THE EFFECT OF *CINNAMOMUM CASSIA* ON TWO BREAST CANCER CELL LINES

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ABSTRACT

The bark of the cinnamon tree (*Cinnamomum cassia*) is a popular culinary spice. It is also used in traditional medicine to maintain health and prevent disease. The antioxidant and anticancer activity of *C. cassia* was investigated using various assays. *C. cassia* bark was sequentially extracted with seven solvents of varying polarity. The acetone extract of *C. cassia*, at 30 µg/ml, protected the mouse fibroblast cell line, 3T3-L1, from DNA damage by 45 %, as estimated by the comet assay. The acetone extract had the highest total phenolic and flavonoid content. The hexane extract of *C. cassia* and the two main components, *trans*-cinnamaldehyde and coumarin, inhibited the proliferation of two breast cancer cell lines, the estrogen-sensitive MCF-7 cells (IC_{50}, 34 ± 3.52 µg/ml) and the estrogen-insensitive MDA-MB-231 cells (IC_{50}, 32.42 ± 0.37 µg/ml). The mechanism of cell death was investigated by determining the activity of the caspases. The expression of particular apoptotic genes such as Bcl2, Akt1, p53 and Bid were investigated by real time RT-PCR. The hexane extract activated initiator caspases-8 and -9 and effector caspases-3 and -7. There was up-regulation of Bid and p53 expression. Akt1 expression was down-regulated in MDA-MB-231cells but up-regulated in MCF-7 cells, indicating partial resistance to apoptosis. The activity of the antioxidant enzymes, catalase and glutathione peroxidase in both cell lines, in response to 100 µg/ml of hexane extract, decreased in a time dependent manner, whereas that of superoxide dismutase decreased in MDA-MB-231 cells but increased in MCF-7 cells, indicating that *C. cassia* bark is a good source of antioxidants. Together with its anticancer and anticarcinogenic properties, it is a good supplement for maintenance of health and prevention of cancer.

ABSTRAK

Kulit kayu manis (*Cinnamomum cassia*) adalah popular dalam rempah ratus masakan. Ia ju ga digunakan dalam perubatan tradisional untuk penjagaan kesihatan dan melindungi daripa da penyakit. Pelbagai ujian telah dijalankan untuk mengkaji aktiviti antioksidan dan anti-ka nser *C. cassia.* Kulit kayu *C. cassia* telah diekstrak secara berurutan dengan tujuh jenis pela rut yang berlainan polariti. Ujian komet menunjukkan ekstrak aseton *C. cassia*, pada 30 µg/ ml melindungi sel fibroblast tikus, 3T3-L1, daripada 45% kerosakan pada DNA. Ekstrak as eton juga menunjukkan jumlah kandungan fenolik dan flavonoid yang paling tinggi. Ujian MTT menunjukkan ekstrak heksana *C. cassia* dan dua komponen utamanya, *trans*-sinamal dehid dan coumarin, telah merencat pertumbuhan dua jenis sel kanser payu dara, iaitu MCF -7 yang peka terhadap estrogen (IC_{50}, 34 ± 3.52 µg/ml) dan MDA-MB-231 yang tidak peka terhadap estrogen (IC_{50}, 32.42 ± 0.37 µg/ml). Mekanisme kematian sel dikaji dengan meng ukur aktiviti kaspase. Ekspresi gen apoptotik tertentu dikaji dengan menggunakan RT-PC R. Ekstrak heksana telah mengaktifkan kaspase pengaktif -8 dan -9 dan kaspase efektor -3 dan -7. Terdapat naik-kawalaturan (up-regulation) pada ekspresi gen Bid dan p53. Ekspresi Akt1 menunjukkan turun- kawalaturan (down-regulation) pada sel MDA-MB-231 tetapi nai k-kawalaturan pada sel MCF-7, menunjukkan rintangan separa terhadap apoptosis. Aktiviti enzim-enzim antioksidan, katalase, dan glutation peroksida untuk kedua-dua jenis sel sebag ai respon pada 100 µg/ml ekstrak heksana, mengurang mengikut masa, manakala aktiviti su peroksida dismutase mengurang dalam sel MDA-MB-231 tetapi meningkat dalam sel MC F-7, menunjukkan yang kulit kayu *C. cassia* adalah bagus sebagai sumber antioksidan. Ber sama dengan ciri-ciri anti-kanser dan anti-karsinogeniknya, *C. cassia* adalah suplemen yang baik untuk mengekalkan kesihatan dan melindungi daripada kanser.

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LIST OF ABBREVIATIONS

Acronym	Definition
AIF	Apoptosis-Inducing Factor
AP	Alkaline Phosphate
AP_1	Activator Protein-1
Apaf-1	Apoptosis Protease Activating Factor-1
Bcl2	B-cell Lymphoma 2
BHA	Butylated Hydroxyl Anisole
BHT	Butylated Hydroxyl Toluene
Bid	BH_3 Interacting Death Domain Against Protein
BRCA	Breast Cancer Susceptibility Protein
C. cassia	*Cinnamomum cassia*
CAT	Catalase
cDNA	Complementary Deoxyribonucleic Acid
Ct	Cycle Threshold
DISC	Death Inducing Signaling Complex
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DPPH	1,1-Diphenyl-2-Picrylhydrazyl
DR	Death Receptor
ER	Estrogen Receptor
FADD	Fas Associated Death Domain
FAS L	Fas Receptor Ligand
FBS	Fetal Bovine Serum
GAE	Gallic Acid Equivalent
GPx	Glutathione Peroxidase
h	Hour
H_2DCFDA	2',7'-Dichlorodihydrofluorescein Diacetate
H_2O_2	Hydrogen Peroxide
HCL	Hydrochloric Acid
HMG	3-Hydroxy-3-Methyl-Glutaryl

Acronym	Definition
HPLC	High Performance Liquid Chromatography
IAP	Inhibitor of Apoptosis Protein
IC_{50}	Half Maximal Inhibitory Concentration
JNK	C-Jun N-terminal Kinases
MCF-7	Michigan Cancer Foundation 7
MDA	Malondialdehyde
MIC	Minimum Inhibitory Concentration
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NED	Naphthyl Ethylenediamine Dihydrochloride
NF-κB	Nuclear Factor kappa-light-chain-enhancer of Activated B Cells
OD	Optical Density
P53	Tumor Protein 53
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PKB	Protein Kinase B
Purpald	4-Amino-3-Hydrazino-5-Mercapto-1, 2, 4-Triazol
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
Smac/ DIABLO	Second Mitochondria-derived Activator of Caspase/ Direct IAP Binding Protein with Low PI
SNP	Sodium Nitroprusside
SOD	Superoxide Dismutase
TFC	Total Flavonoid Content
TNF	Tumor Necrosis Factor
TPC	Total Phenolic Content
TPTZ	2,4,6-Tripyridyl-s-Triazine
TRADD	Tumor Necrosis Factor Receptor Type 1-Associated Death Domain
TRAF	TNF Receptor Associated Factor
TRAIL	TNF-Related Apoptosis Inducing Ligand

CHAPTER I: INTRODUCTION

1.1 Research Background

1.1.1 Medicinal Plants

Medicinal plants or nature's healing herbs are believed to be very useful in healing or relieving diseases and suffering because plants can synthesize beneficial chemical compounds. These chemical compounds using to carry out vital biological functions in the plant. They can defend against attack from predators, such as insects, fungi, and herbivorous mammals. Almost 12,000 of such compounds have been found and isolated (Drews, 2000). Chemical compounds in plants produce their effects on the human body through various processes. Nowadays, many researchers are studying these processes to find out how to use these herbal medicines as drugs against diseases (Lai & Roy, 2004). This characteristic not only enables herbal medicines to be as effective as conventional medicines but also gives them the same potential to cause harmful side effects. Ethno botany (the study of traditional human uses of plants) is recognized as an effective way to discover future medicines. In 2001, researchers identified 122 compounds used in modern medicine that were derived from “ethno medical” plant sources; 80% of these have had an ethno medical use identical or related to the current use of the active elements of the plant (Fabricant & Farnsworth, 2001). Many of the pharmaceuticals currently available including aspirin, digitalis, quinine, and opium to physicians have a long history of being herbal remedies (Liu, 2004). Using herbs to treat a disease is almost universal among nonindustrialized societies. The use of and search for drugs and dietary supplements derived from plants have accelerated in recent years. Pharmacologists, microbiologists,

botanists, and natural-product chemists are combing the earth for phytochemicals and leads that could be developed for the treatment of various diseases. In fact, according to the World Health Organization, approximately 25 % of modern drugs used in the United States have been derived from plants (Lichterman, 2004). All plants produce chemical compounds during their metabolic activities. These phytochemicals fall under two clusters: (1) primary metabolites, such as sugars and fats that are found in all plants; and (2) secondary metabolites, which have more specific functions (Talalay & Talalay, 2001).

There are many laboratory methods to determine the biological activity of herbs. Usually, the first procedure used in the study of herbs is the extraction of constituents from the plants. The different kinds of extraction methods include the following (Green, 2000):

1. Herbal teas: herbs are extracted with hot water.
2. Decoctions: extraction of roots or bark of plants.
3. Alcoholic extraction of herbs; usually, the solvent includes ethanol or methanol.
4. Herbal wine: alcoholic extraction of herbs, usually with an ethanol percentage of 12 % to 38 %.
5. Extracts: extraction of herbs using solvents; the solvents are chosen according to the properties of the components inside the herbs. For instance, water, a very polar solvent, is used for the extraction of the very polar components. Oil and nonpolar solvents such as hexane can be applied for extracting nonpolar components. Some solvents, such as ethyl acetate, fall in between (Gilani *et. al.*, 2009).

1.2 Previous findings and potential of *C. cassia*

Many studies have indicated that the bark of *Cinnamomum cassia* has bioactivities such as antimicrobial (Zu *et. al.,* 2010), antioxidant (Yang *et. al.,* 2012), anticancer (Frydman-Marom *et. al.,* 2011; Koppikar *et. al.,* 2010), anti-diabetic and anti-inflammatory (O'Mahony *et. al.,* 2005).

Our preliminary investigations showed that the *C. cassia* extract inhibits the proliferation of the estrogen receptor positive breast cancer cell line (MCF-7) and the estrogen receptor negative breast cancer cell line (MDA-MB-231). Some extracts of *C. cassia* exhibited high antioxidant activity. Taken together, we believed that further investigation should be carried out to elucidate the medicinal potential of this plant

1.3 Objectives of this Study

As an expansion from our initial study on biological screening of a medicinal plant for anticancer properties, we identified *C. cassia* as a potential candidate for further investigation. The present study was conceptualized with the following objectives:

1. To determine the antioxidant content and activity of the extracts of *C. cassia.*
2. To investigate the effect of extracts of *C. cassia* on breast cancer cell lines, MCF-7 and MDA-MB-231.
3. To isolate and identify the bioactive components of the hexane extract of *C. cassia*.

Chapter II: LITERATURE REVIEW

2.1 Cancer

The word “cancer” was first brought up by the father of medicine, Hippocrates, a Greek physician. Hippocrates used the terms carcinos and carcinoma to describe non-ulcer forming and ulcer-forming tumors also called cancer, “karkinos,” meaning crab-shaped tumor and later the Roman physician, Celsus translated the Greek term into cancer. In cancer, certain body cells grow abnormally and become cancerous, change their appearance, lose their normal function, and give almost all of their energy to multiplication by cell division. The first documented case of cancer can be traced back to ancient Egypt in 1500 BC. The details recorded on papyrus documented eight cases of tumors occurring in the breast. However, the origin and cause were not discovered then. In ancient Egypt, it was believed that cancer was caused by the gods (Fishchenko *et. al.,* 1986).

In recent years, much progress has been made to understand the basic chemistry of living cells: its chemical changes and the abnormal behavior of cancers. When the growth of normal cells is lost genetically, they lead to cancer. Cancer results from DNA mutation at a molecular level, which leads to improper cell proliferation. Most of these mutations are found in somatic cells (Jemal *et. al.,* 2011). Genetic changes can occur at different levels and by different mechanisms. The gain or loss of an entire chromosome (the largest type of mutation) can occur in mitosis, which changes in the nucleotide sequence of genomic DNA (Anand *et. al.,* 2008).

Genomic augmentation occurs when many copies (20 or more) of a small chromosomal locus are added to the cell. These added parts usually include one or more oncogenes and adjacent genetic material. Another example of mutation is translocation,

which occurs when two separate parts of one specific location of chromosomal regions become abnormally combined (Bertram, 2000).

Small mutations such as point mutations, deletions, and insertions occur in the promoter region of one gene and alter its expression, or occur in the gene's coding sequence and modify the function or stability of the resulting protein. The replication of the immense volume of data within the DNA of living cells causes some mutations (Hastings *et. al.,* 2009).

There is a complex system inside cells to remove these mutations, protecting the cell against cancer. If a significant error occurs, then the cell with this mutation can be destroyed through programmed cell death (apoptosis) (Hastings *et. al.,* 2009). Sometimes, this control processes fail, which leads the mutations to remain, passed along to daughter cells. Some environments, such as the presence of disruptive substances which are types of carcinogen, repeated physical injury, heat, ionizing radiation, or hypoxia can make the errors and lead to many diseases like cancer. (Harrison & Gerstein, 2002). The errors which cause cancer are self-amplifying and compounding, for example:

1. A mutation in the error-correcting machinery of a cell might cause that cell to accumulate errors very fast.
2. A further mutation in an oncogene might cause the cell to divide more rapidly and more frequently than normal cells.
3. A further mutation causes loss of a tumor suppressor gene, disrupting the apoptosis signaling pathway and resulting in the cell becoming immortal.
4. A further mutation in the signaling system of the cell may transfer error-causing signals to nearby cells.

The transformation of normal cells into cancer is related to a chain reaction caused by initial mutation, which compounds into more intensive errors (Chenevix-Trench *et. al.*, 2002). Usually, these mutations occur in two classes of cellular genes:

1. Oncogenes. In cancer cells, these genes are often mutated or expressed at higher levels compared with normal cells. Apoptosis is a vital procedure that occurs in most cells. Activated oncogenes can cause those cells that ought to die to survive and proliferate instead.
2. Tumor suppressor genes. The mutation of these genes leads to the loss or reduction of its function, which can result in cancer cells.

Usually, mutation in suppressor genes is in combination with other genetic changes such as the following:

1. Overexpression of the gene or duplication (such as amplification) to produce increased onco-protein.
2. Activation or formation of combination genes by translocation.
3. Alteration of the gene product to produce transforming proteins (Chenevix-Trench, 1959).

According to Table 2.1(Siegel et. al., 2012), prostate cancer is the most common type of cancer, with more than 240,000 new cases expected in the United States in 2012, and pancreatic cancer is the least common, with 43,920 new cases expected in 2012. Table 2.1 gives the estimated numbers of new cases and deaths for each common cancer type.

Researchers believe that breast cancer is one of the oldest known forms of cancerous tumors in humans and it is the most common cancer in women. After skin cancer, breast cancer accounts for 16 % of all female cancers. Mortality from breast cancer is 25 % greater than lung cancer (Sariego, 2010).

Table 2.1. Statistics of cancers in the United States of America in 2012

Cancer type	Estimated new cases	Estimated deaths
Bladder	73,510	14,880
Breast	226,870 (female) and 2,190 (male)	39,510 (female) and 410 (male)
Colon and rectal (combined)	143,460	51,690
Endometrial	47,130	8,010
Kidney (renal cell) cancer	59,588	12,484
Leukemia (all types)	47,150	23,540
Lung (including bronchus)	226,160	160,340
Melanoma	76,250	9,180
Non-Hodgkin lymphoma	70,130	18,940
Pancreatic	43,920	37,390
Prostate	241,740	28,170
Thyroid	56,460	1,780

American Cancer Society (Siegel *et. al.*, 2012).

The Malaysia National Cancer Registry (NCR) reported that 21,773 Malaysians were diagnosed and registered with cancer in 2006 and 18,219 new cancer cases in 2007. It comprised 9,974 males and 11,799 females in 2006 and 8,123 males and 10,096 females in 2007. It reported that cancer prevalence is more in females than males with a ratio of 1:1.2 male to female. In 2007, the five common cancers among Malaysian children (0-14 years) were leukemia, cancers of the brain, lymphoma, cancers of the connective tissue and kidney. In the ages of 50 years and above, cancers of the lung, colon, rectum, nasopharynx, prostate, and stomach were the most common cancers among Malaysian males. While the five most common cancers in Malaysian females were breast, lung, colon, rectal, cervical, and leukemia were reported (Rampal &Yahaya, 2008).

2.1.1 Breast Cancer

Breast cancer caused 460,000 deaths in women in the world in 2008, accounting for 7 % of cancer deaths and almost 1 % of all deaths (Huo *et. al.,* 2009). It has been reported that the incidence of breast cancer is lower in less-developed countries than the more developed countries. The annual age-standardized incidence rates per 100,000 women in 12 word reigns, according to statistical reports (Lacroix, 2006) are as follows: Eastern Asia, 18; South Central Asia, 22; sub-Saharan Africa, 22; Southeastern Asia, 26; North Africa and Western Asia, 28; South and Central America, 42; Eastern Europe, 49; Southern Europe, 56; Northern Europe, 73; Oceania, 74; Western Europe, 78; and North America, 90 (Lacroix, 2006).

There is much evidence to indicate a strong relationship between breast cancer and age; it is said that 5 % of all breast cancers occur in women younger than 40 years (Goss *et. al.,* 2008). In breast cancer, apparent changes in DNA can increase the risk for developing cancer and cause the cancers that run in some families. For instance, BRCA1 and BRCA2 are tumor suppressor genes. The mutation in these two genes leads to an increased risk for breast cancer as part of a hereditary breast-ovarian cancer syndrome. Scientists have identified hundreds of mutations in the BRCA1 gene, which are associated with an increased risk of cancer. Women with an abnormal BRCA1 or BRCA2 gene have up to 80 % risk of developing breast cancer by the age of 90 years (Shaheen *et. al.,* 2011). Most mutations of DNA in breast cancer occur in single breast cells during a woman's life rather than having been inherited (Vadaparampil *et. al.,* 2012). Factors that cause breast cancer are as follows:

- Risk factors that cannot be changed: gender, age, genetic risk factor, family, history, personal history of breast cancer, race, dense breast tissue, certain

beginning breast problems, menstrual periods, earlier breast radiation, and treatment with DES (dietary stilbestrol) for lowering chances of miscarriage.

- Risk factors related to lifestyle choices: not having children, recent use of birth control pills, alcohol, being obese, and lack of exercise.
- Uncertain risk factors: high-fat diet, breast implants, pollution, tobacco, night work (Eliassen and Hankinson, 2008).

In Asia, including Malaysia, breast cancer is the commonest cancer in the two genders combined and its incidence is increasing fast (Sim *et. al.,* 2006; Parkin & Fernández, 2006). The association between breast cancer subtype and common risk factors were studied in breast cancer cases in Malaysia. The age-specific incidence of breast cancer in Malaysia is much lower than in the western world. The second report of the Malaysian National Cancer Registry in 2004 reported that 46.2 in 100,000 population was diagnosed with breast cancer in 2003 (Lim & Halimah, 2004). This is compared to 130 in 100,000 population in the United States. Despite a low incidence as compared to other countries, breast cancer is the commonest cancer amongst Malaysian women, where breast cancer made up 31 % of cancers diagnosed in women that year. Breast cancer is the most common amongst Chinese and Indian women compared to Malay women in Malaysia. Although the incidence is low, breast cancer in Malaysia could be considered as the leading cause of cancer deaths among women. It is very discouraging to know that there is a discrepancy in survival in Malaysia as compared to developed nations. The 5- year relative survival rates in the United States in 2000 approached 90 %. In Malaysia there are no national survival data. In UMMC the 5-year survival rate for patients diagnosed from 1993 to 1997 was only 58.4 % (Yip *et al.,* 2006). Racial discrepancy in the 5-year survival was seen among the

three major ethnic groups, with Malay women surviving only 46 %, Chinese women, 63 % and Indian women having a 57 % 5-year survival rate. Reasons behind this discrepancy could be due to differing screening practices, health seeking behaviour, treatment compliance and health resources available to Malaysian women (Taib *et. al.,* 2007).

2.1.1.1 Breast Cancer Cell Lines

Among all breast cancer cell lines, BT-20-1958 was the first one to have been established. Another breast cancer cell line, MCF-7, is the most studied. MCF-7 cells was established in 1973 by the Michigan Cancer Foundation (Royle, 1946). Hormone sensitivity through the expression of the estrogen receptor (ER) in MCF-7 cells makes this cell line significant. Therefore, this property makes MCF-7 cell an ideal model for studying hormone response. The histological-type, tumor-grade lymph node status and the predictive markers such as ER and, more recently, human epidermal growth factor receptor 2 (HER2) are the factors that have been used to classify breast cell lines (Feller *et. al.,* 1979).

Using DNA microarray and the immounohistochemical expression of ERα, progesterone receptor, and HER2 in breast cancer, breast cancer cell lines are classified into at least five subtypes: luminal A, luminal B, HER2, basal, and normal (Perou, *et. al.,* 2000).

2.1.1.1.1 MCF-7 cells

MCF-7 cell is a cell line that was first isolated in 1970 from the breast tissue of a 69-year-old Caucasian woman. Of the two mastectomies she received, the first revealed the removed tissue to be benign. Five years later, a second operation revealed malignant adenocarcinoma in a pleural effusion from which MCF-7 cells were extracted (Orr *et. al.,* 1955). MCF-7 is derived from breast adenocarcinoma, which retains the characteristics of

differentiated mammary epithelium, including the ability to process estradiol via cytoplasmic ERs (Ruan *et. al.*, 2008).

Radiotherapy and hormonal therapy are usually applied for treating breast cancer. In addition to their estrogen sensitivity, MCF-7 cells are also sensitive to cytokeratin and unreceptive to desmin, endothelin, GAP, and vimentin. The growth of MCF-7 is inhibited by tumor necrosis factor α (TNF-α) and anti-estrogen drugs (Levenson *et. al.*, 1997). MCF-7 cells are a good candidate for detecting mitogen-activated protein kinase and phosphoinositide 3-kinase components, and extracellular signal-regulated kinases and AKT phosphorylation are easily detectable in these cells (Soule *et. al.*, 1973; Charafe-Jauffret *et. al.*, 2006). Many reagents and plant drugs have cytotoxic effects against MCF-7. For example, *Rumput mutiara* (genus of flowering plants in the family Rubiaceae) shows a cytotoxic effect in MCF-7 cells by inducing apoptosis and caspase-8 activities (Franco-Molina *et. al.*, 2010). In another study, it has been shown that *Tinospora cripsa*, a traditional medicinal plant of India, Philippines, and Malaysia, has antiproliferative activity in MCF-7 and MDA-MB-231 cells by activating caspases-8 and -3, inducing apoptosis (Farah, 2005). *Plectranthus rotundifolius*, or *Solenostemon rotundifolius*, a perennial herbaceous plant of the mint family (Lamiaceae) native to tropical Africa, is another example of a plant drug that can inhibit the proliferation of MCF-7 cells *in vitro* by decreasing the expression of nuclear factor kappa B (NF-κB), inducing apoptosis in the cells (Nugraheni *et. al.*, 2011).

2.1.1.1.2 MDA-MB-231 cells

The MDA-MB-231 breast cancer cell line was obtained from a patient in 1973 at MD Anderson Cancer Center (Garcia *et. al.*, 1992). With epithelial-like morphology, the

MDA-MB-231 breast cancer cells appear phenotypically as spindle-shaped cells. This cell line has an invasive phenotype (Fillmore & Kuperwasser, 2008).

It has abundant activity in both the Boyden chamber chemo-invasion and the chemo-taxis assay. The MDA-MB-231 cell line is also able to grow on agarose, an indicator of transformation and tumorigenicity, and displays a relatively high colony-forming efficiency (Shibata, 2012).

Tamoxifen (TAM) is very commonly used to inhibit the proliferation of MDA-MB-231cells. The cytostatic effects of TAM have been attributed to the antagonism of the ER and the inhibition of estrogen-dependent proliferative events. TAM induces the activity of caspase-3 in ER-negative breast cancer cell lines such as MDA-MB-231cells. TAM induces the activity of caspase-3 and JNK1 pathways, which are initiated at the cell membrane by an oxidative mechanism (Chen & Thompson, 2003). Tocotrienols (α, γ, and δ) belong to the vitamin E family, indicating a potent anti-proliferative and apoptotic activity against a variety of cancer cells. In one study that investigated the effect of the tocotrienols (α, γ, and δ) against ER-positive and ER-negative cell lines, it was shown that cell proliferation and clonogenicity in both cell lines were significantly inhibited by γ- and δ-tocotrienols with little effect when the cells were similarly exposed to α-tocotrienol. However, in MDA-MB-231 cells, δ-tocotrienol was more active than α- or γ-tocotrienol (TZE-Chen *et. al.*, 2010).

The organic extracts of the root bark of *Juglans regia,* the Persian walnut, have an inhibitory effect on cell proliferation in MDA-MB-231 cells by altering the expression of Bcl-2, Bax, caspases, Tp53, Mdm-2, and TNF-α (Hasan *et. al.*, 2011).

2.2 Free Radicals and Cancer

Studies show that many types of cancers, especially breast cancer, are diet related. Recent studies prove that just with fat reduction in the daily diet, we can prevent certain

cancers (Palù *et. al.,* 1992). The role of certain bioflavonoid compounds as radical scavengers is just beginning to emerge, and the protective potential of these flavonoids is impressive (Lotito & Frei, 2006). Oxygen in free radicals has two unpaired electrons in separate orbitals in its outer shell. This electronic structure makes oxygen especially susceptible to radical formation. Their random and wild molecular movements within cellular material can create cellular damage, which can eventually result in degeneration or mutation (Friestad, 2001). A free radical can destroy proteins, enzymes, or DNA of cells. Free radicals can multiply through a chain reaction mechanism, resulting in the release of thousands of these cellular oxidants. When this occurs, cells can become so badly damaged that DNA codes can be altered and immunity can be compromised (Lomnicki *et. al.,* 2008).

Free radical damage has been associated with more than 60 known diseases and disorders, one of which is cancer. Some of the more dangerous free radical–producing substances include cigarette smoke, herbicides, high fat, pesticides, car exhaust, certain prescription drugs, diagnostic and therapeutic rays, UV light, gamma radiation, rancid foods, fats, alcohol, some of our food and water supplies, stress, and poor diet (Pacher *et. al.,* 2007).

2.3 Antioxidants

An antioxidant is a molecule with the ability of terminating the chain reactions by removing free radical intermediates that act by being oxidized themselves (Sies, 1997). Antioxidants are classified into two broad categories: the water soluble (hydrophilic) and the lipid soluble (hydrophobic). Water-soluble antioxidants react with cell cytosol and blood plasma, whereas lipid-soluble antioxidants protect cell membranes from lipid peroxidation (Nordberg & Arner, 2001). These two types of antioxidants may be

synthesized in the body or obtained from the diet (German, 1999). Antioxidants and pro-oxidants in the body are continuously balanced by dietary antioxidants and antioxidant enzymatic systems in the body. The imbalance of these systems causes chronic diseases such as cancer (Galli *et. al.,* 2012) or coronary heart disease (Ceriello & Motz, 2004).

2.3.1 Dietary antioxidants

Body fluid and tissues contain a wide range of concentrations of different antioxidants. Some antioxidants are only found in a few organisms, and these compounds can be important in pathogens and can be a virulence factor (German, 1999). Some examples of antioxidants are as follows:

- Vitamin E: α-tocopherol is found in many oils such as wheat germ, sunflower, corn, nuts, and broccoli are good sources of vitamin E. In cells, most of the vitamin E is placed in the membranes, adjacent to unsaturated fatty acids that are vulnerable to free radical attack.
- Vitamin C: ascorbic acid is in high abundance in many fruits and vegetables and also found in cereals, beef, poultry, and fish.
- Carotenoids: the pigmentations in plants and microorganisms are carotenoids. Animals cannot synthesize carotenoids. Lutein, β-cryptoxanthin, lycopene, and α- and β-carotene are the main carotenoids identified in cell plasma of human (Gutteridge & Halliwell, 1993).

Many studies showed the key role of dietary antioxidants to neutralize or trap reactive oxygen species (ROS); therefore, this nutrient acts as a cancer-preventive agent (Valko *et. al.,* 2006). Further studies show that oxidant stress increases the progression of breast cancer, and an antioxidant-rich diet reduces the risk of certain cancers (Nakabeppu

et. al., 2006). New studies show that some dietary antioxidants may have active potential in cancer therapy by their ability to induce programmed cell death (apoptosis) (Raha & Robinson, 2000). Studies in cell cultures show that vitamins E and C, selenium, and some phytochemicals induce apoptosis in cancer cells compared with normal cells (Hirst *et. al.,* 2008).

Dietary antioxidants (or non-enzymatic antioxidants) can be measured by several assays such as ferric-reducing antioxidant power (FRAP) assay (Benzie and Strain, 1996) and the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method (Oki *et. al.,* 2002). The extracted chemicals or phytochemicals of the plant as a source of dietary antioxidants have been well described (Fraga, 2007). These phytochemicals, such as, carotenoids, phenolics, alkaloids, and organosulfur compounds, have been reported to have antioxidant properties and play a role in the prevention of diseases such as cancer (Hsieh *et. al.,* 2012).

Studies have focused on the potential role of phytochemical components, such as the flavonoids, phenylpropanoids and phenolic acids, as important contributing factors to the antioxidant activity of the diet (Pietta, 2000). For instance, flavonoids are polyphenolic compounds that occur ubiquitously in plant tissues in very high concentrations it is anti-microorganism in plants (Galeotti *et. al.,* 2008). Flavonoids have antioxidant (Table 2.2), antiviral and antimicrobial activities; therefore, they should be consumed in a balanced diet.

Table 2.2. List of some important flavonoids and their antioxidant activity

Flavonoid	Antioxidant activity (TEAC, mM)
Quercetin	4.7
Rutin	2.4
Catechin	2.4
Luteolin	2.1
Taxifolin	1.9
Apigenin	1.5
Naringenin	1.5
Hesperetin	1.4
Kaempferol	1.3

(Rice-Evans *et. al.,* 1997)

2.3.2 Enzymatic Antioxidants

There is a network of antioxidant enzymes in cells that can protect cells against oxidative stress. For instance, the superoxide released by processes such as oxidative phosphorylation is first converted to hydrogen peroxide and then further reduced to give water. This detoxification pathway is the result of multiple enzymes, with superoxide dismutase (SOD) catalyzing the first step and then catalase and various peroxidases, removing hydrogen peroxide (Matés *et. al.,* 1999).

2.3.2.1 Superoxide dismutase

Superoxide dismutase is an enzyme that catalyzes the conversion of the superoxide anion oxygen and hydrogen peroxide. SOD enzymes are found in aerobic cells and extracellular fluids and include metal ion cofactors, and depending on the isozyme, can be copper, zinc, manganese, or iron (Brogstahal *et. al.,* 1996, McCord & Fridovich, 1988). In humans, the copper/zinc SOD is present in the cytosol; manganese SOD is present in the mitochondrion. There also exists a third form of SOD in extracellular fluids, which contains

copper and zinc in its active site. The mitochondrial isozyme seems to be the most biologically important of these three (Tainer *et. al.,* 1983). When the human breast cancer cell line, MCF-7 was exposed to H_2O_2, the specific activity of the catalase was elevated threefold; activities of other antioxidant enzymes, such as glutathione peroxidase and SOD, were not increased (Punnonen *et. al.,* 1994).

MnSOD activity decreased in malignant tumors (Kamarajugadda *et. al.,* 2013). The low antioxidant capacity and the oxidant-antioxidant imbalance have been shown to have a key role in multistage carcinogenesis (Rungtabnapa *et. al.,* 2011). Several *in vitro* studies showed lower MnSOD levels in cancer cells compared with normal cells (Rungtabnapa *et. al.,* 2011, 2008; Pani *et. al.,* 2010). The level of the other antioxidant enzymes is highly variable, and CuZn SOD and catalase activities are low in cancer cells (Jauniaux *et. al.,* 2000); for instance, CuZn SOD activity is higher in Wilms' tumor tissue compared with adjacent normal tissue (Gajewska *et. al.,* 1996) but lower in hepatocellular carcinoma than normal liver cells (Liaw *et. al.,* 1997).

2.3.2.2 Catalase

Catalases are enzymes that catalyze the conversion of hydrogen peroxide to water and oxygen, using either an iron or a manganese cofactor. This protein is localized in peroxisomes in most eukaryotic cells. Catalse catalyzes the following two reactions (Chelikani *et. al.,* 2004; Maehly & Chance, 1954).

$$H_2O_2 + \text{Fe (III)-Catalase} \longrightarrow H_2O + \text{O-Fe (IV)-Catalase}$$
$$H_2O_2 + \text{O=Fe (IV)-Catalase} \longrightarrow H_2O + \text{Fe (III)-Catalase}$$

It has been shown that TNF-α-mediated down-regulation of catalase in MCF-7 (a breast cancer cell line), Caco-2 and Hct-116 (epithelial colorectal adenocarcinoma cells),

results in sufficient H_2O_2 being available for appropriate functioning of the NF-κB dependent survival pathway (Lüpertz *et. al.,* 2008). O'shea et al. (1998) has shown that down-regulation of catalase and superoxide dismutase is related to the extent of lipid peroxidation (O'shea *et. al.,* 1998).

2.3.2.3 Thioredoxin and Glutathione Systems

Thioredoxin, thioredoxin reductase, and nicotinamide adenine dinucleotide phosphate (NADP), the thioredoxin system, is ubiquitous from archaea to man. Thioredoxins, with a dithiol/disulfide active site, are the major cellular protein disulfide reductases; they therefore also serve as electron donors for enzymes such as ribonucleotide reductases, thioredoxin peroxidases (peroxiredoxins), and methionine sulfoxide reductases (Jauniaux *et. al.,* 2005).

The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases, and glutathione *S*-transferases. This system is found in animals, plants, and microorganisms. Glutathione peroxidase is an enzyme containing four selenium cofactors that catalyze the breakdown of hydrogen peroxide and organic hydroperoxides. There are at least four different glutathione peroxidase isozymes in animals. Glutathione peroxidase is the most abundant and is a very efficient scavenger of hydrogen peroxide, and glutathione peroxidase 4 is most active with lipid hydroperoxides (Brigelius-Flohé, 1999). Up-regulation of glutathione peroxidase in LNCaP and PC-3 cells (prostate adenocarcinoma cells) treated with genistein was correlated with the inhibition of the proliferation of both cell types (Suzuki *et. al.,* 2000).

Punnonen et al. (1999) investigated cancerous and noncancerous tissue samples from 23 patients with breast cancer. They found that the CuZn SOD and GPx activities were higher in cancer tissues; whereas catalase activity was lower. GPx was up-regulated in

most of the malignant tumors (Tew, 1994). The production of ROS combined with a decreased antioxidant enzyme level is a significant marker for tumor cells (Toyokuni *et. al.* 1995; Oberley *et. al.*, 2005). Studies show that the malignant phenotype of a cancer cell can be suppressed by raising the MnSOD level of the cell (Oberley *et. al.* 2005), and it has been hypothesized that the MnSOD gene is a tumor suppressor (Archer *et. al.*, 2010). Anticancer drugs induce the activity of glutathione-related enzymes (GST, GPx, glutathione reductase, gamma-glutamylcysteine synthetase) and catalase (De Vries *et. al.*, 1989; Cheng *et. al.*, 1997; Hao *et. al.*, 1994).

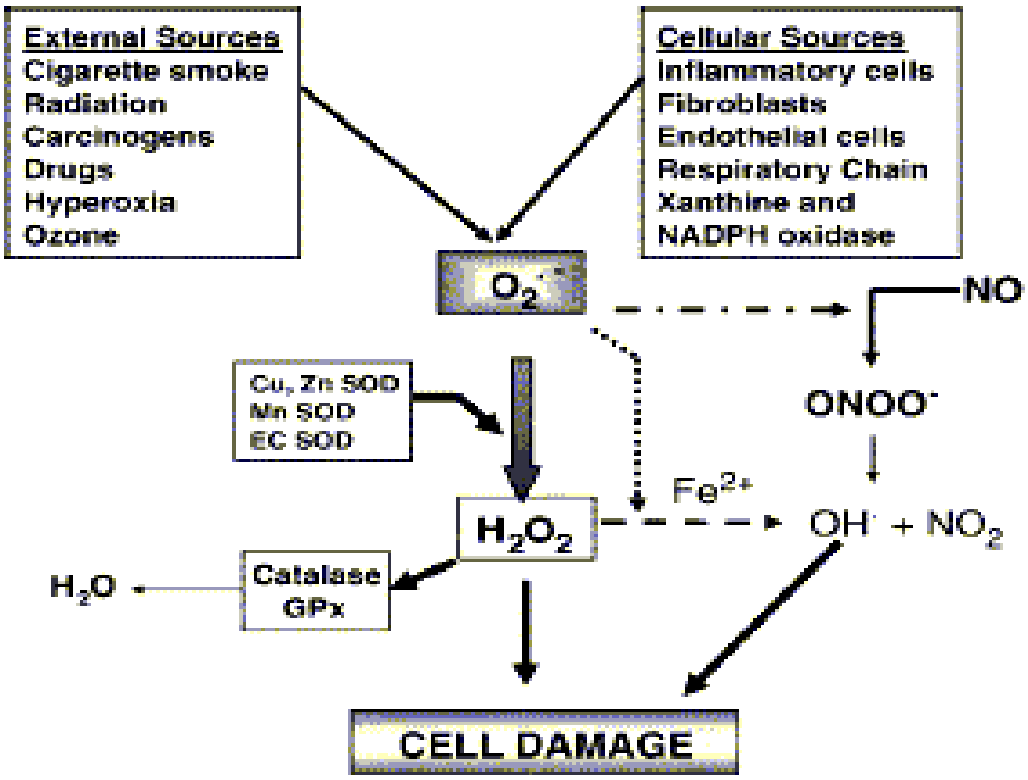


Figure 2.1. Cellular generation of reactive oxygen intermediates/species and antioxidant defences in the body
(Rahman *et. al.*, 2006).

2.4 *Cinnamomum* Species

2.4.1 Nomenclature, Taxonomy and Species

Among the spices, cinnamon is considered as an antioxidant, an anticancer, and an antimicrobial agent, and it has received considerable attention because it is widely used throughout the world as a tasty seasoning in our daily food and confectionery (Kostermans, 1986). Cinnamon is the wooden bark of an evergreen tree, *Cinnamomum aromaticum*, or *Chinese* cinnamon of the *Lauraceous* family. *Cinnamomum aromaticum* originates from southern China, Bangladesh, Uganda, India, and Vietnam (Tieu & Loeffler, 2013). The root word of *cinnamon* comes from the Greek *kinnámōmon* from Phoenician times (Janick & Jules, 2011). *Cinnamomum aromaticum* is related closely to Ceylon cinnamon (*Cinnamomum zeylanicum*). The other types of cinnamon are as follows: Saigon cinnamon (*Cinnamomum loureiroi*, also known as Vietnamese cinnamon), camphor laurel (*Cinnamomum camphora*), Malabathrum (*Cinnamomum tamala*), and Indonesian Cinnamon (*Cinnamomum burmannii*).

2.4.1.1 *Cinnamomum cassia* (*C. cassia*)

As a species, the dried bark or the powder of *cassia* is used. The flavor of *C. cassia* is stronger than Ceylon cinnamon *(Cinnamomum zeylanicum)* (Kostermans, 1986). The bark of *C. cassia* is much thicker than Ceylon cinnamon, which is because all the branches and small trees are harvested for *cassia* bark, and the small shoots are used in the production of *C. cassia* (Tracy, 1997). Ceylon cinnamon, which is produced only from the thin inner bark, has a softer, less dense, and more crumbly texture and is considered to be more aromatic and softer in flavor than *C. cassia*. *C. cassia* has more coumarin (a fragrant organic chemical compound usually found in plants) than Ceylon cinnamon (Kostermans, 1986).

There are many characteristics by which all the cinnamon species can be distinguished from one another. For instance, the bark of a *C. cassia* has many thin layers and can easily be made into powder using a spice grinder, whereas the bark of a *C. cassia* is much harder. Saigon cinnamon (*Cinnamomum loureiroi*) and *C. cassia* has thick barks. The powdered bark is harder to distinguish, but if it is treated with a tincture of iodine (a test for starch), little effect is visible with pure cinnamon, but a deep-blue color is produced with *C. cassia* (Feng *et. al.*, 2013).

C. cassia was initially grown in the southeastern province of China and Vietnam. *C. cassia* was marketed through Canton and Hong Kong. *C. cassia* trees are grown on hillsides, approximately 100 to 300 m above sea level. Peeling of the bark is performed after 6 years of growing (Braudel, 1984).

2.4.2 Flavor, Aroma and Taste

One of the components responsible for the flavor of cinnamon is an aromatic essential oil that makes up 0.5 % to 1 % of its composition (Benencia *et. al.,* 2000). This essential oil is prepared by macerating the bark of cinnamon in water followed by evaporation. One of the significant characteristics of this component is the golden-yellow color and very hot aromatic taste (Khan & Abourashed, 2011). The pungent taste of cinnamon comes from cinnamic aldehyde or cinnamaldehyde (approximately 60 % of the bark oil), which becomes darker in color and gummy because of the absorption of oxygen as it ages (Lungarini *et. al.,* 2008). Some other chemical components of the essential oil are ethyl cinnamate, eugenol (found mostly in the leaves), β-caryophyllene, linalool, and methyl chavicol (Fahlbusch *et. al.,* 2003).

2.4.3 Chemistry of Cinnamon

Cinnamon bark contains approximately 0.5 % to 1.0 % oil, which is light yellow (Chen *et. al.,* 2011). The main components of cinnamon oils are phenols (eugenol) and aromatic aldehyde (cinnamaldehyde), which can be analyzed by high-performance liquid chromatography and UV spectrophotometry (Lubbe & Verpoorte, 2011). The main constituents, besides cinnamic aldehyde and eugenol, are benzaldehyde and α-pinene, l-linalool, phellandrene, esters of isobutyric acid, cinnamyl alcohol, and cymene. Cinnamon is composed of essential oils, resinous compounds, cinnamic acid, cinnamaldehyde, and cinnamate (Api *et. al.,* 2008). The essential oil and its major constituents, such as *trans*-cinnamaldehyde, caryophyllene oxide, l-borneol, l-bornyl acetate, eugenol, β-caryophyllene, E-nerolidol, and cinnamyl acetate in cinnamon, have been reported by Tung et al. (2008). Some other constituents are terpinolene, α-terpineol, α-cubebene, and α-thujene (Jakhetia *et. al.,* 2010). It was reported that the pungent taste and scent of cinnamon comes from cinnamaldehyde (Hahm *et. al.,* 2007). The chemical structures of some important chemical constituents of cinnamon are given in Table 2.3 (Tung *et. al.,* 2008).

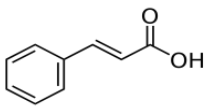
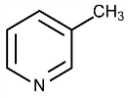
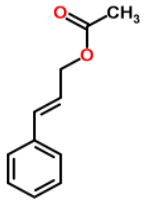
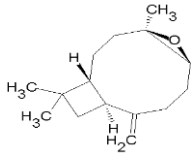
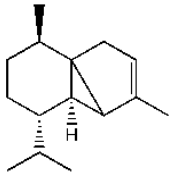
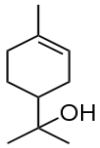
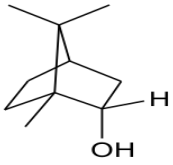
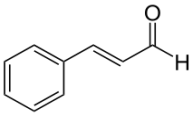
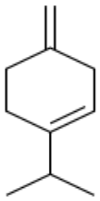
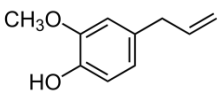
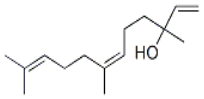
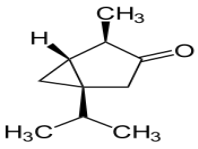
2.4.3.1 Chemistry of *Cinnamomum cassia*

The benzopyrene family consists of natural plant components present in *C. cassia,* such as coumarin. The chemical composition of different cinnamon species varies. For instance, in contrast to *C. cassia*, Ceylon cinnamon contains eugenol and benzyl-benzoate but no coumarin. *C. cassia* contains up to 1% coumarin, whereas Ceylon cinnamon contains only a trace, about 0.004 % (Jayatilaka *et. al.,* 1995; Ulbricht *et. al.,* 2011).

Gas chromatography/mass spectrometry revealed that cinnamaldehyde is the major component (85 %) in the essential oil of hydro-distilled *C. cassia* (Ooi *et. al.,* 2006; Jang *et. al.*, 2007). Oussalah et al. in 2007 compared the chemical composition of several

plants including, *C. cassia* (leaf) and *C. verum* (leaf and bark) using HPLC and GC-MS. They found that cinnamaldehyde (65 %), methoxy-cinnamaldehyde (21 %) in *C. cassia* (leaf), cinnamaldehyde (87 %) and eugenol (63 %), β-caryophyllene (5 %) in *C. verum* (bark) were the main compounds (Oussalah *et. al.*, 2007).

Table 2.3. Chemical Structures of some important constituents of cinnamon

Cinnamic acid 	β-Caryophyllene 	Cinnamyl acetate 
Caryophyllene oxide 	α-Cubebene 	α-Terpineol 
L-Borneol 	Cinnamaldehyde 	Terpinolene 
Eugenol 	E-nerolidol 	α-Thujene 

Source: **(**Oussalah *et. al.*, 2007)

2.4.4 Bioactivity of Cinnamon

Studies on cinnamon *in vitro* and *in vivo* indicates that cinnamon has multiple health benefits and bioactivities such as anti-microbial (El-Baroty *et. al.,* 2010), antioxidant (Jang *et. al.,* 2007), anti-diabetic (O'Mahony *et. al.,* 2005), anti-tumour (Koppikar *et. al.,* 2010), blood pressure-lowering (Hlebowicz *et. al.,* 2007), cholesterol (Al-Kassie, 2009) and gastro-protective properties (Hlebowicz *et. al.,* 2009).

2.4.4.1 Antioxidant Activity of Cinnamon

All the antioxidant and antimicrobial components of *Cinnamomum zeylanicum* and ginger essential oils were extracted and characterized by using TLC and GC-MS. It was found that the essential oil of cinnamon bark (CEO) was found to be a unique aromatic mono-terpene-rich natural source, with *trans*-cinnamaldehyde (45.62 %) as the major constituent that has antioxidant and antimicrobial activity (El-Baroty *et. al.,* 2010). The etheric, methanolic, and aqueous *Cinnamomum zeylanicum* extracts inhibited the oxidative process by 68 %, 95.5 %, and 87.5 %, respectively. Five fractions obtained by column chromatography exhibited antioxidant activity and presence of phenolic compounds (Mancini-Filho *et. al.,* 1998). In one study, which was performed to compare the antioxidant activity of 30 plant extracts, it was found that the aqueous extracts of oak (*Quercus robur*), pine (*Pinus maritima*), and cinnamon (*Cinnamomum zeylanicum*) possessed the highest antioxidant capacities in most of the methods used and, thus, could be potential rich sources of natural antioxidants (Dudonné *et. al.,* 2009). The methanolic extract of *Cinnamomum verum* contains many antioxidant compounds that scavenge ROS, including superoxide anions and hydroxyl radicals significantly, although *Cinnamomum verum* is weak in chelating metal ions (Mathew & Abraham, 2006).

2.4.4.1.1 Antioxidant activity of *Cinnamomum cassia*

The antioxidant activity of leaves of five species of *Cinnamomum*, namely, *C. burmanni, C. cassia, C. pauciflorum, C. tamala* and *C. zeylanicum*, has been investigated. The results indicated that *C. zeylanicum* exhibited the highest total phenolic content while *C. burmanni* had the highest flavonoid content among the five species. Also, *C. zeylanicum* showed the highest DPPH radical scavenging activity, total antioxidant activity and reducing power, while *C. tamala* exhibited the highest superoxide anion scavenging activity (Prasad *et. al.*, 2009).

In 2011, Boga et al. studied the antioxidant activities of several edible plants including, *Apium graveolens, Helianthus tuberosus, Helianthus tuberosus, Spinacia oleracea, Beta vulgaris, Portulaca oleracea, Trachystemon orientalis, Eruca sativa, Brassica oleracea, Tilia tomentosa, Cinnamomum cassia*, and *Rosa canina*. They reported that *C. cassia* showed the best antioxidant activities among the tested pants. Investigation of the antioxidant activity of various parts of *C. cassia* (bark, buds, and leaves), in ethanol and supercritical fluid extraction, showed that the ethanol extracts of cinnamon bark have the most potent antioxidant activity compared to other parts (Yang *et. al.*, 2012).

The ethanol (96.30 % purity) extracts of *C. cassia* showed a higher inhibition than α-tocopherol (93.74 % purity) on rat liver homogenate *in vitro* (From 0.05 to 1.0 mg/ml). The same extract also showed potent antioxidant activity in enzymatic and nonenzymatic assays in liver tissue. In comparison between α-tocopherol and the ethanol extract of *C. cassia*, the IC_{50} value of cinnamon extract (0.24 mg/ml) was lower, in the thiobarbituric acid assay (0.37 mg/ml) (Lin *et. al.*, 2003). Among *C. cassia*, *C. longa* and *C. rhizoma* extracts, the extract of *C. cassia* had the highest antioxidant activities, i.e., 84–90 % (DPPH), 17–33 μmol/l (FRAP), and 53–82 % (FTC) (Jang *et. al.*, 2007).

2.4.4.2 Anticancer Activity of Cinnamon

Frydman-Marom et al. (2011), applied solvent extraction to obtain components from the cinnamon bark and studied the effect on the treatment of amyloid-associated diseases and related disorders. The effect of the anticancer activity of cinnamon against colorectal cancer *in vitro* and *in vivo* in a mouse melanoma model has been investigated. The results showed that the antitumor effect of cinnamon extracts was directly linked with enhanced pro-apoptotic activity (Guimarães *et. al.*, 2010). The antineoplastic activity of cinnamon has also been shown in the cervical cancer cell line, SiHa, by inducing apoptosis in the cells (Koppikar *et. al.*, 2010). The identification of the antitumor effect of cinnamon extracts was linked with enhanced proapoptotic activity through the inhibition of the activities of NF-κB and AP1 in a mouse melanoma model, confirming the anticancer effect of cinnamon associated with the modulation of angiogenesis and effector function of CD^{8+} T cells (Kwon *et. al.*, 2010). Cinnamaldehyde and cinnamon extract strongly up-regulated cellular glutathione levels and also protected HCT116 cells against H_2O_2 genotoxicity and arsenic-induced oxidative insult in human colon cancer cells (HCT116 and HT29) and non-immortalized primary fetal colon cells (FHC) (Wondrak *et. al.*, 2010).

Cinnamon extract has the ability to interact with phosphorylation/dephosphorylation signaling activities to reduce cellular proliferation and block cell growth at the G2/M phase of the Wurzburg, Jurkat, U937 cells (Shimada *et. al.*, 2004; Schoene *et. al.*, 2005). The cell cycle of HL-60 was stopped in G1 when it was exposed to *Cinnamomum zelanicum* extract (Assadollahi *et. al.*, 2013).

2.4.4.2.1 Anticancer activity of *Cinnamomum cassia*

C. cassia also alters the growth kinetics of SiHa cells in a dose-dependent manner. The cells treated with the extract of *C. cassia* exhibited reduced migration potential, due to

the down regulation of MMP-2 expression. Cinnamon extract induced apoptosis in cervical cancer cells through the increase of intracellular calcium signaling as well as loss of mitochondrial membrane potential (Koppikar *et. al.,* 2010). The water extract of *C. cassia* bark significantly protected against glutamate-induced cell death and also inhibited glutamate-induced Ca^{2+} influx. Cinnamaldehyde (Cin), cinnamic acid (Ca), and cinnamyl alcohol (Cal), the major constituents of *C. cassia*, have been shown to possess antioxidant, anti-inflammatory, anticancer, and other activities by Ng and Wu (2009). They showed that these compounds had anti-proliferative activity and induced apoptosis through p53 activation in treated human hepatoma cells in the following order, Cin > Ca > Cal. Cin, with an IC_{50} of 9.76 ± 0.67 μM, demonstrated an anti-proliferative activity as good as 5-fluorouracil (Res, 2000). Water-soluble polymeric polyphenols from cinnamon showed anticancer activity against three myeloid cell lines (Jurkat, Wurzburg, and U937). The percentage of cell distribution in G2/M increased in all three cell lines when the cells were treated with the different concentration of cinnamon extract. At the highest concentration of cinnamon extract, the percentage of Wurzburg cells in G2/M was 1.5- and 2.0-folds higher than those observed for Jurkat and U937 cells, respectively. Induction of the kinase/phosphatase pathway in these cell lines was the cause of cell death especially in Wurzburg cells because they do not have CD45 phosphatase and may be more sensitive to imbalances in signaling through this pathway (Schoene *et. al.,* 2005). *C. cassia* diminished tumor necrosis factor (TNF)-α and prostaglandin PGE-2 in lipopolysaccharide (LPS)-activated mouse leukaemic monocyte/macrophage (RAW264.7) cells and peritoneal macrophages in a dose-dependent manner. It also blocked mRNA expression of inducible NO synthase (iNOS), cyclooxygenase (COX)-2, and TNF-α by suppressing the activation of nuclear factor (NF)-κB (Yu *et. al.,* 2012). The effect of *C. cassia* on liver carcinoma (Hep-2) cells and adenocarcinomic human alveolar basal epithelial cells (A549) revealed

that it did not have any cytotoxicity on both these cells up to a concentration of 1000 µg/ml (Yeh *et. al.,* 2013).

Cinnamon extract strongly inhibited tumor cell proliferation *in vitro* and *in vivo* studies and induced active cell death of tumor cells. It has been shown that cinnamon does not have any cytotoxic effect on normal cells by various reports, including, normal fibroblasts (Chanda *et. al.,* 2011), primary mouse lymphocytes (Kwon *et. al.,* 2010), MCF-10, commonly recognized as a "normal" breast epithelial cell line (Morre *et. al.,* 2002) and normal rat fibroblasts, F2408 cells (Unlu *et. al.,* 2010).

2.4.4.3 Antibacterial Activity of *Cinnamomum cassia*

Cinnamaldehyde (CA) in *C. cassia* revealed a potent inhibition against *Clostridium perfringens, Bacteroides fragilis,* and *Bifidobacterium bifidum* at 1 and 0.5 mg/disk. Activities of 10 essential oils toward *Propionibacterium acnes* and PC-3 (human prostate cancer cell line), A549 (adenocarcinomic human alveolar basal epithelial cells), and MCF-7 (breasr cancer cells) showed that cinnamon extract exhibited strong anticancer activity against these cancer cell lines (Zu *et. al.,* 2010). In the study of the antibacterial effect of 25 plants, including cinnamon against *Helicobacter pylori*, cinnamon was not so effective compared with the rest of the studied plants to inhibit the growth of this bacterium (O'Mahony *et. al.,* 2005). Both extracted oil and pure cinnamaldehyde of *C. cassia* were equally effective in inhibiting the growth of some studied G-positive (*Staphylococcus aureus*), G-negative bacteria (*E. coli, Enterobacter aerogenes, Proteus vulgaris, Pseudomonas aeruginosa, Vibrio cholerae, Vibrio parahaemolyticus* and *Samonella typhymurium*), and fungi including yeasts (*Candida, C. albicans, C. tropicalis, C. glabrata,* and *C. krusei*), filamentous molds (*Aspergillus spp*. and one *Fusarium sp*.) and

dermatophytes (*Microsporum gypseum, Trichophyton rubrum* and *Trichophyton mentagraphytes*). The range of the minimum inhibitory concentration (MICs) of both oil and cinnamaldehyde for bacteria, yeast, filamentous fungi and dermatophytes was 75 µg/ml to 600 µg/ml, 100 µg/ml to 450 µg/ml, 75 µg/ml to 150 µg/ml and 18.8 µg/ml to 37.5 µg/ml, respectively. The antimicrobial activity of *C. cassia* oil and its major constituents is almost equivalent, and it was shown that the broad-spectrum antibiotic activities of *C. cassia* oil are due to cinnamaldehyde (Ooi *et. al.,* 2006). Also, the essential oil of *C. cassia* showed strong antifungal effect (MIC 80 % = 0.169 µl/ml and K_{aff} = 18,544 µl/ml) against *Candida albicans* (Giordani *et. al.,* 2006).

2.4.4.4 Anti-diabetic and Anti-inflammatory Properties of *Cinnamomum cassia*

The anti-diabetic effect of *C. cassia* extract in a type 2 diabetic animal model (C57BIKsj db/db) showed that blood glucose concentration is significantly decreased in a dose-dependent manner, mostly in the 200 mg/kg treated group (n=6) for 32 weeks compared with the control (O'Mahony *et. al.,* 2005). Broadhurst et al. (2000) compared 49 herbs, spices, and medicinal plant extracts for their insulin-like or insulin-potentiating action in an *in vitro* model. The aqueous extract of cinnamon potentiated insulin activity more than 20-fold higher than any other compound tested at comparable dilutions in epididymal fat cells (Broadhurst *et. al.,* 2000). Cinnamon extracts were shown to improve insulin receptor function by activating the enzyme that causes insulin to bind to cells (insulin receptor kinase) and by inhibiting the enzyme that blocks this process (insulin receptor phosphatase), leading to the maximal phosphorylation of the insulin receptor, which is associated with increased insulin sensitivity (Wajant *et. al.,* 2003). Cinnamon has also been shown to prevent the decrease in SOD activity or suppress the increase of MDA-MB-231 cells as well (He *et. al.,* 2011). It has been shown that *C. cassia* is reach of

Procyanidin oligomers type B which are thought to be responsible for the antidiabetic activity of cinnamon (Chen *et. al.,* 2011).

C. cassia simultaneously inhibited the upstream inflammatory signaling cascades, including spleen tyrosine kinase (Syk) and Src (Yu *et. al.,* 2012), and also prevented airway epithelia from HRSV infection by inhibiting viral attachment, internalization and syncytium formation (Yeh *et. al.,* 2013).

2.5 Apoptosis

2.5.1 Apoptosis Pathways

Apoptosis or programmed cell-death is a multi-step pathway in cells of the body. In cancer, the apoptosis-to-cell-division ratio is decreased. Cancer treatment by chemotherapy and irradiation kills target cells primarily by inducing apoptosis (Thorburn *et. al.,* 2003). Apoptosis is inducible by two pathways: the extrinsic pathway and the intrinsic pathway.

2.5.2 Extrinsic Pathway

Extrinsic pathways are associated with the triggering of death receptors (DRs) on the cell surface. The binding of the ligand to the receptor leads to the triggering of DR, and the use of DR proteins can cause the auto activation of caspases via proteolytic domains in the pro–caspases (Alberts *et. al.,* 2008). Three of the known DR ligands are TNF-α, Fas, and TNF-related apoptosis, inducing ligand (TRAIL). TNF-α enhances the proliferation of chemically induced breast cancer cells (Konopleva *et. al.,* 2006). Upon binding, TNF receptor 1 (TNF-R1) forms trimeric clusters, which will recruit TNF-R-associated death domain intracellular adaptor protein (TRADD) to form the death-inducing signaling

complex (DISC). Subsequently, if TNF-associated factor 2 is recruited to the TRADD, it will lead to the activation of NF-κB and c-Jun N-terminal kinase pathway, whereas the recruitment of Fas-associated death domain protein (FADD) will lead to the recruitment and autoproteolytic cleavage of pro–caspase-8 into the active caspase-8 (Seino *et. al.*, 2001). Fas receptor ligand (FasL) binds to the TNF-R1, and FADD is recruited without TRADD. However, TRAIL ligands are regulated by five receptor subtypes, of which only TRAIL-R1 and TRAIL-R2 lead to downstream apoptosis, whereas the other three do not, because of a truncated or lack of a death domain (Schneider & Tschopp, 2000). Activation of both FasL and TRAIL leads to the activation of caspase-8. Active caspase-8 is released from the DISC and is free to cleave pro–caspase-3 to caspase-3. Alternatively, the BH3-interacting domain death agonist (Bid) proteins (from the Bcl-2 protein family) are cleaved to truncated-Bid (Bid) proteins, which induce Bax-mediated mitochondrial cytochrome c release. Both events commit the cell to apoptosis (Sengupta & Harris, 2005).

2.5.2.1 Intrinsic Pathway

The intrinsic pathway involves the mitochondria and is activated by stress events such as DNA damage (Joza *et. al.*, 2001). Mitochondria contain apoptosis-inducing factors, the second mitochondria-derived activator of caspases/direct IAP binding protein with low p*I* (Smac/DIABLO) and cytochrome c, which are all pro apoptotic. The release of these pro apoptotic proteins is facilitated by the formation of permeability transition pores. With the amplification of apoptotic signals from the DRs and the activation of caspase-8, which results in cleavage of Bid (Bcl-2 family) (Decuypere *et. al.*, 2012). The functions of these Bcl-2 family proteins are very critical in the homeostasis of apoptosis, particularly in the intrinsic pathway. Bcl-2 proteins are both pro apoptotic (Bad, Bax, and Bid) and anti-apoptotic (Bcl-2 and Bcl-XL). Bid proteins are present in the cytosol and act as biological

sensors to cellular stress and DR activation. Upon receiving apoptotic stimuli, pro-apoptotic proteins in the cytoplasm bind to the outer mitochondrial membrane, signaling it to release cytochrome c and the mitochondrial intra membrane contents (Decuypere *et. al.,* 2012). The excess accumulation of pro-apoptotic proteins on the mitochondrial membrane is believed to be the cause of the formation of these pores. The apoptotic stimuli from either the intrinsically attributed cellular stress or the extrinsically attributed DR ligands eventually lead to the release of mitochondrial cytochrome c. The released cytochrome c forms a multi-protein complex called the apoptosome, which consists of cytochrome c, apoptotic protease activating factor 1 (Apaf-1), pro–caspase-9, and ATP. The formation of the apoptosome leads to the activation of caspase-9 and eventually the caspase cascade that activates effector caspases (i.e., caspase-3 and caspase-6). These caspases are responsible for the cleavage of the key cellular proteins, such as cytoskeletal proteins, which leads to the typical morphological changes observed in cells undergoing apoptosis (Pace *et. al.,* 2010).

2.5.3 Caspase enzymes

The apoptotic procedure is regulated by a family of cysteine proteases called caspases, which are the main enzymes in cell death. Caspases are divided into two groups: initiator caspases, which have long prodomains and have the ability to autocatalyse on oligomerization, and effector caspases, which have shorter pro domains and are activated by initiator caspases or by activated caspases (Gil-Parrado *et. al.,* 2002). Caspases contribute to the proteolysis of several critical structural and regulatory proteins within the cell, such as poly-ADP-ribose polymerase, gelsolin, cytokeratins, and a DNA fragmentation factor of 45 kDa (Nuñez *et. al.,* 1998). In humans, caspases-8 and -9 are involved in initiating apoptosis by activating effector caspases such as caspase-3 through proteolytic

processing. Consecutively, it cleaves downstream targets and irreversibly commits the cell to undergo apoptosis. For example, caspase-6 is activated by caspase-3. It was demonstrated that caspase-6 is required for the apoptotic cleavage of nuclear lamin A, which is responsible for cell cycle regulation, chromatin organization, DNA replication, and cell differentiation (Takahashi *et. al.,* 1996; Kihlmark *et. al.,* 2011). Caspase-8 is an initiator caspase as its predominant function is mainly associated with DR activation and effector caspase activation (Kumar, 2007). Activated caspase-8 leads to the downstream activation of the apoptosis pathway. Caspase-8 is activated by associating itself with the FADD adaptor protein (Wang & Lenardo, 2013).

Caspase-3 is the main effector caspase in the apoptosis pathway, involved in chromatin condensation and DNA fragmentation (Porter & Jänicke, 1999). It was said that caspase-3 plays a critical role in the development of the mammalian brain (Kuida *et. al.,* 1998). Caspases-3, -6, and -7 are called executioner caspases, as they act directly on nuclear proteins and inhibit DNA repair, which eventually leads to apoptosis. The substrate specificity of caspases-3 and -7 toward the DEVD sequences of proteins is one of the commonalities between these two caspases (Walsh *et. al.*, 2008).

Caspases-3 and -7 cleave DEVD tetra-peptide at the same rate, but not LEHD (inhibitor of caspase-9); caspase-3 was shown to cleave LEHD tetra peptide at a higher rate compared with caspase-7. Caspases-3 and -7 showed differential activities toward natural substrates in cell-free extracts, but caspase-3 was active toward more cellular substrates compared with caspase-7 (Liang *et. al.,* 2001). MCF-7 cells do not express caspase-3 due to a 47–base pair deletion of the gene in the exon region, leading to an introduction of a stop codon that halts the translation of this protease (Węsierska-Gądek *et. al.,* 2011; Ja¨nicke, 2009). However, there are numerous articles that state that the caspase-3 in MCF-7 cells contributed to apoptosis signaling (Zhang *et, al.,* 2006). Interestingly, it was demonstrated

that in MCF-7 cells, caspase-3 is not necessary to develop DNA fragmentation associated with apoptosis but by caspase-7 action (Nuñez *et. al.*, 1998). Hence, although there is a lack of expression of caspase-3 in MCF-7 cells, apoptosis takes place when induced with appropriate stimuli. One feature of many diseases such as cancers, autoimmune diseases, inflammatory diseases, and viral infections is a defect in apoptosis, which decreases cell death. But sometimes, excessive cellular proliferation, which is often characterized by an overexpression of IAP family members, causes these disorders. Among these diseases, cancer is the most common. As a result, the abnormal response to apoptosis in malignant cells can lead to abnormal cycle regulating genes (such as p53, Bid, Bax, ras, or c-*myc*), which are mutated or inactivated in diseased cells, and further genes (such as bcl-2 and Akt1) also modify their expression in tumors (Liu *et. al.*, 2004). Table 2.4 illustrates the fourteen caspases and the relative synonyms.

Table 2.4. Fourteen known caspases and their synonyms

Caspase	*Synonyms*
1	IL-1 -converting enzyme (ICE)
2	IL-1β convertase (ICH-1), Neural precursor cell expressed developmentally down- regulated 2 (NEDD2)
3	Yama, 32-kDa cysteine protease (CPP32), apopain
4	ICE-II, ICH-2, transcript X (TX)
5	ICE-III, transcript Y (TY)
6	Mch2
7	ICE-LAP3, Mch3, CMH-1
8	Flip-in ICE (FLICE), MACH, Mch5
9	ICE-LAP6, Mch6
10	Mch4
11	ICH3 (expressed in murine)
12	Casp12p1
13	Evolutionary rel ICE (ERICE)
14	MICE

Reproduced from (Chowdhury *et. al.*, 2008; Rupinder *et. al.*, 2007).

2.6 Genes Involved in Breast Cancer

Apoptosis in eukaryotic cells is a critical process and depends on the expression of a specific set of genes (Raff, 1992). For instance, wild-type p53 induces apoptosis (Lotem & Sachs, 1993; Roth *et. al.*, 1996). Bcl-2 inhibits apoptosis triggered by several stimuli such as blocking γ-radiation-induced cell death (Sentman *et. al.*, 1991), antioxidant pathways that inhibit lipid peroxidation (Hockenbery *et. al.*, 1993). The Bcl-2 protein family is important in the regulation of apoptosis when the stimuli are intrinsic in nature (Liu *et. al.*, 2000). Treatment of MCF-7 cells with 17-estradiol resulted in increase in Bcl-2 mRNA levels but had no effect on Bax mRNA levels. These changes in the levels of Bcl-2 and Bax induced apoptosis in this cell line (Wang & Phang, 1995). In another study, it was shown that hormonal prevention of apoptosis, induced by treating MCF-7 cells with hydrogen peroxide, was related to Bcl-2 up-regulation (Perillo *et. al.*, 2000). Also, there was overexpression of Bcl-2 in MCF-7 cells treated with chemotherapy agents, VP-16 or taxol, making the cells 5-10-fold more sensitive to apoptosis (Sumantran *et. al.*, 1995). Sodium butyrate is a potent growth inhibitor and differentiating agent for many cell types. MCF-7 cells treated with sodium butyrate resulted in Bcl-2 over-expression and induced apoptosis in the cells (Mandal *et. al.*, 1996). Up-deregulation of Bcl-2 is associated with the enhanced survival of HER2-overexpressing and ER^+ breast cancer cells (e.g., MCF-7) treated with antiestrogens to induce apoptosis (Kumar *et. al.*, 1996). Apoptotic death in resveratrol (RES)-treated MCF-7 cells was mediated by Bcl-2 down regulation which was not related to cytochrome c release, activation of caspases 3/8 or poly (ADP-ribose) polymerase proteolysis. This reduction was related to the mitochondrial membrane potential and increased reactive oxygen species and nitric oxide production (Pozo-Guisado *et. al.*, 2005). Genistein at 0.15 mM induced apoptotic cell death in MCF-7

cells, which was related to cell cycle delay in the G2/M phase and up-regulation of Bcl-2 in this cell line while there was no effect on the level of p53 (Constantinou *et. al.,* 1998).

The genes involved in apoptosis are categorized into two groups: the anti-apoptotic genes (e.g., Bcl-2 family) and the pro-apoptotic genes (e.g., Bax, Bad, Bid, and p53). The ratio of the activity of these two categories of genes in a cell determines the fate of the cell (Toledo & Wahl, 2006). The resulting proteins of these genes exist in a localized manner in different parts of the cellular structures. Bcl-2, for example, is highly localized in the endoplasmic reticulum, mitochondrial membrane, and nuclear envelope. Bax and Bid proteins are mainly found in the cytosol. On the onset of apoptosis, the localization of some Bcl-2 proteins is altered. For example, Bax protein translocates from the cytosol to the mitochondrial membrane after treatment with an apoptotic stimulus (Zhang *et. al.,* 1997). Generally, this gene is up-regulated in MCF-7 cells, and it suppresses apoptotic events. It is usually down-regulated when apoptosis is stimulated. For example, MCF-7 cells treated with taxol showed a decrease in Bcl-2 mRNA expression (Akman *et. al.*, 1990).

Camptothecin (CPT)-induced apoptosis in MCF-7 cells is related to the activation of cathepsin B and compression of Bid and Bax in mitochondria. Bid knockdown can reduce apoptosis and induce autophagy (Lamparska-Przybysz *et. al.,* 2006). Some breast cancers which have the wild-type form of p53 protein can inactivate the tumour-suppressing activity by sequestering this protein in the cytoplasm. This mechanism could explain why some breast cancers inactivate p53 function without mutation (Moll *et. al.,* 1992). Tumour protein p53 is involved in diverse functions, particularly in suppressing the pathogenesis of tumours. It induces cell cycle arrest, apoptosis, senescence, and DNA repair. In normal cells, p53 is poorly expressed. The p53 gene encodes the tumour protein p53, a transcriptional factor that binds to the DNA and activates the expression of

downstream genes that inhibit growth and cell invasion (Angeloni *et. al.,* 2004). It has been demonstrated that MCF-7 cells treated with genistein and activated vitamin D elevated the expression of the p53 gene (Janz *et. al.,* 2002). The expression of this gene is induced by the presence of estrogen (Brekman *et. al.,* 2012). Akt expression on the anti-proliferative and apoptotic effect of tamoxifen (TAM) in MCF-7 cells showed that Akt could confer resistance to anti-estrogen-mediated cell death and inhibition of proliferation (Shin & Arteaga, 2006). Overexpression of p53 in MCF-7 cells induced down-regulation of Bcl-2, both at protein and mRNA level, leading to apoptosis (Haldar *et. al.,* 1994). High expression of mitogen-activated protein kinase phosphatase-1(MKP-1) by NO^{-2} leads to dephosphorylation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and over expression of Akt1 which is the initial factor to induce the cells to the apoptotic pathway in breast cancer cells such as MCF-7 and ZR 75-30 cells. But NO^{-2} cannot induce apoptosis through this pathway in MDA-MB-231cells (Pervin *et. al.,* 2003). Inhibiting a step downstream of caspase-8 activation and Bid cleavage activates TRAIL-induced translocation of Bax from cytosol to mitochondria, release of cytochrome c from mitochondria and activation of caspase-9 which leads to apoptosis in treated MCF-7 cells (Sarker *et. al.,* 2002). The PI3 kinase signaling components Akt1 and Akt2 are expressed at similar levels by both parental wild-type MCF-7 cells and tamoxifen-resistant MCF-7 cells. Akt1 phosphorylation is significantly increased in tamoxifen-resistant MCF-7 cells grown under basal conditions to induce apoptosis in these cells (Jordan *et. al.,* 2004).

2.7 High performance liquid chromatography and GC-MS

High performance liquid chromatography (HPLC) is widely used for analysis of chemicals and drugs. The isolation and purification of phytomolecules of therapeutic and commercial interest is very helpful in the medicinal and aromatic plant industries. Both gas

chromatography and mass spectroscopy is commonly used to identify the bioactive compounds of many plants and herbals. The combination of HPLC with these detectors has become a main way to identify and quantify fractions of interest within a complex mixture before doing biological tests on the fractions. HPLC and GC-MS have been used in many studies to identify the bioactive components in cinnamon. Water-soluble polyphenol polymers from cinnamon increase insulin-dependent *in vitro* glucose metabolism and has antioxidant activity. Nuclear magnetic resonance and mass spectroscopy identified that the polymers were composed of monomeric units with a molecular mass of 288. Two trimers with a molecular mass of 864 and a tetramer with a mass of 1152 were isolated. Their molecular masses showed they are A type doubly linked procyanidin oligomers of the catechins and/or epicatechins. These polyphenolic polymers in cinnamon can function as antioxidants, potentiate insulin action, and may be beneficial in the control of glucose intolerance and diabetes (Ross, 1976). Also, ground cinnamon improves glucose and lipid profiles of people with type 2 diabetes. The water extract of cinnamon (CE) and seven different polyphenols (CP) purified with HPLC, indicated that this polyphenol with doubly linked procyanidin type-A polymers displayed insulin-like activity (Cao *et. al.,* 2007). In 1998, Archer determined cinnamaldehyde, coumarin and cinnamyl alcohol in *C. cassia* by high-performance liquid chromatography (Archer, 1998). HPLC chromatography for the quantitation of cinnamaldehyde showed trace amounts of eugenol and piperine in pepper-contaminated cinnamon, using a combination of ultraviolet-diode array (UV) and electrochemical (EC) detectors (Kermasha *et. al.,* 1994). To identify coumarin, which is a component of natural flavorings, including *C. cassia*, HPLC is a simple and efficient method (Sproll *et. al.,* 2008).

GC–MS analysis indicated that the main compound in the volatile oils of three species and seven habitats of cinnamon is *trans*-cinnamaldehyde (66.28–81.97

%). *Cinnamomum loureirii* had the highest volatile oil yield (3.08 %) and in that yield is a high percentage of *trans*-cinnamaldehyde (81.97 %). *Cinnamomum verum* contained significant quantities of eugenol, and *C. cassia* had more abundant α-guaiene compared *with Cinnamomum loureirii* and *Cinnamomum verum* (Li *et. al.*, 2013).

CHAPTER III: MATERIALS and METHODS

3.1 Research Methods

3.1.1 Overview of Research Methods

This study was separated into several components of interest, which can be generalized into several sections. Table 3.1 illustrates the research methods applied to each section.

Table 3.1. Research methods used in this study

Research aspect	Methods	References
Isolation and identification of bioactive compounds	Extraction HPLC and GC-MS	
Growth modulation study	Cell culture and MTT assay	(Mosmann, 1983)
Cytotoxicity	MTT assay	(Mosmann, 1983)
Antioxidant enzyme activity	CAT, SOD, GPx	Cayman Chemical kit (USA)
Apoptosis	Caspase activity	Promega kit (USA)
Antioxidant activity and phytochemical content	FRAP assay, total phenol, and flavonoid content assay	(Benzie and Strain, 1996) (Singleton and Rossi, 1965) (Ozsoy, 2007)
Free radical scavenging assay	DPPH, superoxide anion, hydroxyl and nitric oxide radical scavenging assay	(Oki *et. al.*, 2002) (Nishikili *et. al.*, 1972) (Halliwell *et. al.*, 1994) (Marcocei *et. al.*, 1994)
Gene expression study	RNA extraction and real-time polymerase chain reaction	MO-BIO kit (USA) TaqMan, Applied Biosystem Life Technology kit (USA)

3.1.2 Schematic Overview of This Study

The following schematic diagram (Figure 3.1) describes the general activities assessed and the corresponding methods.

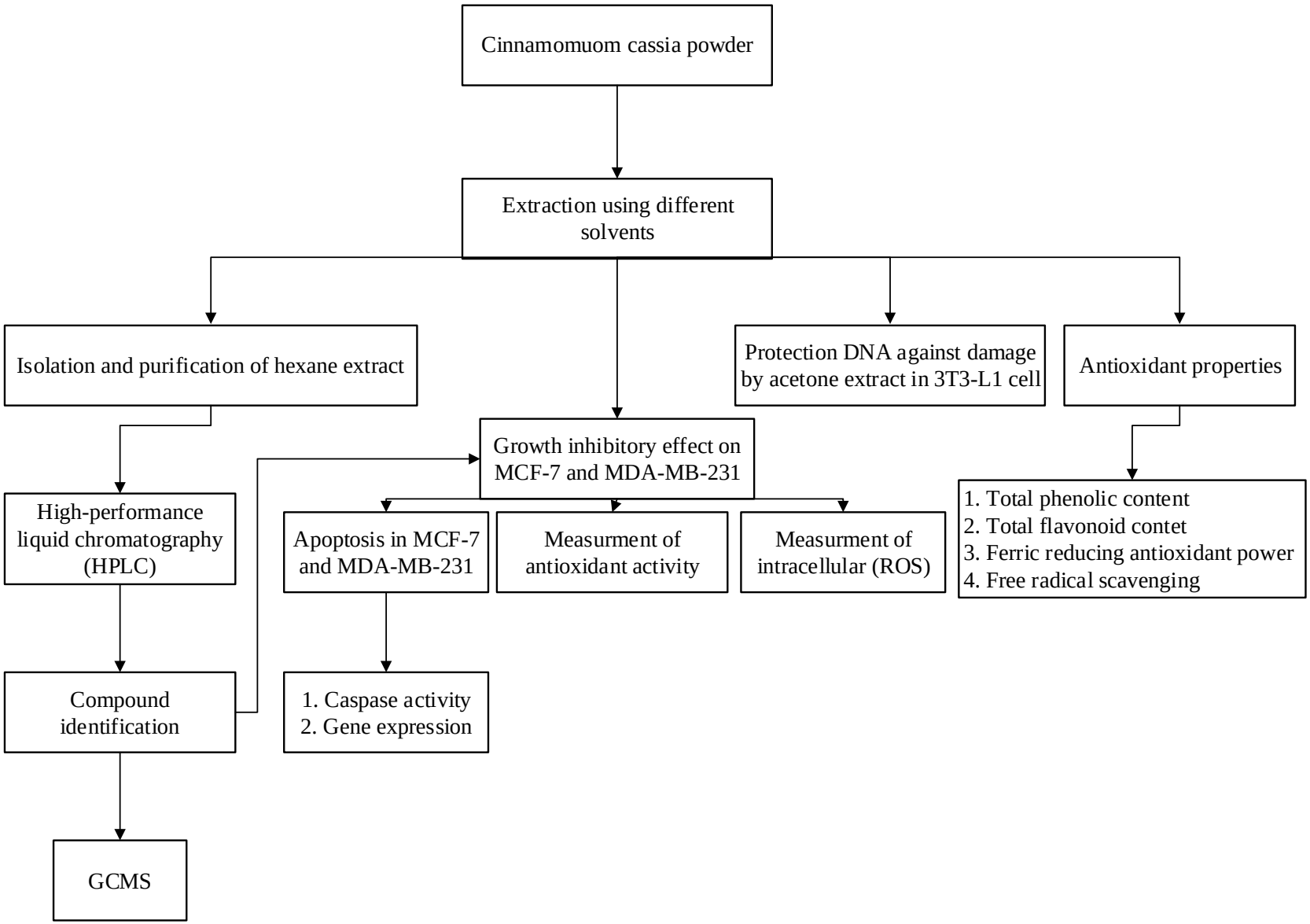


Figure 3.1. Schematic overview of this study

3.2 Materials

The materials and kits used in this study are listed in Tables 3.2 and 3.3, respectively.

Table 3.2. List of materials used in this study

Merck (Germany)	**Used in**
Acetone 99.9 % (HPLC grade)	Extraction
Ethanol 99.9 % (HPLC grade))	Extraction
Ethyl acetate 99.8 % (HPLC grade)	Extraction
Hexane 98.5 % (HPLC grade)	Extraction
Methanol 99.9 % (HPLC grade)	Extraction, DPPH assay
NaOH (1M)	TFC
Quercetin	Free radical scavenging
Rutin	Free radical scavenging
Fisher scientific	**Used in**
Chloroform 99.9 % (HPLC grade)	Extraction
Sigma Aldrich (USA)	**Used in**
1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH)	DPPH assay
2,4,6-tripyridyl-s-triazine (TPTZ)	FRAP assay
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)	MTT assay
Ascorbic acid	HO^- scavenging
Dulbecco's modified Eagle's medium (DMEM)	Cell culture
Foetal bovine serum	Cell culture
Gallic acid	TPC
Glacial acetic acid	FRAP assay
Griess reagent for nitrite	NO^- scavenging
Sodium acetate trihydrate	FRAP assay
Trypan blue dye	Cell culture
2-Deoxy-D-ribose phosphate	HO^- scavenging
2-thioharbituric acid (TBA)	HO^- scavenging

Agarose	Comet assay
Colchicine	Caspase activity assay
Dichlorodihydrofluorescein diacetate (DCFH-DA)	ROS
Ethidium bromide	Comet assay
Mitomycin C	Caspase activity assay
Na_2-EDTA	Comet assay
Nitroblue tetrazolium, phenazine methosulphate (PMS)	SO^{-2} scavenging
Phenazine methosulfate	SO^{-2} scavenging
Sodium nitrate	TFC
Sodium nitroprusside	NO^{-} scavenging
Trichloroacetic acid (TCA)	HO^{-} scavenging
Tris	Comet assay
Trolox (6-hydroxy-2, 5, 7, 8- tetramethylchroman-2-carboxylic acid)	Free radical scavenging
Trypsin-EDTA	Cell culture
RPMI-1640 media	Cell culture
Triton X-100	Comet assay
Sodium dodecyl sulphate (SDS) 1 %	Comet assay
Ferric chloride ($FeCl_3.6H_2O$)	FRAP assay
L-glutamine (200 mM)	Cell culture
Penicillin-streptomycin (10,000 U)	Cell culture
Nunc, USA	**Used in**
75 cm^2 tissue culture flasks	Cell culture
25 cm^2 tissue culture flasks	Cell culture
96- well tissue culture plates	Cell culture
12-well tissue culture plates	Cell culture
6-well tissue culture plates	Cell culture

Table 3.3. List of kits used in this study

Cayman Chemical (USA)
Catalase kit
SOD kit
GPx kit
Promega company (USA)
Caspase Glo 3/7
Caspase Glo 8
Caspase Glo 9
MOBIO (USA)
Total RNA extraction kit
TaqMan, Applied Biosystem Life Technology kit (USA)
cDNA synthesis kit
TaqMan fast advanced master mix kit

3.3 Equipment

The equipment used in this study are listed in Table 3.4.

Table 3.4. List of equipment used in this study

Inverted Microscope (Olympus CK-40, Japan)
Light Microscope (Olympus BX60, Japan)
Inverted Microscope Camera (Olympus XC10, Japan)
Microplate Reader, Model 680 (Bio-Rad Laboratories Ltd, UK)
GloMax Microplate Luminescence Reader (Promega Company, US)
Safety Cabinet (Holten Laminair, Germany)
CO2 Water-jacketed Incubator (Thermo Fisher Scientific , USA)
Autoclave, High Pressure Steam Sterilizer, SX 7 (Tomy Seiko Co.,Ltd, USA)
Centrifuge (Thermo Fisher Scientific , USA)
HPLC-MS (Agilent, USA)
HPLC-MS Column, ZORBAX Eclipse XDB-C18 (Agilent, USA)
Rotary Evaporator System (BUCHI, Switzerland)
Weighing (Sartorius, USA)
Freeze Dry Systems (Labconco, UK)
Hot Plate (Leica Biosystems, Germany)
Waterbath B-480 (BUCHI, Switzerland)
Milli-Q system, Model Purelab Ultra (ELGA LabWater, Chemopharm, UK)
Real-time PCR (StepOne Plus, Applied Biosystems)
Hot Plate Stirrer (Corning, USA)
Pipettes Discovery Comfort Single Channel 0.5–10 µl (HTL, Poland) Discovery Comfort Single Channel 10–100 µl (HTL, Poland) Discovery Comfort Single Channel 100–1000 µl (HTL, Poland) Discovery Multichannel 20–200 µl (HTL, Poland) Eppendorf Multichannel 0.5-10 µl (Eppendorf, Germany) Eppendorf Multichannel 2-20 µl (Eppendorf, Germany)

3.4 Methods

3.4.1 Extraction

Extraction using solvent is a method to sequester compounds in mixtures by exploiting differences such as polarity in the solubility of the components. For this goal, the sample must be shaken or mixed with the chosen solvents (or with two immiscible solvents) to effect the separation. The “like dissolves like” is a useful guide for selecting solvents to use in the extraction. Nonpolar substances are usually successfully extracted into nonpolar solvents such as hexane. Polar and ionic substances are extracted with polar solvents such as alcohol or water (Ban *et. al.,* 2011).

3.4.1.1 Extraction of Plant Components

Organic compounds exist in plants, which can be extracted by some organic compounds such as ethyl alcohol or ethyl acetate for polar components, but some are nonpolar and soluble in ether or hexane. The best way of solvent extraction is to use the nonpolar solvent first and extract the nonpolar compounds then use the polar solvent and extract the polar compounds (Christie, 1993). The extraction and the isolation of plant components require protocols that are essential in isolating biologically active compounds and understanding their role in disease prevention and treatment (Nascimento *et. al.,* 2000).

3.4.1.2 Extraction of Cinnamon Bark

Cinnamomum cassia bark **(**Figure 3.2(A)) was purchased from the local market (NSL Distributor, Malaysia) then was ground into a fine powder. Forty g of the powder was extracted with a volume of 200 ml of seven different solvents starting with hexane (98.5 %) as the most nonpolar and ending with water as the polar solvent. The insoluble residue from

the hexane extraction was then extracted with chloroform (99.9 %), and the extractions continued with the other solvents. The solvents used in this extraction were hexane (98.5 %), chloroform (99.9 %), ethyl acetate (99.8%), acetone (99.9 %), ethanol (99.9 %), methanol (99.9 %), and double-distilled water, which were chosen according to the gradual increase in their polarities. Each extraction was performed in triplicate at 27 °C ± 1 °C, and the mixture was stirred for 6 h and filtered by Whatman filter paper No.1, qualitative circle 110 mm. After combining the solutions in each step, they were transferred to a round-bottom flask and evaporated to dryness in a vacuum by rotary evaporator, except for the water extract which was freeze-dried. The final extract was weighted and dissolved in a minimum volume of dimethyl sulfoxide (DMSO) followed by dilution with water to make the stock solution and stored at –20 °C until further analysis.

A concentration of 1 mg/ml was used for TPC, TFC, and FRAPS assays. Each of the stock solutions was then diluted to 25, 50, 75,100, 200, and 300 µg/ml and used for the hydroxyl, DPPH, nitric oxide and superoxide anion scavenging, and MTT proliferation assays.

3.4.2 Antioxidant and Free Radical Scavenging Assays

In this study, the FRAP, DPPH, hydroxyl radical, nitric radical, superoxide anion radical scavenging assays, the total phenolic content (TPC), and the total flavonoid content (TFC) were used to evaluate the antioxidant activity of the *C. cassia* extract.

3.4.2.1 Ferric Reducing Antioxidant Power (FRAP) Assay

3.4.2.1.1 Principle

The ferric reducing antioxidant power (FRAP) assay was carried out to measure the reduction of the ferric to the ferrous state, according to the protocol by Benzie and Strain (1996). At low pH, the reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (blue color) is monitored by measuring the change in absorbance at 593 nm.

3.4.2.1.2 Procedure

A volume of 2.5 ml of 300 mmol/l acetate buffer (100 ml H_2O_2, 1.6 ml glacial acetic acid, and 0.31 g sodium acetate) was added to 1 mg/ml of the stock solutions, followed by the addition of 2.5 ml TPTZ (0.0625 g + 20 ml H_2O) to the mixtures. Standard and positive controls were $FeSO_4.7H_2O_2$, quercetin, and rutin, respectively.

3.4.2.1.3 Calculation

Calculation of the FRAP value is as follows:

FRAP value of sample (μM) = (change in absorbance of sample from 0 to 60 min/change in absorbance of standard from 0 to 60 min) × the FRAP value of the standard (1000 μM).

3.4.2.2 DPPH Radical Scavenging Activity

3.4.2.2.1 Principle

DPPH radical scavenging activity was carried out using the method described by Gerhauser et al. (2003) with some modifications. In this assay, the ability of antioxidants to reduce 1, 1-diphenyl-2-picrylhydrazyl (DPPH) is measured by a spectrophotometer.

Free radical scavenging activity is determined using 1, 1- diphenyl-2-picryl hydrazyl radical (DPPH), which is a stable free radical having a purple colour. When free radical scavengers are added, DPPH is reduced and its colour is changed to yellow, based on the efficacy of antioxidants.

3.4.2.2.2 Procedure

To each 100 µl of diluted stock solution of each extracts, 0.6 ml of DPPH (0.004 g of DPPH reagent + 100 ml methanol) was added and incubated in the dark for 20 min, and then the absorbance was measured at 517 nm. Trolox was chosen as the standard; quercetin and rutin were the positive controls. The DPPH radical scavenging activity (%) was calculated according to the following equation.

3.4.2.2.3 Calculation

The optical density (OD) at the end of the 20-min incubation was used in the calculation.

DPPH radical scavenging activity (%) = (OD blank – OD sample) / (OD blank) × 100.

3.4.2.3 Superoxide Anion Radical Scavenging Assay

3.4.2.3.1 Principle

A superoxide (hyperoxide) is a compound that contains the superoxide anion with the chemical formula O^{-2}. Superoxide anion is an important product of the one-electron reduction of dioxygen O_2, which occurs widely in nature. Molecular oxygen (dioxygen) is a diradical containing two unpaired electrons, the addition of a second electron fills one of

its two degenerate molecular orbitals leaving a charged ionic species with single unpaired electron and a net negative charge of -1. Both dioxygen and superoxide ions are free radicals which have harmful effects on biosystems (Fridovich, 1995). Superoxide anion was generated by the reaction of NADH and phenazine methosulphate (PMS) coupled to the reduction of nitro blue tetrazolium chloride. This assay was carried out according to the procedure described by Nishikili et al. (1979).

3.4.2.3.2 Procedure

A volume of 50 µl of NADH (468 µM), 50 µl of nitro blue tetrazolium (150 µM), and finally 50 µl of phenazine methosulfate (60 µM) were added to 20–200 mg/ml of the samples. All the above reagents were diluted by adding phosphate-buffered saline (pH 7.4). The mixtures were incubated for 15 min in the dark, and the absorbance was measured at 560 nm. The reference standard in this assay was trolox; quercetin and rutin were used as positive controls. The superoxide radical scavenged (%) was calculated using the following equation.

3.4.2.3.3 Calculation

The optical density (OD) at the end of the 15 min incubation period was used for the calculation:

Superoxide radical scavenged (%) = (OD blank – OD sample) / (OD blank) × 100

3.4.2.4 Hydroxyl Radical Scavenging Assay

3.4.2.4.1 Principle

The hydroxyl radical, $HO^{\cdot}$, is the neutral form of the hydroxide ion (HO^{-}). Hydroxyl radicals are highly reactive and consequently short-lived; however, they form an important part of radical chemistry. Most notably hydroxyl radicals are produced from the decomposition of hydroperoxides (ROHO) or, in atmospheric chemistry, by the reaction of excited atomic oxygen with water (Beckman *et. al.*, 1990). Hydroxyl radicals were generated by phenyl hydrazine in solution which was measured by appearance of pink colour (TBA) – MDA chromogen (due to $HO^{\cdot}$ mediated decomposition of 2- Deoxyribose). This assay was done according to the method of Halliwell et al. (1994).

3.4.2.4.2 Procedure

A volume of 200 µl of $FeCl_3$ (100 mM) was added to 200 µl of 20–200 mg/ml of the samples. Then 200 µl of 1.25 mM H_2O_2 was added to the mixtures. A volume of 200 µl of 2-deoxy-D-ribose (2.5 mM) and 200 µl of (100 mM) vitamin C were added to the mixtures. The mixtures were incubated at 37 ^{O}C for 1 h. Then 0.5 % TBA which was diluted by NaOH (0.025 M) and 2.8 % TCA were added, and the mixture was incubated at 100 ^{O}C for 30 min, followed by cooling on ice, and the absorbance was measured at 532 nm. The standard for this assay was trolox, and quercetin and rutin were chosen as the positive controls.

3.4.2.4.3 Calculation

The hydroxyl radical scavenged percentage was calculated according to the following equation, and optical density (OD) at the end of the 1.5 h incubation period was used for the calculation:

Hydroxyl radical scavenged (%) = (OD blank − OD sample) / (OD blank) X 100

3.4.2.5 Nitric Oxide Radical Scavenging Assay

3.4.2.5.1 Principle

It is well-known that nitric oxide (NO^{-}) has an important role in various types of inflammatory processes in the animal body. The scavenging NO^{-} activity may help in arresting the chain of reactions initiated by excess generation of NO^{-} that are detrimental to human health. It is a potent pleiotropic inhibitor of physiological processes, such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation, and regulation of cell-mediated toxicity (Mufti *et. al.,* 2011). Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions which were measured using the Griess reagent. The experiment was carried out according to the method described by Marcocci et al. (1994).

3.4.2.5.2 Procedure

A volume of 50 µl of sodium nitroprusside (SNP, 5 mM) was added to the 20–200 µg/ml of the samples. The mixture was incubated under visible polychromatic light (light and heat) for 1 h. A volume of 100 µl of Griess reagent (1 % sulfanilamide in 5 % H_3PO_4 + 0.1 % naphthylethylenediamine dihydrochloride, NED) was added to the mixtures and

incubated for a further 5 min before reading the absorbance at 532 nm. The standard for this assay was trolox; quercetin and rutin were chosen as the positive controls.

3.4.2.5.3 Calculation

The nitric oxide scavenged percentage was calculated in the following equation. The optical density (OD) at the end of 1 h incubation period was used for the calculation:

Nitric oxide radical scavenged (%) = (OD blank − OD sample) / (OD blank) X 100

3.4.2.6 Total Phenol Content (TPC)

3.4.2.6.1 Principle

Phenolic compounds are thought to be able to regenerate ά tocopherol in the phospholipid bilayer or lipoprotein particles back to its active oxidant form; the results are reported as gallic acid equivalent (mg GAE/ 100 g dry weight, DW). They are of particular importance in the human diet as they act as antioxidants and as antiviral agents (Hsieh *et. al.*, 2012). The method which was adapted for TPC assays of the extracts was based on spectrophotometric studies on aluminum chloride complex formation. The Folin-Ciocalteu reagent is a solution of complex polymeric ions formed from the combination of phosphomolybdic and phosphotungtic acids. The reagent oxidizes phenolates (ionized phenolics) present in the sample and reduces the acids to form a blue complex. The color develops in a slightly alkaline environment which is provided by the sodium carbonate. The blue chromogen is quantified optically at 765 nm.

3.4.2.6.2 Procedure

The total phenolic content was determined by the method described by Singleton et al. (1999). The samples used in this experiment were the stock solutions obtained after the solvent extractions. They were mixed with Folin-Ciocalteu reagent (10 ml reagent 10 % diluted with distilled water), incubated for 5 min, and then 350 ml Na_2CO_3 solution was added to the mixture and incubated for additional 2 h. The absorbance of the resulting solution was measured at 765 nm. Gallic acid was used as the standard while quercetin and rutin were chosen as the positive controls. The total phenolic content of the sample was expressed in mg GAE/g dried weight.

3.4.2.6.3 Calculation

Gallic acid was used as the standard while quercetin and rutin were chosen as the positive controls. The total phenolic content of the sample was expressed in gallic acid equivalents (GAE) per 100 g of the sample (dry weight, DW).

Total phenolic content (mg GAE/100 g sample) = (mg gallic acid/ g sample) X 100

3.4.2.7 Total Flavonoid Content (TFC)

3.4.2.7.1 Principle

Flavonoids are polyphenolic compounds that occur ubiquitously in plant tissues in relatively high concentrations. The flavonoid functions in plants are believed to be as protective agents against microorganisms (Hsieh *et. al.,* 2012).The total flavonoid content assay was carried out according to the procedure described by (Ozsoy *et. al.,* 2007). Formation of acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols in addition with aluminium chloride. Aluminum

chloride also forms acid labile complexes with the ortho - dihydroxyl groups in the A- or B-ring of flavonoids.

3.4.2.7.2 Procedure

A volume of 10 µl of sodium nitrate (5 % $NaNO_3$ solution) was added to 100 µl of each of the original stock solutions. The mixtures were incubated in the dark for 5 min. Then 10 µl of aluminum chloride (10 %) was added to the mixtures and incubated in the dark for a further 5 min. A volume of 100 µl of NaOH (1M) was added to the resulting mixtures, followed by the addition of 30 µl of distilled water.

3.4.2.7.3 Calculation

The absorbance was measured at 510 nm, and the total flavonoid content of the sample was expressed in quercetin (QE) per g of dried weight.

Total Flavonoid content (mg QE/100 g sample) = (mg gallic acid/ g sample) X 100. The OD of every assay was measured using a microplate reader.

3.4.3 The single cell gel electrophoresis assay (comet assay)

3.4.3.1 Principle

Oxidative DNA damage induced by reactive oxygen species (ROS) and free radicals is important in many human diseases, such as cancer, muscle degeneration, heart disease and ageing. Hydrogen peroxide (H_2O_2), as a potent ROS, causes DNA damage and induces apoptosis and necrosis through activation of caspase-3 in cells (How *et. al.,* 2013).The Single Cell Gel Electrophoresis assay (comet assay) is a sensitive technique for

the detection of DNA damage at the level of the individual eukaryotic cell. This technique was described by Singh et al. (1988).

3.4.3.2 Procedure

The mouse fibroblast cell line, 3T3-L1, was cultured in 25 cm^2 culture flasks. After 24 h of incubation, fresh medium containing varying concentrations of extract was added. After pretreatment for 60 min in a CO_2 incubator, the cells were exposed to 100 µM of H_2O_2 for 30 min on ice. The cells were harvested, centrifuged for 5 min at 1500 rpm and resuspended in phosphate-buffered saline (PBS).

A volume of 24 µl of cell suspension was mixed with 75 µl of 0.6 % low melting agarose. The suspension was spread on a frosted microscopic slide precoated with 0.8 % of normal melting agarose. The cell suspension was covered with a cover slip and kept on ice for 10 min. The cover slips were removed and the slides were incubated in lysis solution containing 1 % SDS, 2.5 M NaCl, 100 mM Na_2EDTA, 1% Triton X-100 and 10% DMSO for 1 h at 4 ^oC. The slides were arranged in an electrophoresis tank filled with pre-chilled electrophoretic buffer (1 mM Na_2EDTA and 300 mM NaOH) and incubated for 20 min. Electrophoresis was carried out at 25V (300 mA) for 20 min using a power supply (CBS). After electrophoresis, the slides were washed with 0.4 M Tris (pH 7.5) and stained with ethidium bromide (20 µg/ml). The slides were viewed using an Olympus BX50 fluorescence microscope (Singh *et. al.*, 1988).

3.4.3.3 Calculation

The comet tail length was measured using the eyepiece micrometer and the DNA damage was calculated as follows:

Comet tail length (μm) = (maximum total length) – (head diameter)

3.4.4 Cell Study

3.4.4.1 Cell lines

Two breast cancer lines, namely, MCF-7 and MDA-MB-231 cells, were used in this study. MCF-7 cell is an (ER^+) cell line because MCF-7cell expresses the estrogen receptor on the surface, whereas MDA-MB-231cell does not express the estrogen receptor (ER^-).

3.4.4.2 Cell Culture

3.4.4.2.1 Principle

After cells are isolated from tissue, they must be maintained in a number of different ways. Cells are grown and maintained at 37 ° C, 5 % CO_2 for mammalian cells in a cell incubator (Figure 3.5 (A)). The most commonly varied factor in culture systems is the cell culture medium. Recipes for growth media can vary in pH, glucose concentration, growth factors, and the presence of other nutrients. To avoid any type of infection, antibiotic media are added. Growth factors used for supplementing media are often derived from animal blood serum, such as fetal bovine serum. Study of the cell and must be done in a safety cabinet Figure 3.5 (B).

3.4.4.2.2 Procedure

RPMI-1640 and DMEM supplemented with 5 % FBS, 100 units/ml of penicillin-streptomycin, 2 mM L-glutamine were the growth media used to maintain MCF-7 and MDA-MB-231 cells, respectively. The supplements were filtered with 0.2 μm cellulose acetate syringe filters before adding to the cells. The cells were seeded in 25 cm^2 and 75 cm^2 flasks. Whenever 70 % confluence was achieved, they were split and transferred into

new flasks. Trypsin-EDTA was used for harvesting the cells from the flask, but sometimes a cell scraper was used. For removing the trypsin from the collected cells, the trypsin including the cells were transferred into a 15 ml centrifuge tube and centrifuged at 1,300 rpm for 5 min.

3.4.4.3 Sub- Culturing

3.4.4.3.1 Principle

The subculture provides an opportunity to expand the population of cells by selectively applying more pressure with a selective medium, and achieve higher growth fraction and allows the generation of replicate cultures for characterization, preservation by freezing, and experimentation. Briefly, subculture involves dissociation of the cells from each other and the substrate to generate a single cell suspension that can be quantified. Reseeding this cell suspension to a reduced concentration in a flask or plate generates a second crop, which can be grown and sub cultured again to give a tertiary culture.

3.4.4.3.2 Procedure

MCF-7 and MDA-MB-231 cells were washed with 1 ml PBS. Then 1-2 ml trypsin EDTA was added to the flask and incubated in the incubator for 5 min. The cells were transferred into a centrifuge tube and centrifuged at 1,300 rpm for 5 min. The supernatant was discarded and 500 µl of media (RPMI-1640 for MCF-7 cells and DMEM for MDA-MB-231 cells) and 500 µl of FBS (10 %) were added to the pellet into the centrifuged tube and transferred into new flask (25 cm^2). Then 4 ml of media was added to the flask including the cells and incubated in the incubator.

3.4.4.4 Cryopreservation

3.4.4.4.1 Principle

To maintain stocks of cells for future experiments, it is important to freeze cells in a systematic and appropriate manner.

3.4.4.4.2 Procedure

MCF- and MDA-MB-231 cells were washed with 1ml PBS and then 1-2 ml trypsin EDTA was added to the flask and incubated in the incubator for 5 min. The cells were then transferred into a centrifuge tube and centrifuged at 1,300 rpm for 5 min. The supernatant was discarded and 700 µl of media (RPMI-1640 for MCF-7 cells and DMEM for MDA-MB-231cells), 200 µl of FBS and 100 µl of DMSO were added to the pellet and transferred to a cryovial. The cells were frozen down at 4 ^{O}C, -20 ^{O}C and -80 ^{O}C for 30 min, 4 h and 24 h, respectively and stored in a liquid nitrogen tank.

3.4.4.5 Counting live cells with Trypan blue

3.4.4.5.1 Principle

Trypan blue is a diazo dye that has been widely used to color dead tissues or cells selectively. The mechanism of trypan blue staining is based on it being negatively charged and not interacting with cells unless the membrane is damaged. Indeed, undamaged cells are very selective concerning the compounds that pass through their membrane, and thus should not take up trypan blue. Therefore, all the cells that exclude the dye are considered viable. By contrast, cells with damaged membranes are stained in a distinctive blue color readily observed under a microscope. Thus, trypan blue dye is described as being a vital stain allowing discrimination between viable cells and cells with damaged membranes that are usually considered to be dead cells. A counting chamber, also known as a

hemocytometer, is a microscope slide that is specifically designed to enable cell counting. The slide has a sink in its middle; the area of the sink is marked with a grid. A drop of a cell culture is placed in the sink to looking at the sample under the microscope. Counting the number of cells in a certain area is done manually. The depth of the sink is predefined, thus the volume of the counted culture can be calculated and with it the concentration of the cells.

3.4.4.5.2 Procedure

10 µl of 1 ml of cells in media (Section 3.4.4.5.2), was added to 10 µl of trypan blue and mixed well. The cells were placed in a hemocytometer and a cover slip placed on top which was done by carefully touching the edge of the cover-slip with the pipette tip and allowing the chamber. The cells were counted in the 1mm center square and the four corner squares. This procedure was repeated using the other chamber of the hemocytometer. Each square of the hemocytometer (with cover slip in place) represents a total volume of 10^{-4} cm^3. Since 1 cm^3 is equivalent to 1 ml, the subsequent cell concentration per ml (and the total number of cells) will be determined using the following calculations.

3.4.4.5.3 Calculation

Number of cells per ml = Average number of cells in one large square x dilution factor* x 10^4

*dilution factor is usually 2 (1:1 dilution with trypan blue).

3.4.5 Antiproliferative activity using MTT Assay

3.4.5.1 Principle

This is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase to dark purple formazan. The cells are then solubilized with an organic solvent (e.g., isopropanol), and the released solubilized formazan reagent is measured spectrophotometrically. The spectrophotometric results indicate the number of the metabolically active cells because the reduction of MTT only occurs in live cells. The inhibition of cancer cell proliferation by cinnamon extracts against the MCF-7 and MDA-MB-231 cells lines was determined by the MTT assay according to the procedure described by Mosmann (1983).

3.4.5.2 Procedure

Cells were seeded into a 96-well plate at 5×10^3 cells/well density and treated with different concentrations of different extracts of cinnamon for 48 h. MTT 10% (5 mg of the powder of MTT in 20 ml of PBS) was filtered with cellulose acetate syringe filter 0.2 µm, then added to each well. After 4 h incubation, the spent media and MTT were aspirated before the addition of 100 µl of 0.1 M HCl/isopropanol to dissolve the MTT formazan. The absorbance was measured at 595 nm in a micro plate reader. The blank which was used as a negative control is the well including the seeded cells with the media.

3.4.5.3 Calculation

All the experiments were performed in triplicate. Growth inhibition of cell was calculated according to the following formula:

$$\text{Inhibition (\%)} = \frac{(\text{OD blank} - \text{OD sample})}{\text{OD blank}} \times 100\%$$

3.4.6 Reactive Oxygen Species

3.4.6.1 Principle

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. Examples include oxygen ions and peroxides. Exogenous ROS can be produced from pollutants, tobacco, smoke, drugs, xenobiotics, or radiation ROS are produced intracellularly through multiple mechanisms and depending on the cell and tissue types, the major sources being the "professional" producers of ROS, NADPH oxidase (NOX) complexes (7 distinct isoforms) in cell membranes, mitochondria, peroxisomes, endoplasmic reticulum. Intracellular reactive oxygen species (ROS) were measured based on intracellular peroxide dependence to oxide of 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to form the fluorescent compound, 2′, 7′-dichlorofluorescein (DCF) was measured, as previously described by Halliwell (1994).

3.4.6.2 Procedure

Two different breast cancer cell lines, MCF-7 and MDA-MB-231cells, were seeded into 96-well plates at a density of 5,000 cells per well and cultured for 24 h. And then various concentrations of the hexane extract of cinnamon were added to the cells (triplicate) for 12 h. After incubation (12 h), cells were washed with PBS, and then 20 μM of 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were added to the each well. The absorbance was measured at 520 nm. H_2O_2 was used as the positive control. The data were collected from the fluorescence reader, and the average was taken from the RFU (Relative Fluorescence Units) replication.

3.4.7 Antioxidant Enzyme Assays

3.4.7.1 Cell Culture for Antioxidant Enzyme Assay

Two different breast cancer cell lines, MCF-7 and MDA-MB-231 cells, were seeded into a 12-well plates containing RPMI-1640 and DMEM, respectively, supplemented with 10 % FBS at 5×10^6 cells/well, and allowed to attach for 24 h. The cells were treated with 100 µg/ml of the hexane extract (IC_{70} concentration determined from MTT assay) at varying time points (6, 9, 1, 24, and 48 h incubation),

3.4.7.2 Protein Extraction for Antioxidant Enzyme Assay

MCF-7 and MDA-MB-231 cells were collected by centrifugation (1300 X g) for 10 min at 4 °C and the pellet was sonicated with the sonicator (Figure 3.9) in 1-2 ml of cold buffer (50 mM potassium phosphate, pH 7.0, containing 1mM EDTA). The cells were centrifuged at 10.000 X g for 15 min at 4 °C again. The supernatant was used for the antioxidant enzyme assay. The protein concentration of the cellular extract was determined by Lowry assay (Lowry *et. al.,* 1951). The same concentration of the extracted total proteins was used for this assay.

3.4.7.3 Catalase Assay

3.4.7.3.1 Principle

Catalase is found in almost all living organisms which are exposed to oxygen. Catalase (CAT) catalyzes the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen; H_2O_2, as an ROS, is a toxic product of aerobic metabolism and is pathogenic. Likewise, catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each

second. Human liver, kidney, and erythrocytes have the highest level of CAT because they are involved in producing H_2O_2 (Maehly & Chance, 1954). The assay is based on the reaction of CAT with methanol in the presence of H_2O_2 producing formaldehyde, which is measured colorimetrically using 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazol (Purpald) as the chromogen. Purpald forms a bicyclic heterocycle with aldehydes which, upon oxidation, changes from colorless to a purple color.

3.4.7.3.2 Procedure

The assay was conducted according to the instruction provided with the commercially purchased kit from Cayman Company (item no. 707002). This assay was performed according to the protocol in the kit. The procedure was done as follows and all the named reagents have been provided.

100 µl of diluted assay buffer, 30 µl of methanol, and 20 µl of the sample (described in Section 3.4.7.2) were mixed together. The reaction was initiated by adding 20 µl of diluted hydrogen peroxide to all the wells being used. The plate was covered with the plate cover and incubated on a shaker for 20 min at room temperature and then, 30 µl of diluted potassium hydroxide and 30 µl of catalase purpalad (Chromogen) were added to each well to terminate the reaction. The well was covered with the plate cover and incubated for 10 min at room temperature on a shaker. A volume of 10 µl of potassium periodate was added to each well and incubated for 5 min at room temperature on a shaker. The absorbance was read up at 540 nm with microplate reader.

3.4.7.3.3 Calculation

CAT activity in each sample was expressed in nmol/min/ml using the following equation:

CAT activity = (µM of sample/20 min) X sample dilution

One unit is defined as the amount of enzyme that caused the formation of 1.0 nmol of formaldehyde per min at 25 °C.

3.4.7.4 Superoxide Dismutase

3.4.7.4.1 Principle

Hydrogen peroxide is converted into oxygen and water in organisms by superoxide dismutases (SOD). Thus, it is important antioxidant defence in nearly all cells exposed to oxygen.

$2O^{-} + 2\ H^{+} + SOD \longrightarrow H_2O_2 + O_2$

Cayman's SOD kit assay utilizes tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine (Deng *et. al.*, 1993).

3.4.7.4.2 Procedure

The assay was conducted according to the instructions provided with the commercially purchased kit from Cayman Company (item no. 706002). The guideline to doing this assay is available online in the Cayman website, www.caymanchem.com. The procedure was done as follows and all the named reagents were provided in the kit.

A volume of 200 µl of the diluted radical detector and 10 µl of the samples (described in Section 3.4.7.2) were mixed together in the 96-well plate. The reaction was initiated by adding 20 µl of diluted xanthine oxidase to all the wells. The 96-well plate was

shaken for a few seconds to mix and covered with the plate cover, then it was incubated on a shaker for 20 min at room temperature. The absorbance was read up at 450 nm using a microplate reader.

3.4.7.4.3 Calculation

SOD activity was calculated using the following formula:

SOD activity (U/ml) = {[(sample LR-y-intercept) / Slop] X (0.23ml/0.01 ml)}

One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radicals.

3.4.7.5 Glutathione Peroxidase

3.4.7.5.1 Principle

Glutathione peroxidase (GPx) is the general name of enzyme groups which have peroxidase activity and protect the organism from oxidative damage. Glutathione peroxidase catalyzes H_2O_2 to H_2O, with oxidation of monomeric glutathione to glutathione disulfide. The mechanism involves oxidation of the selenol of a selenocysteine residue by hydrogen peroxide. This process gives a derivative with a seleninic acid (RSeOH) group. The selenic acid is then converted back to selenol by a two-step process that begins with reaction with GSH to form the GS-SeR and water. A second GSH molecule reduces the GS-SeR intermediate back to the selenol, releasing GS-SG as the by-product. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease in the 340 nm is directly proportional to the GPx activity in the sample (Ran *et. al.*, 2007; Epp *et. al.*, 1983).

3.4.7.5.2 Procedure

The GPx activity was measured through a coupled reaction with glutathione reductase. The assay was conducted according to the instruction provided with the commercially purchased kit from Cayman Company (item no. 703102). The guideline to doing this assay is available online in the Cayman website, www.caymanchem.com. The procedure was done as follows and all the named reagents were provided in the kit.

A volume of 100 µl of assay buffer, 50 µl of co-substrate mixture, and 20 µl of the samples (described in Section 3.4.7.2) were mixed together in the 96-wells. The reaction was initiated by adding 20 µl of cumene hydroperoxide to all the wells being used and shaken for a few second to mix. The absorbance was read once every min at 34 nm using a microplate reader for at least 5 time points.

3.4.7.5.3 Calculation

GPx activity was calculated using two formula:

$$A_{340}/\text{min} = [A_{340}\,(\text{time 2}) - A_{340}\,(\text{time 1})] / [\text{time 2 (min)} - \text{time 1 (min)}]$$

Time 1 = absorbance at 0 min, time 2 = absorbance at 5 min, and A_{340} = change in absorbance per min obtained from the standard curve, so:

The activity of GPx (nmol/min/ml) = $A_{340}\,\text{min}^{-1}$ / 0.00373 μM^{-1}) X (0.19 ml / 0.02 ml) X sample dilution.

3.4.8 Caspase Activity

Many proteins and enzymes are involved in apoptosis. Caspases are protease enzymes synthesized as zymogens and are involved in initiation and execution of apoptosis once activated by proteolytic cleavage. Mammalian caspases are divided in three main groups by function:

- Cytokine activation includes caspases-1, -4, -5, -13
- Apoptosis initiation includes caspases-2, -8, -9, -10
- Apoptosis execution utilizes caspases-3, -6, -7

Caspase assays are based on the measurement of zymogen processing to an active enzyme.

3.4.8.1 Cell Culture for Caspase Activity Assay

Two different breast cancer cell lines, MCF-7 and MDA-MB-231 cells, were seeded into a 96-well plate at 25×10^3 cells. The IC_{50} of the hexane extraction was used for treating the cell. Colchicine was used as the positive control for caspase-3 and mitomycin C was used as the positive control for caspases-8 and -9. The luminescence caspase-Glo assays (8, 9, and 3/7) were optimized by various incubation times (2, 8, 16, 24, and 48 h) to assay the maximum caspase activity.

3.4.8.2 Caspase-3 and -7 Activity

3.4.8.2.1 Principle

Among all caspase proteins, caspase-3 and caspase-7 have almost undetectable activity toward defined synthetic peptide substrates (Pace *et. al.,* 2010). Activation of caspase-3 and caspase-7 is strongly used as a biomarker for evaluation of apoptosis. For detection of caspase-3, the specific substrate of this enzyme, N-Ac-DEVD-N'-MC-R110, is

cleaved by caspase-3 site and generates a highly fluorescent product, which is measured using excitation and emission wavelengths of 485 and 535 nm are used, respectively (Wang & Lenardo, 2000).

In Promega-Glo caspases-3 and -7 activity assay, caspases-3 and -7 cleavage of the luminogenic substrate containing the DEVD sequence, following caspase cleavage, a substrate for luciferase (amino luciferin) is released, resulting in the luciferase reaction and the production of light.

3.4.8.2.2 Procedure

The assay was conducted according to the instructions provided with the fluorometric purchased kit from Promega (item no. TB323).The guideline of this assay is available online in the Promega website, www.promega.com. The 96-well plates used in this assay was white brand SPL Korea (purchased from Next Gene Scientific Sdn Bhd). The plate was read with a Promega luminometer. The procedure was done as follows and all the named reagents were provided in the kit.

Caspase-Glo buffer 3/7 was mixed with the Caspase-Glo substrate to make the Caspase-Glo reagent. Then an equal volume of reagent was added to the samples (described in Section 3.4.8.1), mixed and incubated for 3 h. The luminescence was read using a luminometer.

3.4.8.3 Caspase-8 Activity

3.4.8.3.1 Principle

Apoptosis in mammalian cells is started by activation of proteases/caspases; caspase-8 is one of them. The assay is based on detection of cleavage of IETD to AFC (7-amino-4-trifluoromethyl coumarin). IETD-AFC emits blue light (λmax = 400 nm); upon

cleavage of the substrate by the related caspases, free AFC emits a yellow-green fluorescence (λmax = 505 nm), which can be quantified using a fluorescence microliter plate reader. In Promega-Glo caspase-8 activity assays, caspase-8 cleaves to the luminogenic substrate containing the LETD sequence, following caspase cleavage, a substrate for luciferase (amino luciferin) is released, resulting to luciferase reaction and the production of light.

3.4.8.3.2 Procedure

The assay was conducted according to the instruction provided with the fluorometric purchased kit from Promega (item no. TB332). The guideline to doing this assay is available online in the Cayman website, www.promega.com. The 96-well plates used in this assay was white brand SPL Korea (purchased from Next Gene Scientific Sdn Bhd). The plate was read with Promega luminometer. The procedure was done as follows and all the named reagent were provided in the kit.

Caspase-Glo buffer 8 was mixed with the Caspase-Glo substrate to make the Caspase-Glo reagent. Then an equal volume of reagent was added to the samples (described in Section 3.4.8.1) incubated for 3 h. The luminescence was read using a luminometer.

3.4.8.4 Caspase-9 Activity

3.4.8.4.1 Principle

Caspase-9 is a member of the peptidase family C14 that contains a caspase activation and recruitment domains (CARDs) domain. The pro–caspase of caspase-9 is present in the cytosol and, after activation, translocates to the mitochondria. Caspase-9 is involved in the caspase activation cascade responsible for apoptosis execution and cleaves/activates caspase-3 and caspase-6 (Kihlmark *et. al.*, 2011; Kumar, 2007). In

Promega-Glo caspase-9 activity assays, caspase-9 cleavage to the luminogenic substrate containing the LEHD sequence, following caspase cleavage, a substrate for luciferase (amino luciferin) is released; resulting is the luciferase reaction and the production of light.

3.4.8.4.2 Procedure

The assay was conducted according to the instructions provided with the fluorometric kit from Promega (item no. TB333). The plate was read with Promega luminometer. The procedure was done as follows and all the named reagents were provided in the kit.

Caspase-Glo buffer 9 was mixed with the Caspase-Glo substrate to make the Caspase-Glo reagent. Then an equal volume of reagent was added to the samples (described in Section 3.4.8.1) and mixed and incubated for 3 h. The luminescence was read using a luminometer.

3.4.9 Determination of Gene Expression

TaqMan probes are hydrolysis probes that are designed to increase the specificity of real-time reverse-transcription PCR (real-time RT-PCR) or quantitative-PCR. The TaqMan probe principle relies on the 5′–3′ exonuclease activity of Taq polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence and fluorophore-based detection. In this study the expression of some apoptotic genes were studied using TaqMan probe-based assays in real-time reverse-transcription PCR (real-time RT-PCR).

3.4.9.1 Cell Culture for gene expression assessment

MCF-7 and MDA-MB-231cells were grown in 25 cm^2 flasks. The 5 x 10^6 cells were seeded in 5 ml of growth media. Cells were allowed to attach overnight before the treatment with the extract was initiated. The cells were treated with the IC_{50} concentration of the hexane extract of cinnamon for 24 h before the total RNA was extracted.

3.4.9.2 RNA Extraction

3.4.9.2.1 Principle

RNA extraction procedure is complicated by the ubiquitous presence of ribonucleases in cells and tissues, which can rapidly degrade RNA.

Chaotropic agents, such as guanidium isothiocyanate, are used to protect RNA from endogenous RNases. The sample is then added into a spin column which binds nucleic acids. The column is washed and purified RNA material is eluted with water or buffers.

3.4.9.2.2 Procedure

The total RNA was extracted according to the protocol of Ultra Clean Tissue and Cells RNA Isolation Kit (catalog no. 15000-S (www.mobio.com)).

Kit contents are: Solution TR1, Solution TR2, Solution TR3, Solution TR4, Solution TR5, spin filter and RNase collection tubes.

The matrix of the tissue was dissolved into single cells that were lysed in solution TR1 releasing the RNA. Genomic DNA was sheared into small sizes to enhance removal from the spin filter (Homogenization steps in Solution TR1). Then 1 volume of solution TR2 was added to the lysate and mixed by pipetting (Solution TR2 is 70 % ethanol and prepares optimal binding conditions for RNA capture on the Spin Filter membrane). A

volume of 600 µl of lysate was transferred into the spin filter, centrifuged for 1 min at 10,000 x g. The flow-through was discarded and the spin filter placed back into the 2 ml collection tube. The RNA was bound to the spin filter by passing it through the membrane. The spin filter was then washed with 500 µl of solution TR3 and centrifuged for 1 min at 10,000 x g. The spin filter was transferred to a new 2 ml collection tube (Solution TR3 is a wash buffer that removes protein from the column).In this step, DNA was lysed according to the DNase kit. The procedure has been done according to the protocol of QIAGEN RNase-free DNase set, catalog no. 79254.

The On-Spin Column DNase I kit removed any remaining genomic DNA from the spin filter membrane that did not wash off in Solution TR3. Then the spin filter was washed with 500 µl of solution TR4 and centrifuged for 1 min at 10,000 x g. The flow-through was discarded and the Spin Filter was placed back into the same 2 ml collection tube. Step 5 was repeated (Solution TR4 contains ethanol and removes the salts from the Solution TR3). The spin filter was centrifuged in an empty 2 ml collection tube for 2 min at 13,000 x g and transferred to a new 2 ml collection tube (this dries the membrane completely so that the RNA can be released during the elution step with RNase-Free water (Solution TR5). To elute the RNA, 50-100 µl of solution TR5 was added directly into the spin filter membrane, incubated for 1 min at room temperature and centrifuged for 1 min at 10,000 x g. The total RNA were now extracted and stored at -20 ^{o}C.

3.4.9.2.3 RNA quantity and purity control

The concentration (ng/ml), 260/280 nm ratio and 260/230 nm ratio (quantity and purity) of each extracted RNA were measured using a Nanodrop spectrophotometer (Thermo scientific, USA) as shown in Figure 3.10. The 260/280 nm ratio is a good

indicator of RNA quality as the absorbance at 260 nm will increase as RNA is digested into smaller fragments and single nucleotides. The ratio for pure RNA should be 1.9–2.1. A 260/280 reading below 1.6 may have significant protein contamination.

3.4.9.2.4 RNA quality control

The pure isolated RNA may be contaminated by DNA, protein or phenol that could inhibit further experiments using the RNA. Therefore, evaluation of RNA integrity and quality is necessary. Agarose gel electrophoresis is the most common way to evaluate RNA integrity. Each RNA sample was assessed by integrity of ethidium bromide agarose gel 1 % and evaluated by electrophoresis in TBE buffer. After running the agarose gel electrophoresis for 45 minutes in 90V, the gel was observed in the specific gel documentation system (Vilber Lourmat, Fisher Scientific). RNA sharp and clear bands can be seen in 18S and 28S rRNA. To estimate the size of the RNA bands, a 1 kb DNA ladder was used.

3.4.9.3 cDNA Synthesis

3.4.9.3.1 Principle

Complementary DNA (cDNA) is DNA synthesized from a messenger RNA (mRNA) template in a reaction catalyzed by the enzymes, reverse transcriptase and DNA polymerase. A DNA which has no intron is constructed using “intron-free” mRNA as a template. Therefore, it is a “complementary” copy of the mRNA or cDNA. To obtain expression of the protein encoded by the cDNA, prokaryotic regulatory sequences would also be required (Pérez-Márquez *et. al.,* 2002).

The High Capacity cDNA Reverse Transcription Kits for 200 and 1000 reactions contain all the reagents needed for reverse transcription (RT) of total RNA to single-stranded cDNA using a reaction size of 20 µl.

3.4.9.3.2 Procedure

The concentration of 100 ng/ml of each extracted total RNA was converted to cDNA according to the protocol of TaqMan with catalog no: 4368814. The procedure was done as follows,

The 2X Reverse Transcription Master Mix was prepared according to the protocol of the kit. Then total RNA (Section 3-4-9-2-2) was added to the 2X Master Mix. The mixture was incubated at 25 ^{o}C, 37 ^{o}C, and 85 ^{o}C for 10, 120 and 5 min respectively. The cDNA was stored at 4 ^{o}C to use for gene expression assay.

3.4.9.4 Real time RT-PCR

3.4.9.4.1 Principle

Reverse transcription polymerase chain reaction or RT-PCR is usually used when the goal of study is to discover RNA expression levels in the field of molecular biology. By using real-time RT-PCR and the creation of cDNA from RNA, we can detect gene expression quantitatively. In this technique, the RNA, as a template in real-time RT-PCR, is first converted into (cDNA) using a reverse transcriptase. The cDNA is then used as a template for exponential amplification using PCR. There are four different fluorescent DNA probes available for the real-time RT-PCR detection of PCR products: SYBR Green, TaqMan, Molecular Beacons, and Scorpions (Arya *et. al.,* 2005; Tse & Capeau, 2003). In a real-time PCR assay, a positive reaction is detected by the accumulation of a

fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e., exceeds background level). The Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e., the lower the Ct level, the greater the amount of target nucleic acid in the sample).

3.4.9.4.2 Procedure

The selected genes involved in apoptosis for this study were: Hs00609632-Bid (136 bp), Hs00608023-Bcl2 (81 bp), Hs00178289-Akt1 (66 bp), and Hs01034249-p53 (108 bp). As endogenous controls, Hs01060665-β actin (63 bp) and 18 siRNA (1.9 bp) were used for biological normalizing of the selected genes. All TaqMan probes used in this study were labeled with the 6-carboxyfluorescein acronym (FAM) reporter dye at the 5' end and a TaqMan dihydrocyclopyrroloindole tripeptide minor groove binder acronym (MGB) probe quencher at the 3' end. The real-time polymerase chain reaction (real-time RT-PCR) was done according to the protocol of TaqMan kit (Catalog No: 4333458).

The PCR reaction mix was prepared by adding 1 µl of 20× TaqMan® Gene Expression Assay, 10 µl of 2× TaqMan® Gene Expression Master Mix, 4 µl cDNA template, and 5 µl of RNase-free water into a nuclease-free 1.5-ml microcentrifuge tube. The tubes were capped and inverted several times to mix the reaction components then centrifuged briefly. A volume of 20 µl of PCR reaction was transferred into each well of 96-well reaction plate.

A volume of 1 µl of the gene probes was then added to each well. The plate was covered and centrifuged briefly. The plated was loaded into the instrument (Figure 3.11) and run according to the program which is described in the Table 3.5.

Table 3.5. Programme used for running the RT-PCR

System Applied Biosystems 7500 Fast Real-Time PCR System	Run	Reaction plate	Plate document/ experiment parameters	Thermal cycling conditions		
				Stage	Temp °C	Time mm:ss
	Standard	96-well plate	•Rxn.Volume: 20 µl • Ramp Rate: Standard	Hold§	50	2:00
				Hold	95	10:00
				40 Cycles	95	0:15
					60	1:00

3.4.9.4.3 Calculation

The resulted Ct values were analyzed with the REST 2009 software. Up-regulation and down-regulation or no change in the expression of the genes in the sample group (in comparison with the control group) by a mean factor was determined.

3.5 Purification and identification

3.5.1 High performance liquid chromatography

3.5.1.1 Principle

High performance liquid chromatography (HPLC) is basically an improved technique of column chromatography. The solvent in HPLC is forced to move under high pressure of up to 400 atmospheres which makes it faster. A very small particle can be injected into column packing material which makes a suitable surface area for interactions between the stationary phase and the molecules flowing through it. This interaction allows

greater separation of the components of the mixture used. According to the relative polarity of the solvent and the stationary phase we have two types of HPLC:

1. **Normal phase HPLC**

Like thin layer chromatography, the column in this kind of HPLC is filled with tiny silica particles, and the solvent used is non-polar such as hexane. Polar compounds in the system stick longer to the polar silica than non-polar compounds when they pass through the column. The non-polar ones can pass more quickly through the column.

2. **Reversed phase HPLC**

In this type of HPLC, the column size is the same, but the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them (C18). Polar solvents can be used like a mixture of water and an alcohol such as methanol. There is a strong attraction between the polar solvent used and polar molecules in the mixture which pass through the column. There is not much attraction between the hydrocarbon chains on the surface of silica (the stationary phase) and the polar molecules in the solution. Non-polar compounds of the mixture tend to attract with the hydrocarbon groups. Also, they are less soluble in the solvent. They therefore spend less time in solution and travel down slowly on through the column. In reverse, polar molecules travel through the column more quickly.

3.5.1.2 Procedure

The experiment was performed on a Agilent 1260 infinity HPLC system consisting of a quaternary pump equipped with a 1260 auto sampler (ALS), 1290 thermostat, 1260 thermostatted column compartment (TCC), 1260 diode array detector (DAD VL+), 1260 fraction collector (FC-AS) and Agilent OpenLAB CDS Chemstation for LC software.

The analytical scale analysis was carried out using a binary eluent of chromatographic grade ACN and ultrapure H_2O under isocratic conditions: 40 % ACN in H_2O, the column used was ZORBAX Eclipse XDB-C18 (4.6 x 250mm, 5µm) and temperature was set on 30 °C. The sample was prepared in 5.0 mg/ml in methanol and filtered through a membrane filter (0.45 µM, Sartorius). The sample of 5.0 µl was injected into the column and peaks were detected by monitoring the UV absorbance at 254 nm. Subsequently, a higher loading of sample for preparative scale separation was attempted using the same HPLC method described above. The sample concentration was 40.0 mg/ml in methanol and 100.0 µl of sample was injected into the semi-preparative column, ZORBAX Eclipse XDB-C18 (9.4 x 250mm, 5µm) with a flow rate of 5.0 ml/min. The selected peaks in the resultant chromatography were collected by fraction collector and this separation procedure was conducted repeatedly. Similar fractions from each round of separation were combined and the mobile phases were evaporated in a rotary evaporator at 40 °C. Then the hexane extract of *C. cassia* and collected fractions were subjected to GC-MS analysis for identification of compounds.

3.5.2 Gas chromatography – mass spectrometry (GC-MS)

3.5.2.1 Principle

Gas chromatography-mass spectroscopy (GC-MS) is one of the analytical techniques which are combined to form a single method of analyzing mixtures of chemicals. Gas chromatography is used to separate the components of a mixture and mass spectroscopy is applied to characterize each of the components individually. By combining the two techniques, a solution containing a number of chemicals can be evaluated both qualitatively and quantitatively. In all chromatography types, separation occurs when the sample mixture is injected into the mobile phase. In gas chromatography (GC), the mobile phase is an inert gas such as helium.

3.5.3 Mass Spectroscopy

3.5.3.1 Principle

Compounds are eluted from the GC column individually then bombarded with a stream of electrons causing them to break apart into fragments. These fragments can be large or small pieces of the original molecules.

3.5.3.2 Procedure

Gas chromatography–mass spectrometry (GC-MS) is an analytical method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample.

Applications of GC include drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples.

GC-MS can also be used in airport security to detect substances in luggage or on human beings. Additionally, it can identify trace elements in materials that were previously thought to have disintegrated beyond identification.

GC-MS analysis was performed using an Agilent Technologies 6980N gas chromatograph equipped with a 5975 Mass Selective Detector (70 eV direct inlet) on fused silica capillary column, HP-5ms (30.0 m x 0.25 mm ID x 0.25 μM film thickness). The carrier gas was helium (99.999 %) at a flow rate 1 ml/min and a split ratio of 1:20. The column temperature was initially set at 60 °C and was kept isothermally for 10 min, then increased to 3 °C/min to 230 °C and held for 1 min. The temperature of injector port and interface of mass spectrometer was programmed at 230 °C and 250 °C, respectively. The total ion chromatography obtained was auto integrated by ChemStation and chemical compounds were identified by comparison with the accompanying Wiley 9th edition NIST11 (W9N11) mass spectral library, USA.

3.6 Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analyses were performed by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons and the Student's *t*-test. A P-value of < 0.05 was considered statistically significant. Pearson correlation coefficient was used to assess the correlation between TPC, TFC and FRAP. SPSS, version 18.0 (Chicago, Ill, USA) and Microsoft Excel 2007 (Roselle, Ill, USA) statistical software were used for the statistical and graphical evaluations.

CHAPTER IV: RESULTS

4.1 Extraction

The results of the extractions of 40 g of the cinnamon powder with the different solvents used are listed in Table 4.1. A total of 5.66 g were extracted with the seven solvents used. The extraction started with hexane as the most nonpolar solvent and ended with water as the most polar. The weight of the remaining solid materials was the weight of the original cinnamon powder minus the total weight of all of the components previously extracted in the solvents. The 99.9 % acetone extraction at 27 °C ± 1 °C produced the highest extract yields (1.88 g of 40 g sample or 4.69 %) which were similar to that obtained of the 99.9 % methanol extract at 27 °C ± 1 °C (1.8 g of 40 g sample or 4.49 %). The 98.5 % hexane extraction at room temperature resulted in lowest yields (0.13 g of 40 g sample or 0.32 %) which were similar to that obtained of the double distilled water extract at 27 °C ± 1 °C (0.14 g of 40 g sample or 0.35 %).

As the extraction polarity was increased, the highest amount of the total extracts was yield. The polar-protic solvents (ethanol, methanol and water) dissolved the highest amount of the compounds from 40 g *C. cassia* (2.7 g from 40 g sample), while the non-polar solvent (hexane and chloroform) dissolved the lowest amount of the compounds from 40 g *C. cassia* (0.81 g). The polar-aprotic solvents (ethyl acetate, acetone) dissolved 2.15 g of the compounds from 40 g *C. cassia.*

Table 4.1. Amount of yield extracted from 40 g of *Cinnamomum cassia*

Solvent Polarity	**Nonpolar**		**Polar-aprotic**		**Polar-protic**		
Extract	Hexane	Chloroform	Ethyl acetate	Acetone	Ethanol	Methanol	Water
Weight (g)	0.13	0.68	0.27	1.88	0.76	1.8	0.14
	Total: 0.81 g		Total: 2.15 g		Total: 2.7 g		
	Total: 5.66 g						

Values are means of at least three determinations ± standard deviation and are expressed as grams of freeze-dried water extract and evaporated extract for the rest of extracts from 40 g *C. cassia.* All the extractions were performed at 27 °C ± 1 °C for 6 h.

4.2 Antioxidant activity

In the antioxidant assays, except for the FRAP, where the concentration used was 1 mg/ml of each extract of *C. cassia*, the range of the concentrations used were 25 µg/ml to 1 mg/ml. The IC_{50} for each assays was calculated as described in chapter 3.

4.2.1 Ferric Reducing Antioxidant Power (FRAP) Assay

Figure 4.1 shows that the FRAP values of each of the seven extracts of cinnamon increase in a dose-dependent manner. The acetone extract had the highest FRAP value between 4-60 min (the FRAP value ranging from 1022.8 to 3150.6 mmol Fe^{2+}/ g) compared to the other extracts. Next to the acetone extract, the ethyl acetate extract with a FRAP value of 2505 mmol Fe^{2+}/ g had the highest value compared to the other extracts at 60 min. The

lowest FRAP value belonged to the hexane extract (the FRAP value ranging from 27.2 to 32.8 mmol Fe^{2+}/ g), followed by the chloroform extract FRAP value (the FRAP value ranging from 128.9 to 408-3 mmol Fe^{2+}/ g) between 4-60 min. In the polar-protic extracts, the highest FRAP value belonged to the methanol extract between 4-60 min (the FRAP value ranging from 857.2 to 3275 mmol Fe^{2+}/ g) which was almost twice higher than that obtained of the ethanol extract from 0-60 min (the FRAP value ranging from 451.7 to 1733.9 mmol Fe^{2+}/ g).

The FRAP value of the acetone extract was almost 116 times higher than that of the hexane extract which had the lowest FRAP value compared to the other extracts at 60 min (32.8 mmol Fe^{2+}/ g).

Quercetin (pure flavonoid) which was used as the positive control in FRAP assay, had a FRAP value of 6013.3 ± 0.01 mmol Fe^{2+}/ g at 60 min which was twice higher than that obtained of the acetone extract of *C.cassia*. The exact values at 4 and 60 min were obtained from Table 4.2. $FeSO_4.7H_2O_2$ was used as the standard (Appendix A).

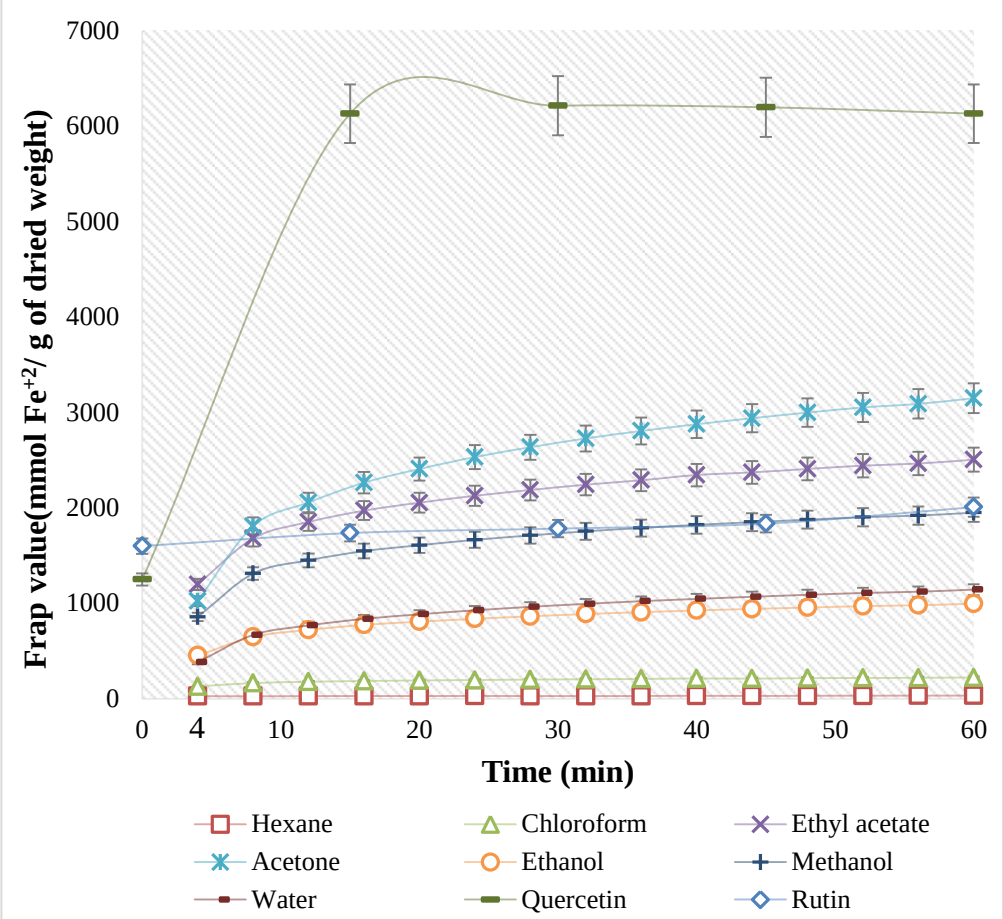


Figure 4.1. FRAP value of the different extracts of *C. cassia*
The ferric reducing antioxidant power (expressed as mmol Fe^{2+} / g sample) of the different extracts. All data are shown as the means ± SD for triplicate determination in same sample. $P < 0.05$ compared to the control (without extract) as tested by the Student's *t*-test.

Table 4.2. FRAP values of the different extracts at 4 and 60 min of the time points

Extract	FRAP value at 4 min (mmol Fe^{2+}/g of DW sample)	FRAP value at 60 min (mmol Fe^{2+}/g of DW sample)
Hexane	27.2±0.02	32.8±0.00
Chloroform	128.9±0.02	222.2±0.00
Ethyl acetate	1197.8±0.01	2505.6±0.01
Acetone	1022.8±0.03	3150.5±0.14
Ethanol	451.7±0.02	1733.9±0.03
Methanol	857.2±0.06	1950.6±0.53
Water	381.7±0.04	1143.9±0.01
Quercetin	1250±0.003	6013.3±0.01
Rutin	1600±0.002	2008.9±0.02

The ferric reducing antioxidant power (expressed as mmol $Fe^{2+/}$g sample) of the tested extracts with different solvents. All data are shown as the means ± SD for triplicate determination in same sample.

4.2.2 Superoxide Anion Radical Scavenging Assay

The activity of each extract in scavenging superoxide anion radicals increase in a dose-dependent manner (Figure 4.2). Table 4.3 shows that the IC_{50} of the chloroform extract, 121.8 ± 1.98 µg/ml, was the lowest among the IC_{50} of the other extracts while rutin with an IC_{50} of 9.1 ± 2.54 µg/ml was much more potent, almost 2.5 times than the chloroform extract. At 200 µg/ml, the chloroform and ethyl acetate extracts with a 72 and 68 % superoxide radical scavenging activity, respectively were the most potent extracts compared to the other extract in this assay. Trolox was used as the standard in this assay (Appendix B).

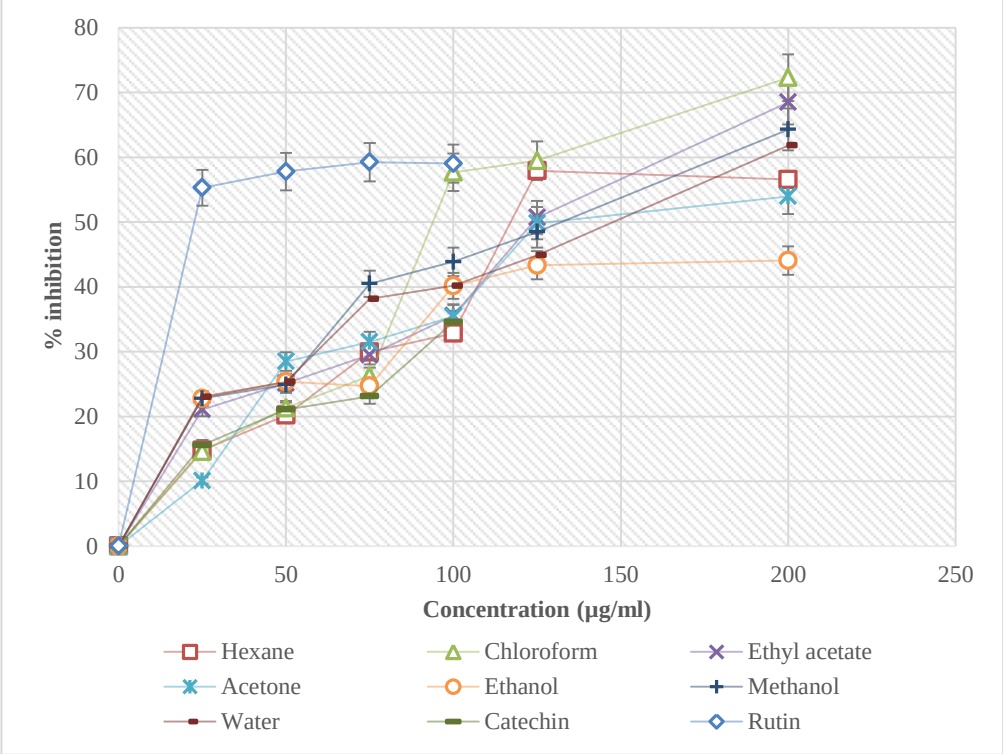


Figure 4.2. Super oxide anion radical scavenging activity of the different extracts

The superoxide anion radical scavenging assay (expressed as % inhibition) of the tested extracts with different solvents. All data are shown as the means ± SD for triplicate determination in same sample. $P < 0.05$ compared to the control (without extract) as tested by the Student's *t*-test.

4.2.3 DPPH Scavenging Assay

In this study, DPPH radical scavenging activity of the tested samples increase with increasing concentrations (Figures 4.3). Figure 4.4 indicates that the ethyl acetate extract had the highest DPPH radical scavenging activity compared to the other extracts, with the highest activity of 91.54 % scavenging at the concentrations of 50 µg/ml which was similar to that obtained of the quercetin (91.7 %) at the same concentration. Rutin showed lower activity at 50 µg/ml (74.58 %) compared to the ethyl acetate extract. The hexane and chloroform extracts, which had the lowest DPPH radical

scavenging activities, exhibited only 8 % and 14 % scavenging at the highest concentration of 300 µg/ml. Next to the ethyl acetate extract, the methanol extract exhibited the highest activity in scavenging the DPPH radical at the concentration of 50 µg/ml (54 %). The exact IC_{50} of different extracts of *C.cassia* in Table 4.3 indicate that the ethyl acetate extract of the cinnamon bark had the highest anti DPPH radical property by inhibiting DPPH radical with an IC_{50} of 69.35 ± 2.23 µg/ml, which was almost comparable with the IC_{50} of quercetin (71.8 ± 2.80 µg/ml) while, the acetone extract with an IC_{50} of 207.88 ± 1.77 µg/ml was the weakest extract in reduction of DPPH radical compared to the other extracts. Trolox was used as the standard in this assay (Appendix C).

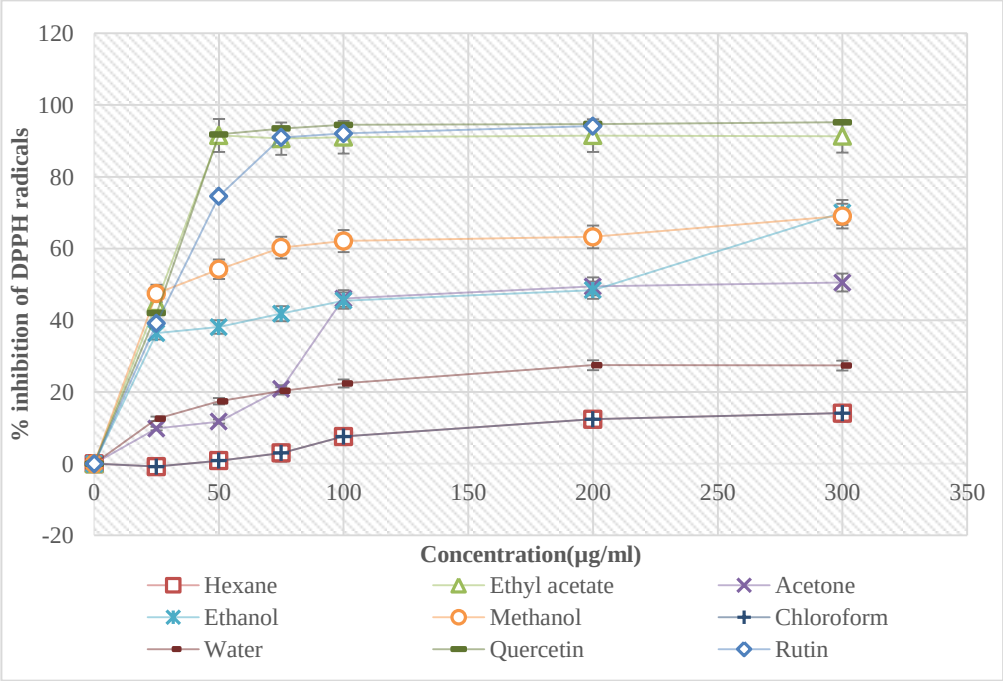


Figure 4.3. DPPH radical scavenging activity of the different extracts
The DPPH radical scavenging assay (expressed as % inhibition) of the tested extracts with different solvents. All data are shown as the means ± SD for triplicate determination in same sample. $P < 0.05$ compared to the control (without extract) as tested by the Student's *t*-test.

4.2.4 Nitric Oxide Radical Scavenging Assay

Each extract under nitric oxide radical scavenging assay showed an increase in a dose-dependent manner (Figure 4.4). Also, it was shown that the hexane and water extracts at 22 µg/ml had the same percentage inhibition of nitric oxide radical (19.2 %). The hexane extract scavenged and suppressed the release of NO^- more powerfully than the other extracts and showed the lowest IC_{50} value of 89.5 ± 2.3 µg/ml. The hexane and water extracts, which had the highest NO^- radical scavenging activity, exhibited 51.9 % and 46.7 % scavenging at the highest extract concentration of 100 µg/ml, respectively. While, the ethyl acetate extract which had the lowest radical scavenging activity, exhibited 31 % scavenging at the highest extract concentration of 100 µg/ml. the IC_{50} value obtained of the hexane extract (89.5 ± 2.3 µg/ml) was lower than that obtained of the pure flavonoids, quercetin and rutin (120.1 ± 2.13 and 104.3 ± 1.88 µg/ml, respectively). Table 4.3 shows the exact amounts of the IC_{50}.

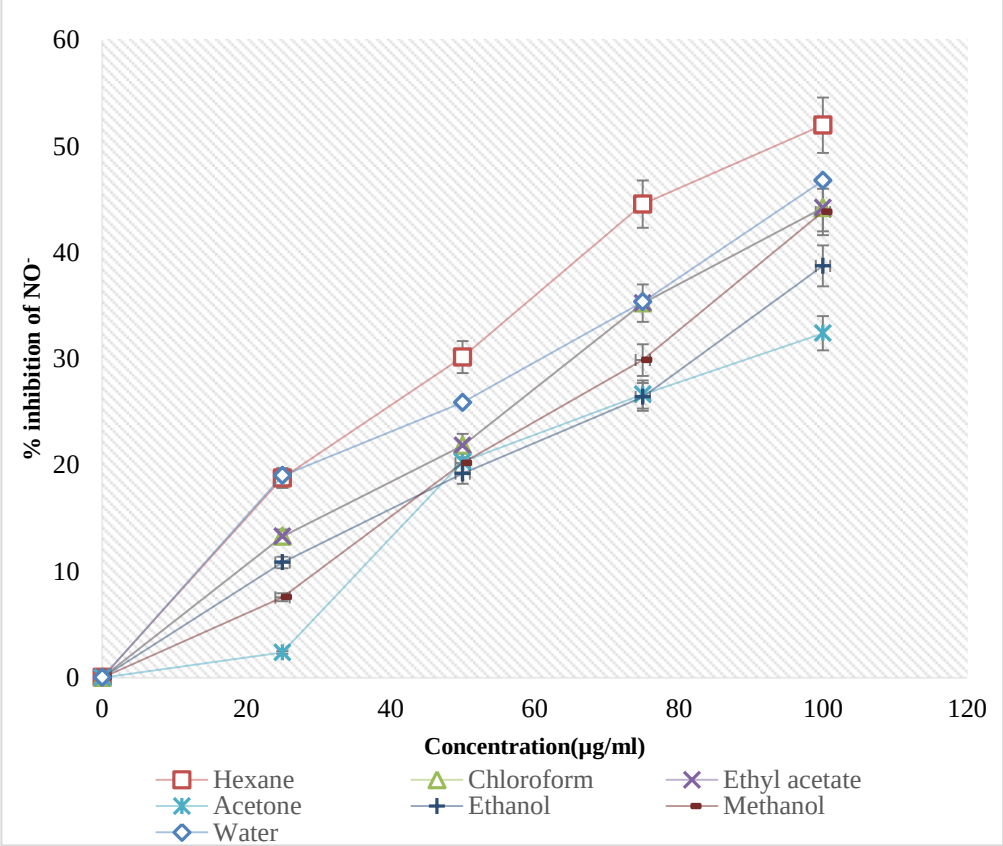


Figure 4.4. Nitric oxide radical scavenging activity of the different extracts

The nitric oxide radical scavenging assay (expressed as % inhibition) of the tested extracts with different solvents. All data are shown as the means ± SD for triplicate determination in same sample. $P < 0.05$ compared to the control (without extract) as tested by the Student's *t*-test.

4.2.5 Hydroxyl Radical Scavenging Assay

Figures 4.5 show, the inhibition of hydroxyl radical of different extracts of cinnamon except the chloroform extract increased in a dose-dependent manner. In chloroform extract, the hydroxyl radical scavenging activity increased to 40.15 % at 50 µg/ml and then remained constant to 125 µg/ml. The ethyl acetate extract showed the highest hydroxyl radical scavenging activity compared to the other extracts which was had highest

activity of 50.6 % hydroxyl radical scavenging at the concentration of 125 µg/ml and it was almost similar to that obtained of the ethanol extract (50.27 %), methanol extract (49.12 %) and water extract (49.12 %) at the same concentration. The exact IC_{50} of each extracts of *C.cassia* in Table 4.3 indicate that the ethyl acetate extract of the cinnamon bark had the highest antiradical property by inhibiting hydroxyl radical with an IC_{50} of 112.77 ± 2.14 µg/ml, which was almost 4 times higher than that obtained of pure flavonoids, cathechin (25.8 ± 2.3 µg/ml) and rutin (26.2 ± 3.01 µg/ml). The acetone extract with the highest activity of 21.92 % at 125 µg/ml was the weakest extract in reduction of hydroxyl radicals compared to the other extract. Trolox was used as the standard in this assay (Appendix D).

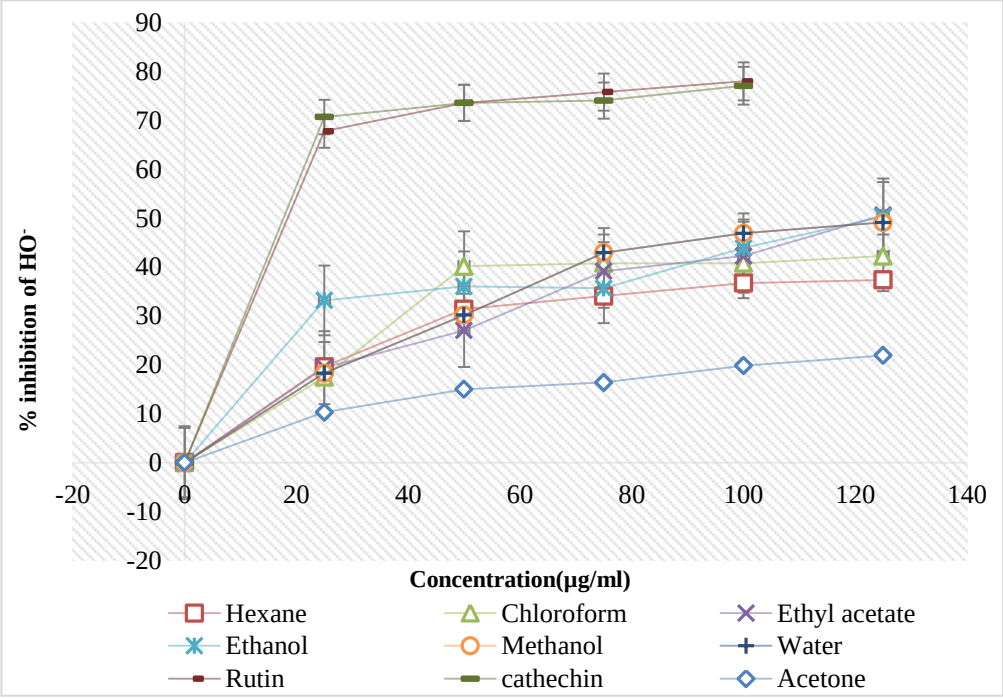


Figure 4.5. Hydroxyl radical scavenging activity of the different extract
The hydroxyl radical scavenging assay (expressed as % inhibition) of the tested extracts with different solvents. All data are shown as the means ± SD for triplicate determination in same sample. $P < 0.05$ compared to the control (without extract) as tested by the Student's *t*-test.

Table 4.3. IC_{50} values of some radicals scavenging activities of the different extracts

Extracts	DPPH radical scavenging (IC_{50}) µg/ml	Superoxide anion radical scavenging (IC_{50}) µg/ml	Nitric oxide radical scavenging (IC_{50}) µg/ml	Hydroxyl radical Scavenging (IC_{50}) µg/ml
Hexane	ND	139.82±6.22	89.5±2.3	ND
Chloroform	ND	121.78±6.22	ND	ND
Ethyl acetate	69.35±2.23	133.23±2.80	ND	112.77±2.14
Acetone	207.88±1.77	146.99±3.20	ND	ND
Ethanol	149.53±2.41	ND	ND	122.79±7.30
Methanol	125.07±2.54	175.73 ±6.10	ND	132.45±2.24
Water	ND	156.14±3.12	ND	144.23±2.44
Quercetin	62.1±3.01	71.8±2.80	120.1±2.13	25.8±2.30
Rutin	188.4±2.22	9.1±2.54	104.3±1.88	26.2.±3.01

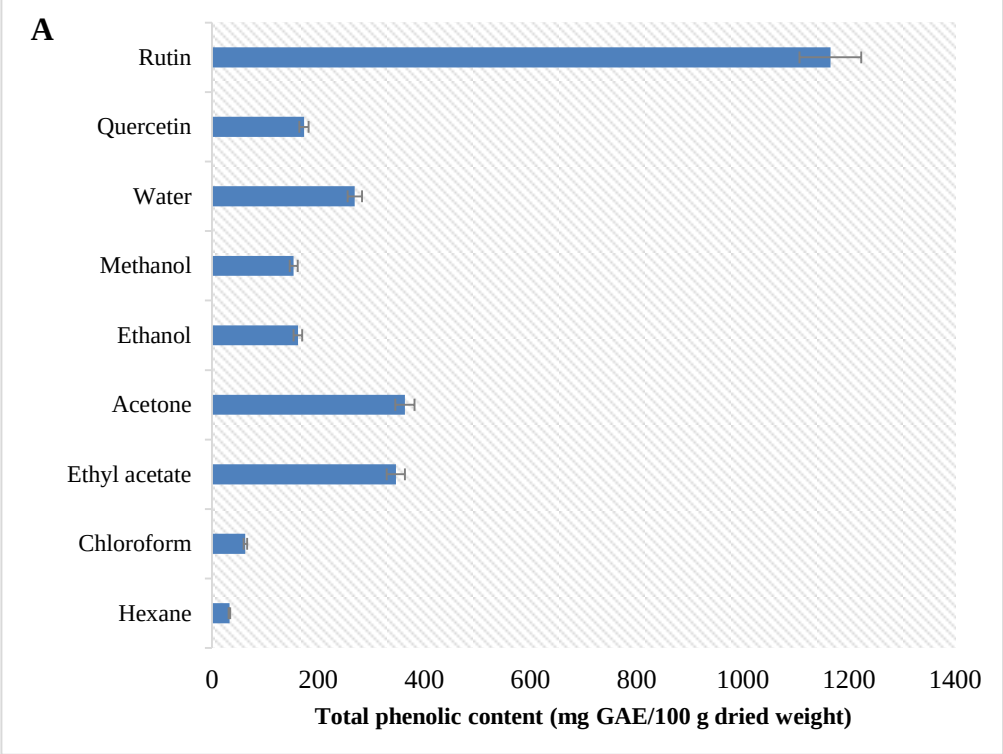
Antioxidant activities of different extracts using different antioxidant radical scavenging assays. Results are expressed as IC_{50} which signifies the effective concentration used to scavenge 50 % of free radicals. Values are expressed as mean ± SD (n=3).
ND = Not Detected

4.3 Total Antioxidant Content (TPC and TFC)

The results of TPC (Table 4.4 and Figure 4.6 (A)) indicate that the acetone extract had the highest content phenolic compound (TPC = 363.33 ± 0.32 mg GAE/ 100 g DW) which was followed by the ethyl acetate extract with 346.25 ± 0.18 mg GAE/ 100 g DW. The hexane extract with 32.7 ± 1.9 mg GAE/ 100 g DW and chloroform with 62.71 ± 0.23 mg GAE/ 100 g DW had the lowest total phenolic content between the other extracts. While, rutin had a TPC value of 1165 ± 0.08.

The results of TFC (Table 4.4 and Figure 4.6 (B)) indicate that the acetone extract had the highest content flavonoid compound (569.43 ± 0.285 mg QE/100 g DW) which was followed by the ethyl acetate extract with 552.48 ± 0.16 mg QE/100 g DW. The hexane and chloroform extract with 14.38 ± 0.1 mg QE/g DW and 47.8 ± 0.16 mg QE/100 g DW, respectively had the lowest total phenolic content among the other extracts. Cathechin and rutin had the TFC values of 942 ± 0.00 and 787.8 ± 0.04, respectively.

Gallic acid and quercetin were used as the standards for TPC and TFC, respectively (Appendix E and F).



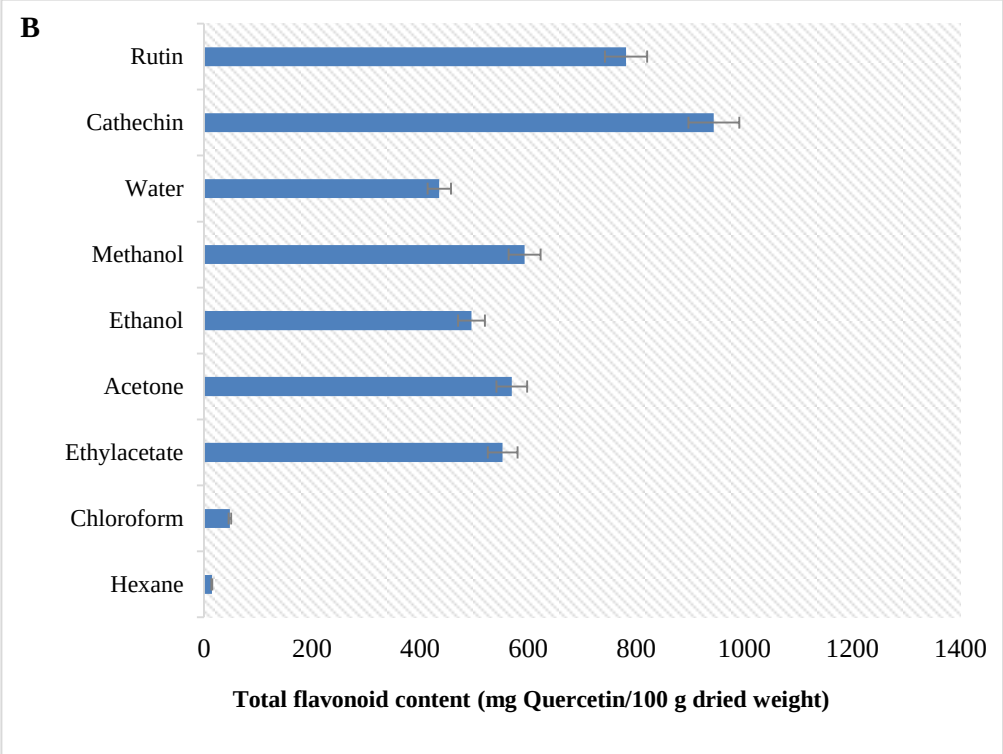


Figure 4.6. TPC and TFC of different extracts

The total phenolic content (expressed as mg QE /100 g DW) (A), the total flavonoid content (expressed as mg GAE / 100 g DW) (B). Values are expressed as mean ± SD (n=3). $P < 0.05$ compared to the control (without extract) as tested by the Student's *t*-test.

Table 4.4. Summary of TPC and TFC values of the extracts

Extracts	Phenolic content (mg GAE/100 g DW)	Flavonoid content (mg QE/100 g DW)
Hexane	32.71 ± 1.9	14.38 ± 0.1
Chloroform	62.71 ± 0.23	47.8 ± 0.16
Ethyl acetate	346.25 ± 0.18	552.48 ± 0.16
Acetone	363.32 ± 0.32	569.43 ± 0.28
Ethanol	161.65 ± 0.38	101 ± 0.44
Methanol	153.75 ± 1.09	494.95 ± 0.86
Water	173.32 ± 1.09	435.32 ± 2.23
Quercetin for TPC **Cathechin for TFC**	1165 ± 3.5	929.43 ± 3.52
Rutin	433 ± 3.01	782.25 ± 3.22

The total phenolic content is expressed as (mg QE /100 g DW). The total flavonoid content is expressed as (mg GAE / 100 g DW). Values are expressed as mean ± SD (n=3).

4.3.1 Correlation of TPC, TFC and FRAP values of the Extracts

4.3.1.1 Correlation of TPC with TFC

As shown in Figure 4.7 and the results of correlations analysis calculated by SPSS (Table 4.5), there was a moderate positive correlation between total phenolic and flavonoid content in the cinnamon extracts (R^2=0.787, $P < 0.05$). Figure 4.7 shows that the TFC value of the ethyl acetate, ethanol, methanol, and water was significant higher than that of obtained of TPC. There was no significant difference between TPC and TFC values in the hexane and chloroform extract.

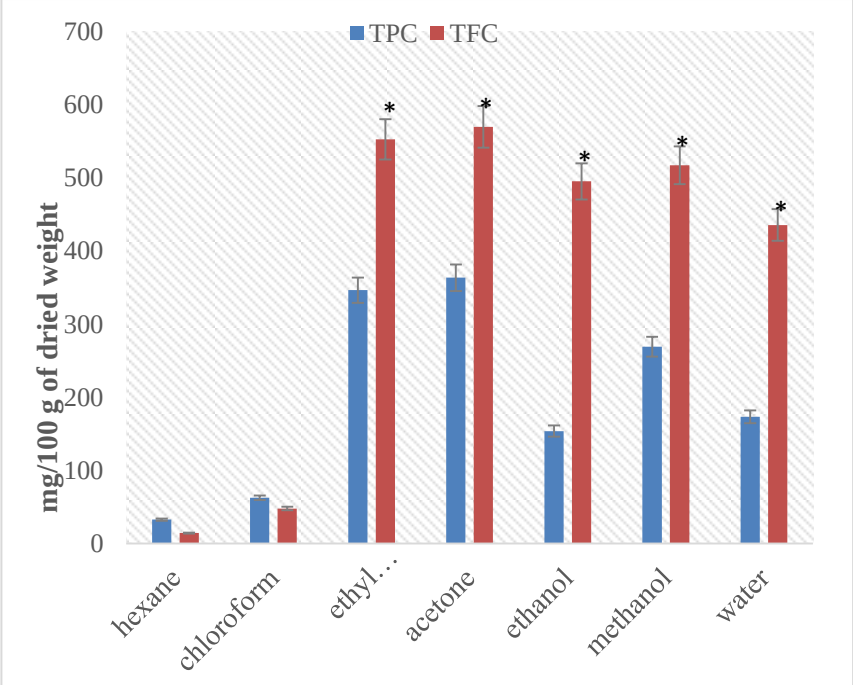


Figure 4.7. TPC and TFC of the different extracts
Values are mean of three replicate determinations (n = 3) ± standard deviation. Total phenolic content (TPC) is expressed as mg GAE /100 g DW of the sample. Total flavonoid content (TFC) is expressed as mg QE /100 g DW of the sample. Values are expressed as mean ± SD of triplicate determinations. * indicates significant difference from TFC and TPC values ($p<0.05$). $P < 0.05$ compared to the control (without extract) as tested by the Student's *t*-test.

Table 4.5. Correlations between TPC, TFC and, FRAP of the extracts

Assay		TPC	TFC	FRAP
TPC	Pearson Correlation	1	.787**	.887**
	Sig. (2-tailed)		.000	.000
	N	21	21	21
TFC	Pearson Correlation	.787**	1	.849**
	Sig. (2-tailed)	.000		.000
	N	21	21	21

** Correlation is significant at the 0.01 level (2-tailed) and $P < 0.05$, calculated using SPSS, version 18.0 (Chicago, Ill, USA).

4.3.1.2 The correlation of TPC and TFC with FRAP

The anti-oxidant activity of the different extracts of *C. cassia* was measured by using FRAP correlated significantly and positively with the total phenolics (R^2=0.887, $P < 0.05$) (Table 4.5). The significant positive correlation between total flavonoid content and FRAP of the different extracts of *C. cassia* is shown in Table 4.5 (R^2=0.849, $P < 0.05$).

4.4 Protection against DNA damage (comet assay)

DNA damage was induced in 3T3-L1 cells by using 100 µM of hydrogen peroxide as the positive control. In treated cells, the effect of the acetone extract on the H_2O_2-induced DNA damage was tested by treating the cells with various concentrations (10 – 50 µg/ml) of the acetone extract.

Figure 4.8 shows all the concentrations of the acetone extract showed a statistically significant DNA protection. With increasing the concentration of the acetone extract to treat the cells from 10 µg/ml to 30 µg/ml, the percentage of DNA protection of 3T3-L1 increased from 32 % to 45 %. No significant change was observed in DNA protection with increasing the concentration from 30 to 50 µg/ml. Therefore, the acetone extract had the highest percentage (44.5 %) of DNA protection at 30 µg/ml.

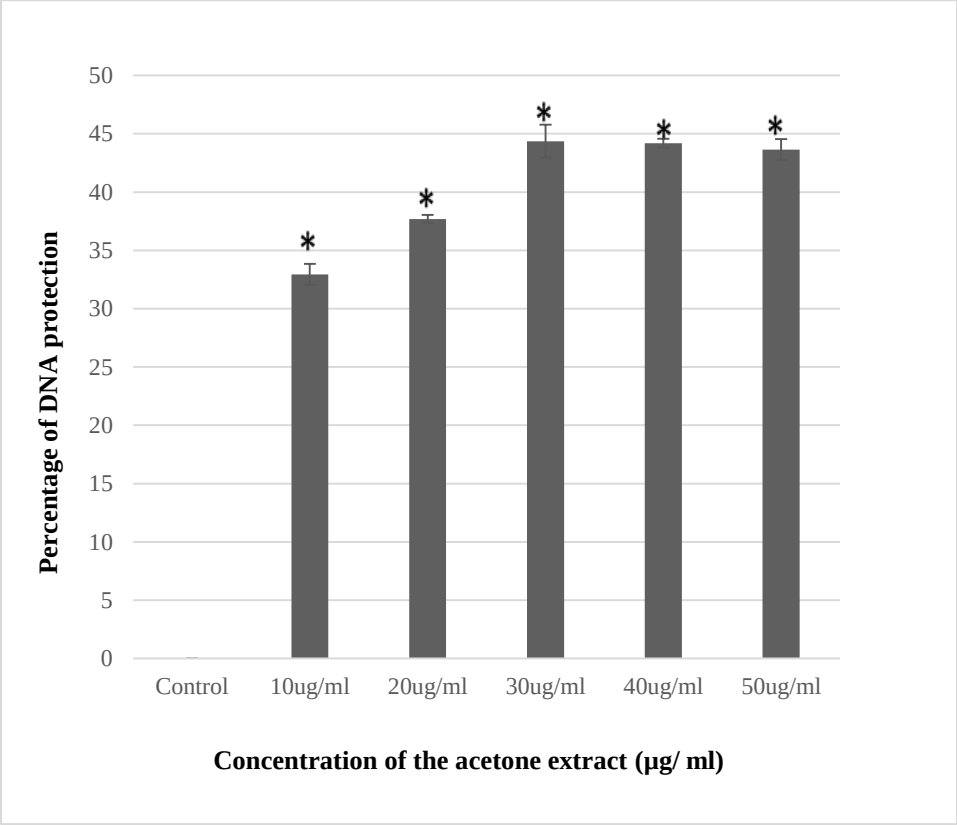


Figure 4.8. Detection of DNA damage by comet assay
The 3T3 L1 cells were cultured in 25 cm^2 culture flasks and incubated with the various concentrations of *C. cassia* for 24 h. Electrophoresis was carried out at 25V (300 mA) for 20 min using a power supply (CBS). The extract showed the highest percentage of DNA protection at 30 µg/ml. * indicates significant difference from untreated cells ($p<0.05$). Results were expressed as mean ± std. dev. $P < 0.05$ compared to the control (without extract) as tested by the Student's *t*-test.

4.5 *In vitro* Inhibition of Cell Proliferation (MTT Assay)

The anti-proliferative activity of the different extracts of cinnamon on MCF-7 and MDA-MB-231 cells by are shown in Figure 4.9 and the IC_{50} values of each extract is shown in Table 4.6.

Figure 4.9 shows that antiproliferative activity of the hexane, chloroform and acetone extracts increase in a dose-dependent manner and the range of percentage of inhibition in treated MCF-7 cells with the hexane extract were from 19.34 % to 94.33 % (Figure 4.9 (A)), while the range in MDA-MB-231 cells treated with the same extract were from 10.16 % to 72.32 % (Figure 4.9 (B)).

Also, Table 4.6 shows that the hexane extract had IC_{50} values of 34 ± 3.52 µg/ml and 32.42 ± 0.37 µg/ml on MCF-7 and MDA-MB-231 cells proliferation, respectively. The IC_{50} of the chloroform extract in MCF-7 and MDA-MB-231 cells were 45.09 ± 2.03 µg/ml and 65.28 ± 2.84 µg/ml respectively, which had the most potent antiproliferative activity compared to the other extracts next to the hexane extract.

The percentage inhibition of the cell proliferation of the ethyl acetate extract in treated MCF-7 cells increased to 50.83 % at 136 µg/ml. The percentage inhibition of the cell proliferation of the ethyl acetate extract in treated MDA-MB-231 cells decreased from 28.4 % at 4.25 µg/ml to 16.23 % at 17 µg/ml, then increased to 32.45 % at 68 µg/ml. The IC_{50} of the ethyl acetate extract in MCF-7 cells was 134.33 ± 2.09 µg/ml.

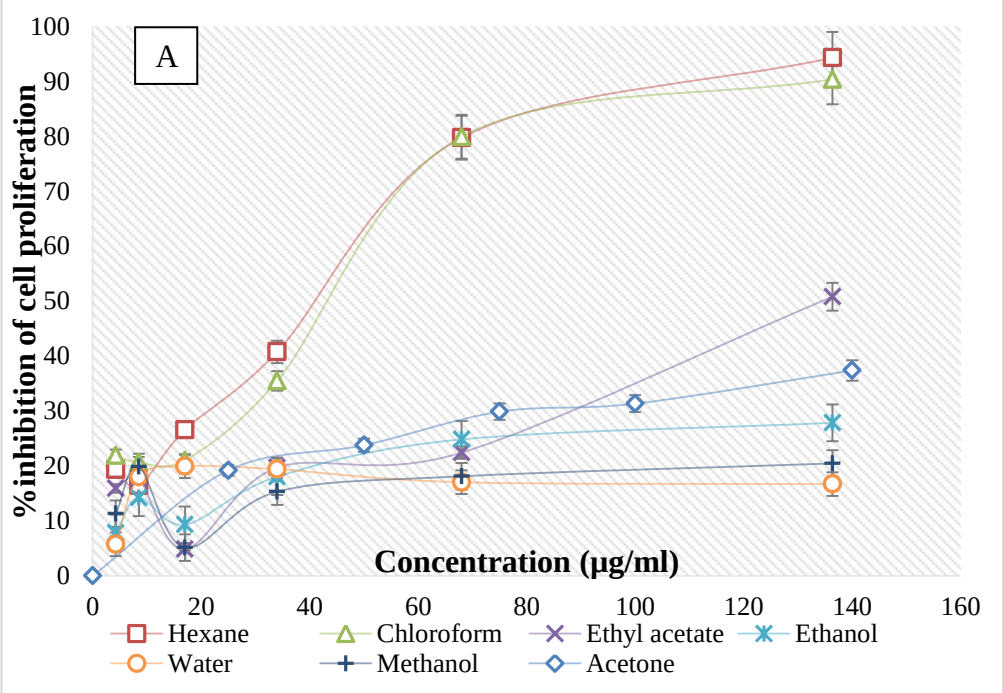
The methanol and water extracts had the lowest antiproliferative activity in both cell lines compared to the other extracts. The highest

percentage inhibition of cell proliferation of the methanol extract at 136 µg/ml in treated MCF-7 and MDA-MB-231 cells were 20.47 % and 2.2 %, respectively. The percentage inhibition of cell proliferation of the water extract at 136 µg/ml in treated MCF-7 cells and MDA-MB-231 cells were 16.7 % and 7.0 %, respectively.

Table 4.6. IC_{50} values in MCF-7 and MDA-MB-231cells treated with the extracts using MTT assay

Extract	IC_{50} (µg/ml) MCF-7 cells	IC_{50} (µg/ml) MDA-MB-231 cells
Hexane	34 ± 3.52	32.42 ± 0.37
Chloroform	45.09 ± 3.02	65.28 ± 2.84
Ethyl acetate	134.33 ± 2.09	ND
Acetone	ND	ND
Ethanol	ND	ND
Methanol	ND	ND
Water	ND	ND

Antiproliferative activities of the different extracts in two breast cancer cell lines are presented. Results are expressed as mean ± std. dev. (n=3).
IC_{50} is defined as concentration of plant extracts that inhibited 50 % growth of the cells.



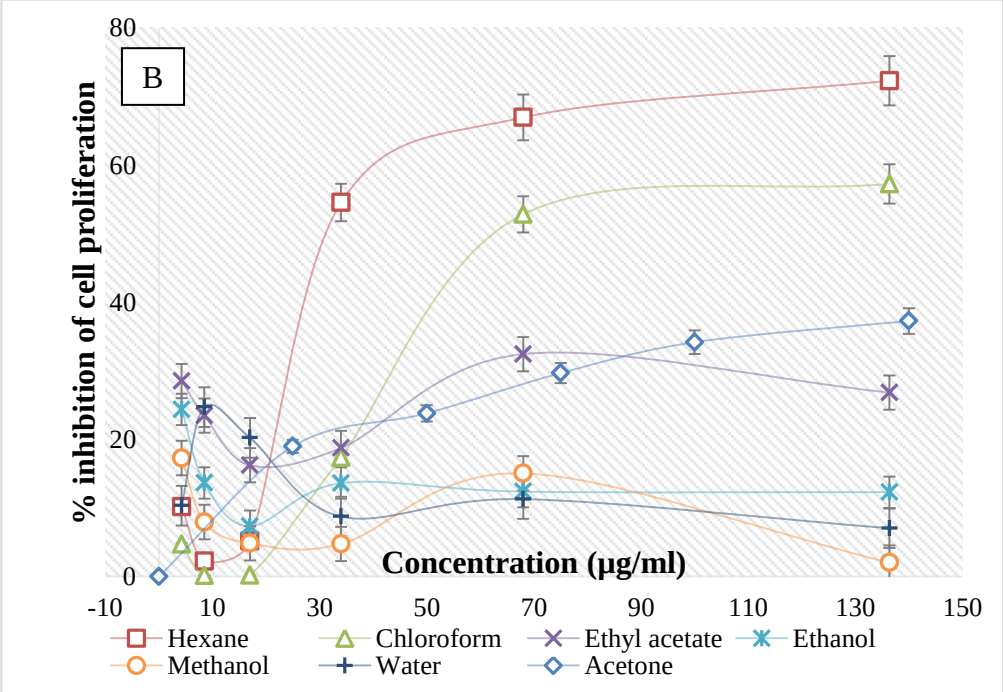


Figure 4.9. Antiproliferative activity of the different extracts using MTT assay

The antiproliferative activity (expressed as % inhibition of cell proliferation) of treated MCF-7 (A) and MDA-MB-231 cells (B) with the different extract. $P < 0.05$ compared to the control (without extract) as tested by the Student's *t*-test.

Figure 4.10 shows the morphology of MCF-7 and MDA-MB-231 cells before and after treatment with the IC_{50} of the hexane extract. In both cell lines, MCF-7 and MDA-MB-231, morphological changes typical of cells undergoing apoptosis, such as shrinkage and blebbing, were observed following the hexane extract treatment at 35 µg/ml for 24 h.

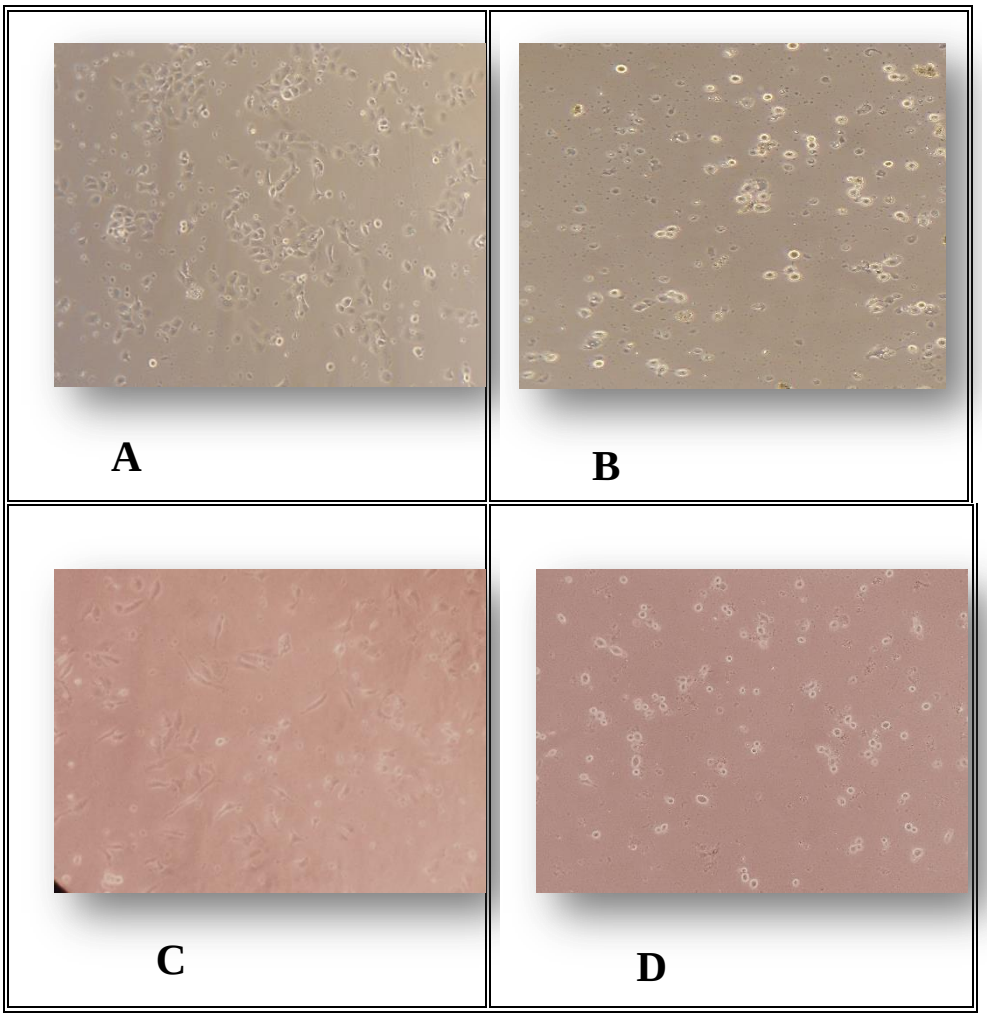


Figure 4.10. Morphology of MCF-7 and MDA-MB-231 cells treated with the hexane extract of *C. cassia*
The morphology of MCF-7 cells in normal media when plated at 70 % confluence in normal media (RPMI-1640, 10 % FBS, 1% antibiotics, and 1 % glutamine) (A). The morphology of MCF-7 cells after being incubated for 24 h in 35 µg/ml (IC_{50}) of the hexane extract (B). The morphology of MDA-MB-231 cells in normal media when plated at 70 % confluence in normal media (DMEM, 10% FBS, 1 % antibiotics, 1 % glutamine) (C). The morphology of MDA-MB-231 cells after being incubated for 24 h in 35 µg/ml (IC_{50}) of the hexane extract (D).

4.6 Reactive Oxygen Species (ROS) Assay

Intracellular ROS generation was evaluated using intracellular peroxide-dependent oxidation of DCFHDA to form fluorescent DCF. H_2O_2 was used as a positive control. Figure 4.11 shows that when MCF-7 cells were treated with the hexane extract, the intracellular ROS increased by 14 % compared to the untreated cells. The highest level of ROS in MDA-MB-231 cells was at a concentration of 16 µg/ml of the hexane extract (16.6 %) compared to the untreated cells.

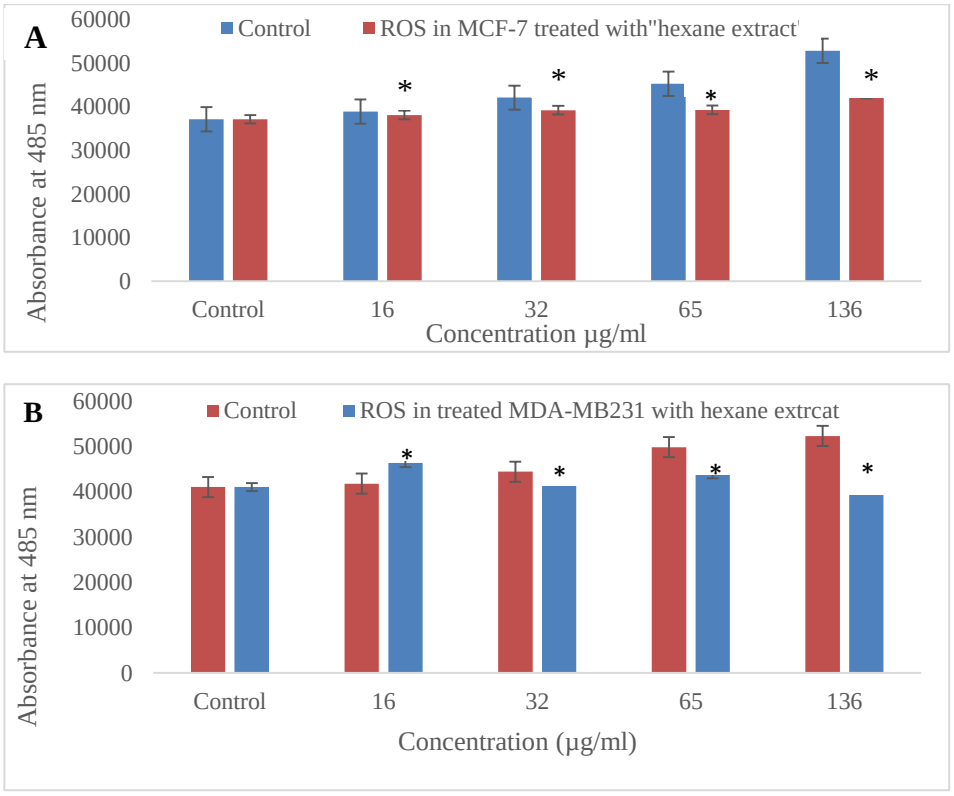


Figure 4.11. Intracellular ROS in MCF-7 and MDA-MB-231cells
Intracellular ROS in MCF-7 cells treated (A) with various concentrations of the hexane extract is dose-dependent manner while, in treated MDA-MB-231cells (B). * indicates significant difference from untreated cells ($p<0.05$). $P < 0.05$ compared to the control (without extract) as tested by the Student's *t*-test.

4.7 Antioxidant Enzyme Assay

The activities of catalase, superoxide dismutase, and glutathione peroxidase in MCF-7 and MDA-MB-231 cells after 0, 6, 9, 15, 24 and 48 h incubation with the IC_{70} concentration of the hexane extract (100µg/ml) are shown in Figure 4.12.

As shown in Figure 4.12 (A), the activity of SOD in MDA-MB-231cells decreased by 31.1 % in 0–48 h, and the activity of GPx (Figure 4.12 (C)) decreased by 38.3 % in 0–48 h incubation. The activity of CAT in Figure 4.12 (B) decreased in 0–9 h then increased in 9–15 h. The CAT activity decreased by 40.2 % between 0–48 h incubation. Also, the data in MCF-7 cells showed that the activity of SOD increased by 37.5 % in 0–6 h. From 9 to 24 h, there was no significant change in the activity of SOD. After 24 h, the activity of SOD increased. The activity of SOD in MCF-7 cells increased by 52 % in 0–48 h, while the activity of GPx decreased by 50 %. The activity of CAT decreased slowly in 0–15 h, and then in 24 h, it increased and then decreased again. In total, the activity of CAT in MCF-7 cells decreased by 62.5 % in 48-h incubation.

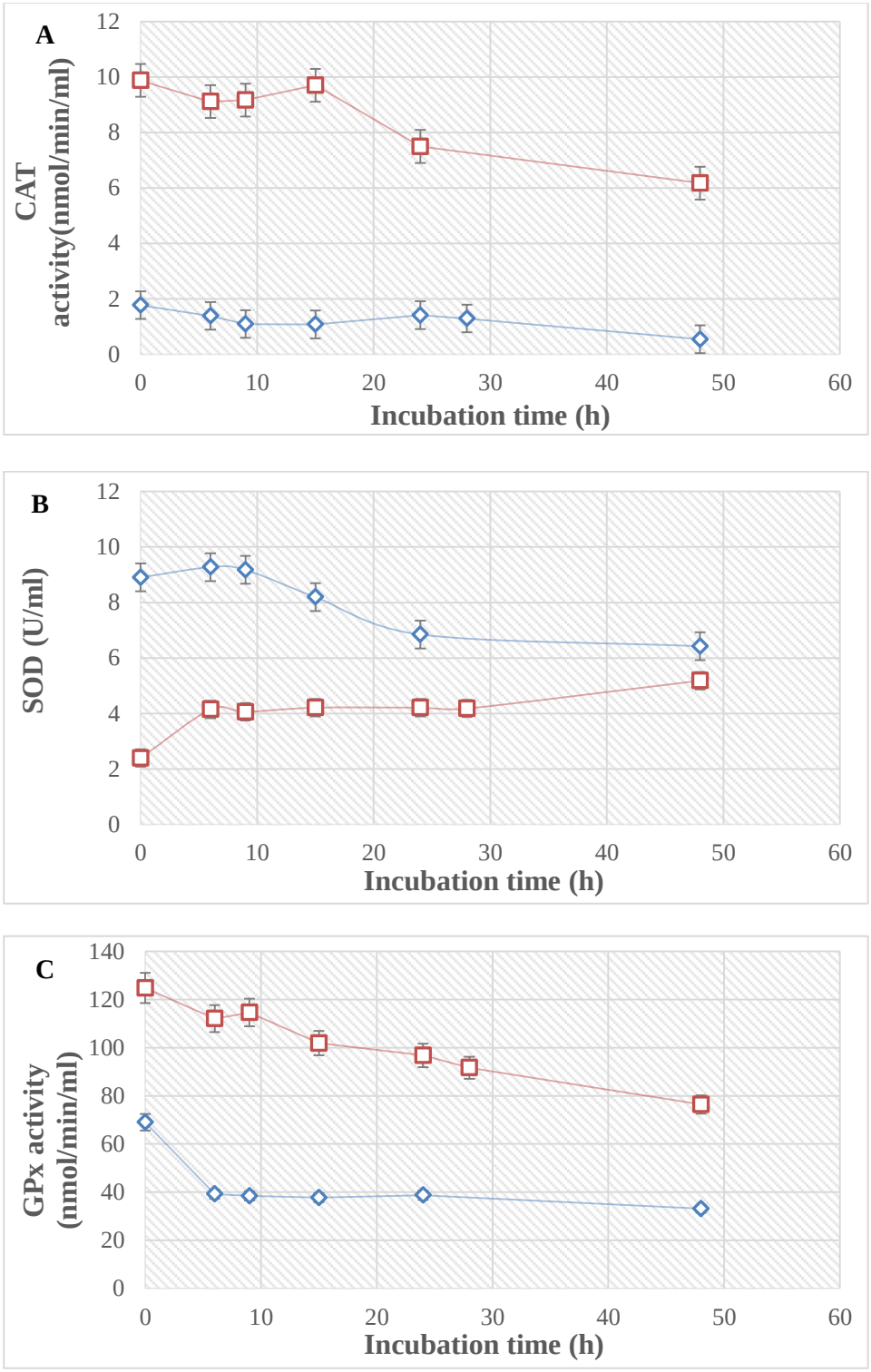


Figure 4.12. Antioxidant enzyme activity in MCF-7 (■) and MDA-MB-231(◆) cells

Activity of catalase (A), superoxide dismutase (B) and glutathione peroxidase (C) was determined using commercial assay kits. Results are expressed as mean ± standard deviation. $P < 0.05$ compared to the control (without extract) as tested by the Student's *t*-test.

4.8 Caspase Activity

To investigate whether (and through which pathways) the antiproliferative activity shown by *C. cassia* induction of apoptosis, the effect of the cinnamon hexane extract on caspase activity was assayed. Caspases-3, -7, -8, and, -9 were assessed.

Figure 4.13 shows the activity of caspase -3/7, -8 and -9 in treated MCF-7 and MDA-MB-231 cells. Mitomycin C and colchicine were used as the controls for the induction of caspase -3 and -8,-9 respectively. As shown in (Figure 4.13 (A)), there was no significant change in the activation of caspase-8 in treated MDA-MB-231 cells with 35µg/ml of the hexane extract during the investigated time points compared to the untreated cells. Mitomycin C as the activator of caspase-8 increased the activation of caspase-8 in a time-dependent manner to a maximum amount at 24 h by 2 folds. However, the activity of caspase-3/7 increased from 2 to 24 h incubation by 7.7 folds compared to the control which is almost 7 times higher than that of colchicine at 24 h. Caspase-9 activity in treated MDA-MB-231 cells, increased in a time-dependent manner to a maximum at 16 h by 3.6 folds and then decreased to 2.3 folds at 48 h. while, the activation of caspase 9 in treated MDA-MB-231 cells with mitomycin C as the activator decreased in a time-dependent manner from 3.2 to 1.2 folds in the investigated time points.

In MCF-7 cells treated with 35µg/ml of the hexane extract of *C. cassia* (Figure 4.13 (B)), the activity of caspase-8 decreased from 2 h to 48 h (3.4 folds to 0.7 folds). But mitomycin C activated caspase-8 from 2 to 48 h incubation by 3 folds. The activity of caspase-7 increased from 2 to 48 h

incubation by 6.7 folds which were almost 2.3 times higher than that of colchicine at 48 h. There was no significant change in the activity of caspase-9 in treated MCF-7 cells in the investigated time point. But the activation of caspase-9 in treated MCF-7 cells with mitomycin C increased from 2 h o 24 h (0.9 to 2.2 folds).

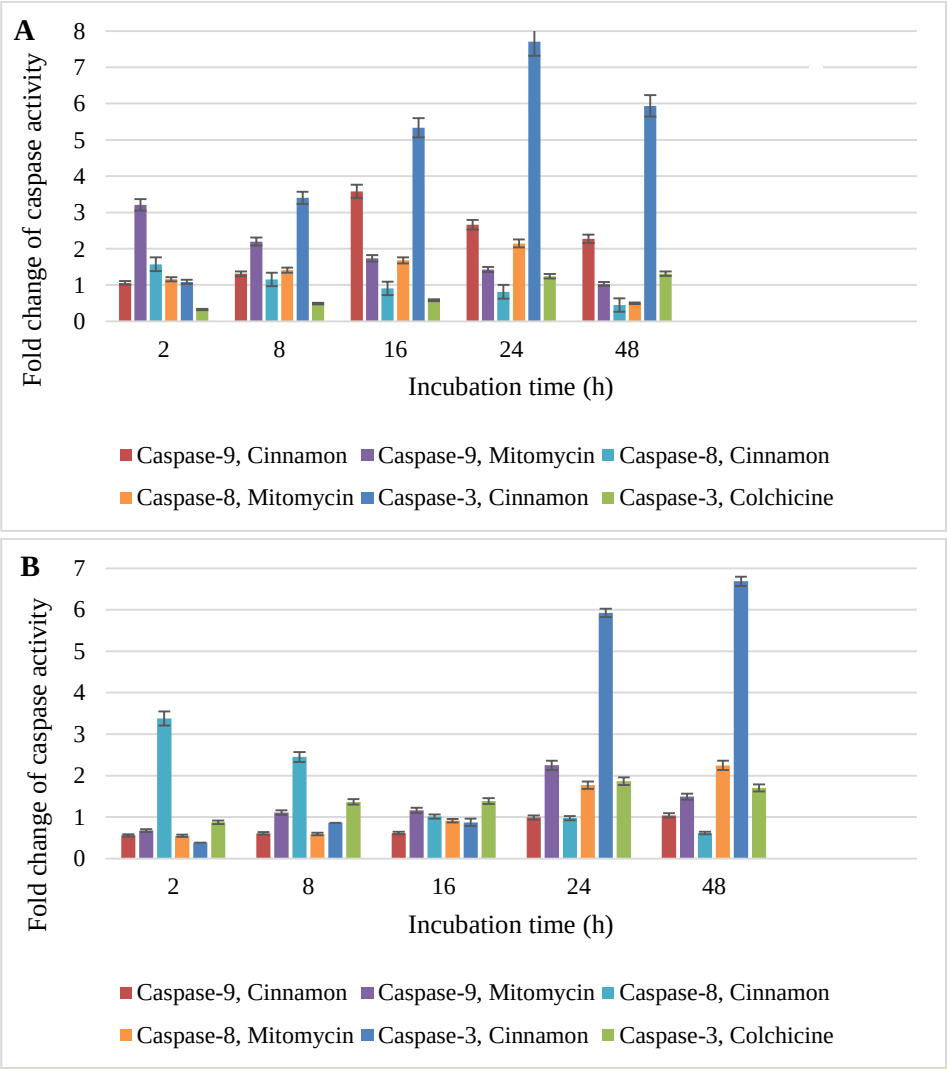


Figure 4.13. Caspase activity in MCF-7 and MDA-MB-231 cells treated with the hexane extract

Figure 4.13(A) indicates the fold change in caspase activity when MDA-MB-231 cells were exposed to 35 µg/ml of the hexane extract. Figure 4.13(B) indicates that the fold change in the caspase activity when MCF-7 cells were exposed to 35 µg/ml of the hexane extract. Results are expressed as mean ± standard deviation. $P < 0.05$ compared to the control (without extract) as tested by the Student's *t*-test.

4.9 Study of Gene Expression by Real Time PCR (RT-PCR)

To further study the pathways involved in the treated MCF-7 and MDA-MB-231 cells, the cells were treated with the IC_{50} concentration of the hexane extract of cinnamon. The expression of Akt1, Bcl2, p53, and Bid was assessed using RT-PCR technique. Figure 4.14 shows that Bcl2, p53, and Bid were significantly overexpressed in treated MDA-MB-231 cells by a mean factor of 2.540, 2.681 and 8.307, respectively. Akt1 was significantly overexpressed in treated MCF-7 cells by a mean factor of 46. The expression of other genes studied; p53, Bcl2, and Bid were not changed significantly.

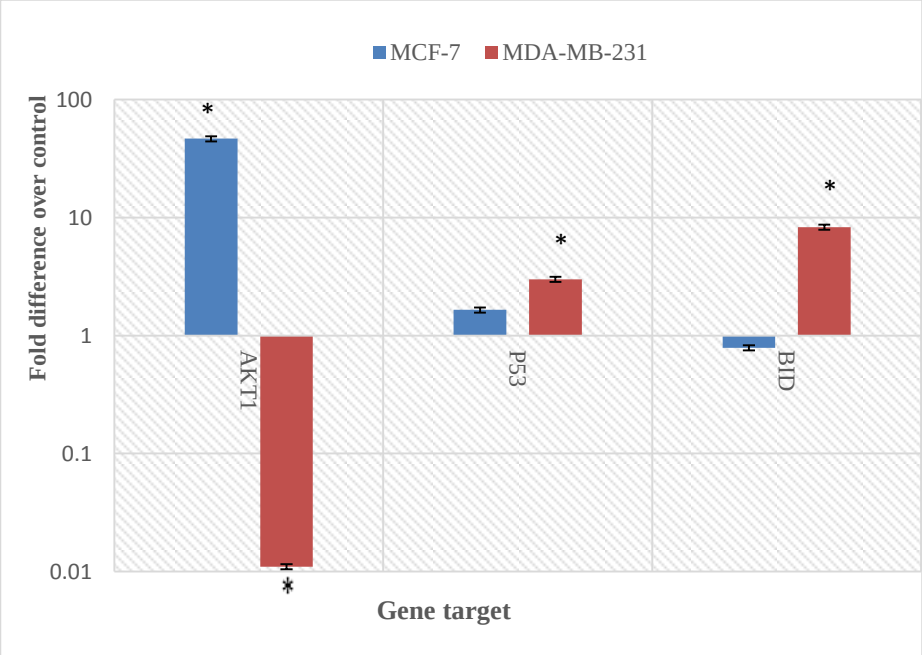


Figure 4.14. Gene expression in treated MCF-7 and MDA-MB-231 cells
Investigation of the expression of p53, Bid, Bcl2, and Akt1 in MCF-7 and MDA-MB-231 cells treated with the IC_{50} of the hexane extract in 24 h. Results are expressed as fold variation over carrier control (blank). Results are expressed as mean ± standard deviation. Statistical significance was calculated based on the mean ΔCt values by the Student's t test. *Indicates significant differences from untreated cells ($p < 0.05$).

4.10 Isolation of Coumarin and *Trans*-Cinnamaldehyde by HPLC and GC-MS

The semi-preparative HPLC chromatography of the hexane extract of *C. cassia* showed two major peaks at retention time 5.831 min and 9.301 min (Figure 4.16). The hexane fractions were subjected to GC-MS analysis and the total ion chromatography (Figure 4.17) showed 2 peaks: identified as coumarin (peak **1**, retention time 34.641 min) and *trans*-cinnamaldehyde (peak **2**, retention time 27.229 min) from their mass spectral data by comparison with those available in the accompanying W9N11 library of database (http://www.sisweb.com/software/ms/wiley.htm). Figure 4.15 shows the chemical structure of *trans*-cinnamaldehyde and coumarin.

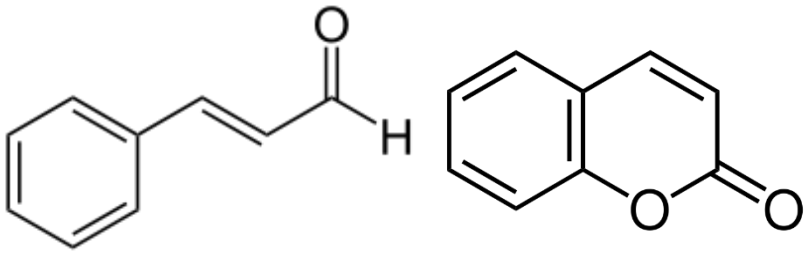


Figure 4.15. Chemical structure of the two main compounds of the hexane extract
Trans-cinnamaldehyde (left) and coumarin (right).

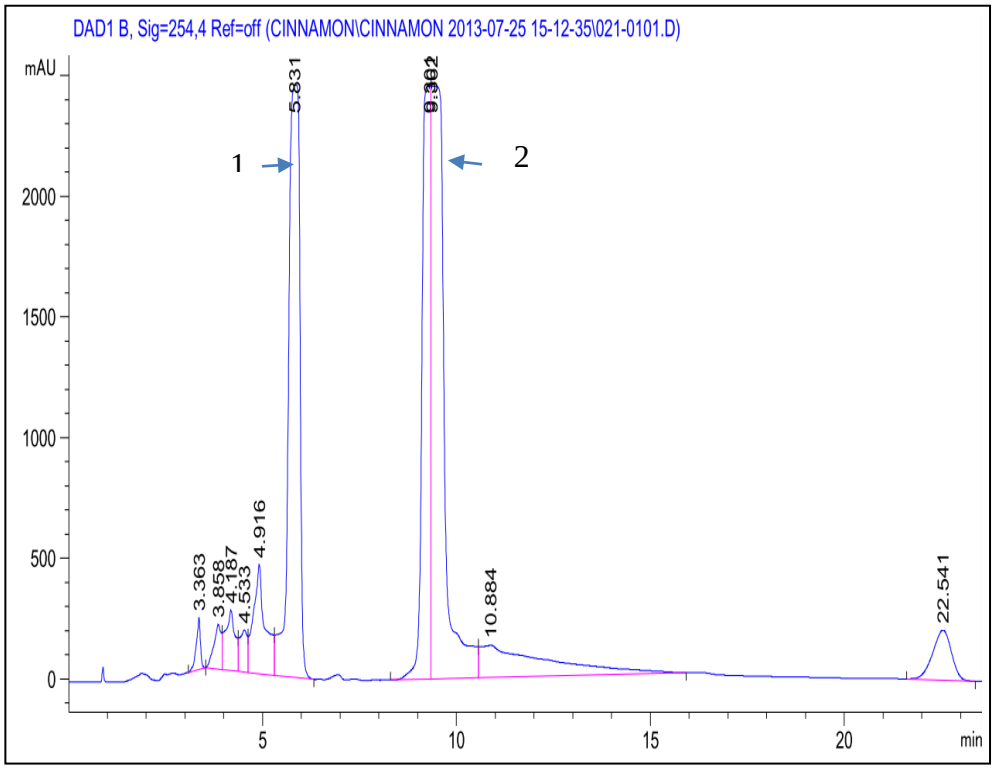


Figure 4.16. Semi-preparative HPLC chromatography of the hexane extract at 254 nm

The two isolated peaks were identified as coumarin (1) and *trans*-cinnamaldehyde (2) through mass spectral library

Source: (http://www.sisweb.com/software/ms/wiley.htm)

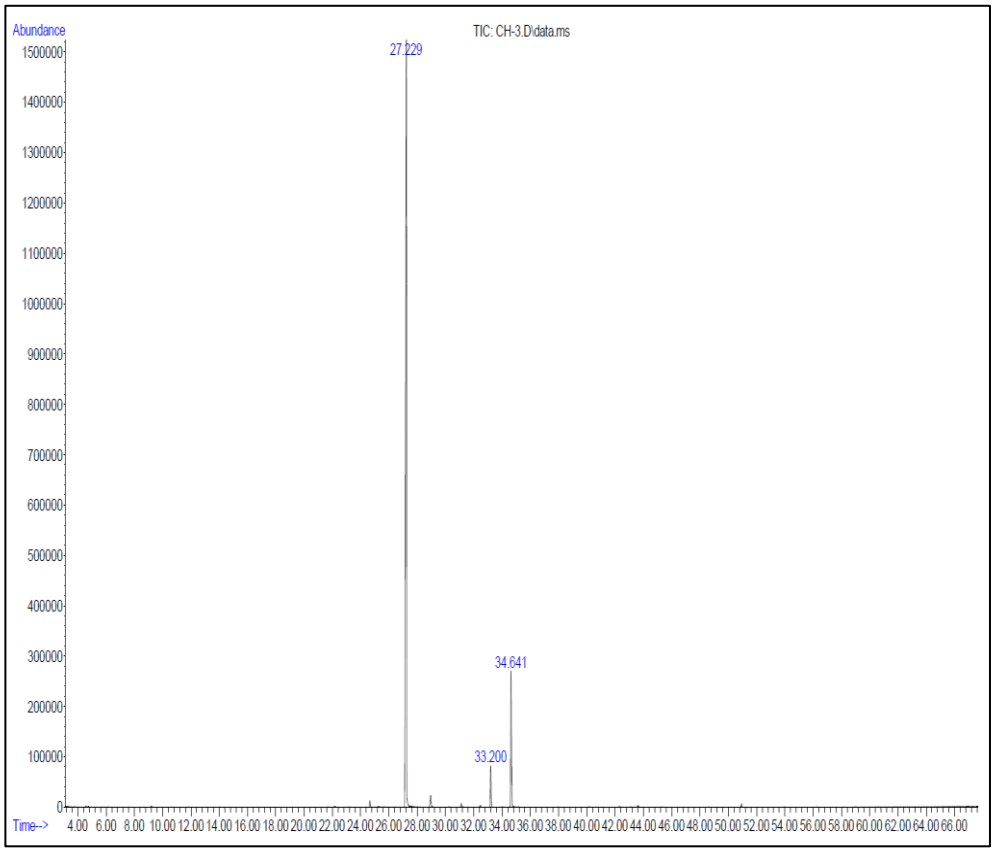


Figure 4.17. GC-MS total ion chromatography profile of the hexane extract

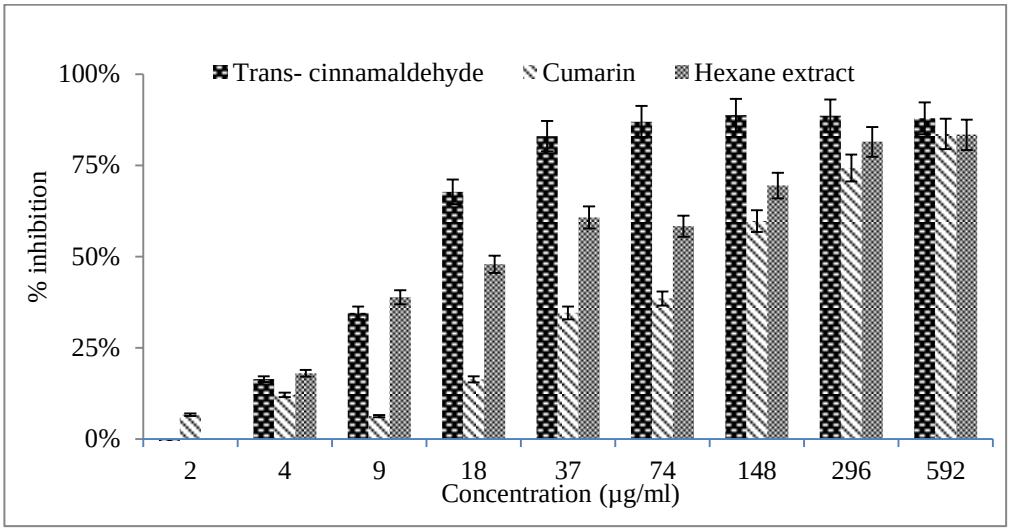
The two major peaks were identified as coumarin (1) and *trans*-cinnamaldehyde (2)

Source: Wiley 9th edition NIST11 Mass Spectral Library, USA.

4.10.1 *In vitro* cell antiproliferative activity of *trans*-cinnamaldehyde and coumarin

Figure 4.18 shows that the antiproliferative activity of the hexane extract containing trans-cinnamaldehyde and coumarin increased in a dose-dependent manner in both treated MCF-7 and MDA-MB-231 cells. As shown in Figure 4.18, and Table 4.7, the hexane extract of *C. cassia*, *trans*-cinnamaldehyde and coumarin revealed different antiproliferative activities in MCF-7 and MDA-MB-231cells. *Trans*-cinnamaldehyde with an IC_{50} values of 9.61 ± 0.07 µg/ml and 14.21 ± 0.02 µg/ml in MCF-7 and MDA-MB-231, respectively exhibited strongest cytotoxicity compared to the hexane extract and coumarin. The hexane extract of *C. cassia* showed an IC_{50} values of 33.42 ± 0.06 and 34 ± 0.07 µg/ml in MCF-7, in MDA-MB-231 cells showed the values were slightly weaker activity than MCF-7 cells. Coumarin was the weakest cytotoxic compound in our test system with an IC_{50} value of 107.98 ± 0.05 µg/ml in MDA-MB-231 cells and 98.15 ± 0.04 µg/ml in MCF-7 cells.

A



B

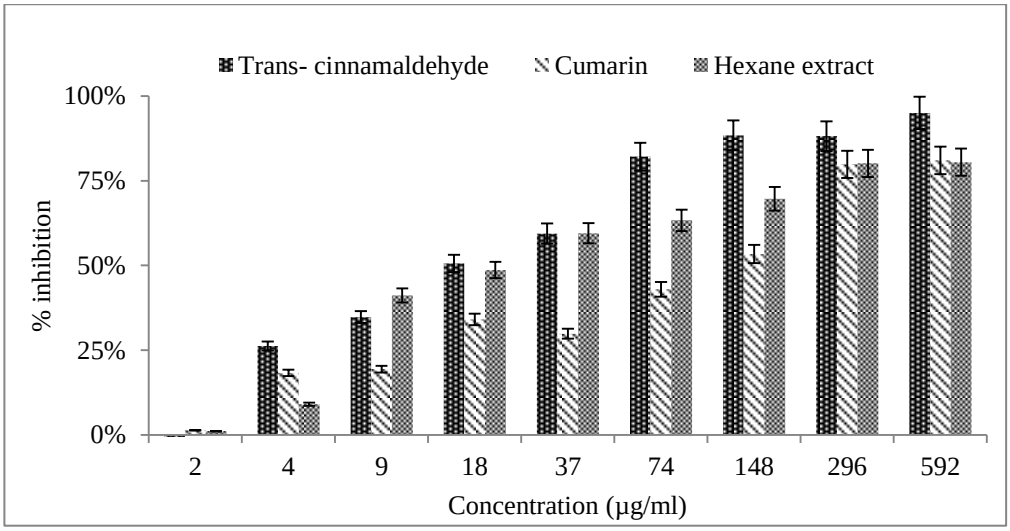


Figure 4.18. Antiproliferative effect of the hexane extract containing *trans*-cinnamaldehyde and coumarin, in MDA-MB-231, MCF-7 cells

Values are expressed as means ± SD of three independent experiments.

Figure A indicates the % inhibition when MDA-MB-231cells were exposed to the various concentrations of the hexane extract and *trans*-cinnamaldehyde and coumarin. Figure B indicates the % inhibition when MCF-7 cells were exposed to the various concentrations of the hexane extract and *trans*-cinnamaldehyde and coumarin. $P < 0.05$ compared to the control (without extract) as tested by the Student's *t*-test.

Table 4.7. IC_{50} values of the hexane extract and two main fractions in MCF-7 and MDA-MB-231 cells

Treatment	MDA-MB-231 cells	MCF-7 cells
	(IC_{50}) µg/ml	**(IC_{50}) µg/ml**
Hexane extract	34 ± 0.07	33.42 ± 0.06
***Trans*-cinnamaldehyde**	14.21 ± 0.02	9.61 ± 0.07
Coumarin	107.98 ± 0.05	98.15 ± 0.04

Antiproliferative activities of the hexane extract, and its components, *trans*-cinnamaldehyde and coumarin in the two breast cancer cell lines are presented. Results are expressed as mean ± std. dev. (n=3). IC_{50} is defined as concentration of plant extracts that inhibited 50 % growth of the cells.

Chapter V: DISCUSSION

5.1 Extraction

The schematic diagram of the extraction and the total yield values of the seven extracts of *C. cassia* barks under analysis are given in Figure 5.1.

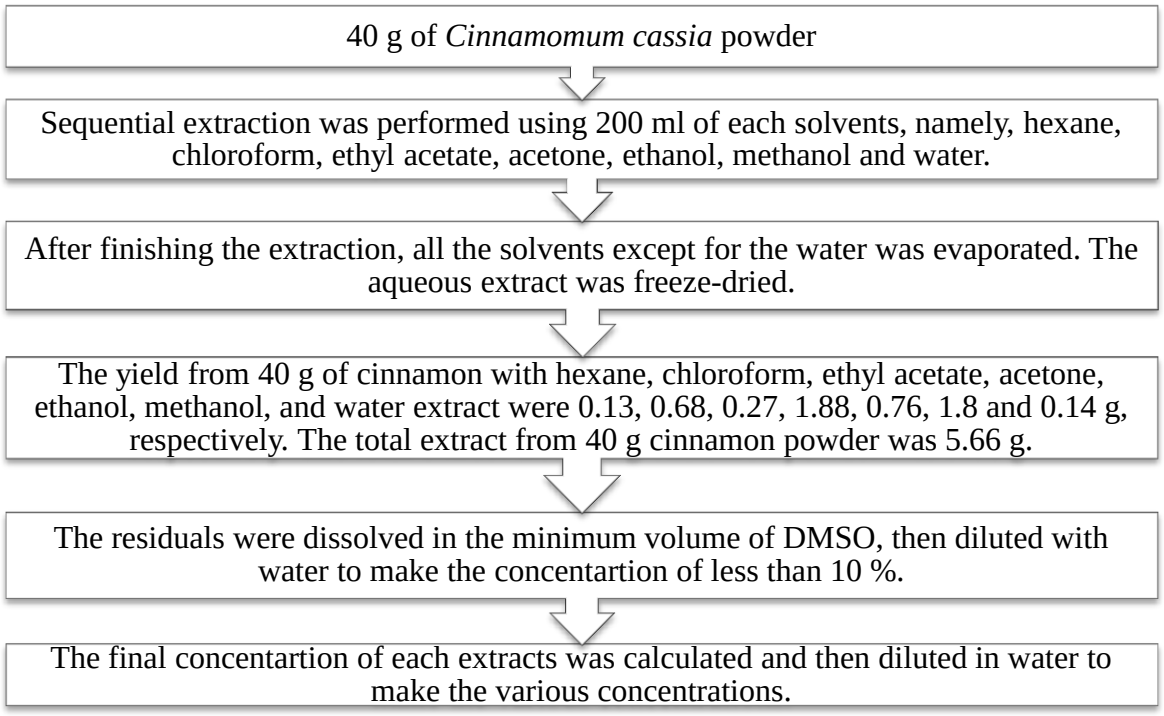


Figure 5.1. Schematic diagram representation extraction and solvent partition of bark of *C. cassia*

The solvents used for the extraction of *C. cassia* were selected based on their polarity: nonpolar (hexane, chloroform), polar-aprotic (ethyl acetate, acetone), and polar-protic (ethanol, methanol, water). Polar aprotic solvents are those polar solvents which do not form hydrogen bonding, while the polar-protic ones do form hydrogen bonding. Table 4.1 and Figure 5.2 indicate that 5.66 g (14.119 %) of the 40 g of cinnamon powder were

dissolved in the seven solvents used. From 5.66 g which was dissolved in the seven solvents, 0.81 g was extracted by the non-polar solvents (hexane and chloroform), 2.15 g was extracted by aprotic-solvents (ethyl acetate and acetone) and the rest (2.7 g) was dissolved in protic-solvents (ethanol, methanol, and water). Acetone, as a polar-aprotic solvent, was the most powerful solvent in dissolving the compounds of the cinnamon which dissolved 4.69 % of the compound of 40 g cinnamon powder, followed by methanol as the second most polar-aprotic solvent which dissolved 4.49 % of the compound of 40 g cinnamon powder, while hexane was the most nonpolar solvent, only dissolving 0.32 % of the 40 g of cinnamon. This shows that most of the components in the cinnamon sample were polar substances. The data shows that 5.35 % of the extracted ingredients of cinnamon were protic- polar and 6.74 % were aprotic-polar and the rest (2.02 %) were non-polar.

The sequential extractions provided information relating to the nature of the compounds in the cinnamon powder. The predominant polar compounds, as reported in the literature, are *trans*-cinnamaldehyde (45.13 %), cinnamyl alcohol (8.21 %), eugenol, methyl eugenol (5.23 %), and also different alcohols, aldehydes, acids, and terpenes, amounting to 1% to 2 %.

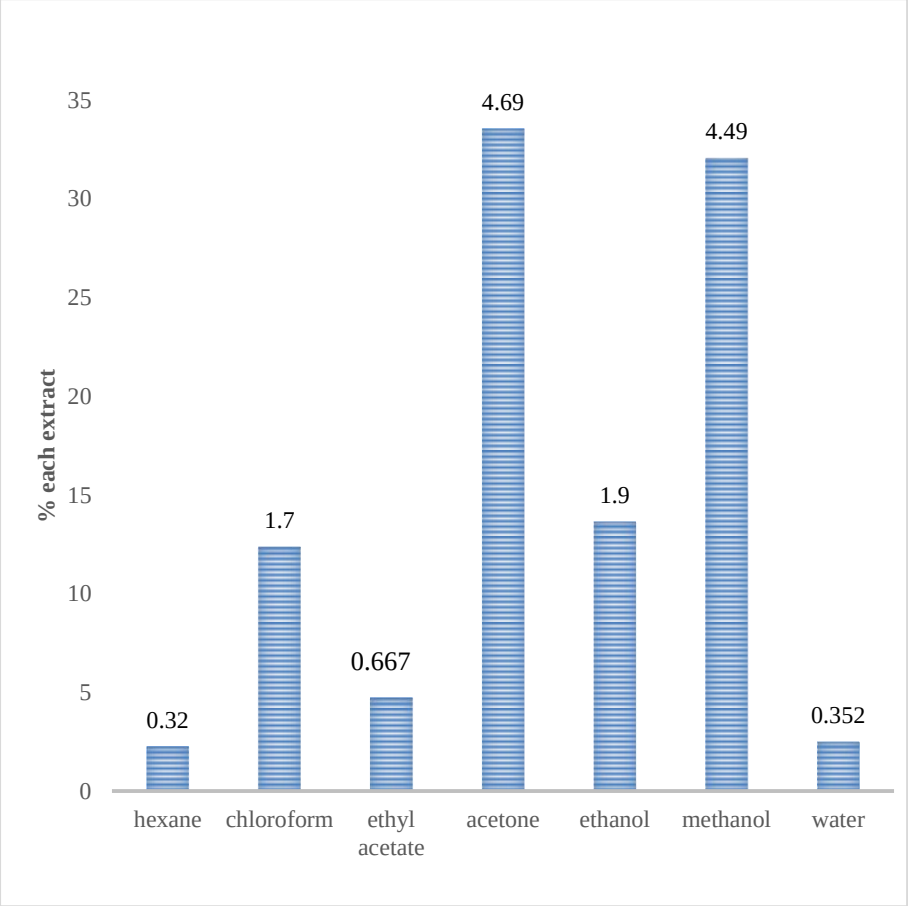


Figure 5.2. The % yield amount of seven extracts of 40 g *Cinnamomum cassia*
The percentage of each extracts were calculated according to the Table 4.1.

5.2 Antioxidant activity

Antioxidants have the ability to delay or inhibit the oxidation processes, which occur under the influence of many types of ROS (Valko *et. al.*, 2006). In the defence system of the organism, antioxidants are active to attack free radicals. In laboratory techniques, a variety of *in vitro* chemical methods such as spectrometry, chromatography, and electrochemical techniques (Stah &Viiia-Ribes, 1998) are used to evaluate the antioxidant activity of products and ingredients. The methods applied to determine the antioxidant capacity and activity of a sample are listed below. These methods differ in the mechanism of generation of different radical species and/or target molecules and in the way end

products are measured. Some antioxidant activity methods which were used in this study are as follows,

1. Ferric reducing antioxidant power (FRAP)
2. 1, 1- diphenyl-2-picryl hydrazyl radical (DPPH)
3. Super oxide anion radical scavenging assay
4. Hydroxyl radical scavenging assay
5. Nitric oxide radical scavenging assay

5.2.1 Ferric Reducing Antioxidant Power (FRAP) Assay

Figure 4.2 and Table 4.2 show that the polar-aprotic components which were dissolved in the acetone and ethyl acetate had the highest antioxidant ability in reducing ferric ions. Then the polar-protic components which were dissolved in the ethanol, methanol and water were potent in antioxidant ability in reducing ferric ions. The nonpolar components which were dissolved in the hexane and chloroform were not potent in the reduction of ferric ions. The power of the acetone extract in reducing ferric tripyridyl (Fe^{+3}) to ferrous form (Fe^{+2}), which was measured by the change of absorbance at 593 nm, was significant. The FRAP value of the acetone extract was almost half of quercetin (6013 ± 0.1 mmol Fe^{+2}/100 mg of dried weight). However, quercetin is a pure flavonoid, whereas the cinnamon extract used was a mixture of many components. The acetone extract had a FRAP value much higher than that of rutin value (2008.9 ± 0.02 mmol Fe^{+2}/100 mg of dried weight). Rutin is also a pure flavonoid. In one study which investigated the antioxidant activity of three Chinese medical plants (*Cinnamomum cassia, Curcuma longa, Coptidis rhizome*), the acetone extract of *C. cassia* showed the highest FRAP value of 17-33 μmol l^{-1} compared to the other studied plant (Jang *et. al.*, 2007). In that study, it was shown that these three plants had a high content of cinnamaldehyde. The higher amount of

cinnamaldehyde in *C. cassia* could lead to high FRAP value and antioxidant activity in *C. cassia*. Although in this study, the hexane extract of *C. cassia* which showed a high content of cinnamaldehyde (Figure 4.16 and 4.17), did not have a high FRAP value. The hexane extract had the lowest ability for reducing the ferric ions. The FRAP value of *C. cassia* extract was in the range of 27.2 ± 0.00 to 3150.5 ± 0.14 mmol Fe^{+2}/100 mg of dried weight as shown in Figure 4.1.

5.2.2 Superoxide Anion Radical Scavenging Assay

Superoxide anion is considered one of the most harmful species to cellular components. Flavonoids are effective antioxidants mainly because they scavenge superoxide anions. In this assay, superoxide radical reduces NBT to a blue colored formazon that is measured at 56 nm (Nishikili *et. al.*, 1972).

Figure 4.2 and the data listed in Table 4.3 indicated that cinnamon was not as strong in scavenging superoxide anion radicals compared to the pure flavonoid, rutin (IC_{50} = 9.1 ± 2.54 µg/ml). The IC_{50} value of the chloroform extract, 121 ± 1.98 µg/ml, was the lowest among the IC_{50} values of the other extracts. However, Prasad et al. (2009) showed that all *Cinnamomum* species exhibited excellent superoxide anion scavenging activity, higher than that obtained of 2, 6-di-tert-butyl-4-methyl phenol (BHT) which was used as the positive control. They showed that among 5 different species of *cinnamomum*, namely, *C. burmanni, C. cassia, C. pauciflorum, C. tamala and C. zeylanica*, *C. cassia* had the weakest activity in reducing superoxide anion radicals. At the highest concentration used (100 µg/ml), the superoxide scavenging activities of *C. burmanni, C. cassia, C. pauciflorum, C. tamala, C. zeylanicum* and BHT were 74.5 ± 0.9, 74.2 ± 0.4, 77.3 ± 2.9, 87.2 ± 1.9, 79.1 ± 0.4 and 25 ± 0.5 %, respectively.

Figure 4.2 showed, at 100 μg/ml, the superoxide scavenging activities of the chloroform extract of *C. cassia* bark was 57.6 ± 1.4 % which was lower than that of the methanol extract of *C. cassia* leaves (79.1 ± 0.4) which was measured by Yang et al. (2012) at the same concentration. These different results could be due to the higher extract yield of the leaves compared to that obtained of the barks. They investigated several antioxidant activity of the ethanol extract of the leaves, buds and barks of *C. cassia.* They showed that the ethanol extract yield of the buds, barks and leaves were 6.85, 12.73 and 23.02 %, respectively.

In this study, the results of HPLC and GC-MS showed that the chloroform extract had the high level of cinnamaldehyde and other flavonoids (data not shown). Phenolic compounds such as flavonoids, phenolic acid and tannins have diverse biological activities which might be related to their antioxidant activity (Tsai *et. al*, 2008). It was reported that the superoxide anion scavenging activity could be affected by the action of a free hydroxyl group of phenolic compounds (Siddhuraju *et. al.*, 2002).

The type of flavonoid molecule could have an effect on the activities of scavenging of superoxide anion radicals. For instance, it was shown that flavonoid molecules with polyhydroxylated substitution on ring A or B and a free 3-hydroxyl substitution show superoxide scavenging activity (Bravo, 1988; Siddhuraju *et. al.*, 2002).

5.2.3 DPPH Scavenging Assay

The effect of antioxidant on DPPH radical scavenging is due to their hydrogen donating ability or radical scavenging activity. When a solution of DPPH is exposed with a substance that can donate a hydrogen atom, it loses of this violet color. So, DPPH assay was used to evaluate the ability of antioxidants to scavenge quenched DPPH radical.

Figure 4.3 and the data listed in Table 4.3 indicated that the cinnamon extract was almost as potent as the pure flavonoids, quercetin and rutin, in scavenging the DPPH radical. The lowest IC_{50} of 69.35 ± 2.23 µg/ml belonged to the ethyl acetate compared to the other extracts of *C. cassia* (Table 4.3). Also, Prasad et al. (2009) studied the antioxidant activities of of five species of *Cinnamomum* leaf, namely *C. burmanni, C. cassia, C. pauciflorum, C. tamala and C. zeylanicum.* Next to *C. zeylanicum* which had the lowest IC_{50} (30 ± 0.06 µg/ml) to scavenge of DPPH radicals, *C. cassia* had the lowest IC_{50} (55 ± 0.03 µg/ml) compared to the other *Cinnamomum* species.

The antioxidant activity of the extracts strongly depends on the extraction solvent. The DPPH radical-scavenging activity of *C. cassia* was close to the results reported by Steenkamp et al. (2005), Tomaino et al. (2005) and Jang et al. (2007). Also, the yielded IC_{50} value of the ethanol extract of *C. cassia* in another study was 72 ± 0.208 µg/ml, while the IC_{50} value of the standard BHT was 27 µg/ml in this study (Yang *et. al.,* 2012). Also, in cinnamon, 80 % methanol indicated to be a better solvent for extracting DPPH radical-scavengers than the acetone with an IC_{50} of 10 µg/ml. Cinnamon had the great capacity to scavenging DPPH (Su *et. al.,* 2007).

Phenolic compounds such as flavonoids have a potent antioxidant activity (Tsai *et. al,* 2008). Parsad et al. (2005) and Zhao et al. (2006) reported that phenolics and flavonoids scavenge DPPH radicals by their hydrogen donating ability. The results obtained in this study showed that the DPPH radical scavenging activities of *C. cassia* might be attributed to the hydrogen donating ability because of the high total phenolic contents in the ethyl acetate extract (346.25 ± 0.18 mg GAE/ 100 g DW) which is comparable with the pure flavonoid, rutin which had the TPC value of 433 ± 0.18 mg GAE/ 100 g DW.

5.2.4 Nitric Oxide Radical Scavenging Assay

It is well-known that nitric oxide (NO^{-}) has an important role in various types of inflammatory processes in the animal body. The scavenging NO^{-} activity may help in arresting the chain of reactions initiated by excess generation of NO^{-} that are detrimental to human health. It is a potent pleiotropic inhibitor of physiological processes, such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation, and regulation of cell-mediated toxicity (Mufti *et. al.,* 2011). It is a diffusible free radical that plays many roles as an effector molecule in diverse biological systems.

Each sample under the assay conditions showed increase of radical scavenging activity in a dose-dependent manner (Figure 4.4). The hexane and water extracts at 22 µg/ml had the same nitric oxide scavenging activity (19.2 %), then the hexane extract scavenged and suppressed the release of NO^{-} more powerfully than the other extracts and showed the lowest IC_{50} value (89.5 ± 2.3 µg/ml). The nitric oxide scavenging capacity of cinnamon was more potent by 1.34 and 1.16 times than that obtained of the pure flavonoids, quercetin and rutin (120.1 ± 2.13 and 104.3 ± 1.88 µg/ml, respectively). The results showed that *C. cassia* had potent activity in scavenging of the nitric oxide radical.

The results analyzed by HPLC and GC-MS (Figure 4.16 and 4.17) showed that the hexane extract of *C.cassia* had 2 major peaks, namely, *trans*-cinnamaldehyde and coumarin (polyphenols).

In the literature, it has been shown that the polyphenol of cinnamaldehyde has shown potent antioxidant activity in scavenging of nitric oxide radical with and IC_{50} value of 437 µg/ml which was compared with the standards like ascorbic acid with an IC_{50} of 342 µg/ml and butylated hydroxy toluene (BHT) with an IC_{50} of 310 µg/ml (D haripriya & Vijayalakshimi, 2013). Also, Miguel (2010) showed that cinnamaldehyde had high radical scavenging activities using several antioxidant tests such as nitric oxide, hydroxyl and

DPPH radical scavenging assays. Cinnamaldehyde displayed strong antioxidant activity towards nitric oxide radicals. Therefore, the high ability of the hexane extract with an IC_{50} of 89.5 ± 2.3 µg/ml could due to a high concentration of cinnamaldehyde and coumarin in this extract.

5.2.5 Hydroxyl Radical Scavenging Assay

Hydroxyl radicals can be considered as the major active oxygen species causing lipid oxidation and enormous biological damage (Gutteridge & Halliwell, 1993). In this assay, free radicals were generated from H_2O_2 in the presence of Fe^{+3}-EDTA at pH 7.4.

Figure 4.5 and the data listed in Table 4.3 indicated that the ethyl acetate extract of *C. cassia* was the most potent in scavenging hydroxyl radicals (IC_{50}, 112.77 ± 2.14 µg/ml) compared to the other extracts which was 4 times higher than those obtained of quercetin and rutin (25.8 ± 2.3 and 26.2 ± 3.01 µg/ml, respectively). Cinnamon was not much potent to scavenge the hydroxyl radical compared to the rutin and quercetin. Md et al. (2013), indicated that cinnamon bark showed the minimum hydroxyl radical scavenging activity (18.18 ± 10.91 µg/ml) compared to the other studied spices such as, fenugreek seeds (*Trigonella foenum*), mustard seeds (*Brassica nigra*), black pepper (*Piper nigram*), poppy seeds (*Papaver somniferum*), coriander seeds (*Coriandrum sativum*), and cumin seeds (*Cuminum cyminum*). The hydroxyl radical scavenging activity of spices that were examined ranged from 18.84 µg/ml to 222.90 µg/ml. In that study, all other selected spices for the study except cinnamon bark showed higher antioxidant activity than that of ascorbic acid.

The scavenging activities of phenolic substances might be due to the active hydrogen donating ability of hydroxyl substitutions (Mathew & Abraham, 2006). Also,

flavonoids are known to be excellent scavengers of oxygen free radicals such as nitric oxide (Vanacker *et. al.,* 1995).

The ethyl acetate next to the acetone extract had the highest flavonoid content (Table 4.3). The TPC and TFC yield of the ethyl acetate were 346.25 ± o.18 mg GAE/ 100 g DW and 552.48 ± 0.18 mg QE/100 g DW, respectively. The highest nitric oxide radical activity of the ethyl acetate extract could be due to the highest yields of the total flavonoids and phenolic content in this extract compared to the other extracts.

The results obtained indicated that the polar-aprotic compounds of *C. cassia* had the highest hydroxyl radical scavenging activity compared to the other compounds. Also, hydroxyl radical scavenging by the extracts were very similar to the scavenging of DPPH by the same extracts. This finding showed that non-polar components which were extracted by these two non-polar solvents had no activity in scavenging hydroxyl and DPPH radicals. This similarity was supported by previous reports (Prakash *et. al.,* 2012).

5.3 Total Antioxidant Content (TPC and TFC)

The methods used for determination of antioxidant content of the extracts are based on estimation by colorimetric assays. Phenolic compounds are thought to be able to regenerate ά tocopherol in the phospholipid bilayer or lipoproteins particles back to its active oxidant form; the results were reported as gallic acid equivalent (mg GAE/ 100 g DW). The results of Figure 4.4 and Table 4.4 showed that the acetone extract had the highest TPC value (363.32 ± 0.32 mg GAE/ 100 g DW) compared to the other extracts which was similar to that of the ethyl acetate extract (346.25 ± 0.18 mg GAE/ 100 g DW) and there was not a significant difference between the TPC values of the acetone extract of *C. cassia* and the pure flavonoid, rutin (433 ± 3.01 mg GAE/ 100 g DW). This finding indicated that *C. cassia* was a good source of phenolic components. Yang et al. (2012),

reported the phenolic content of the ethanol extract of the barks (9.534 g GAE/100 g DW), followed by the leaves (8.854 g GAE/100 g DW) and buds (6.313 g GAE/100 g DW) of *C. cassia.* They resulted the bark of *C. cassia* had a highest content of phenolic content compared to that obtained for the leaves and buds.

Jang et al. (2007) showed that the total phenolic content of the fresh acetone and methanol extracts *C. cassia* was 9.6 and 7.1 mM l^{-1} GAE, respectively which was the highest among the studied herbs. Also, Su et al. (2007) stated that the 50 % acetone extract of cinnamon contained a high level of phenolic groups. Therefore, acetone is an efficient solvent to obtain a high content of the polyphenolics from *C. cassia.*

The method which was adapted for TFC assay was based on spectrophotometric studies on aluminum chloride complex formation. Flavonoids are polyphenol compounds that occur ubiquitously in plant tissues in relatively high concentrations and their functions in plants are believed to be as protective agents against microorganisms. They are of particular importance in the human diet as they act as antioxidants and as antiviral agents (Hsieh *et. al.,* 2012). Table 4.4 and Figure 4.4 showed that the acetone extract had the highest TFC value (569.43 ± 0.28 mg QE/ 100 g DW) compared to the other extracts which was similar to that of the ethyl acetate extract (569.43 ± 0.28 mg QE/ 100 g DW). Yang et al. (2012), reported the phenolic content of the ethanol extract of the barks (2.3 g QE/100 g DW), followed by the leaves (3.34 g QE/100 g DW) and buds (2.69 g QE/100 g DW) of *C. cassia.*

The results showed that the polar-aprotic solvents (ethyl acetate and acetone) were the most effective solvents in dissolving the total phenolic and flavonoid compounds compared to the other solvents used to extract of *C.cassia* bark.

5.3.1 The correlation of the TPC, TFC and FRAP values of the Extracts

5.3.1.1 The correlation of TPC and TFC

Figure 4.7 and Table 4.5 showed that the TFC value was higher than the TPC value which was in contrast to that reported by Yang et al. (2012). They showed that the total phenolic content was almost about two times more than total flavonoid content value for *C. cassia* extract. Biglari et al. (2009) reported there was a strong positive relationship (R^2= 0.99) between TPC and TFC of *Pheonix dactylifera*. They resulted the studied fruits with a high antioxidant capacity generally contain more antioxidant compounds and most of these were shown to be phenolic compounds and in particular flavonoids.

It was shown that medicinal plants have higher total flavonoid content than total phenolic content which supports our findings (Sultana & Anwar, 2008). In this study, the total amounts of TPC and TFC of each group (non-polar, polar-aprotic, and polar-protic) showed that the highest TPC belonged to the polar aprotic extracts with 709 mg GAE/100 g of DW, while the TFC for these extracts is 1.121 QE mg/100 g of DW. In polar-protic group, the TPC and TFC values obtained 489 GAE/100 g of DW and 1030 QE mg/100, respectively. These results showed that the total flavonoid content in these two groups of the extracts of cinnamon was much higher than the total phenol content.

5.3.1.2 The correlation of TPC, TFC and FRAP

Among the extracts of cinnamon, the acetone extract had the highest ability of reducing Fe^{+3} to Fe^{+2} compared to the other extracts. The FRAP value of this extract was almost half of that of quercetin, the positive control. Also, this extract showed the highest amount of total phenolic and flavonoid contents compared to the other extracts.

The results (Figure 4.6, Figure 4.7 and Table 4.4) indicated that *C. cassia* containing high phenolics and flavonoid content may provide a source of dietary anti-oxidants. Also,

the correlation between FRAP and TPC (R^2=0.887) was higher than that of TFC (R^2=0.849). The significant positive correlation between TPC and FRAP in medicinal plants indicated in previous studies (Kaur & Kapoor, 2002; Dodonne *et. al.,* 2009).

This suggests that phenolic compounds are the major contributors to the antioxidant properties of this bark. It was shown that *M. tanarius* with a FRAP value of 12.3 ± 0.7 mg GAE/g was linearly correlated to the TPC values, where the squares of the correlation coefficients was 0.9366 (Lim *et. al.,* 2009). In another study**,** a significant and positive high Pearson's correlations TPC and FRAP assay (R^2 = 0.91) was observed for *Polygonum minus*, *Zingiber officinale*, and *Curcuma longa* extracts (Maizura *et. al.,* 2011). A high significant positive correlation was obtained for TPC with FRAP (R^2 = 1.00) in Thai rice strains which showed a higher polyphenolic content than Chinese or Sri Lankan ones (Sompong *et. al.,* 2011). In all these studies, the strong positive correlation between TPC, TFC and, FRAP indicated that phenolic compounds were the main contributor of antioxidant activity in plants.

5.4 Protection against DNA damage (comet assay)

Oxidative DNA damage induced by reactive oxygen species (ROS) and free radicals is important in many diseases, such as cancer, muscle degeneration, heart disease and ageing. Hydrogen peroxide (H_2O_2), causes DNA damage and induces apoptosis and necrosis through the activation of caspase-3 in cells (How *et. al.,* 2013).

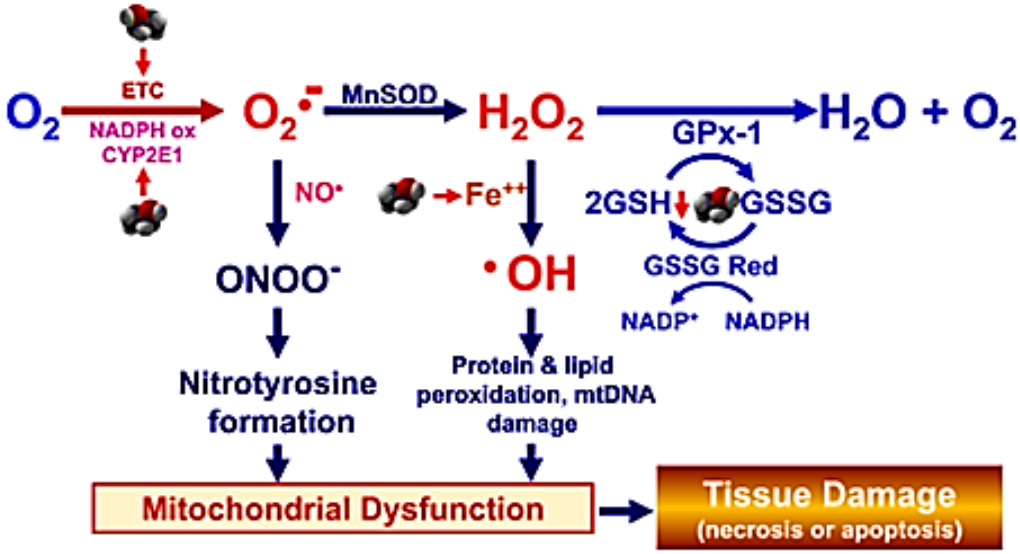


Figure 5.3. Mechanism of how H_2O_2 leades to tissue damage
(Zakhari, 2013)

The DNA protecting effect of the acetone extract of *C. cassia* was assessed using the comet assay. The DNA damage in 3T3-L1 cells was induced using 100 μM of hydrogen peroxide which induced a comet tail length of 10.93 ± 0.65 μm (Figure 4.8). DNA damage protection increased in a dose-dependent manner of the acetone extract.

In our study, the acetone extract of *C. cassia* which showed the highest content of the total phenolic and flavonoids, showed high ability in inhibition of DNA damage in 3T3-L1 cells. Karadağlı (2014) also showed that the water extract of *C. cassia* bark could be beneficial as a prophylactic agent in prevention of oxidative stress-related damage. The high ability of *C. cassia* in DNA damage inhibition was due to the high polyphenol and flavonoid content of this herb (Kumar *et. al*, 2012; Karadağlı, 2014).

5.5 *In vitro* Inhibition of Cell Proliferation (MTT Assay)

The MTT assay has been validated to monitor growth inhibition by phytoestrogens in the MCF-7 cells (Dixon-Shanies & Shaikh, 1999).

These results (Figure 4.9 and Table 4.6) showed the high ability of these extracts to inhibit of proliferation of both cell lines. The ethyl acetate extract inhibited the proliferation of MCF-7 cells with an IC_{50} of 135 ± 2.09 µg/ml. These results showed that MCF-7 cells was more sensitive to be inhibited by cinnamon components compared to MDA-MB-231 cells, which could be due to differences in the structure of the two cell lines. The MCF-7 cell line is dependent on estrogen receptors (ER^+) and carries the wild-type tumor suppressor p53 gene, while the highly aggressive MDA-MB-231cells is an ER-independent and carries the mutant P53 gene. The estrogen receptor of breast cancer cells plays an important role in drug-elicited effects (Yaacob *et. al.,* 2010). In one study it was confirmed that the differential antiproliferative activity using MTT assay of 2′-benzoyloxycinnamaldehyde (BCA) of *C. cassia.*

MCF-7 cells (the normal RK3E-ras) and MDA-MB-231 cells (normal RK3E-ras - mutant) were treated with BCA. BCA induced apoptosis in both cell lines. BCA treatment induced stronger antiproliferative effect in MDA-MB-231cells (IC_{50} 28 µM) than in MCF-7 cells (IC_{50} > 60 µM) (Ock *et. al.,* 2012). Also, Rao et al. (2007) evaluated the hexane, ethyl acetate, and methanol extract of cinnamon bark for their anti-inflammatory in MCF-7 cells. The hexane extract of cinnamon showed the highest antiproliferative activity compared to the other extracts of cinnamon with an IC_{50} of 30 µg/ml was as same as the results of antiproliferative activity in treated MCF-7 cells with the hexane extract of *C. cassia* (Table 4.6).

This data showed the toxicity of the nonpolar extracts of cinnamon on these two types of breast cancer cell lines. It was found that *C. cassia* bark extract had antiproliferative activity in other cancer cell lines as wells as breast cancer cells. For instance, it was found that at almost 80 µg/ml concentration of the aqueous extract of *C.cassia* treatment, there was a significant decrease (~2-fold) in the inhibition of the growth

of SiHa cells (cervical cancer cells) compared to that observed in the untreated control cells (Koppikar *et. al,* 2010). The water-soluble polymeric polyphenols from *C. cassia* showed anticancer activity against three myeloid cell lines (Jurkat, Wurzburg, and U937), with an IC_{50} lower than 50 µg/ml in the cells (Schoene *et. al.,* 2005).

Figure 4.10 showed the morphology of the studied cells before and after treatment. MCF-7 cells are adherent cells that had triangular morphology when adhered to the surface of a plate or dish before treatment ((Figure 4.10 (A)). After incubation of MCF-7 cells with the hexane for 24 h, the cells on the plate were found to detach from the surface and began to alter their morphology and lost cell-to-cell contact ((Figure 4.10 (B)). MDA-MB-231cells were adherent cells with spindle-shape ((Figure 4.10 (C)). After incubation of MDA-MB-231 cells with the hexane extract for 24 h, a rounding-up effect on adherent cells as well the extension of fillipodia-like structures was observed and the cells began to lost cell-to cell contact ((Figure 4.10 (D)). The same morphological change in this investigation was comparable with the morphological change of MCF-7 when exposed to the IC_{50} concentartion of the combination of TNF (30 ng/ml) and cycloheximide (Chx; 10 µg/ml) (Jänicke *et. al.,* 1998). They reported the change in morphology in treated cells was due to the induction of apoptosis by the treatment. Apoptosis is typically accompanied by the activation of a class of death proteases (caspases) and widespread biochemical and morphological changes to the cell. These changes almost invariably involve chromatin condensation and its margination at the nuclear periphery, extensive double-stranded DNA fragmentation, and cellular shrinkage and blebbing.

5.6 Reactive Oxygen Species (ROS) Assay

ROS, in high levels, are toxic but, at low levels, are useful for cell activation or modulation of the signal transduction pathway, modulation of redox-sensitive activities,

transcription factor, regulation of mitochondrial enzyme activity, etc. Cancer cells generally exhibit more intracellular ROS than non-transformed cells. There was one observation that induction of ROS in cancer cell lines is a way to induce apoptosis (Fiaschi *et. al.,* 2005). Understanding the role of reactive oxygen species (ROS) in apoptosis opens new approaches for controlling cancer growth. Many epidemiological studies suggested that increased intake of fruits and vegetables and other foods that contain antioxidants could protect against the DNA damage which initiate carcinogenesis (Liu, 2003).

This biochemical difference between normal and cancer cells may thus be a strategy for modulating cellular ROS to selectively kill cancer cells. It has been hypothesized that ROS associated with oncogenic transformation would make the cells highly dependent on its antioxidant systems to eliminate the harmful effects of ROS (Singh *et. al.,* 2008).

The result obtained when intracellular ROS was estimated showed that when the MCF-7 cells was treated with the hexane extract, the intracellular ROS increased by 14 % compared with the untreated cells. In MDA-MB-231cells, the highest level of ROS was generated with 16 µg/ml of the hexane extract (16.6 %) compared to the untreated cells while the ROS generated in MDA-MB-231cells, 3 % increased when the cells were treated with 136 µg/ml of the extract. However, recent data indicated that cells used reactive oxygen species as part of the signaling process responsible for activating an important mechanism for eliminating cancer cells, programmed cell death (also called apoptosis). Many anticancer agents depend on this form of cell death for their efficacy (Lopaczynski *et. al.,* 2001).

Figure 4.11 shows that the generation of ROS in MCF-7 cells treated with the hexane extract increased in a concentration-dependent manner. A higher ROS in treated MDA-MB-231 was generated when treated at 16 µg/ml of the hexane extract. Increase of ROS induced apoptosis in the cells. Induction of apoptosis should be studied by

investigating the signaling pathway of apoptosis such as activation of caspase proteins (especially caspase-8), releasing of cytochrome c, and etc. ROS activate caspase enzymes which cause apoptosis in cell.

5.7 Antioxidant Enzyme Assay

Tumor cells frequently produce large amounts of reactive oxygen species. This can be explained by the presence of mitochondrial defects and decreased expression of antioxidant enzymes, such as catalase and superoxide dismutase (SOD) (Trachootham *et. al.*, 2009). The reduction in the activity of catalase in cancer cells can cause cell death. The most consistent finding in biochemical studies of SOD, a mitochondrial antioxidant enzyme that it is decreased in most types of primary cancer and cancer cells; polymorphism in the leader sequence of the SOD gene has been correlated with susceptibility to breast cancer. Hence, an increase of SOD in MCF-7 cells can be a good way to inhibit the proliferation of the cell lines (Bartosz, 2005). Some of the newly prepared compounds such as cisplatin and maspin demonstrated inhibitory effects on the growth of MCF-7 cells as compared with the activity of the commonly used anticancer drug, cisplatin. The results of antitumor evaluation revealed that cisplatin compounds inhibited the growth of cancer cells through their effect as free-radical regulators by increasing the activity of superoxide dismutase and depletion of intracellular levels of reduced glutathione, catalase, and glutathione peroxidase activities (Rashad *et. al.*, 2010). Maspin is one of the serine families of protease inhibitors that have been shown to function as a tumor suppressor in human breast epithelium. Maspin expression was up-regulated in MCF-7 cells that overexpress a normal MnSOD gene (Li *et. al.*, 1998). Also, in another study, it was reported that the ferrocenyl ligand, prepared from condensation of 1, 1′-diacetylferrocene dihydrazone with salicylaldehyde, forms 1:1 complexes with Co (II), Ni (II), Cu (II), and Zn (II) in good yield. Anticancer

activity of the prepared ligand and its complexes against MCF-7 cells was determined, and the results were compared with the activity of the commonly used anticancer drug, cisplatin. Treatments of MCF-7 cells with gradually increasing doses (5, 10, 20, and 40 μg/ml) of the prepared complexes revealed that the activity of superoxide dismutase and the level of hydrogen peroxide were significantly increased, while the activities of catalase and glutathione peroxidase and the levels of reduced glutathione were significantly lowered compared with MCF-7 cells harvested from untreated controls. These results (Figure 4.12) indicated that the prepared compounds possessed significant anticancer activity comparable to the activity of cisplatin and may be potent anticancer agents for inclusion in modern clinical trials (Abd-Elzaher *et. al.,* 2010). The obtained results indicated that the SOD level was high in MDA-MB-231 cells when compared to MCF-7 cells. Superoxide dismutase (SOD) is known to play a role in cancer. SOD exerts a tumor-suppressive effect in estrogen-dependent human breast cancer cells. It was shown that estrogen-independent cells expressed a significantly higher basal SOD level compared to estrogen-dependent human breast cancer cell lines (MCF-7). For MDA-MB-231 cells, the high SOD level were accompanied by an overproduction of intracellular hydrogen peroxide (H_2O_2) and by a low expression of the major H_2O_2-detoxifying enzymes, catalase, and glutathione peroxidase compared with MCF-7 cells. Suppression of SOD expression by antisense RNA was associated with a decrease of H_2O_2 content and caused a stimulation of growth with a reduced cell doubling time but induced a decrease of colony formation. Furthermore, treatment of MDA-MB-231 cells with H_2O_2 scavengers markedly reduced tumor cell growth and colony formation. In addition, SOD suppression or treatment with H_2O_2 scavengers reduced the invasive properties of MDA-MB-231 cells up to 43 %. It is known that SOD plays a role in regulating tumor cell growth and invasive properties of estrogen-independent metastatic breast cancer cells. These actions are mediated by SOD-dependent

H_2O_2 production. In addition, these results suggest that SOD up-regulation may be one mechanism that contributes to the development of metastatic breast cancers (Kattan *et. al.*, 2010).

Results showed that the SOD level in MDA-MB-231 cells increased in 9 h compared to the basal level, and the ROS increased as well. After that, the activity of SOD decreased compared to the basal level. So the SOD level decreased because the extract scavenged the free radicals. The extract decreased the levels of SOD that leads to low levels of H_2O_2 production. Decrease in SOD caused decrease of CAT and GPx activity; thus, the cells were not protected from free radicals. These two factors contribute to the inhibition of MDA-MB-231 cells growth. In addition, in MCF-7 cells, the results of antitumor evaluation revealed that the extract of hexane inhibited the growth of cancer cells through their effect as free-radical regulators by increasing the activity of superoxide dismutase and depletion of intracellular levels of reduced glutathione. Reducing these two enzymes causes oxidative stress with high level of H_2O_2.

5.8 Caspase Activity

Apoptosis is typically accompanied by the activation of caspases proteins (Wang & Lenardo, 2000). Caspases, or *c*ysteine-*asp*artic prote*ases* or **c**ysteine-dependent aspartate-directed proteases are a family of cysteine proteases that play essential roles in apoptosis (programmed cell death), necrosis, and inflammation. As shown in Figure 4.13, there was no significant change in the activation of caspase-8 in treated MDA-MB-231 cells during the investigated time points. However, the activity of caspase-3/7 increased from 2 to 24 h incubation by 7.7 folds compared to the control. Caspase-9 activity in treated MDA-MB-231 cells increased in a time dependent manner to a maximum at 16 h by 3.6 folds. Caspase-9 is known to propagate the death signal by stimulating other

caspase activation events. It was suggested that caspase-9 is required for activation of a branch caspase cascade, namely, the intrinsic (mitochondrial mediated) apoptotic pathway (Lamkanfi *et. al.,* 2007). Caspase-3 is activated in apoptotic cells by both the extrinsic and intrinsic pathways. The above results suggest that the apoptosis and activation of caspase-3 occurred through the mitochondrial mediated pathway when MDA-MB-231 cells was treated with the hexane extract of *C. cassia*. The intrinsic pathway involves the mitochondria and is activated by stress events such as DNA damage (Joza *et. al.,* 2001). Mitochondria contain apoptosis-inducing factors, the second mitochondria-derived activator of caspases/direct IAP binding protein with low pI (Smac/DIABLO) and cytochrome c, which are all pro apoptotic. With the amplification of apoptotic signals from the death receptors (DRs) and the activation of caspase-8, BID proteins (a Bcl-2 family protein) are activated (Decuypere *et. al.,* 2012). The functions of these Bcl-2 family proteins are very critical in the homeostasis of apoptosis, particularly in the intrinsic pathway. The released cytochrome C forms a multi-protein complex called the apoptosome, which consists of cytochrome C, apoptotic protease activating factor 1 (Apaf-1), pro–caspase-9, and ATP. The formation of the apoptosome leads to the activation of caspase-9 and eventually the caspase cascade that activates effector caspases (i.e., caspase-3 and caspase-6) (Pace *et. al.,* 2010).

MCF-7 cells are believed to not express caspase-3 (Yamasaki-Miyamoto *et. al.,* 2009). However, Zhang et al. (2006) and Yang et al. (2006) have reported that caspase-3 is present in MCF-7 cells. The different reports on the presence of caspase-3 in MCF-7 could be due to the different variants of MCF-7 cells used. Studies have shown differences in the formation of apoptotic DNA ladders in different variants of MCF-7 cells (Gooch & Yee, 1999, Osborne *et. al.,* 1987). In MCF-7 cells treated with 35µg/ml of the hexane extract of *C. cassia* (Figure 4.13 (B), the activity of caspase-8 decreased from 2 h to 48 h (3.4 folds to

0.7 folds). But the activity of caspase-7 increased from 2 to 48 h incubation by 6.7 folds. Caspase-8 has a critical role in initiating the downstream apoptotic process which includes the activation of caspase-3, -6 and -7 and mitochondrial damage (extrinsic apoptosis or death receptor mediated pathway). The obtained results suggested that apoptosis occurred through extrinsic (death receptor mediated) pathway (Kataoka & Tschopp, 2004). Dietary flavonoids induce apoptosis through caspase-7 in caspase-3-deficient MCF-7 cells (Yang *et. al.,* 2012). The extrinsic pathways are associated with the triggering of death receptors on the cell surface. The binding of the ligand to the receptor leads to the triggering of DR, and the use of DR proteins can cause the auto activation of caspases via proteolytic domains in the pro–caspases (Albert's *et. al.,* 2008). Three of the known DR ligands, TNF-α, Fas, and TNF-related apoptosis induce TNF-related apoptosis-inducing ligand (TRAIL). TNF-α enhances the proliferation of chemically induced breast cancer cells (Konopleva *et. al.,* 2006). In that pathway, activation of two main proteins, namely, FasL and TRAIL, led to the activation of caspase-8. Active heterotetramer caspase-8 is released from the DISC and is free to cleave pro–caspase-3 to caspase-3. Bcl-2 family members share one or more of the four characteristic domains of homology entitled the Bcl-2 homology (BH) domains (named BH1, BH2, BH3 and BH4). Alternatively, the BH3-interacting domain death agonist proteins (BID) (from the Bcl-2 protein family) are cleaved to truncated-BID (BID) proteins, which induce Bax-mediated mitochondrial cytochrome c release. Both events commit the cell to apoptosis (Sengupta & Harris, 2005).

5.9 Study of Gene Expression by Real Time PCR (RT-PCR)

Bcl-2 protein family is important in the regulation of apoptosis when the stimuli are intrinsic in nature (Liu *et. al.,* 2004), Bax and Bid proteins are mainly found in the cytosol.

On the onset of apoptosis, the localization of some Bcl-2 proteins is altered. For example, Bax protein translocates from the cytosol to the mitochondrial membrane after treatment with an apoptotic stimulus (Zhang *et. al.,* 1997). Tumor protein p53 is involved in diverse functions, particularly in suppressing the pathogenesis of tumors, it has been demonstrated that MCF-7 cells treated with genistein and activated vitamin D elevated the expression of the p53 gene (Janz *et. al.,* 2002). Akt expression on the anti-proliferative and apoptotic effect of TAM in MCF-7 human breast cancer cells shows that Akt could confer resistance to anti-estrogen-mediated cell death and inhibition of proliferation (Shin & Arteaga, 2006).

Increased expression of anti-apoptotic Bcl-2 proteins confers therapeutic resistance in various cancer types, and as a result, the Bcl2 family proteins have an important responsibility to regulate apoptosis and are considered a target of anticancer therapy (Tzifi *et. al.,* 2011). For instance, increase in the Bcl2 to Bax ratio has been documented as a key factor showing induced apoptosis (Xiao & Zhang, 2008). Also, up-regulation of p53 induces apoptosis. P53-independent cell death has also been observed following ionizing radiation and DNA damage (Gersende *et. al.,* 2013).

The results obtained suggest that *C. cassia* extract induces apoptosis through activation of the p53 and Bid pathway by caspase-8 (Kutuk *et. al.,* 2009). There was no significant change in the expression of these genes in treated MCF-7 cells (Figure 4.14). Bax protein translocates from the cytosol to the mitochondrial membrane after treatment with an apoptotic stimulus (Zhang *et. al.,* 1997). Generally, this gene is up-regulated in MCF-7 cells, and it suppresses apoptotic events. It is usually down-regulated when apoptosis is stimulated. For example, MCF-7 cells treated with taxol showed a decrease in Bcl-2 mRNA expression (Akman *et. al.,* 1990). Tumor protein p53 is involved in diverse functions, particularly in suppressing the pathogenesis of tumors. It induces cell cycle arrest, apoptosis, senescence, and DNA repair. In normal cells, p53 is poorly expressed.

The p53 gene encodes the tumor protein p53, a transcriptional factor that binds to the DNA and activates the expression of downstream genes that inhibit growth and cell invasion (Angeloni *et. al.,* 2004). It has been demonstrated that MCF-7 cells treated with the flavonoid, genistein, and activated vitamin D elevated the expression of the p53 gene (Janz *et. al.,* 2002). The expression of this gene is induced by the presence of estrogen (Brekman *et. al.,* 2012). Akt expression on the anti-proliferative and apoptotic effect of tamoxifen (TAM) in MCF-7 cells showed that Akt could confer resistance to anti-estrogen-mediated cell death and inhibition of proliferation (Shin & Arteaga, 2006).

To evaluate the downstream signaling pathways induced by *C. cassia* extract, we also studied Protein Kinase B (PKB) or Akt1 gene expression. There was significant up-regulation of Akt1 in treated MCF-7 cells by a mean factor of 46.617. Unlike MCF-7, in treated MDA-MB-231 cells, *C. cassia* extract down-regulated Akt1 by a mean factor of 0.011. These data suggest a possible role of Akt1 in the different patterns seen in the expression of Bcl2, p53 and Bid in the two cell lines. MCF-7 cells, after 24 h incubation with the IC_{50} concentration of *C. cassia* extract, showed a partial resistance behavior to apoptosis. The Akt family of kinases promotes cell survival partly by inhibiting proteins that induce apoptosis. They do this by phosphorylating and inhibiting ASK-1 (apoptosis signal-regulating kinase 1) (Kim *et. al.,* 2001). In one study, the down-regulation of Akt1 in MDA-MB-231 cells removed the inhibition of Ask-1 and promoted apoptosis as suggested by other researchers (Hahm *et. al.,* 2011; Morse *et. al.,* 2005; Blanco-Aparicio *et. al.,* 2007).

In this study, in treated MCF-7 cells, Akt1 mRNA expression was significantly up-regulated. Treated MCF-7 cells showed a partial resistance to apoptosis through the up-regulation of Akt1, thus inhibiting ASK-1, although significant activation of caspase -8, and -3 was observed in MCF-7 cells. It can be suggested that the MDA-MB-231 cells were

more sensitive to apoptosis when induced by the extract, compared to MCF-7 cells. A previous study suggested that the difference in expression of Akt1 in treated MCF-7 and MDA-MB-231 cells was due to the presence or absence of the estrogen receptor, respectively (Dugasani *et. al.,* 2010). Akt1 limits breast cancer cell motility, high proliferation and invasion through nuclear factor of activated T-cells (NF-AT) transcription factor (Yoeli-Lerner *et. al.,* 2005; Badve *et. al.,* 2010). Akt1 also has a role in the phosphorylation and inactivation of downstream proapoptotic molecules such as caspase-9 (Cardone *et. al.,* 1998; Shin & Arteaga, 2006), reflecting our observations in treated MCF-7 cells. For the proliferation assays, only the attached surviving cells were taken into count. However, both floating apoptotic cells and attached healthy cells were combined and used for apoptosis assays (Chakrabarty *et. al.,* 2013). Phosphorylation and activation of downstream pro-apoptotic molecules such as caspase-9 have a critical role in apoptosis (Cardone *et. al.,* 1998).

These observations indicate that compounds present in the hexane extract of *C. cassia* are able to induce the caspases and p53 and Bid dependent apoptosis in MDA-MB-231 cells and also caspase dependent apoptosis in MCF-7 cells, irrespective of Akt1 levels. Based on these results, we suggest that *C. cassia* may be a candidate for further evaluation as a chemotherapeutic agent for cancer.

5.10 Isolation of Coumarin and *Trans*-Cinnamaldehyde by HPLC and GC-MS

Coumarin (1) was isolated from peak 1 at retention time 5.831 min in HPLC chromatography as a colourless chemical component with a sweet and fragrant odour (2.3 mg) (Figure 4.16 and Figure 4.17). *Trans*-cinnamaldehyde (**2**) was obtained from peak 2 at retention time 9.301 min as pale yellowish oil with a pungent and *C. cassia*-like odour (10.0 mg) (Figure 4.16 and Figure 4.17).

Coumarin (Figure 4.15), a class of compounds that contains a 1,2-benzopyrone skeleton, are widespread in plants including many vegetables, spices, fruits, and medicinal plants (Apak *et. al.,* 2007). Most of these compounds are not harmful to humans in the amounts present in edible plants. Coumarin (2H-chromen-2-one) (1), the simplest member of this class, as a pure compound or as a constituent of tonka beans had been used as a flavoring agent in food, alcoholic beverages and tobacco. It was shown that *C. cassia* has the high content of coumarin (Lungarini *et. al.,* 2008).

Many factors, such as, harvesting time of the plant, vegetative cycle stage, climatic and agronomic conditions, age and segment of the plant, the plant part used, extraction processes and assay methods was reported to extract of cinnamaldehyde levels of various C. cassia essential oils (Geng *et. al.,* 2011). In this study the major peak was cinnamaldehyde (the most component identified in the hexane extract of *C. cassia).* Chou et al. (2013) and Giordani et al. (2000) described that the identified compounds in the essential oil of *C. cassia* bark showed high fluctuations in the percentage compositions and the major compounds, *trans*-cinnamaldehyde, percentages varied within 33.95 %–76.4 %. According to a study by Cheng et al. (2012), GC-MS determination of superficial fluid extract of *C. cassia* bark contains, (E)-Cinnamaldehyde (57–69 %), coumarin (4–21 %), and naphthalene (3–14 %); and in the leaf extracts they were eucalyptol (17–24%), bornylene (10–17 %) and n-hexadecanoic acid (7–19 %) which was shown trans-cinnamaldehyde and coumarin was the highest amount of the components of *C. cassia* in bark and leave.

5.10.1 *In vitro* cell antiproliferative activity of the principle fractions of the hexane extract (*trans*-cinnamaldehyde and coumarin)

The cinnamaldehyde derivatives isolated from the bark of *C. cassia* were shown to have various activities such as anti-tumor and anti-angiogenic activity (Koh *et. al*., 1998; Kwon *et. al*., 1998; Shaughnessy *et. al*., 2001). The results of this study (Figure 4. 18, and Table 4.7) also revealed strong cytotoxicity activity of the hexane extract of *C. cassia* which could be due to the presence of trace quantities of *trans*-cinnamaldehyde. It was indicated that cinnamaldehyde inhibits cell proliferation through various important pathways involving alcohol dehydrogenases (Klibanov *et. al*., 1982), glutathione S-transferase inhibition in human melanoma cells (Van *et. al*., 1997) and anti-tyrosinase (Lee *et. al*., 2002). Also it induces apoptotic cell death in many cancer cell lines by down regulation of Bcl-2 and induced myeloid leukemia cell differentiation protein (MCL-1) expression and up-regulation of Bax expression (Wu *et. al*., 2005). In addition, new studies have focused on the bioactivity of derivatives of cinnamaldehyde. For instance, 2-hydroxycinnamaldehyde induced apoptotic cell death through up-regulation of apoptosis-regulating gene, caspase-3, and inhibiting of the anti-apoptosis regulating genes, Bcl-2, in a dose-dependent manner (Banjerdpongchai *et. al*., 2011). GC-MS analysis on the essential oil of *C. cassia* bark led to the identification of cinnamaldehyde, 2-hydroxycinnamaldehyde, and coumarin and cinnamyl acetate. The major volatile flavor in *C. cassia* was found to be 2-hydroxycinnamaldehyde. But, coumarin was first isolated from this plant by phytochemical isolation and spectroscopic analysis (Choi, *et. al.*, 2001).

The effect of cinnamaldehyde and its derivatives on breast cancer has not been well understood. But it was reported that 2′-benzoyloxycinnamaldehyde inhibits cell proliferation in MCF-7 and MDA-MB-435 cells (Kwon *et. al*., 1998). Also, 2′-

benzoyloxycinnamaldehyde was shown to be more effective in inhibiting the proliferation in MDA-MB-231 (IC_{50} 28 μM) compared with MCF-7 cells (IC_{50} > 60 μM) (Ismail *et. al.*, 2012; Kawate *et. al*., 2013).

In our study, it was shown that MCF-7 (ER^+) cells were much more sensitive than MDA-MB-231 (ER^-) cells when they were incubated with the same concentrations of the extracts. Therefore, this difference and the mechanism of the inhibition of the proliferation induced by *trans*-cinnamaldehyde will be studied further in the future. Also, a synergic effect between *trans*-cinnamaldehyde and coumarin or other minor compounds in the hexane extract was suggested.

5.11 Overall discussion

This study determined the anticancer and antioxidant activities of *C. cassia*. In this section, some of the possible interlinking factors between these separate activities are highlighted in an attempt to illustrate the commonalities between them.

Antioxidants have long been known to offer protection against cancer, mainly offering protection against carcinogens and mainly via the prevention of mutation (Hochstein & Atallah, 1988). There are also reports that indicated that oxidative stress leads to the development of cancer (Trueba *et. al.*, 2004). Hence, cancer prevention by preventing DNA damage through the use of antioxidants has been proposed before (Collins, 2005). The acetone extract of *C. cassia* showed the highest DNA damage inhibitory effect (45 %) at 30 μg/ml.

It was reported that induction of ROS in cancer cell lines is a way to induce apoptosis (Fiaschi *et. al.*, 2005). A similar effect was observed with the hexane extract of *C. cassia* in MCF-7 and MDA-MB-231 cells (Figure 4.11). Reducing the activation of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione

peroxidase (GPx) (bartoz, 2005) is another factor to inhibit the proliferation of cancer cells. It was shown that (Figure 4.12) the hexane extract of *C. cassia*, by reduction of the activities of GPX, SD, and CAT in MDA-MB-231cells and induction of the activity of SOD in MCF-7 cells, induced apoptosis in these cell lines.

There are some reports of flavonoids activating caspases (intrinsic and extrinsic pathway) (Yang *et. al.,* 2012) and up-regulating the apoptotic genes and inducing apoptosis in cancer cell lines (Duagasani *et. al.,* 2012).

Similarly, it was shown in this study that the hexane extract of *C. cassia* induced apoptosis via the intrinsic pathway in treated MCF-7 cells and the extrinsic pathway in treated MDA-MB-231 cells (Figure 4.13). Also the up-regulation of the apoptotic genes such as Akt1 in treated MCF-7 cells and p53 and Bcl-2 in treated MDA-MB-231cells induced apoptosis in these cells (Figure 4.14).

5.12 Limitations of the study

Some of the limitations of this study:

- The effect of the bark in an *in vitro* system was studied while in *in vivo* systems, other factors come into play, particularly digestion, absorption, bioconversion and pharmacodynamics. Treatment in an *in vivo* model would provide a better applicability compared to an *in vitro* model. For example, some of the possible *in vivo* experiments that may be considered are the antioxidant enzyme profile of a treated animal, tumor size assessment in cancer inoculated animals, glycemic control in diabetes induced animals, estrogenic activity by assessing the uterine weight and rate of healing in wound-induced animals.

- A gene microarray, if carried out, would have shown the global genomic patterrns of down- regulated genes by *C. cassia* treatment of the cells. However, circumstances did not permit this study.

Chapter VI: CONCLUSIONS

6.1 Conclusion

The medicinal properties of *C. cassia* extract were investigated in this study. The aims of this study were to investigate the medicinal bioactive compounds present in *C. cassia* and delineate the possible biological or molecular mechanisms involved in the activities. The study determined the antioxidant activity and the growth inhibitory effect on breast cancer cell lines of the crude extract. The crude extract demonstrated marked antioxidant activity and had a high phenolic and flavonoid content. *C. cassia* generated intracellular reactive oxygen species (ROS) in treated MCF-7 and MDA-MB-231 cells. The high level of ROS is one the main factors to induce apoptosis in these cell lines. Also, the activation of the antioxidant enzymes, namely, catalase, superoxide dismutase, and glutathione peroxidase were studied in *C. cassia* treated MCF-7 and MDA-MB-231 cells. An increase in the activation of SOD in treated MCF-7 cells and a decrease in the activation of CAT and GPx in treated MCF-7 and MDA-MB-231 cells inhibited cell proliferation. Therefore, using bioactivity guided purification assessed by HPLC and GC-MS and growth inhibitory activity on MCF-7 and MDA-MB-231 cell lines, some chemical compounds were isolated from the hexane extract of *C. cassia*. The major extracted compounds from the hexane extract of *C. cassia* present were *trans*-cinnamaldehyde and coumarin. One of the main discoveries from this study was that the growth inhibitory effect of the crude extract and these two compounds on both MCF-7 and MDA-MB-231 cells. *Trans*-cinnamaldehyde with an IC_{50} of 14.21 ± 0.02 in MDA-MB-231 cells and 9.61 ± 0.07 µg/

ml in MCF-7 cells was the most potent compound to inhibit cell proliferation compared to the hexane extract and coumarin.

This finding indicated the growth inhibitory effect of cinnamon on MCF-7 and MDA-MB-231 cells was caused by apoptosis mediated by the hexane extract of cinnamon and induced cellular caspase activity, and up-expression of some specific genes involved in apoptosis (Akt1, Bcl-2, Bid, and P53).

Evidence provided here showed that besides having strong antioxidant effects, the hexane extract of *C. cassia* could also induce apoptosis through the induction of caspase activities, initiating the intrinsic apoptosis pathway (mitochondrial) in treated MCF-7 cells and the extrinsic apoptosis pathway in treated MDA-MB-231 cells which suggests possible applications of *C. cassia* as an herbal remedy for breast cancer.

The results obtained in this study suggests that *C. cassia* could be used as an easy accessible source of natural antioxidants and protection against cancer and as a possible supplement in the pharmaceutical industry. The major components responsible for the immune modulatory and anticancer activities need to be further investigated. The antioxidant activities of *C. cassia* also warrant its use in supplements and food on a regular basis to prevent many chronic diseases and maintain good health.

The medicinal benefits of *C. cassia* is not limited to what has been presented here and therefore deserves further investigation so that its full potential could be harnessed for the benefit of mankind.

6.2 Future work

One of the main objectives of this study was centered on the isolation of the bioactive compounds and systematic elucidation of the biochemical and molecular events surrounding the activities demonstrated by *C. cassia* extract. Our concept was to provide

new scientific evidences to explain previous reports of medicinal activities of this herb. Here, other possible aspects which are relevant are proposed for future investigation.

- Study of the effect of this bark in an *in vivo* system.
- Other cancer cells can be assessed to determine if *C. cassia* is effective against other type of cancers.
- DNA fragmentation and cell cycle analysis can be investigated
- Study of the effect of other derivatives of *trans- cinnamaldehyde* on cancer cells to determine which is the most effective.
- Synthesis of the other derivatives of *trans- cinnamaldehyde.*

Finally it is strongly believed that the isolated *trans- cinnamaldehyde* has numerous medicinal properties that can be scientifically validated and has good potential for development into viable medicinal products.

Publications

Sima Kianpour Rad[a], M.S Kanthimathi[a,b] *, Jayakumar Rajarajeswaran[a], Sri Nurestri Abd Malek[c], Guan Serm Lee[c], (2014). Bioactive components, antioxidant, anticancer and DNA damage inhibitory of *Cinnamomum cassia*. *Ready for submission in Food Chemistry.*

Proceedings

Posters presented at conferences:

1) Sima Kianpour Rad and M. S. Kanthimathi (2011). Antioxidant actvity of *Cinnamomum Cassia*. 36th Annual Conference of the Malaysian Society for Biochemistry and Molecular Biology: Kuala lumpur, 71
2) Sima Kianpour Rad and M. S. Kanthimathi (2010). The effect of *Cinnamomum Cassia* on breast cancer cell lines. 35th Annual Conference of the Malaysian Society for Biochemistry and Molecular Biology: Kuala lumpur, 71

REFERENCES

Abd-Elzaher, M. M., Moustafa, S. A., Labib, A. A., & Ali, M. M. (2010). Synthesis characterization and anticancer properties of ferrocenyl complex containing a salicylaldehyde Moiety. *Monatshefte für Chemie-Chemical Monthly*, *141*(4), 387-393.

Akman, S. A., Forrest, G., Chu, F. F., Esworthy, R. S., & Doroshow, J. H. (1990). Antioxidant and Xenobiotic-metabolizing Enzyme Gene Expression in Doxorubicin-resistant MCF-7 Breast Cancer Cells. *Cancer Research, 50*(5), 1397-1402.

Alberts, K., Johnson, A., Lewis, J., Raff, M., Roberts, W. P., & Walter, P. (2008). Chapter 18 Apoptosis: progmed cell death eliminates unwanted cells. *Molecular biology of the cell, 5th edn.* 1115. *New York:* Garland Science.

Al-Kassie, G. A. (2009). Influence of two plant extracts derived from thyme and cinnamon on broiler performance. *Pakistan Veterinary Journal, 29*(4), 169-173.

Anand, P., Kunnumakara, A. B., Sundaram, C., Harikumar, K. B., Tharakan, S. T., Lai, O. S., & Aggarwal, B. B. (2008). Cancer is a preventable disease that requires major lifestyle changes. *Pharmaceutical Research, 25*(9), 2097–2116.

Angeloni, S. V., Martin, M. B., Garcia-Morales, P., Castro-Galache, M. D., Ferragut, J. A., & Saceda, M. (2004). Regulation of estrogen receptor- expression by the tumor suppressor gene p53 in MCF-7 cells. *Journal of Endocrinology, 180*(3), 497–504.

Apak, R., Güçlü, K., Demirata, B., Özyürek, M., Çelik, S. E., Bektaşoğlu, B., & Özyurt, D. (2007). Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules*, *12*(7), 1496-1547.

Api, A. M., Basketter, D. A., Cadby, P. A., Cano, M. F., Ellis, G., Gerberick, G. F., & Safford, R. (2008). Dermal sensitization quantitative risk assessment (QRA) for fragrance ingredients. *Regulatory Toxicology and Pharmacology*, *52*(1), 3-23.

Archer, S. L., Marsboom, G., Kim, G. H., Zhang, H. J., Toth, P. T., Svensson, E. C., & Rehman, J. (2010). Epigenetic attenuation of mitochondrial superoxide dismutase 2 in pulmonary arterial hypertension a basis for excessive cell proliferation and a new therapeutic target. *Circulation*, *121*(24), 2661-2671.

Archer, A. W. (1988). Determination of cinnamaldehyde, coumarin and cinnamyl alcohol in cinnamon and cassia by high-performance liquid chromatography. *Journal of Chromatography A, 447*, 272-276.

Arya, M., Shergill, I. S., Williamson, M., Gommersall, L., Arya, N., & Patel, H. R. (2005). Basic principles of real-time quantitative PCR. *Expert Review of Molecular Diagnostics*, *5*(2), 209-219.

Assadollahi, V., Parivar, K., Roudbari, N. H., Khalatbary, A. R., Motamedi, M., Ezatpour, B., & Dashti, G. R. (2013). The effect of aqueous cinnamon extract on the apoptotic process in acute myeloid leukemia HL-60 cells. *Advanced Biomedical Research*, *2*(1), 25.

Badve, S., Collins, N. R., Bhat-Nakshatri, P., Turbin, D., Leung, S., Thorat, M., & Nakshatri, H. (2010). Subcellular Localization of Activated AKT in Estrogen Receptor- and Progesterone Receptor-Expressing Breast Cancers. *The American Journal of Pathology*, *176*(5), 2139–2149.

Ban, Y., Hotoku, S., & Morita, Y. (2011). Selective extraction of U (VI) by counter-current liquid-liquid extraction with N, N-di (2-ethylhexyl)-2, 2-dimethylpropanamide. *Solvent Extraction and Ion Exchange*, *29*(4), 519-533.

Banjerdpongchai, R., Punyati, P., Nakrob, A., Pompimon, W., & Kongtawelert, P. (2011). 4-Hydroxycinnamaldehyde from Alpinia galanga (Linn.) induces human leukemic cell apoptosis via mitochondrial and endoplasmic reticulum stress pathways. *Asian Pacific Journal of Cancer Prevention*, *12*(3), 593-598.

Bartosz, G. (2005). *Superoxide dismutases and catalase*. *In Reactions, Processes,* Vol. 20. 109-149. Berlin: Springer Berlin Heidelberg.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., & Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proceedings of the National Academy of Sciences*, *87*(4), 1620-1624.

Benencia, F., & Courreges, M. C. (2000). In vitro and in vivo activity of eugenol on human herpesvirus. *Phytotherapy Research*, *14*(7), 495-500.

Benzie, I.F.F., & Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry*, *239*(10), 70-76.

Bertram, J. (2000). The molecular biology of cancer. *Molecular Aspects of Medicine*, *21*(6), 167–223.

Blanco-Aparicio, C., Pérez-Gallego, L., Pequeño, B., Leal, J. F., Renner, O., & Carnero, A. (2007). Mice expressing myrAKT1 in the mammary gland develope carcinogen-induced ER-positive mammary tumors that mimic human breast cancer. *Carcinogenesis*, *28*(3), 584–594.

Boga, M., Hacibekiroglu, I., & Kolak, U. (2011). Antioxidant and anticholinesterase activities of eleven edible plants. *Pharmaceutical Biology*, *49*(3), 290-295.

Borgstahl, G. E., Parge, H. E., Hickey, M. J., Johnson, M. J., Boissinot, M., Hallewell, R. A., & Tainer, J. A. (1996). Human mitochondrial manganese superoxide dismutase polymorphic variant Ile58Thr reduces activity by destabilizing the tetrameric interface. *Biochemistry*, *35*(14), 4287–97.

Braudel, F. (1982). *Civilization and Capitalism, 15th-18th Century: The perspective of the world*, Vol. 3. California: University of California Press.

Bravo, I. (1998). Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Review*, *56*(11), 317–333.

Brekman, A., Singh, K. E., Polotskaia, A., Kundu, N., & Bargonetti, J. (2012). A p53-independent role of Mdm2 in estrogen-mediated activation of breast cancer cell proliferation. *Breast Cancer Research*, *13*(1), R3.

Brigelius-Flohé R. (1999). Tissue-specific functions of individual glutathione peroxidases. *Free Radicical Biolology Medicine*, *27*(9–10), 951–65.

Broadhurst, C. L., Polansky, M. M., & Anderson, R. A. (2000). Insulin-like Biological Activity of Culinary and Medicinal Plant Aqueous Extracts *in vitro*. *Journal of Agricultural and Food Chemistry*, *48*(3), 849−852.

Cao, H., Polansky, M. M., & Anderson, R. A. (2007). Cinnamon extract and polyphenols affect the expression of tristetraprolin, insulin receptor, and glucose transporter 4 in mouse 3T3-L1 adipocytes. *Archives of Biochemistry and Biophysics*, *459*(2), 214-222.

Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke,T. F., Stanbridge, E., Frisch, S., & Reed, J. C. (1998). Regulation of cell death protease caspase-9 by phosphorylation. *Science, 282*(5392), 1318 – 1321.

Ceriello, A., & Motz, E. (2004). Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *24*(5), 816-823.

Chakrabarty, A., Bhola, N. E., Sutton, C., Ghosh, R., Kuba, M. G., Dave, B., & Arteaga, C. L. (2013). Trastuzumab-resistant cells rely on a HER2-PI3K-FoxO-survivin axis and are sensitive to PI3K inhibitors. *Cancer Research*, *73*(3), 1190-1200.

Chanda, N., Shukla, R., Zambre, A., Mekapothula, S., Kulkarni, R. R., Katti, K., & Katti, K. V. (2011). An effective strategy for the synthesis of biocompatible gold nanoparticles using cinnamon phytochemicals for phantom CT imaging and photoacoustic detection of cancerous cells. *Pharmaceutical research*, *28*(2), 279-291.

Charafe-Jauffret, E., Ginestier, C., Monville, F., Finetti, P., Adelaide, J., Cervera, N., Fekairi, S., Xerri, L., Jacquemier, J., Birnbaum, D., & Bertucci, F. (2006). Gene expression profiling of breast cell lines identifies potential new basal markers. *Oncogene*, *25*(15), 2273–2284.

Chelikani, P., Fita, I., & Loewen, P. C. (2004). Diversity of structures and properties among catalases. *Cellular and Molecular Life Sciences, 61*(2), 192–208.

Chen, J., & Thompson, L. U. (2003). Lignans and tamoxifen, alone or in combination, reduce human breast cancer cell adhesion, invasion and migration *in vitro*. *Breast Cancer Research and Treatment, 80*(2), 163-170.

Chen, Y. J., L. C., Cheng, S. S., & Chang, S. T. (2011). Phylogenetic relationships of the genus Chamaecyparis inferred from leaf essential oil. *Chemistry Biodiversity*, *8*(6), 1083-1097.

Chen, L., Sun, P., Wang, T., Chen, K., Jia, Q., Wang, H., & Li, Y. (2012). Diverse Mechanisms of Antidiabetic Effects of the Different Procyanidin Oligomer Types of Two Different Cinnamon Species on db/db Mice. *Journal of Agricultural and Food Chemistry*, *60*(36), 9144-9150.

Chenevix-Trench, G., Spurdle, A. B., Gatei, M., Kelly, H., Marsh, A., Chen, X., & Khanna, K. K. (2002). Dominant negative ATM mutations in breast cancer families. *Journal of the National Cancer Institute*, *94*(3), 205-215.

Cheng, X., Kigawa, J., Minagawa, Y., Kanamori, Y., Itamochi, H., Okada, M., & Terakawa, N. (1997). Glutathione S-transferase-π expression and glutathione concentration in ovarian carcinoma before and after chemotherapy. *Cancer, 79*(3), 521-527.

Choi, J., Lee, K. T., Ka, H., Jung, W. T., Jung, H. J., & Park, H. J. (2001). Constituents of the essential oil of the Cinnamomum cassia stem bark and the Biological Properties. *Archives of Pharmacal Research*, *24*(5), 418-423.

Chou, S. T., Chang, W. L., Chang, C. T., Hsu, S. L., Lin, Y. C., & Shih, Y. (2013). Cinnamomum cassia Essential Oil Inhibits α-MSH-Induced Melanin Production and Oxidative Stress in Murine B16 Melanoma Cells. *International Journal of Molecular Sciences, 14*(9), 19186-19201.

Chowdhury, I., Tharakan, B., & Bhat, G. K. (2008). Caspases—an update. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *151*(1), 10-27.

Christie, W. W. (1993). Preparation of lipid extracts from tissue. *In Advances in Lipid Methodology*, *2*, 195-213.

Collins, A. R. (2005). Antioxidant intervention as a route to cancer prevention. *European. Journal of Cancer, 41*(13), 1923-1930.

Constantinou, A. I., Kamath, N., & Murley, J. S. (1998). Genistein inactivates bcl-2, delays the G2/M phase of the cell cycle, and induces apoptosis of human breast adenocarcinoma MCF-7 cells. *European Journal of Cancer*, *34*(12), 1927-1934.

D haripriya, K. N. & Vijayalakshimi, K. (2013). Antioxidant potential of cinnamaldehyde; a *vitro* study. *International Journal of Pharmaceutical Research and Bio-sicence,* 2(5), 270-278.

De Vries, E. G. E., Meijer, C., Timmer-Bosscha, H., Berendsen, H. H., de Leij, L. F. H. M., Scheper, R. J., & Mulder, N. H. (1989). Resistance mechanisms in three human small cell lung cancer cell lines established from one patient during clinical follow-up. *Cancer Research, 49*(15), 4175-4178.

Decuypere, J. P., Parys, J. B., & Bultynck, G. (2012). Regulation of the Autophagic Bcl-2/Beclin 1 Interaction. *Cells, 1*(3), 284-312.

Deng, H. X., Hentati, A., Tainer, J. A., Iqbal, Z., Cayabyab, A., Hung, W. Y., & Roos, R. P. (1993). Amyotrophic lateral sclerosis and structural defects in Cu, Zn superoxide dismutase. *Science*, *261*(5124), 1047–1051.

Dixon-Shanies, D. O. N. N. A., & Shaikh, N. I. S. H. A. T. (1999). Growth inhibition of human breast cancer cells by herbs and phytoestrogens. *Oncology Reports*, *6*(6), 1383-1390.

Drews, J. (2000). Drug discovery: a historical perspective. *Science*, *287*(5460), 1960-1964.

Dudonne, S., Vitrac, X., Coutiere, P., Woillez, M., & Mérillon, J. M. (2009). Comparative Study of Antioxidant Properties and Total Phenolic Content of 30 Plant Extracts of Industrial Interest Using DPPH, ABTS, FRAP, SOD, and ORAC Assays. *Journal of Agricultural and Food Chemistry, 57*(5), 1768–1774.

Dugasani, S., Balijepalli, M. K., Tandra, S., & Pichika, M. R. (2010). Antimicrobial activity of *Bauhinia tomentosa* and *Bauhinia vahlii* roots. *Pharmacognosy Magazine, 6*(23), 204.

El-Baroty, G. S., El-Baky, H. H., Farag, R. S., & Saleh, M. A. (2010). Characterization of antioxidant and antimicrobial compounds of cinnamon and ginger essential oils. *African Journal of Biochemistry Research, 4*(6), 167-174.

Eliassen, A. H. & Hankinson, S. E. (2008). Endogenous hormone levels and risk of breast, endometrial and ovarian cancers. *Advances in Experimental Medicine and Biolology, 630,* 148-165.

Epp, O., Ladenstein, R., & Wendel, A. (1983). The refined structure of the selenoenzyme glutathione peroxidase at 0.2-nm resolution. *European Journal of Biochemistry, 133*(1), 51–69.

Fabricant, D. S. & Farnsworth, N. R. (2001). The value of plants used in traditional medicine for drug discovery. *Environ Health Perspect, 109*(Suppl 1), 69–75.

Fahlbusch, K. G., Hammerschmidt, F. J., Panten, J., Pickenhagen, W., Schatkowski, D., Bauer, K., & Surburg, H. (2003). Flavors and Fragrances. *Ullmann's Encyclopedia of Industrial Chemistry*. 1 nd edn. New York: Willey and Sons.

Farah, I. O. (2005). Assessment of cellular responses to oxidative stress using MCF-7 breast cancer cells, black seed (N. Sativa L.) extracts and H_2O_2. *International Journal of Environmental Research and Public Health, 2*(3-4), 411-419.

Feller, W. F., Stewart, S. E., & Kantor, J. (1972). Primary tissue culture explants of human breast cancer. *Journal of the National Cancer Institute*, *48*(4), 1117-1120.

Feng, F., Hu, P., Chen, L., Tang, Q., Lian, C., Yao, Q., & Chen, K. (2013). Display of Human Proinsulin on the Bacillus subtilis Spore Surface for Oral Administration. *Current Microbiology*, *67*(1), 1-8.

Fillmore, C. M., & Kuperwasser, C. (2008). Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast Cancer Research*, *10*(2), R25.

Fishchenko, A., & Khimich, S. D. (1986). Modification of the Hippocratic cap-shaped bandage. *Klinicheskaia Khirurgiia*, *72*, 72.

Fraga, C. G. (2007). Plant polyphenols: how to translate their *in vitro* antioxidant actions to *in vivo* conditions. *Iinternational Union of Biochemistry and Molecular Biology Life, 59*(4-5), 308-315.

Franco-Molina, M. A., Mendoza-Gamboa, E., Sierra-Rivera, C. A., Gómez-Flores, R. A., Zapata-Benavides, P., Castillo-Tello, P., & Rodríguez-Padilla, C. (2010). Antitumor activity of colloridal silver on MCF-7 human cancer cell. *Journal of Experimental and Clinical Cancer Research, 29*(148), 29-148.

Fridovich, I. (1995). Superoxide radical and superoxide dismutases. *Annual review of Biochemistry*, *64*(1), 97-112.

Friestad, G. K. (2001). Addition of carbon-centered radicals to imines and related compounds. *Tetrahedron*, *57*(26), 5461-5496.

Frydman-Marom, A., Levin, A., Farfara, D., Benromano, T., Scherzer-Attali, R., Peled, S., & Ovadia, M. (2011). Orally Administrated Cinnamon Extract Reduces β-Amyloid Oligomerization and Corrects Cognitive Impairment in Alzheimer's disease animal models. *Plos One*, *6*(1).

Fiaschi, A. I., Cozzolino, A., Ruggiero, G., & Giorgi, G. (2005). Glutathione, ascorbic acid and antioxidant enzymes in the tumor tissue and blood of patients with oral squamous cell carcinoma. *European Review for Medical and Pharmacological Sciences*, *9*(6), 361.

Gajewska, J., Szczypka, M., Izbicki, T., Klepacka, T., & Laskowska-Klita, T. (1996). Antioxidants and glutathione-associated enzymes in Wilms´ tumour after chemotherapy. *Journal of Cancer Research and Clinical Oncology, 122*(8), 483-488.

Galli, F., Battistoni, A., Gambari, R., Pompella, A., Bragonzi, A., Pilolli, F., & Cabrini, G. (2012). Oxidative stress and antioxidant therapy in cystic fibrosis. *Biochimica et Biophysica Acta-Molecular Basis of Disease, 1822*(5), 690-713.

Garcia, M., Derocq, D., FREiss, G. I. L. L. E. S., & Rochefort, H. (1992). Activation of estrogen receptor transfected into a receptor-negative breast cancer cell line decreases the metastatic and invasive potential of the cells. *Proceedings of the National Academy of Sciences*, *89*(23), 11538-11542.

Geng, S., Cui, Z., Huang, X., Chen, Y., Xu, D., & Xiong, P. (2011). Variations in essential oil yield and composition during Cinnamomum cassia bark growth. *Industrial crops and products*, *33*(1), 248-252.

Gerhäuser, C., Klimo, K., Heiss, E., Neumann, I., Gamal-Eldeen, A., Knauft, J., & Frank, N. (2003). N. Mechanism-based *in vitro* screening of potential cancer chemopreventive agents. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 523*(524), 163-172.

German, J. B. (1999). Food processing and lipid oxidation. *Advances in Experimental Medicine and Biology*, 459, 23-50.

Gersende, A., Mira, M., Priscillia, B. M., Dominique, A., Michael, B., Robert, R., & Claire, R. L. (2013). p53-independent early and late apoptosis is mediated by ceramide after exposure of tumor cells to photon or carbon ion irradiation. *Biomed Centeral, Cancer*, *13*(51), 147.

Gilani, A. H., Shah, A. J., Zubair, A., Khalid, S., Kiani, J., Ahmed, A., & Ahmad, V. U. (2009). Chemical composition and mechanisms underlying the spasmolytic and bronchodilatory properties of the essential oil of Nepeta catarial. *Journal of Ethnopharmacol, 121*(3), 405-411.

Gil-Parrado, S., Fernández-Montalván, A., Assfalg-Machleidt, I., Popp, O., Bestvater, F., Holloschi, A., & Machleidt, W. (2002). Ionomycin-activated Calpain triggers apoptosis a problem role for Bcl-2 family members. *Journal of Biological Chemistry*, *277*(30), 27217-27226.

Giordani, R., Regli, P., Kaloustian, J., & Portugal, H. (2006). Potentiation of antifungal activity of amphotericin B by essential oil from Cinnamomum cassia. *Phytotherapy Research*, *20*(1), 58-61.

Gooch, J. L., & Yee, D. (1999). Strain-specific differences in formation of apoptotic DNA ladders in MCF-7 breast cancer cells. *Cancer Letters, 144*(1), 31-37.

Goss, P. E., Ingle, J. N., Alés-Martínez, J. E., Cheung, A. M., Chlebowski, R. T., Wactawski-Wende, J., & Richardson, H. (2011). Exemestane for breast-cancer

prevention in postmenopausal women. *New England Journal of Medicine, 364*(25), 2381-2391.

Green, J. (2011). *The Herbal Medicine-Maker's Handbook: A Home Manual*. *Random House LLC,* 1 nd edn, Newyork: Crossing Press.

Guimarães, F. S., andrade, L. F., Martins, S. T., Abud, A. P., Sene, R. V., Wanderer, C., & Trindade, E. S. (2010). *In vitro* and *in vivo* anticancer properties of a Calcarea carbonica derivative complex (M8) treatment in a murine melanoma model. *Biomed Central, Cancer, 10*(1).

Gutteridge, J. M., & Halliwell, B. (1993). Free radicals in disease processes: a compilation of cause and consequence. *Free Radical Research Community, 19*(3), 141-158.

Hahm, E. R., Moura, M. B., Kelley, E. E., Van Houten, B., Shiva, S., & Singh, S. V. (2011). Withaferin A-Induced Apoptosis in Human Breast Cancer Cells Is Mediated by Reactive Oxygen Species. *PlosOne, 6*(8), e23354.

Haldar, S., Negrini, M., Monne, M., Sabbioni, S., & Croce, C. M. (1994). Down-regulation of bcl-2 by p53 in breast cancer cells. *Cancer Research, 54*(8), 2095-2097.

Halliwell, B. (1994). Free radicals, antioxidants, and human disease: curiosity, cause, or consequence?. *The Lancet, 344*(8924), 721-724.

Hao, X. Y., Bergh, J., Brodin, O., Heltman, U., & Mannervik, B. (1994). Acquired resistance to cisplatin and doxorubicin in a small cell lung cancer cell line is correlated to elevated expression of glutathione-linked detoxification enzymes. *Carcinogenesis, 15*(6), 1167-1173.

Harrison, P., & Gerstein, M. (2002). Studying genomes through the aeons: protein families, pseudogenes and proteome evolution. *Journal of Molecular Biololgy, 318*(5), 1155–74.

Hastings, P. J., Lupski, J. R., Rosenberg, S. M., & Ira, G. (2009). Mechanisms of change in gene copy number. *Nature Reviews, Genetics, 10*(8), 551–564.

He, K., Li, X., Chen, X., Ye, X., Huang, J., Jin, Y., & Shu, H. (2011). Evaluation of antidiabetic potential of selected traditional Chinese medicines in STZ-induced diabetic mice. *Journal of Ethnopharmacology, 137*(3), 1135-1142.

Hirst, J., King, M. S., & Pryde, K. R. (2008). The production of reactive oxygen species by complex I. *Biochemical Society Transactions, 36*(5), 976–80.

Hlebowicz, J., Hlebowicz, A., Lindstedt, S., Björgell, O., Höglund, P., Holst, J. J., & Almér, L. O. (2009). Effects of 1 and 3 g cinnamon on gastric emptying, satiety, and postprandial blood glucose, insulin, glucose-dependent insulinotropic polypeptide,

glucagon-like peptide 1, and ghrelin concentrations in healthy subjects. *The American Journal of Clinical Nutrition*, *89*(3), 815-821.

Hlebowicz, J., Darwiche, G., Björgell, O., & Almér, L. O. (2007). Effect of cinnamon on postprandial blood glucose, gastric emptying, and satiety in healthy subjects. *The American Journal of Clinical Nutrition*, *85*(6), 1552-1556.

Hochstein, P., & Atallah, A. S. (1988). The nature of oxidants and antioxidant systems in the inhibition of mutation and cancer. *Mutation Research, 202*(2), 363-375.

Hockenbery, D. M., Oltvai, Z. N., Yin, X. M., Milliman, C. L., & Korsmeyer, S. J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell*, *75*(2), 241-251.

How, V., Hashim, Z., Ismail, P., Omar, D., Md Said, S., & Bahri Mohd Tamrin, S. (2013). Characterization of risk factors for DNA damage among paddy farmworker exposed to mixture of organophosphate. *Archives of Environmental and Occupational Health*, *68.*

Hsieh, D. S., Lu, H. C., Chen, C. C., Wu, C. J., & Yeh, M. K. (2012). The preparation and characterization of gold-conjugated polyphenol nanoparticles as a novel delivery system. *International Journal of Nanomedicine*, *7*, 1623.

Huo, D., Ikpatt, F., Khramtsov, A., Dangou, J. M., Nanda, R., Dignam, J., & Olopade, O. I. (2009). Population differences in breast cancer: survey in indigenous African women reveals over-representation of triple-negative breast cancer. *Journal of Clinical Oncology*, *27*(27), 4515-4521.

Hsieh, T. C., Elangovan, S., & Wu, J. M. (2010). Differential Suppression of Proliferation in MCF-7 and MDA-MB-231 Breast Cancer Cells Exposed to α-, γ- and δ-Tocotrienols Is Accompanid by Altered Expression of Oxidative Stress Modulatory Enzymes. *Anticancer Research, 30*(10), 4169-4176.

Ismail, I. A., Kang, H. S., Lee, H. J., Kwon, B. M., & Hong, S. H. (2012). 2'-Benzoyloxycinnamaldehyde-mediated DJ-1 upregulation protects MCF-7 cells from mitochondrial damage. *Biological & Pharmaceutical Bulletin*, *35*, 895-902.

Jänicke, R. U., Sprengart, M. L., Wati, M. R., & Porter, A. G. (1998). Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *Journal of Biological Chemistry*, *273*(16), 9357-9360.

Ja¨nicke, R. U. (2009). MCF-7 breast carcinoma cells do not express caspase-3. *Breast Cancer Research Treatment, 117*(1), 219-221.

Jakhetia, V., Patel, R., Khatri, P., Pahuja, N., Garg, S., Pandey, A., & Sharma, S. A. (2010). Cinnamon : A pharmacologicam review. *Journal of Advanced Science Research, 1*(2), 19-23.

Jang, H. D., Chang, K. S., Huang, Y. S., Hsu, C. L., Lee, S. H., & Su, M. S. (2007). Principal phenolic phytochemicals and antioxidant activities of three Chinese medicinal plants. *Food Chemistry, 103*(3), 749-756.

Janz, C., Suesse, S., & Wiesmueller, L. (2002). p53 and recombination intermediates: role of tetramerization at DNA junctions in complex formation and exonucleolytic degradation. *Oncogene, 21*(14), 2130-2140.

Jauniaux, E., Watson, A. L., Hempstock, J., Bao, Y. P., Skepper, J. N., & Burton, G. J. (2000). Onset of maternal arterial blood flow and placental oxidative stress: a possible factor in human early pregnancy failure. *The American Journal of Pathology, 157*(6), 2111-2122.

Jayatilaka, A., Poole, S. K., Poole, C. F., & Chichila, T. M. (1995). Simultaneous micro steam distillation/solvent extraction for the isolation of semivolatile flavor compounds from cinnamon and their separation by series coupled-column gas chromatography. *Analytica Chimica Acta, 302*(2), 147-162.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. (2011). Global cancer statistics. *A Cancer Journal for Clinicians, 61*(2), 69–90.

Jordan, N. J., Gee, J. M., Barrow, D., Wakeling, A. E., & Nicholson, R. I. (2004). Increased constitutive activity of PKB/Akt in tamoxifen resistant breast cancer MCF-7 cells. *Breast Cancer Research and Treatment, 87*(2), 167-180.

Joza, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y., & Penninger, J. M. *(*2001). Essential role of the mitochondrial apoptosis-inducing factor in progmed cell death. *Nature, 4*(10), 549–554.

Kamarajugadda, S., Cai, Q., Chen, H., Nayak, S., Zhu, J., He, M., & Lu, J. (2013). Manganese superoxide dismutase promotes anoikis resistance and tumor metastasis. *Cell Death and Disease*, *4*(2), e504.

Kanno, Y., Jikumaru, Y., Hanada, A., Nambara, E., Abrams, S. R., Kamiya, Y., & Seo, M. (2010). Comprehensive hormone profiling in developing AraBidopsis seeds: examination of the site of ABA biosynthesis, ABA transport and hormone interactions. *Plant and Cell Physiology, 51*(12), 1988-2001.

Karadağlı, S. S. (2014). Investigation of the protective effect of cinnamomum cassia bark extract against H2O2-induced oxidative DNA damage in human peripheral blood lymphocytes and antioxidant activity. *Marmara Pharmaceutical Journal*, *18*(1), 43-48.

Kataoka, T., & Tschopp, J. (2004). N-Terminal Fragment of c-FLIP(L) Processed by Caspase-8 Specifically Interacts with TRAF2 and Induces Activation of the NF-κB Signaling Pathway. *Molecular Cellular Biology*, *24*(7), 2627-2636.

Kattan, Z., Minig, V., Leroy, P., Dauça, M., & Becuwe, P. (2010). Role of Mangenese Superoxide Dismutase on growth and invasive properties of human estrogen-

independent breast cancer cells. *Breast Cancer Researh and Treatment*, *108*(2), 203-215.

Kaur, C., & Kapoor, H. C. (2002). Anti-oxidant activity and total phenolic content of some Asian vegetables. *International Journal of Food Science and Technology*, *37*(2), 153-161.

Kawate, T., Iwaya, K., Kikuchi, R., Kaise, H., Oda, M., Sato, E., & Kohno, N. (2013). DJ-1 protein expression as a predictor of pathological complete remission after neoadjuvant chemotherapy in breast cancer patients. *Breast Cancer Research and Treatment*, *139*(1), 51-59.

Kermasha, S., Goetghebeur, M., & Dumont, J. (1994). Separation and characterization of pepper-contaminated cinnamon Using HPLC analyses. *LWT-Food Science and Technology*, *27*(6), 578-582.

Khan, I. A., & Abourashed, E. A. (2011). *Leung's Encyclopedia of Common Natural Ingredients: Used in Food, Drugs and Cosmetics*. 1nd edn, Canada: John Wiley & Sons.

Kihlmark, M., Imreh, G., & Hallberg, E. (2011). Sequential degradation of proteins from the nuclear envelope during apoptosis. *Journal of Cell Science*, *114*(20), 3643-3653.

Kim, A. H., Khursigara, G., Sun, X., Franke, T. F., & Chao, M. V. (2001). Akt Phosphorylates and Negatively Regulates Apoptosis Signal-Regulating Kinase 1. *Molecular and Cellular Biology*, *21*(3), 893-901.

Klibanov, A. M., & Giannousis, P. P. (1982). Geometric specificity of alcohol dehydrogenases and its potential for separation of trans and cis isomers of unsaturated aldehydes. *Proceedings of the National Academy of Sciences*, *79*(11), 3462-3465.

Koh, W. S., Yoon, S. Y., Kwon, B. M., Jeong, T. C., Nam, K. S., & Han, M. Y. (1998). Cinnamaldehyde inhibits lymphocyte proliferation and modulates T-cell differentiation *International Journal of Immunopharmacology*, *20*(11), 643-660.

Konopleva, M., Contractor, R., Tsao, T., Samudio, I., Ruvolo, P. P., Kitada, S., & Andreeff, M. (2006). Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell*, *10*(5), 375-388.

Koppikar, S. J., Choudhari, A. S., Suryavanshi, S. A., Kumari, S., Chattopadhyay, S., & Kaul-Ghanekar, R. (2010). Aqueous Cinnamon Extract (ACE-c) from the bark of *C. cassia* causes apoptosis in human cervical cancer cell line (SiHa) through loss of mitochondrial membrane potential. *Biomed Central, Cancer*, *10*(1), 210.

Kostermans, A. G. H. (1986). *A monograph of the genus Cinnamomum schaeff. (Lauraceae), Academia Scientific Book*, Vol. 1. Tokyo.

Kuida, K., Haydar, T. F., Kuan, C. Y., Gu, Y., Taya, C., Karasuyama, H., & Flavell, R. A (1998). Reduced apoptosis and cytochrome C-mediated caspase activation in mice lacking caspase 9. *Cell, 94*(3), 325-337.

Kumar, M., Sharma, V. L., Sehgal, A., & Jain, M. (2012). Protective effects of green and white tea against benzo (a) pyrene induced oxidative stress and DNA damage in murine model. *Nutrition and Cancer*, *64*(2), 300-306.

Kumar, R., Mandal, M., Lipton, A., Harvey, H., & Thompson, C. B. (1996). Overexpression of HER2 modulates bcl-2, bcl-XL, and tamoxifen-induced apoptosis in human MCF-7 breast cancer cells. *Clinical Cancer Research, 2*(7), 1215-1219.

Kumar, S. (2007). Caspase function in progmed cell death. *Cell Death & Differentiation, 14*(1), 32-43.

Kutuk, O., Arisan, E. D., Tezil, T., Shoshan, M. C., & Basaga, H. (2009). Cisplatin overcomes Bcl-2-mediated resistance to apoptosis via preferential engagement of Bak: critical role of Noxa-mediated lipid peroxidation. *Carcinogenesis, 30*(9), 1517–1527.

Kwon, H. K., Hwang, J. S., So, J. S., Lee, C. G., Sahoo, A., Ryu, J. H., & Im, S. H. (2010). Cinnamon extract induces tumor cell death through inhibition of NFkappaB and AP1. *Biomed Central, Cancer, 10*(1), 392.

Kwon, B. M., Lee, S. H., Choi, S. U., Park, S. H., Lee, C. O., Cho, Y. K., & Bok, S. H. (1998). Synthesis andin vitro cytotoxicity of cinnamaldehydes to human solid tumor cells. *Archives of Pharmacal Research, 21*(2), 147-152.

Lacroix, M. (2006). Significance, detection and markers of disseminated breast cancer cells. *Endocrine-related Cancer, 13*(4), 1033–67.

Lai, P. K., & Roy, J. (2004). Antimicrobial and chemopreventive properties of herbs and spices. *Current Medicinal Chemistry, 11*(11), 1451-1460.

Lamkanfi, M., Festjens, N., Declercq, W., Berghe, T. V., & Vandenabeele, P. (2007). Caspases in cell survival, proliferation and differentiation. *Cell Death and Differentiation, 14*(1), 44–55.

Lamparska-Przybysz, M., Gajkowska, B., & Motyl, T. (2006). BID-deficient breast cancer MCF-7 cells as a model for the study of autophagy in cancer therapy. *Autophagy, 2*(1), 47-48.

Lee, H. S. (2002). Tyrosinase inhibitors of Pulsatilla cernua root-derived materials. *Journal of Agricultural and Food Chemistry, 50*(6), 1400-1403.

Levenson, A. S., & Jordan V. C. (1997). MCF-7: the first hormone-responsive breast cancer cell line. *Cancer Research, 57*(15), 3071–3078.

Li, J. J., Colburn, N. H., & Oberley, L. W. (1998). Maspin gene in tumor suppression induces by overexpressing manganese containing superoxide dismutase cDNA in tumor breast cancer cells. *Carsinogenesis, 19*(5), 833-839.

Li, Y. Q., Kong, D. X., & Wu, H. (2013). Analysis and evaluation of essential oil components of cinnamon barks using GC–MS and FTIR spectroscopy. *Industrial Crops and Products, 41,* 269-278.

Liang, Y., Yan, C., & Schor, N. F. (2001). Apoptosis in the absence of caspase 3. *Oncogene, 20*(45), 6570-6578.

Liaw, K. Y., Lee, P. H., Wu, F. C., Tsai, J. S., & Lin-Shiau, S. Y. (1997). Zinc, copper, and superoxide dismutase in hepatocellular carcinoma. *American Journal of Gastroenterology, 92*(12), 2260-2263.

Lichterman, B. L. (2004). Aspirin: The Story of a Wonder Drug. *British Medical Journal, 329*(7479), 1408.

Lim, G. C. C., & Halimah, Y. (2004). Cancer incidence in Malaysia 2003. *National Cancer Registry Kuala Lumpur*.

Lim, T. Y., Lim, Y. Y., & Yule, C. M. (2009). Evaluation of antioxidant, antibacterial and anti-tyrosinase activities of four Macaranga species. *Food Chemistry, 114*(2), 594-599.

Lin, C. C., Wu, S. J., Chang, C. H., & Ng, L. T. (2003). Antioxidant activity of Cinnamomum cassia. *Phytotherapy Research, 17*(7), 726-730.

Liu, R. H. (2003). Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *The American Journal of Clinical Nutrition, 78*(3), 517S-520S.

Liu, R. H. (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of action. *The Journal of Nutrition, 134*(12), 3479S-3485S.

Lomnicki, S., Truong, H., Vejerano, E., & Dellinger, B. (2008). Copper oxide-based model of persistent free radical formation on combustion-derived particulate matter. *Environmental Science and Technology, 42*(13), 4982–4988.

Lopaczynski, W., & Zeisel, S. H. (2001). Antioxidant, progmed cell death and cancer. *Nutrition Research, 21*(1), 295-307.

Lotem, J., & Sachs, L. (1993). Hematopoietic cells from mice deficient in wild-type p53 are more resistant to induction of apoptosis by some agents. *Blood, 82*(4), 1092-1096.

Lotito, S.B., & Frei, B. (2006). Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomenon?. *Free Radical Biology Medicine, 41*(12), 1727–46.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biology Chemistry, 193*(1), 265–75.

Lubbe, A., & Verpoorte, R. (2011). Cultivation of medicinal and aromatic plants for specialty industrial materials. *Industrial Crops and Products*, *34*(1), 785-801.

Lungarini, S., Aureli, F., & Coni, E. T. T. O. R. E. (2008). Coumarin and cinnamaldehyde in cinnamon marketed in Italy: a natural chemical hazard?. *Food Additives and Contaminants*, *25*(11), 1297-305.

Lüpertz, R., Chovolou, Y., Kampkötter, A., Wätjen, W., & Kahl, R. (2008). Catalase overexpression impairs TNF-α induced NF-κB activation and sensitizes MCF-7 cells against TNF-α. *Journal of Cellular Biochemistry*, *103*(5), 1497-1511.

Maehly, A. C., & Chance, B. (1954). The assay of catalases and peroxidases. *Methods Biochemstry Anal, 1,* 357–424.

Maizura, M., Aminah, A., & Wan Aida, W. M. (2011). Total phenolic content and antioxidant activity of kesum (*Polygonum minus*), ginger (*Zingiber officinale*) and turmeric (Curcuma longa) extract. *International Food Research Journal*, *18*(2).

Mancini-Filho, J., Van-Koiij, A., Mancini, D. A., Cozzolino, F. F., & Torres, R. P. (1998). Antioxidant activity of cinnamon (*Cinnamomum Zeylanicum*, Breyne) extracts. *Bollettino Chimico Farmaceutico, 137*(11), 443-447.

Mandal, M., & Kumar, R. (1996). Bcl-2 expression regulates sodium butyrate-induced apoptosis in human MCF-7 breast cancer cells. *Cell Growth & Differentiation: The Molecular Biology Journal of the American Association for Cancer Research*, *7*(3), 311-318.

Marcocci, L., Maguire, J. J., Droy-Lefaiz, M. T., & Packer, L. (1994). The nitric oxide-scavenging properties of Ginkgo biloba extract EGB 761. *Biochemical and Biophysical Research Communications, 201*(2), 748−755.

Mathew, S., Abraham, & T. E. (2006). Studies on the antioxidant activities of cinnamon (Cinnamomum verum) bark extracts, through various *in vitro* models. *Food Chemistry, 94*(4), 520–528.

McCord, J. M., & Fridovich, I. (1988). Superoxide dismutase: the first twenty years (1968-1988). *Free Radical and Biology Medicine, 5*(5–6), 363–9.

Md, A. R., Marka, V., & Yalavarthy, P. D. (2013). A study on antioxidant activity of some commonly used species in India. *International Journal of Science Biotechnology and Pharmacology Research*, *2*(4).

Miguel, M. G. (2010). Antioxidant and anti-inflammatory activities of essential oils: a short review. *Molecules*, *15*(12), 9252-9287.

Moll, U. M., Riou, G., & Levine, A. J. (1992). Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. *Proceedings of the National Academy of Sciences*, *89*(15), 7262-7266.

Morre, D. M., Morre, J. D., Cooper, R., & Chang, M. N. (2002). *U.S. Patent No. 20,020,176,898*. Washington, DC: U.S. Patent and Trademark Office.

Morse, D. L., Gray, H., Payne, C. M., & Gillies, R. J. (2005). Docetaxel induces cell death through mitotic catastrophe in human breast cancer cells. *Molucular Cancer Theraphy*, *4*(10),1495-1504.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1–2), 55–63.

Mufti, F. R. S., Amin, S., & Zargar, M. A. (2011). *In vitro and In vivo Validation of Folk Lore Claims of Thymus serpyllum* (Doctoral dissertation).

Nagle, A. A., Gan, F. F., Jones, G., So, C. L., Wells, G., & Chew, E. H. (2012). Induction of Tumor Cell Death through Targeting Tubulin and Evoking Dysregulation of Cell Cycle Regulatory Proteins by Multifunctional Cinnamaldehydes. *Plos One*, *7*(11), e50125.

Nascimento, G. G., Locatelli, J., Freitas, P. C., & Silva, G. L. (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. *Brazilian Journal of Microbiology*, *31*(4), 247-256.

Ng, L. T., & Wu, S. J. (2009). Antiproliferative activity of cinnamomum cassia constituents and effects of pifithrin-alpha on their apoptotic signaling pathways in Hep G2 Cells. *Evidence Based Complementary and Alternative Medicine*, *2011*, 38.

Nakabeppu, Y., Sakumi, K., Sakamoto, K., Tsuchimoto, D., Tsuzuki, T., & Nakatsu, Y. (2006). Mutagenesis and carcinogenesis caused by the oxidation of nucleic acids. *Biological Chemistry*, *387*(4), 373–9.

Nishikimi M., Appaji N., & Yagi K. (1972). The occurrence of super oxide anion in the reaction of reduced Phenazine methosµlphate and molecµlar oxygen. *Biochemistry Biophysics Research Communications*, *46*(2), 849-853.

Nordberg, J., & Arner, E. S. (2001). Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radicical and Biology Medicine, 31*(11), 1287–312.

Nugraheni M, S. U., Suparmo, H., & Wuryastuti, H. I.F.R.J. (2011). Plectranthus rotundifolius: *The Study of Chemical Compositions and Cytotoxic Activity*, *18*, 1471-1480.

Nuñez, G., Benedict, M. A., Hu, Y., & Inohara, N. (1998). Caspases: the proteases of the apoptotic pathway. Oncogene, *17*(25), 3237-3245.

Oberley, L. W. (2005). Mechanism of the tumor suppressive effect of MnSOD overexpression. *Biomedicine and Pharmacotherapy*, *59*(4), 143-148.

Ock, J., Lee, H. A., Ismail, I. A., Lee, H. J., Kwon, B. M., Suk, K, & Hong, S. H. (2011). Differential antiproliferation effect of 2′-benzoyloxycinnamaldehyde in K-ras-transformed cells via downregulation of thiol antioxidants. *Cancer Science*, *102*(1), 212-218.

Oki, T., Masuda, M., Furuta, S., Nishiba, Y., Terahara, N., & Suda, I. (2002). Involvement of anthocyanins and other phenolic compounds in radical-scavenging activity purplefleshed sweet potato cultivars. *Journal of Food Science, 67*(5), 1752-1756.

O Mahony, R., Al-Khtheeri, H., Weerasekera, D., Fernando, N., Vaira, D., Holton, J., & Basset, C. (2005). Bactericidal and anti-adhesive properties of culinary and medicinal plants against Helicobacter pylori. *World Journal of Gastroenterol*, *11*(47), 7499-7507.

Ooi, L. S., Li, Y., Kam, S. L., Wang, H., Wong, E. Y., & Ooi, V. E. (2006). Antimicrobial activities of cinnamon oil and cinnamaldehyde from the Chinese medicinal herb Cinnamomum cassia Blume. *The American Journal of Chinese Medicine*, *34*(03), 511-522.

Orr, M. F., & McSwain, B. (1955). Tissue culture of human breast carcinoma. *The American Journal of Pathology*, *31*(1), 125–141.

Osborne, C. K., Hobbs, K., & Trent, J. M. (1987). Biological differences among MCF-7 human breast cancer cell lines from different laboratories. *Breast Cancer Research and Treatment*, *9*(2), 111-121.

O'shea, M., Stanton, C., & Devery, R. (1998). Antioxidant enzyme defence responses of human MCF-7 and SW480 cancer cells to conjugated linoleic acid. *Anticancer Research*, *19*(3A), 1953-1959.

Oussalah, M., Caillet, S., Saucier, L., & Lacroix, M. (2007). Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: E. coli, O157H7, Salmonella, Typhimurium, Staphylococcus aureus and Listeria monocytogenes, *Food Control*, *18*(5), 414-420.

Ozsoy, N., Can, A., Yanardag, R. & Akev, N. (2007). Antioxidant activity of Smilax Excelsa L. leaf extracts. *Food Chemistry*, *110*(3), 571–583.

Pace, V., Bellizzi, D., Giordano, F., Panno, M. L., & De Benedictis, G. (2010). Experimental testing of a mathematical model relevant to the extrinsic pathway of apoptosis. *Cell Stress Chaperones*, *15*(1), 13-23.

Pacher, P., Beckman, J. S., & Liaudet, L. (2007). Nitric oxide and peroxynitrite in health and disease. *Physiology Review, 87*(1), 315–424.

Palu, G., Valisena, S., Ciarrocchi, G., Gatto, B., & Palumbo, M. (1992). Quinolone binding to DNA is mediated by magnesium ions. *Proceedings of the National Academy of Sciences, 89*(20), 9671-9675.

Pani, G., Galeotti, T., & Chiarugi, P. (2010). Metastasis: cancer cell's escape from oxidative stress. *Cancer and Metastasis Reviews, 29*(2), 351-378.

Parkin, D. M., & Fernández, L. M. (2006). Use of statistics to assess the global burden of breast cancer. *The breast journal*, *12*(s1), S70-S80.

Pérez-Márquez, J., Reguillo, B., & Paniagua, R. (2002). Cloning of the cDNA and mRNA expression of CLRP, a complex leucine repeat protein of the Golgi apparatus expressed by specific neurons of the rat brain. *Journal of Neurobiol, 52*(2), 166-173.

Perillo, B., Sasso, A., Abbondanza, C., & Palumbo, G. (2000). 17β-Estradiol inhibits apoptosis in MCF-7 cells, inducing bcl-2 expression via two estrogen-responsive elements present in the coding sequence. *Molecular and Cellular Biology, 20(*8), 2890-2901.

Perou, C. M., Sørlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., & Botstein, D. (2000). Molecular portraits of human breast tumours, *Nature. 406*, 747-752.

Pervin, S., Singh, R., Freije, W. A., & Chaudhuri, G. (2003). MKP-1-Induced Dephosphorylation of Extracellular Signal-Regulated Kinase Is Essential for Triggering Nitric Oxide-Induced Apoptosis in Human Breast Cancer Cell Lines Implications in Breast Cancer. *Cancer Research*, *63*(24), 8853-8860.

Pietta, P. G. (2000). Flavonoids as antioxidants. *Journal of Natural Products, 63*(7), 1035-1042.

Porter, A. G., & Jänicke, R. U. (1999). Emerging roles of caspase-3 in apoptosis. *Cell Death & Differentiation, 6*(2), 99-104.

Pozo-Guisado, E., Merino, J. M., Mulero-Navarro, S., Lorenzo-Benayas, M. J., Centeno, F., Alvarez-Barrientos, A., & Salguero, P. M. F. (2005). Resveratrol-induced apoptosis in MCF-7 human breast cancer cells involves a caspase-independent mechanism with downregulation of Bcl-2 and NF-κB. *International Journal of Cancer, 115*(1), 74-84.

Prakash, D., Upadhyay, G., Gupta, C., Pushpangadan, P., & Singh, K. K. (2012). Antioxidant and free radical scavenging activities of some promising wild edible fruits. *International Food Research Journal*, *19*(3), 1109-1116.

Prasad, N. K., Divakar, S., Shivamurthy, G. R., & Aradhya, S. M. (2005). Isolation of a free radical scavenging antioxidant from water spinach (Ipomoea aquatica Forsk). *Journal of the Science of Food and Agriculture*, *85*(9), 1461–1468.

Prasad, K. N., Yang, B., Dong, X., Jiang, G., Zhang, H., Xie, H., & Jiang, Y. (2009). Flavonoid contents and antioxidant activities from Cinnamomum species. *Innovative Food Science and Emerging Technologies*, *10*(4), 627-632.

Punnonen, K., Ahotupa, M., Asaishi, K., Hyöty, M., Kudo, R., & Punnonen, R. (1994). Antioxidant enzyme activities and oxidative stress in human breast cancer. *Journal of Cancer Research* and *Clinical Oncology*, *120*(6), 374-377.

Raff, M. C. (1992). Social controls on cell survival and cell death. *Nature*, *356*, 397-400.

Raha, S., & Robinson, B. H. (2000). Mitochondria, oxygen free radicals, disease and ageing. *Trends in Biochemical Sciences, 25*(10), 502–8.

Rahman, I., Biswas, S. K., & Kode, A. (2006). Oxidant and antioxidant balance in the airways and airway diseases. *European Journal of Pharmacology*, *533*(1), 222-239.

Rampal, S., & Yahaya, H. (2008). *Cancer Incidence in Peninsular Malaysia, 2003-2005: The Third Report of the National Cancer Registry, Malaysia*. National Cancer Registry.

Ran, Q., Liang, H., & Ikeno, Y. (2007). Reduction in glutathione peroxidase 4 increases life span through increased sensitivity to apoptosis. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *62*(9), 932–42.

Rao, Y. K., Fang, S. H., & Tzeng, Y. M. (2007). Evaluation of the anti-inflammatory and anti-proliferation tumor cells activities of Antrodia camphorate, Cordyceps sinensis, and Cinnamomum osmophloeum bark extracts. *Journal of Ethnopharmacology*, *114*(1), 78-85.

Rashad, A. E., El-Sayed, W. A., Mohamed, A. M., & Ali, M. M. (2010). Synthesis of New Quinoline Deravitives as Inhibitors of human Tumor cells Growth. *Chemistry in Life Sciences, 323*(8), 440-448.

Res, P. (2000). the water extract of cinnamon cassia bark significantly protected against glutamate-induced cell death and also inhibited glutamate-induced Ca(2+) influx. *Phytother Research, 14*(6), 466-468.

Rice-Evans, C., Miller, N., & Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends in Plant Science*, *2*(4), 152-159.

Ross, M. S. F. (1976). Analysis of cinnamon oils by high-pressure liquid chromatography. *Journal of Chromatography A*, *118*(2), 273-275.

Roth, J. A., Nguyen, D., Lawrence, D. D., Kemp, B. L., Carrasco, C. H., Ferson, D. Z., & Cai, D. (1996). Retrovirus–mediated wild–type P53 gene transfer to tumors of patients with lung cancer. *Nature Medicine*, *2*(9), 985-991.

Royle, J. G. (1946). Some cultural and cytolpgical characteristics of human tumors *in vitro*. *Cancer Research, 6*(5), 225–229.

Ruan, Y., Pei, W., & Wan, M. (2008). Membrane protein analysis of human breast cancer cell line MCF-7 by different membrane washing methods. *Cell Biochemistry Function, 26*(7), 787-796.

Rungtabnapa, P., Nimmannit, U., Halim, H., Rojanasakul, Y., & Chanvorachote, P. (2011). Hydrogen peroxide inhibits non-small cell lung cancer cell anoikis through the inhibition of caveolin-1 degradation. *American Journal of Physiology-Cell Physiolog, 300*(2), C235-C245.

Rupinder, S. K., Gurpreet, A. K., & Manjeet, S. (2007). Cell suicide and caspases. *Vascular Pharmacology, 46*(6), 383-393.

Sariego, J. (2010). Breast cancer in the young patient. *The American Surgeon, 76*(12), 1397–1401.

Sarker, M., Ruiz-Ruiz, C., Robledo, G., & Lopez-Rivas, A. (2002). Stimulation of the mitogen-activated protein kinase pathway antagonizes TRAIL-induced apoptosis downstream of BID cleavage in human breast cancer MCF-7 cells. *Oncogene, 21*(27), 4323-4327.

Schneider, P., & Tschopp, J. (2000). Apoptosis induced by death receptors. *Pharmacochemistry Library, 31*, 281-286.

Schoene, N. W., Kelly, M. A., Polansky, M. M., & Anderson, R. A. (2005). Water-soluble polymeric polyphenols from cinnamon inhibit proliferation and alter cell cycle distribution patterns of hematologic tumor cell lines. *Cancer Letters, 230*(1), 134-140.

Seino, K. I., Setoguchi, Y., Ogino, T., Kayagaki, N., Akiba, H., Nakano, H., & Fukao, K. (2001). Protection Against Fas-Mediated and Tumor Necrosis Factor Receptor 1-Mediated Liver Injury by Blockade of FADD Without Loss of Nuclear Factor-κB Activation. *Annuls of Surgery, 234*(5), 681–688.

Sengupta, S., & Harris, C. C. (2005). p53: traffic cop at the crossroads of DNA repair and recombination. *Nature Reviews Molecular Cell Biology, 6*(1), 44-55.

Sentman, C. L., Shutter, J. R., Hockenbery, D., Kanagawa, O., & Korsmeyer, S. J. (1991). Bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell, 67*(5), 879-888.

Shaheen, M., Allen, C., Nickoloff, J. A., & Hromas, R. (2011). Synthetic lethality: exploiting the addiction of cancer to DNA repair. *Blood, 117*(23), 6074-6082.

Shaughnessy, D. T., Setzer, R. W., & DeMarini, D. M. (2001). The antimutagenic effect of vanillin and cinnamaldehyde on spontaneous mutation in Salmonella TA104 is due to a reduction in mutations at GC but not AT sites. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 480*, 55-69.

Shibata, D. (2012). Cancer. Heterogeneity and tumor history. *Science*, *336*(6079), 304-5.

Shimada, Y., Yokoyama, K., Goto, H., Sekiya, N., Mantani, N., Tahara, E., & Terasawa, K. (2004). Protective effect of Keishi-bukuryo-gan and its constituent medicinal plants against nitric oxide donor-induced neuronal death in cultured cerebellar granule cells. *Phytomedicine*, *11*(5), 404-410.

Shim, H., Park, J., Paik, H., Nah, S., Kim, D. S. H. L., & Han, Y. S. (2007). Acacetin-induced apoptosis of human breast cancer MCF-7 cells involves caspase cascade, mitochondria-mediated death signaling and SAPK/JNK1/2-c-Jun activation. *Molecules and Cells*, *24*(1), 95.

Shin, I., & Arteaga, C. L. (2006). Expression of active Akt protects against tamoxifen-induced apoptosis in MCF-7 Cells. *International Union of Biochemistry and Molecular Biology Life*, *58*(11), 664-669.

Siddhuraju, P., Mohan, P. S., & Becker, K. (2002). Studies on the antioxidant activity of Indian Laburnum Cassia fistula: a preliminary assessment of crude extracts from stem bark, leaves, flowers and fruit pulp. *Food Chemistry*, *79*(1), 61-67.

Siegel, R., Naishadham, D., & Jemal, A. (2012). Cancer statistics, 2012. *CA: A Cancer Journal for Clinicians*, *62*(1), 10-29.

Sies, H. (1997). Oxidative stress: Oxidants and antioxidants. *Experimental Physiology*, *82*(2), 291–5.

Sim, X., Ali, R. A., Wedren, S., Goh, D. L., Tan, C. S., Reilly, M., & Chia, K. S. (2006). Ethnic differences in the time trend of female breast cancer incidence: Singapore, 1968–2002. *Biomed Central, Cancer*, *6*(1), 261.

Singh, A., Boldin-Adamsky, S., Thimmulappa, R. K., Rath, S. K., Ashush, H., Coulter, J., & Biswal, S. (2008). RNAi-mediated silencing of nuclear factor erythroid-2–related factor 2 gene expression in non–small cell lung cancer inhibits tumor growth and increases efficacy of chemotherapy. *Cancer Research*, *68*(19), 7975-7984.

Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exprimental Cell Research*, *175*(1), 184–191.

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteau reagent. *Method Enzymology*, *299*(5), 152−178.

Sompong, R., Siebenhandl-Ehn, S., Linsberger-Martin, G., & Berghofer, E. (2011). Physicochemical and antioxidative properties of red and black rice varieties from Thailand, China and Sri Lanka. *Food Chemistry*, *124*(1), 132-140.

Soule, HD., Vazquez, J., Long, A., Albert, S., & Brennan, M. (1973). A human cell line from a pleural effusion derived from a breast carcinoma". *Journal of the National Cancer Institute, 51*(5), 1409–1416.

Sproll, C., Ruge, W., andlauer, C., Godelmann, R., & Lachenmeier, D. W. (2008). HPLC analysis and safety assessment of coumarin in foods. *Food chemistry*, *109*(2), 462-469.

Stah, W., & Viiia-Ribes, J. (1998). Functional food science and defence against reactive oxidative species. *British Journal of Nutrition, 80*(1), 77- 112.

Steenkamp, V., Grimmer, H., Semano, M., & Gulumian, M. (2005). Antioxidant and genotoxic properties of South African herbal extracts. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *581*(1), 35-42.

Su, L., Yin, J. J., Charles, D., Zhou, K., Moore, J., & Yu, L. L. (2007). Total phenolic contents, chelating capacities, and radical-scavenging properties of black peppercorn, nutmeg, rosehip, cinnamon and oregano leaf. *Food Chemistry*, *100*(3), 990-997.

Sultana, B., Hussain, Z., Hameed, M., & Mushtaq, M. (2013). Antioxidant activity among different parts of Aubergine (Solanum melongena L.). *Pakistan Journal of Botany*, *45*(4), 1443-1448.

Sultana, B., & Anwar, F. (2008). Flavonols (kaempeferol, quercetin, myricetin) contents of selected fruits, vegetables and medicinal plants. *Food Chemistry*, *108*(3), 879-884.

Sumantran, V. N., Ealovega, M. W., Nuñez, G., Clarke, M. F., & Wicha, M. S. (1995). Overexpression of Bcl-XS sensitizes MCF-7 cells to chemotherapy-induced apoptosis. *Cancer Research, 55*(12), 2507-2510.

Suzuki, K., Koike, H., Matsui, H., Ono, Y., Hasumi, M., Nakazato, H., & Yamanaka, H. (2002). Genistein, a soy isoflavone, induces glutathione peroxidase in the human prostate cancer cell lines LNCaP and PC-3, *International Journal of Cancer*, *99*(6), 846-852.

Taib, N. A., Yip, C. H., Ibrahim, M., Ng, C. J., & Farizah, H. (2007). Breast cancer in Malaysia: are our women getting the right message? 10 year-experience in a single institution in Malaysia. *Asian Pacific Journal of Cancer Prevention*, *8*(1), 141.

Tainer, J. A., Getzoff, E. D., Richardson, J. S., & Richardson, D. C. (1983). Structure and mechanism of copper, zinc superoxide dismutase, *Nature, 306*(5940), 284–7.

Takahashi, A., Alnemri, E. S., Lazebnik, Y. A., Fernandes-Alnemri, T., Litwack, G., Moir, R. D., & Earnshaw, W. C. (1996). Cleavage of lamin A by Mch2 alpha but not CPP32: multiple interleukin 1 beta-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. *Proceedings of the National Academy of Sciences*, *93*(16), 8395–400.

Talalay, P., & Talalay, P. (2001). The importance of using scientific principles in the development of medicinal agents from plants. *Academic Medicine, 76*(3), 238–247.

Tarique, N., Grace, L., Shafi, G., Al-Hazzani, A., & Alshatwi, A. (2011). Anti-proliferative effects of organic extracts from root bark of Juglans Regia L. (RBJR) on MDA-MB-231 human breast cancer cells: role of Bcl-2/Bax, caspases and Tp53. *Asian Pacific Journal of Cancer Prevention,12*(2), 525-530.

Tew, K. D. (1994). Glutathione-associated enzymes in anticancer drug resistance. *Cancer Research, 54*(16), 4313-4320.

Thorburn, J., Bender, L. M., Morgan, M. J., & Thorburn, A. (2003). Caspase- and Serine Protease-dependent Apoptosis by the Death Domain of FADD in Normal Epithelial Cells. *Molecular Biology of the Cell*, *14*(1), 67-77.

Tieu, H., & Loeffler, M. F. (2013). Nutraceutical composition and method of use for treatment / prevention of cancer. *U.S. Patent No. 20,130,101,627*. Washington, DC: U.S. Patent and Trademark Office.

Toledo F., & Wahl G. (2006). Regulating the p53 pathway: *in vitro* hypotheses, *in vivo* veritas. *Nature Reviews Cancer, 6*(12), 909-923.

Tomaino, A., Cimino, F., Zimbalatti, V., Venuti, V., Sulfaro, V., De Pasquale, A., & Saija, A. (2005). Influence of heating on antioxidant activity and the chemical composition of some spice essential oils. *Food Chemistry*, *89*(4), 549-554.

Toyokuni, S., Okamoto, K., Yodoi, J., & Hiai, H. (1995). Persistent oxidative stress in cancer. *Federation of European Biochemical Societies letter*, *358*(1), 1-3.

Trachootham, D., Alexandre, J., & Huang, P. (2009). Tegeting cancer cells by ROS-mediated mechanisms: radical; therapeutic approach. *Drug Discovery, 8*(7), 579-591.

Tracy, J. D. (1997). *The political economy of merchant empires: State power and world trade,* Vol. 2. 1350-1750. British: Cambridge University Press.

Trueba, G. P., Sanchez, G. M. & Giuliani, A. (2004). Oxygen free radical and antioxidant defence mechanism in cancer. *A Journal and Virtual Library, 9*, 2029-2044.

Tsai, T.H., Tai, T.H., Chien, Y.C., Lee, C.W., & Tsai, O.J. (2008). *In vitro* antimicrobial activities against *Cariogenic streptococci* and their antioxidant capacities: A comparative study of green tea versus different herbs. *Food Chemistry, 110*(4), 859–864.

Tse, C., & Capeau, J. (2003). Real time PCR methodology for quantification of nucleic acids. *In Annales de Biologie Clinique, 61*(3), 279-293.

Tung, Y. T., Chua, M. T., Wang, S. Y., & Chang, S. T. (2008). Anti-inflammation activities of essential oil and its constituents from indigenous cinnamon (Cinnamomum osmophloeum) twigs. *Bioresource Technology, 99*(9), 3908–3913.

Tzifi, F., Economopoulou, C., Gourgiotis, D., Ardavanis, A., Papageorgiou, S., & Scorilas, A. (2011). The Role of BCL2 Family of Apoptosis Regulator Proteins in Acute and Chronic Leukemias. *Advances in Hematology, 2012,* 15.

Ulbricht, C., Seamon, E., Windsor, R. C., Armbruester, N., Bryan, J. K., Costa, D., & Zhang, J. (2011). An evidence-based systematic review of cinnamon (Cinnamomum spp.) by the Natural Standard Research Collaboration. *Journal of Dietary Supplements*, *8*(4), 378-454.

Unlu, M., Ergene, E., Unlu, G. V., Zeytinoglu, H. S., & Vural, N. (2010). Composition, antimicrobial activity and *in vitro* cytotoxicity of essential oil from Cinnamomum zeylanicum Blume Lauraceae. *Food and Chemical Toxicology*, *48*(11), 3274-3280.

Vadaparampil, S. T., Malo, T., de la Cruz, C., & Christie, J. (2012). Do Breast Cancer Patients Tested in the Oncology Care Setting Share BRCA Mutation Results with Family Members and Health Care Providers?. *Journal of Cancer Epidemiology, 2012*, 10.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry and Cell Biology, 39*(1), 44–84.

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M. M., & Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions, 160*(1), 1-40.

Van, I. M. L., Ploemen, J. P., Lo, B. M., Federici, G., & Van, B. P. J. (1997). Interactions of alpha, beta-unsaturated al- dehydes and ketones with human glu- tathione S-transferase P1-1. *Chemistry Biology Interact*, *108*(1-2), 67-78.

Vanacker, S. A., Tromp, M. N., Haenen, G. R., Vandervijgh, W. J. F., & Bast, A. (1995). Flavonoids as scavengers of nitric oxide radical. *Biochemical and Biophysical Research Communications, 214*(3), 755-759.

Wajant, H., Pfizenmaier, K., & Scheurich, P. (2003). Non-apoptotic Fas signaling. *Cytokine & Growth Factor Reviews*, *14*(1), 53-66.

Walsh, J. G., Cullen, S. P., Sheridan, C., Lüthi, A. U., Gerner, C., & Martin, S. J. (2008). "Executioner caspase-3 and caspase-7 are functionally distinct proteases. *Proceedings of the National Academy of Sciences, 105*(35), 12815-12819.

Wang, J., & Lenardo M. J. (2000). Roles of caspases in apoptosis, development, and cytokine maturation revealed by homozygous gene deficiencies. *Journal of Cell Science*, *113*(5), 753-757.

Wang, T. T., & Phang, J. M. (1995). Effects of estrogen on apoptotic pathways in human breast cancer cell line MCF-7. *Cancer Research*, *55*(12), 2487-2489.

Węsierska-Gądek, J., Hackl, S., Zulehner, N., Maurer, M., & Komina, O. (2011). Reconstitution of human MCF-7 breast cancer cells with caspase-3 does not sensitize them to action of CDK inhibitors. *Journal of Cell Biochemistry, 112*(1), 273-288.

Wondrak, G. T., Villeneuve, N. F., Lamore, S. D., Bause, A. S., Jiang, T., & Zhang, D. D (2010). The Cinnamon-derived Dietary Factor Cinnamic Aldehyde Activates the Nrf2-dependent Antioxidant Response in Human Epithelial Colon Cells. *Molecules, 15*(5), 3338–3355.

Wu, S. J., Ng, L. T., & Lin, C. C. (2005). Cinnamaldehyde-induced apoptosis in human PLC/PRF/5 cells through activation of the proapoptotic Bcl-2 family proteins and MAPK pathway. *Life Sciences, 77*(8), 938-951.

Xiao, D., & Zhang, L. (2008). Upregulation of Bax and Bcl-2 following prenatal cocaine exposure induces apoptosis in fetal rat brain. *International Journal of Medical Sciences, 5*(6), 295-302.

Yaacob, N. S., Hamzah, N., Kamal, N. N. N. M., ABidin, S. A. Z., Lai, C., Navaratnam, V., & Norazmi, M. N. (2010). Anticancer activity of a sub-fraction of dichloromethane extract of Strobilanthes crispus on human breast and prostate cancer cells *in vitro*. *Biomed Central Complementary and Alternative Medicine, 10*(1), 42.

Yamasaki-Miyamoto, Y., Yamasaki, M., Tachibana, H., & Yamada, K. Fucodian induces apoptosis thrugh activation of caspase-8 on human breast cancer MCF-7 cells. (2009). *Journal of Agricultural and Food chemistry, 57*(18), 8677-8682.

Yang, C. H., Li, R. X., & Chuang, L. Y. (2012). Antioxidant Activity of Various Parts of *C. cassia* Extracted with Different Extraction Methods. *Molecules, 17*(2012), 7294-7304.

Yang, P. M., Tseng, H. H., Peng, C. W., Chen, W. S., & Chiu, S. J. (2012). Dietary flavonoid fisetin targets caspase-3-deficient human breast MCF-7 cells by induction of caspase-7 associated apoptosis and inhibition of outophagy. *International Journal of Oncology, 40*(2), 469-478.

Yang, C. H., Li, R. X., & Chuang, L. Y. (2012). Antioxidant activity of various parts of *Cinnamomum cassia* extracted with different extraction methods. *Molecules, 17*(6), 7294-7304.

Yang, H. L., Chen, C. S., Chang, W. H., Lu, F. J., Lai, Y. C., Chen, C. C., & Hseu, Y. C. (2006). Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by *Antrodia camphorata*. *Cancer Letters, 231*(2), 215-227.

Yeh, C. F., Chang, J. S., Wang, K. C., Shieh, D. E., & Chiang, L. C. (2013). Water extract of Cinnamomum cassia Blume inhibited human respiratory syncytial virus by preventing viral attachment, internalization, and syncytium formation. *Journal of Ethnopharmacology, 147*(2), 321-326.

Yip, C. H., Taib, N. A., & Mohamed, I. (2006). Epidemiology of breast cancer in Malaysia. *Asian Pacific Journal L of Cancer Prevention*, **7**, 369-74.

Yoeli-Lerner, M., Yiu, G. K., Rabinovitz, I., Erhardt, P., Jauliac, S., & Toker, A. (2005). Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. *Molecular Cell*, *20*(4), 539–550.

Yu, T., Lee, S., Yang, W. S., Jang, H. J., Lee, Y. J., Kim, T. W., & Cho, J. Y. (2012). The ability of an ethanol extract of *Cinnamomum cassia* to inhibit Src and spleen tyrosine kinase activity contributes to its anti-inflammatory action. *Journal of Ethnopharmacology*, *139*(2), 566-573.

Zu, Y., Yu, H., Liang, L., Fu, Y., Efferth, T., Liu, X., & Wu, N. (2010). Activities of Ten Essential Oils towards Propionibacterium acnes and PC-, A-9 and MCF-7 Cancer Cells. *Molecules*, *15*(5), 3200-3210.

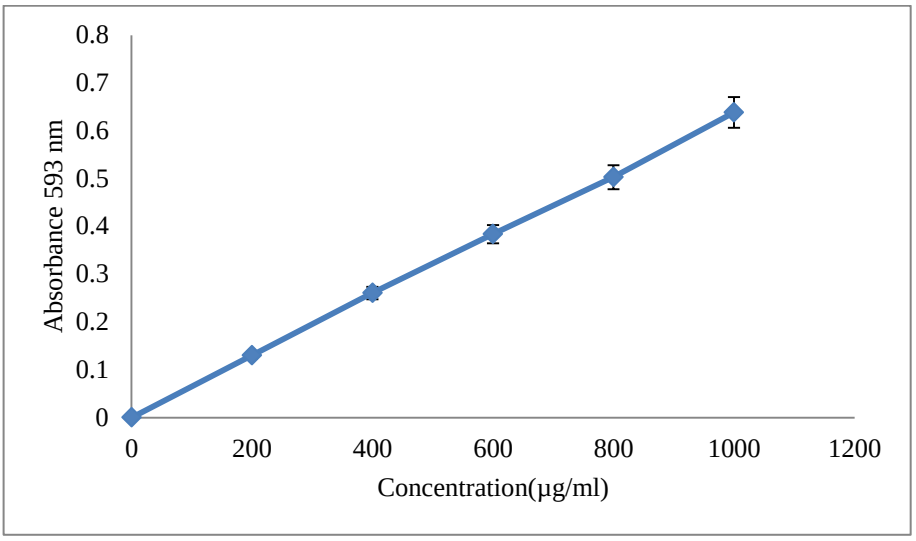
Zakhari, S. (2013). Bermuda Triangle for the liver: Alcohol, obesity, and viral hepatitis. *Journal of Gastroenterology and Hepatology*, *28*(S1), 18-25.

Zhang, G. J., Kimijima, I., Abe, R., Kanno, M., Katagata, N., Hara, K., & Tsuchiya, A. (1997). Correlation between the expression of apoptosis-related bcl-2 and p53 oncoproteins and the carcinogenesis and progression of breast carcinomas. *Clinival Cancer Research, 3*(12), 2329-2335.

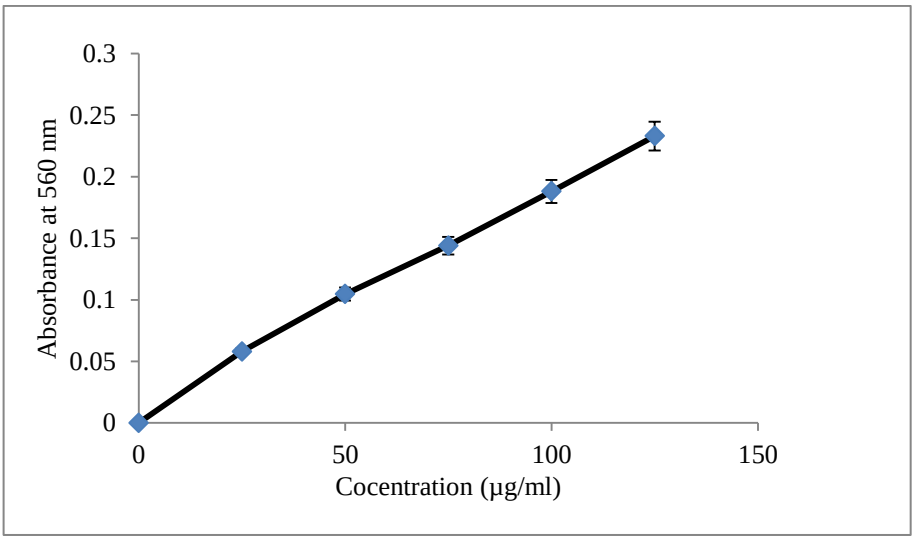
Zhang, G., Lu, Y., Lv, J., & Ou, H. (2006). Effect of ursolic acid on caspase-3 and PARP expression of human MCF-7 cells. *China Journal of Chinese Materia Medica, 31*(2),141-144.

Zhao, M., Yang, B., Wang, J., Li, B., & Jiang, Y. (2006). Identification of the major flavonoids from pericarp tissues of lychee fruit in relation to their antioxidant activities. *Food Chemistry*, *98*(3), 539-544.

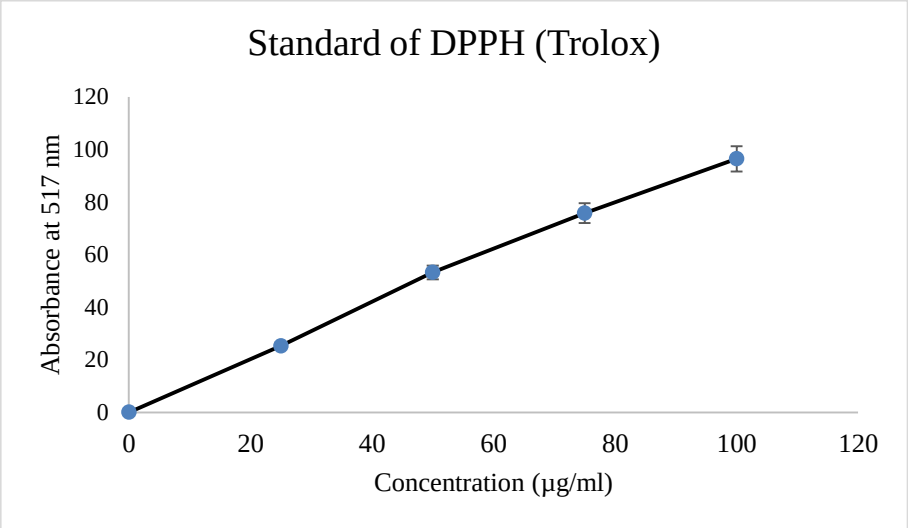
Appendix A: Standard curve of $FeSO_4.7H_2O_2$ in FRAP assay



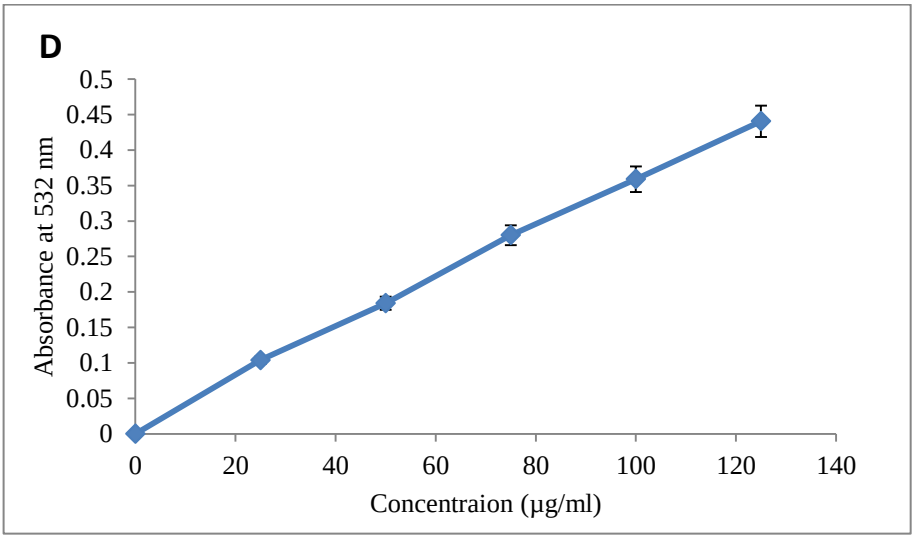
Appendix B: Standard curve of trolox in the super oxide anion radical scavenging assay



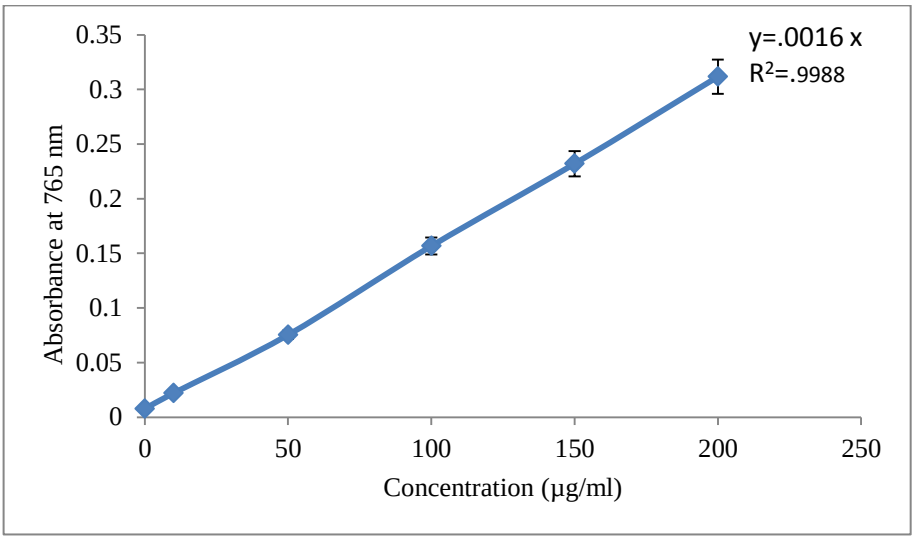
Appendix C: Standard curve trolox in DPPH assay



Appendix D: Standard curve of trolox in hydroxyl radical scavenging assay



Appendix E: Standard curve of gallic acid in TPC (Gallic acid)



Appendix F: Standard curve of quercetine in TFC