.

Siziopikou, K. P. (2013). Ductal carcinoma in situ of the breast. *Archives of Pathology & Laboratory Medicine, 137*(4), 462-466.

Soujanya, J., Silambujanaki, P., & Leela krishna, V. (2011). Anticancer efficacy of holoptelea integrifolia, planch. against 7, 12-dimethyl benz(a)anthracene induced breast carcinoma in experimental rats. *International Journal of Pharmaceutical Sciences, 3*, 103-106.

Sridar, C., Kent, U. M., Notley, L. M., Gillam, E. M., & Hollenberg, P. F. (2002). Effect of tamoxifen on the enzymatic activity of human cytochrome CYP2B6. *The Journal of Pharmacology and Experimental Therapeutics, 301*(3), 945-52.

Storz, P. (2005). Reactive oxygen species in tumor progression. *Frontiers in Bioscience,10,* 1881-96.

Tabassum, H., Parvez, S., Rehman, H., Banerjee, B. D., & Raisuddin, S. (2007). Catechin as an antioxidant in liver mitochondrial toxicity: Inhibition of tamoxifen-induced protein oxidation and lipid peroxidation. *Journal of Biochemical and Molecular Toxicology, 21*(3), 110-7.

Tabassum, H., Rehman, H., Banerjee, B. D., Raisuddin, S., & Parvez, S. (2006). Attenuation of tamoxifen-induced hepatotoxicity by taurine in mice. *Clinica Chimica Acta, 370,* 129- 136.

Vina, J., Borras, C., Gomez-Cabrera, M.C., & Orr, W.C. (2006). Part of the series: from dietary antioxidants to regulators in cellular signalling and gene expression. Role of reactive oxygen species and (phyto) oestrogens in the modulation of adaptive response to stress. *Free Radical Research, 40*(2) 111– 119.

Wade, G. N., & Heller, H. W. (1993). Tamoxifen mimics the effects of estradiol on food intake, body weight, and body composition in rats. *The American Journal of Physiology, 264*(6), 1219-23.

White, I. N. (1999). The tamoxifen dilemma. *Carcinogenesis, 20*(7), 1153-60.

Williams, G. M., Iatropoulos, M. J., Djordjevic, M. V., & Kaltenberg, O. P. (1993). The triphenylethylene drug tamoxifen is a strong liver carcinogen in the rat. *Carcinogenesis, 14*(2), 315-7.

Woo, C. C., Loo, S. Y., Gee, V., Yap, C. W., Sethi, G., Kumar, A. P., & Benny Tan, K. H. (2011). Anticancer activity of thymoquinone in breast cancer cells: Possible involvemen of PPAR-γ pathway. *Biochemical Pharmacology, 82*(5), 464-475.

VITA

Author: Nadiah Salem F. Alotaibi

Place of Birth: Khobar, Saudi Arabia

Undergraduate School Attended: King Faisal University

Degrees Awarded: Bachelor of Science in Botany & Microbiology, 2004, King Faisal University

Honors & Awards: King Abdullah scholarship to United States 2010- 2013

Professional Experience: Internship, Biology Department, 2012, Eastern Washington University, Cheney, Washington